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Production of polyhydroxyalkanoates by hydrocarbonaclastic bacteria

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Production of polyhydroxyalkanoates (PHA) by bacteria isolated from spent engine oil (SEO) contaminated soils was investigated using nitrogen limitation in the production medium. Out of ten isolates, three were selected as the best based on their ability to metabolize SEO effectively and fluoresce orange when stained with Nile Blue A dye. Fourier Transform Infrared spectrophometer was used as a confirmatory test for PHA detection by tracking the carbonyl bands on the spectra. Optimum growth of the isolates occurred at 1% *(v/v)* SEO, pH 7, 37°C for K1+VE and Kar5+VE1 while for Kar5+VE2 was at 1% *(v/v)* SEO, PH 7, 40°C and 150 RPM. Sequencing of 16S rDNA partial genes grouped the isolates into 6 different genera: *Ochrobactrum*, *Pseudoxanthomonas, Bodetella, Achromobacter, Alcaligenes* and *Acinetobacter* species. Isolate K1+VE, identified as an *Ochrobactrum* produced Poly (3-hydroxyheptanoate) 46% (*w/w)*, isolate Kar5+VE1, identified as *Achromobacter* spp. produced poly (3-hydroxybutyrate) 20% *(w/w)*) while isolate Kar5+VE2, identified as an *Alcaligenes* spp. produced a co-polymer poly-3- (3-hydroxybutyrate-Co-3-hydroxyoctanoate) 45% (*w/w*). Orthoxylene and ethylbenzene were the major hydrocarbons in spent engine oil before degradation while ethylhexanol was the major degradation product as identified by Gas Chromatography-Mass Spectrophotometry. The isolates were able to degrade hydrocarbons as well as produce polyhydroxyalkanoates.

Key words: Polyhydroxyalkanoates, spent engine oil, 16S rDNA, co-polymer.

INTRODUCTION

Synthetic plastics pose a big challenge as they generate non-degradable waste products. They remain in water bodies, soil and landfill for many years without decomposing hence generating a high environmental burden. This has resulted in exploration of eco-friendly polyesters such as polylactic acid and polyhydroxyalkanoic acid (Akaraonye et al., 2010). Microbial fermentation produces polymeric materials like polyhydroxyalkanoates (PHA) that are carbonic and a form of energy storage (Maheshwari et al., 2018). PHAs are natural polyesters of 3-, 4-, 5-, and 6-hydroxyalkanoic acids which are biodegradable, biocompatible and thermoplastic

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in nature (Sinha, 2013). Accumulation of PHAs can occur either during nutrient limitation such as phosphorous, nitrogen, oxygen or magnesium, and in excess of carbon source (fed batch culture) or in a single batch culture that does not require any nutrient limitation (Nitschke et al., 2011).

In prokaryotes, the ability to accumulate PHA is broadly distributed among the Gram-negative and Gram-positive organisms. *Bacillus* species are well known for their ability to accumulate poly-3-hydroxybutyrate (PHB) which is the most common and simplest form of PHA found in bacteria (Balakrishna et al., 2017). Fluorescent *Pseudomonas* strains, for example, are well known to accumulate medium chain length (mcl)-PHAs with 6 to 14 carbon atoms (Kim et al., 2007).

Some bacteria species are able to synthesize PHA copolymers depending on the substrate utilized. Among the copolymers, poly(3-hydroxybutyrate-co-3-hydroxyheptanoate (P3HB-co-3HP) is promising due to its beneficial properties such as biodegradability, biocompatibility and thermostability (Andreessen and Steinbuchel, 2010).

Despite their distinct advantage, one of the challenges facing the development of biodegradable polymers as substitutes for conventional plastics is their high cost of production compared to petrochemical derived plastics (Kim, 2000). The major factors that increase the cost of production of PHAs include the type of carbon source, fermentation process, yield on the selected carbon sources and downstream processing (Choi et al., 2013). Spent engine oil is an available and affordable raw material that can be used in the productions of PHAs and the use of microorganisms that do not require media sterilization cuts down the cost of producing PHAs (Alvi et al., 2014).

Soil contamination with petroleum and petroleum-based hydrocarbons result to environmental hazards and health defects (Goudarztalejerdi et al., 2015). Bioremediation methods have received positive publicity because they promise to be environmentally friendly, efficient and cheap treatment technologies for the remediation of hydrocarbon contamination. Bioremediation can be described as the transformation of chemical compounds by living organisms, especially microorganisms, into energy, cell mass and biological waste products (Minai-Tehrani et al., 2015).

Some microorganisms have been reported to degrade crude oil and accumulate PHA in limited nutrients (Goudarztalejerdi et al., 2015). The use of such microorganisms for PHA production would provide several advantages, namely, reduction in the cost of producing PHAs, value addition to waste petroleum by-products, bioremediation and recovery of biodegradable plastics from the cell biomass.

With the ban on manufacturing, importation and use of plastic bags in Kenya, the application of microorganisms in bioremediation in combination with recovery of valuable products like biopolymers (PHA) provides a suitable and unexplored substitute for synthetic plastics. The main objective of this study was to screen for production of polyhydroxyalkanoates by microorganisms isolated from spent engine oil contaminated sites within Nairobi, Kenya.

MATERIALS AND METHODS

Sample collection

Sixteen soil samples were collected from six spent engine oil (SEO) contaminated sites in Kenya, Nairobi County and the coordinates captured on Global Positioning System (GPS). The soil samples were obtained from the surface, at 5 and 10 cm deep. Each soil sample was packaged in a labelled sterile zip lock bag and transported to United States International University, Africa School of Pharmacy and Health Sciences laboratory for storage at 4°C until usage.

Bacterial isolation

This was done in a two phase process as described by Mwaura et al. (2018) with slight modifications. One gram from each of the 16 soil samples was weighed and inoculated in 100 ml of sterile Bushnell Haas broth (BHM) media (containing: (g/l) 0.4; $MgSO_4$, 0.04; $CaCl_2$, 2.0; KH_2PO_4, 2.0; K_2HPO_4, 2.0; NH_4NO_3 and 0.1; $FeCl_3$) supplemented with 1% SEO (v/v) and the pH adjusted to 7. The samples were incubated for three days at 37°C on a thermoshaker (HY-5B cycling vibrator, Wincom, China) at 150 rpm. From each culture, 100 µl was taken and spread on sterile Blood Agar Base (BAB) media on petri plates in triplicates. The BAB media comprised, pancreatic digest of casein (a source of amino acids), papaic digest of soy meal (a source of carbohydrates and vitamins), sodium chloride (for maintenance of osmotic balance) and Agar (Oxoid, Basingstoke, United Kingdom). The media were prepared according to manufacturers' specification of 40 g dissolved in 1 L distilled water with pH adjusted to 7.0 then autoclaved at 121°C for 15 min. The plates were incubated for 48 h at 37°C. Preliminary test for polyhydroxyalkanoate accumulation was then carried out by flooding the culture plates with 1% Nile Blue A aqueous solution and observed under UV light at 365 nm. Ten (10) fluorescing colonies were picked and plated on fresh BAB media in duplicate for further analysis.

Screening for PHA accumulation

In order to confirm PHA accumulation, FT-IR was performed as a rapid qualitative analysis of PHA in intact cells. A scan was done to identify ester carbonyl groups (C=O) that are characteristic for PHA. Hydroxyoctanoic acid was used as a standard to support identification of marker bands. Fluorescing pure isolates were each picked from culture plate using a sterile wire loop and inoculated into 100 ml sterile Luria Bertani (LB) medium. The inocula were then incubated at 37°C and 150 rpm for 24 h on a thermoshaker (HY-5B cycling vibrator, Wincom and China). This was followed by centrifugation at 4032×g (Universal 320R Centrifuge, Germany) for 10 min and the cells were re-suspended in fresh sterile BHM broth, pH 7 supplemented with 1% SEO (v/v) and incubated at 37°C and 150 rpm for 48 h on a thermoshaker. Thereafter, the cells were centrifuged at 4032×g and washed twice with normal saline for 10 s through turning the tube then it was air dried overnight. Spectra of intact air dried bacterial cells were then acquired using (JASCO FT-

IR-4700, Japan) spectrophotometer. Commercial Poly-hydroxyoctanoate (PHO) standards (Sigma Aldrich®, Germany) was run through the same FTIR to provide reference spectrum.

Bacterial morphological characterization

The pure isolates that fluoresced under Nile Blue A dye were subjected to microscopic analysis to determine colony shapes, bacterial cell shapes and arrangements. Gram's stain technique was done to determine cell wall constitution and shape of each bacterial cell according to Garrity et al. (2005).

Biochemical characterization of isolates

Biochemical tests were performed on the ten (10) PHA positive isolates for the determination of biochemical properties. The tests included carbohydrate utilization (where lactose, sucrose, maltose and fructose utilization were tested) (Breed et al., 1975) and enzyme activities where catalase and oxidase tests were done according to Garrity et al. (2005).

DNA extraction and amplification procedure

DNA was extracted from ten PHA producing bacterial isolates using Cetyl trimethylammonium bromide (CTAB) method (Doyle and Doyle, 1990). Partial 16S rRNA genes from each isolate were subsequently amplified by Polymerase Chain Reaction (PCR). The universal primers used for this study: FD1; 5'AGAGTTTGATCCTGGCTCAG3' and RD1; 5' AAGGAGGTGATCCAGC 3' (Weisburg et al., 1991) spanning through 500 bp were synthesized by Inqaba, South Africa. The PCR was carried out in a total volume of 20 µl containing 2 µl of DNA template, 1 µl of each of forward and reverse primers, 4 µl of PCR master mix and 12 µl of nuclease free water (Qiagen, Valencia, USA). Optimisation was carried out for the purpose of primer specificity to suit this current study. Amplification process was performed in MJ Mini Personal Thermal Cycler, BIO RAD, USA and involved 35 cycles of 95°C (30 seconds) denaturation, annealing at 55°C for 1 min 30 s, extension at 72°C for 1 min 30 s and final extension 72°C for 10 min. The initial heating of the DNA was performed at 95°C for 5 min. The PCR products were run through 1% agarose gel electrophoresis after staining with 0.4 µl of ethidium bromide and visualised using Bio-Rad Gel Doc XR+ imaging system, USA. Subsequently, the PCR products were sequenced using Sanger sequencing (ABI 3500XL Genetic Analyzer v3.1, Inqaba, South Africa). The nucleotide sequences were visualised and edited by Chromas software version 2.6.4. The analysed sequences were then deposited in Genbank and assigned accession number MK346108-17. The 16S rRNA partial sequences were aligned with NCBI-generated sequences of related microorganisms to evaluate degree of relatedness and ancestral evolution using MEGA software version 7.0.18.

Optimization of bacterial growth conditions

The growth parameters for three best SEO emulsifiers K1+VE, Kar5+VE 1 and Kar5+VE 2 were optimized through variation of temperature (34, 37 and 40°C), pH (4, 7, and 10) and SEO concentration (1, 2 and 3%), respectively. The initial default parameters were 37°C and pH 7 with varying SEO concentrations at 1, 2 and 3%. This was followed by temperature variation at 34, 37 and 40°C with fixed pH of 7 and 1% SEO. Finally, pH was varied at pH (4, 7, and 10) using 1 M NaOH and 1 M HCl with fixed SEO at 1% and temperature at 37°C for K1+VE, Kar5+VE 1 and 40°C for Kar5+VE 2, respectively. In the optimisation of each parameter (temperature, pH or % SEO), the isolates were cultured in triplicates at 150 rpm on a thermoshaker. Aliquots (4 ml) were taken from each sample and optical density (OD) (600 nm) measured at interval of 24 h for 5 days.

Quantitative estimation of PHAs by crotonic acid assay

Spectrophotometric assay was employed to determine the concentration of PHAs using crotonic acid. Isolates K1+VE, Kar5+VE1 and Kar5+VE2 were cultured at optimum pH, temperature and substrate (SEO) concentration as determined in 2.7 above. The isolates were first cultured in sterile LB medium for 24 h then transferred to BHM medium for 72 h. Extraction of PHA was done using rapid hypochlorite method in triplicates as described by Rawte and Mavinkurve (2002). Each bacteria sample was centrifuged at 1792×g for 10 min. The pellets were suspended in 5 ml 3.85% w/v sodium hypochlorite. The mixture was incubated at 37°C for 10 min on a shaker followed by centrifugation at 1792×g for 20 min. The pellets were washed three times using 2 ml 95% diethyl ether for 5 s by shaking the tube and air dried. The pellet of each sample was suspended in 4.5 ml of concentrated sulphuric acid followed by incubation in a water bath at 100°C for 20 min. The solution was then loaded in a glass cuvette and OD measured at 255 nm against a sulphuric acid blank. This process was performed after 2, 8, 24, 48 and 72 h of incubation.

Hydroxyoctanoic acid standard (Sigma Aldrich®, Germany) was prepared by dissolving 1 mg in 5 ml concentrated sulphuric acid. The solution was then incubated in a water bath at 100°C for 20 min and then cooled to form crotonic acid. A wavelength scan was run in the range of 200 to 500 nm to determine maximum absorbance wavelength. A concentration of 20 to 200 µg/ml crotonic acid standard was prepared and subjected to photometric analysis at a wavelength of 255 nm using (Advanced Microprocessor UV-Vis single Beam Spectrophotometer L1-295). The amount of crotonic acid thus PHA in the samples was then determined from the plotted graph as established by Slepecky and Law (1960).

PHA production under varying nitrogen concentrations

K1+VE, Kar5+VE 1 and Kar5+VE 2 isolates were grown under optimum conditions earlier established in fed-batch culture. The initial culture was in 100 ml sterile LB medium with 10 g/l tryptone as the source of nitrogen at pH 7, 37 and 40°C for 24 h followed by centrifugation. The cells were then re-suspended in 100 ml of fresh sterile PHA production medium (BHM) with modified nitrogen concentration (0.2, 0.5 and 1 g/L ammonium nitrate). The isolates were cultured for 48 h and PHA produced quantified using crotonic acid assay as earlier described.

PHA extraction

PHA recovery from cells was done using rapid hypochlorite extraction method as described by Rawte and Mavinkurve (2002). This method is simple and efficient in the recovery of the polymers from non PHA cell material. In order to recover PHA from the cells, 100 ml bacterial cultures were grown under optimum temperature, pH, carbon concentration and nitrogen concentration as described earlier. Sterile 15 ml falcon tubes treated with acetone and methanol to remove plasticizer were weighed and used for centrifugation of the culture at 1792×g for 10 min. The pellets were air dried and the falcon tubes containing pellets weighed. The pellets were then re-suspended in 5 ml 3.85% w/v sodium hypochlorite and incubated at 37°C on thermoshaker at 150 rpm for

10 min. This was followed by centrifugation at 1792×g for 20 min. Subsequently, the pellets were washed three times using cold 2 ml 95% diethyl ether for 5 s by shaking the tubes. The pellets were later air dried and falcon tubes with extracted PHA extract then weighed as done by Rawte and Mavinkurve (2002).

Formula

The dry weight of cells was determined by subtracting the weight of the dry empty tube from that of the dry tube with cells.

W-X=Y,

where W=weight of the dry falcon tube with cells (g), X= weight of empty dry falcon tube (g), and Y= dry weight of cells (g).

The dry weight of PHA was determined by subtracting the weight of dry empty tube from that of dry tube with PHA.

M-N=O,

where M =dry falcon tube with PHA (g), N= dry empty falcon tube (g), and O= Dry weight of PHA (g).

Percentage of PHA in cell dry weight was determined by [(O/Y)×100].

PHA Gas Chromatography-Mass Spectrophotometry analysis

Dried PHA pellets were treated with (1.7 ml) 98% (v/v) methanol (0.3 ml) of 98% sulphuric acid and 2.0 ml chloroform at 100°C for 140 min followed by the addition of 1.0 ml of water. 1 µl of the lower phase was injected into GC-MS injector port as described by Lee and Choi (1997) with modifications. Analysis was done in a GC-MS (Shimadzu GC-MS 2010 SE) equipped with a capillary column BPX5 (dimensions: 30 m in length, 0.25 mm ID, and 0.25 µm film thickness). Helium was used as the carrier gas and a temperature program set as initial temperature 40°C for 3 min; temperature increased from 40 to 300°C at a rate of 20°C min^{-1}, hold time: 5 min. Data generated was matched with the GC-MS inbuilt standard mass spectra library of NIST-05.

Hydrocarbon analysis of SEO using GC-MS

SEO hydrocarbons, their derivatives and intermediate metabolites in BHM media inoculated with isolate K1+VE was analysed after 10 days of incubation. The inoculum of this isolate was previously cultured overnight in LB media and afterwards washed twice with normal saline. An aliquot (100 µl) of bacterial cells was transferred to a 250 ml volumetric flask containing 100 ml sterile BHM media supplemented with 1% diesel oil (v/v). Un-inoculated BHM media in a flask was kept as a control. After 10 days of incubation, the hydrocarbons were analysed according to the procedure described by Tebyanian et al., (2013). The hydrocarbons were extracted from 30 ml BHM media using an equal volume of dichloromethane with the aid of a separating funnel. This was repeated twice to ensure complete recovery of the hydrocarbons. Analysis was done in a GC-MS (Shimadzu GC-MS 2010 SE) equipped with a capillary column BPX5 (dimensions: 30 m in length, 0.25 mm ID, and 0.25 µm film thickness). Helium was used as the carrier gas and a temperature program consisting of an initial oven temperature of 70°C for 3 min and the temperature increased to 270°C at a rate of 10°C/min and maintained for 5 min. An aliquot of 1 µl was used as the sample. The injector and detector temperatures were maintained at 200 and 250°C, respectively. Split (10.1) injection mode was applied.

RESULTS AND DISCUSSION

Isolation and identification of PHA producing bacteria

Out of the 16 soil samples, 80 distinct colonies were obtained after culturing in 100 ml Bushnell Haas broth media before transfer onto BAB solid agar media. Ten isolates fluoresced orange after staining with 1% Nile blue A aqueous solution (Figure 1A). Three out of the ten PHA positive isolates were selected for PHA production due to their significant fluorescence intensity and their ability to emulsify SEO after 5 days of incubation as shown in Figure 1B.

Nile blue A is a fluorescent dye that stains PHA granules in bacterial cells and gives a characteristic orange glow under UV light of 365 nm. The intensity of the glow is directly proportional to the concentration of PHA accumulation in the cells (Goudarztalejerdi et al., 2015). Ten bacterial isolates from 80 colonies showed fluorescence of different intensities. K1+VE, Kar5+VE1 and Kar5+VE2 were selected as the best isolates because of their high fluorescence which is an indication of sufficiently high PHA accumulation. Dispersion of small oil droplets in the media by these three isolates following 5 days of incubation could be attributed to bio surfactants production. Micro-organisms growing in hydrocarbon-rich environments have previously been reported to produce bio surfactants which increase their access to hydrophobic substrates (Joy et al., 2017).

FT-IR spectra for the commercial standard and intact cell samples are as shown in Figure 2. Purified hydroxyoctanoic acid showed PHA characteristic band at 1710 cm^{-1} (Figure 2A). Ester carbonyl bands are usually visible within 1700 to 1750 cm^{-1} region of FT-IR spectra. The absorption band observed at 1725.01, 1706.69 and 1733.69 cm^{-1} from the samples are PHA marker bands and are assigned to the stretching vibration of carbonyl (C=O) ester bond (Figure 2B, C and D, respectively). Sample bands displayed low peak intensities of C=O peaks (as shown in Figure 2B, C and D) compared to standard C=O peak (as shown in Figure 2A). This could be due to the measurements being done on intact cells hence interference from proteins and lipids (Hong et al., 1999). The infrared absorption spectra observed at 3275.50 cm^{-1} was due to the O-H stretching vibration of the hydroxyl group of the polymer chain while a characteristic peak at 2924.52 cm^{-1} was assigned to asymmetric CH_2 of the lateral monomeric chains as described by Gumel and Annuar (2012).

Morphological, biochemical and genetic characterisation

MacConkey Agar, catalase, oxidase, sucrose, maltose and fructose fermentation tests results are summarized in Table 1.

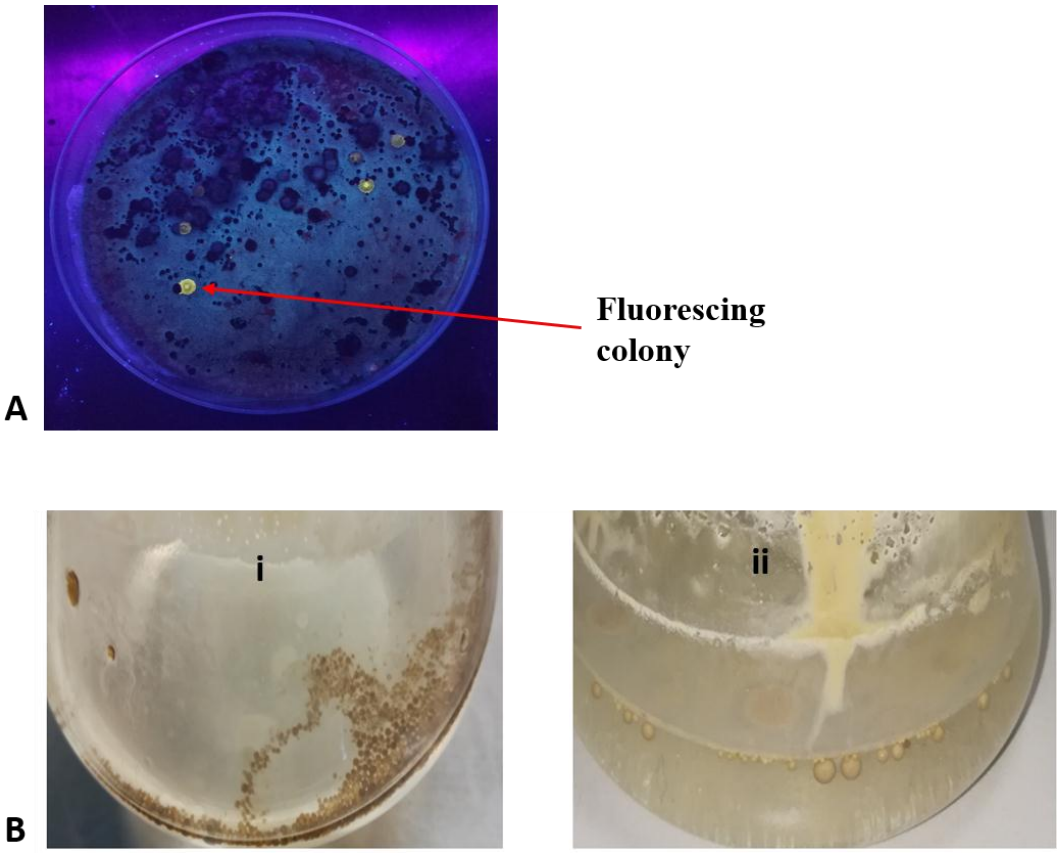

Figure 1. A: Isolates fluorescing orange after staining with 1% aqueous Nile blue A solution. B: Emulsification ability of bacterial isolates, flask (i) shows a good emulsifier (small oil droplets) while flask (ii) indicates a poor emulsifier (big oil droplets).

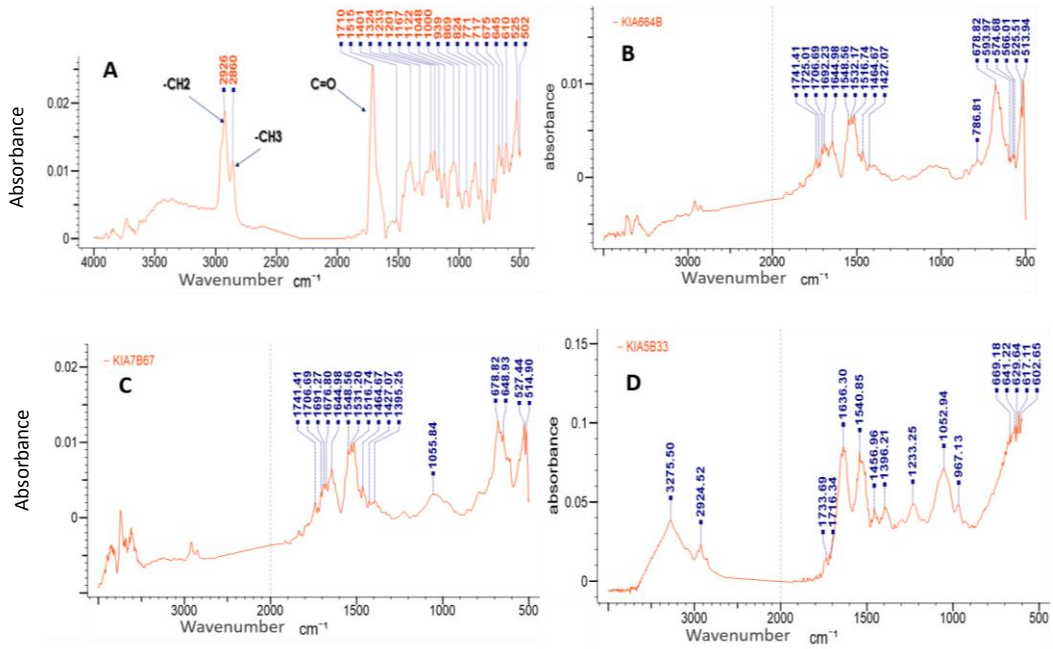

Figure 2. FT-IR spectra of (A), pure Poly-hydroxyoctanoate standard (Sigma Aldrich) spectrum with key bands identified. (B) PHH producing K1+VE intact cells, (C) PHB-co-HO producing Kar5+VE2 intact cells, and (D) PHB producing Kar5+VE1 intact cells.

Table 1. Morphological and biochemical test results of ten isolates from spent engine oil contaminated soils that fluoresced orange on staining with aqueous Nile blue, a specific dye for PHA.

Isolate	K1+VE	N10-2	N10-3	N5+VE	G5-1	GI5-4	GI10-1	Kar5+VE 1	Kar5+VE 2	$Kar_{II}5$-1
Morphology										
Shape	Cocci	Rod	Cocci	Cocci	Cocci	Rod	Rod	Cocci	Cocci	Rod
Colony colour	Cream	Yellow	Cream	Cream white	Light brown	Cream yellow	cream	Yellow	Cream yellow	Cream
Colony form	Circular	Irregular	circular	Circular	Irregular	Irregular	Irregular	Circular	Circular	Irregular
Gram reaction	-	+	-	-	-	-	-	-	-	-
Enzyme activity										
Oxidase	+	+	+	+	-	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+
Carbohydrate fermentation										
Maltose	-	-	-	+	-	+	+	+	-	+
Fructose	-	-	-	+	-	+	+	+	-	+
Sucrose	-	-	-	+	-	+	+	+	-	+
Lactose	+	-	+	+	+	+	+	+	+	+

Nine out of 10 isolates were Gram negative while the shapes of the cells are distributed between cocci and rods. All rod shaped cells formed irregular colonies while cocci cells formed circular colonies. Gram-negative bacteria have previously been reported to be more tolerant to mixtures of saturated, monoaromatic and polyaromatic hydrocarbons which are predominant in spent engine oil than Gram-positive bacteria (Lăzăroaie, 2010, Mwaura et al., 2018). This dominance is attributed to presence of lipopolysaccharide membranes in gram negative bacteria which play a role of accelerating the release of bio surfactants in emulsification of spent engine oil (Mahjoubi et al., 2013).

All the isolates possessed catalase enzyme activity as the bacteria were isolated from oxygenated soil where they are required to neutralize toxic oxidized compounds. This is an indication that the isolates are either aerobic or facultative anaerobes. This is also supported by the positive results of oxidase test with an exception of G5-1 as reported by Taylor and Anchanzar (1972).

The PCR amplification of genomic 16S rDNA from the ten isolates yielded fragments of 500 bp (Figure 3). The DNA sequences were submitted to Genbank and were assigned accession numbers. A homology search of the sequenced DNA fragments in NCBI website identified the ten isolates as shown in Table 2. A phylogenetic tree of the best three PHA accumulating isolates (K1 +VE, Kar5 +VE1 and Kar5 +VE2) was constructed using Maximum Likelihood method as shown in Figure 4. A recent study by Mwaura et al. (2018) reported the isolation of *Ochrobactrum* and *Acinetobacter* species from spent oil contaminated soil in Kenya which are among the isolates identified in this research. The presence of these bacteria in such harsh environment could be attributed to the production of bio surfactants which is evident from the preliminary observation of SEO emulsification in culture flasks. This feature enables the bacteria to mineralize SEO and use it as a source of energy. The ability to accumulate PHAs coupled with the presence of enzyme catalase enables these bacteria to survive in toxic environment with limited nutrient availability (Haytham, 2016).

Isolate growth and optimization of PHA accumulation

Growth optimization was done by growing the isolates in Bushnell Haas medium with variation of growth parameters, that is, temperature, pH and carbon concentration. As shown in Figure 5A, the isolates reached log phase in 8 h and attained maximum growth rate at 72 h which was followed

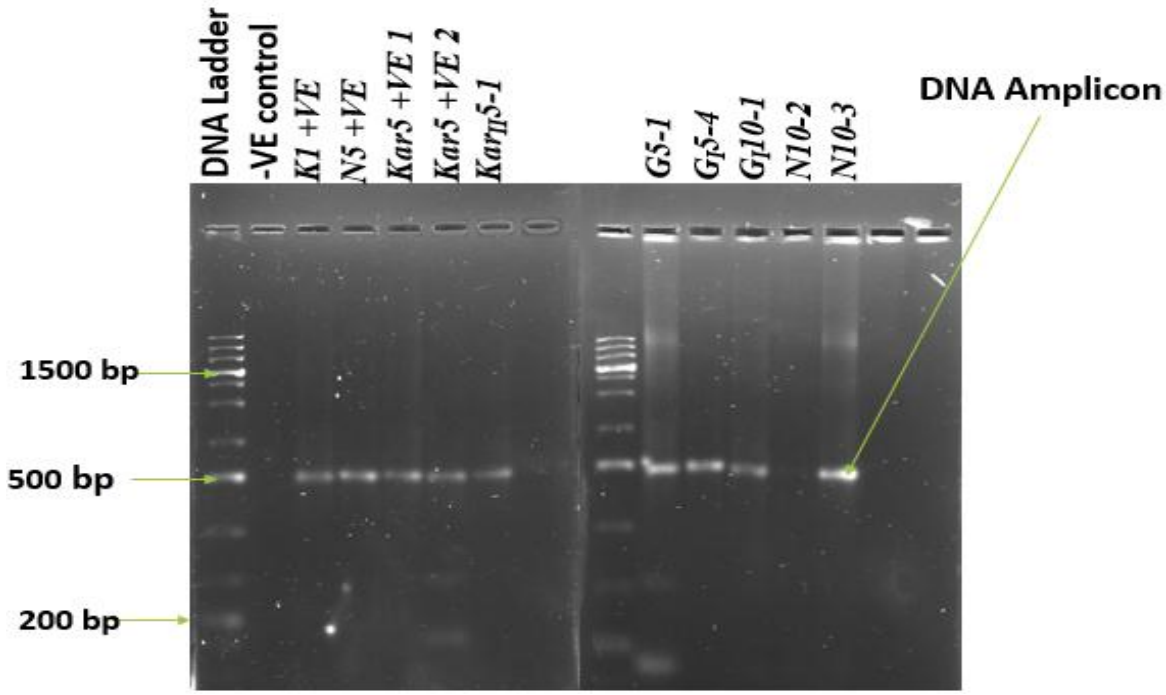

Figure 3. 1% agarose gel with 16S rDNA amplicons run against 1 kb plus DNA ladder (ThermoFisher Scientific, USA). N10-2 amplicon is missing and the sample was re-run.

Table 2. Closest relatives of 16S rDNA gene sequences of selected bacterial isolates with assigned accession numbers.

Isolate	Closest Hit	Identity (%)	Accession No.
K1+VE	*Ochrobactrum* spp.	96	MK346108
Kar5+VE1	*Achromobacter pulmonis*	99	MK346109
Kar5+VE2	*Alcaligenes* spp.	96	MK346110
N5+VE	*Bodetella petrii*	100	MK346111
Kar$_{II}$5-1	*Pseudoxanthomonas Mexicana*	83	MK346112
G5-1	*Bodetella petrii*	99	MK346113
G$_1$5-4	*Acinetobacter* spp.	100	MK346114
G$_1$10-1	*Ochrobactrum anthropi*	91	MK346115
N10-2	*Pseudoxanthomonas* spp.	96	MK346116
N10-3	*Achromobacter* spp.	99	MK346117

by a growth declining phase after 96 h of incubation.

Isolates K1+VE and Kar5+VE1 displayed optimum growth at pH 7, 1% SEO and 37°C while Kar5+VE 2 had optimum growth at pH 7, 1%SEO and 40°C. This is attributed to the fact that K1+VE and Kar5+VE1 were isolated from surface soil while Kar5+VE2 was isolated from 5 cm deep soil. Incubation temperature varied from one microorganism to another, this is because temperature greatly affects all metabolic processes and is expected to have a significant influence on oil degradation as well as explained by Aleer et al. (2011). The growth of the three isolates was optimum at pH 7. The obtained results were consistent with the findings of Jain et al. (2010) who reported that the degradation of petroleum hydrocarbons in crude oil was favourable near neutral pH. Spent engine oil as a sole carbon source varying from 1 to 3% v/v showed that with 1% v/v SEO the isolates have maximum growth and the highest PHA accumulation. Concentration of SEO above 1% v/v had an inhibitory effect on the growth of the isolates which could be as a result of toxicity of SEO and the difficulty to access nutrients in the culture medium as described by

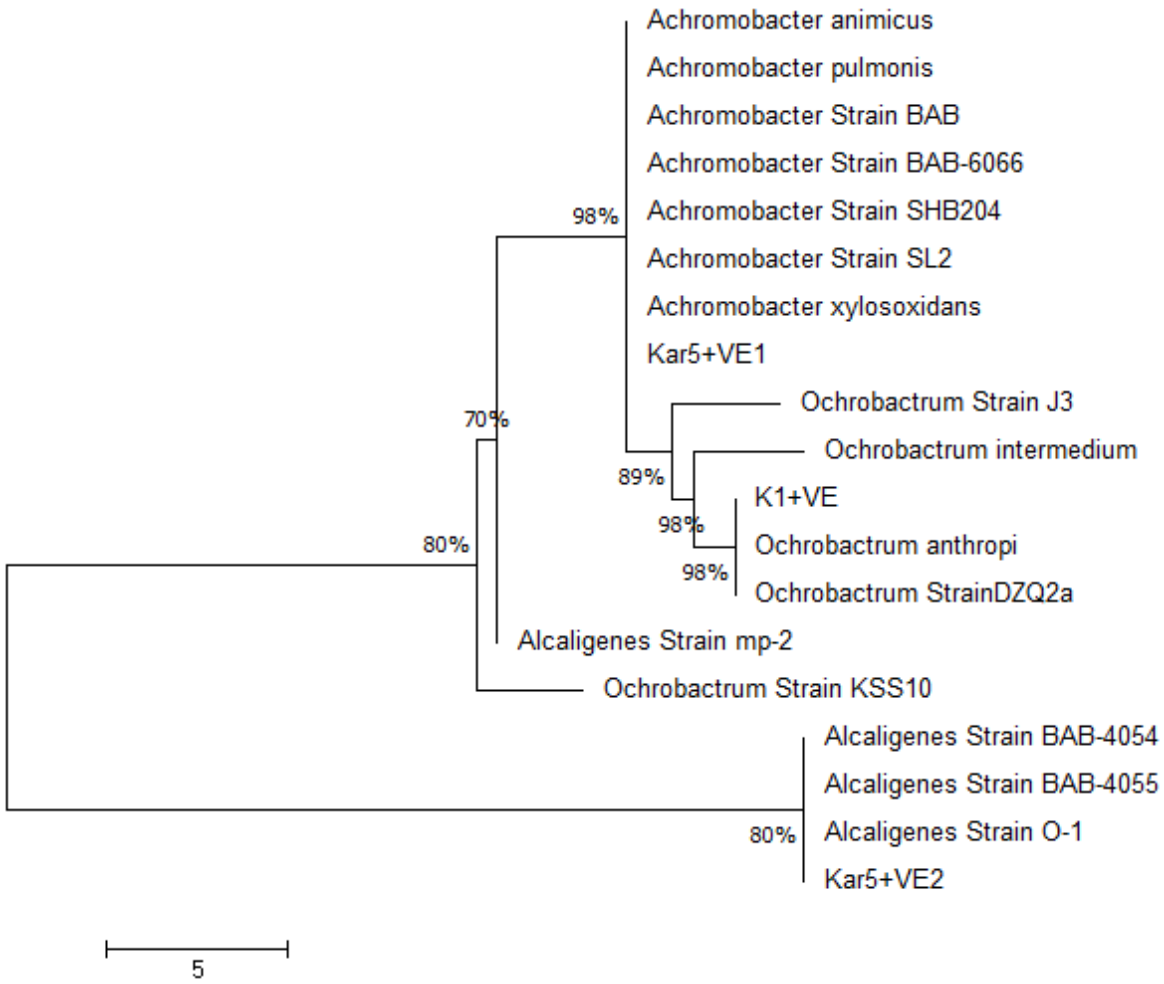

Figure 4. Phylogenetic tree based on 16S rDNA gene sequences. The tree was constructed using comparable 16S rDNA gene sequences of isolates K1+VE, Kar5+VE1, Kar5+VE2 with accession numbers MK346108, MK346109, MK346110 respectively and those retrieved from the NCBI database. The numbers at the node indicate bootstrap values as percentages obtained with 1000 resampling analyses.

Goudarztalejerdi et al. (2015).

The time-course analysis of PHA production revealed that accumulation of PHA in K1+VE, Kar5+VE1 and Kar5+VE2 is growth associated and thus PHA accumulation begins from the log phase and increased exponentially from 8 to 48 h of incubation, thereafter, the PHA accumulation started to decline up to 72 h (Figure 5B). The decline could be as a result of the cells utilizing PHA as energy reserves due to nutrient depletion in the medium as observed by Maheshwari et al. (2018) where PHA accumulation in *Bacillus cereus* SS105 declined after the third day of incubation.

Growth of the isolates at optimum pH, temperature and substrate concentration with limited nitrogen concentration resulted in varied PHA concentrations. For Isolate K1+VE, at 0.2 g/l ammonium nitrate, PHA concentration was 191.448 µg/ml and reduced to 69.172 µg/ml at 1 g/l of ammonium nitrate. The drop in concentration of PHA was also observed in isolates Kar5+VE1 and Kar5+VE2 as shown in Table 3.

Nitrogen limitation provides a stressed environment which facilitates production of PHA (Maheshwari et al., 2018). From the results, at 0.2 g/l nitrogen, K1+VE accumulated the most PHA at 46% *(w/w)* of CDW followed by Kar5+VE2 (45% *w/w*) then Kar5+VE1 (20% *w/w*). Nitrogen concentration at 0.5 and 1 g/l resulted in reduced PHA accumulation (Table 2). Oil-contaminated soils are estimated to contain about 84% carbon, 14% hydrogen, 1 to 3% sulfur, and <1% of nitrogen and other compounds (Atlas, 1995). Excess carbon with <1% nitrogen makes these sites a potential source for isolating PHA producers since the synthesis of PHA is favoured by environmental stress (Di Martino et al., 2014). For that reason, the accumulation of PHA in SEO contaminated soil bacteria might also increase the survival abilities of these bacteria in the toxic environments and can be used

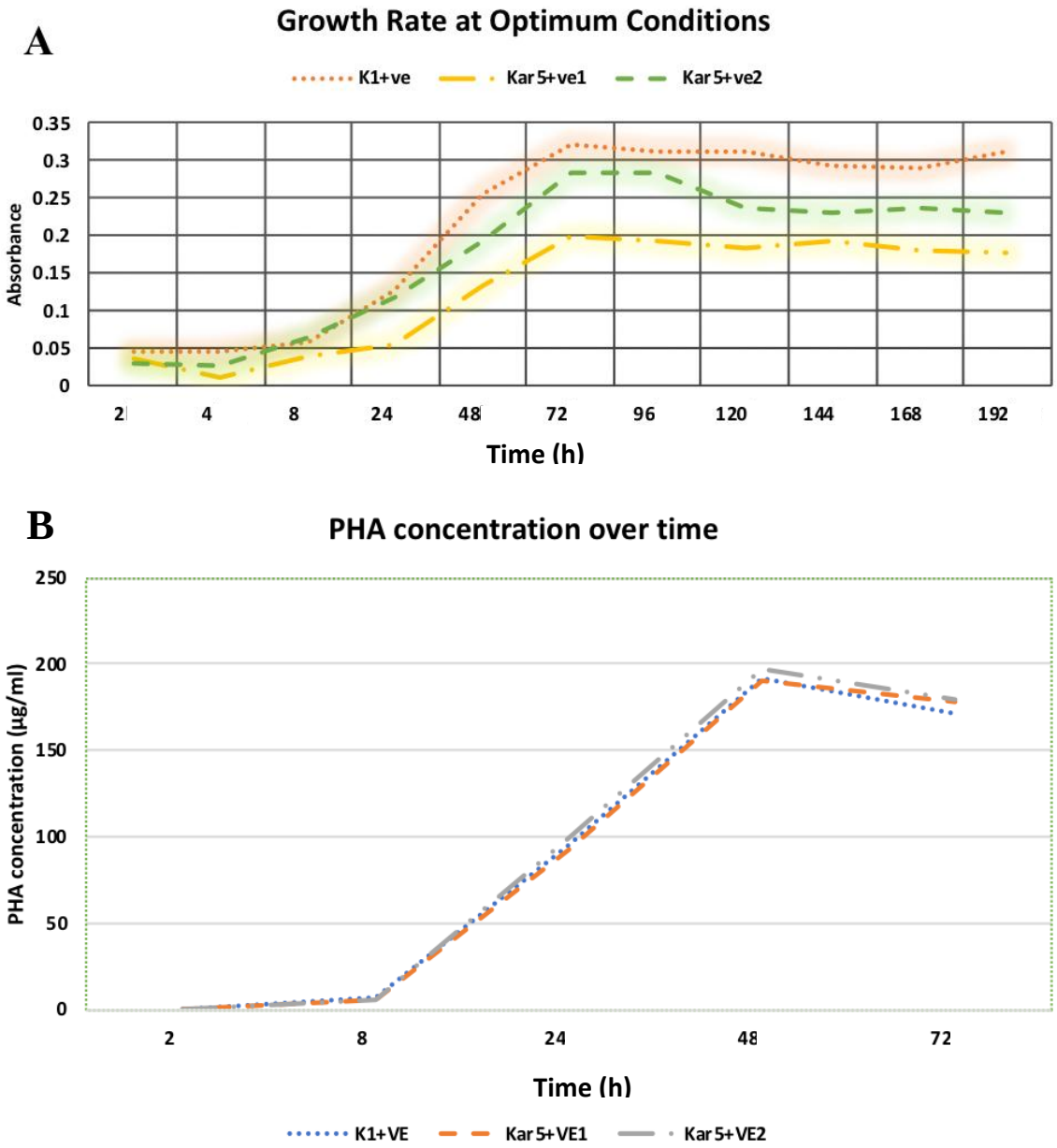

Figure 5. (A). Growth curve for isolates K1+VE and Kar5+VE1 at pH (7), temp (37°C) and SEO concentration (1%SEO) while Kar5+VE2 at pH (7), temp (40°C) and SEO concentration (1% SEO). (B). Time course analysis of PHA concentration in isolates K1+VE, Kar5+VE2 and Kar5+VE1.

Table 3. Amount of PHA produced at 0.2, 0.5 and 1 g/l concentrations of ammonium nitrate and determined through crotonic acid assay.

Sample	Concentration		
	Ammonium nitrate (g/l)		
Nitrogen source	0.2 g/l	0.5 g/l	1 g/l
	PHA (µg/ml)		
K1+VE	191.5	107.7151	69.19053
Kar5+VE1	188.5288	85.49002	42.20479
Kar5+VE2	190.8163	95.0283	56.95958

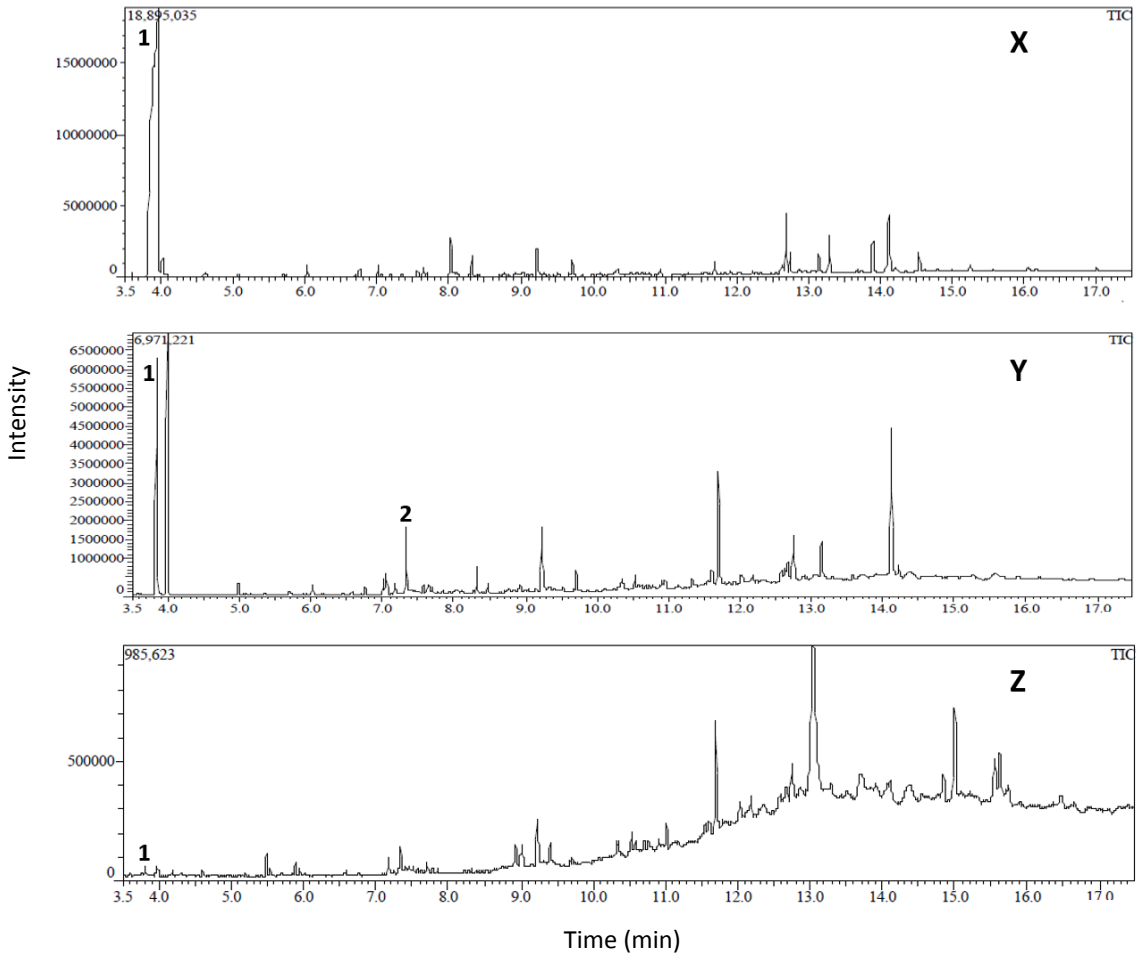

Figure 6. GC-MS chromatogram of extracted PHAs. (X) Isolate K1+VE peak 1 represents 3-hydroxyheptanoate monomer (RT 3.955), (Y) Isolate Kar5+VE2 peak 1 represents 3-hydroxybutyrate monomer (RT 3.838) while peak represents 3-hydroxyoctanoate monomer (RT 7.332), and (Z) Isolate Kar5 + VE1 peak 1 represents 3-hydroxybutyrate monomer (RT 3.821).

for the bioremediation of oil polluted sites.

Identification of PHAs

Characterization of PHAs was done using GC-MS. Esterification of PHA with methanol in the presence of sulphuric acid yielded methyl esters that were analysed by GS-MS. This analysis enabled identification of repeat monomer composition of the polymers produced by selected isolates. From the NIST-05 mass spectra library, isolate K1+VE was shown to produce 3-hydroxyheptanoate monomer. Isolate Kar5+VE1 produced 3-hydroxybutyrate monomers while isolate Kar5+VE2 produced a copolymer 3-hydroxybutyrate-co-3-hydroxyoctanoate (Figure 6). Isolate K1+VE, Kar5+VE1 and Kar5+VE2 belong to *Ochrobactrum*, *Achromobacter* and *Alcaligenes* species, respectively. Interestingly, it has been reported by Byrom (1987) that *Alcaligenes* species produced a copolymer (poly-(3-hydroxybutyrate-co-3-hydroxyoctanoate). Production of copolymer (poly-(3-hydroxybutyrate-co-3-hydroxyoctanoate) by *Sinorhizobium fredii* strain using glucose and sodium dodecanoate substrate has been reported by Liangqi et al., (2006) as well. Three (3)-hydroxybutyrate is a short-chain-length (scl) monomer and is considered to be brittle while 3-hydroxyheptanoate is a medium-chain-length (mcl) monomer which has high flexile elasticity (Sudesh et al., 2000). With 3-hydroxybutyrate-co-3-hydroxyoctanoate therefore is a combination of both scl and mcl monomers. Different combinations of PHAs have been used to form hybrid monomers with suitable quality for extensive use in medicine (Chen, 2010). The PHAs copolymers biocompatible nature, low inflammatory response and biodegradability have attracted application in drug delivery (Shrivastav et al., 2013). Polyhydroxybutyrate and hydroxyapatite based composites have been used and reported to be

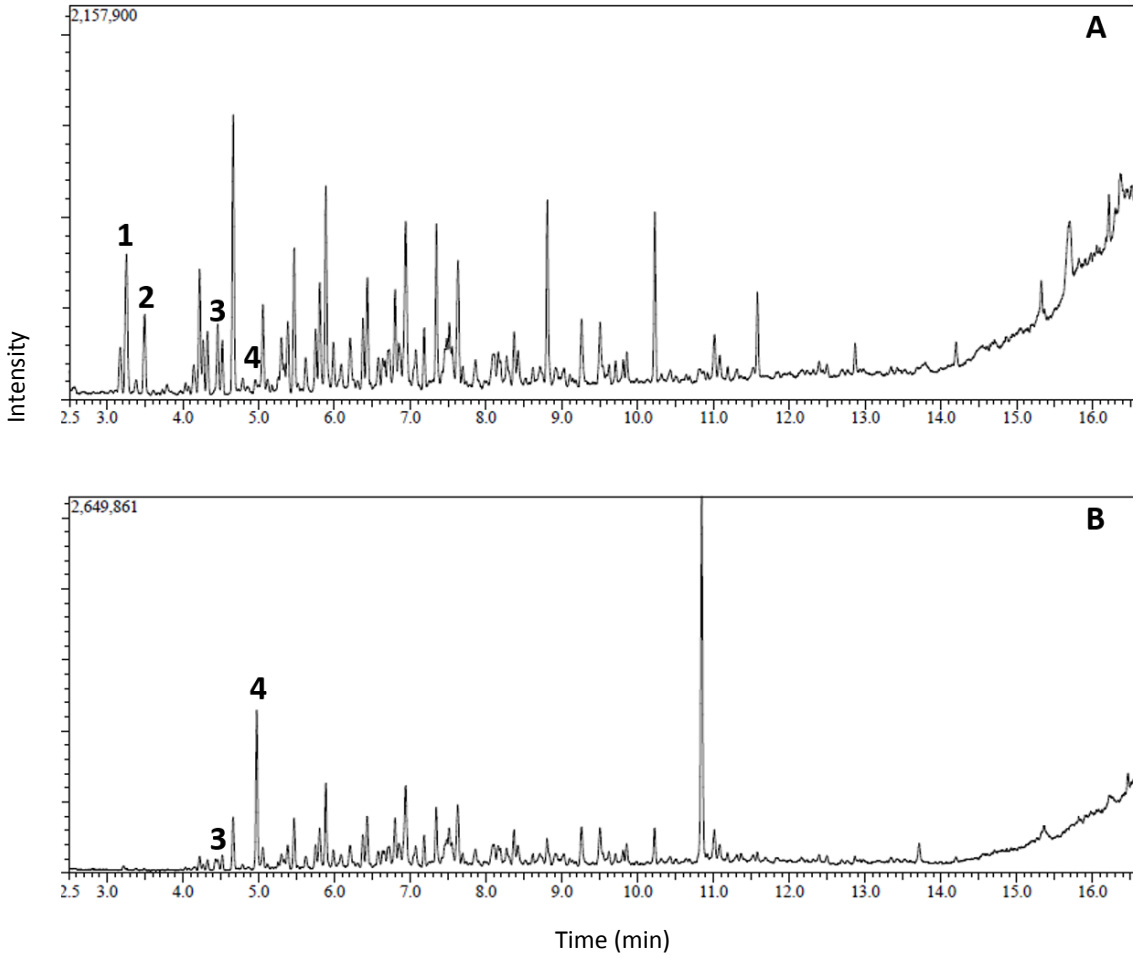

Figure 7. GC-MS chromatogram of spent engine oil extracted from BHM media after 10 days of incubation at pH 7.0 and 37°C with and without inoculation. (A) Control (un-inoculated); (B) Isolate K1+VE inoculum.

compatible for bone replacement in rabbits (Reis et al., 2010). The future of PHAs is promising due to the recent integration of nanotechnology. This has enabled control of matter size at small scale resulting in the formation of nanogels, nanospheres and nanocapsules for controlled drug delivery (Zhang et al., 2010).

Analysis of spent engine oil before and after biodegradation

Biodegradation of SEO was confirmed through GC-MS (Haytham, 2016). The ability of isolate K1+VE to utilize SEO was determined through quantitative comparison of GC-MS chromatogram of SEO extracted from inoculated BHM and un-inoculated BHM (control) as shown in Figure 7. The data obtained shows that the isolate was capable of readily mineralizing most of the hydrocarbons present in SEO. This is indicated by decrease in size of hydrocarbon peaks (low relative abundance) at certain retention times. Comparison of chromatogram profiles of the control and isolate K1+VE (Figure 7) indicate that most of the branched chain alkanes and aromatic hydrocarbons were completely degraded as indicated by disappearance of peaks. Ethylbenzene (RT 3.175) and orthoxylene (RT 3.495) designated as peak 1 and 2 were observed in spectrum A, but are missing in spectrum B as shown in Figure 7. In contrast, peaks for linear alkanes and few branched chain alkanes were still present though in reduced size as in peak 3 [decane (RT 4.520)]. New peaks indicating formation of metabolic intermediates were also observed in peak 4 [ethylhexanol (RT 4.975)] as in spectrum B. Mwaura et al. (2018), reported most branched-chain and cyclic alkanes were totally degraded as compared to straight chain alkanes while Minai-Tehrani et al. (2015) observed a significant reduction of aliphatic fractions in oil sludge after 21 days of incubation.

Hydrocarbon structure is key to their biodegradability (Paria, 2008). Among various classes of hydrocarbons, alkanes and n-alkyl-aromatics with medium length chain (C10-C22) are favourable substrates for microorganisms

hence are rapidly biodegraded. Short-chain alkanes (C5-C9) on the other hand possess high membrane toxicity while long-chain alkanes (>C22) have low water solubility and absorption into surfaces hence reduced bioavailability and consequently reduced biodegradation (Paria, 2008; Tebyanian et al., 2013). Bhattacharya et al., (2015) reported that a newly isolated *Ochrobactrum* sp. C1 could grow in the presence of waste lubricants as the sole carbon source and degrade a wide range of hydrocarbons present in this waste efficiently at pH 7.3 and 36.4°C. These conditions were similar to what was observed in this research.

Conclusion

This study identified bacteria belonging to three genera: *Ochrobactrum, Achromobacter* and *Alcaligenes* as potential candidates for production of PHA which can be used to synthesize bioplastics hence reduce the magnitude of pollution caused by commercial plastics. The isolates were able to withstand harsh SEO contaminated soil environment by readily mineralizing the hydrocarbons and accumulating PHA's as energy reserves. These results show that oil contaminated sites are an important source of PHA accumulating bacteria due to their high stress tolerance capability. The isolates from this study can be used for bioremediation of oil contaminated sites and production of bioplastics which can be used in medical, packaging and pharmaceutical industries. This research has shown that it is possible to use SEO as a single carbon source in production of copolymer, poly (hydroxybutyrate-co-hydroxyoctanoate) by *Alcaligenes* spp.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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ABBREVIATIONS

PHA, Polyhydroxyalkanoate; **PHB,** polyhydroxybutyrate; **SEO,** spent engine oil; **FTIR,** Fourier Transform Infrared; **GC-MS,** Gas Chromatography-Mass Spectrophotometry; **SCL,** short chain length; **MCL,** medium chain length; **LCL,** long chain length; **GPS,** global positioning system.

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