
Biodegradation of Hydrocarbons by *Bacillus cereus* and *Enterococcus casseliflavus* Isolated from Crude oil Contaminated Seafood

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Abstract: This study aims to determine the biodegradability capacity of *Enterococcus casseliflavus* and *Bacillus* isolated from crude oil contaminated seafood. Fresh seafood samples such as crude oil contaminated periwinkle and prawn were obtained. BOD, COD, pH, nitrate, phosphate, heavy metals (Cr, Cd, Pb, Zn), polyaromatic hydrocarbons (PAHs), total petroleum hydrocarbon (TPH) were determined. Standard microbiological methods for total heterotrophic bacterial and *Lactobacillus* count were determined. The isolates were molecularly identified and were subjected for biodegradability test at day 0, 30 and 60 respectively. Total bacterial count ranged 7.13 to 8.05 log₁₀cfu/g; *Lactobacillus* count ranged 5.04 to 6.26 log₁₀cfu/g in the crude oil contaminated seafood. The pH level at day zero ranges from 7.1 (neutral) to 5.8 (acidic), Phosphate ranges from 0.032 to 0.133 (mg/l), Nitrogen level ranged from 0.94 to 0.20%, Cadmium (Cd) ranged from 1.8233 to 1.3322, Zinc (Zn) ranged from 2.5334 to 1.8732, Chromium (Cr) ranged from 7.5133 to 4.7221, Lead (Pb) ranged from 12.3124 to 8.3421 (ppm). PAHs level ranged from 2231.1 to 1211.0 and TPH level ranged from 3342.3 to 2184.2 (ppm) at day zero. The pH level at day thirty ranges from 5.8 to 6.4 (acidic), Phosphate level ranged from 0.383 to 0.110 (mg/l), Nitrogen level ranged from 2.11 to 0.50%, Cadmium (Cd) ranged from 0.6332 to 0.2104, Zinc (Zn) ranged from 1.7322 to 0.1043, Chromium (Cr) ranged from 5.2262 to 2.0122, Lead (Pb) ranged from 7.4102 to 4.611 (ppm), PAHs ranged from 2238.2 to 1312.8, TPH ranged from 2238.2 to 1312.8 (ppm). Whereas at day sixty, the PAHs level ranged from 72.01 to 311.2 and TPH level ranged from 221.6 to 551.2 (ppm). Biodegradation of petroleum hydrocarbons is energetically favorable, therefore indigenous hydrocarbon degrading microorganisms play a significant role in biodegradation process.

Key word: Biodegradation, Crude oil, Heavy Metals, *Lactobacillus*, PAHs, Seafood, TPH

INTRODUCTION

Crude oil is a complicated mixture of solid or gaseous hydrocarbons with molecular weights ranging from 16 to 1800. Polyaromatic hydrocarbons (PAHs), saturated aromatic-ringed hydrocarbons (MAHs), polar oil surface structures, resins dissipated in aromatics, and saturates are the four basic groups of petroleum hydrocarbons (Feng *et al.*, 2021). More than 30 percent of crude oil is formed of poly-aromatic hydrocarbons (PAHs), with the remaining 70 percent made up of a wide variety of hydrocarbons including aromatic compounds, naphthenes, paraffins, organic nitrogen compounds, sulphur compounds, and phenols (Cui *et al.*, 2020). Hydrocarbons originating from crude oil constitute the main category of environmental contaminants on a global basis (Chrzanowski, *et al.*, 2020). The inadvertent discharge of oil has led to an increased concern for the environment worldwide regarding crude oil contamination Singh *et al.* (2020).

The most prevalent sources of oil spills and discharges are explosions during oilfield drilling; leaks from oil and gas pipelines and reservoirs; fuel tankers; well waxing; and refinery and petrochemical manufacturing equipment overhauls Brkić and Praks (2021). Crude oil basic hydrocarbons are the greatest category of environmental pollutants produced globally (Chrzanowski, *et al.*, 2020). Hazardous aromatic organic compounds, such as polycyclic aromatic hydrocarbons (PAHs), chlorophenols, and cresols, which are poisons present in crude oil, are released into the environment during industrial processing activities associated to the crude oil sector (Varjani, 2017).

Petroleum hydrocarbon contamination is caused by human activities such as runoff from factories and cities, effluent leakage, and accidents involving the onshore and offshore petroleum industries. According to Sajna *et al.* (2015), this pollution contaminates the ecosystem, harms all life forms on Earth directly or indirectly, and endangers their health. Since the marine

environment is thought to be the ultimate and greatest sink for pollutants derived from petroleum hydrocarbons, contamination must be prevented (Varjani and Srivastava, 2015). Oleophilic bacteria are employed in biodegradation, one of the main processes for bioremediation, which removes hydrocarbon contaminants from the environment (Varjani and Upasani, 2016c; Varjani, 2017). One of the main methods of bioremediation is biodegradation, which uses oleophilic bacteria to remove hydrocarbon contaminants from the environment (Varjani and Upasani, 2016c). The petroleum sector is principally concerned with two issues namely: the biodegradation of hydrocarbon pollutants and increased oil recovery (Sajna *et al.*, 2015; Varjani *et al.*, 2015; Varjani and Upasani, 2016ac). Different naturally occurring bacteria that are capable of dissolving specific hydrocarbon compounds can degrade hydrocarbons. *Acinetobacter*, *Bacillus*, *Pseudomonas*, *Rhodococcus*, and other microbes are among those linked to the degradation of petroleum (Varjani, 2017). Petroleum hydrocarbon pollution in both terrestrial and aquatic ecosystems can be treated using the commonly utilized method of microbial biodegradation (Abbasian *et al.*, 2015; Varjani and Srivastava, 2015). Several research papers on the biodegradation of hydrocarbon pollutants have been published in the last decades (Sajna *et al.*, 2015; Varjani *et al.*, 2015; Varjani and Upasani, 2016c). Outlining the general mechanisms and pathways for hydrocarbon degradation, several reviews have been published (Abbasian *et al.*, 2015; Meckenstock *et al.*, 2016; Wilkes *et al.*, 2016). It has been documented that the biodegradation of petroleum hydrocarbons is energetically advantageous because microorganisms obtain energy during the process of pollutant degradation that they can use for metabolism and because they also obtain "Carbon," which is a necessary component of all cellular constituents (Abbasian *et al.*, 2015). Native bacteria that break down hydrocarbons are vital to the biodegradation

process (Varjani *et al.*, 2015; Varjani and Upasani, 2016c).

It has been documented that microorganism such fungi, bacteria, and algae can break down hydrocarbon pollutants (Wilkes *et al.*, 2016). According to Varjani and Srivastava (2015), microbes are vital for protecting the biosphere and ecosystem and establishing a sustainable habitat. They are useful for biomonitoring ecosystems, bioremediation, or cleaning up pollutants from the environment, and decreasing the harmful impacts of pollutants (Varjani and Upasani, 2016c). Given that hydrocarbons are naturally occurring compounds, it is not surprising that microbes have the potential to consume and breakdown hydrocarbons when isolated from hydrocarbon-polluted soil, sediments, or water (Varjani and Upasani, 2016b).

Using a single native microorganism or a group of organisms is a mitigation strategy that accelerates the pace of pollution breakdown by utilizing the catalytic characteristics of living things. The study aimed at assessing the biodegradability ability of *Enterococcus casseliflavus* and *Bacillus cereus* on crude oil. Its objectives are the isolation of total heterotrophic bacteria and *Lactobacillus* species from seafood samples and determination of the biodegradation rate of crude oil by bacteria.

MATERIALS AND METHODS

Sample Collection: Fresh seafood from the crude oil-contaminated area of Igbeta-Ewoama Nembe, Bayelsa State, was collected in significant quantities, including periwinkle (*Tympanotonus fuscatus*) and prawn (*Penaeus monodon*). The seafood was sent to the lab for additional examination after being preserved in an ice-cooled container.

Enumeration and Characterization of Bacteria from Seafood: The method of Goveas LC, (2020) was adopted with slight modification. Nutrient Agar (NA) was used to determine the total heterotrophic bacteria present in the samples. De Man Rogosa Sharpe Agar (MRSA) was used to identify

Lactobacillus species. It was prepared in accordance with the manufacturer's instructions, autoclaved for 15 minutes at 120°C, cooled to roughly 45°C, then poured into sterile petri dishes and allowed to solidify. Using the spread plate approach, an exact 0.1 ml aliquot of inoculum was aseptically inoculated in triplicate on an agar plate that had been pre-dried. For 24 hours, inoculated plates were incubated at 37°C. Purification of isolates was done based on their cultural and morphological characteristics and colony appearance, *Gram* Staining and Biochemical Test were also carried out.

Molecular Identification and Characterization of Bacterial Isolates:

The following techniques were utilized for molecular identification, and the approach taken by Reyam-Abdul *et al.* (2022) was modified for the investigation. Zymo Research fungal/bacterial DNA micro prep extraction kit was used for the extraction process. The pure culture that had been separated was suspended in 200 microliters of isotonic buffer and placed into ZR Bashing Bead Lysis tubes. After adding 750 microliters of lysis solution, the tubes were centrifuged at 10,000xg for a duration of one minute. 400 microliters of the supernatant were placed in a collecting tube with an orange-topped Zymo-Spin IV spin filter, and the tube was centrifuged at 8000xg for one minute. The filtrate was combined with 1200 microliters of fungal/bacterial DNA binding buffer to make a final volume of 1600 microliters. Eight hundred microliters of this were then put to a Zymo-Spin.

Using the primers 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3', the isolates' 16S rRNA region was amplified using an ABI 9700 Applied Biosystems thermal cycler for 35 cycles at a final volume of 40 microliters. Primers were added at a concentration of 0.5 µM to an X2 Dream taq Master mix (taq polymerase, DNTPs, and MgCl), and the extracted DNA was also utilized as a template in the PCR mix. The following were the PCR

conditions: Five minutes of initial denaturation at 95 °C, forty seconds of denaturation at 95 °C, forty seconds of annealing at 52 °C, thirty-five cycles of extension at 72 °C, and five minutes of final extension at 72 °C. The 1500 bp product was seen on a blue light UV transilluminator after being resolved on a 1% agarose gel at 200V for 15 minutes. On a 3510 ABI sequencer manufactured by Inqaba Biotechnological in Pretoria, South Africa, sequencing was carried out utilizing the BigDye Terminator kit. The components of the sequencing, which had a final volume of 10µl, were as follows: 0.25µl of BigDye® terminator v1.1/v3.1, 2.25µl of 5 x BigDye sequencing buffer, 10µM primer PCR primer, and 2–10ng PCR template per 100bp. The following sequencing conditions were used: 32 cycles of 96°C for 10 seconds, 55°C for 5 seconds, and 60°C for 4 minutes. Sequences that were obtained were altered using the bioinformatics method Trace edit, and BLASTN was used to download similar sequences from the National Center for Biotechnology Information (NCBI) database. With ClustalX, these sequences were aligned. Richard and Frances (2017) employed MEGA 6.0's Neighbor-Joining approach to infer the evolutionary history. The evolutionary history of the taxa under study is assumed to be represented by the bootstrap consensus tree that was generated from 500 replicates. Xian Ming *et al.* (2023) using the Jukes-Cantor technique to calculate the evolutionary distances.

Proximate Analysis of Crude Oil Contaminated and Non-contaminated Seafood: The following proximate analysis was carried out in this study and the method by AOAC (2016), was adapted to determine the moisture content, crude fat, ash content, crude fiber, crude protein and carbohydrate present in all the crude oil contaminated and non-contaminated seafood.

Determination of Moisture Content: After recording the weight of the pre-dried coded crucible, about 5 grams of the seafood sample was dispersed across the base of the coded crucible. following that, the crucible

was heated to 105 degrees Celsius in a forced draft oven for four hours, following which it was cooled in a desiccator. Once the sample had cooled, its weight was recorded and the percentage of moisture loss used to determine its moisture content.

% Of Moisture Content = $\frac{[\text{Weight of crucible} + \text{Sample (before)}] - \text{Weight of crucible} + \text{Sample (after)}}{\text{Weight of Sample}} \times 100$

Determination of Crude Fat: A thimble was filled with non-absorbent cotton after 5g of the sample was weighed into it. The Soxhlet apparatus's extractor was then used to hold the sample-containing thimble. The next step was to gather a dry Soxhlet equipment and measure out 200 ml of petroleum ether, the solvent for extraction. To aid in the condensation of the organic solvent, the Soxhlet apparatus was prepared with cold water flowing through the condenser. About six hours were given to this procedure to run. Next, put the Soxhlet flask inside an air oven set at 105°C for one hour to dry the fat that has been extracted. After putting the flask in a desiccator and measuring its weight, the percentage of crude fat was the end result of the fat content calculation.

% Of Crude Fat = $\frac{(\text{Weight of flask} + \text{Oil}) - \text{Weight of empty flask}}{\text{Weight of Sample}} \times 100$

Determination of Total Ash Content: The weight of ignited and cooled porcelain crucible was recorded. The base of the crucible was labelled with a pencil for easy identification. With the use of tongs, 2 g of the sample was transferred to the crucible, and then it was allowed to sit in a muffle furnace set at 550⁰ degrees Celsius. The ash was weighed after it had dried in a desiccator and cooled to room temperature. The proportion of total ash was used to determine the ash content.

% Of Ash Content = $\frac{[\text{Weight of crucible} + \text{Sample (before)} - \text{Weight of crucible} + \text{sample (after)}]}{\text{Weight of Sample}} \times 100$

Determination of Crude Fibre: In a round-bottom flask, about 2 grams of defatted sample was combined with 100 ml of 1.25% H₂SO₄ and then heated over a hot plate to

boil for half an hour. Following that, the sample underwent filtration and the flask was rinsed with warm water. Proceeding to pour 100 ml of 1.25% NaOH over the residue, it was thereafter moved to. We cooked the combination for 30 minutes, strained it, and then rinsed it with warm distilled water and 80% alcohol to remove any leftover residue. Following that, the leftover material was heated to 130 degrees Celsius in a hot air oven for two hours before being transferred to a desiccator to cool for around half an hour. A pre-dried crucible (W₁) was used to measure the weight of the dried fiber. Following a 2-hour incineration period at 550⁰C in a furnace, the sample was allowed to cool.

Afterwards, the weight of the crucible and ash (W₂) was measured and the percentage crude fiber was calculated.

% of Crude Fiber = $\frac{[\text{Weight of crucible} + \text{Sample (before)}] - \text{Weight of crucible} + \text{Sample (after)}}{\text{Weight of the sample}} \times 100$

%CF = $\frac{W_2 - W_1}{W_s} \times 100$

Determination of Crude Protein: There was a 1g sample weighted into the digesting tube. To the digesting tube, we put two tablets of catalyst and fifteen ml of concentrated H₂SO₄. Using a Kjeldahl digester, the sample was heated to 420 degrees Celsius for two hours. The digestion process was followed by diluting 10 ml of the digest into a 100 ml volumetric flask. Next, the diluted digest was mixed to 50 ml of 40% NaOH and 200 ml of distilled water, with 20 ml being taken. The distillation unit was set up shortly at a temperature of 150-200°C with the water circulation pump of the condenser turned on. 2-3 drops of a combination of indicators were introduced into a conical flask containing 30ml of a solution containing 4% boric acid. The conical flask was connected to a distillation apparatus and 100-150ml of the distilled liquid was collected back into the conical flask. Following distillation, the resulting solution was subjected to titration using a standardized solution of 0.1 N HCl. A blank test was conducted to ensure that the reported values were free from any

contamination that may have occurred in the laboratory. The percentage protein content was calculated as percentage crude protein.

Kjeldahl Nitrogen % as; $\%N = [(Titre \text{ of sample} - Titre \text{ of blank}) \times N(HCl) \times 14.01] \times 100 \text{ Weight} \times 10$

Crude Protein % as; $\% \text{ Crude Protein} = \% \text{ Kjeldahl N} \times F$, where N = Normality of standardized HCl 14.01 = Molecular weight of Nitrogen 10 = Factor to convert mg/g to % Titre = Volume of standardized acid used to titrate a test F = Conversion factor to protein, F factors are 5.70 wheats, 6.38 dairy products and 6.25 for other feeds.

Determination of Carbohydrate: The method used by Panel Lukas *et al.*, (2023) was adapted to determine the carbohydrate content of the seafood. A solution containing 200mg of anthrone reagent was prepared by dissolving it in 100ml of ice-cold mixture of 95% ethanol and sulfuric acid (H₂SO₄). 100 milligrams (mg) of the prepared seafood samples were measured and placed into a boiling tube. The samples were hydrolyzed in a boiling water bath for 3 hours with 5 ml of 2.5 normal (N) hydrochloric acid (HCl) at room temperature. Solution was neutralized with solid sodium carbonate until bubbling stopped. Adjusting the sample volume to 100ml and centrifuging. The liquid after centrifugation was collected. The material was split into 0.5 and 1ml parts for examination. The standard preparation used 0, 0.2, 0.4, 0.6, 0.8, and 1ml working standard. Blank sample '0' was used. Distilled water is added to all tubes, including the sample tubes, to make 1ml. Then 4ml of anthrone reagent is added. Subject the sample to a boiling water bath for a duration of 8 minutes. Afterward, immediately cool the sample and measure the intensity of the dark green colour at a wavelength of 630nm. The amount of carbohydrate in the sample was calculated thus.

Amount of carbohydrate present in 100mg of the sample = (mg of glucose + volume of test sample) $\times$ 100.

Biodegradation Setup: This procedure was used to find out whether lactobacillus

species that were isolated from seafood that had been polluted with crude oil (periwinkle and prawn) could biodegrade crude oil. Through molecular identification, the isolates were determined to be *Enterococcus casseliflavus* (MF979558.1) and *Bacillus cereus* (OR921907.1). To establish a pure culture, the detected isolates were subcultured into freshly prepared De Man Rogosa broth. The pure culture was then incubated at 37°C for 24 hours, and the crude oil's biodegradation was ascertained. To conduct the biodegradability test, the methodology of Inuwa-Ja'afaru *et al.* (2017) was chosen and used. Different concentrations of crude oil were represented by ratios of 1:1 and 1:5, or 1% and 5%, respectively, into 99 and 95 milliliters of sterile water. The volume of water used was measured using a measuring cylinder and a 500 ml conical flask. Subsequently, a 24-hour bacterial solution containing 1 milliliter was added to the crude oil-contaminated environment (biodegradability setup). This allowed the organism to adjust to its new surroundings. Samples were then collected for physiochemical analysis on days 0, 30, and 60 to detect the presence of heavy metals: zinc (Zn), chromium (Cr), cadmium (Cd), lead (Pb), and polycyclic aromatic hydrocarbons, total petroleum hydrocarbons, H level, phosphate, and nitrate levels.

pH Determination: The biodegradability test sample's pH level was ascertained by adapting the technique utilized by Akomah and Abu (2015). The sample was measured to a volume of 100 milliliters in a clean beaker. The pH meter was then turned on, and the beaker containing the sample was set on top of it. The electrode was then taken out of the buffer 7 solution and placed inside the beaker containing the sample, where it was read to determine the pH level

Nitrate Determination: To ascertain the nitrate content of the biodegradability test sample, the technique employed by Akomah and Abu (2015) was modified. All reagents and standards were prepared at 10-15°C using distilled water devoid of nitrite and nitrate in order to measure the nitrate level.

After dissolving 300g of NaCl in distilled water and diluting it to 1 liter, 125ml of distilled water was carefully mixed with 500ml of H₂SO₄ concentration. To stop moisture from the atmosphere from absorbing, maintain cool and properly sealed. In 70ml of hot distilled water, dissolve 1g of brucine sulfate [(C₂₃H₂₆N₂O₄)₂ H₂SO₄ 7H₂O] and 0.1g of sulfanilic acid (NH₂C₆H₄SO₃H H₂O). Add 3 ml of the HCl concentration, mix well, and then dilute with 100 ml of purified water. Keep at 5°C in a dark bottle.

Phosphate Determination: The phosphate content of the sample was ascertained by adapting the procedure utilized by Beniah and Christian (2020). The water sample was evaluated by pipetting duplicate 25.0 cm³ portions of the diluted sample to two separate 100 cm³ volumetric flasks, followed by the color development. A standard stock solution of phosphorus and calibration curve were also prepared. The reading was taking at a wavelength of 880nm.

Heavy Metal Analysis: Heavy metal such as Chromium (Cr), cadmium (Cd), lead (Pb), and zinc (Zn) analyses were performed using a modified version of the method developed by Balali-Mood *et al.* (2021) using nitric acid and hydrochloric acid digestion (wet digestion). To create an aqua regia solution, a total volume of hydrochloric acid (HNO₃) was added in a 3:1 ratio and mixed together. Subsequently, a conical flask was filled with 1 milliliter of each sample solution, and 20 milliliters of the combined acid were added to each sample. Using a fume cupboard and hotplate, digestion was carried out until white fume formed.

Total Petroleum Hydrocarbon and Polycyclic Aromatic Hydrocarbon Analysis: Gas chromatography was employed for this procedure, and the sample analysis technique employed by Geni *et al.* (2021) was modified. 10 milliliters of well-shake water were weighed into a beaker that had been rinsed with acetone and acid washed. 25 milliliters of 1:1 dichloromethane was then added to the mixture and the mixture was placed in a sonicator for 20 minutes.

The mixture was then centrifuged for 10 minutes, and the solvent phase was extracted using filter paper that had been preconditioned with acetone and 1:1 dichloromethane and contained 5 grams of anhydrous sodium sulfate. The conical flask holding the solution received an additional 50ml of a 1:1 dichloromethane (DCM) and acetone addition. The extraction process was repeated, mixing the extracts in an Erlenmeyer flask and using a rotary evaporator to condense the mixture into 5 ml.

RESULTS

Statistical analysis revealed a statistically significant difference in the mean *Lactobacillus* count for the periwinkle that had been polluted with crude oil (5.28 ± 1.15) and non-contaminated periwinkle (0.133 ± 0.15) at $P < 0.05$ with crude oil contaminated prawn (6.193 ± 0.90) crude oil contaminated periwinkle (5.28 ± 1.15) having the highest mean and crude oil contaminated clams (4.94 ± 0.85) having the lowest mean. There is significant difference between crude oil contaminated prawn (CPN) and crude oil contaminated clams (CC) at $P < 0.05$ as presented in table 4.9 and 4.10 above.

In a megablast search for very similar sequences in the NCBI non-redundant nucleotide (nr/nt) database, the extracted 16s rRNA sequence from the isolates yielded a perfect match. There was a perfect match between the isolates' 16S rRNA and those of other species. The evolutionary distances calculated using the Jukes-Cantor method showed a close relationship to *Enterococcus casseliflavus*, *Bacillus cereus*, and *Kurthia gibsonii*, which was in agreement with the phylogenetic placement of the 16S rRNA of the isolates within the *Enterococcus*, *Bacillus*, and *Kurthia* sp.

The analysis of mean deviation, degrees of freedom (df), and p-values for Polycyclic Aromatic Hydrocarbons (PAHs) in the crude oil contaminated and non-contaminated seafood reveals substantial variations for periwinkle, prawn and indicating a

significant difference at $P < 0.05$. There was significant difference in TPH levels in periwinkle, prawn and clams at $P < 0.05$ and in lead (Pb) levels of periwinkle, prawn and clams at $P < 0.05$.

Also, the analysis of mean deviation, degrees of freedom (df) indicate a significant difference in cadmium (Cd) levels in both periwinkle and clams at $P < 0.05$. Prawn has a p-value above the conventional significance level of 0.05 with 3 degrees of freedom indicating no significant difference at $P < 0.05$. There was significance difference in chromium (Cr) levels in periwinkle, prawn and clams at $P < 0.05$. The analysis of mean deviation, degrees of freedom (df) suggest a significant difference in zinc (Zn) levels of periwinkle and clams at $P < 0.05$ but indicates no significant difference in Zn levels at $P < 0.05$ for prawn. Mean ash content reveals no significant difference in crude oil contaminated and non-contaminated

periwinkle, prawn and clams at $P > 0.05$. Mean moisture reveals an exceptionally low p-value indicating a significant difference in periwinkle, clams and prawn at $P < 0.05$. Mean protein content reveals a remarkably low p-value indicating a significant difference in periwinkle, clams and prawn at $P < 0.05$. Mean lipid content reveals a low p-value indicating a significant difference in periwinkle, clams and prawn at $P < 0.05$. Mean crude fiber content reveals a low p-value indicating a significant difference in periwinkle, clams and prawn at $P < 0.05$. Mean carbohydrate content reveals a low p-value suggesting a significant difference in periwinkle, clams and prawn at $P < 0.05$. The percentage degradation of polyaromatic hydrocarbons: A, 82.8%; B, 94 %; C, 94.01 %; D, 94.3 %; E, 94.7 % while the total petroleum hydrocarbon percentage degradation was A, 80.5 %; B, 88.3 %, C, 90.3 %; D, 90.2 %; E, 88.2 %.

Table 1: Total Heterotrophic Bacterial and *Lactobacillus* Count and Log₁₀CFU/g

S/N	Seafood Code	THBC	Log ₁₀ CFU/g	<i>Lactobacillus</i> (cfu/g ¹)	Log ₁₀ CFU/g	% LB/THBC
1.	CPK	1.34×10^7	7.13	1.5×10^5	5.18	27.3
2.	CPN	1.11×10^8	8.05	1.8×10^6	6.26	22.2

Keys: CPK = Crude oil Contaminated periwinkle; CPN = Crude oil Contaminated Prawn, LB = *Lactobacillus*

Table 2: Biochemical Characteristics of the Bacteria Isolates

S/N	SC	GR	CM	C a t	O x i d	I n d	C i t	M e t	V o l	G a s	H ₂ S	S l a n t	B u t t	G l u	L a c	M a l	X y l	Tentative Organism
1.	CPK	+	R	+	-	-	-	+	-	-	-	A	A	A	-	-	-	<i>Enterococcus casseliflavus</i>
2.	CPN	+	R	+	-	-	-	-	-	-	-	-	-	-	-	-	-	<i>Bacillus cereus</i>

Keys: CPK-Crude oil Contaminated periwinkle; CPN- Crude oil Contaminated Prawn; GR = Gram's reaction, CM = Citrate: Cat = catalase; OX = Oxidase; Ind = Indole; MR = Methyl red; VP = Voges Proskauer; Glu = glucose; Lac = Lactose; Mal = Maltose; Xylose

Table 3: PAHs, TPH and Heavy Metals on the Seafood Samples

S/N	PARAMETERS (ppm)	SAMPLE A ₁	SAMPLE A ₂	SAMPLE B ₁	SAMPLE B ₂	SAMPLE C ₁	SAMPLE C ₂
1.	PAHs	43.96	2.24	56.31	1.26	111.58	7.80×10^{-1}
2.	TPH	102.74	7.01	102.86	38.65	93.91	22.40
3.	Pb	4.232	0.032	2.657	1.102	2.113	0.432
4.	Cd	2.001	0.112	3.321	1.421	1.873	1.435
5.	Cr	1.361	0.135	2.455	0.432	2.745	0.122
6.	Zn	3.251	1.334	3.751	1.112	1.352	0.743

Keys: Sample A₁ = Crude oil contaminated Periwinkle; sample A₂ = Non-contaminated Periwinkle, sample B₁ = Crude oil contaminated Clams; sample B₂ = Non-contaminated Clams; sample C₁ = Crude oil contaminated Prawn; sample C₂ = Non-contaminated Prawn

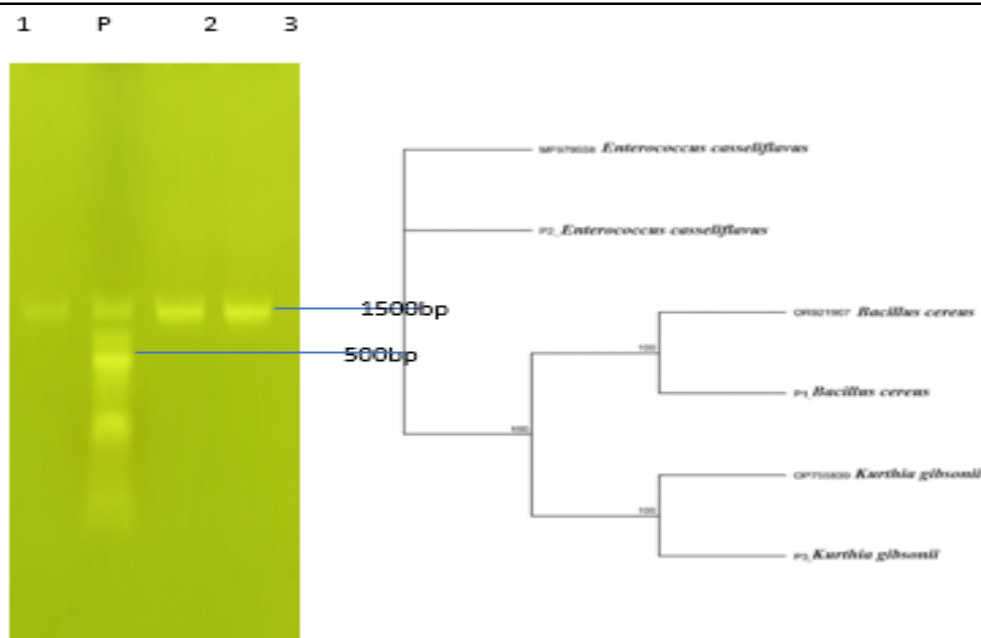


Figure 1: Agarose Gel Electrophoresis of *Lactobacillus* spp and Phylogenetic Tree Showing the Evolutionary Relationship between the Bacterial Isolates

Table 4: Proximate Values of Crude Oil Contaminated and Non-Crude Oil Contaminated Seafoods

S/N	PARAMETERS (%)	SAMPLE A ₁	SAMPLE A ₂	SAMPLE B ₁	SAMPLE B ₂	SAMPLE C ₁	SAMPLE C ₂
1.	Ash	3.69	3.34	3.89	3.01	0.78	0.88
2.	Moisture	23.38	33.15	70.01	75.21	65.51	61.49
3.	Protein	20.21	54.54	10.11	12.51	20.78	24.26
4.	Lipid	15.25	1.45	7.18	1.66	5.50	2.28
5.	Crude Fibre	14.33	0.89	5.10	2.10	5.01	2.91
6.	Carbohydrate	3.14	6.63	3.71	5.51	2.42	8.18

Keys: Sample A₁ =Crude oil contaminated Periwinkle; sample A₂ =Non-contaminated Periwinkle
sample B₁ =Crude oil contaminated Clams; sample B₂ =Non-contaminated Clams; sample C₁ = Crude oil contaminated Prawn; sample C₂ =Non-contaminated Prawn

Table 5: pH, Salts and Heavy metals Concentration of Crude Oil Biodegradation Set-ups

S/N	PARAMETERS	SAMPLE A	SAMPLE B	SAMPLE C	SAMPLE D	SAMPLE E
1.	pH day 0	7.1	5.7	5.3	5.5	5.2
	Day 30	5.8	6.4	6.0	6.1	6.3
2.	Phosphate (mg/l) day 0	0.032	0.128	0.133	0.112	0.125
	Day 30	0.110	0.311	0.383	0.221	0.278
3.	Nitrogen (%) day 0	0.20	0.94	1.02	0.81	0.88
	Day 30	0.50	2.11	2.26	1.22	1.67
4.	Cd (ppm) day 0	1.6412	1.5232	1.3322	1.8233	1.5411
	Day 30	0.4421	0.5663	0.2104	0.6332	0.3324
5.	Zn (ppm) day 0	2.2032	2.1312	1.8732	2.8231	2.5334
	Day 30	1.7322	1.4502	0.9432	0.9332	0.1043
6.	Cr (ppm) day 0	5.8311	5.1323	4.7221	7.5133	5.8912
	Day 30	3.2921	2.8323	2.0122	5.2262	4.8342
7.	Pb (ppm) day 0	10.3212	9.7214	8.3421	12.3124	11.7512
	Day 30	6.7512	5.3521	4.6311	7.1102	6.9113

Keys: Sample A = Control, Sample B = 1% Crude + *Bacillus cereus*, Sample C = 1% Crude + *Enterococcus casseliflavus*, Sample D = 5% Crude + *Bacillus cereus*, Sample E = 5% Crude + *Enterococcus casseliflavus*

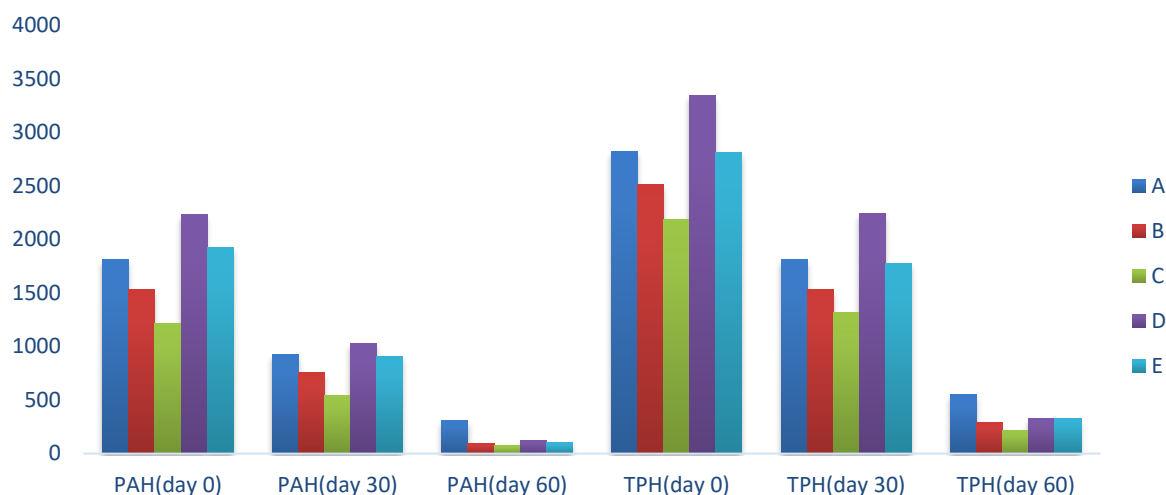


Figure 2: Biodegradation of Polyaromatic Hydrocarbons and Total Petroleum Hydrocarbons by Various Microbial Set-ups

Keys: Sample A = Control, sample B = 1% Crude + *Bacillus cereus*, sample C = 1% Crude + *Enterococcus casseliflavus*, sample D = 5% Crude + *Bacillus cereus*, sample E = 5% Crude + *Enterococcus casseliflavus*

DISCUSSION

The utilization a variety of techniques, the microbiological, physiochemical, and biodegradability capacities of the bacteria isolated from the seafood on crude oil were evaluated in relation to seafood obtained from contaminated water, such as periwinkle (*Tympanotonus fuscatus*) and prawn (*Penaeus monodon*). The prawn polluted with crude oil had a higher total heterotrophic bacterial and Lactobacillus count than the periwinkle contaminated with crude oil.

Two strains of Lactobacillus species were recovered from prawn and periwinkle seafood that had been polluted with crude oil. Following a 24-hour incubation period at 37°C, growth was seen on the plates. Table 1 and 2 displays the results of the count and biochemical test. Figure 1 shows the results of the Lactobacillus spp. The results of the agarose gel electrophoresis of Lactobacillus species are shown in Figure 1, which shows that p1, p2, and p3 which represent the isolates were examined using 16S rRNA in order to determine the dominant gene. The amplified 16srRNA at 1500 bp is shown in lanes 1-3, and the 100 bp DNA ladder is shown in lane P. The 500 bp molecular

ladder is shown. In order to verify the identification of two bacterial isolates from seafood culture samples, DNA was isolated, measured, and the 16S rRNA region of the isolates' rRNA gene was amplified. During the megablast search for extremely similar sequences from the NCBI non-redundant nucleotide (nr/nt) database, the retrieved 16s rRNA sequence from the isolates yielded a perfect match. The isolates' 16S rRNA displayed a 100% similarity rate to other species. The Jukes-Cantor method was utilized to compute evolutionary distances, which were found to be in agreement with the isolates' phylogenetic placement within the *Enterococcus*, *Bacillus*, and *Kurthia* sp. The results indicated a close relatedness to *Enterococcus casseliflavus*, *Bacillus cereus*, and *Kurthia gibsonii* (Figure 1).

The findings of this study are consistent with those of Mohammed *et al.* (2023), who investigated the degradation of crude oil and the pure hydrocarbon fractions by naturally occurring soil microorganisms. They also isolated 17 bacterial isolates from soil and reported that, in order to determine the taxonomic origin of the 17 16S rDNA sequences, independent computerized alignments were carried out against readily

accessible prokaryotic sequences of 16S rDNA using NCBI BLAST. The biodegradability test was applied to the bacterial isolates that were identified. The biodegradability test's result reveals the various pH values from day 0 to day 30. At day zero, sample A's pH level in the biodegradability test samples is neutral, whereas samples B, C, D, and E continue to be acidic. Samples A, B, C, D, and E's pH levels are still at an acidic level at day thirty. According to Husnain (2021), the pH of a microbiological environment is less suited when an element is drained, raising the pH and creating ground. When compared to sample A, the control, the phosphate and nitrogen levels in samples B, C, D, and E, at days 0 and 30 demonstrate an increased rate of biodegradation; in contrast, the concentration of PAHs and TPH declines as the number of days increases in the biodegradation process. Day thirty (30) has a greater increase in phosphate and nitrogen levels than Day Zero (0) due to the microorganisms been able to produce these growth limiting nutrients. Additionally, compared to day zero (o), there is an increase in the breakdown of heavy metals at day thirty (30). The following elements are affected been metabolized and utilized by microorganisms either through adsorption or absorption. The heavy metals include: lead (Pb), zinc (Zn), chromium (Cr), and cadmium (Cd) in parts per million (ppm). Day sixty (60) shows a high rate of breakdown of TPH and PAHs.

This study's hydrocarbon biodegradation results indicate that *Enterococcus casseliflavus* is more capable of breaking down hydrocarbons than *Bacillus cereus*. Hydrocarbons can be biodegraded and broken down by bacteria, although this process takes time. The rate at which the organism is degrading increases with its exposure to crude oil. The findings of the present investigation bear similarities to those of a study conducted by Inuwa-Ja'afaru *et al.* (2017), which investigated the biodegradation of crude oil by *Bacillus* species isolated from various sources and

discovered that after 14 days of incubation, the microorganism grew well in 1% crude oil mineral salt medium. Mohammed *et al.* (2017) also investigated the biodegradation of crude oil by *Bacillus* species isolated from various sources and came to the conclusion that changes in optical density, pH, and gas chromatographic and mass spectrometer (GC-MS) analysis could indicate the potential for crude oil degradation by *Bacillus subtilis* isolated from non-oil contaminated sites.

According to Falade *et al.* (2017), a variety of microorganisms, particularly bacteria, produce enzymes like peroxidases and laccases that can break down petroleum hydrocarbons. Additionally, it has been discovered that microorganisms are crucial to the cycling of carbon (Bugg and Rahmanpour, 2015). Toxins are broken down into non-toxic compounds when appropriate microorganisms are used in the degradation of petroleum hydrocarbons, according to Ghoreishi *et al.* (2017). According to Xiumei *et al.* (2019) research, a consortium of bacterial strains can degrade petroleum hydrocarbons more quickly than a single strain. This finding is consistent with previous research by Ghorbannezhad *et al.* (2018) and Khan *et al.* (2018), which also reported that single bacterial strains were ineffective for bioremediation. Additionally, According to Wang *et al.* (2019), a "microbial consortium" is a grouping of more than two symbiotically interacting microbial species. Because of the synergistic connections created by the complementary roles and metabolic capacities of each species, a consortium frequently outperforms a single microbe.

CONCLUSION

The results of the study demonstrate the ability of *Bacillus cereus* and *Enterococcus casseliflavus* to biodegrade petroleum hydrocarbons in an environment contaminated with crude oil. Microorganisms are considered a good source of crude oil biodegradation therefore they can also be used to achieve crude oil

biodegradation. This makes microbially mediated biodegradation of crude oil a

viable strategy for biodegradation of crude oil contamination in aquatic environments.

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