World Journal of Advanced Research and Reviews
eISSN: 2581-9615 CODEN (USA): WJARAI
Cross Ref DOI: 10.30574/wjarr
Journal homepage: https://wjarr.com/

(Research Article)

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Isolation and identification of cyclohexane degrading microorganisms from hydrocarbon contaminated soils

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World Journal of Advanced Research and Reviews, 2024, 22(03), 2108–2120

Publication history: Received on 02 May 2024; revised on 10 June 2024; accepted on 13 June 2024

Article DOI: https://doi.org/10.30574/wjarr.2024.22.3.1763

Abstract

Objective: One of the major problems the world is facing nowadays is hydrocarbons pollution due to its detrimental spillage effects on water and soil during transportation. Therefore, assessing and identifying hydrocarbon-degrading bacteria will contribute to the biodegradation of the toxic compounds.

Methods: Pure bacterial cultures were isolated from two different locations, Petrol Depot R57 and Sasol Truck Stop. The soils were excavated from cyclohexane-contaminated areas and then transported to the laboratory for biological analysis. The ability of the bacterial isolates to use cyclohexane as the sole source of carbon was used as a requirement for the identification of the above-mentioned isolates, followed by confirmation of such identity after performing several tests.

Results: Bacterial isolates from Sasol Truck Stop and Petrol Depot R57 were assessed for cyclohexane biodegradation. Bacterial growth was observed at 6% cyclohexane for the Sasol sample but not the Petrol sample due to its toxicity. Both locations required biostimulation at lower concentrations. Morphological and Gram staining differentiated the isolates as followed: Petrol samples had varied bacterial types, while Sasol samples had bacteria only constituted of Gram-positive cocci. Biochemical tests identified varied oxidase, catalase, citrate, indole, lactose fermentation, and motility results. DNA analysis showed contamination during extraction, but effective 16S rRNA amplification and sequencing identified *Staphylococcus warneri* and *Bacillus subtilis*, indicating promising bioremediation applications.

Staphylococcus warneri and *Bacillus subtilis* showcased the potential to degrade cyclohexane as they were able to grow in the highest concentrations.

Conclusion: Identifying bacteria capable of degrading hydrocarbons, like *Staphylococcus warneri* and *Bacillus subtilis*, can significantly contribute to bioremediation efforts in reducing hydrocarbons pollution in affected environments.

Keywords: *Bacillus subtilis*; Bioremediation; Cyclohexane; Hydrocarbons; Pollution; *Staphylococcus warneri*

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1. Introduction

Pollution is a major problem in the environment and it can be caused by petroleum-based products from petrochemical industry (Eren & Güven, 2022; Malatova, 2005). Petroleum pollution has reached several millions of tons annually and it has been classified as a priority pollutant due to its serious hazard to human health and environmental deterioration (Baburam, 2020; Yuniati, 2018; Antwi-Akomeah et al., 2018).

Petroleum is one of the most important energy resources and a raw material of the chemical industry. The world depends on oil and the use of oil as fuel has contributed to intensive economic development. Although petrochemical plants and oil refineries are beneficial to society, they produce a large amount of hazardous waste. Moreover, oil spills during exploration, transportation, and refining, have caused serious environmental problems (Silva et al., 2014; Dan et al., 2019).

The advance of sustainable technologies has driven the search for natural, biodegradable compounds to remediate sites contaminated by hydrocarbons. This has led to the discovery of surfactants of a natural origin. Most of these surfactants are synthesized by living organisms, such as, saponins produced by plants, glycolipids produced by microorganisms, and bile salts produced by animals. Compounds with surfactant properties produced by microorganisms are denominated biosurfactants (Souza et al., 2014; Silva et al., 2014).

Biosurfactants are mainly produced by aerobic microorganisms in aqueous media with a carbon source feedstock, such as carbohydrates, hydrocarbons, fats, and oils. It is believed that biosurfactants are secreted into the culture medium to assist in the growth of the microorganism by facilitating the translocation of insoluble substrates across cell membranes (Campos et al., 2013; Silva et al., 2014).

Hydrocarbons are organic pollutants which are difficult to remove from a contaminated site thus, microorganisms can be used effectively in the bioremediation process of hydrocarbon contaminated soil because of their ability to use hydrocarbons as a sole carbon source (Baburam & Feto, 2021). Moreover, the mechanical and chemical method used to remove pollutants are limited and expensive (Edenshaw, 2017).

Hydrocarbons are rich compounds composed of carbon and hydrogen, found in fossil fuels like petroleum and coal, they serve as energy source for industry and daily life. However, petroleum Hydrocarbon has been identified as one of the causes of pollution around the world (Shukor et al.,2009) because of its detrimental spillage effects on water and soil during transportation. The spillage usually comes from tankers, pipelines, refineries and storage tanks (Singh & Chandra, 2014). Petroleum hydrocarbon also occurs in seepage from natural oil reservoirs and domestic waste including runoff from road surfaces. Most of the world's largest oil spills fit into three categories which are acts of war, out- of-control wells and tanker accidents. Transportation oil spills constitute about 13% of oil pollution in the oceans worldwide (Zhang et al., 2019; Board & Board, 2003). Oil spills from tankers are one of the most hazardous pollution among marine pollution. The oil spills during shipping not only threaten the life of crew members (Galieriková & Materna, 2020) but also result in huge economic loss and resource damage along with serious negative impact to the marine ecological environment and ecosystems in coastal countries (Chen et al., 2019; Kollo et al., 2017; Brekke & Solberg, 2005).

The first large petrol spill that affected South African coastline was when the Appolo Sea sank near Cape Town in June 1994.The oil spill from the vessel caused a major environmental disaster and killed thousands of seabirds like the endangered. It has been reported that over 2000 penguins were killed due to the effect of hydrocarbon pollution in South Africa. It is a proof that the effects of hydrocarbon pollution may be cancergenic, mutagenic and neurotoxic on human while causing death of aquatic animals and infertility of soil (Mbhele, 2007; Church, 2015; Elijah, 2022).

According to News 24, in May 2013, oil pollution was reported off Bloubergstrand on the South African coastline when a Turkish bulk carrier, Seli One vessel which ran aground in 2009 started to release oil after being battered by rough seas causing one of the tanks to rupture underwater. This posed a major threat to the marine life and the Peninsula's most popular beaches (Baburam, 2020).

Another case of oil spills occurred in May 2022 in Algoa Bay near the port of Ngqurha on the eastern seaboard of South Africa, the South African Maritime Safety Authority (SAMSA) reported that oil spill occurred during a ship-to-ship transfer of oil. All the relevant pollution response units have been activated, and booms deployed to contain the oil around the vessels (SAMSA, 2022).

Cyclohexane is a saturated hydrocarbon which belong to the group of cycloalkane, its ring structure makes it difficult to remove from a contaminated environment. Hence, there are microorganisms able to produce enzymes that break down the structure into a simple covalent bonds and use carbons as energy sources. Cyclohexane can be breakdown into cyclohexanol→cyclohexanone→e-carprolactone→6-hydroxycarproate (Savich & Novik, 2016).

Cyclohexane (C6H12) also possesses flammable, immiscible and volatile aromatic properties. It is used to synthesize intermediate products, to produce nylon while being present in some consumer products such as cleaning agents, coatings and fuel paints. It is recommended to use the products with safety procedure because of its toxic properties. Since cyclohexane, one of the recalcitrant pollutants, has been listed as one of the major environmental problems due to the persistence of such pollutant in soil, and proved difficult to remove as stated by Bocos et al. (2015), engineering field of biotechnology discovered a new approach by making use of microorganisms capable of degrading pollutants of the contaminated soil. The approach is called bioremediation.

Bioremediation of hydrocarbon pollutants and microbial enhanced oil recovery are the two main burning issues of the application of biotechnology in the petroleum industry (Silva et al. 2014; Dan et al., 2019).

Bioremediation is the transformation or breakdown of compounds, usually organic into simpler components through the biochemical reactions of microorganisms such as bacteria, yeast and fungi. Bioremediation using the microorganisms degrading hydrocarbons has major advantages to remove contaminants from environments (Endeshaw, 2017; Adams et al., 2015). It is used as a natural process to remove toxic chemicals from nature with low energy requirements as compared to other technologies and less manual supervision (Das & Chaillan, 2010). On the other hand, bioremediation is said to be time consuming during the removal of heavy metals because days to months are taken for such process. Additionally, high permeability is needed for soil during in-situ bioremediation.

Bioremediation can be classified into three categories which are microbial remediation, phytoremediation and enzymatic remediation. Microbial remediation involves the use of microorganisms to remove pollutants; these microorganisms are capable to break down (or metabolize) contaminants by using them as a food source (Zhang et al., 2019), phytoremediation is the process of using a living plant directly on the pollutants (Yavari et al., 2015) and enzymatic remediation occurs when the enzymes produced by microbial cells are extracted and applied on a contaminated environment (Osuoha & Nwaichi, 2021).

There are some factors affecting the efficiency of microbial bioremediation such as nutrient availability where inorganic nutrient namely nitrogen and phosphorus are necessary for microbial activity and cell growth (Yuniati, 2018), pH of soil is important for the survival of microorganisms, excess of moisture is undesirable because it reduces the amount of available oxygen for aerobic respiration, temperature influences rate of biodegradation by controlling rate of enzymatic reactions within microorganisms; those that grow above 55 °C and below 20 °C are called thermophiles and psychrophiles, respectively, the remainder being called mesophiles. Extreme thermophiles, also called hyperthermophiles, can tolerate and thrive above 80 °C. All cellular macromolecules such as RNA, DNA and proteins must be stable and functional in the temperature range in which these species live (Dutta & Chaudhuri, 2010).

Microorganisms capable of degrading hydrocarbons are commonly and widely distributed in nature (Van et al., 2003). They degrade hydrocarbons in order to produce energy and biomass, reduce toxicity and perform other functions. Microorganisms are involved in bioremediation through their enzymatic pathways act as biocatalysts and facilitate the progress of biochemical reactions that degrade the desired pollutant (Endeshaw, 2017).

Microorganisms are suited to the task of contaminant destruction because they possess enzymes that allow them to use environmental contaminants as a food. The aim of bioremediation is encouraging them to work by supplying optimum levels of nutrients and other chemicals essential for their metabolism in order to degrade/ detoxify substances which is hazardous to environment and living things (Endeshaw, 2017). Microorganisms able to degrade the contaminant increase in numbers when the contaminant is present; when the contaminant is degraded, the biodegradative population declines (Sardrood et al., 2013).

Aerobic bacteria use the contaminants as the sole source of carbon and energy. Example of such microorganisms is Pseudomonas, Alcaligenes, Rhodococcus, Mycobacterium and Sphingomonas (Chaillan & Das, 2010). Anaerobic microorganisms, on the other hand, are not frequently used as aerobic bacteria, they are mainly used for the bioremediation of polychlorinated bisphenyls. It is necessary that bacteria and the contaminants be in contact during biodegradation, but this is usually difficult to achieve because it can happen that they are not uniformly spread in the soil.

Many microorganisms, such as *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Bacillus subtilis*, *Bacillus cereus*, *Bacillus licheniformis* and *Bacillus laterospor* excrete emulsifiers that increase the surface area of the substrate. On the other hand, these microorganisms modify their cell surface to increase its affinity for hydrophobic substrates and, thus facilitate their absorption (Malatova, 2005; Cybulski et al., 2003; Carvalho and Fonseca, 2004).

Culture based which involves growing microorganisms on appropriate media (Goldman & Green, 2015) is a method that can only confirm the presence of microorganisms able to grow on a selected media. Therefore, it may not be effective at identifying the presence of novel organisms or known but uncultivable. Nevertheless, culture based technique remains the most widely used diagnostic method because of its extensive validation and cost – effectiveness (Goldman & Green, 2015).

Metagenomics has the ability to improve diagnosis and this need to be compared and validated by culture methods (Dunne et al., 2012). Microorganisms that were not able to grow under culture based technique can be detected using the metagenomics pathway. Metagenomics sequencing is much more expensive but very efficient, especially when identification goes as deep as the genes levels such as 16S RNA or 23S RNA. 16S rRNA genes have conserved and variable regions where conserved areas reflect phylogenetic relationship among species (and are used as sites for PCR priming) and highly variable regions reflecting differences between species (Wang et al., 2015). Sequencing the entire 16S gene provides real and significant advantages over sequencing commonly targeted variable regions (Johnson et al., 2019).

The aims of the present study are to isolate and identify cyclohexane degrading bacteria from hydrocarbon contaminated soils in two different locations: the Sasol Truck Stop in Sasolburg Freestate and the Petrol Depot on the R57 Vanderbijlpark, Gauteng, South Africa. The steps included isolation of hydrocarbon-degrading bacteria, identification of the bacterial isolates based on morphological and biochemical characterization and 16S rRNA gene sequence analysis.

2. Material and Methods

2.1. Samples

For isolation of cyclohexane-degrading bacteria, samples were collected from two different locations, the Sasol Truck Stop in Sasolburg Freestate and the Petrol Depot on the R57 Vanderbijlpark, Gauteng, South Africa. Samples were collected from a depth of 10 cm using a spade and kept in a sterile plastic bag. The samples were stored at 4 °C in the laboratory until further analysis.

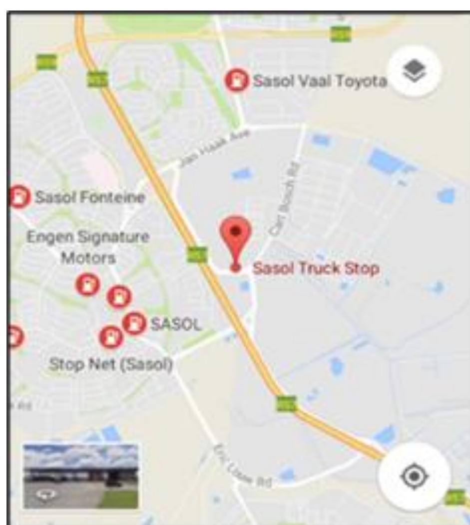

Figure 1 Sasol Truck Stop.

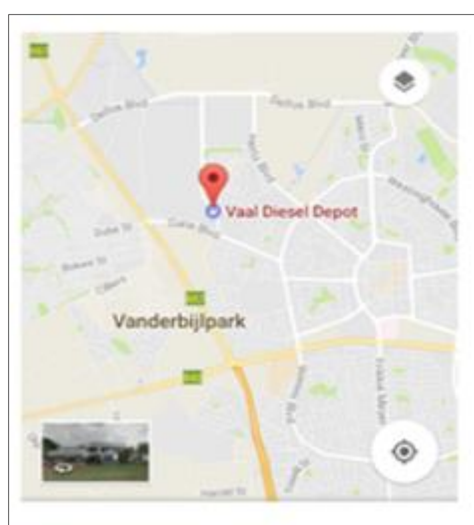

Figure 2 Petrol Depot R57

2.2. Screening for hydrocarbon degrading bacteria

One gram of the soil sample was diluted using saline solution (0.85% NaCl) then 1ml of the diluted sample was serially diluted up to 10^{-6} saline test tube. The serial dilution factors 10^{-2}, 10^{-4} and 10^{-6} were spread plated on minimum salt medium (MSM) plates containing cyclohexane at 2% (v/v), 4% (v/v) and 6% (v/v) concentrations and uninoculated

plate was serve as media control. After 10 days of incubation at 37 °C, single colony from different minimum salt medium plates was aseptically subcultured for further preliminary identification and biochemical tests.

2.3. Morphological and Biochemical tests

The bacteria isolates were characterized by their morphological properties through gram staining technique and visualized using an optical microscopy. The biochemical tests such as catalase, oxidase, motility, Simmon's citrate, indole, lactose were carried out according to Varghese & Joy, (2014); Sagar, (2022).

2.4. Molecular identification

The 16S ribosomal RNA (rRNA) gene was analysed to identify the selected bacteria strains. For each location, two candidate clones from the highest cyclohexane concentration were selected for DNA extraction. A modified CTAB extraction protocol was followed as described by Worden, (2009) and CTAB- Genomic DNA Isolation, (2021).

The concentration and quality based on ratio (260/280 nm:1.8) of the DNA samples were observed using the NanoDrop. Before running the gel, the DNA of each candidate clones (4 µl) was stained with a loading dye (2 µl) which plays roles such as increasing the density of the sample, allowing it to sink into the gel, providing colour and indicating the distance that DNA fragments have migrated. The isolated DNA containing the loading dye with a total volume of 6 µl along with the universal Kappa Ladder (4 µl) was separated on a 1% (w/v) agarose gel stained with ethidium bromide (0.5 µg/ml). The electrophoresis was filled with a buffer (1XTAE) where an electric current of 100 Volts was applied for 45 minutes to pull DNA samples through the gel from negative electrode to positive electrode. The observation of the bands was done using computer with imager Doc. The molecular weight of the DNA samples was compared to the molecular weight marker to find the corresponding base.

2.4.1. Separation of PCR amplicons

Amplification of the 16S rRNA region was carried out using a PCR protocol as described by Pichler et al., (2018). The PCR amplicons were separated on a 1% (w/v) agarose gel and compared to a molecular weight marker to confirm that the correct size band was amplified.

Polymerase chain reaction was performed from the DNA extracted sample where a volume of 12.5 µl of master mix was aliquoted to each PCR tubes followed by 2 µl of DNA template. 2 µl of the reverse (907R) and forward (27F) primes were added in the tubes, and a volume of 6.5 µl was added to make up a total volume of 25 µl.

The first step of the cycle was denaturation, brought about by heating the target DNA to about 95°C this process separates the double-stranded DNA into two single strands. The second step was annealing, that occurs with the cooling of the DNA in the presence of 27 forward and 907 reverse primers, which anneal and hybridise to complementary sequences in the two DNA strands. The optimal temperature for annealing varies between 40 °C to 60 °C. The third step was the extension, where Taq DNA polymerase at 72 °C enables nucleotides to be added to the 3' end of the annealed primers and extend in the 5' to 3' direction (Pichler et al., 2018). The new synthesised fragments serve as templates for the next cycle and within a short period of time, many copies of the original can be produced.

The PCR products were analysed after running the agarose gel at 100 volts for 45 minutes. The PCR products were sent for sequencing at Inqaba BiotecTM PTY LTD.

2.5. Sanger sequencing and BLAST search

Sanger sequencing was carried out on the amplified DNA fragments for the two candidate clones using the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) this was carried out to identify the two selected positive clones.

2.6. Glycerol stock preparation

Glycerol serves as microbial preservative where it is used as carbon and energy source for microbial growth. To preserve the hydrocarbon degrading bacteria, 10ml of glycerol was pipette into micro tubes then 10ml of culture was added. The micro centrifuge tubes were kept refrigerated at -20 °C for longer storage.

3. Results and Discussions

3.1. Isolation and identification of isolated bacteria

Bacteria isolates were screened for their ability to biodegrade cyclohexane, and they were tested in duplicates. The minimum salt media (MSM) revealed growth of colonies capable to grow in the presence of cyclohexane used as the sole source of carbon.

The plate count of viable bacteria in the highest concentration of cyclohexane (6%) was observed from the sample of Sasol Truck Stop. The number of colonies was determined to be in order of 8, 3 and 2 as the dilution factor increases at a concentration of 6% of cyclohexane as shown in table 2. On the other hand, no bacteria were able to grow in the sample of Petrol Depot R57 at a concentration of 6% due to the degree of toxicity that inhibits the growth of bacteria. However, the topmost concentration for bacteria growth in Petrol Depot R57 sample is 4% where the number of colonies was determined to be in order of 10, 8 and 2 respectively as indicated in table 1.

This case explained how some bacteria in petrol Sasol Truck Stop develop the ability to survive in a concentration above 4%, therefore becoming resistant to cyclohexane.

Furthermore, no growth was observed at a concentration of 1 and 2% in Sasol Truck Stop as shown in table 2 due to some factors affecting the growth of bacteria such as the poor availability of nutrients. Their growth and activity must be stimulated. Biostimulation usually involves the addition of nutrients and oxygen to help indigenous microorganisms to grow (Kensa, 2011).

Table 1 Number of colonies obtained at 37 ºC from Petrol Depot R57 Sample after incubation period of 5-10 days.

Concentration %.	Dilution factors.	Number of colonies
1	10^{-2}	50
2		40
3		25
4		10
1	10^{-4}	20
2		15
3		10
4		8
1	10^{-6}	10
2		5
3		3
4		2

Table 2 Number of colonies obtained at 37 ºC from Sasol Truck Stop Sample after incubation period of 5-10 days

Concentration %.	Dilution factors.	Number of colonies
3	10^{-2}	18
4		15
5		10
6		8
3	10^{-4}	14

4		9
5		5
6		3
3	10^{-6}	7
4		4
5		2
6		2

The above tables are represented in a form of a chart as shown in figure 3 and 4 respectively, where the numbers of colonies are decreasing due to the degree of toxicity of the substrate. Cyclohexane sample from Petrol Depot R57 becomes more toxic at a concentration above 4% thus inhibit the growth of bacteria while at Sasol Truck Stop some bacteria were able to survive and use cyclohexane as carbon source; at a concentration above 4% bacteria were able to adapt to the environment. In fact, Petrol Depot R57 area, is more contaminated than Sasol Truck Stop, this can be shown by the growth rate of bacteria, where, in figure 4, the concentration of bacteria is lower than those in figure 3 with a higher concentration of bacteria.

Concentration of cyclohexane has effect on the growth of bacteria by reducing the number of colonies as the concentration increases. The highest concentration of isolate bacteria was chosen to distinguish between the isolate bacteria with the potentiality to degrade cyclohexane at a high degree of toxicity against those that are not able to use cyclohexane as a source of energy in the highest concentration.

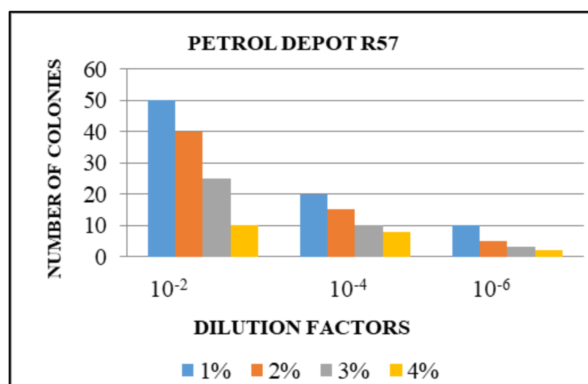

Figure 3 Effect of cyclohexane concentrations on the number of colonies isolated from Petrol Depot R57 sample incubated for 5-10 days

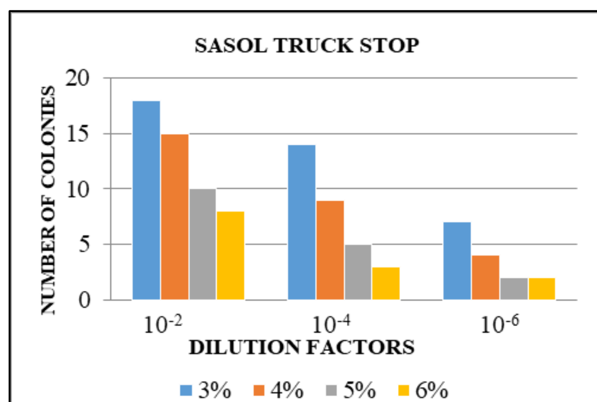

Figure 4 Effect of cyclohexane concentrations on the number of colonies isolated from Sasol Truck Stop sample incubated for 5-10 days

3.2. Identification of the isolates using morphological characteristics and staining techniques

The isolated bacteria were identified using their morphological characteristics based on shape, size, colour, elevation and texture on nutrient agar plates. All isolates were examined using Grams staining reaction to differentiate between Gram positive from Gram negative bacteria. Bacteria were found to be either gram positive or gram negative after Gram staining with different morphology on the plate as shown on table 3 of Petrol Depot R57 sample; and all bacteria were identified Gram positive for location of Sasolburg as indicated on table 4.

Table 3 Morphological characteristic of colonies and Gram staining of the cells from Petrol Depot R57 sample

Isolates	Size	Color	Form	Texture	Elevation	Gram stain	Shape
Cy1R	small	yellow	Round	smooth	raised	-	Rods
Cy2R	Tiny	Creamy white	Round	smooth	flat	+	Cocci
Cy3R	medium	Creamy white	Round	smooth	flat	-	Rods
Cy4R	medium	Creamy white	Circular	smooth	flat	+	Cocci
Cy5R	small	Creamy white	Round	smooth	flat	+	Rods
Cy6R	small	Creamy white	Round	smooth	flat	-	Rods
Cy7R	medium	Creamy white	Irregular	rough	raised	+	Cocci
Cy8R	medium	Creamy white	Round	smooth	flat	-	Rods

(+) positive results and (-) negative results of Gram staining from Petrol Depot R57 sample

Table 4 Morphological characteristic of colonies and Gram staining of the cells from Sasol Truck Stop sample

Isolates	Size	Color	Form	Texture	Elevation	Gram stain	Shape
Cy1S	small	Creamy white	round	Smooth	flat	+	Cocci
Cy2S	small	Creamy white	round	Smooth	flat	+	Cocci
Cy3S	medium	Creamy white	round	Smooth	flat	+	Cocci
Cy4S	medium	Creamy white	round	Smooth	flat	+	Cocci

(+) positive results of Gram staining from Sasol Truck Stop sample

3.3. Identification of isolates using biochemical tests

After sub-culturing the isolates on the nutrient agar plates and incubate for 24 hours, the biochemical characteristics of the isolates were determined using some tests. The positive results of oxidase test show that bacteria are aerobic or facultative anaerobic since they were able to produce cytochrome c oxidase except for those that were oxidase negative. The indole test indicates that all the bacteria were not able to split the tryptophan to form the compound indole because of the absence of the tryptophanase enzyme therefore they were tested negative. Some bacteria were able to ferment lactose since they were positive for lactose fermentation test. The following results were insufficient to identify microbes at the species level. Therefore, reliability on molecular methods was a breakthrough and answer to this study.

Table 5 Biochemical characteristics of the isolates from Petrol Depot R57 sample

Isolate	Oxidase	Catalase	Simmon's citrate	Indole	Lactose fermentation	Motility
Cy1R	+	+	+	-	+	+
Cy2R	+	-	+	-	+	+
Cy3R	+	+	+	-	+	-
Cy4R	+	-	+	-	+	-
Cy5R	+	-	+	-	+	-

Cy6R	+	+	+	-	+	+
Cy7R	-	+	+	-	+	-
Cy8R	+	-	+	-	+	-

(+) positive results and (-) negative results of biochemical tests from Petrol Depot R57 sample

Table 6 Biochemical characteristics of the isolates from Sasol Truck Stop sample

Isolate	Oxidase	Catalase	Simmon's citrate	Indole	Lactose fermentation	Motility
Cy1S	-	+	-	-	-	+
Cy2S	-	+	-	-	+	+
Cy3S	-	+	-	-	+	-
Cy4S	+	+	+	-	-	+

(+) positive results and (-) negative results of biochemical tests from Sasol Truck Stop sample

3.4. Quantitative and qualitative analysis

Table 7 Concentration and ratio of the DNA isolates

Isolates	Concentration (260-280 nm)	Ratio
Cy4R	65.1 ng/µl	1.53
Cy7R	110.3 ng/µl	1.45
Cy2S	49.8 ng/µl	1.61
Cy4S	68.5 ng/µl	1.51.

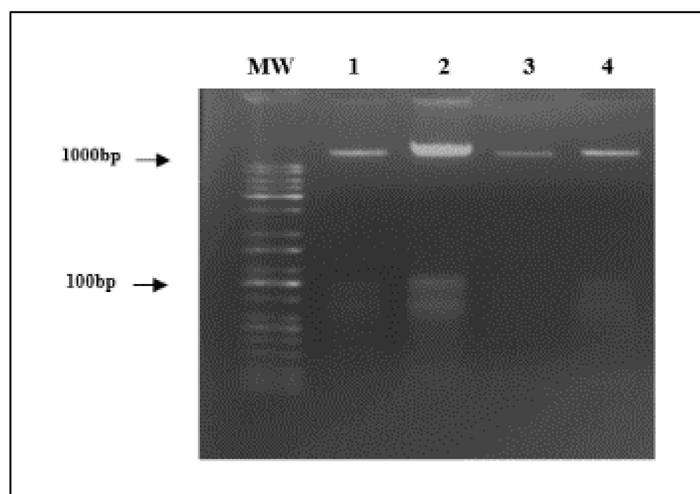

Figure 5 Agarose gel (1% w/v) stained with ethidium bromide. Lane MW represents 1kb Kappa universal marker. Lane 1 to 4 are examined isolates. Lane 1 and lane 2 are DNA isolates from Petrol Depot R57 sample (Cy4R and Cy7R respectively). Lane 3 and lane 4 are DNA isolates from Sasol Truck Stop sample (Cy2S and Cy4S respectively). The presence of the smears indicates that the DNA isolates were contaminated during the extraction with carbohydrate, therefore the quality of the DNA is affected

The concentration and ratio of the isolated DNA samples were determined using a NanoDrop as shown in table 7 bellow. The isolate Cy7R was found to have the highest concentration of 110.3 ng/µl in the range of 260 - 280 nm with a ratio

of 1.45 below the standard ratio of DNA which is 1.8; the DNA sample might have been contaminated during the extraction with carbohydrates.

3.5. Separation of PCR amplicons

Although the presence of smears on the gel which indicate contamination of the DNA isolates, the PCR products were sequenced because of the 16S rRNA region amplified so the contaminant couldn't inhibit the PCR process.

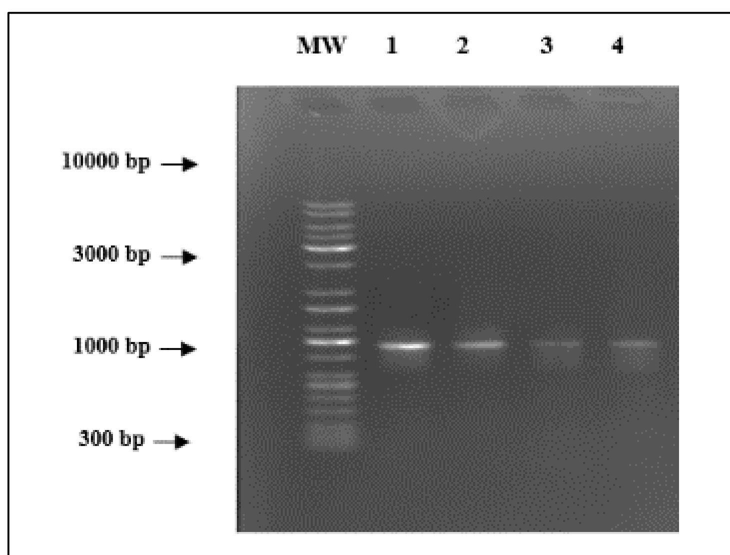

Figure 6 Illustrating agarose gel 1% (w/v) stained with ethidium bromide with four amplified products. Lane MW represents the Kappa universal marker. Lane 1 and lane 2 contain aplicon from Petrol Depot R57 sample in Vanderbijlpark (Cy4R and Cy7R respectively). Lane 3 and lane 4 contain aplicon from Sasol Truck Stop sample in Sasolburg (Cy2S and Cy4S respectively). The size of the band was found to be 1000 bp, clear and sharp, as compared to the size of the Kappa universal marker

Isolated cloned were identified by molecular analysis of 16S rRNA. The 16S rRNA genes were amplified by polymerase chain reaction PCR using 27F (AGAGTTTGATCMTGGCTCAG) and 907R (CCGTCAATTCCTTTRAGTTT). Taxonomic analysis was conducted using the BLAST program and sequences were deposited in the NCBI GenBank.

3.6. Sanger sequencing and BLAST search

The sequences were edited using the BioEdit software. A BLAST search was carried out on the NCBI website for the identification of the two isolates Cy7R and Cy4S which indicate the highest concentration of DNA, as shown in Table 7. The 16S rRNA achieved 99% and 98% of similarity with *Staphylococcus warneri* and *Bacillus subtilis* respectively. The two isolates have the potentiality to use cyclohexane as carbon source of energy in a high concentration.

Table 8 Identification of bacterial isolates after BLAST search

Isolates	Strains	Similarity (%)	Accession number
Cy7R	*Staphylococcus warneri*	99	MK005281.1
Cy4S	*Bacillus subtilis*	98	CP029461.2

Staphylococcus warneri belongs to the family of staphylococcaceae and genus of Staphylococcus, according to Godini et al. (2018) this species was identified based on the 16S rRNA to degrade polyaromatic hydrocarbons and was reported to be isolated from lab soil polluted with aromatic hydrocarbon derivatives and heavy metals using an enrichment media.

Degtyareva et al. (2020) worked with the full genome sequencing of the *Staphylococcus warneri* strain isolated from oil-contaminated soil and state that this species contains 2535 protein coding sequencing therefore, the role of this bacterial type in the degradation of hydrocarbons remains poorly understood.

Recent studies have demonstrated that some secondary metabolites, produced by certain species of Staphylococcus isolated from the natural environment, have biotechnological and biomedical significance, including those involved in the production of biosurfactants (Degtyareva et al., 2020).

The microbial biodegradability of mineral oil and other hydrocarbons, namely hexane, decane and tetradecane was determined using the Warburg constant volume respirometer where *S. warneri* showed the highest value (2.895) when exposed to hexane (Saadoun et al., 1999). However, this result ensures the ability of this isolate to grow effectively in a hydrocarbon contaminated area.

Bacillus subtilis belongs to the family of bacillales and genus of Bacillus, this species has the potential to degrade petroleum compound because it's able to exhibit growth by breaking down petroleum hydrocarbon, thus, can grow in the highest concentration of toxic oils (Darsa et al., 2014; Ghorbannezhad et al., 2022).

According to Darsa et al., few researchers have reported on Bacillus sp. for oil degradation potential, *Bacillus subtilis* is more tolerant to high levels of oil toxicity due to their resistant endospore. During petrol degradation process by *Bacillus subtilis* the pH level decrease, thus, cause the formation of organic acids.

Then again, Bo Wu et al., have investigated on the co-culture system of *B. subtilis* and *P. aeruginosa* on the degradation of crude oil; they noticed that the combination of *B. subtilis* and *P. aeruginosa* can effectively promote the degradation and utilization of crude oil, which may provide a new idea for the improvement of bioremediation strategies; the degradation efficiency of crude oil was enhanced from 32.61% and 54.35% in individual culture to 63.05% by the defined co-culture (Bo et.al, 2023). This investigation shows that the B. subitils strain can be combined with another biosurfactant bacteria strain for an efficiency crude oil biodegradation.

B.subtilis has the potential to be used for the bioremediation of hydrocarbon pollutants and could have promising applications in the petrochemical industry since they are able to degrade 65% of crude oil under optimal condition (Dan et al., 2019). On the report of Punniyakotti et al., the isolate had the ability to synthesize degradative enzymes such as alkane hydroxylase and alcohol dehydrogenase at the time of biodegradation of hydrocarbon (Punniyakotti et al., 2017).

4. Conclusion

The identified bacteria, *Staphylococcus warneri* and *Bacillus subtilis* have shown the potential to use cyclohexane as a carbon source and grow in an environment containing high concentrations of hydrocarbons. *Staphylococcus warneri* and *Bacillus subtilis* could be the choice for microbial bioremediation as the study demonstrated that they have high bioremediation potential and are likely candidates to be used for degradation of highly toxic cyclohexane in contaminated area.

Compliance with ethical standards

Acknowledgments

I am grateful to Vaal University of Technology '**VUT**' along with the department of Biotechnology for making this project possible. To those with whom I have had the pleasure to work during this project especially, Mrs. Cindy Baburam from Vaal University of Technology, department of Biotechnology, VanderbijlPark, South Africa.

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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