Isolation and characterization of *Bacillus* spp. for use in the remediation of petroleum waste residues

Submitted in fulfilment of the degree of Master of Applied Science: Biotechnology in the Faculty of Applied Sciences at the Durban University of Technology

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Declaration

I, Wendy Snoyolo Masika do hereby declare that in respect of the dissertation and the content herein is entirely my own work. It has never before been submitted for any diploma, degree or examination to any other University, Technikon or Tertiary Educational Institute.

Wendy Snoyolo Masika Date

As the candidate's supervisors we agree to the submission of this thesis

Prof Tukayi Kudanga Date

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Dedication

I dedicate this thesis to my beloved parents:

To my late mother who encouraged me to embark on this journey, your love and support for my education has carried me through this journey mama. It's sad that you are not here to witness and celebrate this milestone with me. I'm the person I am today because of you. You live forever in my heart. Ndiyabulela mama, Enkosi Jola.

To my father who has been a sole provider to our family, my pillar of strength and my personal hero. It's no secret that you are a perfect example of a good father to us as your children, and you continue to do so in so many ways. I'm where I am today because of you. Ndiyabulela Tata, Enkosi Sbewu.

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Abstract

Petroleum hydrocarbons are toxic to all forms of life; therefore, environmental pollution caused by petroleum is of great concern. Environmentally friendly strategies are required for the remediation of the contaminated sites. Microbial populations comprising of several different genera have been detected in soil and water environments that have been contaminated with petroleum. This suggests that these organisms are able to use hydrocarbon compounds as a substrate for survival and could be harnessed in bioremediation of contaminated sites. The first stage of this research was focussed on the isolation, purification, screening and selection of putative *Bacillus* spp from environmental samples. Samples were collected from different sites around the Gauteng province in South Africa. Samples from both soil and water were obtained from selected sites including environments that were contaminated by oil. Isolate selection was based on the growth rate of the isolates, the degree of sporulation and the rate of oil degradation. The identities of the potential isolates as well as their safety status were clarified in order to reduce possible risk to end users or the environment. Once suitable isolates were identified, those that possessed inherently strong biodegradation ability were assessed for their efficacy as well as compatibility to perform in a consortium. Various organism combinations were assessed and compared to the efficacy of individual isolates, in order to formulate a bioremediation consortium.

Of the 115 isolates, the top performing isolates, identified as GPA 11.2, GPA 7.1, GPA 3.5, GPA 8.3 and GPB 4.4, were obtained from a car workshop in Midrand and a taxi rank in Silverton. GPA 8.3 and GPA 4.4 were, however, eliminated due to their low sporulation efficiency.

The selected *Bacillus* isolates were identified using 16s rDNA sequencing and GPA 7.1 and GPA 11.2 were identified as *B. subtilis*, while GPA 3.5 was identified as *B. methylotrophicus*. These isolates were further assessed for enterotoxin production and the presence of anthrax virulent plasmids pX01 and pX02. After conducting the biosafety assays, the isolates were rendered safe for use.

The bioremediation potential of the consortium was evaluated using industrial effluents that contained hydrocarbons. Degradation of hydrocarbons using all three consortiums (Gen 3.1, Gen 3.2, Gen 3.3) in the respective industrial effluents were determined by measuring the rate of degradation for each hydrocarbon compound using Gas Chromatography (GC). Results indicated that the bulk of the contaminants were removed during the first 48 hours; and removal (%) did not increase significantly after 72 hours. The total petroleum hydrocarbons (TPH) (C8-C28) removal rates from synthetic effluent after 48 hours of treatment using the Gen 2, Gen 3.1, Gen 3.2 and Gen 3.3 consortia were 0.8, 0.26, 0.07 and 0.58 $mg.L^{-1}.h^{-1}$, respectively. The TPH (C8-C28) removal rate from true effluents after 48 hours of treatment using the Gen 2 (benchmark), Gen 3.1, Gen 3.2 and Gen 3.3 consortia were 0.23, 0.25, 0.12 and 0.17 $mg.L^{-1}.h^{-1}$, respectively. The results showed that the best performing consortium was Gen 3.1.

This study has demonstrated the potential application of *Bacillus* as bioremediation agents for the treatment of hydrocarbon-contaminated sites. This technology could potentially also be utilised for addressing the challenges of a wider range of different hydrocarbon effluents.

Preface

In the accomplishment of this master's degree I am submitting a project report on

"**Isolation and characterization of *Bacillus* spp. for use in the remediation of petroleum waste residues**"

This whole project has been divided into 5 chapters:

1. Introduction – a brief background on environmental pollution and the purpose of the study with aims and objectives.
2. Literature Review – a detailed literature overview on hydrocarbon degradation, microorganisms that are involved and the mechanisms used in the degradation of hydrocarbons.
3. Research methodology on how the samples were collected, and how microorganisms were isolated, purified and screened for hydrocarbon degrading properties.
4. Results and discussion – presents the results, discusses meaning of the results and how they relate to literature.
5. Conclusion and recommendations – summarises the major findings of the study and proposes recommendations for further study.

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Chapter 1

GENERAL INTRODUCTION

Petroleum hydrocarbons are toxic to all forms of life; therefore, environmental pollution caused by petroleum is of great concern. Crude oil contamination is relatively common because of its widespread use, accidental spillages and the associated disposal operations (Pavel and Gavrilescu, 2008). Most oil components are lethal to humans and wildlife as can easily enter the food chain. Natural leakage accounts for almost 47% of the crude oil leaks into the marine environment (Pavel and Gavrilescu, 2008).

Previous published work states that the major mechanism for the removal of hydrocarbon from contaminated sites occurs via biodegradation by naturally occurring organisms (Chibueze et al., 2016). Biodegradation is a natural process wherein microorganisms are able to modify and breakdown organic molecules into other substances. This process may occur as one reaction or a series of reactions. Mineralization is one of the reactions that occurs, and involves the breakdown of petroleum hydrocarbons into carbon dioxide and/or methane and water. Bioremediation refers to the addition of materials to spill sites to speed up of the natural biodegradation process (Das and Chandran, 2011).

In the past decades, bioremediation has been found to be a relatively cost effective and better way to manage waste from petroleum systems, and bioremediation processes have several advantages over traditional chemical processes. The advantages include flexibility when treating several types of waste in the soil and water environments; lower operational and capital costs, and hydrocarbons are almost

completely converted to nontoxic products. However, one shortcoming of bioremediation technologies is that it is not always possible to predict the success or failure of the operation based on observations from another operation, due to system variations (Chibueze et al., 2016).

The success of any hydrocarbon bioremediation process is dependent on the specific waste composition, the presence of microorganism able to degrade waste and the respective environmental conditions. These unique site specifications are required in order to predict success or otherwise of the bioremediation system. It usually creates a need for laboratory scale testing (Chibueze et al., 2016).

Various consortia, or groups of microorganisms have been proven to degrade mineral oil hydrocarbons under laboratory or field conditions (Abatenh et al., 2017a). A consortium contains a specific mixture of organisms that are mutualistic hydrocarbon utilizing microorganisms. Microorganisms involved in the bioremediation process can produce enzymes which are capable of attacking almost any size or type of hydrocarbon. Other microorganisms can only produce enzymes that attack and breakdown one specific type or size of molecule and no single species of microorganisms are able to degrade all of the different components of hydrocarbon in oil products. As a result, several different enzymes and metabolic pathways are required to degrade a significant number of compounds which are contained in petroleum and other related products. In the event of a petroleum spillage, microorganisms that are part of the system will show fast growth, as a result of the high availability of hydrocarbons which are easy to degrade. These fast growing species may cause the delay in the growth of other species by causing a depletion of

oxygen and nutrients in the specific environment. When those easily degradable hydrocarbons are depleted, microorganisms are nutrient limited and may die off. Other microorganisms which are able to utilise the residual hydrocarbons will then exhibit growth in the system. As a result, the cycle continues, as species flourish and retreats as the hydrocarbons they excel at degrading become available and thereafter become depleted (Mcguinness and Dowling, 2009).

Nearly 100 species of bacteria, representing 30 microbial genera, have hydrocarbon that has ability to oxidize compounds. However, limited studies have been done that show a bacterial consortium comprising only of a known population of microorganisms, specifically *Bacillus sp. Bacillus* organisms have been shown to degrade hydrocarbons and their efficacies have been reported by Malik and Ahmed (2012) and Janaki et al. (2016). *Bacillus* candidates are suitable as bioremediation agents because they display good bioremediation characteristics; grow rapidly, able to tolerate a range of physiological conditions, able to sporulate, relatively cheap to produce and can be processed into products with exceptional shelf-life (Lalloo et al., 2007).

This study focusses on the isolation (from oil contaminated sites), identification, characterisation and evaluation of *Bacillus* spp. for their ability to degrade hydrocarbons. Based on the ability to utilize petroleum products as their growth substrate, the top performing isolates were selected for further testing individually and in consortia.

Aim and objectives

Aim

To isolate, characterize and evaluate *Bacillus* spp. for their ability to degrade hydrocarbons.

Objectives:

- To Screen, isolate and identify *Bacillus* spp. that are potentially capable of degrading hydrocarbons.
- To demonstrate the efficacy of selected *Bacillus* spp. strains, in consortia, for hydrocarbon degradation using laboratory and synthetic industrial wastewater containing motor oil.

Chapter 2

1. LITERATURE REVIEW

This chapter reviews literature relevant to the study. The first part is an introduction to petroleum hydrocarbons which consists of the history and background information on petroleum hydrocarbons. It also includes the characteristics and compositions (chemical and physical) of petroleum hydrocarbons. The next part reviews the types of bioremediation technologies which are available and the mechanisms which are associated with hydrocarbon degradation. The last part of the chapter reviews the effects of biosurfactants on biodegradations and also the factors influencing petroleum hydrocarbon.

2.1 Petroleum hydrocarbon exploration

Petroleum is said to have first been discovered during 20,000 B.C. by the Mesopotamian peoples (Clark et al., 2013). The Chinese have used petroleum from around 5,000 B.C and used it in the production of medication, waterproofing as well as in warfare applications. In the Middle East, petroleum use became widespread around 3,000 B.C, and numerous oil leaks were discovered underground in abundance (Pourfakhraei et al., 2018).

To access oil, a well is formed by drilling a long hole into the ground with an oil rig (El Hanafy et al., 2016). The first oil well was dug in Titusville, west of Pennsylvania during 1859 by a railroad conductor named Colonel Edwin Drake. The oil business currently provides almost half of the world's power, including petrochemicals raw materials. Regions such as the Persian Gulf, the United States (U.S), Russia, Northern and

Western Africa, Mexico, Indonesia and Venezuela are the major oil producers (Gutierrez et al., 2016).

2.2 Petroleum hydrocarbon pollution

Upon initiation of the petroleum industry, the demand for petroleum as well as its derivative products have increased worldwide. Petroleum has been distinguished as a potential hazard post the 20th century (Co-operation and Agency, 2006). The extensive use and petroleum storage has led to petroleum hydrocarbons becoming the most dangerous contaminants in the environment, because of the imposed threat on the health and sustainability of the environment. Contamination from petroleum constitutes has led to it becoming the leading source of environmental pollution in the commercialized countries (Xu et al., 2018).

It has been reported that there are two main causes or sources of petroleum pollution that are a contributing factor when it comes to pollution of marine environments (Zakaria et al., 2002). Dumping and spillage from excess oil, including the leakage of oil tanks on vehicles and onto motorway surfaces is one of these causes (Zakaria et al., 2002). Human activities is another cause of petroleum contamination and includes manufacturing of engineering products and machinery, spills from shipping accidents, accidental spillage of industrial oil, activities from motor industries, uncontrolled disposal of oil brines, cleaning of bilges, domestic and industrial wastes, recreational boating, overflow from terrestrial environments, and atmospheric depositions (Petrikevich et al., 2003). These activities have severe effects in the plant and animal ecosystems as well as human health (Pourfakhraei et al., 2018).

A number of effects of oil contamination have been identified by Canadian authorities regarding fresh water, marine eco-systems and ecological habitats (Torres et al., 2008). Large components of the spilled oil spreads above the exterior of the marine environment, and results in an anaerobic environment in the water below. This leads to the depletion of oxygen which causes death of the naturally occurring plants and animals as oxygen is their main element for respiration. Aquatic birds are also impacted as they may suffer from hypothermia, drown due to loss in flight, and also be poisoned. When exposed to crude oil, aquatic birds and animals may have damage caused to their kidneys, lungs, liver, intestines, reproductive systems, and other inner organs (Helm et al., 2015). Oil pollution can also cause changes in the marine ecosystem which include proliferation of seashore algae when limpets that usually consume algae die.

Several scientists have assessed the effects of oil contamination on human health (Knafla et al., 2011, Lee et al., 2006, Chen et al., 2008). Humans and other animals can suffer from severe diseases caused by crude oil contamination (Govind, 2014). Polycyclic aromatic hydrocarbons (PAHs) are amongst the main components of crude oil and are extremely hazardous. These causes skin cancer, skin erythema (reddening), nasal cancer, bladder cancer and gastrointestinal cancer (Mandal et al, 2011). Further inhalation of vapours from hydrocarbons may cause headache, nausea, dizziness, and respiratory irritation (Khatoon et al., 2017). Benzene, xylene (BTEX), and ethylbenzene toluene, are the main components of crude oil which cause alterations, birth defects, different cancers, disruption of endocrine systems, liver disease, nervous disorders, depression, and irregular heartbeats (Mandal et al., 2011).

Contamination from petroleum-derived products is indirectly linked to harm imposed on wild life. During a contamination event, key factors that require consideration include oil type, weather and environmental conditions (Kingston, 2002). Upon exposure to oil contamination in a marine environment, seabirds, marine mammals, turtles, and fish are physically coated by oil. As a result, their fur and feathers become imbalanced. This affects their mobility and may ultimately lead to death of the animal (Wilson and LeBlanc, 2000; Kingston, 2002).

2.3 Petroleum hydrocarbon components and characteristics

Petroleum is a substance that is naturally occurring and forms as a result of decaying living material. These residues which are organic in nature are converted into oil (petroleum) due to pressure and heat. This produced material then moves upward towards the earth surface, or it may get trapped in reservoirs (Kingston, 2002). Petroleum is composed of various hydrocarbons and non-hydrocarbons; the latter component is less abundant (Wilson and LeBlanc, 2000). Non-hydrocarbons are comprised of: (1) sulphurs, (2) nitrogen, (3) oxygen, and (4) metallic compounds (Leahy and Colwell, 1990). Hydrocarbons can be divided into four classes: (1) aromatics, (2) saturates, (3) resins (pyridines, sulfoxides, and amides). (4) and asphaltenes (phenols, fatty acids, ketones, esters, and porphyrins).

Hydrocarbon compounds are made of linked carbon atoms forming a backbone to which hydrogen atoms can then bind to the remaining sites. The carbon backbone can be cyclic, branched, or straight (Lamberth, 1994). Compounds that only contain carbon and hydrogen are known as the main compounds. Derivative compounds are compounds that contain other elements or hydrocarbon branches. Hydrocarbons are

then classified based on their physical properties or on their chemical structure of main compounds. The taxonomy of the chemical structure is based on cycloalkanes, cycloalkenes, alkanes, alkenes, and alkynes. Alkanes, or paraffin, have a straight backbone structure which contains single carbon to carbon bonds. Cycloalkanes have a ring structure with a sole carbon to carbon bonds. Alkenes, or olefins, have a normal backbone structure which contains a double carbon to carbon bonds. Cycloalkanes, or aromatics, are a cyclic structure with double bonds. Alkynes, which are also known as olefins, comprise of a three carbon bonds in their backbone. The double and triple carbon to carbon bond represent unsaturated hydrocarbons (Derek, 2016).

Petroleum components and mixtures thereof are highly complex and they are defined as any mixture of natural gas, condensate, and crude oil. Crude oil contains a massive amount of individual compounds with hydrocarbons expressing a percentage of 50% to 98% of the total weight of crude oil. When crude oil is released into the environment, the compounds go through biological changes chemical, and physical, which are referred to as weathering. The amount to which the numerous types of petroleum hydrocarbons are degraded under these changes depends on the physical and chemical properties of the hydrocarbons (Gallego et al., 2001; Scragg, 2005; Okoh, 2006; Williams et al., 2006 and Hogan, 2010).

The classes of hydrocarbons mentioned above are based on physical properties from crude oil separation. Industrial compounds derived from hydrocarbons are mostly produced from crude petroleum oil, natural gas and coal. Naturally hydrocarbons are mostly produced by bacteria, animals and plants (El-Naggar et al., 2014). It is therefore

expected that these hydrocarbons compounds can be biodegradable by using a number of organisms.

Petroleum hydrocarbons comprises of compounds in complex combinations, therefore the rate petroleum degradation does not occur at the same. The rate at which microorganisms are able to degrade hydrocarbons to completion depends highly on their concentration and chemical structure.

Petroleum hydrocarbons are usually characterized into four fractions which includes (i) saturates, (ii) asphaltenes, (iii) resins and (iv) aromatics. It has been noted n-alkanes of intermediate length (C10-C25) are the preferred substrates for microbial degradation as they tend to be the most readily degradable compounds. Shorter chain compounds tend to be more toxic. Longer chain alkanes (C25-C40) are hydrophobic solids and therefore more difficult to degrade due to their poor water solubility and bioavailability. Branched chain alkanes and cycloalkanes also have a slower rate of degradation in comparison to normal alkanes.

The compounds that offer the highest resistance to biodegradation includes condensed aromatic and cycloparaffinic structures, bitumen, tars and asphaltic materials. These compounds have been reported to have the high boiling points which makes remediation efforts more expensive (Loeher et al., 2001; Ivancev-Tumbas et al., 2004; Brassington et al., 2007; Stroud et al., 2007).

2.3.1 Total petroleum hydrocarbons (TPHs)

TPHs are difficult to dispose of as they contain a mixture of nitrogen, aromatic, sulphur, alkane, asphaltenes fractions and compounds containing oxygen (Bhattacharya et al., 2003). Most TPHs that contaminate the environment are diesel, gasoline, and fuel oils and have similar chemical and physical properties and range between C6 and C25 (APIP, 2012). They are viscous and insoluble and as a consequence, any improper disposal of TPHs has dire negative environmental impact (Yuste et al., 2000).

2.3.2 Chemical composition of petroleum hydrocarbons

Petroleum comprises of a combination of various hydrocarbons. Alkanes (paraffin), aromatic hydrocarbons, cycloalkanes, and chemicals such as asphaltenes are the most commonly found molecules (Fig 1).

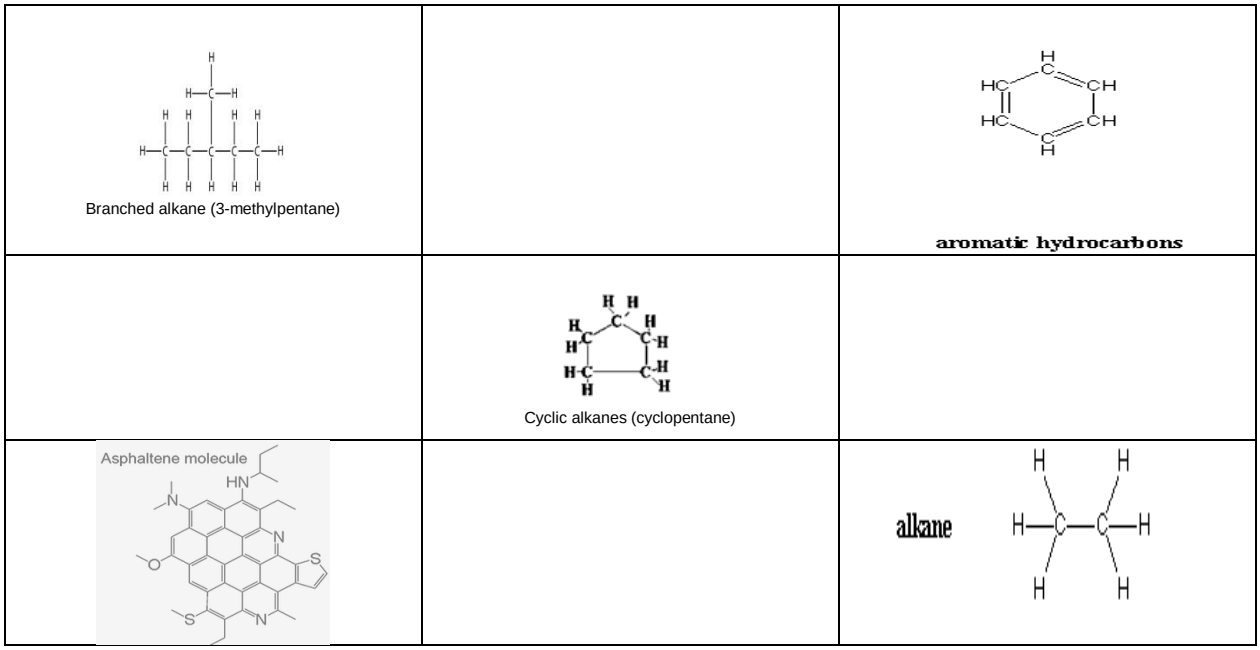


Figure 1: Chemical structures of common hydrocarbon molecules (Cava et al., 2011).

The alkanes generally called paraffins are referred to as saturated hydrocarbons. These compounds contain only carbon and hydrogen atoms and are either straight or branched chains, with a general formula of C_nH_{2n+2}. Molecules that contain 4 or less

carbon atoms are termed petroleum gasses. These are found in a gaseous state at room temperature. Gasoline is an alkanes ranging from pentane (C_5H_{12}) to octane (C_8H_{18}). Alkane compounds that range from nonane (C_9H_{20}) to hexadecane ($C_{16}H_{34}$) are refined to form diesel fuel, kerosene and jet fuel (Islam, 2015). Fuel and lubricating oil are formed by refining alkanes with more than 16 carbon atoms. Paraffin wax is an also an alkane, and has approximately 25 carbon atoms, whereas asphalt contains 35 or more carbon atoms. Modern refineries split these higher carbon-containing compounds into more valuable products (ThemaNord, 2003).

Cycloalkanes have similar properties to alkanes and are known as naphthenes. These compounds have higher boiling points due to them containing one or more carbon rings, to which hydrogen atoms are then attached as per the formula C_nH_{2n}. (ThemaNord, 2003).

Aromatic hydrocarbons consist of more than one planar six-carbon rings which are referred to as benzene rings, where hydrogen atoms are attached and have a formula C_nH_{2n-6} (Islam, 2015). They usually to burn with a black flame, and most may have a sweet aroma. Some aromatic hydrocarbons have been found to be carcinogenic. Fractional distillation is a process that separate these different molecules at an oil refinery to produce gasoline, kerosene, jet fuel, and other hydrocarbons (Hoel, 1981). Isooctane, is an aromatic hydrocarbon (2, 2, 4-trimethylpentane) and is used widely used in gasoline or petrol production. Its chemical formula is C_8H_{18} and is known to reacts with oxygen in an exothermic reaction (Surena, 2016).

Based on the chemical structure, various hydrocarbons vary in susceptibility to degradation. Various compounds have ranging orders of decreasing susceptibility from n-alkanes (e.g. ethane and methane), branched alkanes (e.g. 3-methylpentane), low-molecular-weight aromatics (e.g. naphthalene), to cyclic alkanes (e.g. cyclopantane) (APIP, 2012).

2.3.3 Physical state of petroleum hydrocarbons

Oil dissipation is an outcome of numerous chemical and physical processes that alter the oil components when a spill occurs. The processes are collectively known as weathering. Wind and wave action has the ability to cause oil-in-water or water-in-oil emulsions (Xhelilaj and Sinanaj, 2010).

When the hydrocarbons are dispersed in water as oil-in-water emulsions, there is an increase in the surface area of the oil, which makes it bioavailable for microbial attack. Contrastingly, large masses (or plates) of mousse can also form in the water body thereby adversely affecting microbial degradation as a result of low surface to volume ratios. Tar balls are huge masses of weathered oil and this form of oil slick also restrict the advent of microbial attack due to their limited surface area. This causes the oil to be weathered in different ways. Natural weathering may involve evaporation or water in oil emulsions which may ultimately lead to reduction in the oil slick (Xhelilaj and Sinanaj, 2010). Fig 2 and 3 show examples of oil spills.

Figure 3: Oil spill on land (Helm, 2015)

Figure 2**:** Oil spill on sea (Branork, 2008)

2.3.4 Effect of concentration of petroleum hydrocarbons

The rate of mineralization of the organic compounds by microbial populations in the aquatic environment depends on the concentration of the compound. In general, this conforms to Michaelis-Menten kinetics (Osei Owusu, 2015). These kinetics have been established for microbial uptake and the oxidation of toluene. This compound is a low molecular-weight aromatic hydrocarbon that has a relatively high water solubility. Therefore, Michaelis-Menten equations may not be applicable to more insoluble hydrocarbons. The rate in which higher molecular-weight aromatic hydrocarbons are mineralized, such as naphthalene and phenanthrene, are related to aqueous solubilities rather than total substrate concentrations (Derek, 2016). Microbial degradation of long (C12) alkanes chains that have solubilities which are less than 0.01 mg/L, occurs at rates which goes beyond the rates of hydrocarbon degradation (Sawadogo et al, 2014). The degradation rate also depends on surface area available on the hydrocarbon for emulsification or physical attachment by cells. As a result, rate of biodegradation of many hydrocarbons are not dependent on concentration. High concentrations of hydrocarbons can be related to heavy, undispersed oil slicks in water, this causes biodegradation to be inhibited by nutrient or oxygen restriction or

through toxic effects exerted by volatile hydrocarbons. Fusey and Oudot (1984) reported that contamination that occurs at seashore sediments with crude oil above a threshold concentration prevented biodegradation of the oil due to oxygen and/or nutrient limitation. It is expected that high concentrations of oil have similarly negative effects on biodegradation rates following oil spills in other calm, low-energy environments such as the beach, river, harbour, and small lakes or ponds, where the oil is rather protected from spreading due to wind and wavy weathers. There is a high of degradation rate of spilled crude oil that occurs from tankers in protected bays, and the most highest rate occurs areas here there is an extreme wave energy (Das and Chandran, 2011).

The theory of a maximum or starting concentration for microbial degradation of hydrocarbons may apply also to the soil ecosystems. Dibble and Bartha (1979) stated that increases in CO_2 evolution across a range of 1.25 to 5% hydrocarbon mass per dry weight of soil, was noted when organisms were applied to soil. No increase in CO_2 evolution was observed at a level of 10% supplementation, and the rates declined when supplementation was done to 15%. In the event of high oil loading concentrations, a decrease in activity was noted, and this inhibition could be ascribed to the toxic components present in the oil sludge.

2.4 Remediation technologies which enable hydrocarbon degradation

The remediation of oil discharged in water sources occurs via various mechanisms. These include mechanical processes, dispersion, washing and evaporation (Das and Chandran, 2011). These processes in some instances simply change the properties and behaviour of the oil and are not very effective in contaminant removal (Bako et al., 2008).

It has been observed that almost 47% of crude oil that has leakage into the marine environment, is cleaned up (Zabbey and Olsson, 2017). This raises the question on what the mechanisms that nature uses to clean-up these spills. Biological organisms including plants and bacteria have the ability to remediate toxic organic pollutants from both freshwater and environmentally polluted sites to levels that are acceptable for discharge (Nilesh and Hardik, 2013). It has been found that bioremediation technology has been strongly advocated as a suitable technique for treating oil contaminated sites due to its low cost, non-invasive and environmentally friendly (Jain and Bajpai, 2012). This technique has resulted in the complete degradation of organic (oily) contaminants into carbon dioxide, energy and water which are harmless to the environment or any water resources (Ojuederie and Babalola, 2017).

A redox reaction within microbial cells during biodegradation produced energy as an end product. This biodegradation process is dependent on the availability of nutrients and the optimum presence of other factors that support biological functions (Panda et al., 2013). The occurrence of microorganisms containing metabolic capabilities to remediate an oil spill is the most important requirement for bioremediation (Abatenh et al., 2017b). It has been found that communities which are exposed to hydrocarbons

in the environment are able to degrade more hydrocarbons than newly exposed species because of selective enrichment and genetic changes (Adams et al., 2015).

The modified bacterial populations can respond to the presence of hydrocarbon pollutants in a short period and display higher biodegradation rates than populations with no history of hydrocarbon contamination. Crude oil contains a mixture of compounds therefore a combination of different bacterial groups or consortium is necessary to degrade a broader range of hydrocarbons (Brzeszcz and Kaszycki, 2018). Table 1 shows a selection of bioremediation systems that have been used in different countries.

Table 1: Bioremediation carried out in different countries

COUNTRY	BIOLOGICAL SYSTEMS USED
USA	Pure or mixed cultures of *Bacillus, Clostridium, Pseudomonas,* and Gram-negative rods; mixed cultures of hydrocarbon degrading bacteria; mixed cultures of marine source bacteria; spore suspension of *Clostridium*; indigenous stratal microflora; slime-forming bacteria; ultra microbacteria
Russia	Pure cultures of *C. tyrobutiricum* bacteria mixed cultures; indigenous microflora of water injection and water formation; activated sludge bacteria naturally occurring microbiota of industrial (food) wastes
China	Mixed enriched bacterial cultures of *Bacillus*, *Bacteroides, Eubacterium, Fusobacterium, Pseudomonas* slime-forming bacteria: *Brevi bacterium viscogenes, Corynebacterium gumiform, Xanthomonas campestris*
Australia	Ultra microbacteria with surface active Properties
Bulgaria	Indigenous oil-oxidizing bacteria from water injection and water formation
Canada	Pure culture of *Leuconostoc mesenteroides*
Former Czechoslovakia	Hydrocarbon oxidizing bacteria(predominant *Pseudomonas sp*.);sulphate-reducing bacteria
England	Naturally occurring anaerobic strain, high generator of acids; special starved bacteria, good producers of exopolymers
Former East Germany	Mixed cultures of thermophilic *Bacillus* and *Clostridium* from indigenous brine microflora
Hungary	Mixed sewage-sludge bacteria cultures(predominant: *Clostridium*, *Desulfovibrio, Pseudomonas*)
Norway	Nitrate-reducing bacteria naturally occurring in North Sea water
Oman	Autochthonous spore-forming bacteria from oil wells and oil contaminated soil
Poland	Mixed bacteria cultures (*Arthrobacter*, *Clostridium*, *Mycobacterium*, *Peptococcus, Pseudomonas*)
Romania	Adapted mixed enrichment cultures(predominant: *Bacillus*, *Clostridium*, *Pseudomonas*, and other Gram-negative rods)
Saudi Arabia	Adequate bacterial inoculum according to requirements of each technology
The Netherlands	Slime-forming bacteria(*Beta coccus dextranases*)
Trinidad-Tobago	Facultative anaerobic bacteria high producers of gases
Venezuela	Adapted mixed enrichment cultures

Source: Lazar et al., 2007; Al-Wahaibi et al., 2012; Bahry et al., 2013, Al Sulaimani 2010, Alsulaimani, 2011, Al-Wahaibi, 2013)

2.5 Effect of hydrocarbon availability on bioremediation

Biodegrading hydrocarbons is a composite process which mainly depends on the amount of the hydrocarbons that are present in the environment and the physical nature. The main limiting factor in the degradation of petroleum pollutants is that they are less available to microorganisms (Al-Hawash et al., 2018).

Microorganisms are capable of transporting bioavailable compounds such as alkanes, aromatics, heterocyclic polar compounds and asphaltenes through the membrane during degradation. These compounds are typically attached to the organism. These hydrocarbons are nonpolar compounds, which means their solubility in water is quite low; they are therefore simply adsorbed onto soil, which makes them less available (Pourfakhraei et al., 2018).

2.6 Types of bioremediation

Bioremediation can be clustered into 4 groups based on the organisms/ species actively involved in the process. These are:

2.6.1 Phycoremediation

Phycoremediation is a type of remediation that uses macro or micro-algae to remove or bio transform pollutants from wastewater and carbon dioxide from waste air. The phycoremediation process can be operated as a continuous, a semi continuous batch or batch wise (Hanumantha Rao et al., 2011). A research study was carried out by Jamaian et al. (2017) to access the depletion of the nutrient by microalgae *Botryococcus sp*. from the wastewater using a Verhulst model. This model has been certified with the experiments of microalgae *Botryococcus sp*. grown in domestic and

palm oil wastewater. The results suggested that microalgae *Botryococcus sp*. could be cultured in domestic and palm oil wastewater while nutrients are reduced from these wastewaters (Jamaian et al., 2017).

2.6.2 Phytoremediation

Phytoremediation is the action of using plants to treat environmental pollution without isolating or digging it out from the site of pollution. This remediation includes bioaccumulation of pollutants such as crude oils and its derivatives metals, explosives, solvents, and pesticides (Meagher, 2003).

2.6.3 Mycoremediation

Mycoremediation is a form remediation that uses fungi to degrade contaminated environmental sites. Fungi use mycelia to decompose pollutants. Certain fungi are hyper-accumulators, which enables them to absorb and concentrate heavy metals. Fungi are able to degrade PAH, chlorinated compounds, aromatic pollutants, and other toxic wastes (Ma et al., 2018).

2.6.4 Bioremediation using microorganisms

Bioremediation is the use of bacteria to degrade hydrocarbon. Some bacterial isolates have the ability to biodegrade or alter the chemical substances that are part of petroleum products to maintain their life cycle. There are numerous genus that are reported as hydrocarbon degraders, such as *Bacillus, Flavobacterium, Proteus, Pseudomonas, Aeromonas, Micrococcus, Klebsiella, and Acinetobacter* (Ripley et al., 2002).

Some microorganisms can utilize the hydrocarbons as sole carbon sources for getting their energy and metabolic activities (Jyothi et al., 2012). Biodegradation is a complex process that is highly dependent on the environment and also on the quantity of the hydrocarbons that are present (Das and Chandran, 2011).

2.7 Hydrocarbon bioremediation mechanisms and products

Biodegradation uses microorganisms to mineralize organic contaminants, this is done through metabolic or enzymatic processes, they are converted into less harmful or non-hazardous substances, which are then included into natural biogeochemical cycles. There are two biodegradation mechanisms that are responsible for the degradation of organic material: (1) aerobically, with oxygen, or (2) anaerobically, without oxygen. The anaerobic process is conducted by anaerobic microorganisms and this pathway of biodegradation is very slow (Truskewycz et al., 2019). Anaerobic biodegradation follows different biochemical pathways which depend on the electron acceptor used by the microorganism (Vogt et al., 2011).

The most effective and complete degradation process of organic pollutants is under aerobic conditions. The initial intracellular occurrence of organic pollutants is an oxidative process; the activation and the incorporation of oxygen is the key enzymatic reaction which is catalyzed by oxygenases and peroxidases (Widdel and Rabus, 2001). Degradation mechanism transforms organic pollutants methodically into intermediates of the central intermediary metabolism, for example, the tricarboxylic acid cycle. Specific enzyme systems can mediate the degradation of petroleum hydrocarbons. Other mechanisms involved include the attachment of microbial cells to the substrates as well as biosurfactant production. The first mechanism regarding

is still largely unknown however, biosurfactant production has been extensively (Kothari et al., 2014).

2.8 Enzymes participating in the degradation of hydrocarbons

The presence of a xenobiotic metabolising enzymes permits microbial populations to degrade composite hydrocarbons. Some enzymes modify or degrade petroleum hydrocarbons (Das and Chandran, 2011). Their genomic variety contributes to the metabolic flexibility of microorganisms for the conversion of contaminants into less-toxic final products, which are then included into natural biogeochemical cycles. The principal benefit of the contaminant-degrading process is the complete mineralisation of compounds, as well as biomass formation (Al-Hawash et al., 2018). The biodegradation efficiency of petroleum contaminants is influenced by several biotic and abiotic factors. These factors include the existence, activity and effectiveness of petroleum-degrading microorganisms in the environment, the availability and concentration of petroleum as well as nutrient, salinity and temperature conditions (Depot, 2017).

Various microorganisms, such as bacteria, fungi, cyanobacteria, and green algae, are able to degrade different components of petroleum under diverse environmental conditions (e.g. aerobic and anaerobic conditions at varied salinities and pH). The enzymatic apparatus provides these capabilities to microorganisms. The degradation of Petroleum take place progressively by sequential metabolism of its compounds. The genes involved in petroleum-degrading enzyme production may be located on chromosomal or plasmid DNA (Akpe et al., 2013).

Enzymes that degrade alkanes are called alkane hydroxylases and they are dispersed among many different species of yeast, fungi, bacteria, and algae (Van Beilen and Funhoff, 2007). Additionally, van Beilen and Funhoff (2007) projected three classifications of alkane-degrading enzyme systems: C1–C4 (methane to butane, oxidised by methane-monooxygenase-like enzymes), C5– C16 (pentane to hexadecane, oxidised by integral membrane non-heme iron or cytochrome P450 enzymes), and C17+ (longer alkanes, oxidised by essentially unknown enzyme systems). They then reported the structures, cofactors, substrate ranges, and existence of the core groups of alkane hydroxylases; soluble methane monooxygenase (sMMO), particularly methane monooxygenase (pMMO), AlkB-related alkane hydroxylases, eukaryotic P450 (CYP52, class II), bacterial P450 oxygenase system and dioxygenase (CYP153, class I). Furthermore microorganisms that are capable of degrading alkanes can contain numerous alkane hydroxylases and can therefore consume different substrate ranges (Ehsan et al., 2014). One of the most studied alkane degradation pathways is that described for *Pseudomonas putida* Gpo1, encoded by the OCT plasmid (Grant et al., 2014). In this instance, the conversion of an alkane into an alcohol is first facilitated by a membrane monooxygenase, soluble rubredoxin, and rubredoxin reductase. Van Hamme and colleagues (Singh et al., 2015) presented a model for alkane metabolism in gram-negative bacteria and described the locations and functions of the ALK gene products.

The catechol dioxygenase group of bacterial enzymes that contain irons is an example of an enzyme class which is involved in the degradation of aerobic aromatic hydrocarbons. These enzymes are capable of catalysing the addition of molecular oxygen atoms to 1, 2-dihydroxybenzene (catechol) and its derivatives, with successive

cleavage of the aromatic ring (Singh et al., 2015). Catechol dioxygenases enzymes are responsible for the cleaving in aromatic ring and also for the wide selection of microorganisms which are capable of degrading aromatic compounds (Fathepure et al., 2014).

Although biodegradation of petroleum is faster in aerobic environments than in anaerobic environments, biodegradation in anaerobic conditions is necessary because oxygen is limited in certain environments, such as in mangroves, aquifers, and sludge digesters (Peixoto et al., 2011). In anaerobic metabolism, usually aromatic compounds are transformed into benzoyl-CoA, which is the aim of the benzoyl-CoA reductase (BCR) action (Hosoda et al., 2006). It has been found that the degradation pathways converge to benzoyl-CoA, and is dependent differing environmental conditions and terminal electron acceptors such as sulphate, nitrate and iron (Fe III). (Truskewycz et al., 2019). The respective enzymatic reactions involved in the degradation of hydrocarbons are presented in Figure 4.

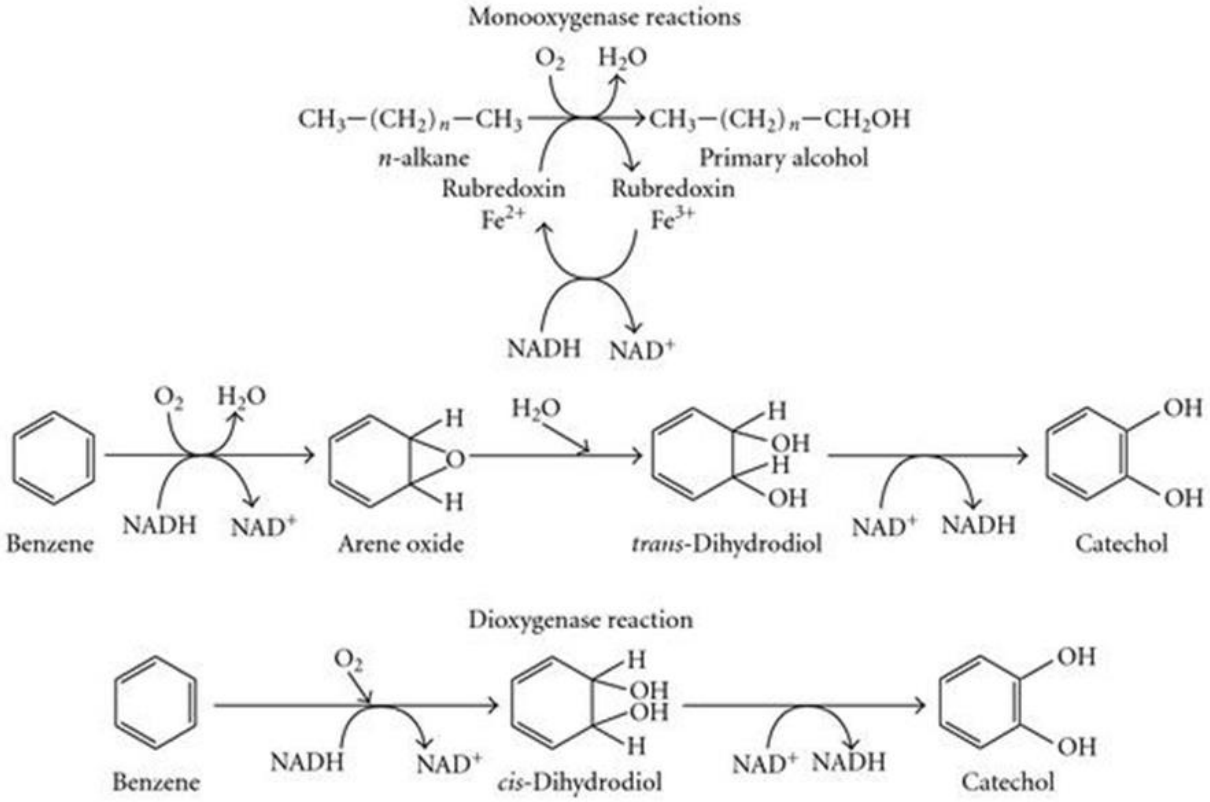


Figure 4: Enzymatic reactions involved in the processes of hydrocarbons degradation (Truskewycz et al., 2019) .

Numerous enzymes are accountable for the biodegradation of hydrocarbons. Methane monooxygenase is part of the degradation of short-chain length alkanes (C_2–C_4), while the degradation of medium n-alkanes (C_5–C_{17}) is carried out by the activity of Alk-B gene which encodes the enzyme non-heme alkane monooxygenase. Multiple alkane hydroxylases have been listed in the degradation of long n-alkanes > C_{18} (Parthipan et al., 2017).

Cytochrome P450 alkane hydroxylases is an enzyme that constitute a super family of ubiquitous heme-thiolate monooxygenases, which they play an significant role in the microbial degradation of oil, chlorinated hydrocarbons, fuel additives, and many other compounds (Van Beilen and Funhoff, 2007). Depending on the length of the chain, enzyme which are involved in the system are required to introduce oxygen in the substrate to initiate biodegradation. Higher eukaryotes mostly comprise of numerous different P450 families that consist of an excessive quantity of distinct P450 forms that may contribute as a collective of isoforms to the metabolic conversion of a given substrate. In microorganisms such P450 multiplicity can only be found in limited species (Ehsan et al., 2014). Cytochrome P450 enzyme systems were found to be involved in biodegradation of petroleum hydrocarbons.

Peixoto et al., (2011), conducted a study where the role of the degradative enzymes in biodegradation of the crude oil was studied. *Bacillus subtilis* A1 produced great quantities of hydrocarbon degrading enzymes and biosurfactants in the existence of crude oil as a substrate. Optimum growth conditions for biosurfactant production included sucrose and yeast extract as best carbon and nitrogen sources, respectively and pH 7.0, 40°C. The amount of biosurfactants that was produced with these enhanced conditions was 4.85 g l^{-1} and the produced biosurfactant was lipopeptide in nature and exhibited high emulsification activity. The efficiency of biodegradation of the crude oil was 87% which was related with high production of biosurfactant, alkane hydroxylase and alcohol dehydrogenase enzymes. This strain had degraded the low molecular weight hydrocarbons to completion (C10-C14) and displayed up to 97% degradation of high molecular weight hydrocarbons range between C15-C19. These results demonstrate that *B. subtilis* A1 is an effective crude oil degrading bacterium,

and that the respective crude oil hydrocarbons that were available for degradation as a result of hydrocarbon degrading enzymes, or emulsification or biosurfactant production capabilities of the organism. Furthermore, this organism A1 could be applied in the bioremediation of crude oil or environments contaminated with PAH (Peixoto et al., 2011).

2.9 Microbes associated with petroleum bioremediation and their effect on bioremediation

Hydrocarbons naturally occur in low concentration environments, and a number of hydrocarbon degrading organisms are present in different sites such as soil, marine, and fresh water (Das and Chandran, 2011). These organisms include bacteria, cyanobacteria, yeast, as well as fungi. Hydrocarbon degraders which are bacteria are isolated from soil using enrichment techniques include *Arthrobacter, Pseudomonas, Rhodococcus and Nocardia,*species (Spini et al., 2018). The microorganisms that are able to degrade oil include *Pseudomonas*, various *Corynebacterium*, Mycobacteria and some yeast (Sasikumar and Papinazath, 2016). These hydrocarbon degraders develop and become a principal fraction of the population in these environments. Microorganisms are the environment's creative recyclers. Their ability to convert synthetic chemicals into compounds which can be used to generate energy and raw materials for their own development means that costly chemical or physical degradation processes can be interchanged with biological processes that are cost effective and are more environmentally friendly.

Microbes can be an attractive source of innovative environmentally based biological technologies. Studies continues to authenticate the biodegradation prospective of

microorganisms (Sasikumar, 2016). These findings propose that further research of bacterial diversity will probably lead to the discovery of other numerous organisms with exclusive characteristics useful in biodegradation. Microbes that are able to biodegrade pollutants tend to increase in numbers when the contaminant is present. Such microbes will continue to be important because oil leakage into the aquatic environment and contaminated wastewater is now a continuous occurrence (Sasikumar and Papinazath, 2003). Numerous microorganisms can use oil as a nutrient and a lot of the organisms produce non-toxic and biodegradable emulsifiers that facilitate the remove of oil in water.

The rate at which the organism grow and hydrocarbon degradation ability are not detected immediately. The microorganisms must first adapt to the new environment (Alrumman et al., 2015). When there is no change in hydrocarbon concentration due to microbial activity, the stage is called the lag phase. The lag phase is different and depends on the type and number of microorganisms and type and concentration of hydrocarbon present. For the lag phase to be reduced, identified organisms which are able to degrade hydrocarbons are incorporated into the contaminated environment. The effects of adding an organism differs and it depends on the type of the microorganism as well as the conditions of the environment which includes the growth substrate. (Kanaly, 2000). When adding commercial bioaugmentation products, the rate of hydrocarbon degradation in soil may decrease if a carrier molecule is introduced which is also a source of carbon for the organism. Only after the carrier is degraded do the organisms turn to oil degraders (Ramadass and Catz, 2016).

2.10 Effect of biosurfactant on biodegradation

Biosurfactants are compounds that are produced by microorganisms on a surface. They express a variation of surface activities that increase the bioavailability of organic pollutants, including carbon and oxygen components, and as a result to increase biodegradation (Antoniou et al., 2015). Biosurfactants increases the rate of biodegradation of hydrocarbons that have low solubility hydrocarbons which produced only when there is a limited amount of low solubility hydrocarbon substrates that are available. These organic compounds are part of a structurally diverse group of amphiphilic biomolecules, and they have both hydrophobic and hydrophilic properties. They generally are clustered either as low or high molecular weight biosurfactants; the anterior consisting of glycolipids and lipopeptides and the latter of high molecular weight polymeric biosurfactants. Due to their high rate of biodegradability and low toxicity levels they are capable for use in remediation technologies as an alternative to the synthetic surfactants (Jain et al., 1992).

The key purpose of biosurfactants when it comes to the degradation of hydrocarbon it is to increase the solubility and bioavailability of hydrocarbon. The addition of the biosufactant into the solution, the surfactant molecule increases the solubility of the hydrocarbon by facilitating the oil water interface (Patowary et al., 2016). In biosufactants, the hydrophobic end is aligned with hydrocarbon molecule, whereas the hydrophilic end of the molecule is aligned with the water forming micelle (Ward, 2010). A high level of solubility permits for quicker *in-situ* biodegradation resulting in more effective pump and treat methods. The advantages of using biosurfactants instead of synthetic surfactants is because of their biodegradability, resistance to environmental changes, diversity, and low production costs (Mulligan, 2005). Synthetic surfactants

are frequently unsuccessful in bioremediation studies because they modify the hydrophobicity of the surfaces, solubilises organic matter and hydrocarbons within the micelles, thus intensely complicating an already complicated system (Banat et al., 2010). Foght and Westlake (1986) investigated the result of adding a synthetic surfactant on degradation of crude oil. The results indicated that the surfactant provides another source of carbon for the microorganisms and slowed down the degradation process. Shreve et al. (1995) reported that a purified biosurfactant cab be a better surfactant as it produces a better micro emulsion. It has also been shown that biosurfactants had lower toxicity to the organism (Foght et al., 1996).

2.11 Factors Influencing petroleum hydrocarbon bioremediation

An effective application of bioremediation technologies in a contaminated area is mainly dependent on the nature of the contaminated site due to the composite system of factors that affect the biodegradation process (Yadav and Hassanizadeh, 2011). The primary factors that affect the overall rate of biodegradation include, oxygen, temperature, pressure, water activity, salinity, pH and availability of nutrients (Liu et al., 2017). It is important to understand the dynamics of this complex system and thereafter plan and implement a particular bioremediation strategy (Liu et al., 2017).

2.11.1 Effect of temperature on biodegradation

The main factor in hydrocarbon degradation is temperature among other physical factors, it directly affects the diversity of the microbial flora, the physiology as well as the chemistry of the pollutants (Duran and Cravo-Laureau, 2016). Even though hydrocarbon biodegradation can occur over an extensive range of temperatures, the rate of biodegradation usually decreases with decreasing temperature. In addition to

viscosity and volatility, temperature also affects the solubility of hydrocarbons (Yuniati, 2018). The viscosity of the oil increases at low temperatures, while the volatility of the toxic low molecular weight hydrocarbons is reduced, therefore delaying the biodegradation (Nilanjana and Chandran, 2010). High temperatures increase the rates of hydrocarbon degradation extremely, usually in the range of 30 to 40°C, above which the membrane toxicity of hydrocarbons is increased. Thermophilic alkane-utilizing bacteria do exist, however, environmental conditions and season would be probable to select for different populations of hydrocarbon-utilizing microorganisms which are modified to ambient temperatures (Xu et al., 2018)

2.11.2 Effect of oxygen on biodegradation

Bacteria and fungi require molecular oxygen to catabolise cyclic, aliphatic and aromatic hydrocarbons using oxygenases for the oxidation of the substrate (Das, 2011). In the upper levels of the water column, in both marine and freshwater environments, oxygen limitation does not usually prevail. However, in aquatic sediments, conditions are anoxic with the exception of a thin layer towards the surface of the sediment (Pakhomova et al., 2014). Oxygen availability in soils is dependent on soil type, whether soil is waterlogged or not, oxygen consumption rate of organisms as well as the presence of substrates that could be used and as a result lead to the depletion of oxygen. The concentration of oxygen in either land based or aquatic systems has been found to be a critical rate-limiting variable involved in petroleum degradation in soil as well as groundwater (Neira et al., 2015).

The significance of anaerobic biodegradation of aromatic hydrocarbons in the environment is unidentified, and further research is essential to elucidate anaerobic pathways, and also to determine whether other hydrocarbons, such as alkanes, and hydrocarbon mixtures, such as crude oil, can be degraded to completion under denitrifying or methanogenic conditions (Laso-Pérez et al., 2019). Anaerobic degradation of petroleum hydrocarbons by microorganisms has been noted in some studies to occur only at insignificant rates, and its environmental significance has generally been considered to be minor (McGenity et al., 2012a).

2.11.3 Effect of salinity on biodegradation

Several studies have demonstrated that a suitable salt concentration can promote the biodegradation of hydrocarbons or crude oil (Xu et al., 2018). However, there are limited sources of literature that focusses on the effects of salinity on hydrocarbon degradation by microorganisms. In general, a positive correlation has been found in estuarine sediments between salinity and mineralization rates for both phenanthrene and naphthalene (Shiaris, 1989).

Olajide and Ogbeifun, (2010) isolated a *Proteus vulgaris* strain from a fish sample and found that the *P. vulgaris* could tolerate 20 g/L NaCl and showed the best degradation efficiency of light crude oil at 10 g/L NaCl. *Rhodococcus* sp. isolated from contaminated soil was studied by Li et al. (2011). This bacterium degraded crude oil as the carbon source over a range of salinities from 5 to 30 g/L NaCl. The biodegradation efficiency decreased from 65% at 5 g/L NaCl to 50% at 30 g/L. Another halotolerant strain, *Alcanivorax* sp. was isolated by Dastgheib et al. (2011) from saline soil to utilize TPH at a wide range of salt concentrations. They observed the

degradation was 24.8% in a medium without added salt and around 10% at 125 g/L NaCl, reaching the highest biodegradation (26.1%) at 25 g/L. Chen et al. (2012) isolated a strain of *Virgibacillus* sp. from drilling wastewater and checked the effect of salt on biodegradation of crude oil at salinities from 5 to 200 g/L NaCl. The results showed that optimal degradation occurred at 50 g/L NaCl; when the salinity was lower than 50 g/L, the degradation rate increased with increasing salt concentration. However, the degradation rate decreased dramatically once the salinity was higher than this value. Wang et al. (2014) investigated the ability of *Acinetobacter sp*. to degrade crude oil at 10-50 g/L NaCl. The best degradation result occurred at 10 g/L NaCl (58%), however, it decreased to 38% degradation at 50 g/L NaCl. These results demonstrated that the best biodegradation result was usually not achieved at the no salt condition, but probably at a physiological salt concentration due to the optimal activity of some enzymes being reached at the optimal salt concentration.

2.11.4 Effect of pressure on biodegradation

The significance of pressure as a variable in the biodegradation of hydrocarbons is most probably limited to the deep-sea environment (Ferguson et al., 2017). Studies conducted by Schedler et al. (2014) have reported the effects of pressure on biodegradation in which the degradation of, hexadecane, tetradecane, and a varied hydrocarbon substrate by a mixture of culture deep-sea sediment bacterial culture of was checked at 1 atm (ca. 101 kPa) and 495 or 500 atm (ca. 50,140 or 50,650 kPa). At 4°C, 94% of the hexadecane was used only after incubating for 40-week under conditions of high pressure, compared with 8 weeks at 1 atm.

2.11.5 Effect of water activity on biodegradation

The water activity (a_w) of soils can range from 0.0 to 0.99, contrary to aquatic environments where water activity is steady at a value near 0.98. Hydrocarbon biodegradation in terrestrial environments may consequently be limited by the available amount of water for microbial growth and metabolism (Das, 2011). Diniz (2016), in a study of oil sludge degradation in soil, reported optimal rates of biodegradation at 30 to 90% water saturation.

2.11.6 Effect of pH on degradation

Contrary to most marine environments, soil pH can be highly adjustable, ranging from 2.5 in mine spoils to 11.0 in alkaline deserts (Chandra et al., 2013). Most heterotrophic bacteria and fungi support a pH near neutrality, with fungi being more tolerant of acidic conditions (Sangeetha et al., 2017). An increase in pH, as can be detected in some soils, would as a result be expected to have a negative effect on the ability of microbial populations to degrade hydrocarbons (Chandra et al., 2013). Affandi et al. (2014) reported an immediate expansion of rates on the biodegradation of gasoline in an acidic (pH 4.5) soil by altering the pH to 7.4. Rates decreased significantly, however, when the pH was further increased to 8.5. Similarly, an optimal pH of 7.8 was observed for the mineralization of oily sludge in soil (Leahy and Colwell, 1990). Special or extreme environments such as salt marshes, may have extreme conditions for pH, and in some instances the pH of sediments may around 5.0 (King and Lester, 1995). Studies conducted by Sawadogo et al. (2014) indicated that the microbial mineralization rates of octadecane and naphthalene decreased at pH 5.0 in comparison to a pH level of 6.5. Rate of octadecane mineralization increased when

the pH of the environment was increased from 6.5 to 8.0, contrastingly, the rate of naphthalene mineralization did not improve.

2.11.7 Effect of nutrients on biodegradation

In general, microorganisms need various amounts of nutrients to live and grow. The most important nutrients required for metabolic activity are carbon, hydrogen, nitrogen, and oxygen (Chen et al., 2003).Other micronutrients that are required by organisms include, calcium, phosphorus potassium, sodium, sulphur, magnesium, and iron. Other trace requirements include, manganese, tungsten, molybdenum, cobalt, copper, nickel, zinc, and selenium (Chitturi et al., 2015).

Hydrocarbons only provides carbon and hydrogen to microbial cell as their feed substrate. In order for these organisms to grow and multiply they must obtain the remaining nutrients from environment. (Joutey et al., 2014). These nutrients, if in low-level amounts could affect the biodegradation processes. In the instance of an oil spill, carbon is highly available, however, nitrogen and phosphorus concentration could be the limiting factor for oil degradation (McGenity et al., 2012b). In freshwater systems, such as wetlands, the environmental plants in that specific ecosystem may have a high nutrient demand, as a result, there could be limited availability of nutrients for microbes (Bornette and Puijalon, 2011). Therefore, in order to enhance the biodegradation of oil pollutant in a freshwater oil spill, augmentation of additional nutrients are necessary. The presence of essential nutrients in the contaminated environment significantly improves degradation rates (Tyagi et al., 2011). Nutrients can be added to the contaminated site in order to increase the rate and extent of oil degradation. The non-availability of phosphate and nitrogen significantly delays time

and reduces the degree of oil degradation (Das and Chandran, 2011). In conclusion, the existence of macro and micronutrients in an environment is vital for a successful and complete hydrocarbon biodegradation. Addition of nutrients by these hydrocarbons to the environment has shown to increase biodegradation rates in many cases (Lang, 2016).

Conclusion

The purpose of this chapter was to review petroleum pollution and the disadvantages it has on the environment. Many technologies have been developed to clean-up the environments contaminated by petroleum. It is clear from the research reviewed that one of the most popular and cost-effective technologies is bioremediation, which uses microorganisms to degrade hydrocarbons. This review has provided the types of microorganisms that are involved in the bioremediation technology and some studies show the different consortiums of microorganisms used in hydrocarbon degradation. These include fungi, yeast and bacteria. In this study, the focus was on the use of *Bacillus* consortia to degrade petroleum hydrocarbons.

Chapter 3

3. METHODOLOGY

3.1 Sampling, isolation and basic characterization of indigenous *Bacillus spp* that could be used to degrade hydrocarbons

3.1.1 Environmental sampling

Samples were collected from various petroleum oil contaminated sites around the Tshwane region in South Africa (Figure 5).

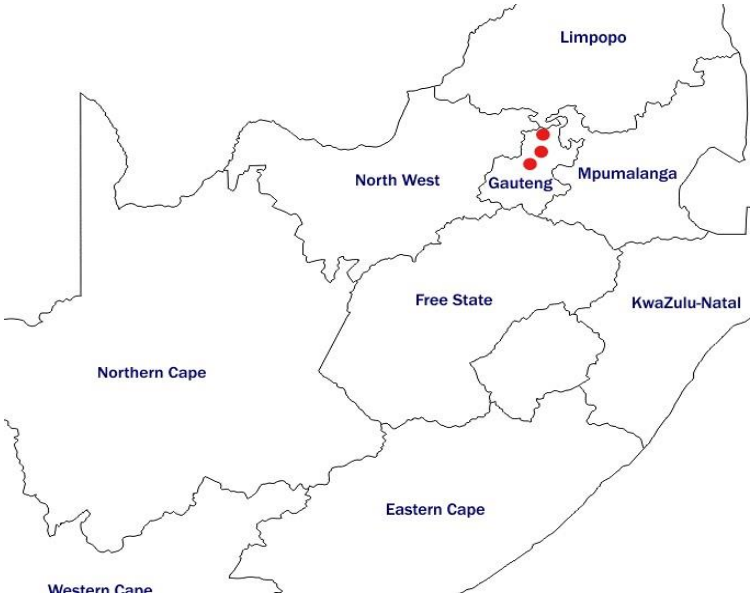


Figure 5: South African map with location of strain isolation

The sites included bus and taxi ranks, car workshops, city drains and harbours. Example of sampling sites are shown in Figure 6. Before sample collection, a sampling kit was prepared which included personal protective equipment (PPE) and sampling equipment (Table 2). Water samples were collected using sterile and labelled Falcon tubes. Soil samples were collected using a spatula wiped clean with 70% ethanol

(Proton Chem, Durban, South Africa) and transferred into sterile and labelled McCartney bottles. Liquid samples such as oil spills from car workshops were collected using a sterile swab kit. After collection, all the samples were labelled according to the sample site and GPS coordinates. All the samples were placed in a cooler box containing ice to keep them at the desired temperature and brought back to the laboratory then stored at 4°C until further analysis.

Bus and taxi ranks **Car workshops** **City drains** **Harbour**

Figure 6 : Petroleum contaminated environmental sites

Table 2: Sampling kit and personal protective equipment

Equipment Name
Personal Protective Equipment
Lab coat
Gloves
Goggles
Mouth mask
Sample collection
50 mL falcon tubes
Sterile McCartney bottles
Swabs
Spatula
70% ethanol
Paper towel
Styrofoam cooler
Stationary kit
Sample Labels
Pen
Marker
Log sheet

3.1.2 Isolation of spore-forming *Bacillus* isolates from environmental samples

A sterile (autoclaved for 15 min at 121ºC) 1000 mL Erlenmeyer flask containing 100 mL of sterilized sporulation media that was prepared using the following components: 10 $g.L^{-1}$ yeast extract (8013-01-2, Biolab, Merck, South Africa), 5 $g.L^{-1}$ peptone water buffered (1072280500, Biolab, Merck, South Africa), 50 $mg.L^{-1}$ $MnSO_4.4H_2O$ (CAS10101-68-5, Merck, South Africa), 100 $mg.L^{-1}$ $CaCl_2.4H_2O$ (CAS25094-02-4

,UniLAB, South Africa) and 500 mg.L^{-1} $MgSO_4.7H_2O$ (CAS10034-99-8, Minema, South Africa) adjusted to pH 7 was used to cultivate all the collected samples.

All the collected samples were inoculated into flasks containing sporulation media under the biosafety cabinet. Liquid samples were mixed using a vortex mixer and 1 mL of the sample was inoculated into the flasks containing sporulation media using a sterile disposal pipette. The solid samples were weighed to 1 g and aseptically inoculated in the Erlenmeyer flasks. Swab samples were inoculated by cutting the cotton bud part using a scissor that was wiped with 70% ethanol. The swab samples were then aseptically transferred to the Erlenmeyer flasks containing sporulation media. All the inoculated Erlenmeyer flasks were then incubated at 30°C on a rotary shaker set at 180 rpm. After 24 hours, the samples were checked for sporulation efficiency by taking a sample aseptically once daily until a range of 90-100% was reached. Sporulation efficiency was counted via a microscope using a haemocytometer (Thoma® counting chamber, Hawksley and Sons, London). Sporulation efficiency was calculated using the formula below:

$$Sporulation\ efficiency\ = \frac{No\ of\ spores}{Total\ no\ of\ cells\ (no\ of\ spores + no\ of\ cells)} \qquad \textit{Equation1}$$

Flasks containing spore cultures were used to isolate only spore forming *Bacillus* spp. A volume of 4 mL from each flask was transferred into 50 mL corning® centrifuge tubes. Ethanol (50% $v.v^{-1}$) was added to each falcon tube and made up to a volume of 20 mL. The contents were centrifuged at 10 000 x*g*, the supernatants were decanted and the resultant pellets were incubated at 105°C in a convection oven (Series 2000, Scientific) for 5 minutes. This step facilitated the evaporation of any residual ethanol.

The dry pellets were then reconstituted into 20 mL of sterile distilled water and serially diluted to 10^{-1} in 10^{-4} increments.

Thereafter, aliquots (0.1 mL) of each serial dilution were spread onto nutrient agar (Biolab, Merck) plates and incubated at 30°C and incubated base-up for 48 hours. Nutrient agar was prepared in Duran® laboratory bottles by adding 28 g of nutrient agar (Merck, Darmstadt, Germany) to 1000mL distilled water and sterilized for 15 minutes at 121°C in an autoclave. After sterilization the agar was left to cool to approximately 60°C then supplemented with antibiotic polymyxin B (10 $mg.L^{-1}$) (Sigma Aldrich, USA). Thereafter it was poured into petri dishes and left to solidify at room temperature. The method used to isolate spore forming *Bacillus* spp from environmental samples was adapted from methods outlined by Lalloo et al. (2007).

3.1.2.1 Purification of spore forming *Bacillus* isolates

Colonies formed on culture plates from isolation were first purified using a three stage passage method to get pure colonies. Passaging involves using a four way streaking technique to isolate single distinct colonies. Colonies were purified by streaking onto agar plates until only one colony type was visible on the agar plates. Purification was done by flaming a loop then a single colony was picked from the plate with mixed culture. A single colony was transferred into a fresh agar plate by performing a four-way streak. This colony was purified by repeating the streaking methods three times (Figure 7).

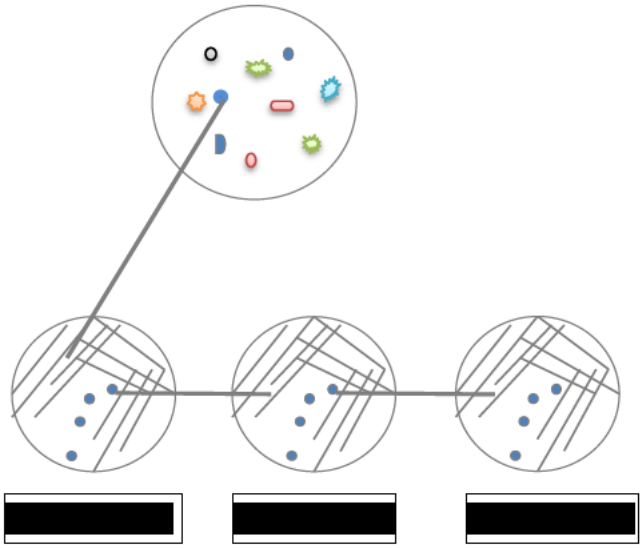


Figure 7 : An illustration of how microorganisms are purified from a mixed culture on an agar plate to a pure culture.

Using the information in figure 8, the morphology of each colony was classified. The Shape, margin, elevation, sixe, texture, appearance, pigmentation and optical property of each colony were explained according to figure 8 below.

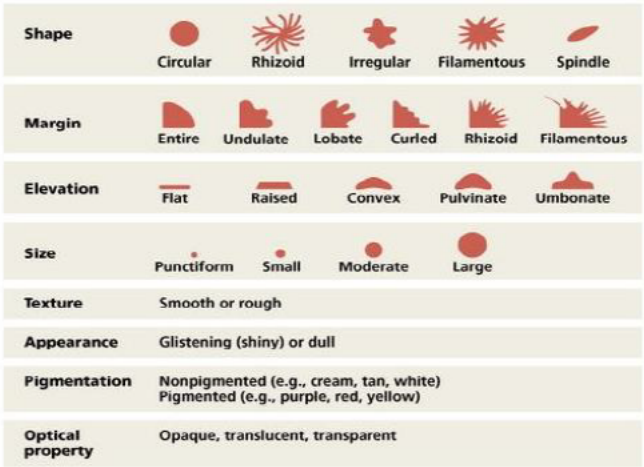


Figure 8: Morphological description of colonies (Nishiyama et al., 2010)

3.2 Screening and selection of indigenous *Bacillus* spp that could be used to degrade hydrocarbons

3.2.1 Presumptive tests to screen for *Bacillus* spp (primary screen)

Gram staining also referred to as "Gram's Method" is a technique involving the staining of cells that can be used to differentiate bacterial species into two large groups: Gram positive and Gram negative. The required components are presented in Table 3. This technique was employed to potentially differentiate *Bacillus* (Gram positive) from non-*Bacillus* bacteria (Gram negative).

Table 3: Components of Gram's Method

Solution	Role
Crystal violet (Merck)	Primary stain
Gram's Iodine (Merck)	Binds to, and traps crystal violet inside the bacterial cell
Ethyl alcohol (Merck)	Decolouriser
Safranin (Merck)	Counterstain

Purified isolates were subjected to gram staining which separates bacteria into two categories based on cell wall composition (Claus, 1992). Gram stain was carried out as described by (Beveridge, 2001). Bacteria able to retain the crystal violet-iodine complex and remain violet are referred to as gram positive while bacteria that get decolourised and subsequently take up the counterstain (Safranin) are referred to as gram negative. *Bacillus* species are gram positive (Amin et al., 2015).

All purified colonies were subjected to a qualitative catalase activity test used to determine the presence of the catalase enzyme in bacteria. This involved saturating the colonies with 3% hydrogen peroxide (H_2O_2). The 3% solution was prepared by dissolving 3 mL of 100% hydroxide peroxide (Barrs Pharmaceutical Industries) solution in 97 mL of distilled water. A drop of water was transferred to a clean glass slide. Using a loop, a colony was picked, and mixed with the drop of water on the slide. A few drops of 3% hydrogen peroxide were added on the smear. Presence of the *catalase* enzyme was indicated by the evolution of oxygen bubbles. The enzyme reaction on the slide is represented by the equation below:

$$2H_2O_2 + catalase \rightarrow 2H_2O + O_2 \qquad \textit{Equation 2}$$

This technique was employed to potentially differentiate *Bacillus* (catalase positive) from non-*Bacillus* bacteria (catalase-negative).

3.2.1.1 Spore form test

Purified single colonies were used to inoculate 100 mL of sporulation media (as described in Section 3.1.2). After 48 hours of growth, each sample was microscopically investigated for the presence of spores as described in Section 3.1.2. This technique was employed to potentially differentiate *Bacillus* spore formers from non-spore forming bacteria.

3.2.2 Secondary screen assay for hydrocarbon utilising bacteria isolated from selected areas in Gauteng Province

3.2.2.1 Determination of culture age and optical density (OD)

Culture age (hours) was determined by recording the time of inoculation until the time at which the screen was performed. The OD of the culture was measured at wavelength 660 nm by diluting appropriately in order to obtain OD below 0.900. If the OD of the culture was higher than 4.0, the culture was diluted using the following equation in order to adjust to the correct OD at which the screen is performed:

$$\left(\frac{desired\ OD}{Actual\ OD}\right) * 2 = \textit{Amount of whole broth suspension to be made up to 2 mL with Bushnell Haas media}$$

Equation 3

3.2.2.2 Rapid colorimetric screening using the dye 2,6 dichlorophenol-lindophenol (DCPIP)

The method as described by Hanson et al. (1993) was used to determine hydrocarbon degrading activity. The original method is a qualitative assay used to screen isolates that are capable of degrading hydrocarbons. However, the rate of decolourisation can potentially be an indication of the organism's ability to degrade hydrocarbons. In order to extract more information from the method, a semi-qualitative assay was devised in order to follow the rate of reduction of the 2,6 DCPIP dye. In addition to determination of the rate of dye breakdown, different fractions of the culture broth were used in order to elucidate where the activity lies; in the supernatant (i.e. an extracellular enzyme or biosurfactants capability) or in the actual cells (i.e. cell surface components able to

make the cell wall more hydrophobic). The experimental outlay is presented in Figure 9.

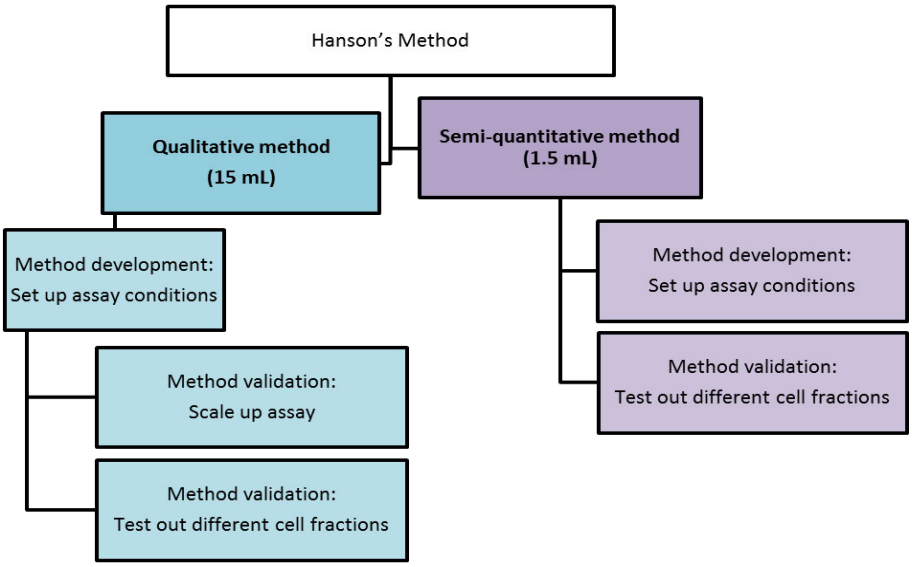


Figure 9 : Experimental outlay of the planned activities in order to develop the rapid colorimetric assay to screen for potential hydrocarbon degrading organisms (Hanson et al., 1993).

All the purified and primarily selected isolates were cultivated on prepared and sterilised (15 minutes at 121°C (Steridium Micro Digital) Tryptone Soy Broth (TSB; 3% $m.v^{-1}$; Biolab, Merck) containing diesel (Sasol, South Africa) that was filter-sterilised using a 0.22 µm filter into the sterile TSB to give a 1% $v.v^{-1}$ effective diesel concentration.

After inoculation, each flask was incubated on a platform shaker (Innova 2300, New Brunswick) at 180 RPM and 32°C and allowed to grow for 12 to 18 hours, or until an OD_{660nm} of minimum 4.0 units was reached.

3.2.2.3 Bushnell Haas Medium

Bushnell Haas medium is a cultivation medium which is recommended for use to assess the microbial hydrocarbon degradation ability of microorganisms.

Bushnell Haas medium was prepared by dissolving 0.2 $g.L^{-1}$ $MgSO_4$ (CAS 7487-88-9 | 106067, Minema, South Africa), 0.02 $g.L^{-1}$ $CaCl_2$ (CAS 10043-52-4 | 102378,Minema, South Africa), 1 $g.L^{-1}$ KH_2PO_4 1 $g.L^{-1}$ NH_4NO_3 (6484-52-2, Minema, South Africa), 1 $g.L^{-1}$ K_2HPO_4 (7758-11-4, Minema, South Africa) and 0.05 $g.L^{-1}$ $FeCl_3$ (7705-08-0, Minema, South Africa) in 1 L of water and adjusting the pH to 7.2 with either 32% $v.v^{-1}$ HCl or 20% $m.v^{-1}$ NaOH. The media was autoclaved for 15 minutes at 121°C.

3.2.2.4 Hydrocarbon substrate

The hydrocarbon substrate used, was diesel purchased at a local SASOL garage. The diesel was filter sterilized and kept in the fridge at 4°C until use.

3.2.2.5 2,6 DCPIP redox indicator

A stock solution (0.004 $g.mL^{-1}$) of 2,6 DCPIP was prepared. Bushnell Haas media (section 3.2.2.3) was added to a Falcon tube (50 mL) containing assay components in Table 4 below, and vortexed (Reax Top, Heidolph Instruments). The cell suspension was added, and a timer started. The tubes were incubated on a platform shaker at 32°C. Any changes in colour were noted at 10, 20, 40 and 60 minutes into the assay. The tubes were allowed to stand overnight so that a 24-hour time point could also be collected. The change in colour was captured using the key presented in Table 5. Attenuated bacterial cells, prepared by autoclaving at 121 °C for 15 minutes were used as the control.

Table 4: Method of Hanson et al., (1993) and adapted CSIR concentrations

Assay component	Hanson's concentrations	Adapted CSIR concentrations
1.) Cell suspension	25 µL	50.0 µL*
2.) BH medium	1.5 mL	1.5 mL
3.) Hydrocarbon substrate	10 µL	20.0 µL*
4.) 2,6 DCPIP	1.5 µg	32 µg*

Table 5: Colour rating to complete discolouration of sample.

Colour	Rating
Clear	1
Blue: lighter than control, but not clear	2
Blue: same as control	3

3.2.3 Selection of top performing isolates

Three limiting measures were introduced to the results in order to narrow down the list of screened isolates. The results were sorted according to the following three measures:

Sort 1: Culture ages were sorted from low to high. All isolates requiring cultivation time of more than 20 hours were excluded from the selection list.

Sort 2: All catalase negative, Gram negative and non-spore-forming isolates were eliminated from the selection.

Sort 3: Hydrocarbon utilisation (HU)-Indexes were sorted from low to high, after which the top 10 isolates were selected.

3.3 Evaluation of top selected strains studies to determine consortium suitability in hydrocarbon degradation.

3.3.1 Cultivation of hydrocarbon degrading isolates

Cryopreserved cultures (2 mL) of the respective isolates tested in this study were used to inoculate the test medium.

3.3.1.1 Identification of isolates by genetic evaluation - 16s RNA sequences

The selected isolates that were obtained from section 3.3.1 were visually verified as monoseptic cultures and then sent to Inqaba Biotechnical Industries (Pty) LTD, Pretoria, South Africa in order to confirm isolate identities by amplification of the 16s gene. Consensus sequencing was performed in CLC bio main workbench V5 using the Fw and Rw sequence. Sequence alignments were performed using BLASTN, which is available on the NCBI server (Klindworth et al., 2013). The results are shown in Table 16.

Genomic DNA was extracted using the Quick DNA™ Fungal/Bacterial Miniprep kit (Zymo research, catalogue no D6005). The target region was amplified using OneTaq ® Quick-Load ® 2X master mix (NEB, Catalogue No. M0486) with the relevant primers. The PCR products were run on a gel extracted with the Zymoclean ™ Gel DNA recovery kit (Zymo research, catalogue No. D4001). The extracted fragments were sequenced in the forward and reverse direction (Nimagen, BrilliantDye ™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) and purified (Zymo research,

ZR-96 DNA Sequencing Clean-up Kit ™ Catalogue No. D4050). The purified fragments were analyzed on the ABI 3500 XL Genetic analyzer for each reaction sample (Applied Bio-systems, Thermo Fisher Scientific). CLC Bio Main Workbench V7.6 was used to analyze the files generated and the results were obtained by conducting a Blast search (NCBI).

3.3.1.2 Minimal media preparation

Bushnell Haas (BH) minimal media was prepared according section 3.2.2.3. The medium (200 mL) was then autoclaved for 15 minutes at 121 °C. Subsequent to sterilization, 2 mL of filter sterilized engine oil was added to the cooled BH media. The resultant substrate concentration of the medium was 1% ($v.v^{-1}$).

3.3.1.3 Inoculum preparation

Each isolate listed in Table 11 was inoculated into 500 mL Erlenmeyer flasks containing 100 mL of pre-sterilized TSB containing filter sterilized engine oil (1% $v.v^{-1}$).

The flasks were incubated at 32 °C on a rotary platform shaker at 180 rpm, for a period of 8 hours, or until an OD_{660nm} of 2.0 was reached.

3.4. Evaluation of hydrocarbon degradation of the top performing isolates

The study was conducted in 6-well microtitre plates. The cell cultures prepared in section 3.3.1.3 were diluted accordingly with sterilized water in order to achieve a cell concentration of 1×10^9 cells.ml^{-1} in 10 ml of the test media in each well. The study was performed in triplicate. After inoculation, the microtitre plates were incubated at 32 °C and 180 RPM on an orbital shaker (Innova 2300, New Brunswick Scientific, Edison, NJ, USA) and grown for 48 hours. Uninoculated BH medium was used as a negative control.

3.4.1 Sample extraction and GC analysis

Samples (10 mL) were extracted at age 8, 16, 24 and 48 hours and were transferred to 50 mL falcon tube containing 10 mL hexane. Each sample was vortexed for 1 minute, and then centrifuged for 5 minutes at 12 000 x *g*. The top organic layer (containing the extracted hydrocarbons) of the sample was then transferred to an Eppendorf tube (2 mL) containing a pinch of $MgSO_4$. The sample was then vortexed and centrifuged at 12 000 x *g* for 5 minutes. The supernatant was then transferred into a pre-labelled GC vial for analysis using the GC. The GC SOP used in this study was as described by Smit et al. (2017).

The analysis was performed using the Agilent 7890A GC system coupled with a 7693 auto sampler, and equipped with flame ionisation detector (FID). A fused silica capillary column Rxi-1ms with dimensions of 30 m x 0.25 mm x ID 1.0 µm (Restek, United States) was used. The operating parameters are detailed in Table 6.

Table 6: GC analytical method (TPH PUB)

Parameter	Unit	Value
Injection volume	µL	2
Inlet heater temperature	°C	250
Inlet pressure	kPa	109.19
Inlet total flow	mL.min^{-1}	18.4
Inlet septum purge flow	mL.min^{-1}	3
Inlet mode	#	10:1
Initial oven temperature	°C	70 (hold for 1.4 minute)
Oven ramp to	°C	330 (at 19.7 °C.min^{-1}; hold for 2 minutes)
Front Detector (FID) temperature	°C	350
FID H_2 flow	mL.min^{-1}	35
FID air flow	mL.min^{-1}	350
FID make up (N_2) flow	mL.min^{-1}	30

The standard used was the DRO calibration mix standard (Catalog no.: 861287) from Sigma-Aldrich. A chromatogram of the analytical standard is presented in Figure 10 below:

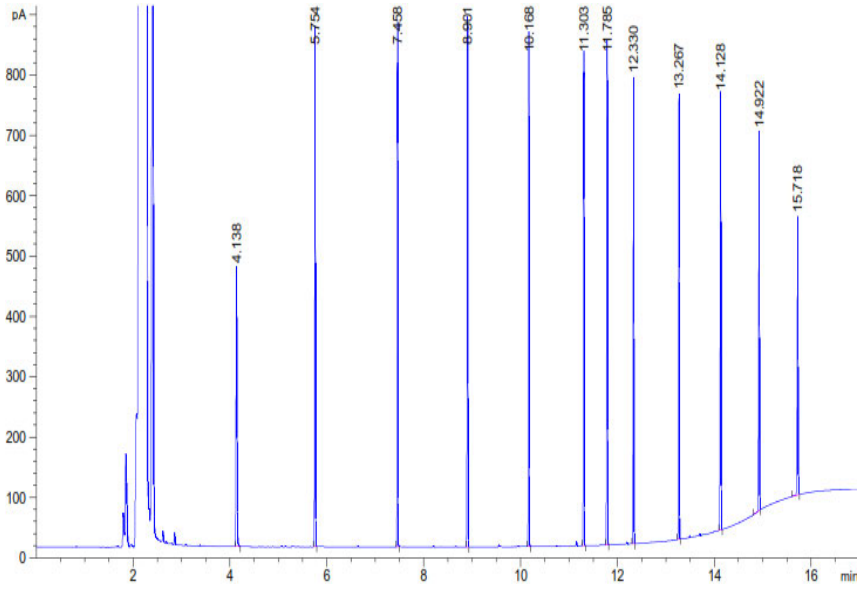


Figure 10: Chromatogram of 250 µg.mL^{-1} DRO calibration mix standard

The retention times (RT) and peak identities are listed in Table 7. Carbon chain lengths of C10 to C28 were separated using the method as described in Table 7.

Table 7: Identification of peaks using the DRO calibration mix standard

Chemical formula	IUPAC name	Retention time (min)	Calibration equation	Regression (R^2)
$C_{10}H_{22}$	N-Decane	5.754	Area = 3.353*amount + 3.968	0.9995
$C_{12}H_{26}$	N-Dodecane	7.458	Area = 3.424*amount + 0.932	0.9998
$C_{14}H_{30}$	N-Tetradecane	8.901	Area = 3.473*amount - 1.711	0.9999
$C_{16}H_{34}$	N-Hexadecane	10.168	Area = 3.454*amount - 5.281	0.9999
$C_{18}H_{38}$	N-Octadecane	11.303	Area = 3.390*amount - 10.126	0.9999
$C_{18}H_{14}$	O-Terphenyl	11.785	Area = 3.766*amount - 4.031	0.9999
$C_{20}H_{42}$	N-Eicosane	12.330	Area = 3.368*amount - 17.121	0.9999
$C_{22}H_{46}$	N-Docosane	13.267	Area = 3.324*amount - 27.190	0.9998
$C_{24}H_{50}$	N-Tetracosane	14.128	Area = 3.272*amount - 42.304	0.9995
$C_{26}H_{54}$	N-Hexacosane	14.922	Area = 3.090*amount - 59.696	0.9985
$C_{28}H_{58}$	N-Octacosane	15.718	Area = 2.765*amount - 76.367	0.9948

The calibration of all the compounds was done using a total of 4 calibration points. The coefficient of determination (R^2) for the linear regression was greater than 0.994 for all 11 compounds. The area under the peak was determined using the response (pA) or peak height.

3.4.2 Assessment of hydrocarbon degradation

Resultant chromatograms were used to determine the total petroleum degradation (C18 – C28) per compound of the various hydrocarbons identified in the sample. Peak area of each compound was used to determine the percentage degradation at a specific time point and the rate at which each hydrocarbon compound was degraded by the respective organism. The average (mean) TPH (C18 – C28) degradation was then calculated. To ensure that the top performing isolates were selected, the data for each compound was clustered using a standard deviation threshold rationale of ±1.0 of the mean of the dataset. The scoring was done by comparing the degradation percentage to the threshold limits (Table 8). The 48-hour sample was used to select

the top performing isolates based on the degradation of >70% of the known compounds. The 48 hour time point was also the maximum time point of the entire study. Table 8: Scoring matrix used to evaluate degradation of each hydrocarbon compound

Level of scoring	**Scoring value**
> 1 SD	5
Above average	4
Average	3
Below Average	2
< 1 SD	1

The final scoring for each isolate can therefore be calculated as follows:

$$S_{o_i} = \sum_{j=18}^{j=28} S_{cj}$$ Equation 4

Where,

S_{oi} = Scoring for organism i (as per Table 9)

S_{cj} = scoring for the hydrocarbon compound

j = hydrocarbon number

3.4.3 Evaluation of the sporulation efficiency of the top five performing isolates

Following the scoring of the isolates according to Table 8, the top 5 isolates were selected to perform a sporulation efficiency test. Five Erlenmeyer flasks (1 L) were used to prepare 200 mL each of sterile sporulation media (containing 5 $g.L^{-1}$ yeast Extract, 5 $g.L^{-1}$ glucose, 50 $mg.L^{-1}$ $MnSO_4.4H_2O$, 100 $mg.L^{-1}$ $CaCl_2.4H_2O$, and 500 $mg.L^{-1}$ $MgSO_4.7H_2O$). The flasks were incubated at 32 °C on a rotary platform shaker

at 180 rpm for a period of 5 days. After 5 days, a sporulation efficiency count was done on each of the 3 selected isolates.

3.5. Lab and bench scale evaluation and testing of hydrocarbon-degrading bacterial consortia using synthetic and industrial effluent

3.5.1. Consortium preparation

Six 2 L Erlenmeyer flasks were each prepared with 700 mL of sterile TSB. Each flask was inoculated with a cryovial of the respective isolate – P401, P402, D014, GPA 11.2, GPA 7.1 and GPA 3.5. P401 P402 and D014 is CSIR's existing bioremediation consortium that was used as a benchmark for this study. The inoculum flasks were incubated on a platform shaker at 32 °C and 180 rpm for approximately 4 – 5 hours until the cultures reached a microscopic cell concentration of approximately 1 x 10^9 cell.mL^{-1}. Each culture was then diluted to exactly 1 x 10^8 cell.mL^{-1} using sterile water and used for efficacy testing as described in section 3.5.2 below.

3.5.2. Efficacy testing

3.5.2.1 Synthetic and Industrial effluent preparation

Approximately 1.5 L of synthetic effluent was prepared using Bushnell Haas media according to Table 9.

Table 9: Concentration of synthetic effluent media components

Components	**Amount**
$MgSO_4$	0.200 $g.L^{-1}$
$CaCl_2$	0.020 $g.L^{-1}$
KH_2PO_4	1.000 $g.L^{-1}$
NH_4NO_3	1.000 $g.L^{-1}$
K_2HPO_4	1.000 $g.L^{-1}$
$FeCl_3$	0.050 $g.L^{-1}$

The media was autoclaved at 121 °C for 15 minutes. The media was decanted into five sterile 1 L Erlenmeyer flasks. Each flask, four consortia test flasks and one control flask, contained 250 mL of the synthetic effluent. A volume of 1.25 mL (0.5% $v.v^{-1}$) of diesel was added to each flask, including the control flask, just prior to inoculation. A sample of the neat effluent (T_0) was taken and prepared according to section 3.4.1.

The industrial (real) effluent was obtained from a Chevron site via Improchem. Approximately 1.5 L of the bulk effluent was mixed for 5 minutes using an overhead stirrer. The pH was measured and manually adjusted to 6.8 using 10% $m.m^{-1}$ NaOH and 5% $v.v^{-1}$ HCl solutions. The effluent was decanted into five sterile 1 L Erlenmeyer flasks. Each flask, four consortia test flasks and one control flask, contained 250 mL of the real effluent. Similar to the synthetic effluent study, diesel was added to the flasks and a neat sample prepared for analyses.

3.5.2.2 Product testing

Each test flask, containing synthetic or real effluent, was inoculated with the prepared cultures (section 3.4.1) as follows:

1. Gen 2: P401, P402 and D014 – 0.83 mL of each culture was added
2. Gen 3.1: GPA 11.2 and GPA 7.1 – 1.25 mL of each culture was added
3. Gen 3.2: GPA 11.2, and GPA 3.5 – 0.83 mL of each culture was added
4. Gen 3.3: GPA 11.2, GPA 7.1 and GPA 3.5 – 0.83 mL of each culture was added

The control flasks were not inoculated. A sample was taken at time point 0 (T_0) and duplicate samples prepared according to section 3.4.1. All flasks were placed on a platform shaker at 30°C, as recommended by Banerjee & Ghoshal , (2016), at 180 rpm. Samples of the synthetic and real effluents were taken for each consortium and the negative control at 12 hourly intervals and duplicate samples prepared for gas chromatography analyses (section 3.4.1)

3.5.2. Sample analyses

3.5.2.1 Gas chromatography method

The Agilent 7890A GC system coupled to a 7693 auto sampler and flame ionization detector (FID) was used for the purpose of GC analyses. The column used was a fused silica capillary column Rxi-1ms with dimensions of 30 m x 0.25 mm x ID 1.0 µm (Restek). The operating parameters used are detailed in Table 10.

Table 10: GC analytical method (TRPH Florida standard)

Parameter	Unit	Value
Injection volume	µL	2
Inlet heater temperature	°C	250
Inlet pressure	kPa	177.31
Inlet total flow	$mL.min^{-1}$	65.92
Inlet septum purge flow	$mL.min^{-1}$	3
Inlet mode	#	Splitless
Initial oven temperature	°C	40 (hold for 2 minute)
Oven ramp to	°C	330 (at 20 $°C.min^{-1}$; hold for 10 minutes)
Front Detector (FID) temperature	°C	350
FID H_2 flow	$mL.min^{-1}$	40
FID air flow	$mL.min^{-1}$	400
FID make up (N_2) flow	$mL.min^{-1}$	2

3.5.2.1.1 Standard calibration curve

A GRO/DRO calibration mix (SV Mix UST method TRPH Std (Florida); 1 mL/ampul containing 500 $µg.mL^{-1}$ of each compound in hexane) consisting of the following compounds: n-octane (C8), n-decane (C10), n-dodecane (C12), n-tetradecane (C14), n-hexadecane (C16), n-octadecane (C18), n-eicosane (C20), n-docosane (C22), n-tetracosane (C24), n-hexacosane (C26), n-octacosane (C28), n-triacontane (C30), n-dotriacontane (C32), n-tetratriacontane (C34), n-hexatriacontane (C36), n-octatriacontane (C38), n-tetracontane (C40) was used. The calibration of all the compounds was done using a total of 8 calibration points. Concentrations of the standard, 1.25; 2.5; 5.0; 10.0; 25.0; 75.0; 150 and 250 $µg.L^{-1}$ were prepared using hexane. The various samples were processed using gas chromatography and standard calibration curves (represented as a standard calibration table) generated per compound.

3 Calculating hydrocarbon removal

The chromatograms for the initial and 12 hourly samples were analysed and the peaks of the identified compounds integrated to obtain peak area per compound. Using the calibration table (Table 10), the concentration of each compound at each time point was determined.

TPH (C8-C28) was calculated as follows:

$$\text{TPH (C8-C28)}_t = \sum_t (C_{Ci}, C_{C(i+2)} \ldots\ldots\ldots\ldots\ldots C_{C28}) \qquad \textit{Equation 5}$$

Where,
C = Concentration, $mg.L^{-1}$
i = Carbon number
t = time point the analyses is conducted at

Removal (%) was therefore calculated as follows:

$$\text{Removal (\%)} = \frac{TPH_{final} - TPH_{initial}}{TPH_{initial}} \times 100 \qquad \textit{Equation 6}$$

The rate of TPH removal was calculated as follows:

$$\textbf{Rate of removal } (mg.L^{-1}.h^{-1}) = \frac{TPH_{initial} - TPH_{final}}{(t_{final} - t_{initial})} \qquad \textit{Equation 7}$$

Chapter 4

4 Results and Discussion

4.1 Sampling, isolation, and basic characterization of indigenous *Bacillus* spp for use in the degradation of hydrocarbons

4.1.2 Environmental sampling

In this study, a total of 38 environmental samples were collected in Silverton (Region A), Centurion (Region B) and Midrand (Region C) (Figure 11). Sites where samples were collected were car service shops, rivers and dams, as well as other oil spillage sites, as a result of human activity.

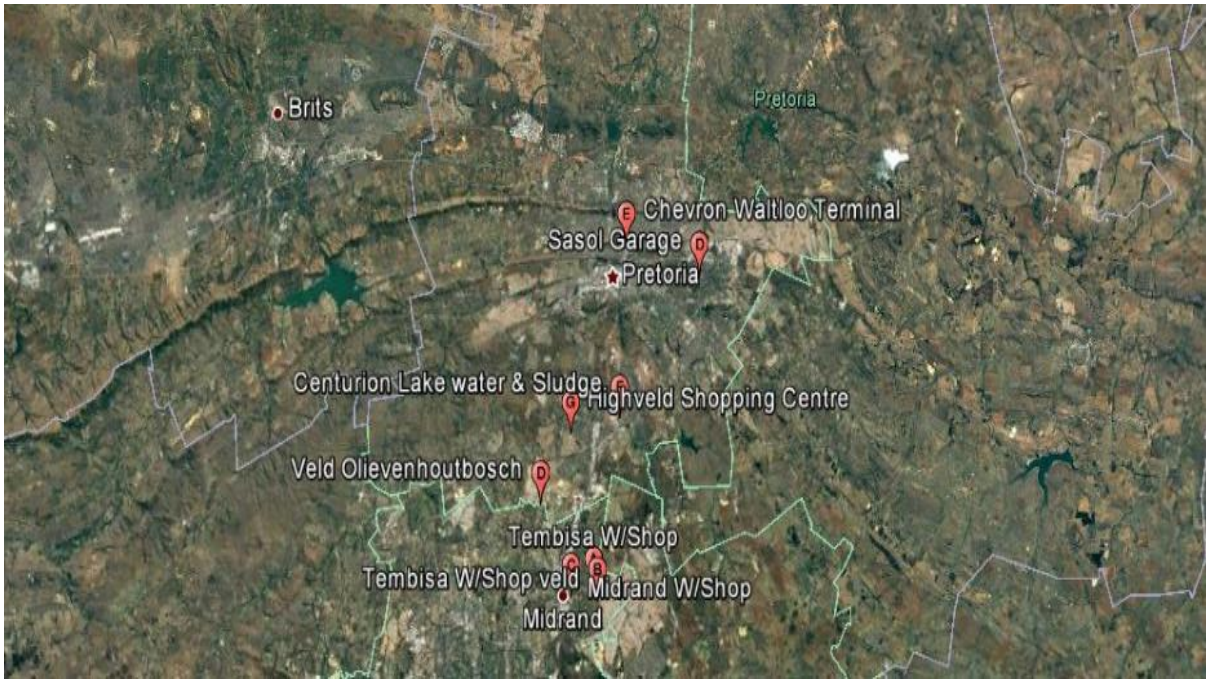


Figure 11 : Representation of various sampling points based on their global positioning system (GPS) coordinates

4.1.3 Isolation of spore forming *Bacillus* isolates from environmental samples

Post collection, the samples were enriched as described in Section 3.1. Microscopic analysis was conducted on each sample to determine the contents of each sample (Figure 12). The relative proportion of microbial community members is subject to the physical and chemical properties of the environment, as well as physiological and metabolic adaptations to the environment (Zengler, 2009).

4.2 Screening and selection indigenous *Bacillus* spp that could be used to degrade hydrocarbons

Primary screen assays were used to identify isolates as *Bacillus* spp (Figure 12). Spore-forming organisms are displayed in Figure 12 (a) and (b). Figure 12 (c) is a representation of non-spore forming organisms.

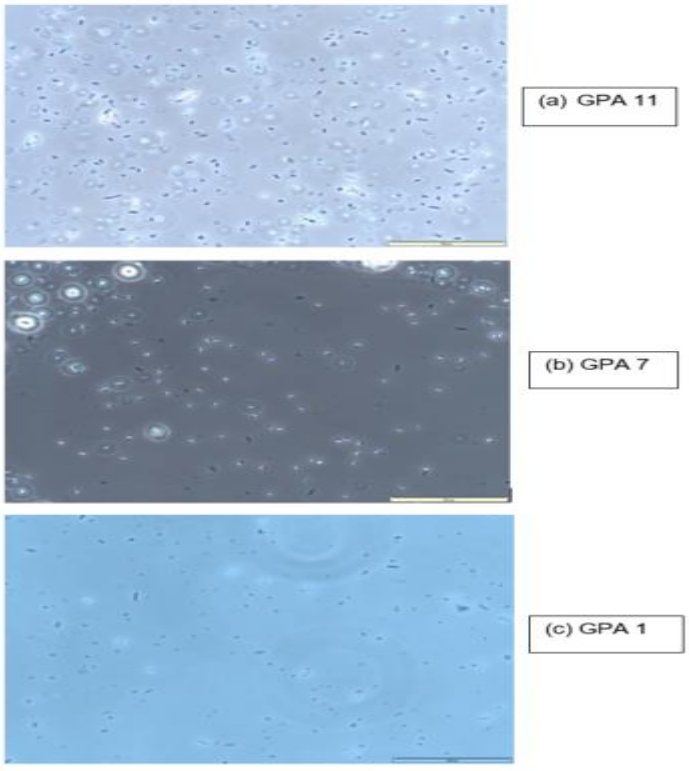
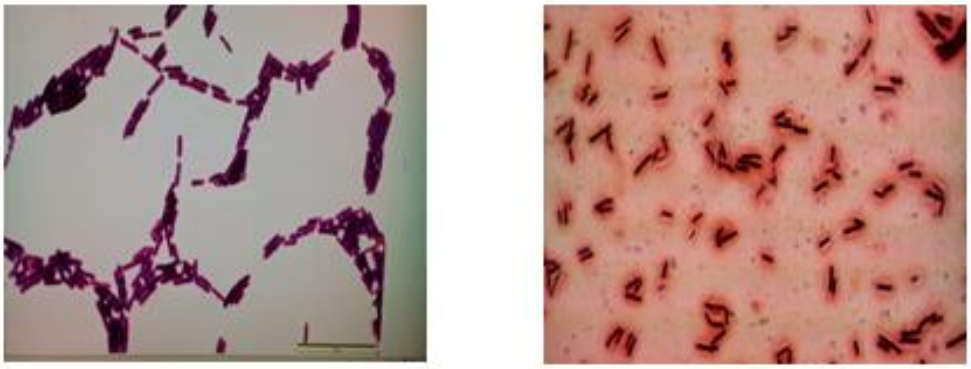


Figure 12 : Preliminary microscopic analysis of crude samples obtained in Regions A, B and C in the Gauteng Province.

Gram staining assays (Figure 13a and b) and catalase tests (Figure 14) were also performed on the individual isolates.



(a) GPA 11.2 (b) GPA 7.1

Figure 13 : Gram stain results of the isolated and purified samples.

A positive gram stain and catalase results are shown in Figure 13 and 14. The catalase enzyme is responsible for the breakdown of hydrogen peroxide (H_2O_2) into H_2O and O_2. H_2O_2 is currently used as a disinfectant for wounds, and the effervescing that occurs is due to the evolution of O_2 gas. H_2O_2 is a powerful oxidizing agent that can cause disorder in a cell, because of this, any cell that uses O_2 or can live in the presence of O_2 must have a mechanism to get rid of the peroxide. Catalase tests were performed on all isolates and all organisms tested positive for catalase. According to literature, Gram staining and catalase test are primary assays used to screen for *Bacillus* isolates (Adel et al., 2012).

Figure 14 : Positive catalase test results of isolated and purified samples.

Assessment of the population using microscopy, showed that a total of 70% of the population was rod like cells, 24% were rods that had sporulated and 3% of the population were short rods. Only 3% of cells were found to be cocci- like cells. Spores were observed in ~58% of the cells (Figure 15). Even though different cell lengths were observed in the rod shaped cells, microscopy alone was not sufficient to make a definite conclusion as to the amount of different populations present in each sample. Gram positive isolates made up 96% of the population, and only 4% of the isolates were gram negative cells (Figure 16).

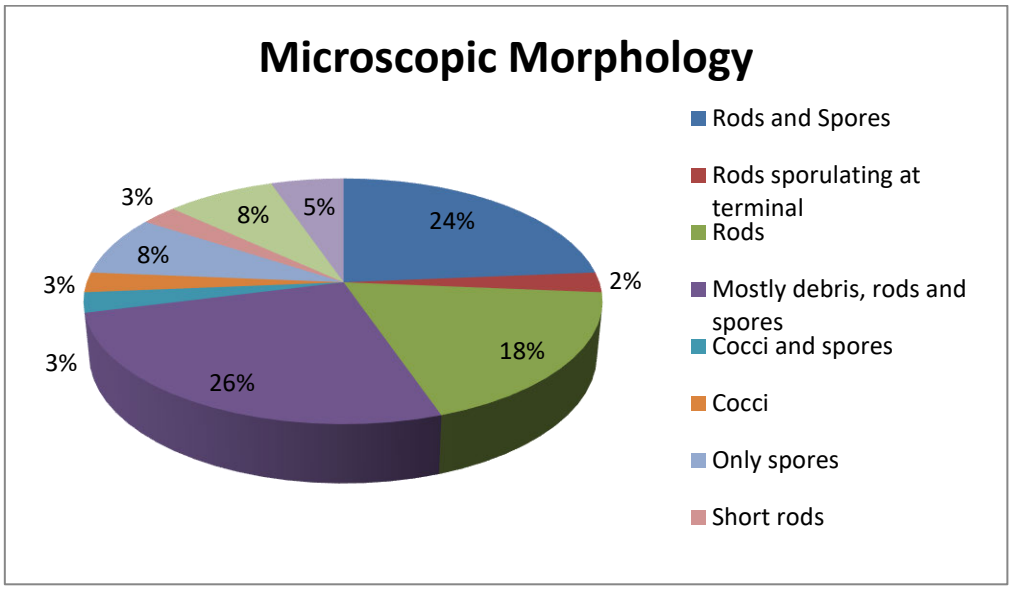


Figure 15 : Microscopic analysis for each crude sample prior to isolation

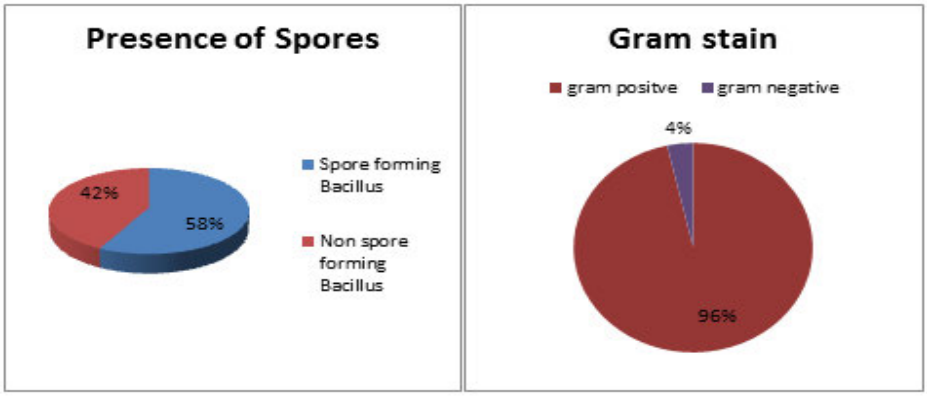


Figure 16 : Microscopic analysis of sporulation efficiency and gram stain of each isolate

Of the 115 colonies tested, 96 % were Gram-positive, 100% were positive for *catalase* activity, and 58% were spore-forming bacteria. Aerobic Gram-positive bacteria are mainly chemoorganotrophs that can produce spores due to harsh environmental conditions (Srivastava et al., 2014). Gram-positive bacteria are famous for their diverse morphologies, metabolic properties, and physiologies which allows them to grow in an extensive range of environments (Narancic et al., 2012). The diverse morphologies of each environmental sample (Table 11) are in support of this statement. Using the key provided in Figure 8, Section 3.2.2; each colony was described according to its margin, pigmentation, shape, elevation, optical property, size, appearance, and texture.

The production of the catalase enzyme is an important component for the cell to act as a defence mechanism against oxidative stress, as it can change hydrogen peroxide to oxygen and water (Sooch et al., 2014). Aerobic microorganisms have developed mechanism which is able to induce repairing, in the form of the *catalase* enzyme, to ease the damaging effects of active oxygen (Sooch et al., 2014). Aerobic Gram-

positive bacteria able to produce the catalase enzyme include the genus *Bacillus* (Sooch et al., 2016). Spores are formed by harsh changes in the bacterial environment, this is an example of an extreme survival strategy. Endospores are formed by bacteria in a complex process within their mother cells this process enables bacteria to remain inactive for extended periods. They are entirely formed by low G+C Gram-positive bacteria, most notably the genera *Bacillus* (Wohlgemuth and Kämpfer, 2014).

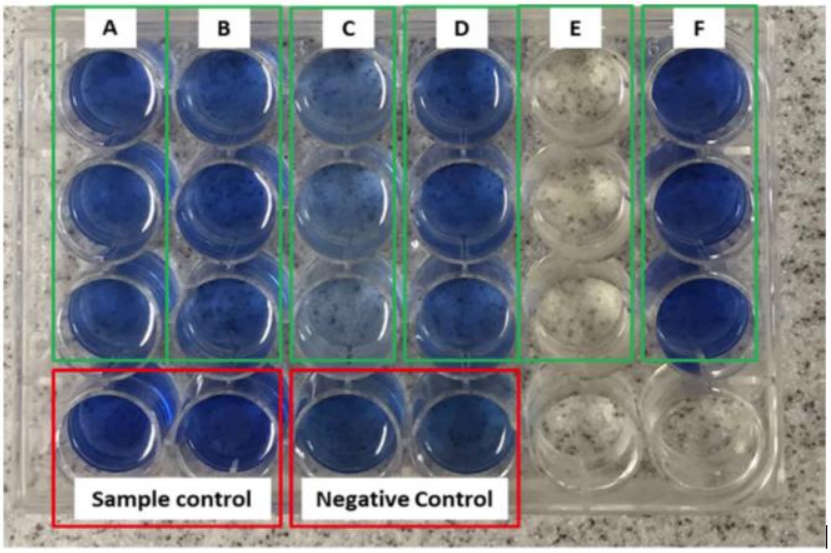
Table 11: Sample designation and basic characterisation of Tshwane Region samples

Sample Number	Shape	Margin	Elevation	Size	Texture	Appearance	Pigmentation	Optical Property	Isolates obtained
1	Irregular	Undulate	Flat	Large	Smooth	Dull	Nonpigmented	Opaque	3
2	Circular with a centre	Undulate	Flat	Large	Smooth	Dull	Nonpigmented	Opaque	5
3	Circular	Undulate	Flat	Small	Smooth	Shiny	Pigmented	Opaque	4
4	Irregular	Undulate	Flat	small	Smooth	Dull	Nonpigmented	Opaque	2
5	Irregular	Undulate	Flat	Medium	Smooth	Dull	Nonpigmented	Opaque	7
6	Circular	Entire	Flat	Small	Smooth	Shiny	Pigmented	Opaque	9
7	Circular	Entire	Flat	Small	Smooth	Dull	Nonpigmented	Opaque	8
8	Irregular	Undulate	Umbonate	Small	Smooth	Dull	Nonpigmented	Opaque	12
9	Circular	Entire	Raised	Medium	Smooth	Dull	Nonpigmented	Translucent	14
10	Circular	Entire	Flat	Medium	Smooth	Dull	Nonpigmented	Opaque	7
11	Circular	Undulate	Flat	Large	Smooth	Dull	Nonpigmented	Opaque	13
13	Irregular	Undulate	Flat (pin head)	Small	Smooth	Dull	Nonpigmented	Opaque	3
14	Irregular	Undulate	Raised	Small	Smooth	Dull	Nonpigmented	Opaque	8
15	Circular with centre	Entire	Flat	Medium	Smooth	Dull	Nonpigmented	Opaque	8
16	Irregular	Entire	Slightly raised	Small	Smooth	Dull	Nonpigmented	Translucent	9
17	Circular	Entire	Flat	Medium	Smooth	Dull	Nonpigmente		6
18	Circular	Entire (Slimy)	Convex	Large	Smooth	Dull	Nonpigmented	Opaque	4
19	Irregular	Undulate	Flat	Small (pin head)	Smooth	Dull	Nonpigmented	Opaque	4
20	Circular	Entire	Flat (defined centre)	Medium	Smooth	Dull	Nonpigmented	Opaque	14
21	Circular	Entire	Concave	Large	Smooth	Dull	Nonpigmented	Transparent	10

4.3 Secondary screen assay for hydrocarbon utilizing bacteria isolated from the Tshwane region in the Gauteng province

4.3.1 Determination of culture age and optical density (OD)

After 8 hours of growth, each isolate OD was measured and reported. Cultures not at OD 4.0, were further cultivated and assessed after 12 or 24 hours. A growth rate estimate was calculated using OD at wavelength 660 nm over the total cultivation time. In the DCPIP rapid test, the biodegradation mechanism of crude oil is assessed by introducing of an electron acceptor (DCPIP) to the culture medium. In this colorimetric method, the capability of bacterial strains to degrade crude oil is evaluated by detecting the change in DCPIP colour from blue (oxidized) to colourless (reduced) (Mariano et al., 2008). Each isolate was screened using the 2.6 DCPIP screen and the semi quantitative result reported as the change in optical density per minute. GPA 11.2 caused a colour change from blue to colourless, indicating ability to degrade crude oil (Figure 17). The principle of this redox indicator lies on the oxidation of the carbon source (hydrocarbon substrates) whereby electrons are transferred to electron acceptors (Nallin et al., 2015).



A= GPA 6.2, **B**= GPA 3.5, **C**=GPA 7.1, **E**= GPA 11.2, **F**= GPA 4.2.

Sample control = assay without any bacteria,

Negative control = assay with attenuated bacteria.

Figure 17 : DCPIP rapid screen results.

A hydrocarbon utilisation (HU) index was estimated by calculating the growth estimate over the ability to degrade the 2,6 DCPIP dye. Therefore, a smaller HU index is indicative of a more competitive hydrocarbon degrading ability. The results are summarised in Tables 12-14.

Table 12: Rapid screen results for Gauteng Province Region A

Designation	Culture age	Culture OD	Growth rate estimate	Semi quant result	HU Index
#	hours	OD #	OD#/h	Δ OD/min	#
GPA 1.1	16	11.1	0.715	0.004	179.6
GPA 1.2	20	11.6	0.582	0.006	97.0
GPA 2.1	20	14.7	0.736	0.011	66.9
GPA 2.2	20	9.5	0.473	0.007	67.6
GPA 2.3	20	10.7	0.536	0.015	35.8
GPA 2.4	15	4.9	0.339	0.018	18.6
GPA 2.5	15	4.9	0.325	0.004	81.2
GPA 3.1	15	9.7	0.650	0.006	108.3
GPA 3.3	19	1.2	0.061	0.012	5.1
GPA 3.4	15	9.8	0.656	0.008	82.0
GPA 3.5	15	4.8	0.321	0.019	16.9
GPA 4.2	16	4.7	0.305	0.027	11.5
GPA 5.1	17	9.2	0.560	0.009	60.4
GPA 5.2	13	7.6	0.583	0.004	145.8
GPA 5.3	20	12.2	0.611	0.015	40.7
GPA 6.1	13	7.4	0.572	0.003	190.5
GPA 6.2	17	1.8	0.111	0.011	9.9
GPA 6.3	40	9.6	0.241	0.009	26.8
GPA 7.1	17	9.1	0.551	0.020	6.3
GPA 7.2	18	9.1	0.520	0.020	26.3
GPA 8.3	13	3.9	0.302	0.023	13.1
GPA 8.4	23	10.2	0.442	0.008	55.2
GPA 8.5	16	9.4	0.588	0.007	84.0
GPA 9.1	15	9.1	0.627	0.004	177.2
GPA 9.3	16	9.2	0.572	0.005	114.4
GPA 9.4	15	4.4	0.305	0.023	13.2
GPA 10.2	16	4.1	0.264	0.002	125.0
GPA 10.3	7	3.3	0.476	0.013	36.6
GPA 11.1	16	5.5	0.357	0.007	53.0
GPA 11.2	17	2.5	0.151	0.014	10.8

Table 13: Rapid screen results for Gauteng Province Region B

Designation	Culture age	Culture OD	Growth rate estimate	Semi quant result	HU Index
#	hours	OD #	OD#/h	Δ OD/min	#
GPB 1.1	20	6.0	0.301	0.019	15.9
GPB 1.2	16	12.0	0.752	0.005	150.5
GPB 2.1	16	5.1	0.328	0.025	13.3
GPB 2.2	9	1.8	0.197	0.018	10.9
GPB 2.2	40	19.0	0.474	0.004	118.5
GPB 3.1	16	15.8	0.987	0.004	246.8
GPB 3.3	39	10.9	0.280	0.009	31.1
GPB 4.1	9	3.0	0.338	0.026	13.0
GPB 4.2	6	6.1	1.022	0.009	113.5
GPB 4.3	24	9.2	0.382	0.006	63.7
GPB 4.4	8	1.5	0.194	0.015	12.9
GPB 5.1	6	5.0	0.826	0.015	55.0
GPB 6.1	10	6.8	0.683	0.012	56.9
GPB 6.2	9	1.4	0.157	0.005	31.3
GPB 6.3	8	5.3	0.707	0.009	78.5
GPB 6.4	34	5.5	0.162	0.005	32.4
GPB 7.1	8	6.1	0.815	0.008	101.9
GPB 7.2	8	7.5	0.997	0.005	199.4
GPB 8.1	24	37.3	1.554	0.003	518.0
GPB 8.2	9	2.7	0.301	0.005	60.2
GPB 8.3	8	7.9	1.054	0.006	175.7
GPB 9.1	10	7.5	0.746	0.004	186.6
GPB 10.1	10	7.8	0.781	0.004	195.3
GPB 10.2	10	8.4	0.841	0.004	210.2
GPB 11.1	10	5.2	0.521	0.007	74.5
GPB 11.2	10	5.0	0.502	0.012	41.9
GPB 11.3	10	7.6	0.757	0.006	126.2
GPB 11.4	34	9.3	0.274	0.008	34.2
GPB 12.1	9	2.7	0.294	0.003	98.1
GPB 12.2	34	7.6	0.224	0.011	20.3

GPB 12.3	8	5.1	0.681	0.008	85.2
GPB 12.4	8	6.7	0.893	0.006	148.9
GPB 12.5	9	1.1	0.126	0.007	17.9
GPB 13.1	29	15.9	0.550	0.004	137.4
GPB 13.2	29	6.0	0.207	0.020	10.4
GPB 13.3	29	6.3	0.216	0.011	19.7
GPB 14.1	29	18.5	0.636	0.005	127.2
GPB 14.2	29	7.3	0.252	0.012	21.0
GPB 15.1	29	13.6	0.469	0.007	66.9
GPB 15.2	29	7.1	0.243	0.014	17.4
GPB 15.3	29	2.4	0.084	0.017	4.9
GPB 15.4	29	5.0	0.171	0.012	14.3

Table 14: Rapid screen results for Gauteng Province Region C

Designation	Culture age	Culture OD	Growth rate estimate	Semi quant result	HU Index
#	hours	OD #	OD#/h	Δ OD/min	
GPC 1.1	15	8.0	0.533	0.012	44.4
GPC 1.2	16	4.8	0.312	0.028	11.2
GPC 1.3	15	7.6	0.505	0.007	72.1
GPC 1.4	37	4.2	0.114	0.014	8.1
GPC 2.1	37	4.4	0.118	0.029	4.1
GPC 2.2	37	3.8	0.103	0.012	8.6
GPC 2.3	15	7.5	0.497	0.010	49.7
GPC 3.1	15	13.4	0.892	0.008	111.5
GPC 3.2	37	3.8	0.103	0.014	7.3
GPC 4.1	37	7.2	0.194	0.017	11.4
GPC 4.2	50	6.9	0.138	0.002	68.9
GPC 4.3	50	9.0	0.180	0.001	179.6
GPC 5.1	15	7.8	0.540	0.006	90.1
GPC 5.2	17	4.7	0.285	0.030	9.5
GPC 5.3	15	5.7	0.393	0.003	130.9
GPC 5.4	15	2.6	0.178	0.007	25.4
GPC 6.1	15	6.8	0.471	0.010	47.1
GPC 6.2	15	5.1	0.349	0.008	46.2
GPC 6.3	13	1.7	0.134	0.005	26.8
GPC 7.1	15	6.1	0.420	0.018	23.1
GPC 7.2	15	8.0	0.554	0.012	47.3
GPC 7.3	13	5.9	0.453	0.007	64.7
GPC 7.4	11	7.8	0.747	0.009	83.0
GPC 8.2	11	7.1	0.675	0.009	75.0
GPC 9.1	11	5.9	0.566	0.008	70.7
GPC 9.2	34	3.8	0.113	0.016	7.0
GPC 11.1	11	5.4	0.511	0.009	56.8
GPC 11.2	11	5.8	0.556	0.008	69.5
GPC 12.1	34	2.8	0.081	0.015	5.4
GPC 12.2	50	7.6	0.153	0.001	152.6

4.3.1.1 Selection of top performing isolates

Three key measures were introduced to the results in order to identify promising isolates. These included (i) shortest growth period to reach an OD of 4.0, (ii) high capability of degrading the 2,6 DCPIP dye and (iii) being catalase positive, Gram positive spore-forming isolates. Upon application of these three filtering characteristics, 35% of the isolates were eliminated based on their performance. The remaining 65% of organisms were assessed further.

Performance data indicated that isolates GPC 5.2 and GPC 1.2 where not capable of sporulation, and therefore were removed from the study. Based on performance, the top six isolates obtained from this study were GPA 3.5, GPA 7.1, GPA 6.2, GPA 11.2, GPB 2.2, GPA 4.2 and GPB 4.4 (Table 15). These isolates were cryo-preserved and stored on the -80°C and used in further studies.

Table 15: Sorted results according to the limitations identified in the screening process.

Designation	Catalase test	Gram stain	Spore test	Culture age	Culture OD	Growth estimate	Semi quant	HU Index
GPA 3.5	+	+	Y	19	1.2	0.061	0.012	5.1
GPA 7.1	+	+	Y	17	9.1	0.551	0.020	6.3
GPA 6.2	+	+	Y	17	1.8	0.111	0.011	9.9
GPA 11.2	+	+	Y	17	2.5	0.151	0.014	10.8
GPA 4.2	+	+	Y	16	4.7	0.305	0.027	11.5
GPB 4.4	+	+	Y	8	1.5	0.194	0.015	12.9

4.3.2 Molecular identification and biosafety evaluation

In an attempt to identify the members of the population, the BLASTN results from Inqaba Biotech were interrogated and returned several possible identity matches for each isolate (Figure 18). The E value or “expect” value is a parameter that describes the number of hits expected by chance when conducting a BLASTN search (Hu and Kurgan, 2019). The lower the E-value, or the closer it is to zero, the more "significant" the match is.

(a) GPA 3.5

Sequences producing significant alignments:		Score (Bits)	E Value
gi|942056516|gb|KT895269.1|	Bacillus sp. DY58 16S ribosomal R...	2019	0E00
gi|944383016|gb|EU567055.2|	Uncultured Bacillus sp. clone TCC...	2019	0E00
gi|944383005|gb|EU567039.2|	Uncultured Bacillus sp. clone TCC...	2019	0E00
gi|940620291|gb|KR709231.1|	Bacillus subtilis strain DMS_K04 ...	2019	0E00
gi|940413535|gb|KR185989.1|	Bacillus sp. Q3(2015) 16S ribosom...	2019	0E00
gi|940413534|gb|KR185988.1|	Bacillus sp. Q2(2015) 16S ribosom...	2019	0E00

ALIGNMENTS
>gb|KT895269.1| Bacillus sp. DY58 16S ribosomal RNA gene, partial sequence

(b) GPA 4.2

Sequences producing significant alignments:		Score (Bits)	E Value
gi|930435162|gb|KR149334.1|	Bacillus amyloliquefaciens strain...	2560	0E00
gi|920782987|gb|KP860311.1|	Bacillus amyloliquefaciens strain...	2560	0E00
gi|920782984|gb|KP860308.1|	Bacillus amyloliquefaciens strain...	2560	0E00
gi|612541014|gb|KJ528275.1|	Bacillus amyloliquefaciens strain...	2560	0E00
gi|532529844|gb|KF482863.1|	Bacillus sp. CZB22 16S ribosomal ...	2560	0E00
gi|532529837|gb|KF482856.1|	Bacillus sp. CZB13 16S ribosomal ...	2560	0E00

ALIGNMENTS
>gb|KR149334.1| Bacillus amyloliquefaciens strain JK6 16S ribosomal RNA gene, partial sequence

(c) GPB 4.4

Sequences producing significant alignments:		Score (Bits)	E Value
gi|930435162|gb|KR149334.1|	Bacillus amyloliquefaciens strain...	2571	0E00
gi|920782987|gb|KP860311.1|	Bacillus amyloliquefaciens strain...	2571	0E00
gi|920782984|gb|KP860308.1|	Bacillus amyloliquefaciens strain...	2571	0E00
gi|612541014|gb|KJ528275.1|	Bacillus amyloliquefaciens strain...	2571	0E00
gi|532529844|gb|KF482863.1|	Bacillus sp. CZB22 16S ribosomal ...	2571	0E00
gi|532529837|gb|KF482856.1|	Bacillus sp. CZB13 16S ribosomal ...	2571	0E00

ALIGNMENTS
>gb|KR149334.1| Bacillus amyloliquefaciens strain JK6 16S ribosomal RNA gene, partial sequence

(d) GPA 7.1

```
                                                                    Score     E
Sequences producing significant alignments:                         (Bits)  Value

gi|943635831|gb|KT962248.1|  Bacillus sp. N311(2015) 16S ribos...   1996    0E00
gi|940836443|gb|KR855694.1|  Bacillus methylotrophicus strain ...   1996    0E00
gi|940836442|gb|KR855693.1|  Bacillus methylotrophicus strain ...   1996    0E00
gi|940836441|gb|KR855692.1|  Bacillus methylotrophicus strain ...   1996    0E00
gi|940836440|gb|KR855691.1|  Bacillus methylotrophicus strain ...   1996    0E00
gi|940813636|gb|KT935670.1|  Bacillus amyloliquefaciens strain...   1996    0E00

ALIGNMENTS
>gb|KT962248.1| Bacillus sp. N311(2015) 16S ribosomal RNA gene, partial sequence
```

(e) GPA 11.2

```
                                                                    Score     E
Sequences producing significant alignments:                         (Bits)  Value

gi|942056516|gb|KT895269.1|  Bacillus sp. DY58 16S ribosomal R...   2075    0E00
gi|939467347|gb|KR780414.1|  Bacillus sp. CR10 16S ribosomal R...   2075    0E00
gi|939467324|gb|KR780391.1|  Bacillus subtilis strain CL8 16S ...   2075    0E00
gi|939467323|gb|KR780390.1|  Bacillus subtilis strain CL7 16S ...   2075    0E00
gi|939467322|gb|KR780389.1|  Bacillus subtilis strain CL6 16S ...   2075    0E00
gi|939467317|gb|KR780384.1|  Bacillus sp. CL1(2015) 16S riboso...   2075    0E00

ALIGNMENTS
>gb|KT895269.1| Bacillus sp. DY58 16S ribosomal RNA gene, partial sequence
```

(f) GPA 6.2

```
                                                                    Score     E
Sequences producing significant alignments:                         (Bits)  Value

gi|1110862328|gb|KY287777.1|  Bacillus amyloliquefaciens strai...   2510    0E00
gi|1100252325|gb|KX931099.1|  Bacillus sp. strain MN2 16S ribo...   2510    0E00
gi|1093394037|gb|KU973624.1|  Bacillus velezensis 16S ribosoma...   2510    0E00
gi|1057718622|gb|KX682280.1|  Bacillus amyloliquefaciens strai...   2510    0E00
gi|1039700913|gb|KU356171.1|  Bacillus amyloliquefaciens strai...   2510    0E00
gi|1039700908|gb|KU356166.1|  Bacillus amyloliquefaciens strai...   2510    0E00

ALIGNMENTS
>gb|KY287777.1| Bacillus amyloliquefaciens strain MI7SQ 16S ribosomal RNA gene, partial seque
          Length=1425
```

Figure 18 : Sequencing results for the selected isolates including scores and alignments.

GPA 4.2, GPA 6.2, and GPA 6.2, were matched to the *Bacillus amyloliquefaciens.* Isolates that were matched to *Bacillus subtilis* were, GPA 7.1 and GPA 11.2. GPA 3.5 was matched to the *Bacillus methylotrophicus* (Table 16). All these isolates were a significant match as their E-value was 0. Some members of the *Bacillus* species are

considered not to be bio-safe (Zeigler, 2011). These include the species *B. cereus and B. anthracis* due to their ability to produce an enterotoxin (Ehling-Schulz et al., 2019). It was therefore important to conduct a biosafety assessment of each member of these species, in order to confirm the safety status and class level of the organism (Lefevre et al., 2017). Table 16 summarises each isolate and their respective identities as confirmed by molecular sequencing. The phylogenomic analysis in figure 19 shows how these strains are closely related to each other. Based on the data obtained, none of the identified organisms belonged to the non-biosafe species, and no further safety assessments were required. For example, *B. subtilis* is classified as a biosafety level (BSL) 1 bacteria according to the American Type Culture Collection (ATCC) (http://www.atcc.org). The BSL classification is based on the U.S Public Health Service Guidelines. As a result, it was deemed unnecessary to conduct further biosafety tests on the organisms. The choice is further supported by the fact that Bacilli, specifically *B. subtilis,* are well-studied organisms that are regarded by the US Food and Drug Administration (USFDA) as "generally regarded as safe" (GRAS), that is, they are recognized as non-pathogenic (Harwood & Wipat, 1996).

Table 16 : Identity of top 6 isolates tested in this study.

Isolate / Product designation	Species Identity / Description
GPA 6.2	*Bacillus methylotrophicus*
GPA 7.1	*Bacillus subtilis*
GPA 3.5	*Bacillus methylotrophicus*
GPB 4.4	*Bacillus amyloliquefaciens*
GPA 4.2	*Bacillus amyloliquefaciens*
GPA 11.2	*Bacillus subtilis*

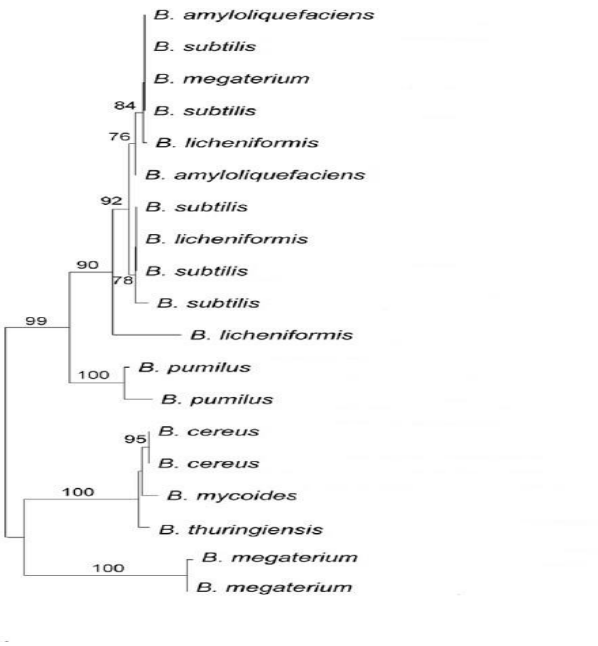


Figure 19: Phylogenetic tree of *Bacillus* species based on 16S rRNA gene sequences.

4.4 Evaluation of top selected strains studies to determine consortium suitability in hydrocarbon degradation

The performance of each isolate was evaluated by calculating the final score according to equation (4). All isolates were assessed for their hydrocarbon degrading capabilities. Hydrocarbons C10 - C16 were considered to be light/volatile compounds that would evaporate within the first 8 hours. Research has shown rate of biodegradation is increased as these organisms are utilizing simple compounds (C10-C16) (Dussán and Numpaque, 2012). The ability of petroleum hydrocarbons to volatilize naturally is not a new concept and is thought to be a benefit to natural remediation during oil spillages in the ocean (Jain, 2010). Although this degradation occurs naturally, all hydrocarbons present in the sample do not degrade to completion. To eliminate the effect of volatility, the sum of the medium (C18 - C22) and heavy (C24 - C30) compounds were assessed to determine degradation capability of the isolates.

4.4.1 Assessment of hydrocarbon degradation using a scoring matrix

Table 17 shows the total scoring obtained for degradation of medium-to-heavy end hydrocarbons for each of the 9 isolates after 48 hours. The top 5 scores of 34, 33, 30, 29 and 28 were achieved for GPA 11.2, GPA 8.3, GPA 3.5, GPA 4.4 and GPA 7.1, respectively. Research showed that hydrocarbon degradation was potentiated by the co-existence and cooperation variety of microorganisms, same as in the consortium (Xu et al., 2018; (Patowary et al., 2016). In the next section different consortium consisting of the top performing isolates were put together and the best performing consortium was selected.

Table 17: Total score obtained for each hydrocarbon degrading isolate after 48 hours

Isolates	Hydrocarbon compounds							Total score
	C18	C20	C22	C24	C26	C28	C30	
GPA 11.2	5	5	5	5	5	4	5	34
GPA 8.3	5	5	5	4	5	4	5	33
GPA 3.5	5	4	5	5	5	3	3	30
GPB 4.4	5	3	5	1	5	5	5	29
GPA 7.1	5	1	5	3	5	5	4	28
GPA 2.3	5	3	5	4	5	2	2	26
GPA 4.2	5	2	5	0	5	4	3	24
GPA 2.4	5	3	5	3	5	1	1	23
GPA 6.2	5	1	5	2	5	1	1	20

4.4.2 Frequency distribution of hydrocarbon degrading efficiency ranges of the top performing isolates after 48 hours

The hydrocarbon degrading efficiencies (%) across all compounds (C18 – C30) were assessed for each isolate. The ranges of efficiencies that were assessed were < 0, 0 – 50, 50 – 75, 75 – 90, and 90 – 100%. The frequency distribution (Figure 20) shows the most predominant and least predominant ranges achieved across the compounds per isolate.

A frequency (distribution) is a function that is able to show different measurement categories including the number of observations of each category. A frequency

distribution should have a minimum range and maximum range values. The range is divided into random intervals called “class interval.” If the class intervals are high, there won’t be a decrease in the bulkiness of data and minor deviations also become noticeable. Also, if they are limited, then the shape of the distribution will not be able to be determined.

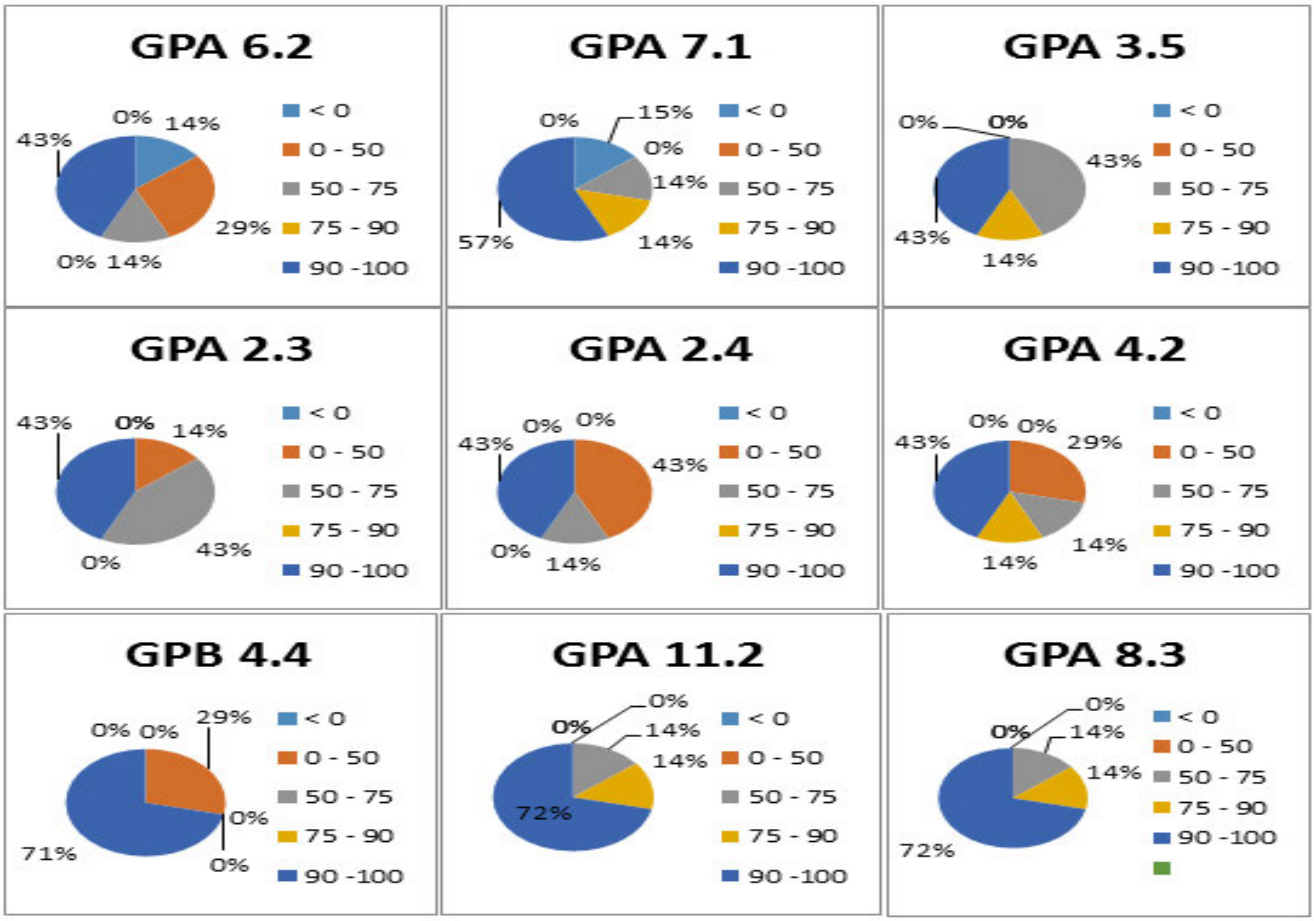


Figure 20 : Frequency distribution of hydrocarbon degrading efficiency ranges (%), < 0%, 0 – 50%, 50 – 75%, 75 – 90%, 90 – 100%, after 48 hours for each isolate.

The satisfactory hydrocarbon degradation level aimed for was at least between 75 - 90% across all compounds. This was required to ensure significant degradation of the compounds and intermediates. With respect to the top performers selected in Section 4.4.1, Figure 20 shows that isolates GPB 4.4, GPA 11.2 and GPA 8.3, were able to degrade the hydrocarbon at the satisfactory range of 50-75% and the degradation was between 90 -100% within 48 hours which is desirable. For GPA 7.1, 57% of the measured hydrocarbons were degraded between 90 – 100%, and 14% were degraded between 75 – 90%, which was considered satisfactory for this assessment (in other words 71% of hydrocarbons were degraded at least between 75 – 90%). Similarly, for GPA 3.5, 67% of hydrocarbons were degraded between 75 – 90%.

4.4.3 Sporulation efficiency test

According to the scoring matrix tabulated in Table 8, the top 5 isolates, GPA 11.2, GPA 8.3, GPA 3.5, GPB 4.4 and GPA 7.1 were tested for their sporulation efficiency. Table 18 represents the sporulation efficiency of each selected isolate.

Table 18: Sporulation efficiency of top performing isolates after 5 days

Isolates	Sporulation efficiency (%)[a]
GPA 11.2	93.6 ± 0.2
GPA 7.1	92.6 ± 0.3
GPA 3.5	77.4 ± 0.3
GPA 8.3	33.5 ± 0.2
GPB 4.4	28.2 ± 0.2

[a] Values are means ± standard deviation (n=3)

Isolates that obtained high sporulation efficiencies (> 90% as per fermentation product standards) were GPA 11.2 and GPA 7.1. The sporulation efficiency obtained for GPA 3.5 of 77.4% was considered satisfactory, whereas, GPA 8.3 and GPB 4.4 obtained low sporulation efficiencies of 33.5% and 28.2%, respectively. Isolates GPA 8.3 and GPB 4.4 were therefore eliminated as top performing isolates due to issues of yield and product viability that are foreseen, related to low sporulation efficiency.

4.4.4 Consortium selection

Selection was based on the following performance areas:

1. The scoring achieved which represents the ability of the isolate to degrade medium-to-heavy hydrocarbons.
2. The ability of the isolate to achieve degradation efficiencies between 90 – 100%, for most of the hydrocarbons measured.
3. The ability of the isolate to achieve high sporulation efficiency (> 90% preferred) for the purpose of production and formulation of a viable product.

Based on the results achieved and the scoring criteria described in Section 3.4.2; Table 9; the isolates listed in Table 19 were selected as the top performing isolates. Isolate GPA 11.2 had the highest results across for sporulation efficiency (SE), degradation efficiency and hydrocarbon degradation percentage. On the contrary, GPA 3.5 had the lowest results for SE amongst the top three performing isolates but had a high result for degradation efficiency and hydrocarbon degradation percentage.

Table 19: Top 3 performing isolates for hydrocarbon degrading consortium

Isolates	SE (%)[a]	Degradation efficiency (%)[a]	Hydrocarbon degradation (%)[a]
GPA 11.2	93 ±0.2	70 ±0.5	34 ±0.2
GPA 7.1	92 ±0.3	57 ±0.4	28 ±0.2
GPA 3.5	77 ±0.2	67 ±0.5	30 ±0.2

[a] Values are means ± standard deviation (n=3)

4.5 Selection of isolates for consortium

The top performing isolates (Table 19) were used to form three different combinations of consortia. These isolates were put together to evaluate the ability to grow and proliferate in the industrial and synthetic effluent by measuring the degradation rate of hydrocarbons. These isolates were selected based on SE percentage, their degradation efficiency and also their hydrocarbon degradation rate. The combination of these isolates were Gen 2: P401+ P402+D014; Gen 3.1: GPA 11.2 + GPA 7.1; Gen 3.2: GPA 11.2+GPA 3.5; Gen 3.3: GPA 11.2+GPA 7.1+GP A3.5.

4.5.1 Standard calibration curve

A standard calibration curve was generated by analyses of a set of standard samples of known concentration of total petroleum hydrocarbons. The retention times corresponding to the identified compounds are listed in Table 20. The resultant standard calibration table, which was used to generate standard calibration curves for each compound, and regression obtained per compound, is given.

Table 20: Identification of peaks and peak compounds using the GRO/DRO calibration mix standard

Chemical formula	IUPAC name	Retention time (min)	Calibration equation	Regression (R^2)
C10	N-Decane	5.047	Area = 19.899*amount - 2.592	0.99983
C12	N-Dodecane	8.44	Area = 21.097*amount - 2.982	0.99978
C14	N-Tetradecane	11.548	Area = 21.393*amount - 6.388	0.99976
C16	N-Hexadecane	14.287	Area = 22.265*amount - 9.948	0.99973
C18	N-Octadecane	16.722	Area = 22.237*amount - 13.86	0.99970
C18	O-Terphenyl	18.915	Area = 21.351*amount - 17.551	0.99967
C20	N-Eicosane	20.903	Area = 18.614*amount - 20.732	0.99962
C22	N-Docosane	22.721	Area = 14.081*amount - 20.161	0.99961
C24	N-Tetracosane	24.394	Area = 9.285*amount - 9.929	0.99963
C26	N-Hexacosane	25.946	Area = 4.904*amount - 2.263	0.99953
C28	N-Octacosane	27.027	Area = 1.459*amount - 2.088	0.99867
C30	N-Triacontane	28.713	Area = 1.785*amount +2.129	0.99955
C32	N-Dotriacontane	29.974	Area = 1.574*amount +2.506	0.99956
C34	N-Tetratriacontane	31.157	Area = 1.638*amount +3.152	0.99929
C36	N-Hexatriacontane	32.475	Area = 1.865*amount - 1.347	0.99919
C38	N-Octatriacontane	34.157	Area = 2.156*amount - 11.314	0.99913
C40	N-Tetracontane	36.374	Area = 2.453*amount - 18.198	0.99860

The coefficient of determination (R^2) was greater than 0.998 for all compounds which demonstrates that the respective calibration curves had a high degree of linearity. The peak area was used to estimate concentrations ($mg.L^{-1}$).

4.5.2 Efficacy testing for TPH degradation

No results were generated for TPH C28-C40 compounds due to the insignificant concentrations present in the respective effluents. The following comparative assessment was performed based on TPH C8-C28 results. A study done by Malatova (2005) also showed that the best degradation (approximately 80 %) was observed on

hydrocarbons with 10-14 carbons. The degradation percentage slightly decreased with increasing numbers of carbons.

There was an increase in the removal (%) of TPH (C8-C28) from synthetic effluent over 72 hours for the Gen 2, Gen 3.1, Gen 3.2 and Gen 3.3 consortia. All consortia performed better than the control. The removal (%) from the synthetic effluent after 72 hours using the Gen 2, Gen 3.1, Gen 3.2 and Gen 3.3 consortia were 84.0, 64.9, 30.0 and 79.8%, respectively. Of the Gen 3 products, the top performing consortium on synthetic effluent was Gen 3.3.

4.5.3 TPH removal (%) from Bushnell-Haas synthetic media

Figure 21 shows TPH C8-C28 removal (%) from Bushnell-Haas synthetic media for the different consortia at 12, 48 and 72 hours.

There was an increase in the removal (%) of TPH (C8-C28) from synthetic effluent over 72 hours for the Gen 2, Gen 3.1, Gen 3.2 and Gen 3.3 consortia. All consortia performed better than the control. The removal (%) from the synthetic effluent after 72 hours using the Gen 2, Gen 3.1, Gen 3.2 and Gen 3.3 consortia were 84.0, 64.9, 30.0 and 79.8%, respectively.

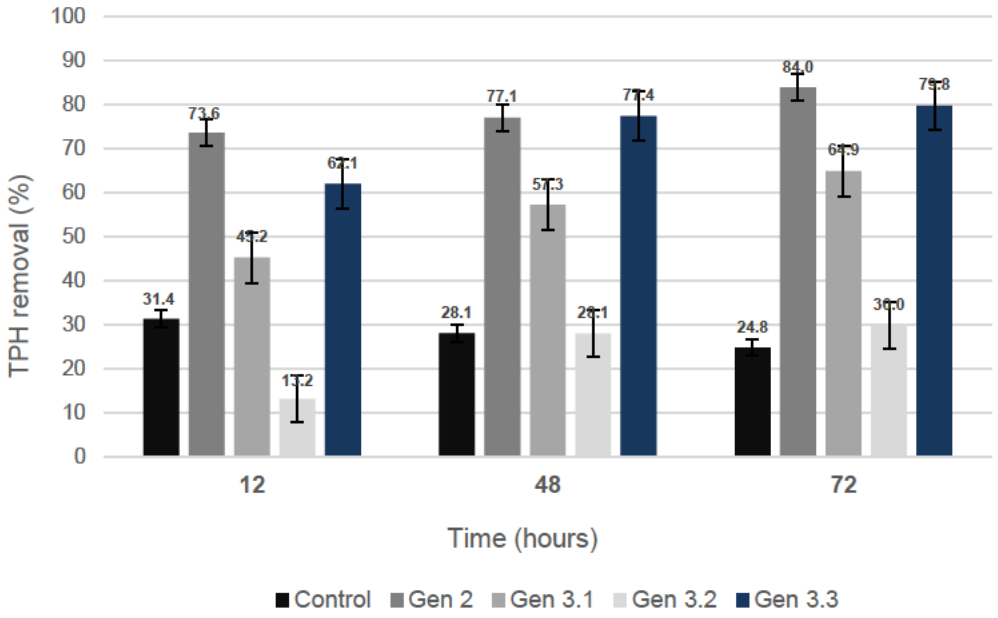


Figure 21 : TPH C8-C28 removal (%) from Bushnell-Haas synthetic media for the control, Gen 2, Gen 3.1, Gen 3.2 and Gen 3.3 at 12, 48 and 72 hours. Values are means ± standard deviation (n=3). Gen 2: P401+ P402+D014; Gen 3.1: GPA 11.2 + GPA 7.1; Gen 3.2: GPA 11.2+GPA 3.5; Gen 3.3: GPA 11.2+GPA 7.1+GPA 3.5.

4.5.4 TPH removal (%) from industrial effluent

An increase in the removal (%) of TPH (C8-C28) from real (industry) effluent over 72 hours for the Gen 2, Gen 3.1, Gen 3.2 and Gen 3.3 consortia, was observed (Figure 22). All consortia also performed better than the control. The removal (%) from the real effluent after 72 hours using the Gen 2, Gen 3.1, Gen 3.2 and Gen 3.3 consortia were 52.0, 64.5, 53.6 and 52.7%, respectively.

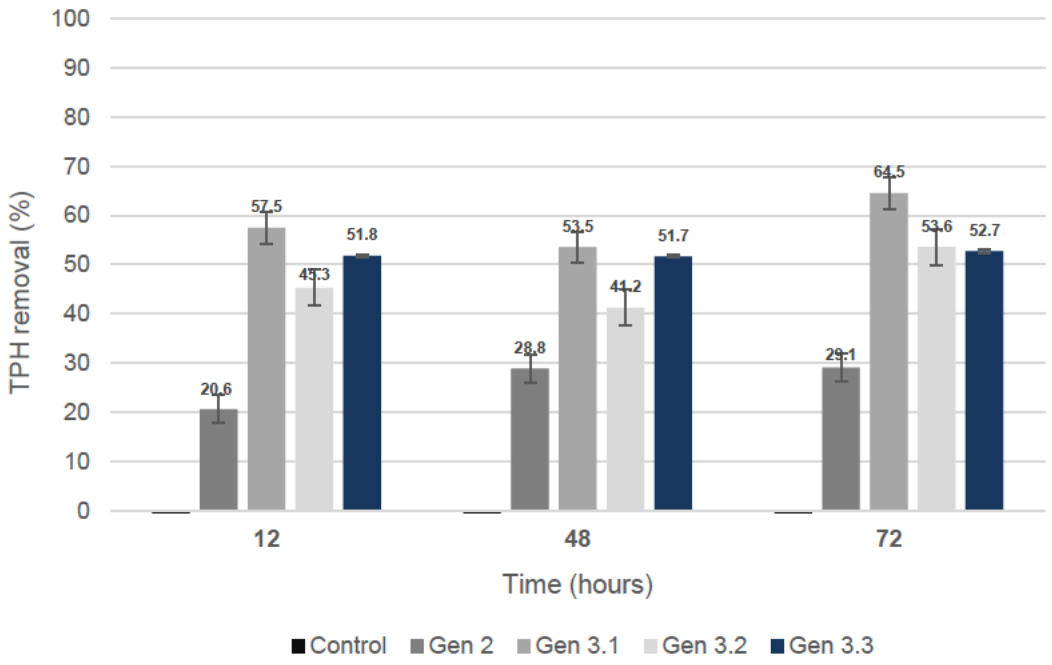


Figure 22 : TPH C8-C28 removal (%) from industry effluent for the control, Gen 2, Gen 3.1, Gen 3.2 and Gen 3.3 at 12, 48 and 72 hours. Values are means ± standard deviation (n=3).

An increase in the removal (%) of TPH (C8-C28) from real (industry) effluent over 72 hours for the Gen 2, Gen 3.1, Gen 3.2 and Gen 3.3 consortia was observed. All consortia also performed better than the control in this case. The removal (%) from the real effluent after 72 hours using the Gen 2, Gen 3.1, Gen 3.2 and Gen 3.3 consortia were 29.1, 64.5, 53.6 and 52.7%, respectively.

Of the Gen 3 products, the top performing consortium on real effluent was Gen 3.1 (GPA 11.2 and GPA 7.1). The removal (%) across all consortia was lower in real effluent which can be attributed to the limited nutrients present for bacterial growth. According to literature, hydrocarbons as a feed substrate are only able to provide carbon and hydrogen to the microbial cell (Singh et al., 2015). The remaining nutrients

must be obtained from the environment for the organism to grow (Joutey et al., 2014). Low levels of nutrients can be a limiting factor thus affecting the biodegradation processes (Abatenh et al., 2017b). Previous studies demonstrating the efficacy of a hydrocarbon-degrading product on industry effluent made use of the consortium (Gen 3) in a formulated dry product containing active ingredients; frequent dosing was performed and the studies proved successful with removal efficiencies of > 90% achieved (Kachieng and Momba, 2017). For an efficient removal of oil in any contaminated environment, speed and efficient process development are the main factors when it comes to kinetics of the biodegradation process. A study done by Venosa (2007), indicated that when evaluating the biodegradation activity, the following factors are taken into account:, surfactant formulation, type of oil, dilution effects of oil, effect of surfactants on microbial attachments towards oil droplets, differences in uptakes of the various hydrocarbon constituents, and dispersant efficiency in biodegradation processes. The breakdown of hydrocarbon compounds is accelerated by enzymes that breakdown different sizes of each compound. These enzymes are activated by the production of a biosufactant that is formed during the growth of these *Bacillus* isolates using petroleum as a carbon and energy source. According to literature (Chikere et al., 2011) and the results from the GC about the rate at which these consortium breakdown the hydrocarbon compound, it is then assumed that these isolates are able to activate the specified enzymes that degrade these hydrocarbons.

This study also showed how effective a dispersant is in improving bioavailability of hydrocarbons and also indicated the biodegradation capability of a consortium of *Bacillus* spp. Following the assessment of the removal efficiencies, the rate of TPH removal across all consortia was greatest between 0 – 48 hours. This indicates the removal of bulk contaminants during the first 48 hours where the removal (%) did not increase significantly after 48 hours. The TPH (C8-C28) removal rates from synthetic effluent after 48 hours of treatment using the Gen 2, Gen 3.1, Gen 3.2 and Gen 3.3 consortia were 0.8, 0.26, 0.07 and 0.58 $mg.L^{-1}.h^{-1}$, respectively. The TPH (C8-C28) removal rate from real effluent after 48 hours of treatment using the Gen 2, Gen 3.1, Gen 3.2 and Gen 3.3 consortia were 0.23, 0.25, 0.12 and 0.17 $mg.L^{-1}.h^{-1}$, respectively.

Performance was based on the removal (%) achieved across consortia in synthetic and industrial effluents. For assessment and selection, the performance on real effluent, however, was preferred as it demonstrated the consortiums' ability to survive and remediate under challenging conditions. The best performing consortium in industrial effluent was Gen 3.1 (containing organisms GPA 11.2 and GPA 7.1) where a removal (%) of 64.5% was achieved after 72 hours. In addition, this consortium achieved a similar degradation efficiency in synthetic effluent (64.9%).

It is recommended to proceed with Gen 3.1 as a hydrocarbon-degrading product prototype for market testing.

Chapter 5

5. Conclusions and recommendations

Petroleum hydrocarbons are the most common pollutants in the environment and this type of pollution poses a major concern for the global ecosystem. Bioremediation using microorganisms constitutes a promising strategy for the clean-up of oil contamination. This study presents a complete focus on the ability of indigenous *Bacillus* isolates that can be used in a consortium to degrade hydrocarbons.

Major conclusions drawn from this study include:

- A total of 115 isolates from 34 environmental samples were obtained and tested positive for their ability to degrade hydrocarbons

- Of the 115, the top performing isolates which were identified as GPA 11.2, GPA 7.1, GPA 3.5, GPA 8.3 and GPB 4.4 were obtained from a car workshop in Midrand and taxi rank in Silverton.

- The top three performing isolates were tested against existing CSIR consortium used for bioremediation called Gen 2 which was used a benchmark for this part of the study. The new consortium of indigenous isolates from this study outperformed the original product (consortium) benchmark.

- Gen 3.1 consortium which is the top performing consortia degradation rate of 64.5% and the TPH removal rate of 0.25mg.L^{-1}.h^{-1}. Whereas Gen 2 which was

using as a benchmark in this study had a degradation rate of 29.1% and a TPH removal rate of 0.23 $mg.L^{-1}.h^{-1.}$ This clearly shows that Gen 3.1 outperformed Gen 2.

- This study has demonstrated an environmentally friendly technique that involved the application of *Bacillus* consortia as bioremediation agents to enhance the degradation of hydrocarbons.

Recommendations:

- A detailed mechanism of the degradation of hydrocarbons, microbial genomics and elucidation of the biodegradative pathways of the hydrocarbons by *Bacillus* isolates could be further investigated.

- Product formulation options wherein product efficacy and shelf life is maintained can be assessed to enable ease of use and promote adoption by industry.

- A production technology for each of the organisms is being developed at lab and commercial scale manufacture. This will enable the commercial implementation of this technology at a production scale. Furthermore, a full technology package would need to be compiled and the necessary technology transfer completed.

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