2021

Assaying Bacillus Amyloliquefaciens Isolates 1BA and 1D3 for Biosurfactant Production and Utilization of Petroleum Hydrocarbons and Phenolic Acids

Pavan Kulkarni
South Dakota State University

ASSAYING *BACILLUS AMYLOLIQUEFACIENS* ISOLATES 1BA AND 1D3 FOR BIOSURFACTANT PRODUCTION AND UTILIZATION OF PETROLEUM HYDROCARBONS AND PHENOLIC ACIDS

BY

PAVAN KULKARNI

A dissertation submitted in partial fulfillment of the requirements for the

Masters of Science

Majoring in Biological Sciences

Specializing in Microbiology

South Dakota State University

2021

THESIS ACCEPTANCE PAGE

Pavan Kulkarni

This thesis is approved as a creditable and independent investigation by a candidate for the master's degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Bruce Bleakley
Advisor Date

Dr. Radhey Kaushik
Department Head Date

Nicole Lounsbery, PhD
Director, Graduate School Date

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Bruce Bleakley for his invaluable supervision, support, and patience in guiding me through the process. My gratitude extends to the Faculty of Dr. Douglas Raynie and Ahsan Ahmed for providing me an avenue to explore the project in detail. Thanks to Mom, Dad, Prasanna Kulkarni, Kerry Drager, Rachel McDaniel, John McMaine for providing emotional support. My appreciation also goes out to my other family members and friends for their encouragement and support all through my studies

Contents

ABBREVIATIONS

PAHs	Poly cyclic aromatic hydrocarbons
DNRA	Dissimilatory nitrate reduction to ammonium
GHG	Greenhouse gases
VOC	Volatile organic hydrocarbons
DMSO	Dimethyl sulfoxide
PHC	Petroleum hydrocarbons
HPLC	High Pressure Liquid Chromatography
GC	Gas Chromatography
MM	Minimal media
NB	Nutrient Broth
TSB	Tryptic Soy Broth
TSA	Tryptic Soy Agar

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

ASSAYING *BACILLUS AMYLOLIQUEFACIENS 1BA AND 1D3 FOR* UTILIZATION OF PETROLEUM HYDROCARBONS AND BIOSURFACTANT PRODUCTION

PAVAN KULKARNI

2021

Several *Bacillus amyloliquefaciens* isolates obtained from wheat residue have been studied as biological agents to control wheat diseases. Notable traits of isolates 1BA and 1D3 include growth with high salt (10% NaCl), at temperatures up to 50°C and over a wide pH range. BIOLOG Gen III plates were used to study and further characterize *Bacillus amyloliquefaciens* 1BA and 1D3. Both isolates showed tolerance to high salt concentration supporting previous studies. They also grew in the presence of lithium chloride, potassium tellurite, and sodium bromate. Both isolates grew at pH 5, with almost identical carbon source utilization fingerprints. However, D-serine, quinic acid, and N-acetylglucosamine were utilized by 1BA but not by 1D3.

Potential for the bacteria to carry out dissimilatory nitrate reduction was studied using nutrient broth (NB) media and tryptic soy broth (TSB) amended with 5 mM potassium nitrate. The isolates were able to reduce nitrate better in richer (tryptic soy broth) culture medium compared to the less nutrient rich medium (nutrient broth), suggesting that the isolates may carry out dissimilatory nitrate reduction to ammonium.

Utilization of phenolic acids that are known to occur in plant and soil habitats, including, cinnamic acid, ferulic acid, salicin, caffeic acid, vanillin, and benzoic acid, was assayed for

both the bacterial isolates. Both the isolates were able to utilize caffeic acid and ferulic acid as sole carbon source, indicating the possibility of utilizing phenolic compounds that are found in soil and plant materials.

The isolates were also assayed for their ability to metabolize of volatile aromatic hydrocarbons. The isolates were incubated in a sealed glass a desiccator on a carbon-free agar growth medium in the presence of toluene, phenol, or kerosene. After a 30-day incubation in the dark at 27°C, soluble proteins of bacterial growth were measured using a bicinchonic acid (BCA) assay. *Bacillus amyloliquefaciens* isolates 1BA and 1D3 showed greater growth with kerosene compared to the individual volatile aromatic hydrocarbons (VOCs) toluene and phenol.

In broth studies, isolates 1BA and 1D3 were grown in TSB amended with sterile (v/v) 1.0 % kerosene, and in minimal broth media (MSM) amended with sterile (v/v) 1.0% kerosene. The petroleum degradation/utilization was tracked every two days Gas Chromatography (GC) connected with an Agilent Mass spectrometer. There was no significant reduction in total kerosene observed. Using the same broth media production of surfactin was tracked using a High-Pressure Liquid Chromatography (HPLC). Of the two isolates only 1D3 was found to produce the biosurfactant surfactin.

In the TSB medium isolate 1D3 produced 1,021 ppm of surfactin after 5 days and 1,055.5 ppm at 10 days incubation in the presence of kerosene. In the absence of kerosene 3,310 ppm was produced after 5 days of incubation, and 3,271.3 ppm after 10 days incubation. The reduction in surfactin concentration in flasks with kerosene compared to media without kerosene could be due to surfactin-kerosene interaction. The concentration of surfactin was

higher in the TSB broth compared to minimal broth medium. Surfactin was detected in the minimal medium, but could not be quantified.

Bacillus amyloliquefaciens isolates 1BA and 1D3 were found to utilize some of the volatile compounds and phenolic acids that are commonly found in plant and soil environments. They also were found likely to carry out dissimilatory nitrate reduction, and to produce surfactin. Future studies could evaluate the impact of surfactin on degradation of long-chain hydrocarbons; and examine their utilization of other volatile compounds which can be harmful to human and environmental health.

INTRODUCTION

Every microorganism that can be isolated and studied by humans has its own unique evolutionary history, and role it plays in its natural habitat. Many human researchers seek to isolate pure cultures of microbes that have specific capabilities that are beneficial to humanity and/or the environment. Once researchers reveal capability of a culturable microorganism, they might further investigate phenotypic traits and metabolic abilities of the microbe. The *Bacillus* isolates that are the focus of this thesis were originally isolated from wheat residues on soil surfaces in the field and used for biological control of certain plant-pathogenic fungi. It became evident during that work that these bacteria might have additional capabilities other than biological control of target organisms, that touched on other research areas, such as bioremediation and biosurfactant production. The work described in this thesis explores that possibility.

During the biocontrol work conducted by previous students in our research lab, it became evident that the *Bacillus amyloliquefaciens* isolates 1BA and 1D3 were able to produce extracellular lipopeptides. These lipopeptides have biosurfactant activity, and sometimes play a role in environmental bioremediation, such as is the needed for oil spills that impact the petroleum industries and flora and fauna around the world.

Bioremediation is a process in which certain microorganisms metabolize contaminants either through oxidative or reductive processes. Under favorable conditions, some microorganisms can oxidatively degrade organic contaminants completely into nontoxic end-products which can include carbon dioxide gas and water, as well as other organic acids and methane (1).

The primary contaminants in oil spills include poly cyclic aromatic hydrocarbons (PAHs), impact both soil and water quality. PAHs are classified as carcinogenic and pose a threat to human health. Their presence in drinking water, soils, and other sensitive environments and their bioconcentration and biomagnification within food chains are of major concern (2).

Awareness of the negative effects of the PAHs has encouraged governments around the world to take a closer look at their bioremediation and the costs associated with the oil spills. The costs associated with oil spills and oil cleanup have increased significantly in the past decade. The cost of oil spill cleanups is influenced by the location of the spills. Oil spills occurring along shorelines are costlier to cleanup, than those occurring offshore. The type of oil spilled is another significant factor in determining cleanup costs. The more persistent and viscous the oil, the more widespread the contamination and the more difficult removal will be.

The composition and physical properties of the oil will affect the degree of evaporation and natural dispersion, as well as the ease of removal. Lighter fraction crude and refined oils evaporate and disperse to a greater extent than heavier oils, except when water-in-oil emulsions form. Heavier crude, fuel oils, and emulsions are difficult to remove using dispersants, skimmers, and pumps resulting in considerably higher cleanup costs from using manual methods.

When oil spills occur near a potentially sensitive coastline or resource, (and/or near a potentially sensitive public areas) cost-effective approach to a cleanup operation is to invest as much equipment, personnel, and energy as feasible to ensure the oil does not reach the shoreline or sensitive resource. One unpublished study suggests that in spill

incidents in which the oil impacts a coastline, as much as 90–99% of the cost of cleanup is associated with shoreline cleanup procedures, especially when manual methods are employed (Franken 1991). This study showed that the cost of removing oil offshore (by either dispersants or mechanical recovery) averaged $7,350/ton, whereas shoreline cleanup costs as high as $147,000–$294,000/ton (2).

In recent years, several bioremediation techniques have been developed by bacteria isolated from soil and other environments. These methods have often proven to be more cost-effective and less time-consuming than other approaches. However, in some instances these methods have had a negative impact on the environment.

One objective described in this thesis was to research the two *Bacillus* isolates to see if they might have potential for use as bioremediation agents for cleanup of petroleum fractions. Even though the environment they were isolated from did not seem to have experienced petroleum contamination. We were interested to see if the isolate's had the metabolic abilities, including biosurfactant production that would allow them to metabolize petroleum fractions to a measurable degree.

Petroleum can contain a verity of recalcitrant compounds that can prove challenging for microorganisms to degrade. Another type of naturally occurring, recalcitrant molecule is lignin, a polymer found in plant cell wall. The hydrolysis of lignin can release its components phenolic acids, such as coumaric acid, ferulic acid and vanillic acid(3). Phenolic acids are produced naturally by terrestrial green plants and some microorganisms, and also by human activities including the paper-manufacturing and olive oil industries (4). Adverse effects from the phenolic molecules have been observed on the

growth of green plants, aquatic organisms, and bacteria exposed to phenolics molecules at concentrations ranging from 0.1 to 6 mM (4-7).

The most consistently reported single-ring aromatic molecule produced from lignin hydrolysis is vanillic acid, ferulic acid and coumaric acid. These phenolic acids can be metabolized via the protocatechuate branch of the β-ketoadipate pathway (8).

The two *Bacillus* isolates of this study were isolated from decomposing wheat residues on soil surfaces, and so were likely adapted to an environment that experienced decomposition of lignin that could release phenolic acids. For that reason, we investigated the ability of these isolates to grow on sealed phenolic acids.

Synthetic compounds produced by humans for agricultural use can also include phenolics. Compounds structurally similar to benzoic acid are produced by the breakdown of some synthetic pesticides in the environment and can cause harm to non-target organisms such as fish and algae (7).

Chemical methods used for breaking down aromatic and phenolic fractions of polluted industrial effluents involve intense oxidation with molecular oxygen and ozone, or ultraviolet radiation, and can also require high energy and sometimes harsh chemicals such as hydrogen peroxide (9).

The high phenolic concentration in these waste streams can be harmful to the bacteria used for treatment at typical wastewater treatment plants, making clean-up for lignocellulose-processing industries more difficult. The wastewater produced from the industries also impact soil due to its high phenolic concentration. When such wastewater is applied to soil the phenolic compounds can negatively impact seed germination, growth, and yield of plants (10).

Bacillus spp have often been used for bioremediation in wastewater treatment (11-13). The work described in this thesis looks at the capabilities of the *Bacillus amyloliquefaciens* isolates to use selected phenolic compounds as their sole carbon source (13).

In the different natural environments these *Bacillus* isolates inhabit, such as soil and aerial portions of wheat that experience variable water availability, the ability to function both aerobically and anaerobically could be advantageous. The ability to ferment, and/or carry out biological denitrification and/or dissimilatory nitrate reduction to ammonium (DNRA) would be phenotypic traits allowing the bacteria to function in anaerobic environments.

One of the products of both denitrification and DNRA can be nitrous oxide gas (N_2O), a greenhouse gas (GHG), that is the most abundant stratospheric ozone depleting substance (14, 15). Nitrate is the primary terminal electron acceptor for both biological denitrification and DNRA. Levels of nitrate in soil and water can be greatly affected by agricultural use of commercial synthetic fertilizers, a practice which not only contributes to GHG emissions but also impacts water and soil quality. Farmland use of fertilizer is predicted to increase 35%–60% by 2030, and extrapolations are that agriculture could contribute up to 59% of total N_2O emissions by then (EPA US, 2015).

Certain microorganisms present in water and soil can help in reducing high nitrogenous chemical-species concentrations in water and air. Such microorganisms are frequently used in bioremediation and wastewater treatment facilities. Many *Bacillus spp.* present in soil and water improve not only water quality but can act as probiotics in aquaculture to promote water quality and prevent diseases (12).

For this thesis, laboratory studies were conducted using two *B. amyloliquefaciens* isolates to examine their metabolic capabilities in the presence of long-chain hydrocarbons and phenolic compounds. These bacterial isolates were previously used in our laboratory in experiments for biocontrol studies of plant diseases. The same study found that they produce surfactants, likely lipopeptides (16). These bacteria were originally isolated from wheat residue, with little to no soil material present, and were likely not previously exposed in any major way to petroleum hydrocarbons. Their production of biosurfactants encouraged us to conduct the present study to see if these bacteria had any measurable capability of breaking down selected recalcitrant compounds.

The hypotheses we wanted to test in this study included the following:

We hypothesized that isolates 1D3 and 1BA might have some ability to metabolize petroleum fractions due to their production of biosurfactants. Also, we hypothesized they had the ability to metabolize one or more phenolic acids found in the lignin components of plant lignocellulose. In addition, it was hypothesized that they would be able to either denitrify or carry out DNRA under, anaerobic conditions when nitrate was present as an exogenous terminal electron acceptor.

1. LITERATURE REVIEW

1.1. Previous works with *Bacillus amyloliquefaciens* isolates 1BA, 1BE, 1BC and 1D3

Several research projects using *Bacillus amyloliquefaciens* isolates 1BA, 1BE, 1BC and 1D3 have been conducted in our laboratory where they have been used as bio-control agents against Fusarium Head Blight caused by the fungus *Fusarium graminearum* (17, 18). As biocontrol agents *Bacillus* strains have the potential to reduce dependence on chemical pesticides, which can impact human health and cause ground-water contamination (17).

1.2. Characterization of *Bacillus amyloliquefaciens* 1BA, 1BE, 1BC and 1D3:

In past studies the bacterial isolates were further characterized to see what phenotypic differences could be observed between then, including differences in colony morphology. Colonies of isolates 1BA and 1BC appear shiny with an undulated margin, whereas colonies of 1BE are shiny but not wrinkled. The colonies from isolate 1D3 are dotted and dull-colored. Some phenotypic and microscopic results suggest the isolates are closely related to *Bacillus firmus,* but the initial 500-bp of their 16s RNA sequence shows they are closely related to *Bacillus amyloliquefaciens* (16).

Bacillus amyloliquefaciens isolates are closely related to the *Bacillus subtilis* group, whose members are known to produce antibiotics and cyclic lipopeptides such as iturin and surfactin (16). Previous studies in out laboratory found that purified iturin A inhibits growth of *F. graminearum* at a concentration of 50μg/ml (19).

1.3. Biosurfactants and lipopeptides

Biosurfactants are a class of microbial metabolites with surface-active properties, forming spontaneous assemblies at the air–water or water–oil interface thereby reducing surface/interfacial tensions due to their hydrophilic and hydrophobic structural components. Based on their chemical structures, biosurfactants are divided into five major classes: lipopeptides, glycolipids, phospholipids, neutral lipids, and polymeric compounds (4).

Many biosurfactant-producing microorganisms have been isolated and described on the literature. They include: *Bacillus*, *Agrobacterium*, *Streptomyces*, *Pseudomonas*, and *Thiobacillus* isolates that produce amino acid-containing biosurfactants; *Pseudomonas, Torulopsis, Candida, Mycobacterium, Micromonospora, Rhodococcus,* and *Corynebacterium* strains, that produce glycolipids; and *Thiobacillus*, *Aspergillus*, *Candida*, *Corynebacterium*, *Micrococcus*, and *Acinetobacter* isolates that produce phospholipids and fatty acids (20, 21).

Bacillus subtilis strains have frequently been used in biodegradation studies, such as one where biodegradation of aliphatic hydrocarbons increased from 20.9 to 35.5% and in the case of aromatic hydrocarbons from zero to 41%, compared to the culture without biosurfactant. The biodegradation-enhancement effect due to biosurfactant addition was more noticeable in the case of long-chain alkanes. Pristane and phytane isoprenoids were degraded to the same extent as n-C17 and n-C18 alkanes and, consequently, no decrease in the ratios n-C17/pri and n-C18/phy was observed. Sufficient rapid production of

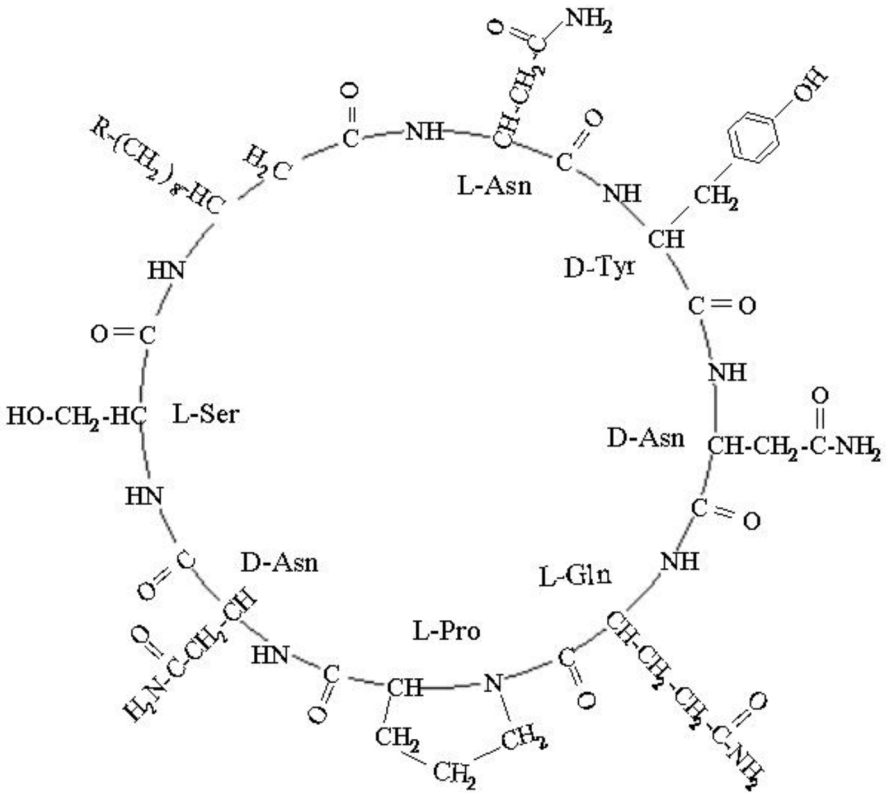
surfactin crude preparation could make it practical for use in bioremediation of ship bilge wastes (22).

1.4. Lipopeptide production by *Bacillus amyloliquefaciens* 1BA in defined media

Isolate *Bacillus amyloliquefaciens* 1BA produce lipopeptides including surfactin and iturin in a *Bacillus* defined medium (BDM). The highest production of surfactin and iturin was observed around 136 hr. after inoculation of BDM (16).

Iturin:

Iturin consists of several isomers, primarily iturin A, iturin B, and iturin C which are known to have antibiotic and antifungal activities(23). Iturin A consists of eight isomers: iturin A1, A2, A3, A4, A5, A6, A7, and A8. These can be observed via HPLC separation. Iturin A is constituted of cyclic lipopeptide with seven α-amino acids, and one unique β-amino fatty acid. The fatty acid chain of lipopeptides varies from 13 to 17 carbon atoms (24). In nature, iturin A is produced as a mixture of up to eight isomers named as iturin A1−A8. The iturin operon is 38–40 kb in size and consists of four open reading frames, namely, ItuA, ItuB, ItuC, and ItuD (24, 25).



Iturin A isomers

A2 CH3CH2CH2-

A3 CH3CH2CH-CH3

A4 (CH3)2CHCH2-

A5 CH3CH2CH2CH2-

A6 (CH3)2CHCH2CH2-

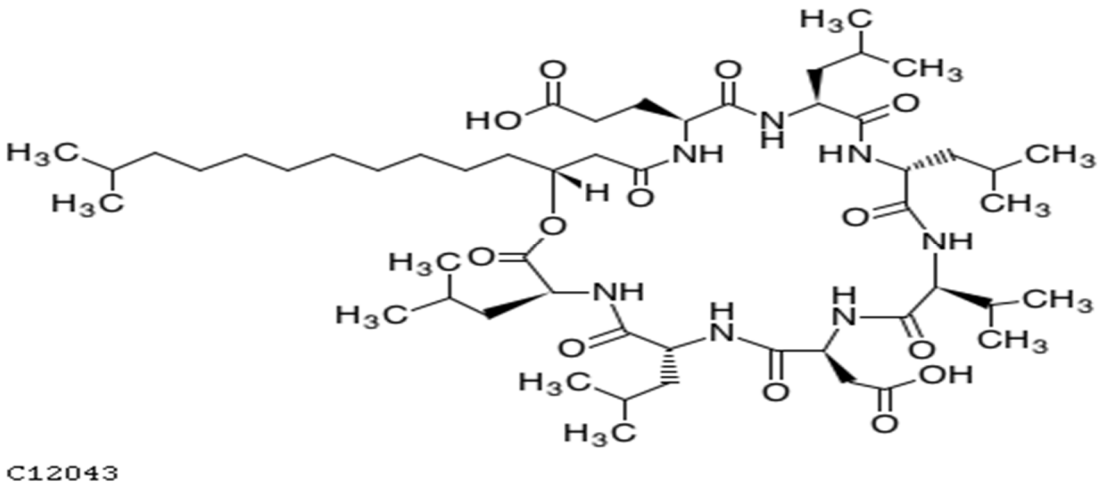
A7 CH3CH2CH2CH2CH2-

A8 CH3CH2CHCH2CH2-CH3-

Figure 1. Chemical structures of iturin A isomers with cyclic lipopeptide and fatty acid chains (26).

Surfactin:

Surfactin is a cyclic lipopeptide produced by several *Bacillus* species (27). It contains both hydrophobic and hydrophilic moieties (making it amphipathic), a property that helps in the reduction of surface tension (20). Surfactin contains hydroxy fatty acids and amino acids; and exhibits strong antibacterial and antifungal properties. It is one of the biosurfactant produced by *Bacillus spp.* in biosurfactant group and is claimed to be the most powerful biosurfactant ever discovered (28). It also plays a significant role in colonization on surfaces and acquiring nutrients. Surfactin is a cyclic compound which contains hydroxy fatty acid and amino acids; and exhibits strong antibacterial and antifungal properties. In one study two novel surfactin molecules isolated from cell-free cultures of *Bacillus subtilis* HSO121 showed strong self-assembly ability to form sphere-like micelles and larger aggregates at very low concentrations (20).



CH3(CH2)n CH CH2CO L-Glu L-Leu D-Leu OL-Leu D-Leu L-Asp L-Val (28)

Figure 2: Surfactin structure

1.5. Degradation and bioremediation of long-chain petroleum hydrocarbons by microorganisms:

Several microbes isolated from oil refineries and oil fields can degrade petroleum hydrocarbons present in crude oil. Most of these organisms also produce biosurfactants. The biosurfactants help in the adsorption as well as absorption of the long-chain hydrocarbon (n-alkanes) present in the crude oil (29).

Strains of *Arthrobacter, Burkholderia, Mycobacterium, Pseudomonas, Sphingomonas, and Rhodococcus* have been found to be involved in alkyl-aromatic degradation. (29, 30).

A *Dietzia* CN-3 isolate from a petroleum-contaminated sediment in Bohai Bay, China was found capable of utilize a wide range of alkanes (C14-C31), aromatic compounds and crude oil as the sole carbon and energy source (31, 32). Some *Acinetobacter sp.* utilize n-alkanes of chain length C10–C40 as a sole source of carbon (29, 33).

Some *Bacillus subtilis* strains are able to grow on and degrade n alkanes and other aromatic compounds in the presence of sufactin. Lower concentrations of surfactin did not affect biodegradation or microbial growth, but the higher concentration gave higher cell densities and increased biodegradation of aliphatic hydrocarbons from 20.9 to 35.5%. The aromatic hydrocarbons were degraded from nil to 41%, compared to the culture without biosurfactant. The enhancement effect on biodegradation due to biosurfactant addition was more noticeable in the case of long-chain alkanes (22).

In a phylogenetic analysis done on a consortium of bacteria isolated from an oil field in Daqing, China 11 bacterial strains were found to degrade the total petroleum hydrocarbons (TPHs) of diesel oil by more than 70% in 7 days. Phylogenetically, 19 of the bacteria related to *Bacillus spp.* (34). *Bacillus* and other soil bacteria found in oil fields have shown

the ability to degrade petroleum hydrocarbons; multiple experiments employing mixtures of multiple bacterial species are known to result in enhanced breakdown of hydrocarbons (35, 36). Biosurfactants secreted by some bacteria are more effective than chemical surfactants in enhancing the solubility and biodegradation of petroleum hydrocarbons, including PAHs (37, 38).

In GC/FID analyses *B. subtilis* and *P. aeruginosa* strains demonstrated that n-alkanes (C14–C30) were preferentially degraded compared to PAHs present in crude petroleum-oil by all the bacteria studied (35). Production of biosurfactant is related to the utilization of available hydrophobic substrates by the biosurfactant-producing microbes in their natural habitat, presumably by increasing the surface area of substrates and increasing their apparent solubility (27, 39-41).

A *Bacillus amyloliquefaciens* strain BC was found to be very proficient at biodegradation of general petroleum hydrocarbons, because it can act n-alkanes, n-alkylcyclohexanes, isoprenoids, and aromatic hydrocarbons (41).

1.6. Assaying growth of *Bacillus* on volatile aromatic compounds as sole carbon source:

Volatile organic compounds are low molecular weight carbon-containing compounds that have low boiling points and measurable vapor pressures at Standard Temperature and Pressure. (42, 43). Those originating from or interacting with organisms ranging from bacteria to humans have numerous effects in medicine, environmental sciences, the food industry, and plant sciences.

In soil and plant environment certain VOCs compounds are used for communication between organisms, acting as chemical signals affording communication between members of the same species and/or between members of different species as well. Some *Bacillus*

spp. which are found ubiquitously on/in soil and on plants are known to improve plant health and reduce fungal infections by producing various VOCs (44, 45).

In an ecological context these *Bacillus* strains help provide plant protection against some pathogens, using direct control of phytopathogens based on effect of both VOCs and antibiotics of benefit for the plant (46, 47). *Bacillus amyloliquefaciens* and other *Bacillus sp* are known to promote/support growth of plants in laboratory environments by helping facilitate interactions between the plant and the VOCs produced by the plants and other associated organisms (46-48).

1.7. BIOLOG studies:

BIOLOG plates have frequently used in verity of studies to characterize physiological capabilities of bacteria. Since both the isolates in this study belong to *Bacillus* species, BIOLOG GEN III plates that allow testing of either Gram positive or Gram negative bacteria were used. Attempts were also made to study the degradation of long-chain petroleum hydrocarbons using BIOLOG MT2 plates which allow the investigator to add their own carbon source of interest to basal medium contained in the plate.

A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
Negative Control	Dextrin	D-Maltose	D-Trehalose	D-Cellobiose	Gentiobiose	Sucrose	D-Turanose	Stachyose	Positive Control	pH 6	pH 5
B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
D-Raffinose	α-D-Lactose	D-Melibiose	β-Methyl-DGlucoside	D-Salicin	N-Acetyl-DGlucosamine	N-Acetyl-ƃ-DMann	N-Acetyl-DGalactosamin	N-Acetyl Neuraminic	1% NaCl	4% NaCl	8% NaCl
C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
α-D-Glucose	D-Mannose	D-Fructose	D-Galactose	3-Methyl Glucose	D-Fucose	L-Fucose	L-Rhamnose	Inosine	1% Sodium Lactate	Fusidic Acid	D-Serine
D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
D-Sorbitol	D-Mannitol	D-Arabitol	myo-Inositol	Glycerol	D-Glucose-6-PO4	D-Fructose-6-	D-Aspartic Acid	D-Serine	Troleandomycin	Rifamycin SV	Minocycline
E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
Gelatin	Glycyl-L-Proline	L-Alanine	L-Arginine	L-Aspartic Acid	L-Glutamic Acid	L-Histidine	L-Pyroglutamic	L-Serine	Lincomycin	Guanidine HCl	Niaproof 4
F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
Pectin	D-Galacturonic	L-Galactonic acid lactone	D-Gluconic Acid	D-Glucuronic Acid	Glucuronamide	Mucic Acid	Quinic Acid	D-Saccharic Acid	Vancomycin	Tetrazolium Violet	Tetrazolium Blue
G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
p-Hydroxy-Phenylacetic Acid	Methyl Pyruvate	D-Lactic Acid Methyl Ester	L-Lactic Acid	Citric Acid	alpha-Keto-Glutaric	D-Malic Acid	L-Malic Acid	Bromo-Succinic Acid	Nalidixic Acid	Lithium Chloride	Potassium Tellurit
H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12
Tween 40	γ-Amino-Butyric	α-Hydroxy-Butyric	β-Hydroxy-D,Lbut	α-Keto-Butyric	Acetoacetic Acid	Propionic Acid	Acetic Acid	Formic Acid	Aztreonam	Sodium Butyrate	Sodium Bromate

Figure 3: BIOLOG GEN III plate layout, showing different carbon sources, nitrogen sources and other chemicals.

1.8. Phenolic compounds as carbon source.

Phenolic compounds are present in terrestrial plant material and also present in human food; and depending on the concentration of the compounds, they can affect the health of human beings ingesting them. Phenolic compounds can also be introduced into the environment as intermediates during the biodegradation of natural polymers containing aromatic rings, such as lignins and tannins, and from aromatic amino acid precursors; and may also be part of synthetic xenobiotic compounds produced by humans. The phytotoxicity of phenolic compounds is dependent on their chemical and/or physical properties and the presence of other toxic or non-toxic biomolecules (49).

Phenol released into the environment can lead to serious pollution, with the primary sources of phenol including pulp mills, coal mines, refineries, wood preservation plants, wastewater from cities and pharmaceutical industries. Due to its antimicrobial properties and inhibitory effects it not only impacts the water quality but also soil quality (50).

Phenolic compounds play important roles in green terrestrial plants, particularly the low molecular weight compounds. Phenolic compounds are used by plants to communicate and interact with a verity of microorganisms, including microbial symbionts and pathogens. Some phenolics can accumulate in plants in response to the attack of pathogens. *Sinorhizobium meliloti* and *Agrobacterium tumefaciens* interact with the plant phenolic and related compounds in complex plant-microbe associations (44, 51, 52).

Some low molecular phenolic compounds have been shown to induce expression of bacterial genes encoding the type III secretion system. For example, ortho-coumaric acid and trans-cinnamic acid induce expression of genes that encode type III secretion system in the soft-rot plant pathogenic bacterium *Dickeya dadantii* (53).

1.9. Dissimilatory nitrate reduction

Denitrification is a widespread process of global importance, for reasons including its role in emission of nitrous oxide gas that can act as GHG (43). Denitrification is a type of anaerobic respiration carried out by several bacterial groups where NO_3^- is reduced to dinitrogen gas with sequential reduction of nitrate, nitrite, nitric oxide, and nitrous oxide in a complete classic denitrification metabolic pathway (54-56). The process is facilitated by functionally similar but structurally different enzymes– the microbial diversity performing denitrification *in situ* is functionally significant (43, 57). Unlike denitrification, dissimilatory nitrate reduction to ammonia (DNRA) acts to conserve bioavailable nitrogen in the system where it occurs, producing water-soluble ammonium compared to denitrifications that produces largely unreactive dinitrogen gas (57, 58).

Bacillus species include several well-known denitrifiers. Andon the DNARA side of nitrogen cycle, *Bacillus halodenitrificans sp.* are able to carry out DNRA at high salt concentration without the involvement or presence of nitrous oxide reductase (43, 59-62). There is also evidence showing strains of *Bacillus amyloliquefaciens* participate in DNRA. *Bacillus amyloliquefaciens* strains are found to promote plant growth in soil and are also known to inhibit plant infections in agriculture (16, 18, 63). It is possible that some of the strains can carry out DNAR in anaerobic situations, and part of their beneficial effects on plant growth may be due to their production of ammonium. In one study, the addition of *Bacillus amyloliquefaciens* to the soil promoted plant growth and increased the total nitrogen contents in the plants. The same study also indicated a reduction of N_2O gas released from the treated soil (64).

The biofilm produced by some *Bacillus amyloliquefaciens* strains has been shown to reduce nitrate concentration in wastewater and can adsorb nitrite (65). Such biofilms forming in soil or plant habitats may facilitate either denitrification or DNRA by the bacteria and could aid in the bioremediation capability of the bacteria.

2. MATERIALS AND METHODS

2.1. Culture Mediums

2.1.1. Seed Culture:

Both *Bacillus amyloliquefaciens* 1D3 and 1BA were grown in 150 ml Erlenmeyer flasks containing 50.0 ml Bacto Tryptic Soy Broth (TSB) at 27°C on a rotary shaker at 150 rpm. The cells were harvested after 18- 24 hr. incubation by centrifugation at 5000 X g and the pellet was resuspended /washed in sterile 0.8% NaCl to help remove traces of the TSB culture broth. The step of washing was performed three times to remove any residual TSB. The washed cells were used as inoculum for the studies described below.

2.1.2. Minimal Salt Medium (MSM):

The formulation of minimal salt media (MSM) was: 0.1 g NH_4NO_3, 0.2 g $MgSO_4$, 0.1 mg $FeSO_4$, 0.02 g K_2HPO_4 H_2O, 0.9 mg KH_2PO_4. 7 H_2O, 4.0 mg KCl, 1.5 mg $CaCl_2$ and 0.01 g of yeast extract were added to 100 ml of deionized water. Additionally, 1.0 ml/L of 1000 X Nitsch's trace element solution was supplemented to the media before autoclaving. Nitsch's trace element solution contained: $MnSO_4$,2.2g; $ZnSO_4$,0.5g; H_3BO_3,0.5g; $CuSO_4$,0.016g; Na_2MoO_4, 0.025g; CoC_{l2} 6 H_2O, 0.046 g;deionized water, 1L (66).

Solid MSM was prepared by the addition of 1.8% (w/vol) Bacto– Noble agar.

2.2. Kerosene degradation and lipopeptide analysis:

2.2.1. Tryptic Soy Broth and kerosene:

Kerosene purchased from a local store was filter sterilized by passing thorough a 0.2-micron nylon syringe filter (Tisch Scientific), then triplicates of 20 ml of TSB in a 150 ml Erlenmeyer flask were aseptically amended with a final concentration of 1% (v/v) of filter-sterilized kerosene. The controls were setup with the addition of 1% (v/v) filter sterilized deionized water. This was then inoculated with 500 µL of bacterial cell suspension from a seed culture. The flasks were sealed with rubber stoppers and covered with parafilm in an attempt minimize the loss of kerosene through evaporation. Flasks were incubated in the dark on a rotary shaker at 120 rpm at 25°C. Flasks containing TSB but lacking kerosene and bacteria, and flasks with TSB and bacteria but lacking kerosene were used as controls.

2.2.2. MSM amended with Kerosene:

Triplicates of 20.0 ml of sterile MSM housed in a 150 ml Erlenmeyer flask were aseptically amended with a final concentration of 1% (v/v) of filter-sterilized kerosene. The controls were setup with addition of 1% (v/v) filter sterilized deionized water. The flasks were then inoculated with 1.0 ml of concentrated cell suspension each of isolate and were incubated in the dark on a rotary shaker at 120 rpm at 25°C. The flasks were sealed with rubber stoppers and covered with parafilm in an attempt to minimize the loss of kerosene through evaporation. Flasks containing MSM but lacking kerosene and bacteria and flasks with MSM with bacteria but, lacking kerosene were used as controls.

2.3. Lipopeptide extraction:

After 30 days of incubation the cultures were harvested for lipopeptide extraction. Bacterial cells were pelleted by centrifugation at 13,000 X g. Using the supernatant, lipopeptides

were precipitated by acidification (pH 2) using 2 N HCl and stored overnight at 4°C. The supernatant was then centrifugated at 13,000 X g to obtain the biosurfactant pellet. The lipopeptide pellet was then dispersed in absolute ethanol (67).

2.4. Analytics

Analytics methods were developed and performed by Ph.D. student Ahsan Ahmed of the Raynie laboratory in the Department of Chemistry and Biochemistry at SDSU.

2.4.1. Gas GC-MS method:

GC-MS instrument	Agilent Technologies 7890 A gas chromatograph (Agilent Technologies. Little Falls, DE), coupled to an Agilent Technologies 5975C triple-axis mass detector, operated in EI mode.
Column	Rxi-1301 Sil MS from Restek, 30 m × 0.25 mm i.d., with 0.25 μm film thickness.
Oven temperature program	60 °C for 3 min, then ramped at 12 °C/min to 200°C (1 min he 30 °C/min to 250°C (1 min held)
Total run time	18.33 min
Carrier gas	Hydrogen
Carrier gas flow rate	2.4 ml/min
Injection volume	1ul
Injection mode	Split (10:1)
Transfer line temperature	280 °C
MS temp.	Ion source 230 °C and quadrupole 150 °C
Scan range	50-550 u

2.4.2. UHPLC method:

Standards used: Iturin A, purity minimum 97% and surfactin, purity ≥ 98% (Sigma-Aldrich) were used as standards. All the standards were dissolved in methanol

Sample preparation: Extracted lipopeptides were evaporated to dryness with rotary evaporation. The residue then, dissolved in 1.0 mL methanol, and then further diluted 1/2 dilution with methanol (in case of TSB media with ID3 isolates), and injected onto the UHPLC system after filtration through 0.2 μm membrane filter

Preparation of standards: 3000 ppm standard surfactin stock solution - prepared in methanol. The ppm of prepared calibration standards from stock solution: 100, 200, 400, 600, 800 and 1000, 1200, 1400, 1600, 1800, 2000 prepared in methanol with 0.1% TFA.

2.4.3. Chromatographic conditions:

<table>
<tr><td>UHPLC instrument</td><td>UltiMate 3000 UHPLC chromatographic system by Thermo Scientific Dionex, USA equipped with a Diode Array detector</td></tr>
<tr><td>Column</td><td>ZORBAX Eclipse Plus C18 column (5 µm, 150 mm×4.6 mm) (Agilent Technologies. Little Falls, DE)</td></tr>
<tr><td>Gradient program</td><td>
<table>
<tr><th rowspan="2">Time, min</th><th rowspan="2">Flow rate, mL/min</th><th colspan="2">Mobile phase</th></tr>
<tr><th>A[a]</th><th>B[b]</th></tr>
<tr><td>0</td><td>0.3</td><td>70</td><td>30</td></tr>
<tr><td>13</td><td>0.3</td><td>49</td><td>51</td></tr>
<tr><td>18</td><td>0.3</td><td>30</td><td>70</td></tr>
<tr><td>35</td><td>0.3</td><td>0</td><td>100</td></tr>
<tr><td>37</td><td>0.3</td><td>0</td><td>100</td></tr>
<tr><td>39</td><td>0.3</td><td>70</td><td>30</td></tr>
<tr><td>41</td><td>0.3</td><td>70</td><td>30</td></tr>
</table>
A[a]-Water with 0.1%TFA, B[b]- Acetonitrile with 0.1% TFA
</td></tr>
<tr><td>Wavelength</td><td>205 nm</td></tr>
<tr><td>Injection volume</td><td>5ul</td></tr>
<tr><td>Column Temp.</td><td>40 °C</td></tr>
</table>

2.5. Growth of *Bacillus* on volatile aromatic compounds:

The isolates were grown on MSM solidified with 2% Noble agar housed in glass Petri plates contained in a glass desiccator. The plates were streaked in triplicates using single colonies as inoculum and placed in an individual desiccator. Then either 4.5 g of phenol or 5.0 ml of toluene, or 500 µL of kerosene were dispersed in 50.0 ml of cold deionized water in a 50.0 ml beaker and placed in the desiccator along with the plates. Triplicates of inoculated control plates were incubated in a desiccator that contained deionized water with no VOCs. The plates were incubated in darkness for 30 days at 25°C in a sealed glass desiccator.

The bacterial cells were harvested by aseptically pipetting 5.0 ml of sterile 0.8 % NaCl on the agar surface of the Petri plates. The bacterial cells were dislodged from the agar surface using a sterile plastic spreader. A bicinchoninc acid (BCA) assay was performed following methods described by the manufacturer on the pellet obtained after centrifugation at 5,000 X g at 27°C for 10 min.

2.6. Denitrification studies/DNRA studies:

Two culture mediums were used in testing for dissimilatory nitrate reduction capabilities of the *Bacillus amyloliquefaciens*.

Medium 1: 0.8% nutrient broth (Difco) amended with 5.0 mM of KNO_3.

Medium 2: 1.5% tryptic soy broth (Bacto) amended with 5.0 5mM of KNO_3.

Each of the broth media above was prepared in deionized water, then 10.0 ml of each was dispended per standard sized screw-cap glass test tube and autoclaved to sterilize. For inoculation 1.0 ml of seed culture was used. Five controls were set up with no bacterial

inoculum for each medium. After 10days incubation in darkness at 27°C, broth culture tubes were tested using diphenylamine reagent to qualitatively assess for the presence or absence of nitrate according to standard procedure (68).

2.7. Phenolic compounds as sole carbon source:

Two hundred mg of phenolic compounds tested (benzoic acid, cinnamic acid, vanillin, salicin, ferulic acid and caffeic acid) were dissolved in 2ml (w/v) DMSO. Then 200µl of the 0.2 µm nylon (Tisch Scientific) sterile filtered concentrate was added to 20 ml MSM in a 150 ml Erlenmeyer flask, the controls were setup with 0.2 µm sterile filtered DMSO without phenolic acids. Flasks were incubated in the dark on a rotary shaker at 120 rpm at 25°C for 10 days.The BCA assay was performed on pellet extracted after centrifugation 5000 X g at 27°C for 10 min.

2.8. Soluble protein extraction and bicinchoninic acid assay:

2.8.1. Soluble protein extraction:

The bacterial cells were pelleted by centrifugation at 5,000 X g for 10 min. The supernatant was discarded, and the pellet was frozen at -20° C for 24 hr. Five ml room temperature B-PER complete reagent (Thermo scientific B-PER Complete Bacterial Protein Extraction Reagent) was added to the pellet and incubated with gentle rocking on a shaker at 20 rpm for 15 min. The lysate was centrifuged at 16000 X g for 20 min to separate soluble proteins.

2.8.2. Bicinchoninic acid assay (BCA):

A Thermo scientific Pierce BCA Protein Kit was used to quantifying the extracted soluble proteins. Protein standards prepared using various dilutions of bovine albumin provided in standard ampules as part of the kit.

Dilution scheme for standard test tube protocol and microplate procedure (working range of 20-200µg/ml)			
vail	volume of diluent (µL)	volume and source of BSA (µL)	Final BSA concentration (µL)
A	0	300 of stock	200
B	125	375 of stock	1500
C	325	325 of stock	1000
D	175	175 of vail B dilution	750
E	325	325 of vail C dilution	500
F	325	325 of vail E dilution	250
G	325	325 of Vail F dilution	125
H	400	100 of Vail G solution	25
I	400	0	0= Blank

Table 1: Dilution scheme for standard curve BCA assay

The working reagent (WR) was prepared by mixing 50 parts BCA reagent A to 1part of BCA reagent B. 0.1 ml of the standards (Table1) was added to a vial containing 2.0 ml of WR and vials were incubated at 37°C for 30 min. After cooling the vials to room temperature, the absorbance was measure on a photo spectrometer at 590 nm after blanking with blanking solution. The absorbance was plotted to yield a standard curve (Figure 20) and slope equation ($y = 0.0007x + 0.0408$) to calculate the soluble protein concentration for the BCA assay.The BCA Assay was used for determining bacterial cell growth for isolate 1BA and 1D3.

2.9. BIOLOG GEN III and MT2 Plates:

2.9.1. BIOLOG GEN III plate protocol:

Bacillus amyloliquefaciens isolates 1BA and 1D3 were grown on TSA plates at 30°C for 48 hr. Cells were dispersed in Inoculating Fluid (BIOLOG) using a sterile cotton swab to achieve 85 % turbidity (T). Then 100 µL of the turbid Inoculation Fluid containing bacterial cells was transferred to each microplate well aseptically. The plates were incubated at 27°C for 24 hr. Absorbance was measured at 590m using spectrophotometer microplate reader (BioTek Synergy 2 Multi-detection Microplate Reader). The microplates were then returned to 27°C for another 24 hr. incubation and the absorbance was measured again at 48 hr. of incubation with the same microplate reader and parameters as used above.

2.9.2. BIOLOG MT2 Plates

Bacillus amyloliquefaciens isolates 1BA and 1D3 grown on TSA plates at 30°C for 48 hr. were dispersed in Inoculating Fluid (BIOLOG) using sterile cotton swab to achieve 85 % turbidity (T). Then 100 µL of the turbid Inoculation Fluid was transferred to each microplate well aseptically. The plates were incubated at 27°C for 30 days in a sealed desiccator that contained cold 50.0 ml of deionized water in a 50.0 ml beaker with either 4.5g of phenol or 5.0 ml of toluene, or 500 µL of kerosene. Absorbance was measured at 590 nm using spectrophotometer plate reader as described above.

2.10. Statistical analysis:

Statistical analysis was performed using Microsoft 2016 excel (Microsoft Corporation, 2016. Microsoft Excel, Available at: https://office.microsoft.com/excel) and R studio

program (RStudio Team (2020). RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL: http://www.rstudio.com/)

3. RESULTS

3.1. Petroleum hydrocarbon degradation and surfactant production in TSB

There was no significant difference observed between the control samples with kerosene and no bacterial cells, compared to samples containing bacteria and kerosene. The natural degradation of kerosene was similar to the degradation observed in TSB inoculated with isolates (Figure 4).

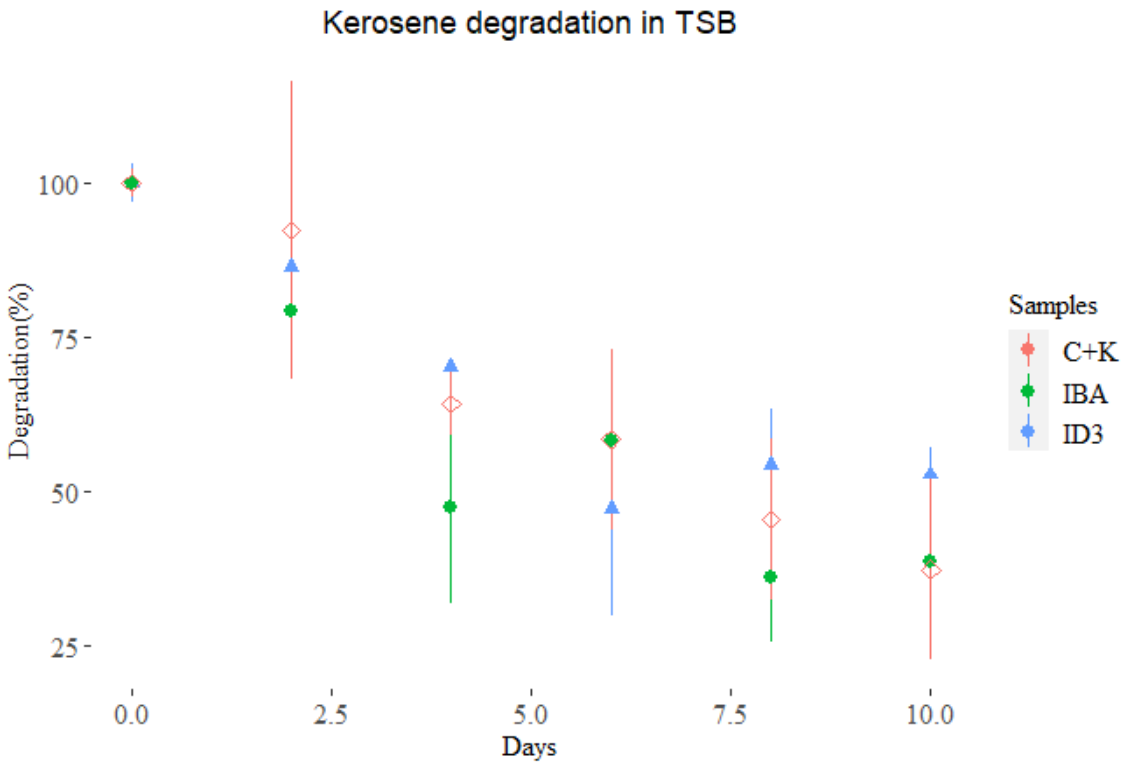


Figure 4: Kerosene degradation in TSB by *Bacillus amyloliquefaciens* 1BA and 1D3. C+K: control (no bacteria) with kerosene, 1BA with kerosene and 1D3 with kerosene.

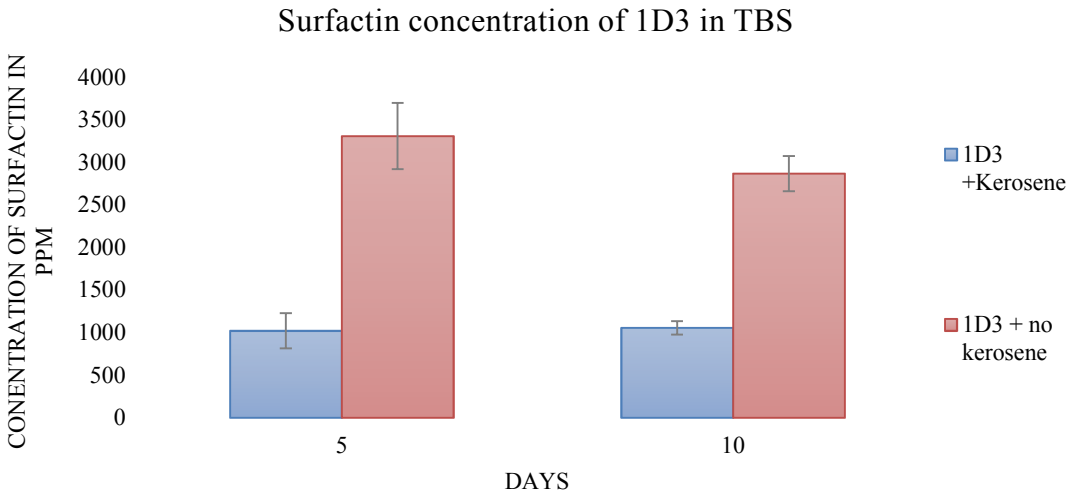


Figure 5: Surfactin production by isolate 1D3 in TSB with and without Kerosene.

The surfactin was only detected in incubations of the isolate 1D3, but not in incubations with isolate 1BA. There was a significant difference between the samples containing kerosene and media without kerosene. A higher concentration of surfactin was found in the media without kerosene (3,310.18 ± 389.74 on day 5 and 2,868.54 ± 206.64 ppm on day 10). The TSB media with kerosene had a concentration of 1,021.18 ± 206.36 ppm on day 5 and 1,055.53± 78.58 ppm on day 10 (Figure 5). No iturin was detected in incubations with either isolate.

3.2. Petroleum hydrocarbon degradation and surfactant production in minimal media

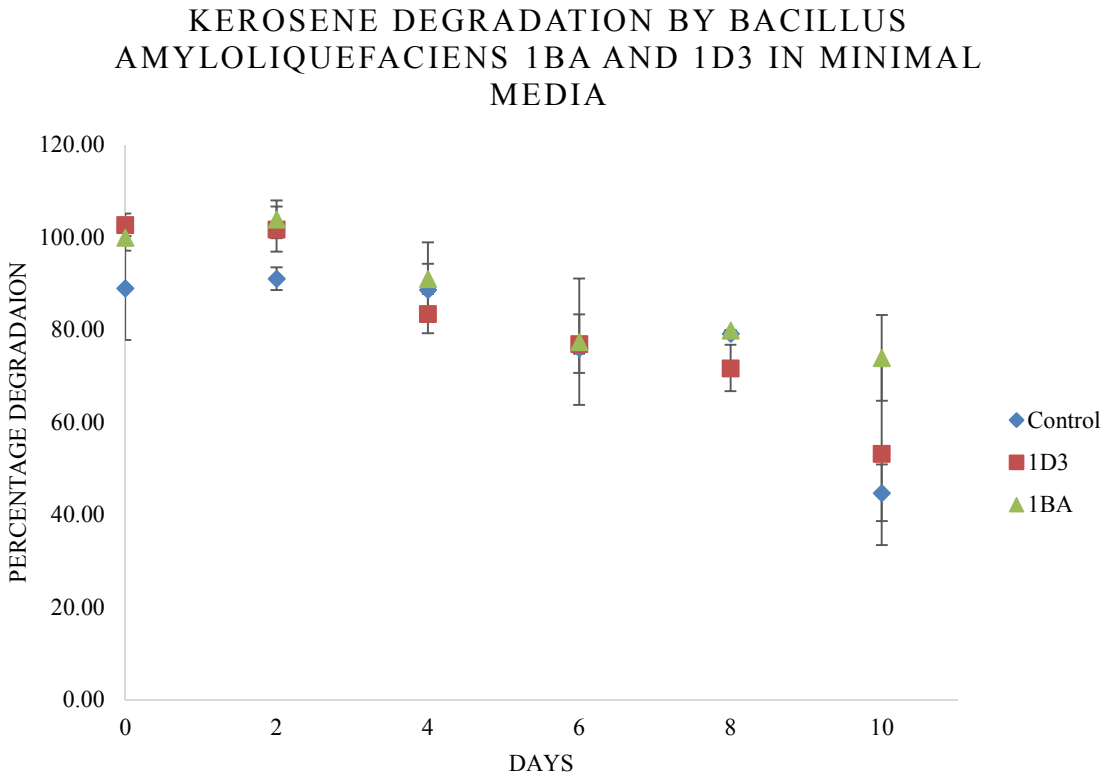


Figure 6: Kerosene degradation by *Bacillus amyloliquefaciens* 1BA and 1D3. C+K: control with kerosene (no bacteria), 1BA with kerosene and 1D3 with kerosene

Similar to TSB media, there was no significant decrease in the reduction of kerosene compared to samples with bacteria and samples without bacteria in minimal media (Figure 6). The reduction of kerosene concentration was observed in all the samples (including the controls).

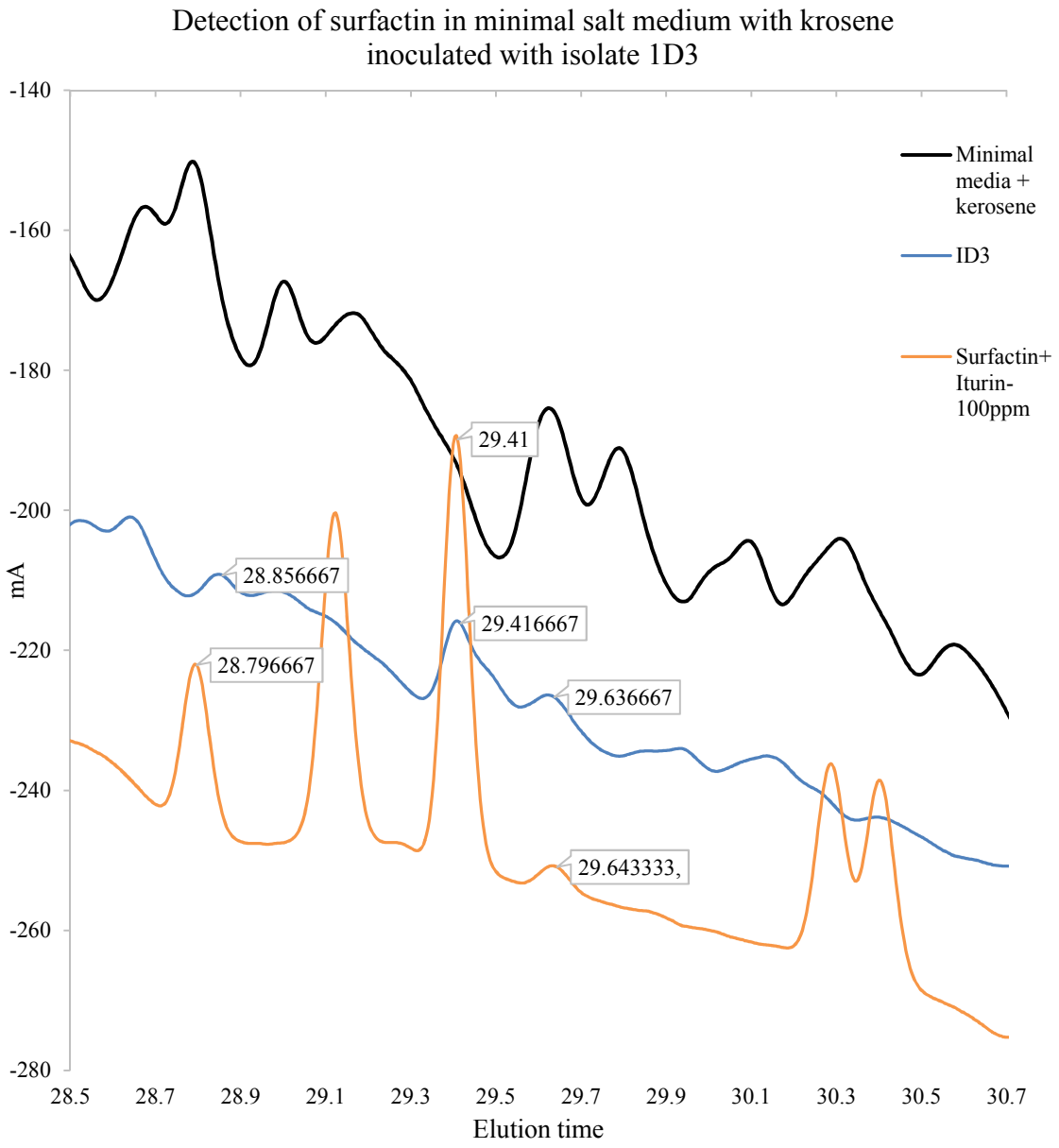


Figure 7: Presence of surfactin in minimal media with *Bacillus amyloliquefaciens* 1D3

Surfactant production in was not as robust as it was observed in the TSB medium. Surfactin was detected in samples containing isolate 1D3 (Figure 7). The concentration of surfactin was below the detection limit and was not quantified. Iturin production was not observed in either of the isolates.

3.3. Growth of *Bacillus* on volatile aromatic compounds

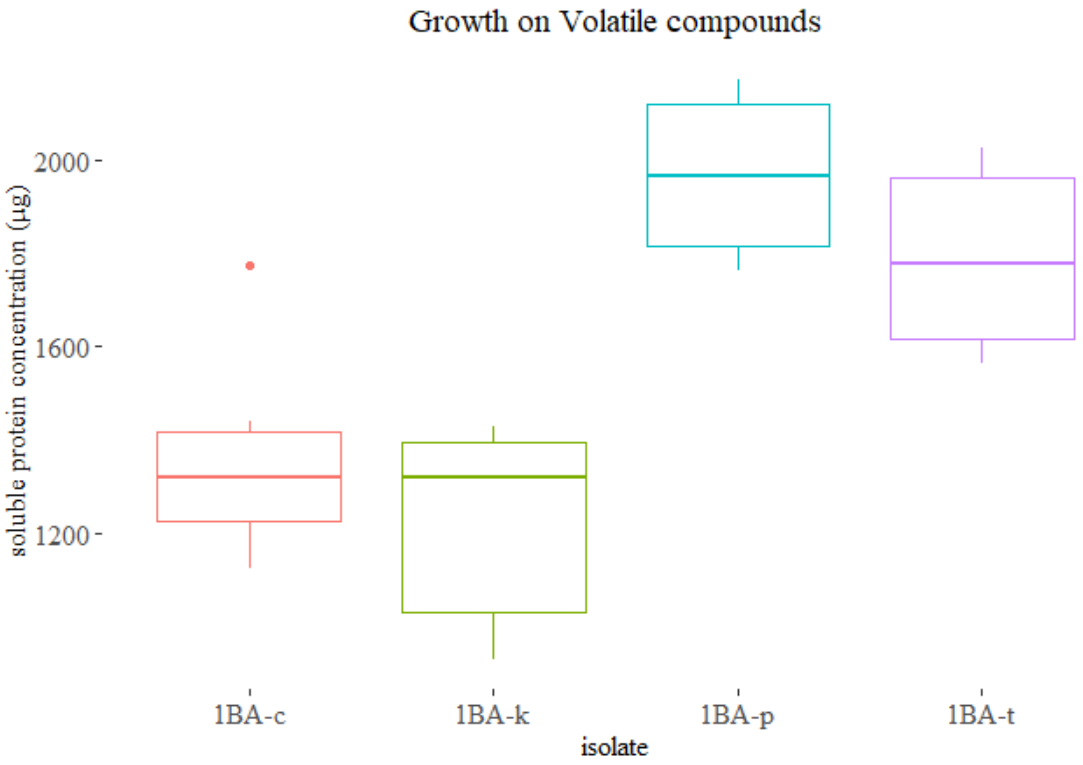


Figure 8: Growth of *Bacillus amyloliquefaciens* 1BA isolate on minimal media plates in presence of volatile compounds. 1BA-c :1BA in the presence of control (no carbon source), 1BA-k: 1BA in the presence of kerosene, 1BA- p: 1BA in the presence of phenol, 1BA-t: 1BA in the presence of toluene

Bacillus amyloliquefaciens 1BA yielded higher concentration of soluble proteins, indicating growth in the presence of toluene, kerosene and phenol when compared to the soluble protein concentration of control (Figure 8). The soluble protein concentrations measured using the BCA assay in the presence of volatile carbon compounds as sole source of carbon were 1,788.86 µg/L for toluene, 1,966.71 µg/L for phenol, 1372.07 µg/L for kerosene and for the control without any carbon source the concentration was 1,348.50 µg/L.

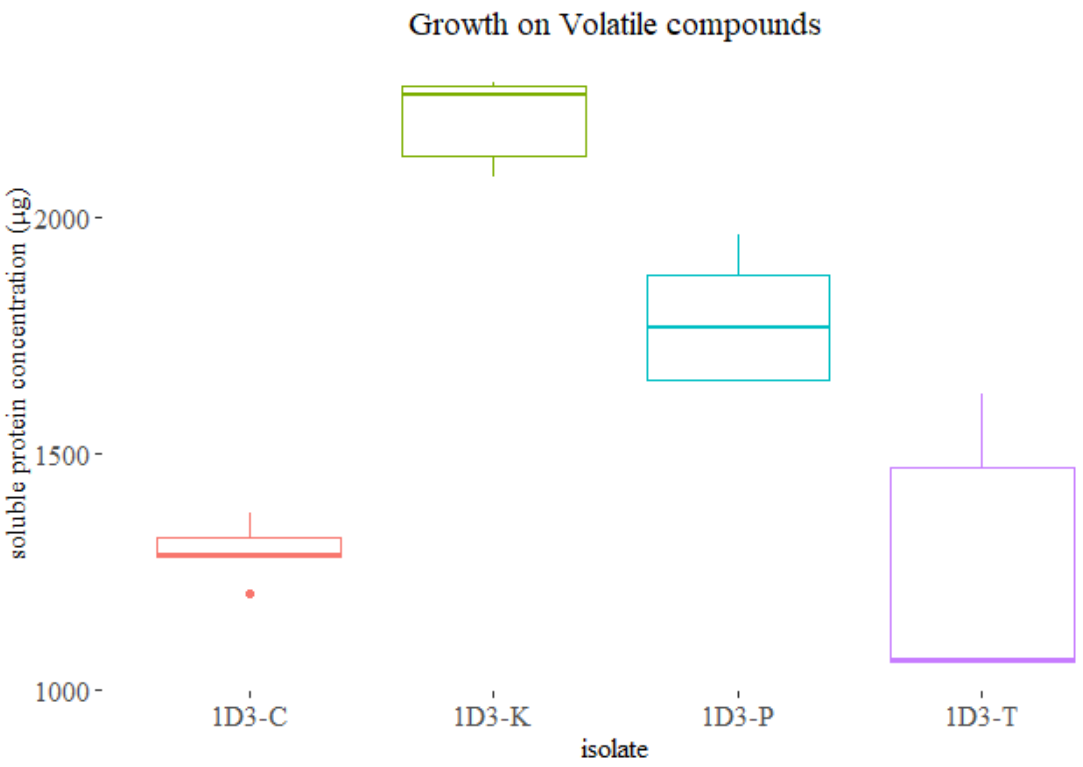


Figure 9*:* Growth of B*acillus amyloliquefaciens* 1D3 isolate on minimal media plates in presence of volatile compounds.1D3 -C: isolate 1D3 in the presence of control (no carbon source), 1D3-K: isolate 1D3 in the presence of kerosene, 1D3- P: isolate 1D3 in the presence of phenol, 1D3-T: isolate1D3 in the presence of toluene

Bacillus amyloliquefaciens 1D3 also yielded a higher concentration of soluble proteins indicating growth in the presence of toluene, kerosene and phenol when compared to soluble protein concentration of the control (Figure 9). The soluble protein concentrations measured using the BCA assay in the presence of volatile carbon compounds as sole source of carbon were 1,615.29 µg/ml for toluene, 1,779.57 µg/ml for phenol;2,183.14 µg/ml for kerosene; and for the control without any carbon source the concentration was 1,306.71 µg/ml.

Isolate	Toluene	phenol	Kerosene	Control
1BA	1788.9	1966.7	1372.0	1348.5
1D3	1615.3	1779.6	2183.1	1306.7

Table 2: Average soluble proteins concentration (µg/ml) of *Bacillus amyloliquefaciens* 1BA and 1D3, indicating growth in the presence of volatile carbon compounds as sole source of carbon.

Statistical analysis (t-test) for isolate 1BA showed was significant statistical difference (p-value 0.07) in bacterial growth between toluene and phenol, compared with the control, but no significant difference between the soluble protein concentrations obtained with kerosene and the control. The statistical trends remained similar for the 1D3 isolate, with the exception of toluene and the control, where no significant statistical difference was observed (p-value 0.71). Significant statistical difference between the 1BA and 1D3 isolates was found for kerosene and toluene as sole carbon source (p-value 2.99E-06), where isolate 1D3 yielded a higher soluble protein concentration compared to isolate 1BA. This statistical difference was not observed for phenol as sole carbon source.

3.4. Phenolic compounds as sole carbon source

Phenolic compounds	1BA (µg/ml)	1D3 (µg/ml)
DMSO (control)	377.5	400.6
Cinnamic acid	262	71.8
Ferulic acid	676.5	643.7
Salicin	379.4	181.1
Caffeic acid	1381.1	1087.2
Vanillin	370.8	347.2
Benzoic Acid	58.4	113.2

Table 3: Average soluble proteins concentration of *Bacillus amyloliquefaciens* 1BA and 1D3, indicating growth in the presence of phenolic compounds as sole source of carbon.

Both the isolates grew only on ferulic and caffeic acid (Figures 10 and 11).

Subtracting values of control from the phenolic acid for isolate 1BA only caffeic acid, (1,003.6 µg/ml), ferulic acid, (299.1 µg/ml) and vanillin (1.9 µg/ml) showed positive numbers. Salicin, cinnamic acid and benzoic acid showed negative numbers, indicating no growth or loss of bacterial cells.

For the 1D3 isolate subtracting the treatment values from the sterile control, only caffeic acid (686.7 µg/ml), and ferulic acid (243.1 µg/ml) showed positive numbers. Salicin, cinnamic acid, vanillin and benzoic acid showed negative numbers indicating no growth had occurred.

The growth on ferulic acid and caffeic acid was statistically significant compared to the controls (that only contained DMSO but no other carbon source).

The statistical analysis (t-test) showed there was no difference (p-value 0.7 and 0.6) between the growth observed for both isolates 1BA and 1D3 on caffeic acid or ferulic acid. However, there was a significant statistical difference (p-value <0.05) between growth on ferulic acid and caffeic acid as a sole carbon source. Isolate 1D3 had higher soluble protein concentration which could indicate higher cell density/biomass when utilizing caffeic acid compared to isolate 1BA.

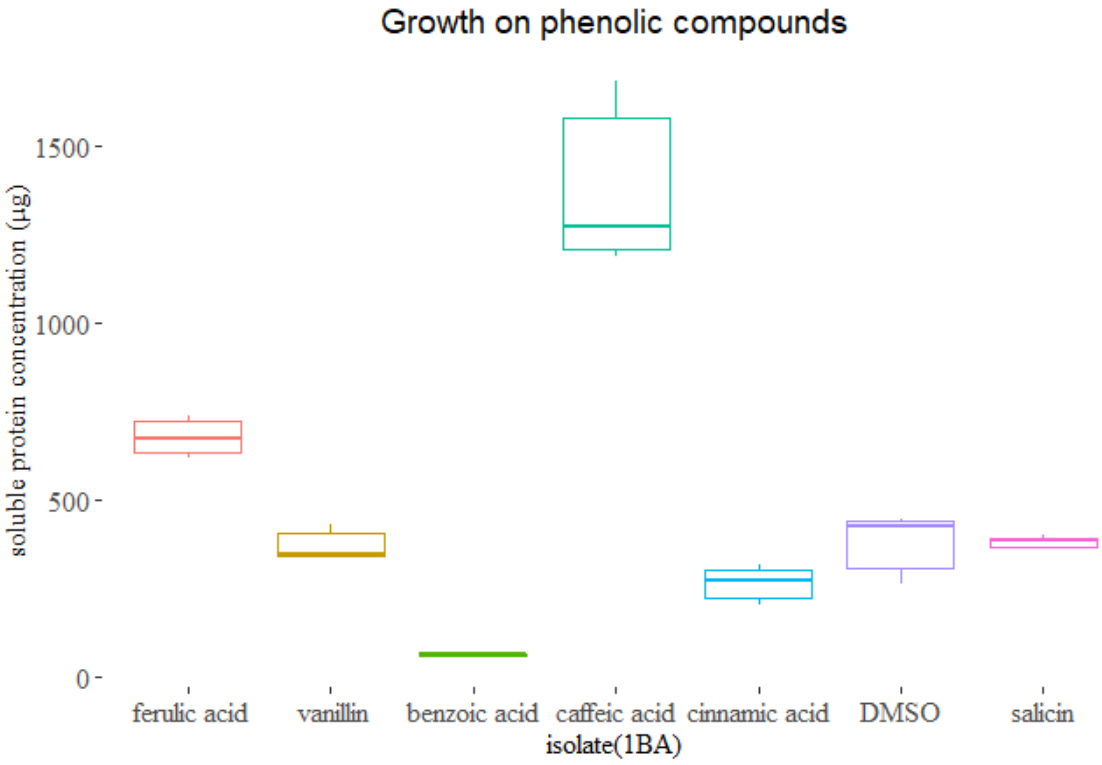


Figure 10: Growth of the 1BA isolate on minimal media plates in the presence of phenolic compounds.

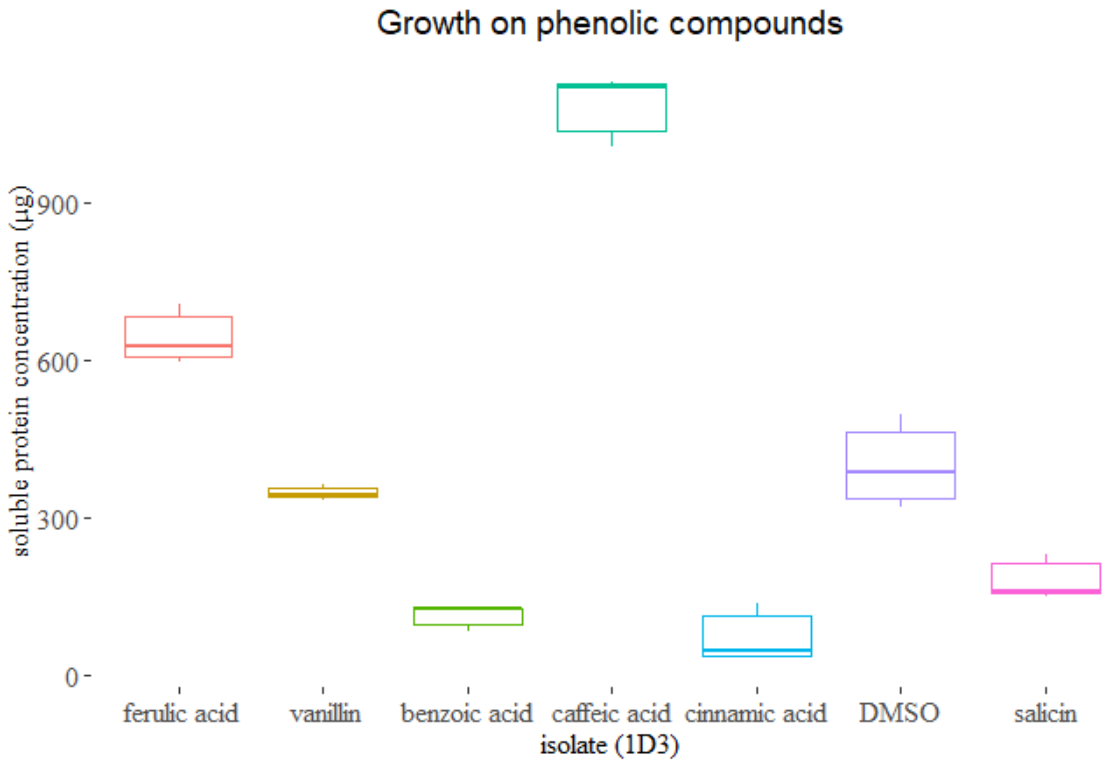


Figure 11: Growth of the 1D3 isolate on minimal media plates in the presence of phenolic compounds.

3.5. BIOLOG results

3.5.1. GEN III plates

Table 4: BIOLOG GEN III absorbance classification at 590 nm

	590 nm
Negative	0.00-0.099
weak Positive	0.100-0.400
Strong positive	0.401-1.5

Table 5: Absorbance 590 nm of BIOLOG GENII plates for isolate 1BA

A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
Negative Control	Dextrin	D-Maltose	D-Trehalose	D-Cellobiose	Gentiobiose	Sucrose	D-Turanose	Stachyose	Positive Control	pH 6	pH 5
B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
D-Raffinose	α-D-Lactose	D-Melibiose	β-Methyl-DGlucoside	D-Salicin	N-Acetyl-DGlucosamine	N-Acetyl-ß-DMannosamine	N-Acetyl-DGalactosamine	N-Acetyl Neuraminic Acid	1% NaCl	4% NaCl	8% NaCl
C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
α-D-Glucose	D-Mannose	D-Fructose	D-Galactose	3-Methyl Glucose	D-Fucose	L-Fucose	L-Rhamnose	Inosine	1% Sodium Lactate	Fusidic Acid	D-Serine
D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
D-Sorbitol	D-Mannitol	D-Arabitol	myo-Inositol	Glycerol	D-Glucose-6-PO4	D-Fructose-6-PO4	D-Aspartic Acid	D-Serine	Troleandomycin	Rifamycin SV	Minocycline
E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
Gelatin	Glycyl-L-Proline	L-Alanine	L-Arginine	L-Aspartic Acid	L-Glutamic Acid	L-Histidine	L-Pyroglutamic Acid	L-Serine	Lincomycin	Guanidine HCl	Niaproof 4
F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
Pectin	D-Galacturonic	L-Galactonic acid lactone	D-Gluconic Acid	D-Glucuronic Acid	Glucuronamide	Mucic Acid	Quinic Acid	D-Saccharic Acid	Vancomycin	Tetrazolium Violet	Tetrazolium Blue
G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
p-Hydroxy-Phenylacetic Acid	Methyl Pyruvate	D-Lactic Acid Methyl Ester	L-Lactic Acid	Citric Acid	alpha-Keto-Glutaric	D-Malic Acid	L-Malic Acid	Bromo-Succinic Acid	Nalidixic Acid	Lithium Chloride	Potassium Tellurit
H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12
Tween 40	Y-Amino-Butyric	α-Hydroxy-Butyric	β-Hydroxy-D,Lbutyric	α-Keto-Butyric	Acetoacetic Acid	Propionic Acid	Acetic Acid	Formic Acid	Aztreonam	Sodium Butyrate	Sodium Bromate

Table 6: Absorbance at 590 nm for GEN III BIOLOG plates of isolate 1D3

A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
Negative Control	Dextrin	D-Maltose	D-Trehalose	D-Cellobiose	Gentiobiose	Sucrose	D-Turanose	Stachyose	Positive Control	pH 6	pH 5
B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
D-Raffinose	α-D-Lactose	D-Melibiose	β-Methyl-DGlucoside	D-Salicin	N-Acetyl-DGlucosamine	N-Acetyl-ß-DMannosamine	N-Acetyl-DGalactosamine	N-Acetyl Neuraminic Acid	1% NaCl	4% NaCl	8% NaCl
C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
α-D-Glucose	D-Mannose	D-Fructose	D-Galactose	3-Methyl Glucose	D-Fucose	L-Fucose	L-Rhamnose	Inosine	1% Sodium Lactate	Fusidic Acid	D-Serine
D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
D-Sorbitol	D-Mannitol	D-Arabitol	myo-Inositol	Glycerol	D-Glucose-6-PO4	D-Fructose-6-PO4	D-Aspartic Acid	D-Serine	Troleandomycin	Rifamycin SV	Minocycline
E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
Gelatin	Glycyl-L-Proline	L-Alanine	L-Arginine	L-Aspartic Acid	L-Glutamic Acid	L-Histidine	L-Pyroglutamic Acid	L-Serine	Lincomycin	Guanidine HCl	Niaproof 4
F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
Pectin	D-Galacturonic	L-Galactonic acid lactone	D-Gluconic Acid	D-Glucuronic Acid	Glucuronamide	Mucic Acid	Quinic Acid	D-Saccharic Acid	Vancomycin	Tetrazolium Violet	Tetrazolium Blue
G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
p-Hydroxy-Phenylacetic Acid	Methyl Pyruvate	D-Lactic Acid Methyl Ester	L-Lactic Acid	Citric Acid	alpha-Keto-Glutaric	D-Malic Acid	L-Malic Acid	Bromo-Succinic Acid	Nalidixic Acid	Lithium Chloride	Potassium Tellurit
H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12
Tween 40	Y-Amino-Butryric	α-Hydroxy-Butyric	p-Hydroxy-D,Lbutyri	α-Keto-Butyric	Acetoacetic Acid	Propionic Acid	Acetic Acid	Formic Acid	Aztreonam	Sodium Butyrate	Sodium Bromate

Table 7*:* Similarities and differences between the *Bacillus amyloliquefacines* 1BA and 1D3.

Bacillus amyloliquefaciens	1D3	standard deviation	1BA	standard deviation	
Positive Control	1.330	0.021	1.020	0.02998	Similarities
4% NaCl	1.319	0.033	1.138	0.29118	
8% NaCl	1.121	0.094	1.410	0.07444	
1% NaCl	0.990	0.035	0.915	0.28923	
pH 6	1.096	0.003	0.992	0.04232	
pH 5	0.742	0.025	0.963	0.16023	
Lithium Chloride	0.973	0.064	0.994	0.01811	
Potassium Tellurit	1.265	0.029	1.083	0.04232	
Sodium Butyrate	1.011	0.024	1.268	0.26544	
Sodium Bromate	0.357	0.036	0.285	0.08912	
D-Serine	-0.044	-0.021	0.283	0.006	differences
quinic acid	0.214	-0.003	0.295	0.045	
N-acetylglucosamine	0.317	0.016	0.303	0.12843	

BIOLOG Gen III plate results showed more similarities than differences in responses to chemicals between 1BA and 1D3 (Table 6). Both the isolates utilized a variety of sugars including α-D-glucose, D-mannose, D-fructose, D-sorbitol, and glycerol. Both the isolates were able to utilize L-alanine, L-arginine, L-aspartic acid, and L-glutamic acid. D-Serine was utilized only by isolate 1D3.

3.6. Denitrification/DNRA

The diphenylamine reagent turns blue in the presence of nitrate/nitrite. The incubations of the isolates in NB + 5 mM KNO_3 that should enrich denitrification/DNRA assumed a blue color after the addition of diphenylamine, due to the presence of nitrate/nitrite in the media, indicating there was evidence of denitrification/DNRA. The incubations in TSB + 5 mM KNO_3 medium that should enrich isolates for denitrification/DNRA remained colorless after the addition of diphenylamine, indicating there was no nitrate/nitrite present and DNRA had likely occurred.

DISCUSSION

BIOLOG GEN III plates were used to characterize the *Bacillus amyloliquefaciens* isolates 1BA and 1D3. The BIOLOG Gen III plates showed more similarities than differences between the isolates. The utilization of D-Serine indicates presence of enzymes which might differ between the isolates. Both the isolates utilized a variety of sugars including α-D-glucose, D-mannose, D-fructose, D-sorbitol, and glycerol. The isolates were able to utilize L-alanine, L arginine, L-aspartic acid, and L-glutamic acid. Both of the isolates were tolerant of high salt concentration (Table 6 and 7). Future work with these bacterial isolates could make use of this information in formulating growth media.

The BIOLOG MT2 plates were used to test if the isolates were capable of utilizing the petroleum hydrocarbons and aromatic volatile hydrocarbons that we tested. Neither of these experiments yield useful results, as the opacity of plastic plates was changed by the petroleum hydrocarbons and the volatile hydrocarbons, which changed the absorbance values and thus could not be obtained (69-71).

The isolates were also capable of DNRA in TSB amended with nitrate, but not denitrification in NB amended with nitrate. This indicates that these bacterial strains in their DNRA functionality may produce more ammonium than nitrous oxide and help to minimize GHG emissions to some degree. The growth habit of these *Bacilli* on plant surfaces and residues may occur as biofilms. There is evidence to suggest the biofilm formation by some *B. amyloliquefaciens* strains assists denitrification by adsorbing nitrate ions onto the biofilm surface (65).

Regarding use of phenolic acids, both isolates could utilize caffeic acid and ferulic acid. Soluble protein concentration was higher in the presence of caffeic acid compared to the ferulic acid (p-value 1.81E-03 for 1BA and p-value 1.20E-05 for 1D3). Caffeic acid and ferulic acid both belong to the hydro-cinnamic acid family and are by-products of lignin degradation commonly found in plant material. Interestingly both the phenolic acids have antioxidant properties and can possibly protect human skin against UV radiation, they do not appear to be potentially harmful to the environment or human health. (72-75)

Certain *Bacillus spp.* along with other species can produce surfactin in presence of the petroleum hydrocarbons (27, 35). Most of these bacteria are isolated from soil contaminated with petroleum. Petroleum degradation by bacteria is usually associated with production of surfactin. There is evidence that lipopeptides assist the degradation of long-chain petroleum hydrocarbons (41, 76).

Isolate 1D3 can produce surfactin in both TSB and in minimal media (Figure 5). A higher concentration of cells in the TSB media compared to MSM might indicate higher concentration of cells, which may produce higher concentration of surfactin. A higher

concentration of surfactin was detected in the TSB medium for isolate 1D3 compared to the medium TSB+1% Kerosene at both five day and 10 day (Figure 5).

Although there was no visible decline in the area of kerosene observed in GC compared to the controls, the reduced concentration of surfactin in the TSB media with kerosene (1,021.2 ± 206.36 ppm on day 5) could be due to the breakdown of petroleum hydrocarbons. In one study, introduction of surfactin to samples containing crude oil and bacteria show higher reduction in the steranes (C27 > C28 > C29) present in the crude oil (41, 76). The same study also shows the removal rate of alkylaromatic and saturated hydrocarbons was significantly enhanced when surfactin was added to the samples in addition to the bacteria used in the study (20, 41).

Both the isolates grew in the presence of volatile hydrocarbons (Figure 8 and 9). In the presence of kerosene both the isolates performed better compared to growth on the individual volatile carbon compounds (Figure 9). Das and Mukherjee (27, 35) indicated bacteria seem to prefer aromatic compounds over long-chain hydrocarbons. Statistical analysis of the results from isolate 1BA showed it grew better to phenol and toluene compared to kerosene. Statistical analysis of the results from the isolate 1D3 showed it grew better in the presence of kerosene and phenol, compared to toluene. Some *Bacillus spp*. are also capable of utilizing other volatile aromatic compounds like benzene, ethylbenzene, and xylene which are similar to toluene and phenol; and seem to prefer growth on a mixture of volatile aromatic carbon compounds compared to growth on individual compounds (27, 29, 77). The ability to produce surfactin could have facilitated the growth observed in the presence of kerosene and toluene. Surfactin has shown to help with emulsification when associated with kerosene oil and toluene (78, 79).

4. CONCLUSIONS

The results of these studies demonstrate that *Bacillus amyloliquefaciens* isolates 1BA and 1D3 are capable of utilizing some VOCs and some phenolic acids found commonly as components of plants and soil. They also appear to have the ability to carry out DNRA, which may be valuable to them in anaerobic environments where exogenous nitrate is available. The diphenylamine test that was carried out has been shown to be a reliable indicator for the ability of bacteria to carry out DNRA. More detailed studies are needed to clearly elucidate the ability of these bacteria to perform dissimilatory nitrate reduction.

Regarding surfactin, future studies could examine the impact of surfactin addition on the growth of cells and reduction of long-chain and cyclical hydrocarbons found in petroleum. The utilization of phenolic compounds and the VOCs also indicates that these *Bacillus amyloliquefaciens* isolates could have a role in their natural habitat in decomposition of dead plant materials. Future studies could also examine the impact of the isolates 1BA and 1D3 in plant-microbe communication and minimization of nitrogenous GHG emissions.

The isolate's ability to tolerate high temperatures and salt concentration, utilize various carbon and nitrogen sources, utilize some VOCs, carry out DNRA, and produce biosurfactant production, could indicate that the isolates are candidates for bioremediation studies.

The methods that were developed and used in this thesis to grow bacteria in closed glass desiccators and to assay the concentration of bacterial protein that develops on the agar surfaces of growth media are relatively simple but are rarely described or used in most of the related scientific literature, when describing similar studies. It is suggested that these

simple methods developed and used in this thesis can be more widely used in a variety of studies of microbial metabolism of phenolic acids and petroleum fractions.

The isolates have been used in field trials to biologically control *Fusarium* Head Blight of wheat and barley. More studies would be needed to fully understand the potential for each isolate to produce lipopeptides under different conditions in the laboratory and in the field. Further studies are required to clarify the role of surfactin production and the ability of *Bacillus amyloliquefaciens* 1BA and 1D3 to degrade a complex amalgam of petroleum hydrocarbons, such as kerosene.

5. APPENDIX

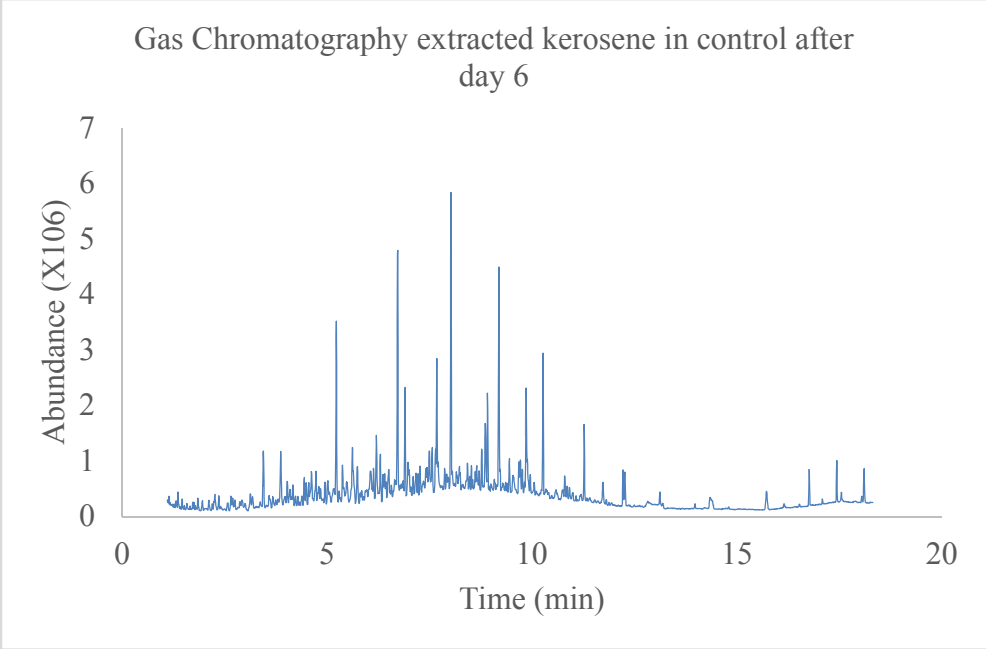


Figure 12: Gas Chromatography of kerosene after six days in TSB medium.

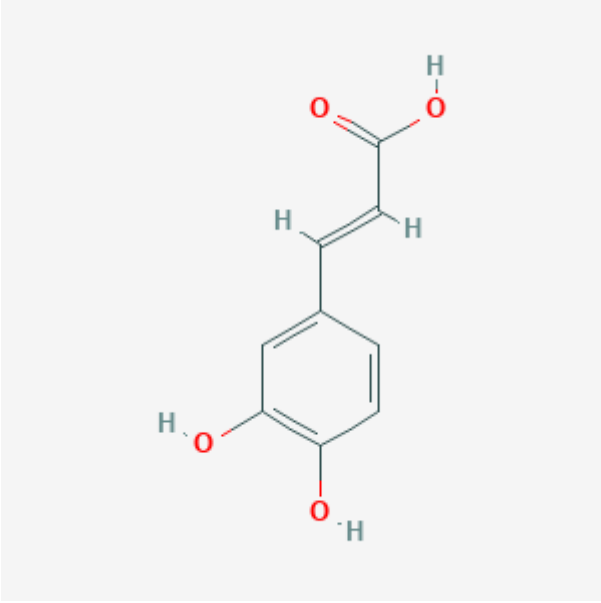


Figure 13: 2D-structure of caffeic acid. Formula: $C_9H_8O_4$

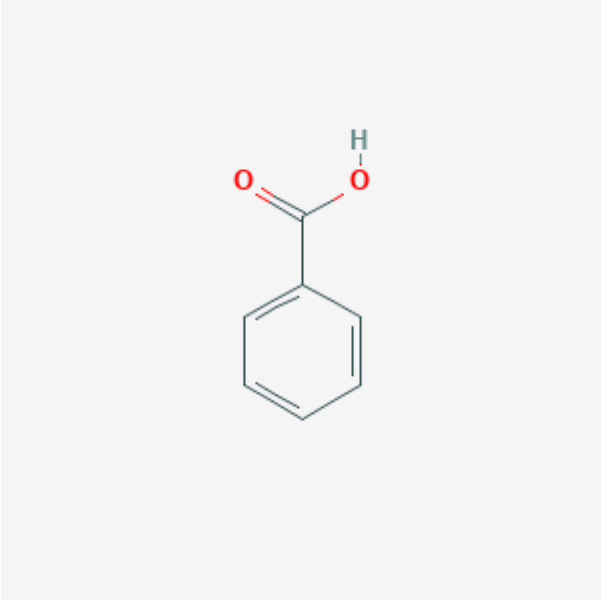


Figure 14: 2D-structure of benzoic acid and formula: C_6H_5COOH

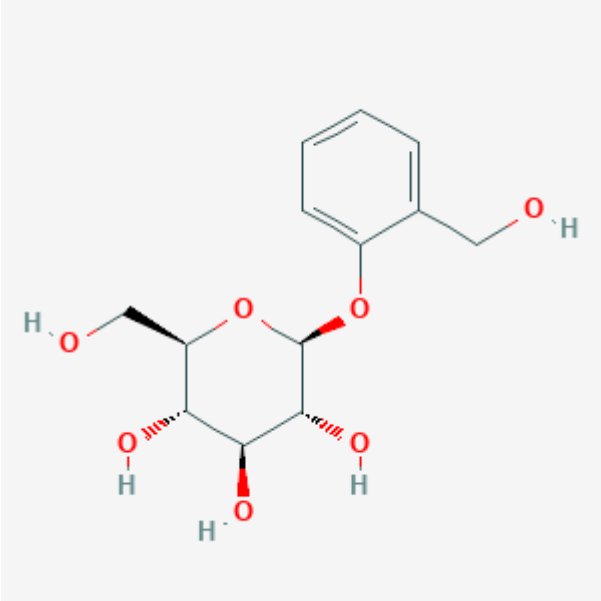


Figure 15: 2D-structure of salicin and formula: $C_{13}H_{18}O_7$

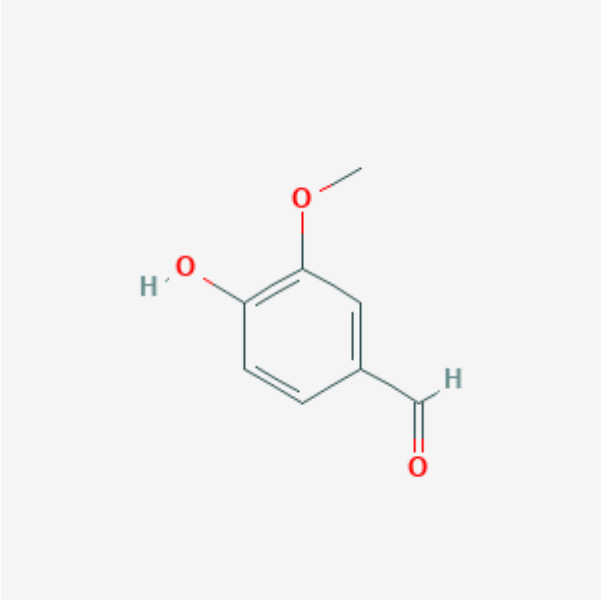


Figure 16: 2D structure of vanilin formula: $C_8H_8O_3$

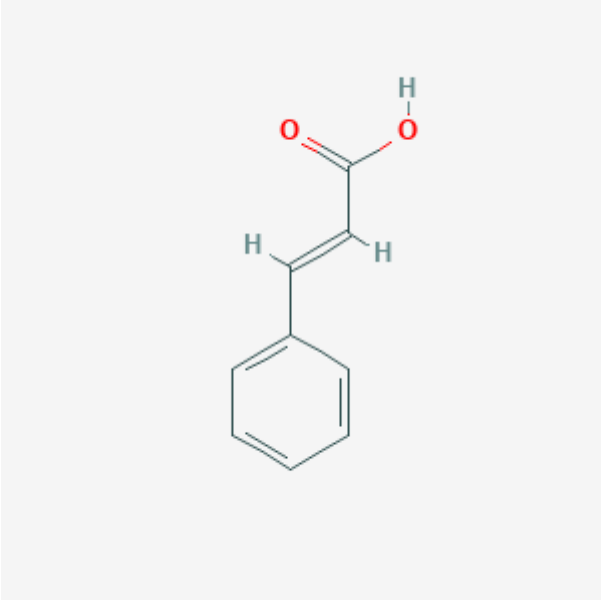


Figure 17: 2D-structure of cinnamic acid. Formula: $C_9H_8O_2$

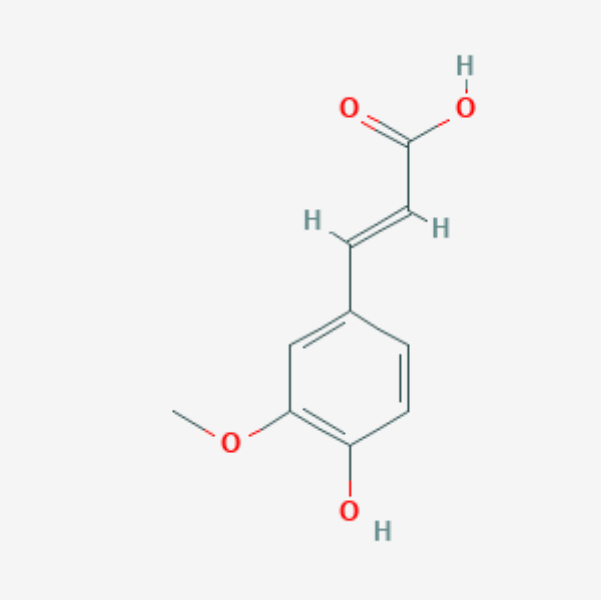


Figure 18: 2D-structure of ferulic acid. Formula: $C_{10}H_{10}O_4$

5.1. Statistical analysis

t-test 1BA volatile compounds	
p value	arrays
0.07	Toluene Vs phenol
1.36E-05	Toluene Vs kerosene
0.001	phenol vs Kerosene
0.04	toluene Vs control
0.01	phenol vs control
0.395916	kerosene vs control

Table 8: t-test and p values *Bacillus amyloliquefaciens* isolate 1BA for volatile hydrocarbons

BA volatile compounds	
p-values	arrays
0.008707	Toluene Vs phenol
0.000193	Toluene Vs kerosene
5.55E-05	phenol vs Kerosene
0.709784	toluene Vs control
0.000203	phenol vs control
2.99E-06	kerosene vs control

Table 9: t-test results and p values *Bacillus amyloliquefaciens* isolate 1D3 for volatile hydrocarbons

Phenolic acid t-test			
Phenolic acid	p-values		
	1BA vs 1D3	1BA vs Control	1D3 Vs Control
ferulic acid	0.07244	6.1897E-05	6.36E-05
caffeic acid	0.06085	0.00064673	1.75E-07

Table 10: Phenolic acids t-test results and p values for *Bacillus amyloliquefaciens* isolates 1BA and 1D3

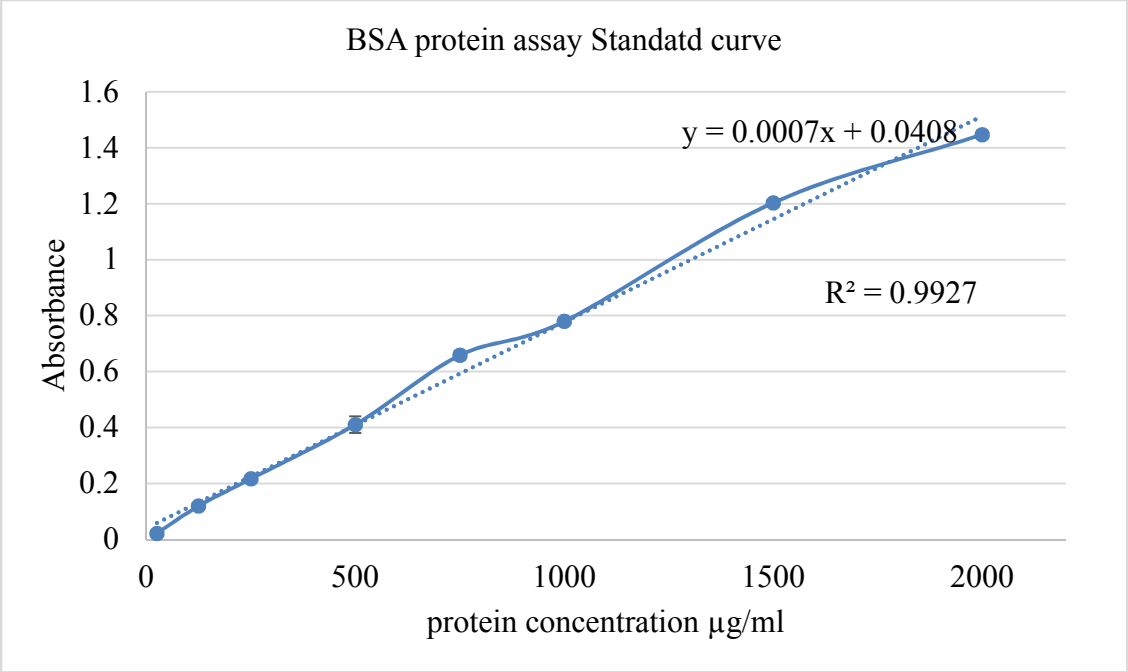


Figure 19: BCA assay Standard curve and equation

6. LITERATURE CITED

1. Etkin DS. 1999. ESTIMATING CLEANUP COSTS FOR OIL SPILLS. International Oil Spill Conference.

2. Elliot R, Singhal N, Swift S. 2010. Surfactants and Bacterial Bioremediation of Polycyclic Aromatic Hydrocarbon Contaminated Soil—Unlocking the Targets. Critical Reviews in Environmental Science and Technology 41:78-124.

3. Albaugh CE. Degradation of monoaromatic compounds by an aerobic halotolerant alkaliphilic bacterium.

4. Di Gioia D, Bertin L, Fava F, Marchetti L. 2001. Biodegradation of hydroxylated and methoxylated benzoic, phenylacetic and phenylpropenoic acids present in olive mill wastewaters by two bacterial strains. Res Microbiol 152:83-93.

5. Pacheco-Ordaz R, Wall-Medrano A, Goni MG, Ramos-Clamont-Montfort G, Ayala-Zavala JF, Gonzalez-Aguilar GA. 2018. Effect of phenolic compounds on the growth of selected probiotic and pathogenic bacteria. Lett Appl Microbiol 66:25-31.

6. Patial PK, Cannoo DS. 2020. Evaluation of volatile compounds, phenolic acids, antioxidant potential and DFT study of essential oils from different parts of Araucaria columnaris (G. Forst.) Hook. from India. Food Chem Toxicol 141:111376.

7. Shalaby S, Horwitz BA, Larkov O. 2012. Structure-activity relationships delineate how the maize pathogen Cochliobolus heterostrophus uses aromatic compounds as signals and metabolites. Mol Plant Microbe Interact 25:931-40.

8. Haribabu B, Kamath AV, Vaidyanathan CS. 1984. Degradation of substituted benzoic acids by a Micrococcus species. FEMS microbiology letters 21:197-200.

9. Benitez FJ, Beltran-Heredia J, Acero JL, Gonzalez T. 1996. Degradation of protocatechuic acid by two advanced oxidation processes: Ozone/UV radiation and H2O2UV radiation. Water research (Oxford) 30:1597-1604.

10. Kain MCSR (ed). 2006. A JOHN WILEY & SONS, INC., PUBLICATION,

11. Gatesoupe FJ. 1999. The use of probiotics in aquaculture. Aquaculture 180:147-165.

12. Hlordzi V, Kuebutornye FKA, Afriyie G, Abarike ED, Lu Y, Chi S, Anokyewaa MA. 2020. The use of Bacillus species in maintenance of water quality in aquaculture: A review. Aquaculture reports 18:100503.

13. Zhao S, Hu N, Chen Z, Zhao B, Liang Y. 2009. Bioremediation of reclaimed wastewater used as landscape water by using the denitrifying bacterium Bacillus cereus. Bull Environ Contam Toxicol 83:337-340.

14. Portmann RW, Daniel JS, Ravishankara AR. 2012. Stratospheric ozone depletion due to nitrous oxide: influences of other gases. Philos Trans R Soc Lond B Biol Sci 367:1256-1264.

15. Ravishankara AR, John SD, Robert WP. 2009. Nitrous Oxide (N□0): The Dominant Ozone-Depleting Substance Emitted in the 21st Century. Science 326:123-125.

16. Baye NL. 2007. Bacillus strains used for biological control of Fusarium head blight : identification, growth studies, and lipopeptide productionThesis (M.S.)--Biology and Microbiology Dept., South Dakota State University, 2007.

17. Gill A. 2006. Development of an isolation method that uses tolerance to salt and heat, to select for Bacillus strain 1BA, a biocontrol agent used to control Fusarium head blightThesis (M.S.)--Biology and Microbiology Dept., South Dakota State University, 2006.

18. Luo Y. 2000. Bacillus strains as biological control agents of tan spot and Fusarium head blight of wheatThesis (M.S.)--Biology and Microbiology Dept., South Dakota State University, 2000.

19. Du T, Niu J, Su J, Li S, Guo X, Li L, Cao X, Kang J. 2018. SmbHLH37 Functions Antagonistically With SmMYC2 in Regulating Jasmonate-Mediated Biosynthesis of Phenolic Acids in Salvia miltiorrhiza. Front Plant Sci 9:1720.

20. Liu JF, Mbadinga SM, Yang SZ, Gu JD, Mu BZ. 2015. Chemical structure, property and potential applications of biosurfactants produced by Bacillus subtilis in petroleum recovery and spill mitigation. Int J Mol Sci 16:4814-37.

21. de Cassia FSSR, Almeida DG, Rufino RD, Luna JM, Santos VA, Sarubbo LA. 2014. Applications of biosurfactants in the petroleum industry and the remediation of oil spills. Int J Mol Sci 15:12523-42.

22. Mor´an AC, Olivera N, Commendatore M, Jos´eL.Esteves, Siñeriz F. 26 May 2000. Enhancement of hydrocarbon waste biodegradation by addition of a biosurfactant from Bacillus subtilis O9. Kluwer Academic Publishers

23. Peypoux F, Guinand M, Michel G, Delcambe L, Das BC, Lederer E. 1978. Structure of iturine A, a peptidolipid antibiotic from Bacillus subtilis. Biochemistry 17:3992-6.

24. Meena KR, Kanwar SS. 2015. Lipopeptides as the antifungal and antibacterial agents: applications in food safety and therapeutics. Biomed Res Int 2015:473050.

25. Tsuge K, Akiyama T, Shoda M. 2001. Cloning, sequencing, and characterization of the iturin A operon. J Bacteriol 183:6265-73.

26. Rao Y, Lin H-Y, Wu W-S, Tzeng Y-M. 2008. Evaluation of HPLC and MEKC methods for the analysis of lipopeptide antibiotic iturin A produced by Bacillus amyloliquefaciens. International Journal of Applied Science and Engineering 6:85-96.

27. Das K, Mukherjee AK. 2005. Characterization of biochemical properties and biological activities of biosurfactants produced by Pseudomonas aeruginosa mucoid and non-mucoid strains isolated from hydrocarbon-contaminated soil samples. Appl Microbiol Biotechnol 69:192-9.

28. Balhara M, Ruhil S, Dhankhar S, Chhillar AK. 2011. Bioactive Compounds Hold Up- Bacillus amyloliquefaciens as a Potent Bio-Control Agent. The Natural Products Journal 1:20-28.

29. Das N, Chandran P. 2011. Microbial degradation of petroleum hydrocarbon contaminants: an overview. Biotechnol Res Int 2011:941810.

30. Adebusoye SA, Ilori MO, Amund OO, Teniola OD, Olatope SO. 2007. Microbial degradation of petroleum hydrocarbons in a polluted tropical stream. World Journal of Microbiology & Biotechnology 23:1149-1159.

31. Wang XB, Chi CQ, Nie Y, Tang YQ, Tan Y, Wu G, Wu XL. 2011. Degradation of petroleum hydrocarbons (C6-C40) and crude oil by a novel Dietzia strain. Bioresour Technol 102:7755-61.

32. Chen WW, Li JD, Sun XN, Min J, Hu XK. 2017. High efficiency degradation of alkanes and crude oil by a salt-tolerant bacterium Dietzia species CN-3. International Biodeterioration & Biodegradation 118:110-118.

33. Throne-Holst M, Wentzel A, Ellingsen TE, Kotlar HK, Zotchev SB. 2007. Identification of novel genes involved in long-chain n-alkane degradation by Acinetobacter sp. strain DSM 17874. Appl Environ Microbiol 73:3327-32.

34. Zhang Z, Gai L, Hou Z, Yang C, Ma C, Wang Z, Sun B, He X, Tang H, Xu P. 2010. Characterization and biotechnological potential of petroleum-degrading bacteria isolated from oil-contaminated soils. Bioresour Technol 101:8452-6.

35. Das K, Mukherjee AK. 2007. Crude petroleum-oil biodegradation efficiency of Bacillus subtilis and Pseudomonas aeruginosa strains isolated from a petroleum-oil contaminated soil from North-East India. Bioresour Technol 98:1339-45.

36. Maier RM. 2003. Biosurfactants: evolution and diversity in bacteria. Adv Appl Microbiol 52:101-21.

37. Ahimou F, Jacques P, Deleu M. 2000. Surfactin and iturin A effects on Bacillus subtilis surface hydrophobicity. Enzyme Microb Technol 27:749-754.

38. Ron EZ, Rosenberg E. 2001. Natural roles of biosurfactants. Environ Microbiol 3:229-36.

39. Cybulski Z, Dziurla E, Kaczorek E, Olszanowski A. 2003. The Influence of Emulsifiers on Hydrocarbon Biodegradation by Pseudomonadacea and Bacillacea Strains. Spill science & technology bulletin 8:503-507.

40. Wong JW, Fang M, Zhao Z, Xing B. 2004. Effect of surfactants on solubilization and degradation of phenanthrene under thermophilic conditions. J Environ Qual 33:2015-25.

41. Wang XW, Cai T, Wen WT, Ai JZ, Ai JY, Zhang ZH, Zhu L, George SC. 2020. Surfactin for enhanced removal of aromatic hydrocarbons during biodegradation of crude oil. Fuel 267:117272.

42. Reese KL, Rasley A, Avila JR, Jones AD, Frank M. 2020. Metabolic Profiling of Volatile Organic Compounds (VOCs) Emitted by the Pathogens Francisella tularensis and Bacillus anthracis in Liquid Culture. Sci Rep 10:9333-9333.

43. Verbaendert I, Boon N, De Vos P, Heylen K. 2011. Denitrification is a common feature among members of the genus Bacillus. Syst Appl Microbiol 34:385-91.

44. Bhattacharya A, Sood P, Citovsky V. 2010. The roles of plant phenolics in defence and communication during Agrobacterium and Rhizobium infection. Mol Plant Pathol 11:705-19.

45. Ortiz-Castro R, Contreras-Cornejo HA, Macias-Rodriguez L, Lopez-Bucio J. 2009. The role of microbial signals in plant growth and development. Plant Signal Behav 4:701-12.

46. Asari S, Matzén S, Petersen MA, Bejai S, Meijer J. 2016. Multiple effects of Bacillus amyloliquefaciens volatile compounds: plant growth promotion and growth inhibition of phytopathogens. FEMS Microbiol Ecol 92:fiw070-fiw070.

47. Choong-Min R, Mohamed AF, Chia-Hui H, Munagala SR, Han-Xun W, Paul WP, Joseph WK. 2003. Bacterial Volatiles Promote Growth in Arabidopsis. Proc Natl Acad Sci U S A 100:4927-4932.

48. Lee B, Farag MA, Park HB, Kloepper JW, Lee SH, Ryu C-M. 2012. Induced resistance by a long-chain bacterial volatile: elicitation of plant systemic defense by a C13 volatile produced by Paenibacillus polymyxa. PLoS One 7:e48744-e48744.

49. Blum U. 1996. Allelopathic interactions involving phenolic acids. J Nematol 28:259-67.

50. Chen Y, Chen W, Lan Y, Wang K, Wu Y, Zhong X, Ying K, Li J, Yang G. 2019. Determination of 18 phenolic acids in tobacco and rhizosphere soil by ultra high performance liquid chromatography combined with triple quadrupole mass spectrometry. J Sep Sci 42:816-825.

51. Ehrhardt DW, Wais, R. and Long, S.R. () Calcium spiking in plant root hairs responding to Rhizobium nodulation signals. 1996. Spiky responses to Rhizobium. Trends in Plant Science 1:293.

52. Fisher RF, Long SR. 1992. Rhizobium - Plant Signal Exchange. Nature 357:655-660.

53. Samer Shalaby BAH, and Olga Larkov. 2012. Structure–Activity Relationships Delineate How the Maize Pathogen Cochliobolus heterostrophus Uses Aromatic Compounds as Signals and Metabolites. 25:931–940.

54. Kraft B, Strous M, Tegetmeyer HE. 2011. Microbial nitrate respiration--genes, enzymes and environmental distribution. J Biotechnol 155:104-17.

55. Lam P, Kuypers MM. 2011. Microbial nitrogen cycling processes in oxygen minimum zones. Ann Rev Mar Sci 3:317-45.

56. Woehle C, Roy AS, Glock N, Wein T, Weissenbach J, Rosenstiel P, Hiebenthal C, Michels J, Schonfeld J, Dagan T. 2018. A Novel Eukaryotic Denitrification Pathway in Foraminifera. Curr Biol 28:2536-2543 e5.

57. Duxbury JM, Bouldin DR, Terry RE, Tate RL. 1982. Emissions of Nitrous-Oxide from Soils. Nature 298:462-464.

58. Marchant HK, Lavik G, Holtappels M, Kuypers MM. 2014. The fate of nitrate in intertidal permeable sediments. PLoS One 9:e104517.

59. Heo H, Kwon M, Song B, Yoon S. 2020. Involvement of NO3 (-) in Ecophysiological Regulation of Dissimilatory Nitrate/Nitrite Reduction to Ammonium (DNRA) Is Implied by Physiological Characterization of Soil DNRA Bacteria Isolated via a Colorimetric Screening Method. Appl Environ Microbiol 86.

60. Heylen K, Keltjens J. 2012. Redundancy and modularity in membrane-associated dissimilatory nitrate reduction in Bacillus. Front Microbiol 3:371.

61. Hoffmann T, Frankenberg N, Marino M, Jahn D. 1998. Ammonification in Bacillus subtilis utilizing dissimilatory nitrite reductase is dependent on resDE. Journal of Bacteriology 180:186-189.

62. Tomlinson GA, Jahnke LL, Hochstein LI. 1986. Halobacterium denitrificans sp. nov., an extremely halophilic denitrifying bacterium. Int J Syst Bacteriol 36:66-70.

63. Idris EE, Iglesias DJ, Talon M, Borriss R. 2007. Tryptophan-dependent production of indole-3-acetic acid (IAA) affects level of plant growth promotion by Bacillus amyloliquefaciens FZB42. Mol Plant Microbe Interact 20:619-26.

64. Wu S, Zhuang G, Bai Z, Cen Y, Xu S, Sun H, Han X, Zhuang X. 2018. Mitigation of nitrous oxide emissions from acidic soils by Bacillus amyloliquefaciens, a plant growth-promoting bacterium. Glob Chang Biol 24:2352-2365.

65. Hui C, Guo X, Sun P, Khan RA, Zhang Q, Liang Y, Zhao YH. 2018. Removal of nitrite from aqueous solution by Bacillus amyloliquefaciens biofilm adsorption. Bioresour Technol 248:146-152.

66. Gibson KA. 2015. Isolation and characterization of cellulase-producing microorganisms from the Homestake Mine and Hot Springs, South DakotaThesis (Ph. D.)--Biology and Microbiology Department, South Dakota State University, 2015.

67. Smyth TJP, Perfumo A, McClean S, Marchant R, Banat* IM. 2010. Isolation and Analysis of Lipopeptides and High Molecular Weight Biosurfactants. doi:10.1007/978-3-540-77587-4_290:3687-3704.

68. Tiedje JM, Sexstone AJ, Myrold DD, Robinson JA. 1983. Denitrification: ecological niches, competition and survival. Antonie van Leeuwenhoek 48:569-583.

69. Palmroth MR, Munster U, Pichtel J, Puhakka JA. 2005. Metabolic responses of microbiota to diesel fuel addition in vegetated soil. Biodegradation 16:91-101.

70. Mansur AA, Adetutu EM, Kadali KK, Morrison PD, Nurulita Y, Ball AS. 2014. Assessing the hydrocarbon degrading potential of indigenous bacteria isolated from crude oil tank bottom sludge and hydrocarbon-contaminated soil of Azzawiya oil refinery, Libya. Environ Sci Pollut Res Int 21:10725-35.

71. Taha M, Kadali KK, AL-Hothaly K, Smith AT, Ball AS, Adetutu EM. 2015. An effective microplate method (Biolog MT2) for screening native lignocellulosic-straw-degrading bacteria. Annals of Microbiology 65:2053-2064.

72. Srinivasan M, Sudheer AR, Menon VP. 2007. Ferulic Acid: therapeutic potential through its antioxidant property. J Clin Biochem Nutr 40:92-100.

73. Zhang D, Nie S, Xie M, Hu J. 2020. Antioxidant and antibacterial capabilities of phenolic compounds and organic acids from Camellia oleifera cake. Food Sci Biotechnol 29:17-25.

74. Benteldjoune M, Boudiar T, Bakhouche A, Del Mar Contreras M, Lozano-Sanchez J, Bensouici C, Kabouche Z, Segura-Carretero A. 2019. Antioxidant activity and characterization of flavonoids and phenolic acids of Ammoides atlantica by RP-UHPLC-ESI-QTOF-MS(n). Nat Prod Res doi:10.1080/14786419.2019.1619722:1-5.

75. Saija A, Tomaino A, Cascio RL, Trombetta D, Proteggente A, De Pasquale A, Uccella N, Bonina F. 1999. Ferulic and caffeic acids as potential protective agents

against photooxidative skin damage. Journal of the science of food and agriculture 79:476-480.

76. Wardroper AMK, Hoffmann CF, Maxwell JR, Barwise AJG, Goodwin NS, Park PJD. 1984. Crude oil biodegradation under simulated and natural conditions—II. Aromatic steroid hydrocarbons. Organic Geochemistry 6:605-617.

77. Wongbunmak A, Khiawjan S, Suphantharika M, Pongtharangkul T. 2020. BTEX biodegradation by Bacillus amyloliquefaciens subsp. plantarum W1 and its proposed BTEX biodegradation pathways. Sci Rep 10:17408.

78. Al-Wahaibi Y, Joshi S, Al-Bahry S, Elshafie A, Al-Bemani A, Shibulal B. 2014. Biosurfactant production by Bacillus subtilis B30 and its application in enhancing oil recovery. Colloids and Surfaces B: Biointerfaces 114:324-333.

79. Nitschke M, Pastore GM. 2006. Production and properties of a surfactant obtained from Bacillus subtilis grown on cassava wastewater. Bioresource Technology 97:336-341.