QATAR UNIVERSITY

COLLEGE OF ARTS AND SCIENCES

DEVELOPMENT OF BIOSURFACTANTS FROM INDIGENOUS HYDROCARBON-

DEGRADING BACTERIA FOR ENHANCING REMEDIATION OF WEATHERED

OILY-SOILS AND OIL RECOVERY IN QATAR

BY

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DEDICATION

I would like to dedicate this thesis to myself first, as I reached the end of my Masters'

journey and proudly achieved one of my life goals. Secondly, I dedicate it to my loved

ones; my family and my friends who supported me along this journey and Finally, to

my father who would be proud of me if he was here, I could not make it without your

great love that I received and kept me all alive and ambitious.

Thank you.

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ABSTRACT

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Title: Development of Novel Biosurfactants from Indigenous Hydrocarbon-Degrading Bacteria for Enhanacing Remediation of Weathered Oily-Soils and Oil Recovery in Qatar

Supervisor of Thesis: Nabil, Middle Initial, Zouari.

Bacterial strains were isolated from highly weathered oil-contaminated sites, identified, and differentiated based on their gene expression with the use of matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI TOF MS). They were clustered using principle component analysis (PCA). PCA and proteodendrogram showed high diversity within same subspecies. This is reflected in the emulsification activity (EA) and solubilization activity (SA) of their adapted biosurfactants. PCA allowed a further clustering of the biosurfactants based on FTIR spectra. Vegetative oils were utilized as carbon source for *Bacillus* and *Pseudomonas* strains, showing strong effect on bioemulsifiers production. Moreover, using seven selected strains of *Bacillus*, high toxicity by hydrocarbons and/or carbon catabolite repression (CCR) was showed. Similarly, bioemulsifiers production by several strains was shown regulated by CCR. These findings showed a type of adaptation occurring with hydrocarbons-degrading bacteria, related to the bacterial cell composition maintaining the biosurfactants composition.

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INTRODUCTION

Microbial biosurfactants are surface-active molecules, which are produced by microorganisms, especially bacteria, to improve the bioavailability of hydrophobic substrates. If appropriately produced and formulated, they can be applied in various fields ranging from hydrocarbons remediation to food industry (Czaplicka & Chmielarz, 2009). The surface-active biomolecules have special properties such as reduced toxicity and the relatively simple preparation process. Therefore, these characteristics have expanded their demand in a broad range of industries of petrochemicals, pharmaceuticals, agrochemicals, fertilizers, cosmetics and beverages as well as in petroleum and mining industries (Pele et al., 2019). Biosurfactants have gained huge interests by the oil industry because of their characteristic of reducing surface tension, which allows their use in bioremediation of oil pollution and oil recovery. In bioremediation, the major roles played by biosurfactants comprise the increase of solubilization and desorption of the hydrophobic pollutants, increasing thus their bioavailability (Kaczorek et al., 2018). In fact, the future of bioremediation of soils contaminated by hydrocarbons is expected to be based on the surfactant-enhanced bioavailability of the contaminants. Biosurfactants have two sides; hydrophobic and hydrophilic, which end up with the formation of an interface zone between fluids with different polarities such as water and oil hydrocarbons (Czaplicka & Chmielarz, 2009). In comparison with other synthetic or chemical surfactants, biosurfactants have various advantages including their biodegradability, biocompatibility and digestibility making them more attractive and effective in cleaning the environment and in the bioremediation of polluted soils (Vijayakumar & Saravanan, 2015).

Biosurfactants are categorized based on their composition and origin. The molecules are grouped based on their molecular weight: High Molecular Weight (HMW) and Low Molecular Weight (LMW). The low molecular weight biosurfactants include lipopeptides, phospholipids and glycolipids. The high molecular weight biosurfactants include polymeric

and particulate biosurfactants (Fenibo, Douglas, & Stanley, 2019). Glycolipid biosurfactants are characterized by the property of a polysaccharide on their head groups. When the head group is affected by changes in pH or by electrolytes, the micellar structure of these biosurfactants changes (Jahan et al., 2020). Among the glycolipid biosurfactants, various hydrophobic fatty acids have the ability to reduce Kraft temperature at which the solubility of a surfactant matches the surfactant's critical micelle concentration (CMC) (Fenibo et al., 2019). Then, fatty acids ensure that their structure is maintained. On the other hand, rhamnolipids are made up of rhamnoses and hydroxyl fatty acids. Sophorolipids are made of sophorose, which forms the hydrophilic part of the surfactant. The hydrophobic part is made of fatty acids that contain a long chain of carbon atoms (Shu et al., 2021). Surfactin, a lipopeptide biosurfactant is composed of molecules that consist of seven amino acids and fatty acids that form the hydrophobic part. High molecular weight biosurfactants majorly function in solubilization of hydrophobic molecules and they have a wide diversity in their structure and functions (Jahan et al., 2019). *Bacillus, Pseudomonas* and *Acinetobacter* are some of the bacteria among others which are biosurfactants producing bacteria. Synthesis of biosurfactants is not only achieved intracellularly but can also be carried out extracellularly by the use of biocatalysts, which are specific enzymes. The hydrophilic and hydrophobic moieties can either be synthesized independently following different pathways and they can both be dependent on a substrate or one can be induced by the substrate and the others are synthesized a new (Nurfarahin et al., 2018). In the amphiphilic structure of the biosurfactants, the hydrophobic moiety can be either a hydroxyl-fatty acid or a long-chained fatty acid. The hydrophilic moiety can be either a carboxylic acid, amino acid, carbohydrate, phosphate, alcohol or cyclic peptide. The synthesis of these moieties involves the carbohydrate metabolic pathway, which synthesizes the hydrophilic moiety, and the hydrocarbon metabolic pathway, which synthesizes the hydrophobic moiety. Various factors influence biosurfactants synthesis and they affect the rate

of production and their properties. Some of the factors that influence the optimum production of biosurfactants include the carbon and nitrogen sources, temperature, pH, oxygen availability, carbon-nitrogen ratio and agitation (Yalaoui-Guellal et al., 2020). Consequently, all the reported parameters affect production, composition and activity. However, in bioremediation approaches, the recalcitrance of certain petroleum compounds to biodegradation results from their strong adsorption on soil particles. Here, adsorption is understood as the retention of a solute in solution by the surface of a solid material, whereas the absorption refers to the retention of the solute within the mass of the solid (Talley, 2016). The strength of this adsorption depends on the contaminant and the matrix on which it is adsorbed (Abdel-Shafy & Mansour, 2016).

On the other hand, it is important to note that the climate of the Gulf region is characterized by harsh conditions, resulting in accelerated weathering of oil components. Under such conditions, it is anticipated that indigenous microorganisms have acclimatized/ adapted to synthesize specific biosurfactants that are effective for these weathered oils, both to mobilize the hydrocarbons and to enhance their bioavailability and biodegradation. Indeed, a lot of failures of bioremediation applications in regions characterized by harsh weather and soils can be attributed to the use of un-acclimated bacteria and their associated biosurfactants.

The novelty of this work resides in the ability of the indigenous Qatari strains to produce biosurfactants that have potential to enhance biodegradation of weathered hydrocarbons or washing of weathered soil. These biosurfactants may be more active under the harsh physical and chemical conditions with Qatari soils, which makes them appropriate for the use in enhanced oil recovery and bioremediation. The objectives of this work are:

- Isolation of highly adapted hydrocarbon-degrading bacteria from the soils which are highly polluted with weathered oil: Construction of a collection of bacteria

- Identification by MALDI TOF MS and differentiation by Principal Component Analysis (PCA) of proteins profiles of the isolated bacterial strains: Diversity at the level of the genus and species. Hypothesis 1: local Qatari bacteria may produce biosurfactants with novel characteristics, making them more adapted to weathered oil, harsh soil conditions and more suitable to soil bioremediation.

- Evaluation of the potentiality of the isolated bacterial strains to produce biosurfactants (Emulsification and Solubilization): Diversity at the level of the biosurfactants production and activity, and selection of candidates. Hypothesis 2: a large biodiversity of biosurfactants will be identified, allowing the establishment of biosurfactants catalog for native Qatari bacteria, including characterization of biosurfactants parameters for application in hydrocarbon bioavailability and bioremediation.

- Extraction purification and characterization of the biosurfactants of the selected strains: Diversity at the level of biosurfactants structure

- Investigation of regulations of production of biosurfactants by *Bacillus* strains. Hypothesis 3: overproduction by overcoming the limitations of biosurfactants production.

LITERATURE REVIEW

1. Microbial Biosurfactants

Microbial biosurfactants are biomolecules that are surface-active, produced by several microorganisms and can be applied broadly in various fields (Ohadi, M.,2020). The surface-active biomolecules have different unique properties including specificity and low toxicity, high biodegradability and they are very easy to prepare (Thavasi, R., 2011). Therefore, these unique properties have expanded their demand in a broad range of industries of petrochemicals, pharmaceuticals, agrochemicals, fertilizers, cosmetics and beverages as well as in petroleum and mining industries (Pele, M., 2019). The three major roles performed by biosurfactants include enlarging the hydrophobic substrates surface area through desorption and solubilization, increasing the bioavailability of the hydrophobic substrate for removal and attachment of microorganisms to surfaces (Chen, 2007).

While anthropogenic surfactants can enhance hydrocarbon bioavailability, the most important class of surfactants for environmental remediation – and the least understood – is the microbially-produced surfactants. Microorganisms have evolved to utilize biosurfactants for enhancing biodegradation of hydrophobic organic compounds and this makes biosurfactants ideal for the engineered bioremediation of hydrocarbon contamination. It is significant to note that the specific chemical composition and yield of biosurfactants depends on the microbial species and physical and chemical growth conditions. This is especially important in the Gulf region, where the climate places severe stress on the microbial ecosystems, and little is known on how this stress affects hydrocarbon biodegradation.

2. Structure of microbial biosurfactants (types, characterization and classification)

Since biosurfactants have diverse origins, they possess a wide variety of chemical structures. The diverse structural composition of these molecules leads to the multifunctional properties of biosurfactants. These molecules have amphiphilic properties which enables them

to distribute between two immiscible liquids causing a reduction of the surface tension thus causing solubility of the two fluids. The hydrophilic part of biosurfactants consists of amino acids, proteins, sugars, peptides or carboxylic acid which is a polar functional group. While, the hydrophobic part can be composed of either unsaturated, saturated or hydroxylated fatty alcohols or fatty acids (Ohadi, M.,2020). Accordingly, biosurfactants are classified based on their composition as chemicals and the origin of microorganism. The molecules are classified into two classes based on molecular weight. These classes are the Low Molecular Weight (LMW) and the High Molecular Weight (HMW). Biosurfactants that are classified under the low molecular weight include glycolipids [Trehalose lipids, rhamnolipids, Mannosylerythritol (MEL), Sophorose lipids, Mannosylarabitol lipid (MAL), Mannosylribitol lipid (MRL)], lipopeptides [Polymixins, surfactin, iturin lichenysin, fengycin, and serrwettin] and phospholipids [spiculisporic acid]. Biosurfactants classified under high molecular weight include polymeric biosurfactants [Emulsans, Biodispesans, alasans, liposans] and particulate biosurfactants [vesicles, Sulphated polysaccharides, whole-cell, and food emulsifiers] (Fenibo et al., 2019). Glycolipid biosurfactants are characterized by the property of a polysaccharide on their head groups. When the head group is affected by changes in pH or by electrolytes the micellar structure of these biosurfactants changes (Iglesias, 2019). Among the glycolipid biosurfactants, various hydrophobic fatty acids have the ability to reduce Kraft temperature to ensure their structure is maintained. The Kraft temperature is the minimum temperature that is required for the formation of micelles. On the other hand, rhamnolipids are made up of rhamnoses and hydroxyl fatty acids. Sophorolipids are made of sophorose which forms the hydrophilic part of the surfactant. The hydrophobic part is made of fatty acids that contain a long chain of carbon atoms (Purwasena et al., 2019). Surfactin, a lipopeptide biosurfactant is composed of molecules that consist of seven amino acids and fatty acids that form the hydrophobic part. High molecular weight biosurfactants majorly function in solubilization of

hydrophobic molecules and they have a wide diversity in their structures and functions (Jahan et al., 2019).

Biosurfactants are produced by microorganisms like *Bacillus, Pseudomonas* and *Acinetobacter* species among others. This is achieved through the process of fermentation and the enzyme-substrate reaction. Synthesis of biosurfactants is not only achieved intracellularly but can also be carried out extracellularly by the use of biocatalysts which are enzymes. The hydrophilic and hydrophobic moieties can either be synthesized independently following different pathways or they can both be dependent on a substrate or one can be induced by the substrate and the others are synthesized anew (Thavasi, R., 2011). In the amphiphilic structure of biosurfactants, the hydrophobic moiety can either be a hydroxy fatty acid or a long-chained fatty acid. While the hydrophilic moiety can either be a carboxylic acid, amino acid, carbohydrate, phosphate, alcohol or cyclic peptide. The synthesis of these moieties involves the carbohydrate metabolic pathway which synthesizes the hydrophilic moiety and the hydrocarbon metabolic pathway which synthesizes the hydrophobic moiety. In most cases, the first enzymes used in the process of precursor synthesis are regulatory enzymes (Desai & Banat, 1997).

The physical characteristics of biosurfactants are similar to those of their synthetic ones. They lower the surface tension for the water by 74.66 mN/m to 27.26 mN/m and show CMC of 40 mg/L values lower to those of synthetic surfactants (Liu et al., 2016). CMC values of 10 mg/L for glycolipids are reported, while sodium dodecyl sulphate (SDS) and Triton X-100 have CMC values of 2100 mg/L and 130 mg/L. In addition, biosurfactants can withstand more extreme environmental conditions (pH, temperature, biodegradability, toxicity) than chemical surfactants (Vijayakumar and Saravanan, 2015).

3. Effects of factors on microbial biosurfactant production

Various factors influence biosurfactants synthesis and they affect the rate of production and biosurfactants properties. Some of the factors that impact the optimum production of biosurfactants include carbon sources, nitrogen sources, temperature, pH, oxygen availability carbon-nitrogen ratio and agitation (Guellal, D., 2020). Though, the nature of the carbon substrate affects the production of biosurfactants. Good carbon sources for biosurfactants synthesis include crude oil, sucrose, glucose, diesel, and glycerol. Nitrogen compounds like urea, yeast extract, sodium nitrate, ammonium nitrate, malt extract, and meat extracts are used in biosurfactants production (Md, 2012). The most used source of nitrogen is the yeast extract, but its usage is dependent on the culture medium and organism used. Environmental factors have a great effect on yield and the characteristics of the molecules. Thus, ensuring high yield can be achieved by optimization of the environmental factors in the bioprocess of production. Some of these factors include pH, temperature, agitation speed, and aeration. Aeration and agitation have a big influence on the process of production as they are both involved in facilitating the passage of oxygen from the gas phase to the aqueous phase (Ohadi, M.,2018). Aeration is also connected to the physiological functioning of the microbial emulsifiers by which bioemulsifiers have been identified to enhance the solubilization of insoluble substrates. The concentration of salt in the medium also has an effect on the production of biosurfactants since salts affect the cellular activity of microorganisms (Md, 2012).

4. Biosurfactants and bioemulsifiers production

Major categories of biosurfactants include glycolipids, lipoproteins and lipopeptides, phospholipids and polymeric surfactants (Kaloorazi & Choobari, 2013). Among these categories, the biosurfactants charges are mainly anionic (rhamnolipids and sophorolipids acids). They can be intracellular, as in the case of monosaccharide and mycolates (Karlapudi et al., 2018), but are generally extracellular and promote emulsion or dispersion of the

hydrophobic phase. Bioemulsifiers are often classified with biosurfactants and terms are frequently inverted (Fracchia et al., 2012). Thus, Mnif and Ghribi (2015) described Emulsan, a lipopolysaccharide of high molecular weight, as a biosurfactant, while specifying that it does not lower the surface tension. Shekhar et al. (2015) also reported emulsan and biodispersion as microbial surfactants but as dispersant or emulsifier. On the other hand, Tripathi et al., (2018) clearly recognized the mixture of terms using the biosurfactant / bioemulsifier. To differentiate between the two groups, it must be noticed that a biosurfactant can act as an emulsifier in stabilizing the emulsion. On the other hand, a bioemulsifier, if it stabilizes the immiscible mixture, does not possess the property of lowering the surface tension nor of defined molecular structure as for the biosurfactants (McClements and Gumus, 2016). If they are so present, it is because bioemulsifiers, generally biopolymers such as polysaccharides, are frequently produced in parallel with biosurfactants (Shekhar et al., 2015) and form a solution with emulsifying and surface-active properties. Glycolipids form the largest class of biosurfactants and include rhamnolipids, sophorolipids and trehalolipids (Santos et al., 2016). The prefix attached to the word lipid refers to identity the carbohydrate of the hydrophilic end of the surfactant: sugar rhamnose, sophorose or trehalose (Nurfarahin, A., 2018). The use of pure products is not essential in these processes; this statement is the basis of the simplifications of alternative. Thus, the use of unpurified biosurfactants and non-sterile production media could satisfy the needs of the process. Moreover, the use of industrial wastes as a base for the production of biosurfactants would make it possible to reduce the costs related to the substrates used for their biosynthesis while promoting the valorization of these residues.

5. Origin of diversity of microbial biosurfactants and their importance in Qatar

Concerning the production of microbial biosurfactants, it is now well-established in environmental microbiology, that microorganisms can adapt their metabolism to overcome

many regulations of metabolic pathways, induction, stressors on microbial cells, oxidative regulations and others which limit their performance in their natural habitat. All these considerations explain the diversity of microbial biosurfactants, as biomolecules and physical and chemical characteristics and activities. In Qatar, the endogenous bacteria are under stress of weathered and harsh soil conditions. Al Disi et al. (2017) and Attar et al. (2017) demonstrated the ability of Qatari hydrocarbon-degrading bacteria to shift their metabolic pathways towards specific biological activities even by slight changes in substrates or growth conditions. Some of them were identified, but most of the factors affecting the cell, considered here as cell factory, can be monitored, identified and alleviated only in bioreactors, fully controlled.

Qatar is a peninsula with just over 11,000 km2 of land and with 900 km of coastline. It is a hot, dry, flat, rocky landscape with sand dunes in the south, and maximum temperatures can reach over 47ºC.   The average rainfall is only 8.1 cm and it is believed that the available groundwater resources accumulated 10,000 to 30,000 years ago (Mamoon & Rahman, 2016). This combination of high temperature and low rainfall makes Qatar one of the most hostile – and unique – environments on earth. Groundwater protection is a vital concern, and given the extent of hydrocarbon extraction and processing, a mean to remediate soil and groundwater contamination in the harsh Qatari environment is required.

The preferred method for remediation of hydrocarbon contamination in the subsurface is bioremediation. It does not require disturbance and removal of the contaminated soil and ultimately results in complete mineralization of the hydrocarbon contamination. PAH-degrading bacteria have been isolated from polluted soil in Qatar (Al-Thani et al., 2009), indicating that bioremediation is possible in the harsh Qatari environment. A key issue in the application of engineered bioremediation is the reduced bioavailability of hydrocarbons due to their low aqueous solubility. This results in low biodegradation rates and thus long times for

remediation of the hydrocarbon contamination.

6. Applications of surfactants in the petroleum industry

6.1. Comportment of petroleum hydrocarbons when discharged to the soil

The recalcitrance of certain petroleum compounds to biodegradation results from their strong adsorption on soil particles. Here, adsorption is understood as the retention of a solute in solution by the surface of a solid material, whereas the absorption refers to the retention of the solute within the mass of the solid (Talley, 2016). During a spill, the hydrocarbons migrate, together with the groundwater, into the pores of the soil, which eventually reach the aquifer. During this migration, some low-soluble compounds will partition to other non-polar phases such as soil, sediments, and other organic materials such as humic acids (Xu, 2015). The strength of this adsorption depends on the contaminant and the matrix on which it is adsorbed. In general, the tendency of a compound to adsorb to a material is evaluated by octanol /water partition coefficient (Abdel-Shafy and Mansour, 2016), a coefficient independent of the adsorption matrix but inversely proportional to the aqueous solubility of the compound and directly proportional to its molecular weight (Attar et al, 2017). It represents the proportion of the compound that is found in the organic part of an equal mixture of octanol and water. The more the compound is soluble in the organic phase (octanol), the more it will tend to adsorb to the soil or organic matter particles.

6.2. Available treatments of soils, polluted with oil

Among the recommended treatments for soil remediation (solid phase) there are disposal, encapsulation / stabilization and incineration (Huang et al., 2018). However, cost and increasing reluctance of the public to incineration limit its use (Ndimele et al., 2018). On the other hand, stabilization and discharge procedures cannot be accepted as long-term solutions

since they do not constitute treatment solutions. It should therefore be aimed at the application of alternative technologies for the restoration of contaminated soils. These include, but are not limited to, solvent extraction, vacuum extraction, thermal desorption, soil washing, and bioremediation (Ball et al., 2012). They represent almost 40% of the US market, a percentage that has remained relatively stable since 1992. The bioremediation treatment of soils that are contaminated with high molecular weight hydrocarbons such as lubricating oils remains laborious due to the physical properties of the pollutants. However, it is essential to remember that if they are difficult to biodegrade, these compounds are still biodegradable and that it is the non-availability of organic pollutants to degrading microorganisms that limits the scope of biological treatments (Chen et al., 2015). In this case, combination of bioprocesses and physicochemical technologies may be advantageous. The aim is to integrate biotechnological options with the established physicochemical processes: the concept of treatment chain is particularly important and appropriate in case of complex recalcitrant hydrocarbons contamination (Varjani, 2017). Nevertheless, the typical problem of generating byproducts of physicochemical processes treatment, such as toxic acids and washing solutions, remains, as does their high application costs.

6.3. The usage of surfactants for washing the contaminated soil

Soil washing technology (*ex-situ*) with surfactants is an innovative treatment pathway specifically to address the weathering problem (Mao et al., 2015). Synthetic surfactants are classified based on the ionic nature of their hydrophilic head, which controls their potential adsorption on soil particles. They are therefore divided into four groups: ionic (acidic hydrophilic head), cationic (basic hydrophilic), nonionic (no charge) and amphoteric (acidic and basic hydrophilic head) (Alwadani and Fatehi, 2018). In general, the solubilization capacity via micelles follows the following order: nonionic> cationic> anionic, for surfactants having the same length of hydrophobic chain (De Almeida, D. G., 2016). However, the nonionic

surfactants are the best in the restoration of soils because they do not tend to adsorb to soil particles which reduces the effectiveness of the surfactant (Trellu et al., 2016).

Surfactants currently used for soil washing or bioremediation are synthetic in nature, often toxic and non-biodegradable, thereby generating a wash solution rich in contaminant(s) (De et al., 2015). Moreover, if surfactants are effective for soil washing (Trellu et al., 2016), their use is severely limited by their excessive cost which can be up to 69% of the total cost of a process (Gharibzadeh et al., 2016). Substitution of synthetic surfactants by biological surfactants, biosurfactants, is the ideal integration of a biological component into the physicochemical washing process. However, since these are available neither in industrial quantities nor at competitive prices to meet industrial requirements, it is necessary to study biosurfactants production that is economically acceptable for soil washing or enhancement of microbial remediation of adsorbed oil components. Substitution of synthetic surfactants by biological surfactants produced by microorganisms responds to the latter. Their integration into soil restoration processes has been widely reported by the scientific community (Liu et al., 2018). In spite of everything, the current market of surfactants is almost entirely dominated by surfactants of synthetic origin (Karlapudi et al., 2018).

6.4. Microbial Enhancement of Oil Recovery technology (MEOR) using surfactants

Although the use of surfactants (chemicals or biomolecules) in oil field is thought to be only in soil restoration by washing or bioremediation, an additional attractive use is Microbial Enhancement of Oil Recovery (MEOR). It is a tertiary recovery of oil with the usage of microbes or their metabolites to improve the yields of recovery of the residual oil, trapped in wells. It is now well established that it is actually less-expensive, in contrast with the chemically-enhanced oil recovery (CEOR), with the condition that the used microorganisms produce large quantities of polymers and/or biosurfactants in low-cost raw materials used as substrates (Sarafzadeh, 2014).

1. The *ex-situ* MEOR is based on *ex-situ* production of these microorganisms and their biosurfactants in industrial bioreactors with batch or continuous modes. Then, they are injected into the well with a water flood (Al-Bhary, 2013; Bachmann, 2014). So, the composition of the medium providing the hydrophobic substrate is crucial and needs a lot of basic development and industrial investigations. In general, the yield carbon/energy is the main factor for production of biosurfactant agents (Fallon, 2011).

2. Bioaugmentation by injecting biosurfactants-producing microorganisms in the reservoir. This is based on the cell/oil interface, with metabolically active cells to permit *in-situ* spreading, in which these cells occupy a role in the interactions on their surfaces at interphases oil/water (Bachmann et al., 2014). They also reported that the formed emulsions are proportional to the total cell surfaces.

3. Biostimulation by injection of nutrients and essential elements. Here, sometimes, growth inhibitors are also injected to inhibit unwanted microbial strains. Thus, the growth of desired indigenous bacteria, able to produce biosurfactants is stimulated (Al-Bahry et al., 2013).

6.5. Other applications in oil field

Besides MEOR, the application of biosurfactants in oil field may be extended to:

1. Enhancement of oil transportation in pipelines: As example, Amani and Kariminezhad (2016) described the use of emulsan from by *Acinetobacter calcoaceticus* PTCC1318 to remove crude oil from stainless steel pipes, showing suitability to the extrapolation to pipeline transportation.

2. Cleaning of oil Storage Tanks: Diab & El Din (2013) showed the benefits of using biosurfactants from *P. aeruginosa* SH 29 in purifying vessels contaminated with oil.

3. Biosurfactants act as anti-corrosive agents: Araujo and Freire (2013) showed that the majority of microbial biosurfactants possess anti-corrosion activities and may delay the corrosion of metals.

4. Control of Sulfate Reducing Bacteria (SRB): it has been demonstrated that the potential of *Bacillus licheniformis* biosurfactants to inhibit growth and even totally killing SRBs with 1.0% crude biosurfactant in few hours (El-Sheshtawy, 2016).

7. Mechanisms of Bioremediation using Biosurfactants

The theoretical model of the action of microbial biomolecules such as biosurfactants and bioemulsifiers that are responsible for the breakdown of oil molecules has been clearly illustrated by many reports. Kazemzadeh et al. (2020) investigated the way by which the surface molecules from microbial communities interact with the molecules of hydrocarbons to degrade the oil molecules and reduce their environmental toxicity. The high surface activation as well as the reduction of surface tension of the hydrocarbon molecules was reported by Kazemzadeh et al. (2020) who studied the production of glycolipid biosurfactants. The research demonstrated an effective consumption of crude oil in contaminated soil through the biosurfactant action. Moreover, Marchut-Mikolajczyk et al. (2018) investigated the bioactivity of microbial surfactants of *Bacillus pumillus* and demonstrated its effective emulsification effect. The previously hydrocarbon-contaminated soil investigated by Marchut-Mikolajczyk et al. (2018) was shown favorable for seed germination after the treatment with biosurfactant molecules. Similar studies demonstrated the reduction of environmental pollution and hydrocarbon toxicity by the use of novel biosurfactants (Karlapud et al., 2018).

Some of the literature comparing active bioremediation and self-cleaning of contaminated soils have yielded insight on how biosurfactants can fasten the degradation process of hydrocarbons. Soil that recovers from the contamination, toxicity, and pollution of

hydrocarbons is called self-cleaned (Ali et al., 2020). Self-cleaning has been demonstrated to proceed at a normal rate if microbial communities are responsible for producing biodegrading molecules highly adapted to the conditions created by the soil contaminants as well as the prevailing soil conditions such as pH, salinity, temperature, and alkalinity (Ali et al., 2020). However, microbial communities that act on oil molecules need biostimulation to produce biosurfactants or bioemulsifiers that are known to be surface-active molecules that have been demonstrated to reduce the surface tension of oil and consequently, to degrade toxic oil molecules. Therefore, Ali et al. (2020) demonstrated the need for identification and deployment of bacteria for the production of biosurfactants responsible for oil degradation.

Biodegradation of oil molecules in contaminated soils and in oil recovery using biosurfactants produced by microbes is essential. Alkaabi et al. (2020) studied the use of novel molecules including biosurfactants that are produced by hydrocarbon-degrading bacteria in contaminated Qatari soils. Accordingly, the harsh conditions created by hydrocarbons on contaminated soils are coupled with slow action of soil bacteria for activation of biosurfactant-producing microbial communities (Alkaabi et al., 2020). When biostimulation and bioaugmentation are combined in the treatment of hydrocarbon-contaminated soils, effective bioremediation is achieved through the production of biosurfactants (Alkaabi et al., 2020). Active bioremediation of hydrocarbon-contaminated soils is effectively and efficiently achieved when specific strains of microbial communities are selected and stimulated to produce biosurfactants.

A proper deployment of biomolecules for bioremediation through the production of biosurfactants requires a thorough consideration of several factors. Given that the harsh conditions of the soil, some of other factors related to the diversity of hydrocarbon-degrading bacteria and their produced surfactants are to be investigated to understand the reasons that some bioremediation efforts fail. In Qatar, oil-contaminated beach soils have been found to

produce conditions that only the use of novel microbial biomolecules is effective in the recovery of the contaminated soils (Alkaabi et al., 2020; Ali et al., 2020). Soil systems affect the way self-cleaning or active bioremediation occurs in hydrocarbon-contaminated soils.

8. Biosurfactant versus Synthetic surfactants in applications at harsh soil conditions

Bioremediation of petroleum-derived hydrocarbons is now validated in comparison with the synthetic degradation of these molecules (Al-Kaabi et a., 2017). Patowary et al. (2018) compared the effectiveness of biosurfactants produced by bacteria and synthetic surfactants in terms of crude oil degradation. Both petroleum hydrocarbons and polycyclic aromatic hydrocarbons were degraded using biosurfactants more efficiently compared to synthetic surfactants (Patowary et al., 2018). The use of *Pseudomonas aeruginosa*-producing rhamnolipid biosurfactants was effective in bioremediation of contaminated soil. A similar comparison was conducted using modified conditions (Chaprao et al., 2015). They demonstrated that the degradation of petroleum hydrocarbons is more efficient and cost-effective with the use of bioremediation with yeast and bacterial biosurfactants compared to chemical surfactants (Chaprao et al., 2015). The researchers used a yeast strain of *Candida sphaerica* and a bacterial strain of *Bacillus sp.* to produce biosurfactants (Chaprao et al., 2015). The biosurfactants were emerged as the efficient agents of the degradation of hydrocarbons and combined with their cost-effectiveness, so they appeared to be more promising for use in remediation than chemical ones.

An integrated method of bioremediation using biosurfactants that utilized biochar, rhamnolipids, and nitrogen was investigated by Wei et al. (2020). The soil condition was coastal marshy environments which are characteristic of the harsh environment created by salinity and the toxicity of petroleum. The best removal of petroleum hydrocarbons occurred when the integrated method of using biosurfactants and nitrogen was used (Wei et al., 2020).

In wetland oil treatment, the use of biochar and rhamnolipid biosurfactants in combination with nitrogen is also an effective remediation method. However, other combinations of biosurfactant treatment methods of petroleum hydrocarbons have been investigated but only in the laboratory conditions (Gidudu and Chirwa, 2020). In the study involving the use of an integrated method of bacteria-produced biosurfactants and electro-kinetic remediation, Gidudu and Chirwa (2020) demonstrated an effective extraction and degradation of the petroleum contaminant of soil. However, this study has not been conducted in a field replication study for remediation of petroleum-contaminated soil.

It is to be reminded that some soil conditions that are typical of coastal Qatar have been investigated regarding the best method for biodegradation of petroleum hydrocarbon contaminants (Al-Kaabi et al., 2018; 2020).

9. Surfactant mode of action

One means to address the low bioavailability is through the use of surfactants, which are molecules that have hydrophobic and hydrophilic moieties (Figure 1). Surfactants are used extensively in consumer and industrial applications to increase the apparent aqueous solubility of hydrophobic organic compounds. This is accomplished through the formation of surfactant micelles (Figure 1), the center of which is an oily phase that allows hydrophobic organic compounds partitioning. When surfactant micelles are present, the apparent hydrophobic organic compounds aqueous concentration is the sum of the aqueous hydrophobic organic compounds and the hydrophobic organic compounds partitioned into the micelles. In this manner, the apparent hydrophobic organic compounds aqueous concentration can be increased well above the aqueous solubility and this is one of the reasons why surfactants are used extensively in consumer products, such as laundry and dish detergents, shampoos, soaps, and household cleaners. This increase in hydrophobic organic compounds solubility led environmental engineers to examine the potential for surfactants to solubilize hydrophobic

organic compounds and to remove hydrophobic organic compounds sorbed to soils (Deshpande, S., 1999). The results were promising, and this facilitated the application of surfactant solutions to wash hydrophobic organic compounds-contaminated soil *in situ* (Duffield et al., 2003). Researchers then focused on the logical question regarding hydrocarbon recalcitrance to biodegradation: i.e., is the micellar-phase hydrophobic organic compounds bioavailable, and if so, can surfactants be used to enhance the biodegradation of recalcitrant hydrophobic organic compounds.

Many studies have been performed to examine the effects of surfactants on biodegradation of different hydrophobic organic compounds (Al-Kaabi et a., 2017). The results are mixed, with some showing enhanced biodegradation, some showing inhibited biodegradation, and some showing no change in biodegradation. The reasons for these apparently conflicting results remained elusive until the scientific community developed the surfactant-enhanced bioavailability theory and put this theory into context with microbial kinetics. This theory applies micellization kinetics, surfactant sorption, and hydrophobic organic compounds solubilization to describe surfactant-enhanced hydrophobic organic compounds bioavailability. It has been successfully demonstrated for a range of surfactant structures and it provides a tool to aid in the development of surfactant-enhanced bioremediation schemes.
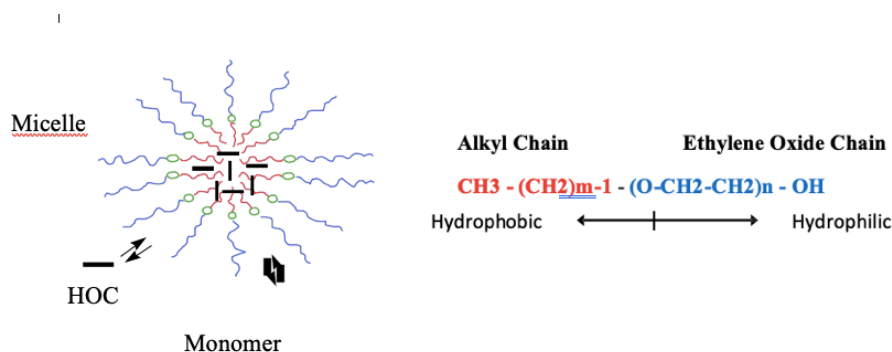
Figure 1: Surfactant molecules combination.

Surfactant molecules consist of a hydrophobic group and a hydrophilic group. Depicted above is a generic linear polyoxyethylene alcohol surfactant, CmEn. At low aqueous concentrations the surfactants are present as individual molecules, called monomers. When the concentration is increased more than the critical micelle concentration (or CMC), there are some things called surfactant monomers which aggregate to form three-dimensional structures called micelles. The simplest and most common structure is a sphere, depicted to the right, with the hydrophobic groups on the inside and the hydrophilic groups on the outside. The hydrophobic core of the micelle is available for hydrophobic organic compounds (HOC) to partition into, thus increasing the HOC apparent aqueous solubility.

The application of biosurfactants to bioremediation in Qatar, whether intrinsic or engineered, is a viable option for increasing hydrocarbon bioavailability and biodegradation rate. However, there is no available data on biosurfactant-producing bacteria, indigenous to Qatar or how the indigenous hydrocarbon-degrading bacteria may be able to utilize biosurfactants if they are made available. In order to develop and apply biosurfactant-enhanced bioremediation (BSEB) in Qatar, we need to know the following:

1- Which biosurfactant-producing bacteria are indigenous to Qatar?

2- Under what conditions do they grow and produce biosurfactants?

3- What are the biosurfactants' physiochemical properties?

4- Are the biosurfactants capable of enhancing hydrocarbon bioavailability and biodegradation?

5- Do the biosurfactants enhance a range of hydrocarbon structures or are they optimized for a specific type of hydrocarbon (e.g., n-alkane versus a PAH)?

These knowledge gaps hamper our ability to (i) select and produce biosurfactants that can be applied in the Qatari environment and to (ii) predict their effects on hydrocarbon bioavailability in engineered systems. Obtaining answers to these questions is necessary for the development

of biosurfactant protocols and methods for the remediation of hydrocarbon contamination. The focus of this study is to answer these questions, with the goal to provide a roadmap that will enable the rational design of biosurfactant-enhanced bioremediation systems.

MATERIALS AND METHODS

1. Soil samples collection

Soil samples were collected from different areas in Qatar, characterized by aged pollution with petroleum hydrocarbons. Two dumpsites in Dukhan industrial area were selected because solid and liquid wastes from the oil industry were discharged and left for self-purification for more than three years in the open air. One sampling site was chosen away from the sea line and another in the intertidal zone of Dukhan. All these selected sites are characterized by pollution with weathered oil as previously demonstrated (Al-Kaabi et a., 2017). An automotive workshop in Doha industrial area was also selected with recent and aged pollution with diesel and lubricants. All samples were collected from the surface soil layer in sterile 50 mL tubes and preserved at 4 $^\circ$C until use (Table 2).

2. Enrichment culture with native bacteria

Hydrocarbon-degrading bacteria were enriched from soil samples using a standard enrichment method (Al Disi et al., 2017; Al-Kabbi et al., 2018; Aparna et al., 2011) as shown in (Figure 2). In 50 mL sterile tube, 1 g of soil sample was added to 25 mL Minimal Salt Medium (MSM) that contains (w/v) : 0.1% NH_4Cl, 0.1% KH_2PO_4, 0.4% $Na_2HPO_4.2H_2O$, 0.006% KCl and 0.04% $MgSO_4.7H_2O$ with pH set at 7.0 before sterilization. Before inoculation, the medium was added with 1% (v/v) of a trace element solution. The trace element solution was contained of (g/100 mL): EDTA, 0.1; $ZnSO_4$, 0.042; $MnSO_4$, 0.178; H_3BO_3, 0.05;

NiCl$_2$, 0.1. All media were autoclaved for 20 min at 121°C. Solid MSM was obtained by adding 15 g/L agar. 5% (v/v) of diesel – the sole carbon source- later on added in a final volume of the culture which is 20 mL. The diesel stock was gently supplied by Mesaieed Refinery (Qatar) with a complete analysis, indicating hydrocarbons structure ranging from n-C$_{12}$ to n-C$_{25}$. It contained 750 g/L carbon. All cultures were incubated at 30 °C for two weeks in a shaker set at 200 rpm. After one week of incubation, 2 mL of each culture was used to inoculate a fresh medium, as performed in the first culture. Three subsequent sub-culturing were then performed, before proceeding to the isolation of the enriched hydrocarbon-degrading bacteria.

3. Isolation and purification of bacterial strains from the enrichment cultures

100 µL of the enriched cultures were plated on MSM solid media and then coated with 100 µL diesel, spread on the surface of the plate which was then sealed with PARAFILM® and incubated at 30 °C. Colonies of different aspects (form, color, shining, and size) were spread on LB solid agar plates since it performed faster growth than MSM. The isolated colonies were separately displaced by streaking in LB solid agar plates. Six purification steps of the isolated colonies were performed. Strains were preserved at -80ºC in LB medium containing 30% glycerol.

4. Sample processing and protein extraction for MALDI-TOF-MS analysis

In order to produce the most reliable results, a techniques was used to prepare the samples for matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (Nacef et al., 2017; Abdel Samad et al., 2020). The extraction was performed using equivalent volumes of ethanol and formic acid (Wang et al., 2012). A separate colony of a strain from an LB plate was suspended in 300 µL sterile water and then re-suspended in 900 µL absolute ethanol. After centrifugation at 10,000 rpm for 5 min, the pellet was supplemented with 1 mL of formic acid (70%) and then 1 mL acetonitrile (100%). After centrifugation, 1 µL of the supernatant was introduced into the biotarget of 48 sample spots. Then, 1 µL of alfa-

cyano-4-hydroxycinnamic acid (HCCA) matrix solution containing (50% acetonitrile and 2.5% trifluoroacetic acid in ultra-pure water) was added for protein extraction. Triplicates were performed by spotting separate colonies in three wells.

5. Identification of the bacterial isolates by MALD TOF MS

The mass spectra generated by MALDI-TOF MS were analyzed for similarities to the database entries. Proteins with an m/z between 2,000 and 20,000 m/z are considered in generating the protein profiles for each strain. The mass peaks corresponding to specific ribosomal proteins are used for the identification of the isolates by similarities establishment. The results were in form of log(scores) as generated by default by the Biotyper software. A log scale from 0.000 to 3.000 was obtained for each strain. If the score ranges between 2.300 and 3.000, the identification is at the highly probable species level with high confidence. However, scores ranging between 2.000 and 2.299 provide high accurate identification at the genus and probable correct at species level. The scores in the range between 1.700 -1.999 provide probable genus-level identification.

6. Data processing

The protein profiles were generated by the Bruker Flex Control software as mass spectra (linear and positive mode, at 60 Hz laser frequency and 35% intensity) using an acceleration and a source voltage set at 20 and 18.7 kV, respectively. For every spectrum, 240 laser shots in 40-shot steps were generated from various areas of the sample spot, then analyzed by the use of the default settings. The obtained protein profiles were examined by using a Flex Analysis and a Biotyper RTC 3 software. Therefore, the mass spectra, which were created by MALDI-TOF MS, are multivariate data. Each mass signal is from a distinct molecular dimension. The multivariate statistical processes are applied to differentiate between bacterial strains. The principal component analysis (PCA) also used to reduce the dimensionality and

keep the original information. The peaks of each MALDI-TOF MS spectrum were the basis of the PCA analysis. The peaks can be of proteins or peptides. The PCA led to the generation of assembled groups of spectra exhibiting similar properties and differences. A 2D or a 3D coordinate system can be generated with the data. However, the 2D system is more recommended since it plots PC1 against PC2 and offers in most cases, more than 80% of the total variance between the studied spectra. Besides, the hierarchical relationship between the isolates was investigated by establishing the dendrogram using the MALDI Biotyper Compass Explorer software that adopts default settings as per the manufacturer's instructions. However, the analysis by dendrogram and PCA was performed according to the procedure's standard operation of the instrument and software. The spectra generated using triplicates were processed for smoothing and subtracting the baseline. Spectra with a strong background noise or high/low intensities were not used and then excluded. A main spectral projection (MSP) was created with the automated MSP creation functionality within the MALDI Biotyper 3.0 software by using the considered spectra. Indeed, the MSP provides information on the means of the peak frequencies, peak masses, and peak intensities. The generated MSPs for each isolate were given to the functionality of PCA or dendrogram for analysis and generating graphs.

7. Biosurfactants production

5% (v/v) diesel was utilized as a carbon source to produce the biosurfactants in MSM liquid medium. The production medium consisted of 19 mL MSM liquid medium added with 1 mL diesel in a 50 mL sterile falcon tube, tightly sealed with parafilm foil and incubated at 30°C, in a shaker set at 200 rpm for one week or two weeks, as specified with results. The cultures were inoculated with a suspension of cells from colonies of each isolate, formed overnight on LB plates. The initial cell density at the inoculation time corresponded to an optical density (OD) at 600 nm of 0.15. Cell density and the growth of each strain was evaluated by determination of the colony-forming Unit (CFU).

8. Colony-forming Unit (CFU) determination

CFU was determined by plating 100 µL of serial dilutions of the cultures on plates of LB, thus using CFU exhibits more accurate results than by using Optical Density (OD). The dilution corresponding to a number of colonies between 30 and 100 was considered. Then the CFUs were calculated for each ml of the corresponding culture.

9. Determination of the diesel solubilization activity

The procedure employed to determine the solubilization activity produced by each strain was based on the method described by Mnif et al. (2013) using diesel. Biosurfactants solution was prepared by centrifugation of 1 mL of the cultured MSM (10,000 rpm for 5 min.). The supernatant which was produced was then separated (0.9 mL) and added to 10 mL of Tris-HCl 20 mM (pH 7.0). Then, 0.2 mL of diesel was added to the tube to have a final 2% (v/v) diesel. Control Number 1 corresponded to a similar mixture except that 0.2 mL Tris-HCl buffer was used instead of diesel to determine the quantity of diesel solubilized by the strains in the culture medium. Control Number 2 was performed using 0.9 ml Tris-HCl instead of the culture's supernatant (without biosurfactants) and 0.2 mL diesel to determine the spontaneous solubilization of diesel in the aqueous phase. All tubes were incubated overnight at a vertical position in a shaker set at 30ºC and 300 rpm, in the dark. After incubation, tubes were left out for 0.5 h to separate the diesel top layer. Then, 4 mL of the aqueous solution were mixed with an equal volume of pure hexane to extract the diesel with vigorous vortexing during 2 min and then centrifugation for 15 min at 4,500 rpm. The optical density of the hexane phase was measured at 295 nm. Hexane was used as a blank. The concentration of diesel in the hexane phase was calculated from the slope of a calibration curve of different diesel concentrations in hexane, extending from 0.3 to 1.25 µL of diesel/mL. The amount of solubilized diesel and percent of solubilization were calculated using equations 1 and 2:

(1): Solubilized diesel = OD of the supernatant - OD of control 1 – OD of control 2

The OD values were obtained from the calibration curve.

(2): % Solubilization= (Solubilized diesel/Initial diesel concentration) ×100

The Initial concentration of diesel was 18.349 µL/mL. The solubilization activity of the freeze-dried biosurfactants was determined in 1 mg solubilized in 0.9 mL Tris-HCl and a similar method was employed.

10. Determination of the emulsification activity

MSM cultured broth of each strain was centrifuged for 15 min at 10,000 rpm. 1 mL of the supernatant was supplemented with 0.15 mL diesel. It was vortexed for 2 min and left for 1 h for separating diesel from the aqueous phase (Jagtap *et al.*, 2006). The aqueous phase was used to measure the optical density at 400 nm. Control Number 1 was performed with the fresh MSM instead of culture broth. Control Number 2 was performed with the culture broth and 0.15 distilled water instead of 0.15 ml diesel. The emulsification activity (EA) was calculated as units of emulsification per milliliter (EU/mL), where each 0.01 absorbance is considered as one activity unit according to Patil and Chopade, (2001 a, b; 2003), as the following equation:

Emulsification Activity (EU/mL) = (OD both – OD control 1 – OD control 2 / 0.01) × dilution factor.

The emulsification activity of the freeze-dried biosurfactants was determined in 1 mg solubilized in 1 mL Tris-HCl and a similar method was employed.

11. Extraction of biosurfactants

Biosurfactants were extracted from each culture broth after centrifugation for 15 min at a 10,000 rpm at 5°C. The employed method was that described by Javaheri et al. (1985). The pH of the supernatants was adjusted to 2.0 using 6.0 M HCl, and the solution was incubated at 4°C for 24 h. Then, $CHCl_3/CH_3OH$ (2:1) was added to an equal volume, vigorously mixed, and incubated overnight at room temperature. Next is centrifugation at 10,000 rpm for 15 min, after that the pellet was suspended in Milli-Q water. The concentrate was neutralized to pH 7.0 by

using 1 M NaOH solution, then freeze-dried.

12. Analysis of the freeze-dried biosurfactants by Fourier transform infrared (FTIR)

The extracts of biosurfactants which were dried of each culture were analyzed by FTIR. The FTIR Perkin Elmer 400 FT-IR/FT-NIR spectrometer was used. The spectra were recorded in the range of 400–4000 cm^{-1}. The method was described by Alkaabi et al. (2018) and Oualha et al. (2019).

13. Culture of selected strains on vegetative oils

3 strains of *Pseudomonas* were selected from our previous collection in the lab, and 3 strains of *Bacillus* were selected from the newly isolated ones to perform biosurfactants production in different carbon sources such as vegetative oils; almond oil, castor oil, and sunflower oil. In addition to that, diesel was also performed on these 6 strains for comparison between diesel and vegetative oils. The production medium consisted of 19 mL MSM liquid medium added with 1 mL from each carbon source in a 50 mL sterile falcon tube, tightly sealed with parafilm foil and incubated in a shaker set at 200 rpm at 30°C, for one week. The cultures were inoculated with a suspension of cells from colonies of each isolate, formed overnight on LB plates with an initial OD at 600 nm of 0.15. After 1 week, emulsification and solubilization activity were determined for each strain as well as the corresponding CFU. The used strains are shown in Table 1.

Table 1: Bacterial Strains Cultured on Vegetative Oils

Strain code	MALDI Score	Identification	Location of isolation
SH1	2.24	*Pseudomonas aeruginosa*	GTL-process water
D5D1	2.22	*Pseudomonas aeruginosa*	Dukhan dumping area
ZA9	2.07	*Pseudomonas aeruginosa*	Automotive workshop working site
S5	1.88	*Bacillus subtilis*	Dukhan Sealine
SA16	1.92	*Bacillus subtilis*	Dukhan IZ
SA29	1.8	*Bacillus mojavensis*	Dukhan DS2

14. Production of biosurfactant on different concentrations of carbon source

Three sources of carbon were used on 7 selected *Bacillus* strains (S5, SA6, SA16, S27, SA28, SA29, S32); diesel oil, corn oil and used corn oil (heated 5 times). The production medium consisted of 19 mL MSM liquid medium supplemented with 0.2 mL, 0.5 mL, 0.8 mL, 1 mL, 1.2 mL, 1.5 mL, 1.8 mL, 2.2 mL from diesel oil and burned oil in a 50 mL sterile falcon tube, tightly sealed with parafilm foil and incubated at 30°C, in a shaker set at 200 rpm for one week. Emulsification and solubilization activities were determined as well as the CFU.

RESULTS AND DISCUSSION

Chapter I: Evaluation by MALDI TOF and PCA of the diversity of biosurfactants and their producing bacteria, as adaption to weathered oil components

Indigenous Qatari bacterial strains were isolated from highly weathered oil-contaminated sites, identified, and differentiated based on their protein profiles using MALDI-TOF MS. Their clustering using the PCA analysis and proteodendogram showed high diversity even within the same subspecies. This high diversity is reflected in the emulsification and solubilization activities of their adapted biosurfactants. The highest emulsification activity was obtained with a strain of *Lysinibacillus fusiformis* (SA4) in MSM- diesel (5%), while the highest solubilization activity was produced by *Bacillus subtilis* (SA6). PCA allowed a further clustering of the biosurfactants based on their composition obtained by FTIR. These findings showed two types of adaptations occurring with hydrocarbons degrading bacteria in weathered-oily soils, one related to bacterial cell composition maintaining biosurfactants composition and one to biosurfactants, the primary tools for interaction with the weathered oil.

I-1. Isolation of hydrocarbon-degrading bacterial strains from weathered hydrocarbons-samples

Since the objective of this research was to establish a collection of bacterial strains isolated from highly polluted soils with weathered hydrocarbons in Qatar, we developed the strategy of isolation and screening shown in Fig.2. It is expected that the diversity of the hydrocarbon-degrading bacterial strains is affected by nature and structure synthesis of the carbon source. However, weathering status of the hydrocarbons and their adsorption to the soil matrix should affect their availability, and thus the produced biosurfactants by the adapted bacterial community (Kostka et al, 2011). A total of 19 bacterial strains were isolated from differently selected locations (Table 2). All these strains are expected to be highly adapted to weathered hydrocarbons and tolerant to high toxicity exhibited by the 5% diesel employed in the enrichment and isolation steps.
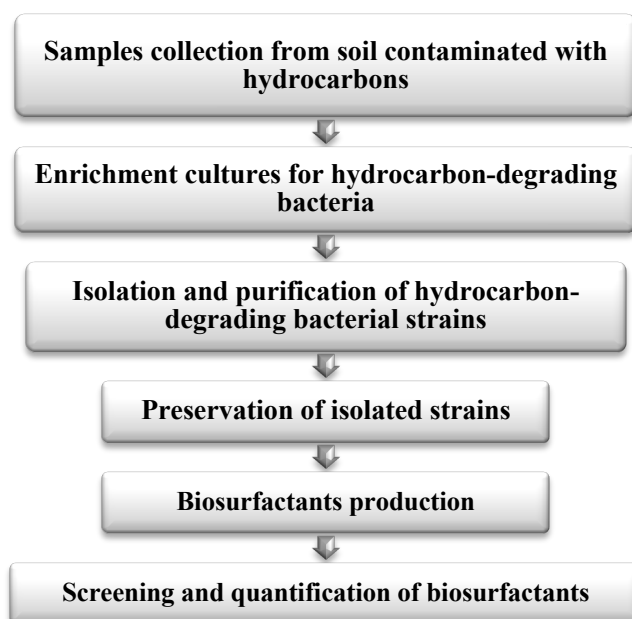

Figure 2: Strategy employed to isolate and screen biosurfactants producing bacteria.

Table 2: Isolated Hydrocarbon-Degrading Bacterial Strains from the Oily-Soils Sampled from Different Locations in Qatar

Soil sample locations	Strains code

Dukhan, away from sea line (Dukhan Sealine)	SA2, SA3, SA4, S5
Dukhan, intertidal zone (Dukhan IZ)	SA9, SA10, SA11, SA12, SA14, SA16
Dukhan, site 1 of dumpsite (DS1)	SA6, SA17
Dukhan, site 2 of dumpsite (DS2)	SA28, SA29, SA31, S32, S33
Automotive Workshop (AWS)	S27, S24

I-2. Identification of the isolated strains by using the MALDI TOF MS technique

The 19 isolated strains were identified by MALDI-TOF MS protein profiling, using the available database in the used machine. Indeed, a MALDI score and a reproducible protein profiles were acquired for each strain by MALDI TOF MS profiling (Table 3). It is interesting to notice that 15 isolates were identified at the level of *Bacillus* genus (five *Bacillus subtilis*, four *Bacillus cereus*, two *Bacillus atrophaeus*, one *Bacillus licheniformis*, one *Bacillus sonorensis*, and one *Bacillus mojavensis*). Three isolates belong to the *Lysinibacillus* genus (two *Lysinibacillus boronitolerans*, and one *Lysinibacillus fusiformis*). One isolate was *Enterococcus faecium*.

Table 3: Identification of the 19 Isolated Strains Based on their MALDI Scores

Strain code	MALDI Score	Identification	Location of isolation
S5	1.88	*Bacillus subtilis*	*Dukhan Sealine*
S24	1.96	*Bacillus cereus*	*AWS*
S27	2.08	*Bacillus subtilis*	*AWS*
S32	2.00	*Bacillus cereus*	*Dukhan DS2*
S33	2.07	*Bacillus licheniformis*	*Dukhan DS2*
SA2	1.8	*Bacillus atrophaeus*	*Dukhan Sealine*
SA3	2.07	*Lysinibacillus boronitolerans*	*Dukhan Sealine*
SA4	2.06	*Lysinibacillus fusiformis*	*Dukhan Sealine*
SA6	2.14	*Bacillus subtilis*	*Dukhan DS1*
SA9	1.76	*Bacillus sonorensis*	*Dukhan IZ*
SA10	1.84	*Lysinibacillus boronitolerans*	*Dukhan IZ*
SA11	1.81	*Bacillus sonorensis*	*Dukhan IZ*
SA12	2.42	*Enterococcus faecium*	*Dukhan IZ*
SA14	1.84	*Bacillus atrophaeus*	*Dukhan IZ*
SA16	1.92	*Bacillus subtilis*	*Dukhan IZ*
SA17	2.19	*Bacillus cereus*	*Dukhan DS1*

SA28	1.95	*Bacillus subtilis*	*Dukhan DS2*
SA29	1.8	*Bacillus mojavensis*	*Dukhan DS2*
SA31	2.06	*Bacillus cereus*	*Dukhan DS2*

In Literature, some strains of *Bacillus cereus* were described as hydrocarbon-degrading bacteria ((Oualha et al.,2019); Al-Kaabi et al., 2018). Other studies reported the biosurfactants of *B. cereus* (Durval, et al., 2018). Biosurfactants of *Bacillus lichenifomis* (*B. licheniformis*) JF-2 were formulated for the petroleum industry (Kumar, et al., 2016). *Bacillus mojavensis* is known for forming biofilms and producing biosurfactants (Ghazal et al., 2018). Surfactants produced by *Bacillus atrophaeus* were reported (Rodriguez et al., 2018). *Bacillus sonorensis* is known as a hydrocarbon-degrading bacterium (Oualha et al., 2019) and biosurfactant producer (Chopra et al., 2014). The genus *Lysinibacillus* is distinguished but relatively close to *Bacillus* as phylogeny, composition of peptidoglycan, and physiology (Ahmed et al., 2007). *Lysinibacillus fusiformis* and *Lysinibacillus boronitolerans* were originally known as Bacillus fusiformis and *Bacillus boronitolerans* before 2007 (Ahmed et al., 2007). Chen et al. (2010) reported the potential of *Bacillus* fusiformis in the biodegradation of naphthalene. Recently, Li et al. (2020) showed the survival of *Lysinibacillus fusiformis* in petroleum environments. While *Lysinibacillus sphaericus* and *Geobacillus sp* are also shown activity in the biodegradation of petroleum hydrocarbons and biosurfactants production (Lina Manchola and Jenny Dussán, 2014), however, the involvement of *Lysinibacillus (Bacillus) boronitolerans* in oil hydrocarbons degradation was never reported. It was only associated with tolerance to boron (Ahmed et al., 2007). Ozyurek and Bilkay (2017) reported the isolation of *Enterococcus faecium* as a hydrocarbon-biodegrading bacterium in crude oil, waste mud pit, and drilling fluid.

These results also show that some well-known bacteria with their high degradation

activity of oil hydrocarbons were not isolated from the highly weathered oil-soils used in this work. By considering the potential of biosurfactants to be applied to oil degradation and recovery, Rhamnolipids produced by *Alcaligenes eutrophus* have been shown to be effective in increasing the solubility of Polychlorinated Biphenyls (PCBs) and their mineralization (Robinson et al., 1996). Commercialized Rhamnolipids of *Pseudomonas aeruginosa* (*P. aeruginosa*) enhanced extraction of hexadecane residues in a sand column, compared to sodium dodecyl sulfate (SDS) and sorbitan monooleate, both are synthetic surfactants (Pacwa-Płociniczak et al., 2011). *Rhodococcus* ST-5 and *Badus* AB-2 surfactants allowed recovery of 95% of the crude oil residues. Biosurfactants of *Bacillus subtilis* were reported but not in the field of oil remediation or recovery (Mnif et al., 2012). *B. licheniformis* is also able to produce biosurfactants. Crude biosurfactant production by *B. licheniformis* can reach 1 g/L, with emulsification power increased up to 96% (El-Sheshtawy et al., 2016). *Bacillus flexus* was also able to produce biosurfactants, even with a low emulsification index.

I-3. Differentiation of the isolated strains using PCA and dendrogram analysis

Further information on the relationships between the highly related isolates can be obtained upon combining the PCA and MALDI-TOF MS analysis. By decreasing the dimensions of objects being demonstrated, linear combinations could be created for variables, representing the studied objects. The PCA results are shown in Fig. 3A. The PCA clustering revealed large biodiversity between the studied strains at the protein level. However, overall variance of the 10 principal components is shown in (Figure 3B). PC1 (34%), PC2 (25.5%) and PC3 (10.5%) combine to show a total of 70% variability in the data. Using the first three principal components, five clusters were obtained. The distances between the clusters indicate the variations at a group level, while the distance between the strains (within each cluster) shows the differences in protein profiles at the strain level. Clusters I and II include *B. subtilis* strains (S5, S27, SA16, SA6, and SA28). The strain *B. mojavensis* SA29 falls within-cluster I,

indicating high similarity in their protein profiles. Similarly, *B. licheniformis* (S33) falls within Cluster III that includes the two *B. sonorensis* (SA9 and SA11). Whereas cluster IV includes three *B. cereus* (SA17, SA24, and SA31). Cluster V includes the three *Lysinibacillus* strains (SA3, SA4, and SA10). Interestingly, the strain *B. cereus* S32 is located at a huge distance from Cluster IV, demonstrating a large variation in its protein profile in comparison to the other *B. cereus* strains in cluster IV. Similarly, the *Enterococcus faecium* SA12 is distinct from any of the five clusters.
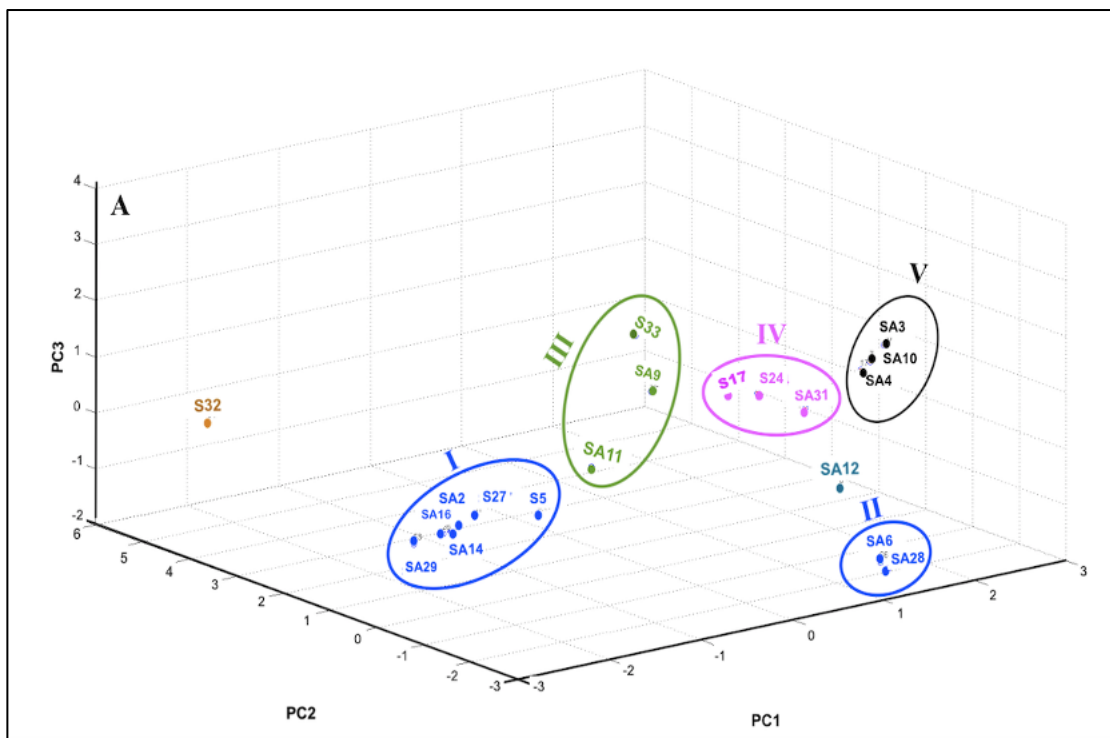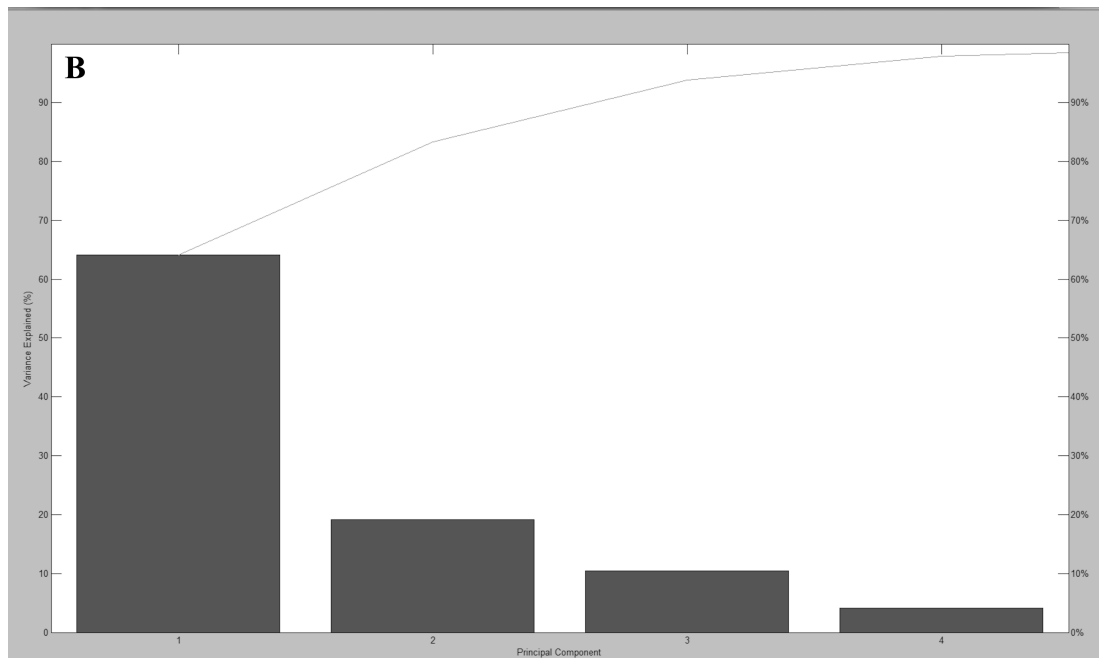
Figure 3: Classification of the studied strains using PCA (A) PCA plot and (B) percentage of variance explained.

The hierarchical relationship between the isolates was investigated by establishing the class dendrogram (Figure 4). The dendrogram revealed two major clusters (**I, II**). Cluster **I** is formed of 9 strains and cluster **II** of 10 strains.   In each cluster, two distinct clades are distinguished. Cluster **I** includes clades **Ia** formed with two strains of *Bacillus subtilis* (SA6 and SA28) and the strains *Enterococcus faecium* (SA12). Clade **Ib** is further subdivided into the sub-clades **Ib1** formed with the three *Lysinibacillus* strains (SA3, SA4, and SA10), and sub-clade Ib2 formed with the three *Bacillus cereus strains* (SA17, SA24, SA31). Cluster **II** includes 2 clades (**IIa, IIb**). Clade **IIa** is formed with the strain *Bacillus licheniformis (*S33) and clade **IIa1** is formed with the two *Bacillus sonorensis* strains (SA9 and SA11). Clade **IIb2** includes one *Bacillus cereus* strain (SA32). Clade **IIb1** is further divided into sub-clade **IIb1a** formed with the two *Bacillus subtilis* strains (S5 and S27) and sub-clade **IIb1b** formed with the strains *Bacillus subtilis (*SA16), *Bacillus mojavensis* (SA29), and *Bacillus atrophaeus* (SA2). The phyloproteomic classification of the studied strains allowed the differentiation and separation of strains belonging to the same *Bacillus* species. Two *B. subtilis* strains (SA6 and

SA28) were classified in clade **Ia** while other strains (S5, S27, and SA16) were classified in IIbIa. Similarly, three strains belonging to *Bacillus cereus* (SA17, S24, and SA31) belong to cluster **Ia**, while the strain B*acillus cereus* S32 is under cluster **IIb**. The approach of using protein profiles may be informative in differentiating strains belonging to the same species (Fernández-No, et al., 2013). Indeed, MALDI-TOF MS could be used to characterize isolates with much higher precision than that of 16S rRNA sequencing. The resolving capability of MALDI-TOF MS is higher than that of 16S rRNA sequencing because it covers a wider range of proteins than the 16S ribosomal subunit. The ability to accurately characterize differences on the strain level is valuable in understanding differences among redundantly isolated strains identified as the same species, while isolated at different occurrences, such as those isolated from different contaminated soils. MALDI-TOF MS is useful for the identification and grouping of isolates based on the strain-level variations (Seuylemezian, et al., 2018). Here, it allowed differentiation and categorization of newly isolated strains from highly weathered oily soils. It also demonstrates the high diversity of the strains as a consequence of their adaptation to harsh conditions (chemical and physical).
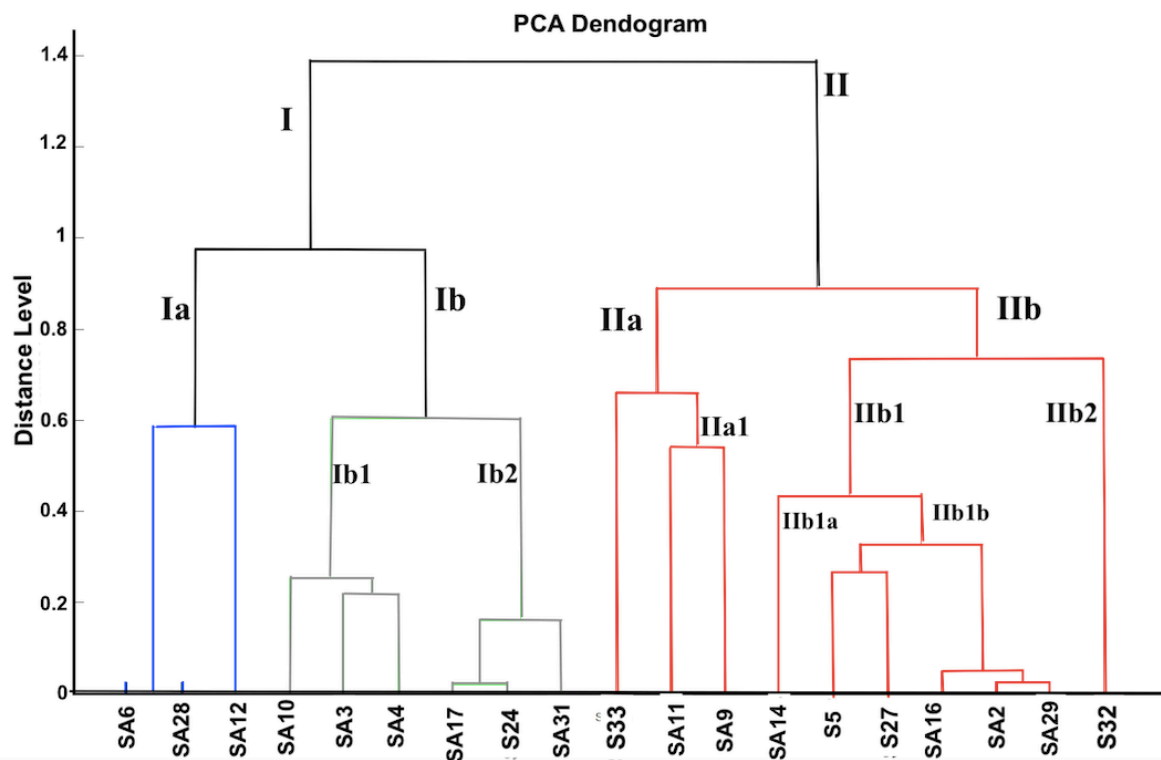
Figure 4: PCA dendrogram of the studied strains.

I-4. Diesel solubilization and emulsification activities of the isolated strains

All the isolated strains were cultured in the MSM medium consisting 5% (v/v) diesel as the sole carbon source. After one week and 2 weeks of incubation at 30 °C, the supernatants were collected and used to assess the diesel solubilization (SA) and emulsification (EA) activities of each strain (Table 4). These results show that SA and EA activities are fluctuating in a wide range of 3.2 U/ml to 42.1 U/ml for EA and 1.17 (%) to 8.6 (%) for SA. Moreover, some strains exhibited high EA activity and low SA or vice versa. They confirm the diversity of the strains. The growth of the strains was also varying from 11 to 99 10^8 CFU/mL, which was almost the same or strongly reduced after 2 weeks of incubation. The specific activity for EA and SA activity would be the characteristics of each strain. The strains *Bacillus subtilis* S5 exhibited the highest growth (99 10^8 CFU/mL) and SA (8.6%) and the second-highest EA (31.7

U/mL). The strain *Lysinibacillus fusiformis* SA4 exhibited the highest EA (42.1 U/mL) but low

SA activity (1.17%) and low growth (11 10^8 CFU/ml). However, the strain *Lysinibacillus*

boronitolerance SA10 produced 2.6 (%) as SA and 23.2 U/mL as EA with 33 10^8 CFU/mL. In

contrast, *Lysinibacillus boronitolerance* SA3 providing a similar growth (30 10^8 CFU/mL)

produced 6.7 U/mL of EA and 4.6 % as SA. All these results confirm the high diversity among

the collection of the isolated strains from weathered oily-soils. The diversity is not matching

with the diversity shown in the dendrogram.

Table 4: Growth, Diesel Solubilization, and Emulsification Capacity, and Specific Activities of the Isolated Strain.

Strains	1 week incubation					2 weeks incubation				
	CFU (10^8/mL)	EA (U/mL)	SA (%)	EA/CFU 10^{-3}	SA/CFU 10^{-3}	CFU (10^8/mL)	EA (U/mL)	SA (%)	EA/CFU 10^{-3}	SA/CFU 10^{-3}
S5	99 ± 5	32 ± 2	8.6 ± 0.4	341 ± 17	93 ± 5	37± 2	14.4 ± 0.7	4.2 ± 0.2	389 ± 19	114 ± 6
S24	75 ± 4	19 ± 1	2.4 ± 0.1	251 ± 13	32 ± 2	50 ± 3	6.7 ± 0.3	3.0 ± 0.2	134 ± 7	61 ± 3
S27	29 ± 1	28 ± 1	1.9 ± 0.1	966 ± 48	66 ± 3	30 ± 2	15.8 ± 0.8	1.4 ± 0.1	527 ± 26	48 ± 2
S32	21 ± 1	11 ± 6	1.97 ± 0.1	533 ± 27	94 ± 5	7 ± 1	2.9 ± 0.2	1.6 ± 0.1	414 ± 21	230 ± 12
S33	23 ± 1	17 ± 1	2.6 ± 0.1	757 ± 38	114 ± 6	27 ± 1	1.3 ± 0.1	2.4 ± 0.1	48 ± 2	90 ± 5
SA2	18 ± 1	1.4 ± 0.1	4.3 ± 0.2	78 ± 4	238 ± 12	15 ± 1	6.8 ± 0.3	5.7 ± 0.3	453 ± 23	378 ± 19
SA3	30 ± 2	6.7 ± 0.3	4.86 ± 0.2	223 ± 11	162 ± 8	37 ± 2	18.2 ± 0.9	2.6 ± 0.1	492 ± 25	71 ± 4
SA4	11 ± 1	42 ± 2	1.2 ± 0.1	3827 ± 191	106 ± 5	1.2 ± 0.1	2.3 ± 0.1	3.0 ± 0.2	2300 ± 115	3040 ± 152
SA6	75 ± 4	22 ± 1	4.5 ± 0.2	217 ± 11	127 ± 6	40 ± 2	28 ± 1	9.5 ± 0.5	693 ± 35	87 ± 4
SA9	17 ± 1	10 ± 1	5.8 ± 0.3	565 ± 28	225 ± 11	10 ± 1	12.4 ± 0.6	2.4 ± 0.1	1240 ± 62	242 ± 12
SA10	33 ± 2	23 ± 1	2.6 ± 0.1	703 ± 35	77 ± 4	27 ± 1	4.3 ± 0.2	1.3 ± 0.1	159 ± 8	48 ± 2
SA11	75 ± 4	9.5 ± 0.5	6.0 ± 0.3	87 ± 4	81 ± 4	33 ± 2	19 ± 1	2.2 ± 0.11	576 ± 29	68 ± 3
SA12	38 ± 2	26 ± 1	1.3 ± 0.1	687 ± 34	34 ± 2	42 ± 2	19 ± 1	3.4 ± 0.2	457 ± 23	81 ± 4
SA14	38 ± 2	3.2 ± 0.2	5.7 ± 0.3	84 ± 4	149 ± 7	36 ± 2	10.3 ± 0.5	2.5 ± 0.1	286 ± 14	68 ± 3
SA16	22 ± 1	34 ± 2	3.1 ± 0.2	1550 ± 78	142 ± 7	20 ± 1	5.8 ± 0.3	3.5 ± 0.2	290 ± 15	174 ± 9
SA17	33 ± 2	8 ± 0.4	5.1 ± 0.3	248 ± 12	153 ± 8	50 ± 3	27 ± 1	7.3 ± 0.4	548 ± 27	67 ± 3
SA28	26 ±1	21 ± 1	7.5 ± 0.4	823 ± 41	287 ± 14	20 ± 1	18 ± 1	3.2 ± 0.2	900 ± 45	162 ± 8
SA29	25 ± 1	17 ± 1	4.0 ± 0.2	668 ± 33	161 ± 8	60 ± 3	20. ± 1	2.2 ± 0.1	337 ± 17	36 ± 2
SA31	32 ±1.6	3.8 ± 0.2	4.8 ± 0.2	119 ± 6	149 ± 7	18 ± 1	23 ± 1	2.5 ± 0.1	1272 ± 64	137 ± 7

Bacteria employ biosurfactants as one of multiple adaptation mechanisms to use hydrocarbons as substrates. The adaptations largely express specific physiological responses to specific microenvironments of the cell and its nutritional requirements (Perfumo et al., 2010). Indeed, some bacteria developed a strategy of pseudo-solubilization to increase the solubility of poorly soluble hydrocarbons. Therefore, they produce a high capability of self-assembly in micelles, hemi-micelles or aggregates, by using highly dynamic low-molecular-mass molecules of biosurfactants. However, other bacteria develop a direct interaction with hydrocarbons by a different tool through the wall-bound biosurfactants. Thus, the cell surface becomes appropriately hydrophobic. Indeed, the high molecular mass molecules are called bioemulsifiers, which adsorb tightly to the hydrocarbons and thus increase their apparent solubility by covering them in the aqueous phase. Biosurfactants share few traits, although their wide variety of specialization and mechanisms to deal with hydrocarbons. All mechanisms are around the interactions between the three phases (cell physiology, cell surface, hydrocarbons that are the substrates for the cell). To achieve the goal of passing the hydrocarbons across the wall, the cell develops reversible and temporary modifications of the membrane adapted to the nature, composition, and type of hydrophobicity of the available substrates, in addition to making the hydrocarbons more soluble (Perfumo et al., 2010). It is not excluded that a "substrate effect" can be developed during the growth of the cell. However, the synthesis pathways of most of the biosurfactants are not yet elucidated. In contrast, the hydrophobic substrates are known to influence the structural variations of biosurfactants to make them particularly active on the same substrate. In addition, it is now established that biosurfactants stimulate the growth of their producing strains, playing a vital role in the interaction between the microbial communities and their micro-environment (Perfumo et al., 2010).

I-5. Production and stability of the biosurfactants activity during growth of the strains

The concomitant production of the EA and SA by four selected strains based on the results of Table 4 was investigated. The strain *Bacillus subtilis* S5 is characterized by high growth and SA and EA activities. The strain *Bacillus subtilis* SA6 exhibited lower growth and activities than *Bacillus subtilis* S5. The strain SA28 is *Bacillus subtilis* strain characterized by a much higher specific activity of SA and EA activities. The strain SA9 is *Bacillus sonorensis* selected because it is characterized by shining colonies, embedded in the extensive production of exopolymeric substances.

The SA and EA activities of the four strains were evaluated during dynamic growth in 5% diesel-MSM. Results are shown in Figure 5.
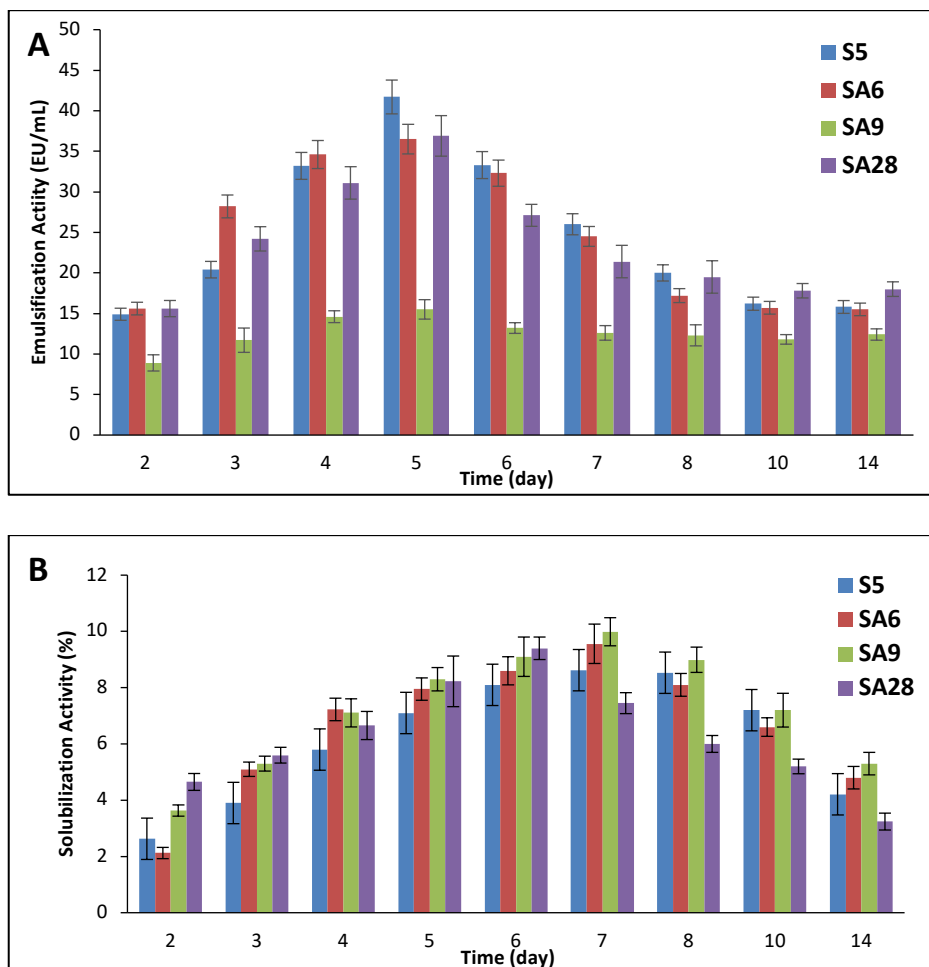

Figure 5: Concomitant production of **A**) EA and **B**) SA activities by the 4 selected strains.

The four strains exhibited, during their growth, a maximum of production of the emulsification activity after 5 days incubation, while their solubilization activities were maximal after 7 days incubation. The activities were remarkably unstable with a continuous and rapid decrease after reaching the maximum. The behavior of the 4 *Bacillus* strains was almost similar.

This result confirms that all the bacterial strains develop reversible and temporary modifications of the membrane adapted to the available substrates while making a more continuous activity of solubilization of the hydrocarbons. The concomitant production of both activities is necessary to achieve the goal of passing the hydrocarbons across the wall. The bio-emulsification touching the cell surface structure and functionality is normal in that it attains its highest activity during the vegetative growth of the cells, after which, the cells enter into sporulation phase with loss of and disintegration of the cell membranes, and thus of the related activities. The solubilization activity can continue to be produced by the sporulating cells or the remaining vegetative ones.

I-6. Analysis of the freeze-dried biosurfactants by Fourier transform infrared (FTIR)

The FTIR absorption spectra of the biosurfactants produced by the studied strains revealed the presence of protein, polysaccharide, ester, and carbonyl groups indicating the presence of lipopeptides (Fig.6 A-D). The strong absorption peak at 3270 cm^{-1} corresponds to the stretching vibrations of –NH and –OH groups related to peptides (Sharma, Singh, & Verma, 2018). The absorption peaks in the range from 2960 cm^{-1} and 2860 cm^{-1} are due to the asymmetric and symmetric stretching of the methylene groups of lipids (–CH_2) (Antoniou et al., 2015; Ricciardi et al., 2020). The absorption peak at 1640 cm^{-1} - 1630 cm^{-1} can be attributed to the CO-NH bend (due to the stretching vibrations of C=O and C-N groups), which confirms the presence of a peptide group in the biosurfactants (Habib et al., 2020). The absorption peaks at 1451 cm^{-1} and 1360 cm^{-1} appear due to the presence of alkyl (-CH_2 and –

CH$_3$) groups (Al-Dhabi, Esmail, & Arasu, 2020). The presence of ether moiety is confirmed due to the presence of an absorption peak at 1230 cm^{-1}. Hence, the biosurfactants were expected to contain fatty acids and peptide moieties indicating their lipopeptides nature (Sharma, Singh, & Verma, 2018).

I-7. PCA analysis of the FTIR spectra

To have more insights into the variations in the obtained FTIR spectra for the biosurfactants, PCA analysis was performed (Figure 7). The FTIR-PCA clustering revealed three main groups. Group 1, the largest one, contained the biosurfactants obtained from nine bacterial strains; three *B. subtilis* (S5, S27and SA16), three *B. cereus* (SA17, S24 & S33), one *B. sonorensis* (SA9)*,* one *B. atrophaeus* (SA14), and one *L. boronitolerans* (SA3). Group 2 contained biosurfactants of one *B. cereus* (SA31), one *B. sonorensis* (SA11), one *B. mojavensis* (SA29), and one *L. boronitolerans* (SA10), Group 3 contained biosurfactants of two *B. subtilis* (SA6 & SA28) and one *L. fusiformis* (SA4). Each of the biosurfactants of *Enterococcus faecium* (SA12), *B. atrophaeus* (SA2) and *B. licheniformis* (S33) is located in separate large distances from any of the groups.
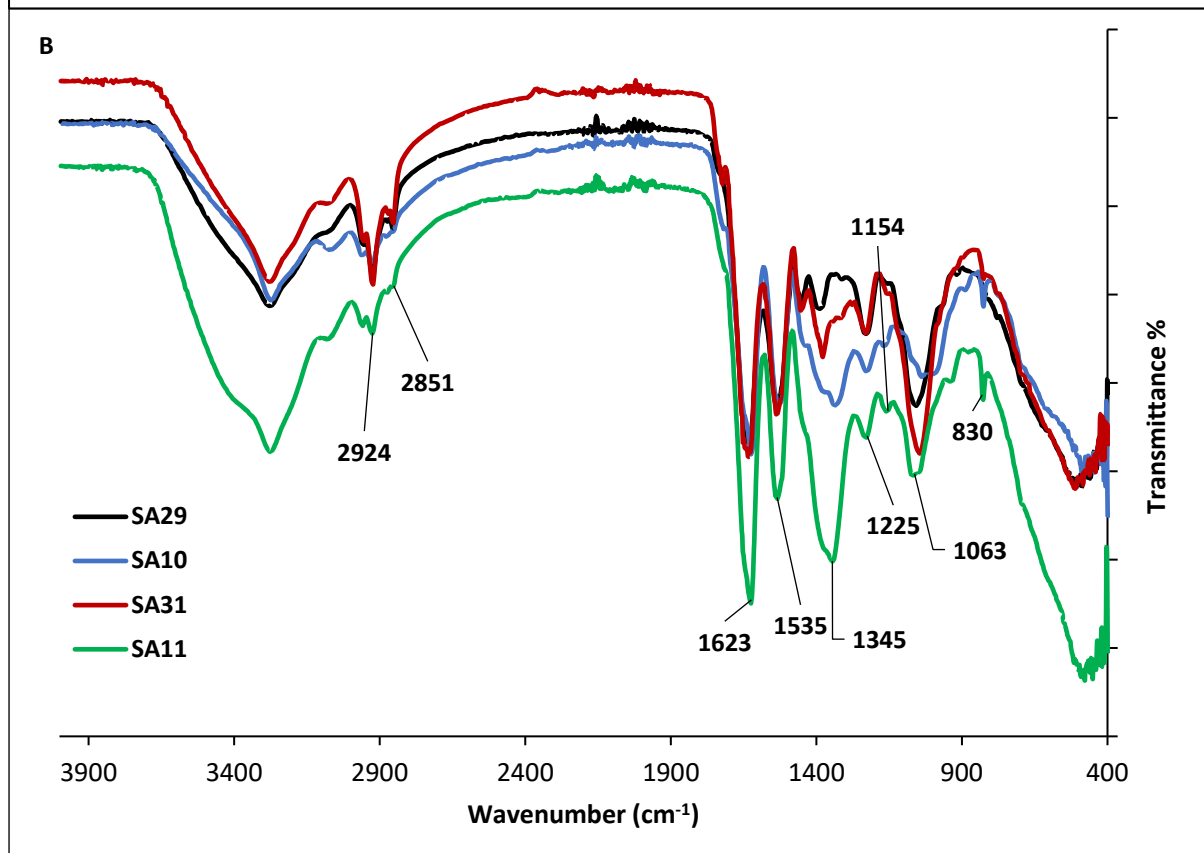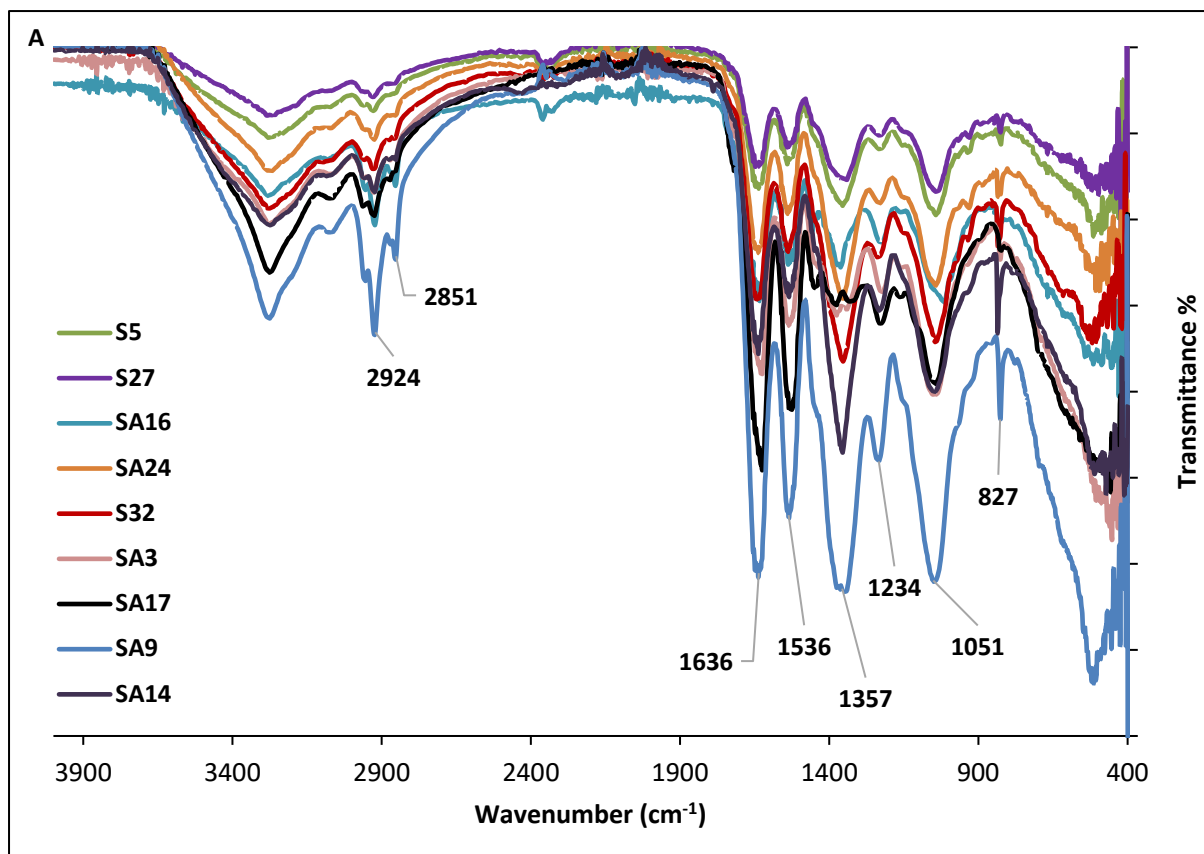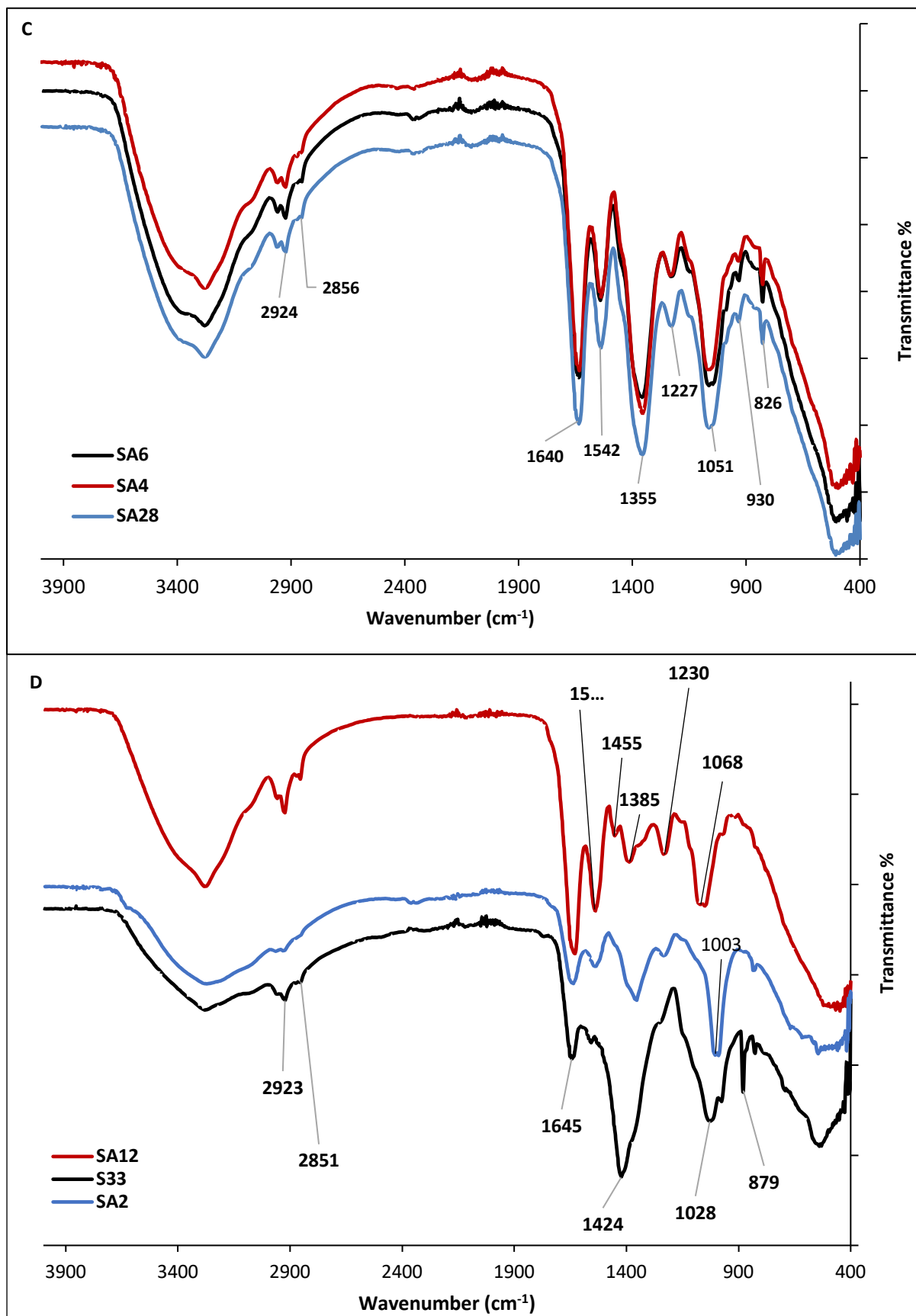
Figure 6: : FTIR spectra of biosurfactants of isolated strains, A) Group 1, B) Group 2, C) Group 3
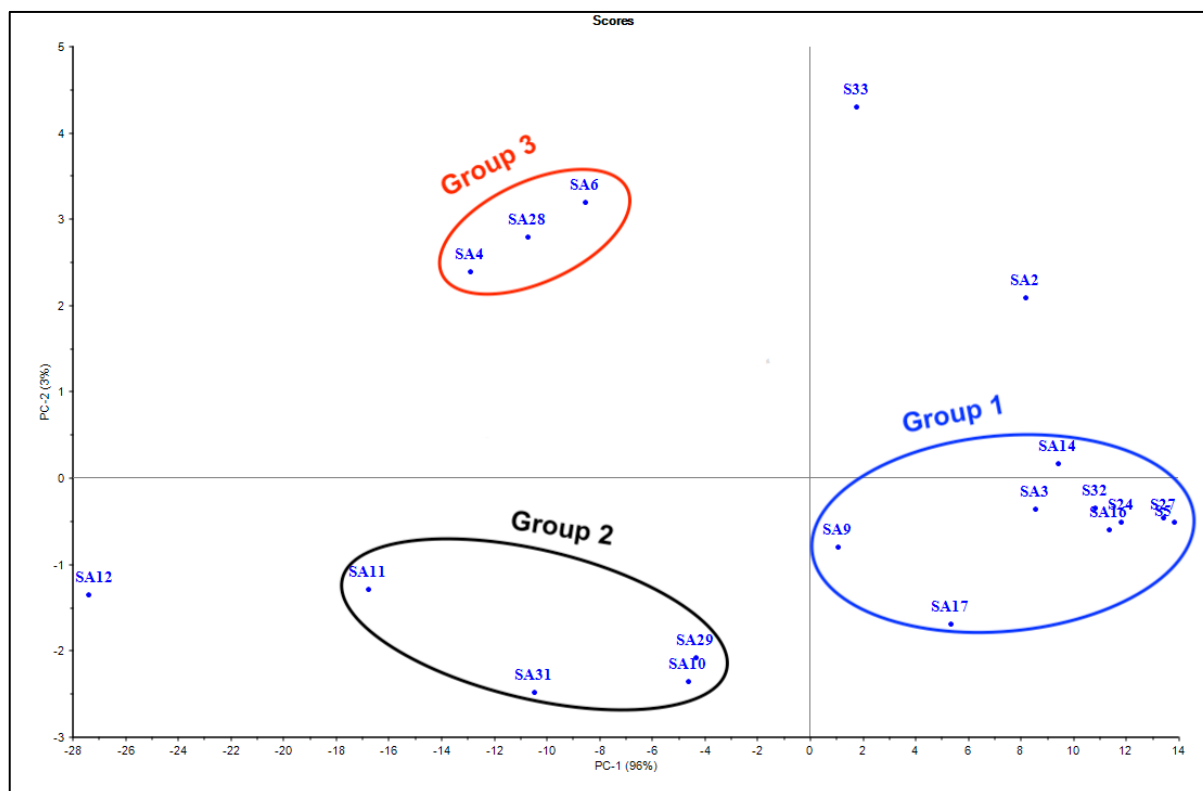
Figure 7: PCA classification for the FTIR spectra of the biosurfactants obtained from the studied strains.

The FTIR bands at 2924 cm^{-1}, 2850 cm^{-1} were preserved in all FTIR spectra of the studied, biosurfactants. However, variations were observed in FTIR bands in the region from 800 cm^{-1} to 1640 cm^{-1}. Obvious shifts in the amide I peaks from 1645 cm^{-1} to 1623 cm^{-1} were observed in the FTIR spectra of the biosurfactants assembled in PCA-Group 2 (Fig 6B & Fig 7). These shifts are attributed to changes in protein secondary structure (Kong & Shaoning, 2007; Chaber et al., 2021). Moreover, the peaks at 1154 cm^{-1} that may be ascribed presence of ester bonds (Hamza et al., 2017) are characteristics of the biosurfactants categorized in PCA-Group 2 (Fig.6B & Fig.7). The weak bands at 930 cm^{-1} which corresponds to phosphorus and oxygen stretching in aliphatic and aromatic molecules (Behzadnia et al., 2020) were clearly observed in the FTIR spectra of biosurfactants clustered in PCA-Group 3 containing biosurfactant produced by *B. subtilis* (SA6 & SA28) and *L. fusiformis* (SA4) (Figure 6C),

which may indicate the presence of phosphate in these biosurfactants. The FTIR peak at 1028 cm^{-1} attributed asymmetric and symmetric C–O–C stretching of ester (Morais et al., 2017; Camargo et al., 2018) was only observed in the FTIR spectra of the biosurfactants of B. lichenifomis S33. The FTIR peak 1003 cm^{-1} observed -only- in the FTIR spectra of *B. atrophaeus* SA2 (Fig. 6D) may be assigned to O–C–O extend vibrations of carboxylic acids. This is a remarkable indication of the oxidation of the hydroxyl groups in the hydrolysates from the medium peptides (Elazzazy et al., 2015).

Indeed, even with the same group of isolates clustered based on their protein profiles, the corresponding biosurfactants are clustered in different groups based on their FTIR spectra. This means that each isolate was able to adapt differently its biosurfactant composition in response to the existing weathered oil components and the weather conditions. However, the three strains, *Enterococcus faecium* (SA12), *Bacillus atrophaeus* (SA2) and *B. licheniformis* (S33) which were not within any of the isolates' clusters produce biosurfactants with structure that are strongly different from those of all the clustered ones, although several similarities were observed. The strain S32, which is not clustered with the other isolates produces biosurfactants highly similar to those of FTIR-cluster **I**, grouping 9 out of the 19 surfactants.

I-8. Conclusion

The ability of the indigenous Qatari strains to produce biosurfactants with great potential to enhance the biodegradation of weathered hydrocarbons was investigated. The obtained findings showed that two types of adaptations occur with hydrocarbons degrading bacteria in the weathered-oily soils, one related to the bacterial cell composition maintaining the biosurfactants composition and one to the biosurfactants which is the primary tool employed by the bacterial cell to interact with the weathered oil. Indeed, the phyloproteomic classification of the studied strains allowed the differentiation and separation of strains belonging to the same *Bacillus* species. High diversity of emulsification and solubilization

activities were recorded among biosurfactants produced by the studied strains. Moreover, combining FTIR data with PCA analysis resulted in further classification of the biosurfactants produced by the studied bacterial isolates.

Chapter II: Evaluation of the limitations and regulation of biosurfactants production by selected isolated strains

Biosurfactants are produced by microorganisms like *Bacillus* and *Pseudomonas* genera among others. This is achieved through the process of fermentation and the enzyme-substrate reaction. Synthesis of biosurfactants is not only achieved intracellularly but can also be carried out extracellularly by the use of biocatalysts, which are enzymes. The synthesis of these molecules involves the carbohydrate metabolic pathway, which synthesizes the hydrophilic moiety, and the hydrocarbon metabolic pathway, which synthesizes the hydrophobic moiety. In most cases, the first enzymes used in the process of precursor synthesis are regulatory enzymes. For these reasons, it can be expected that growth of bacteria will require specific surfactants, which attract specific hydrocarbons, which exhibit the lowest level of toxicity. Second, since carbohydrate metabolic pathways are involved in the biosurfactants synthesis, it can also be expected that several metabolic regulations could be responsible of limiting production of such metabolites. In addition, various factors influence biosurfactants synthesis and affect the rate of production and their properties. Some of the factors that influence the optimum production of biosurfactants include the carbon and nitrogen sources, temperature, pH, oxygen availability, carbon-nitrogen ratio, and agitation. Consequently, all the reported parameters affect production, composition, and activity.

The preferred method for remediation of hydrocarbon contamination in the subsurface is bioremediation. In the previous chapters, we showed the high biodiversity of the isolated

bacterial strains from contaminated soils with weathered hydrocarbons in Qatar as well as diversity of their biosurfactants, in term of activity and structure. These results showed that bioremediation would be possible in the harsh Qatari environment. A key issue in the application of engineered bioremediation is the reduced bioavailability of hydrocarbons due to their low aqueous solubility. This may be achieved by adding biosurfactants to the process, for stimulation. The application of biosurfactants to bioremediation, whether intrinsic or engineered, is then dependent on the mass-production of biosurfactants by suitable bacterial strains. However, there is no available data on biosurfactant-producing bacteria indigenous to Qatar or how the indigenous hydrocarbon-degrading bacteria may be able to utilize biosurfactants if they are made available.

II-1. Investigation of the production of biosurfactants by selected strains

In order to develop and apply biosurfactant-enhanced bioremediation (BSEB) in Qatar, appropriate bacteria need to be selected based on their potential of producing biosurfactants. Those bacterial strains should be indigenous to Qatar and able to overcome metabolic regulations limiting growth and synthesis of biosurfactants at wide range of hydrocarbons and concentrations: Here, 7 bacterial strains were selected based on the results of the previous chapter:

- o S5: *Bacillus.subtilis*
- o SA6: *Bacillus subtilis*
- o SA16: *Bacillus subtilis*
- o SA27: *Bacillus subtilis*
- o SA28: *Bacillus subtilis*
- o SA29: *Bacillus mojavensis*
- o SA32: *Bacillus cereus*

Five of these selected strains are *B. subtilis*. This species is known in literature by biosurfactants applicable in many industries in addition to bioremediation. One strain is *B. cereus*. This strain showed high biosurfactant activity with potential possibility of overproduction. The strain of *B. mojavensis* showed special characteristics of biosurfactants, which may be a result of high adaptation to the harsh conditions. It is noticed that all the isolated strains are belonging to the *Bacillus* genus.

In parallel, three strains of *Pseudomonas aeruginosa* were selected from our collection of hydrocarbon-degrading bacteria (available in our laboratory). The objective is to compare Gram-negative bacterium (*Pseudomona*) to the Gram-positive bacteria (*Bacillus*) because the newly isolated strains were shown to produce surfactin-like biosurfactants highly similar to those of *Pseudomonas*.

An investigation of the production of biosurfactants was observed by using different substrates (hydrophobics): Here, different oils were used to investigate the role of the hydrocarbons and their origin on growth and production of biosurfactants.

These knowledge gaps hamper the ability to (i) select and produce biosurfactants that can be applied in the Qatari environment and to (ii) predict their effects on hydrocarbon bioavailability in specific conditions related to the structure and toxicity of the hydrocarbons. Obtaining answers to these questions is necessary for the development of biosurfactant protocols and methods for the remediation of hydrocarbon contamination.

II-2. Investigation of the effect of the source of hydrocarbons on growth and biosurfactants production

In this study, 3 strains of *Bacillus* were preferred to be used and the 3 other strains of *Pseudomonas* which were previously isolated from harsh and weathered soils, in our laboratory. The objective was to compare the Gram-positive bacteria (*Bacillus*) to the Gram-negative bacteria (*Pseudomonas*). Moreover, it is known that the bacterium *Pseudomonas aeruginosa* produces Surfactins as biosurfactants. Since most of our strains also produce

surfactin-like molecules, the effect of hydrocarbons structure on their production would be different among Gram positive and Gram-negative bacteria. In addition, different sources of hydrocarbons were used: Sunflower, Castor, Almond, corn and five-times used Corn oil. These oils were compared to diesel at 5% (v/v) in MSM. Results are shown in Fig.8 and 9. It is clear that growth of all the strains was much higher with the vegetative oils than with diesel, with few exceptions of similar growth. It is also noted that the solubilization activities of the *Bacillus* strains are always below 10% SA, except with corn oil. However, the biosurfactants activities of *Pseudomonas* strains are much higher. In general, all the biosurfactants exhibit mainly the emulsification activities, which in turn depends a lot on the origin of the hydrocarbons. One appropriate oil, which is favorable to produce high emulsification activity with one strain, may not be favorable for another strain.
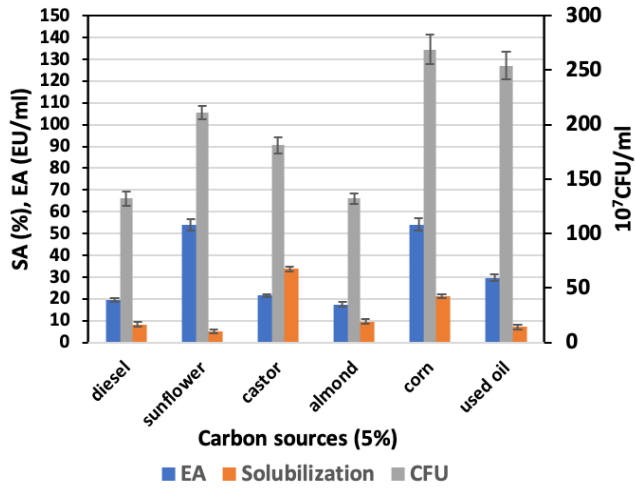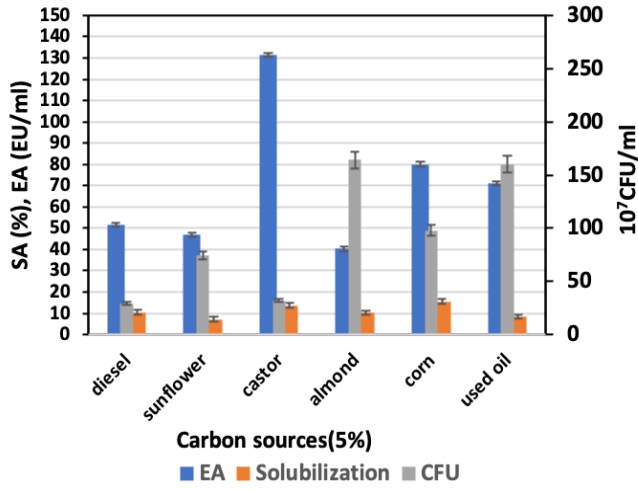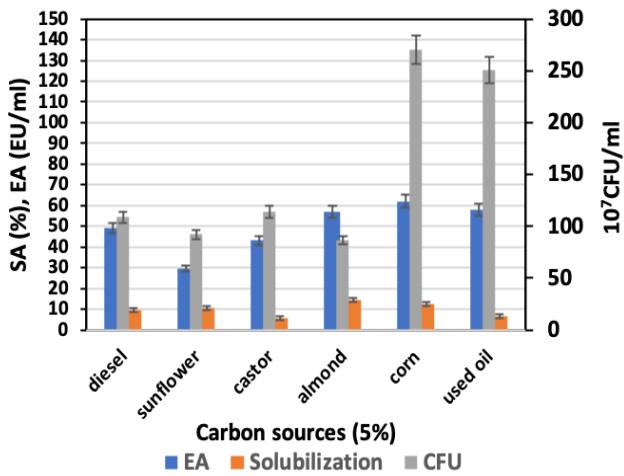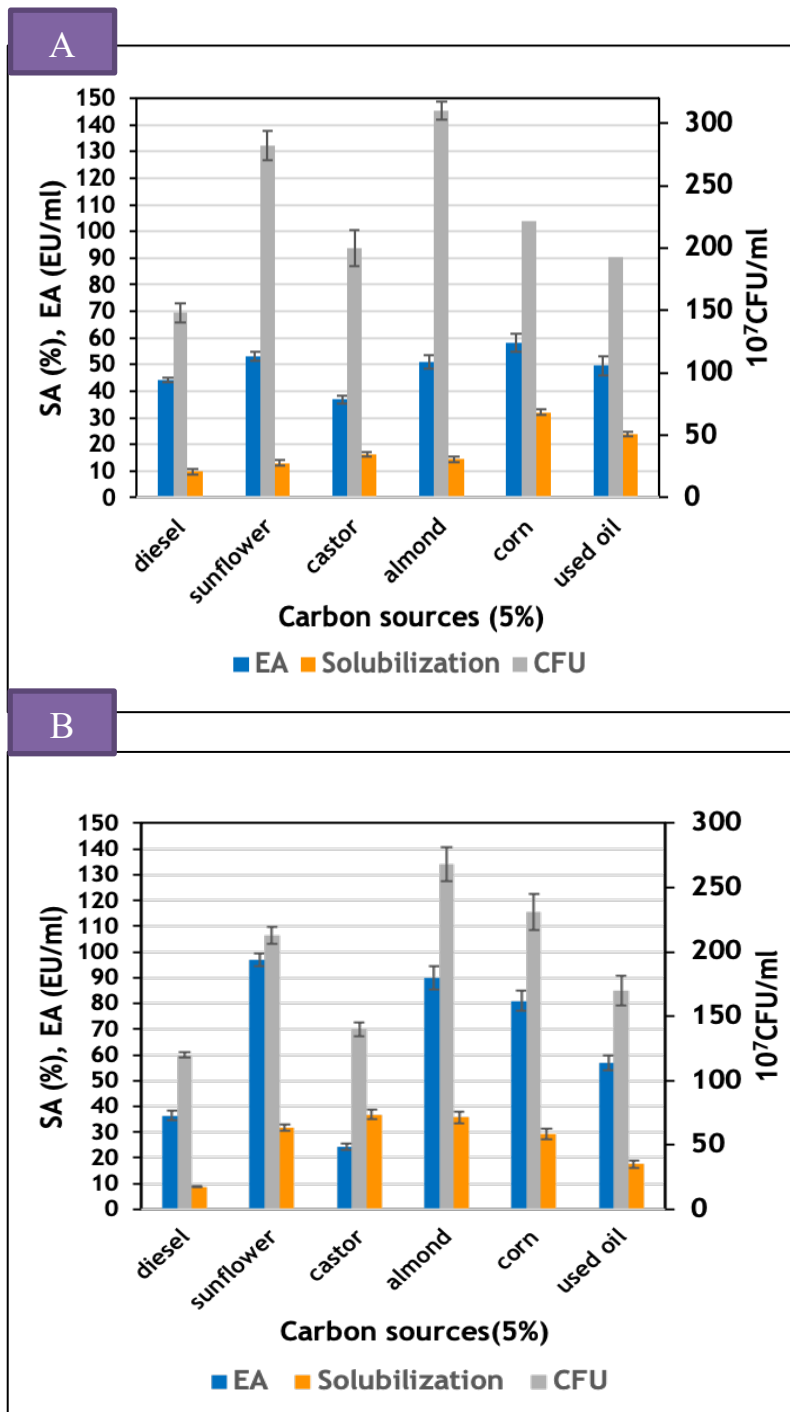
Figure 8: Growth and production of biosurfactants activities by selected *Bacillus* strains: (A): *B.subtilis* S5; (B): *B.subtilis* SA16 and (C): *B.mojavensis*.
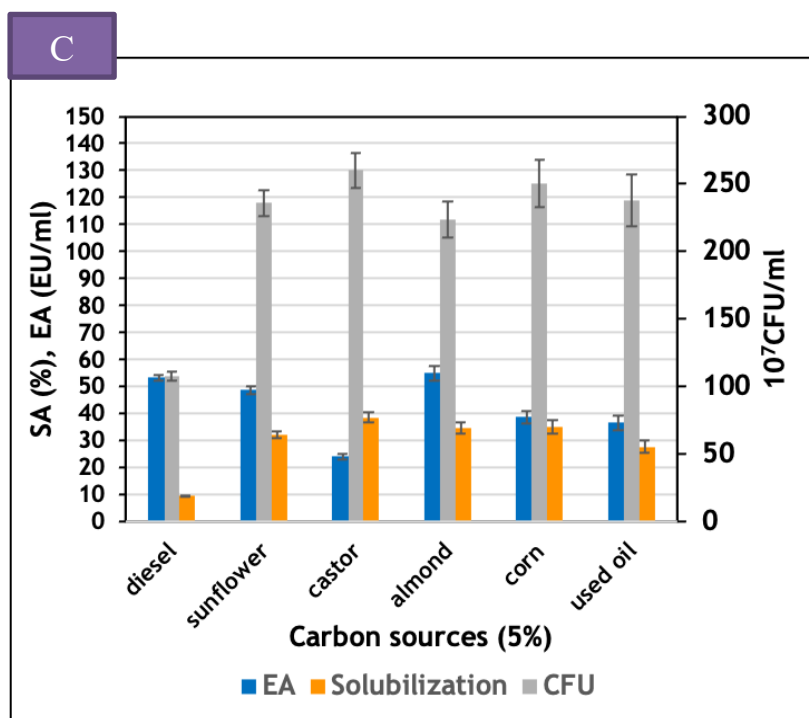
Figure 9: Growth and production of biosurfactants activities by selected *Pseudomonas aeruginosa* strains: (A): SH1; (B): D5D1 and (C): ZA9.

These findings demonstrate that the biosurfactants of all the 6 strains (3 *Bacillus* and 3 *Pseudomonas aeruginosa*) are emulsifiers, with low solubilization activities. This also confirms the results of the previous chapter showing that all of them seem to be surfactin-like molecules. The production of these surfactants depends a lot on the producing strain and the origin of the hydrocarbons. Moreover, these conclusions are applicable to both *Bacillus* and *Pseudomonas* genera.

II-3. Investigation of the effect of hydrocarbons concentrations on growth and biosurfactant production

The source of carbon is one of the most influential parameters in the production of biological surfactants. Thus, the success of the use and production of biosurfactants necessarily requires a reduction in production costs. This reduction could be achieved through the enhancement of growth using low-cost products (Lawniczak, L., 2020). Substrate can represent

up to 50% of total production costs, hence the importance of choosing inexpensive alternative carbon sources (De Almeida et al., 2016). A number of renewable and cheap wastes have been explored as substrates for the production of biosurfactants, which made it possible to develop an effective cost reduction strategy, associated with waste management by reducing the quantities of waste to be treated generated by various companies (Makkar et al., 2011; Geetha et al., 2018). Many inexpensive materials are used as substrates for the production of biosurfactants. Oil selection should ensure a good balance of nutrients to allow microbial growth and production of biosurfactants. It is also well known that high carbohydrate concentrations can cause inhibition of growth due to toxicity or strong metabolic regulations, which can limit growth or biosurfactants production (Singh et al., 2018). The ideal carbon sources and their concentrations should be investigated.

Indeed, seven *Bacillus* strains were selected and the production was investigated by using diesel, corn oil and five-times used corn oil. These are the sources of hydrocarbons which were selected for the potential production of biosurfactants by the selected strains, and to study the limitations of production.

II-4. Investigation of the effect of diesel hydrocarbons concentrations on selected strains

In the previous results, diesel was used at 5% (v/v) in MSM. Here different concentrations corresponding to increasing volumetric proportions in the MSM medium ranging from 1% to 11 % (v/v) were used. The results shown in (Figure 10) indicate that all the seven strains of *Bacillus* had some diesel hydrocarbon-degrading ability. This ability is shown by the fact that all the bacterial strains recorded Emulsification activity (EA), Solubilization activity (SA), and Growth in terms of colony-forming units (CFU). Nonetheless, each strain had its rates, as shown by the different growth rates and activities production rates. This is not novel since it is known that many *Bacillus* strains were reported to produce natural

surfactants that promote desorption and solubilization of organic compounds by increasing their bioavailability hence their ability to degrade diesel hydrocarbons (Nanganuru et al., 2012).

B

B.subtilis (SA6)

EA — Solubilization — CFU

C

B.subtilis (SA16)

EA — Solubilization — CFU

55
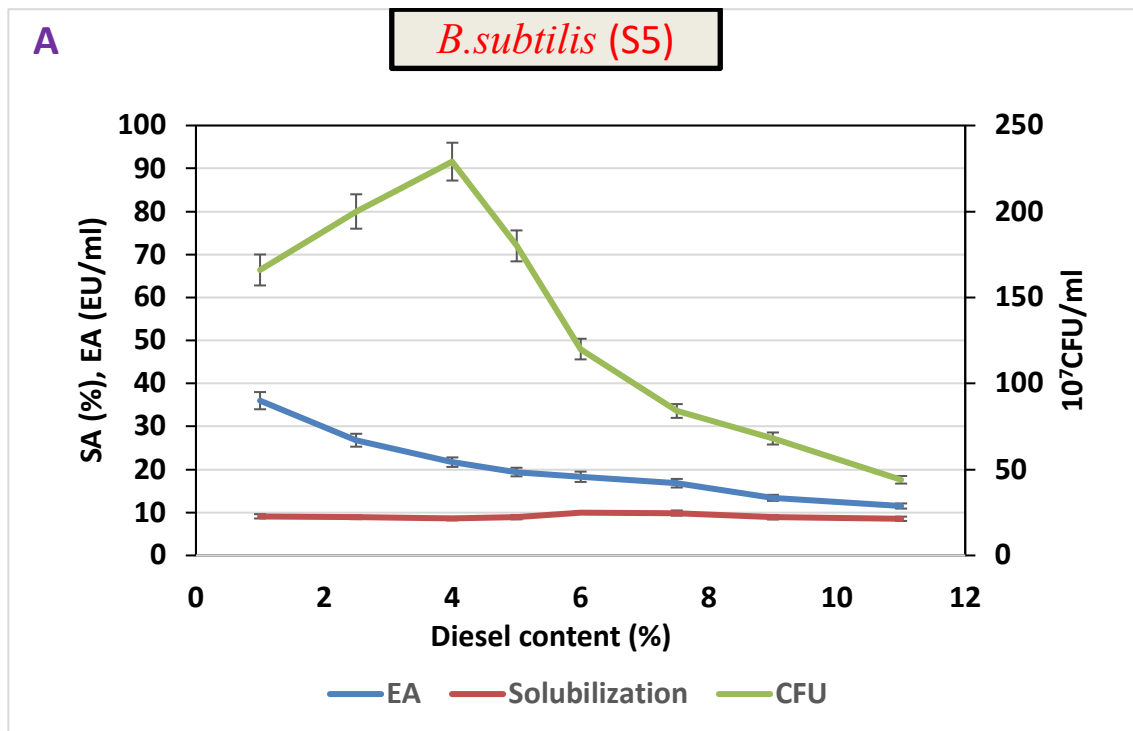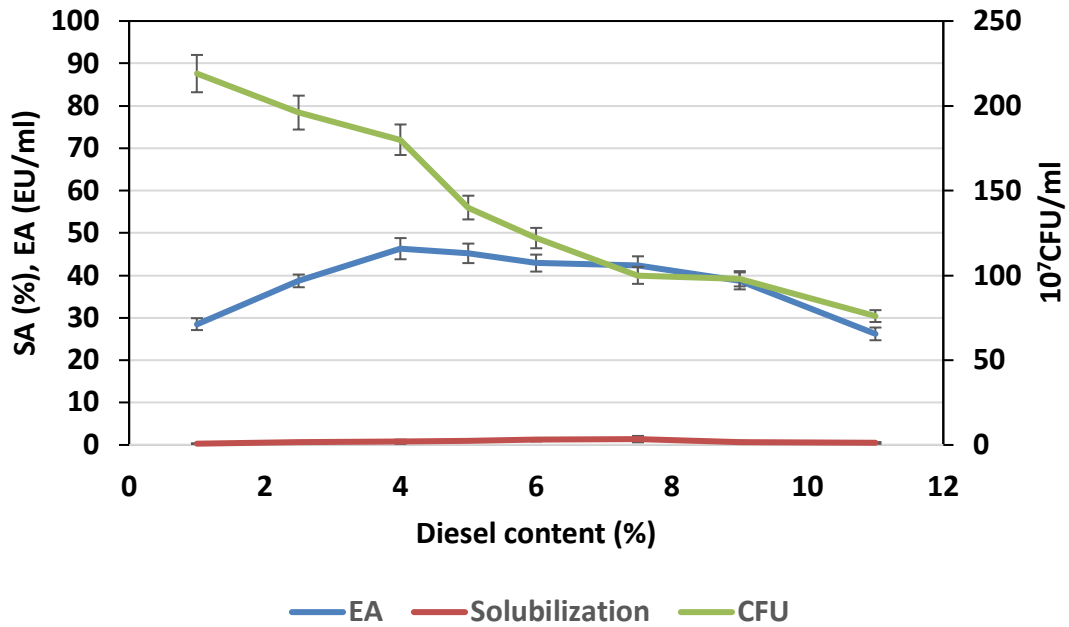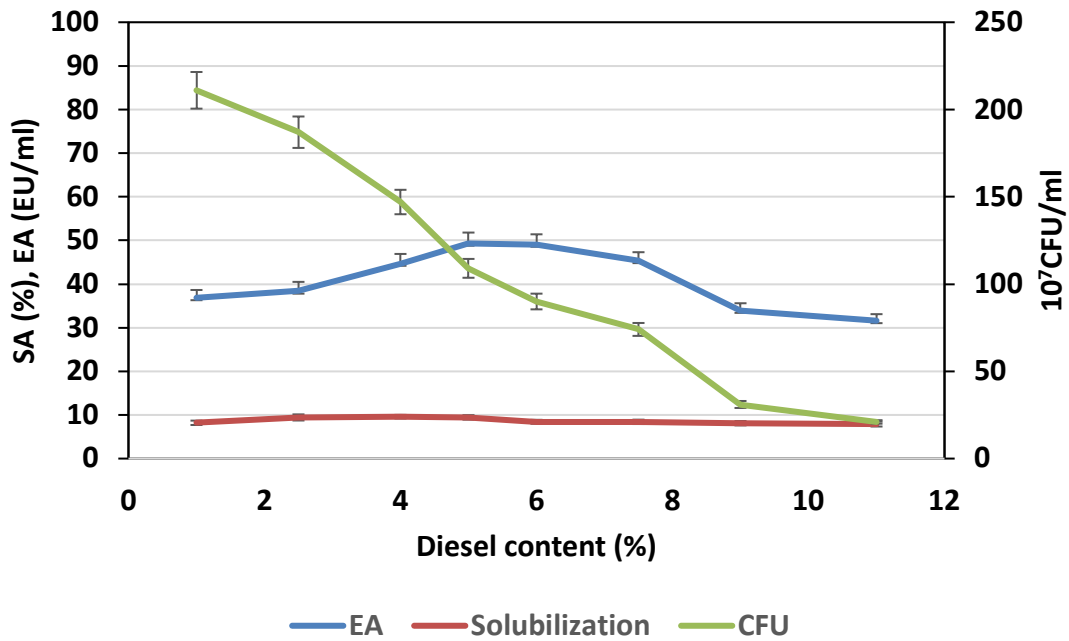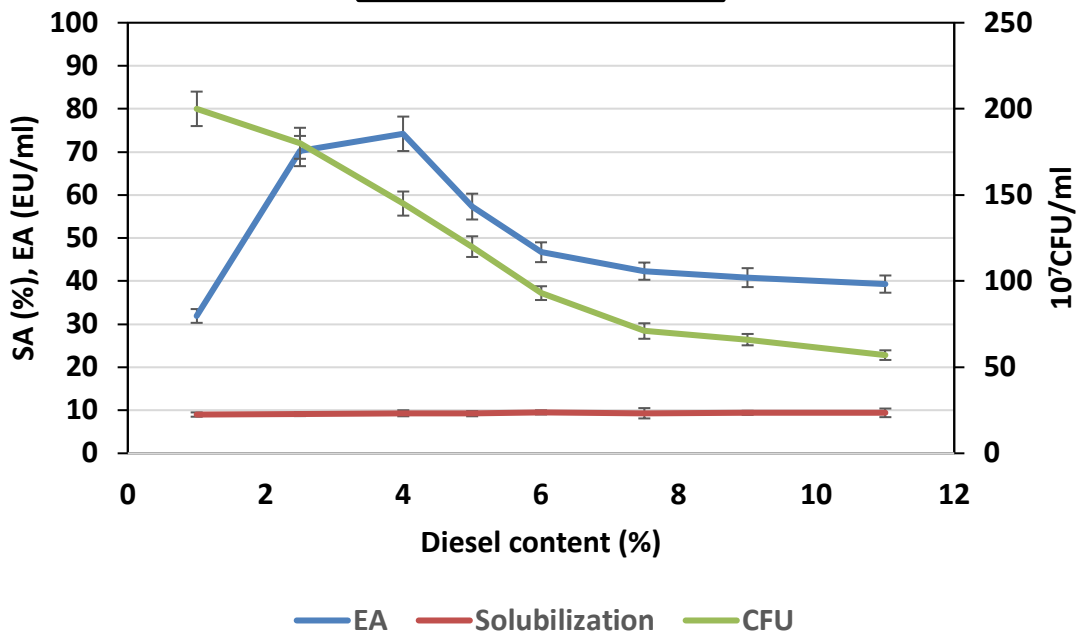
D **B.subtilis (S27)**
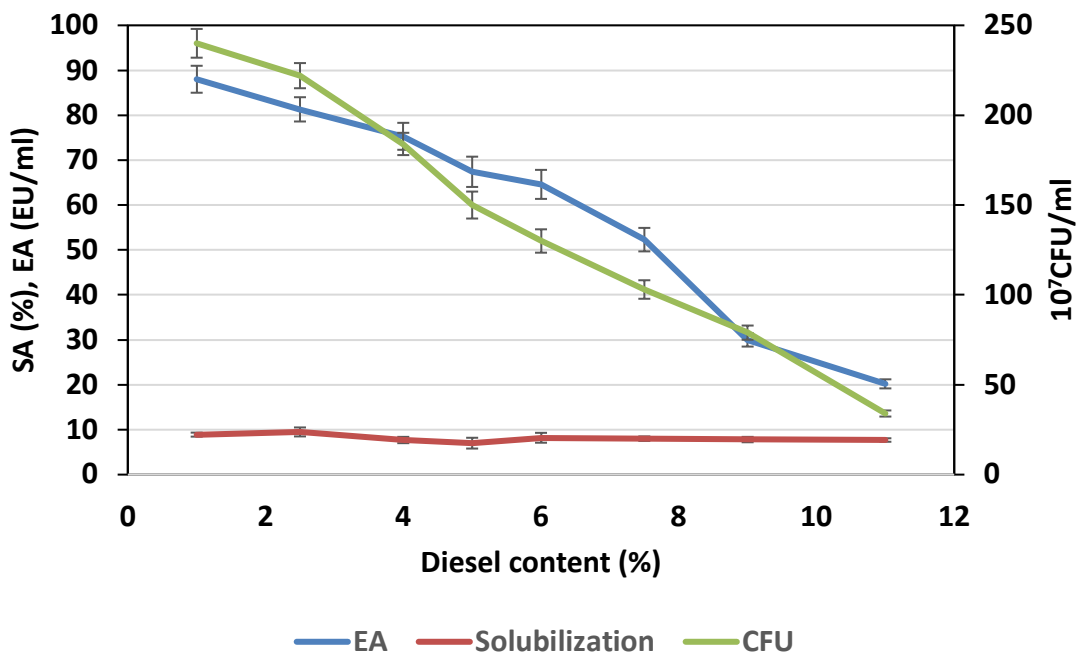
E **B.subtilis (SA28)**

Figure 10: Effect of diesel concentration on growth and emulsification and solubilization potentiality by the selected strain: A: S5 (*B. subtilis*), B: SA6 (*B. subtilis*), C: SA16 (*B. subtilis*), D: S27 (*B. subtilis*), E: SA28 (*B. subtilis*), F: SA29 (*B. mojavensis*), G:S32 (*B.cereus*)

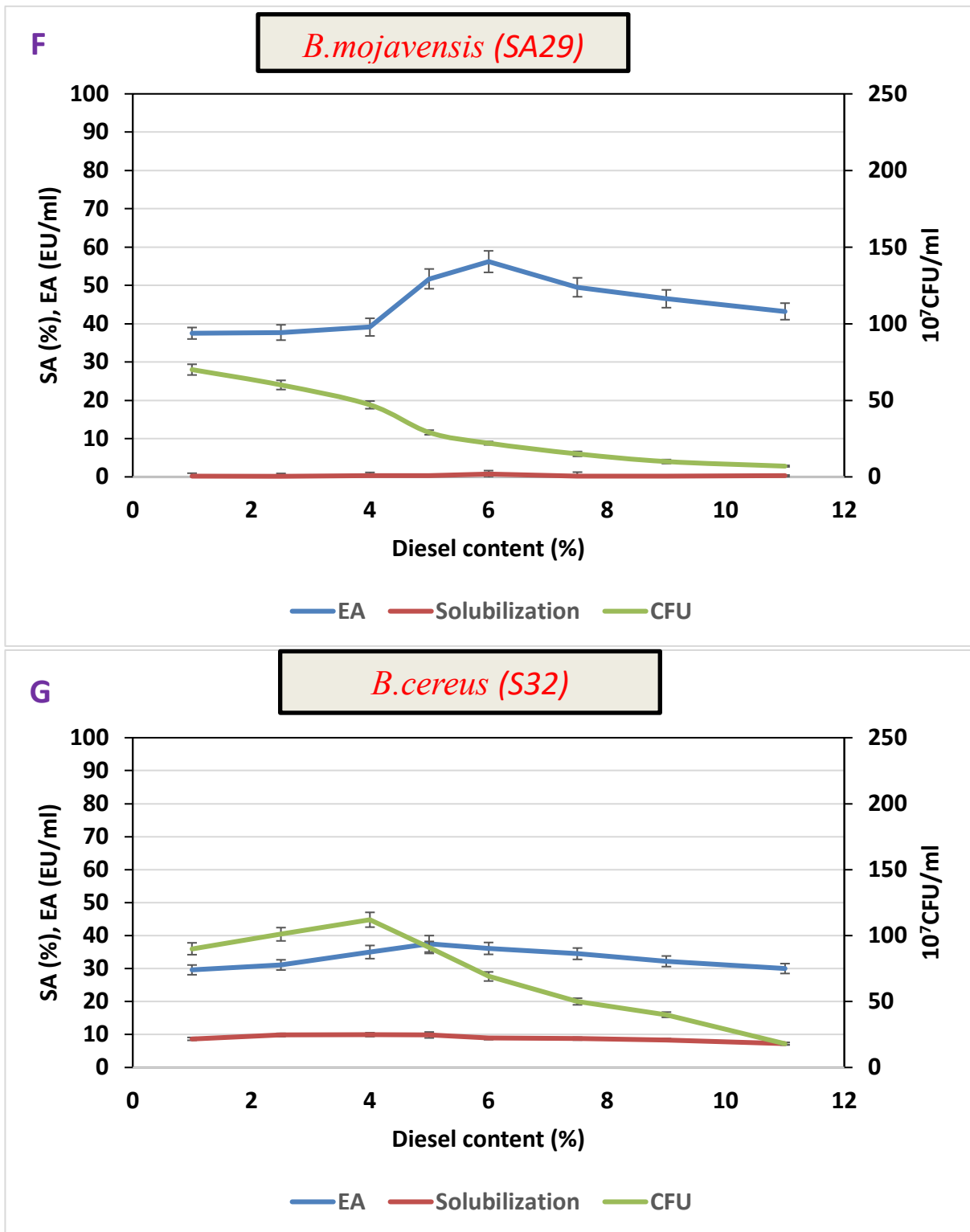With all the used strains, there were strong differences registered in the biosurfactants activity (EA and SA) and in growth. The profiles evolution of these three parameters at different diesel concentrations were enough different to conclude about the high diversity among the selected strains, as previously demonstrated at the level of their protein's profiles and biosurfactants activities and structures.

In addition, the strain *B. subtilis* S5, originated from the sea line, showed high emulsification activity of 36 EA at 1% diesel. Increased diesel content caused continuous decrease of EA, leading to the lowest activity of 11.5 EA obtained with 11% diesel. However, there were no significant differences in the solubilization activity, which was maintained almost constant at around 8%-10% at each hydrocarbon's concentration. In contrast, growth of *B. subtilis* S5 was at its high level with 4% diesel, although the CFU was continuously dropping with higher diesel contents up to 11% diesel. The results obtained with *B. subtilis* S5 clearly show that its growth was inhibited at high diesel concentrations above that corresponding to 4% diesel. While this growth sensitivity to potential toxicity caused by increased hydrocarbons concentrations, the continuous decrease of the emulsification should not be explained by such toxicity.

Bacteria have a mechanism known as carbon catabolic repression (CCR) that enables the selective uptake and metabolism of carbon sources that allow the most rapid growth (Meyer et al., 2011). From the results, the bacterial strains rapidly attain maximum number of viable units and then the number of colonies continues to gradually decrease as the less desirable sources of carbon are availed. However, synthesis of metabolites is continuously decreasing with the increase of the readily assimilable substrates, through CCR. Therefore, one of the potential causes of such effect would be the carbon catabolite repression exerted on synthesis of the emulsifiers. This limiting regulation is expressed as continuous decrease of the

production of metabolites or even of growth by increasing the substrate concentration in the medium (Zouari et al., 1988).
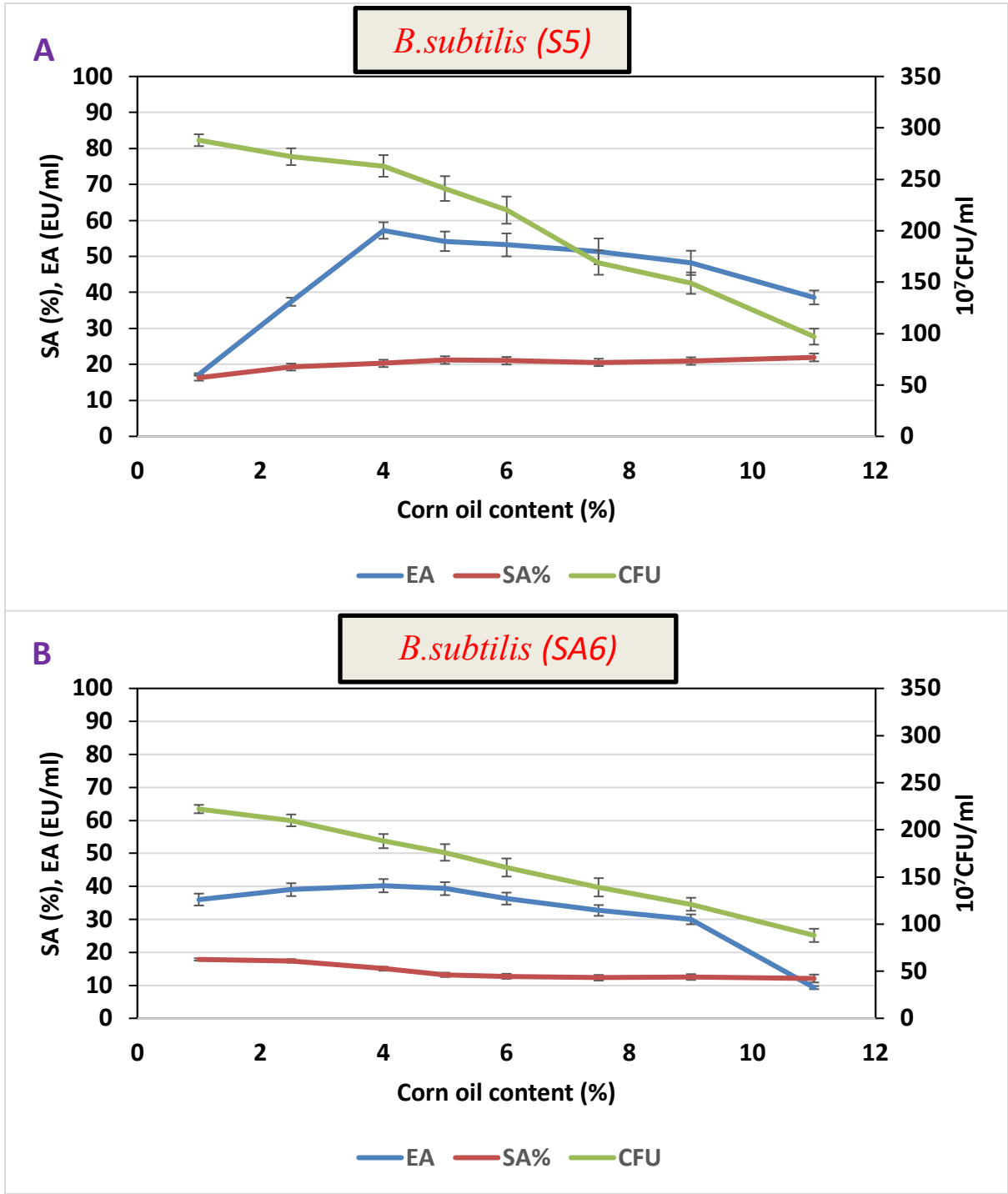
Interestingly, it is clearly shown that the three strains of *B. subtilis* (SA6, SA16 and S27) exhibited the opposite profiles of EA and growth, compared to the strain S5. Indeed, these strains showed a continuous decrease of the biomass production with the increase of the carbon source (hydrocarbons) concentrations. This is in favor of a metabolic regulation expressed as carbon catabolite repression, as same phenomenon occurring with the synthesis of emulsifiers by *B. subtilis* strains S5. On the other hand, the strain SA28 of *B. subtilis* isolated from an oil dumpsite produced an EA activity, which continuously decreases with increasing the diesel content. At 1% diesel content, EA is of 88 EA, while at 11% diesel it is of 20.2 EA. Similarly, increasing the diesel concentrations reduced the growth of the bacterial cells as biomass production. This strain SA28 shows limitations of growth and emulsification activity production due to toxicity and/or carbon catabolite repression, while the other strains of *B. subtilis* are affected either in their growth or emulsifiers production.

It seems that *B. subtilis* strains develop several growth and metabolic regulations as a response to toxicity or substrate excess. These adaptations of *B. subtilis* were shown with strains isolated from different locations. Indeed, while *B. subtilis* strains S5 was from the sea line, SA6 and SA28 were isolated from oil dumpsites, SA16 from an intertidal zone, and S27 from automotive workshop respectively. However, *B. mojavensis* and *B. cereus* also showed an ability to degrade diesel hydrocarbons but recorded a lower CFU compared to the *Bacillus subtilis* species. By studying growth and emulsification activities production by other species of *Bacillus*, it is clearly shown, that the strain *B. mojavensis* SA29 exhibited limitations of growth (toxicity or carbon catabolite repression) due to increasing the hydrocarbons concentrations, while production of emulsifiers is affected only at high concentrations exceeding 6% diesel.

Notably, the strain *B. cereus* S32 that was isolated from an oil dumpsite, performed weakly, compared to the others. The emulsification activities did not exceed 40 EA, it dropped after 5% diesel. It showed an increase of growth up to 4% diesel, then reduction, which can be attributed to toxicity. This strain seems to be not highly sensitive to carbon catabolite repression. However, the solubilization activity is nearly stable with all the strains, which proves the ability of *Bacillus* strains to emulsify oils rather than solubilize them. Therefore, *B. subtilis* species proved to be powerful degraders of diesel hydrocarbons compared to other species. This result is consistent with Parthipan et al., (2017) who established with a strain of *B. subtilis* that producing biosurfactant is linked to the efficient degradation of crude oil.

II-5. Investigation of the effect of corn oil hydrocarbons concentrations on selected strains

By using corn oil as source of hydrocarbons for production of biosurfactants, the results of Fig.11 were obtained. They clearly showed that the selected strains under the effect of increased concentrations of corn oil hydrocarbons exhibit similar behavior as that observed with diesel. All the seven strains of *Bacillus* showed Emulsification activity, Solubilization activity, and growth in terms of CFU. However, each bacterial species had its rate of using and degrading corn oil hydrocarbons, as shown by the different growth profiles.

A B.subtilis (S5)

B B.subtilis (SA6)

61

C *B.subtilis (SA16)*

D *B.subtilis (S27)*

E B.subtilis (SA28)
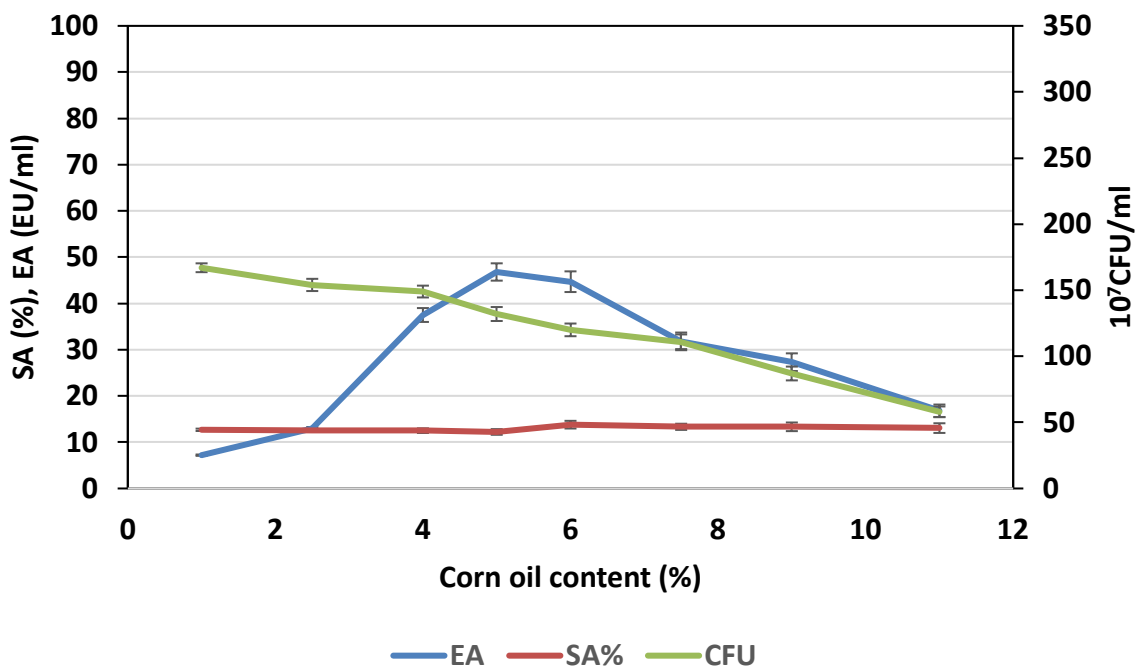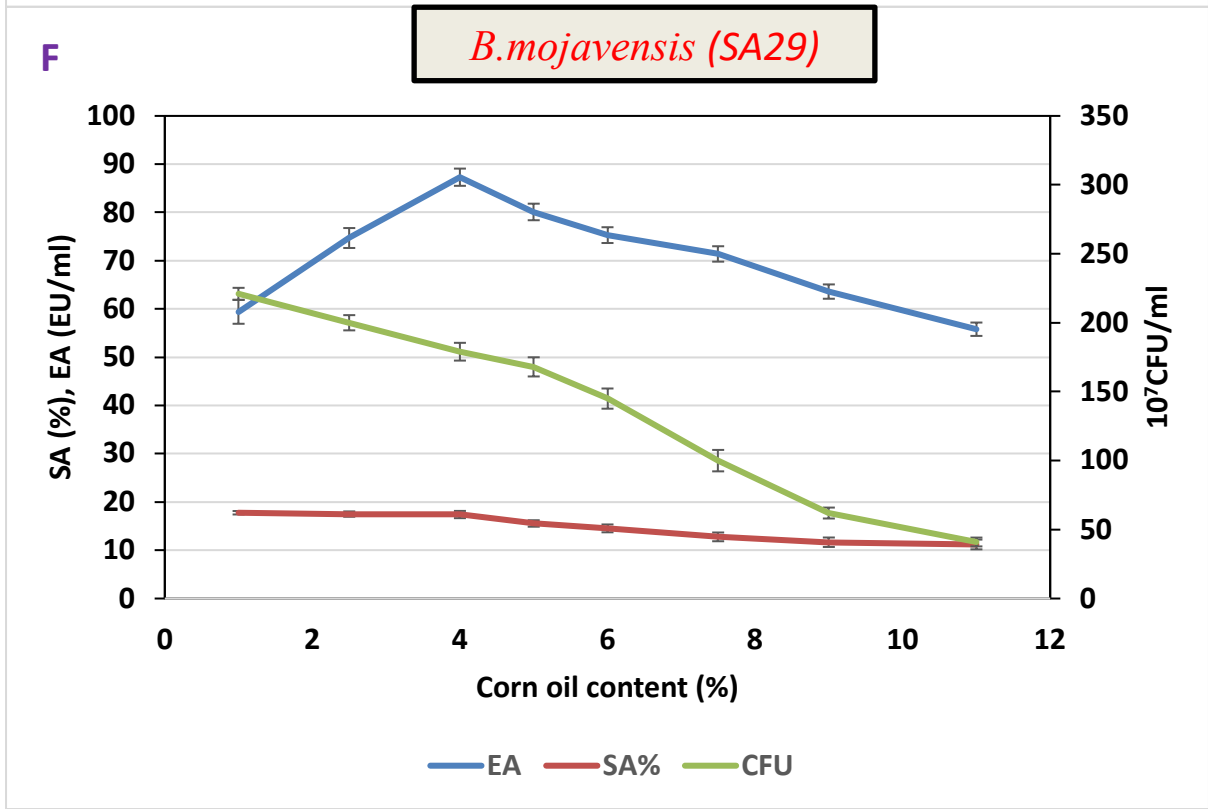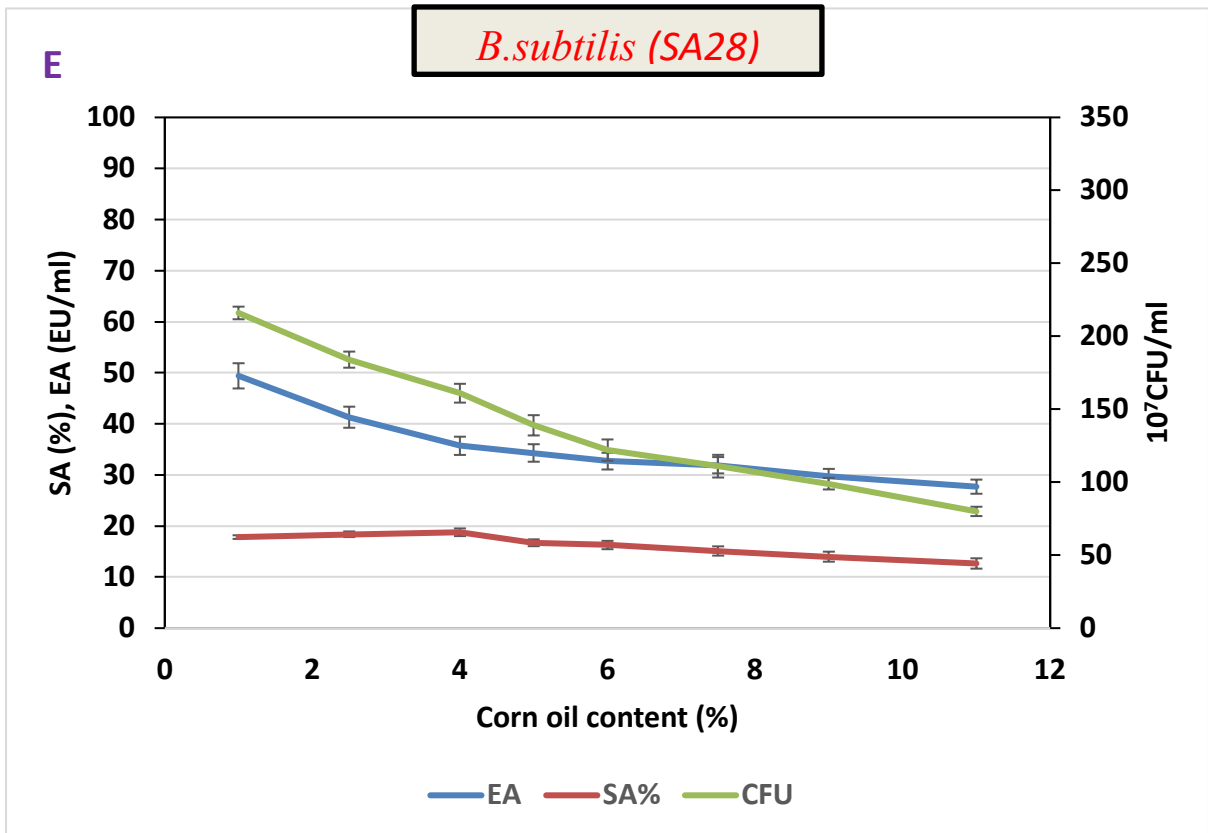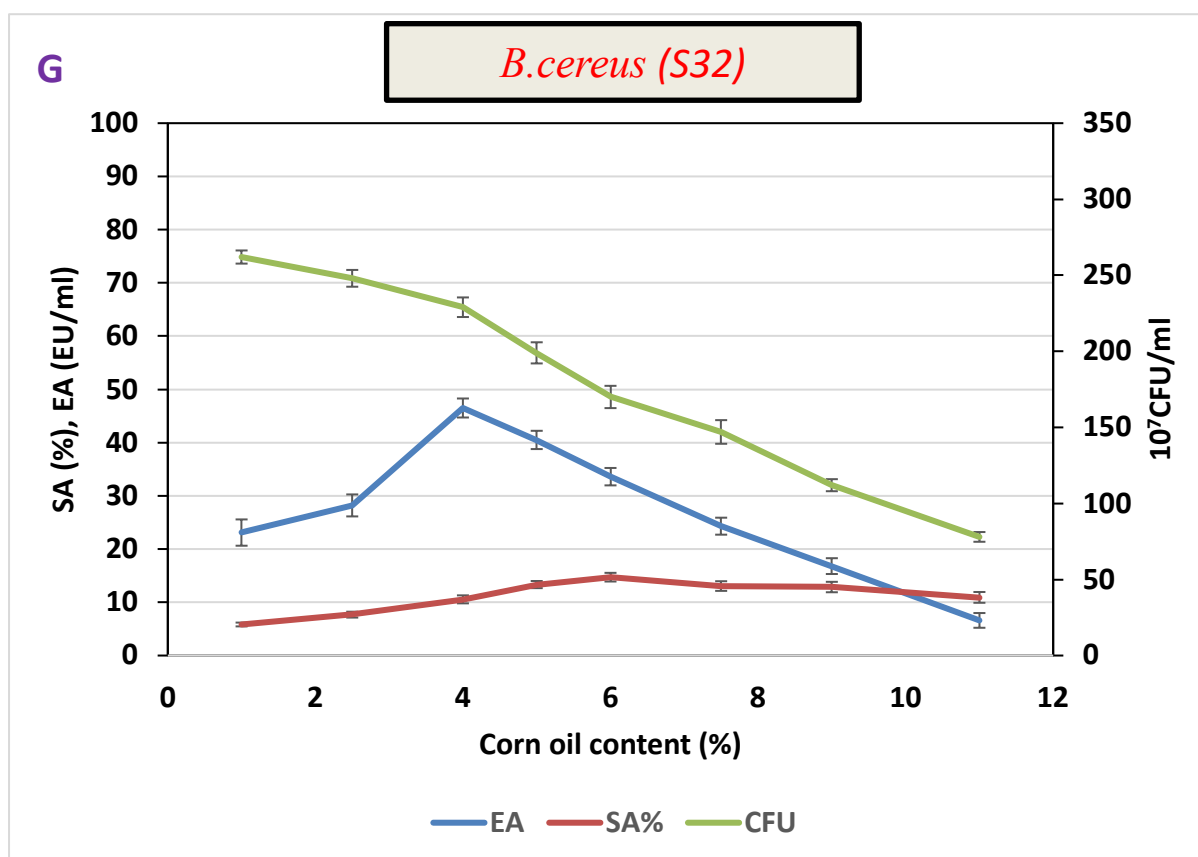
F B.mojavensis (SA29)

Figure 11: Effect of corn oil concentration on growth and emulsification and solubilization potentiality by the selected strain: A: S5 (*B.subtilis*), B: SA6 (*B.subtilis*), C: SA16 (*B. subtilis*), D: S27 (*B.subtilis*), E: SA28 (*B.subtilis*), F: SA29 (*B.mojavensis*), G:S32 (*B.cereus*)

The strain SA16 showed the highest growth with approximately 280 CFU 10^{-7}/ml, followed by the S5 with approximately 275 CFU 10^{-7}/ml. Compared to the bacterial growth ability in diesel, the seven strains displayed superior growth in corn oil, as evidenced by the high CFU recorded in corn oil. SA16 strain recorded the highest CFU followed by S5, S27 then SA28 in the *B. subtilis* species. From the results, *B. mojavensis* and *B. cereus*, which recorded lower CFUs in diesel has recorded higher CFUs in corn oil than *B. subtilis*. From the results, it can be hypothesized that the carbon catabolic repression mechanism avails carbon needed for bacterial growth more efficiently from the corn oil substrate than the diesel substrate. Markedly, catabolic carbon repression significantly contributes to the selective uptake of

bacterial cells from complex media and is activated when the bacterial cell growth rate is high (Zhou et al., 2013). The other assumption could be that the by-products of metabolism accumulate less fast in the corn oil experiment, promoting a higher CFU. Moreover, *B. mojavensis,* and *B. cereus* showed similar emulsification which means they are similar emulsifiers although they belong to different species but have the same genus '*Bacillus*'. Another explanation for their similarity in the performance of emulsification is that they are assorted under the same group when classified by PCA dendrogram in Figure 4.

II-6. Investigation of the effect of used corn oil hydrocarbons concentrations on selected strains

In order to investigate the regulations of growth and biosurfactants production by the selected strains, the 5-times used corn oil was used as source of hydrocarbons. The results of Fig. 12 indicate that all seven strains of *Bacillus* have ability to grow of such weathered hydrocarbons. However, each bacterial strain has its growth profile. The strains of *B. subtilis* species recorded superior ability to grow compared to the other two *Bacillus* strains. The results clearly show that the growth of all the selected strains was continuously decreased by increasing the oil concentrations. This is reflected by the increased toxicity exhibited by several hydrocarbons in the used oil. However, production of emulsification activities is restricted mainly to the emulsification activities since the solubilization activities are below 10 SA%. Production of the emulsification activities by all the 7 strains is not regulated by the carbon catabolite repression, since it increased with the increase of oil content up to a maximum reached with 4 to 7% diesel depending on the strain, then dropped. This drop may be due also to the drop in the growth requiring less emulsified hydrocarbons. The strain *B. subtilis* SA28 showed that the carbon catabolite repression is less accentuated that with vegetative corn oil. This result is very interesting from the practical point of view, showing that the "burned hydrocarbons" in corn oil become less readily assimilable than the vegetative one, conducting

to the overcome of the carbon catabolite repression. This means that the used corn can be an appropriate carbon source to produce bioemulsifiers by *Bacillus* strains adapted to weathered oil.

C — *B.subtilis (SA16)*

D — *B.subtilis (S27)*

Charts showing SA (%), EA (EU/ml) and 10^7 CFU/ml versus Used oil content (%) for B.subtilis (SA16) and B.subtilis (S27), with legend EA, SA%, CFU.

E *B.subtilis (SA28)*
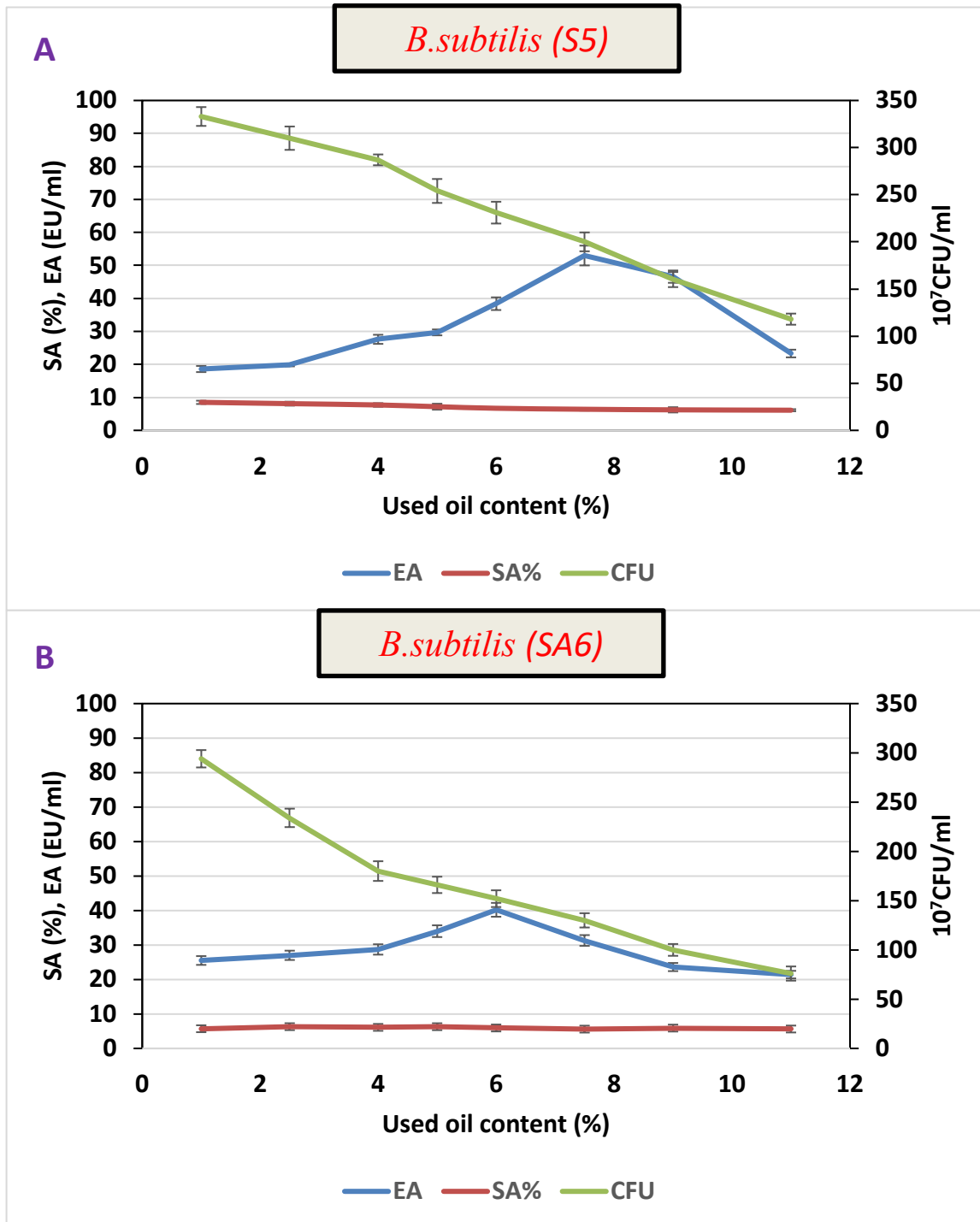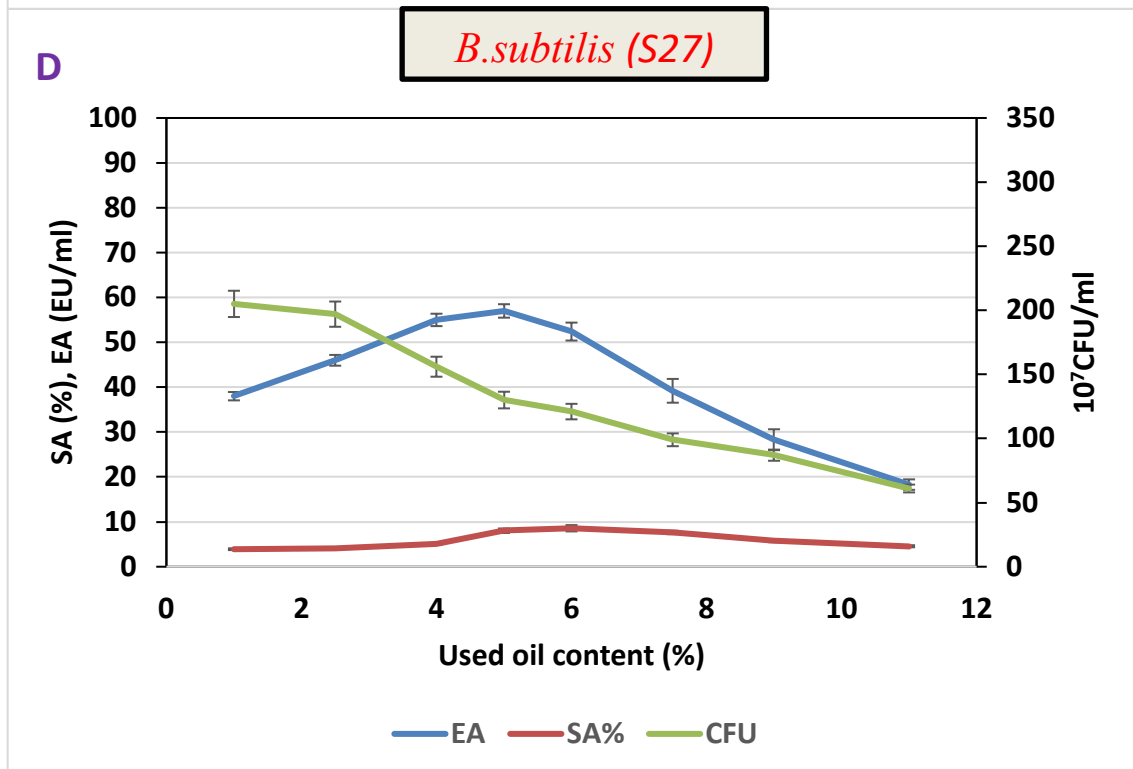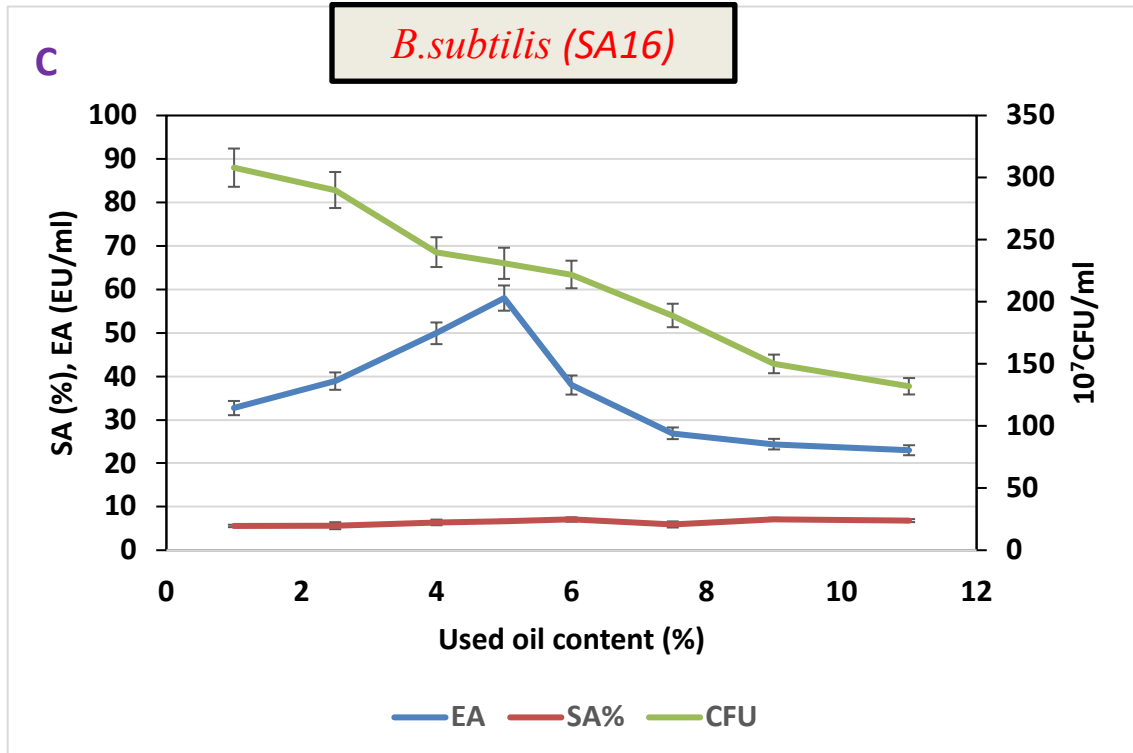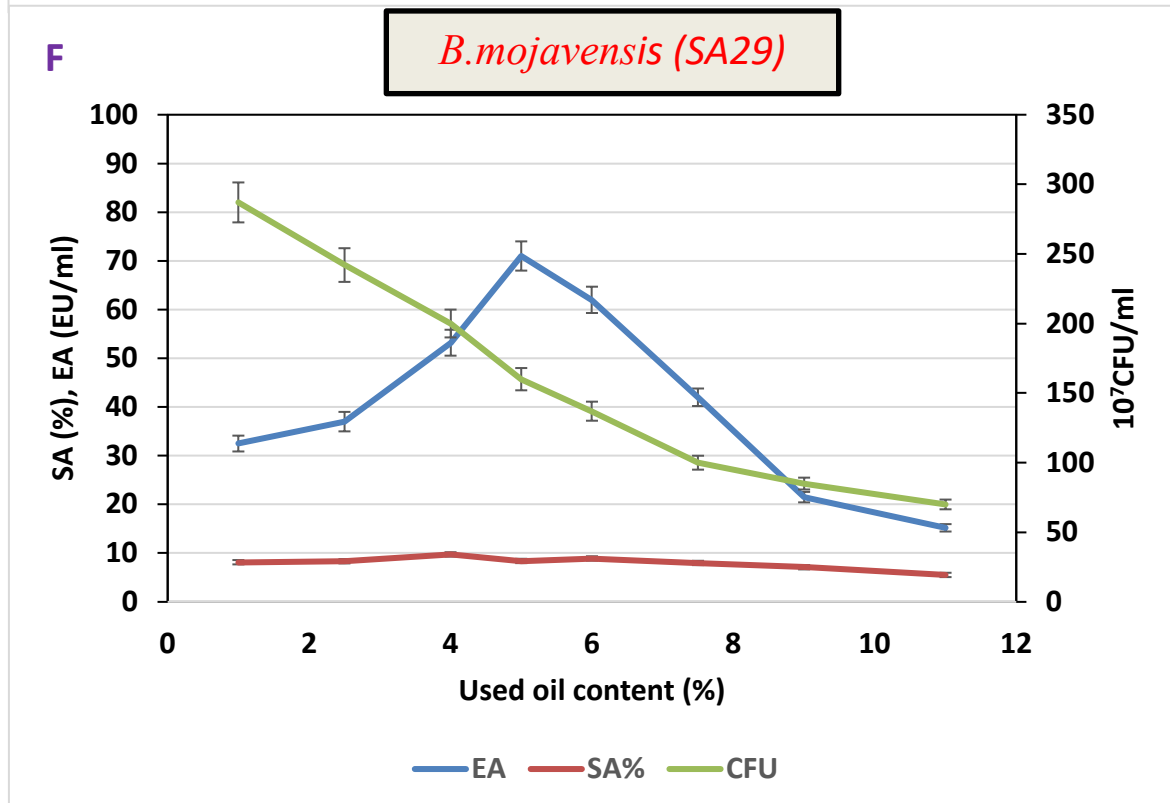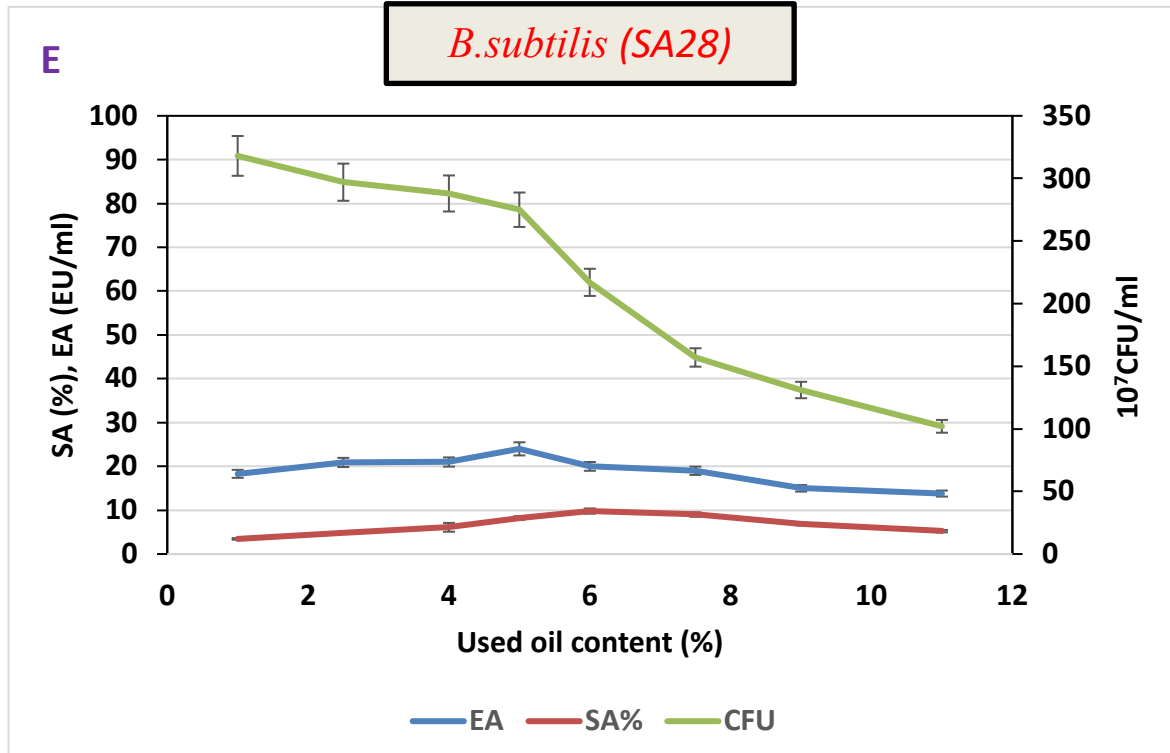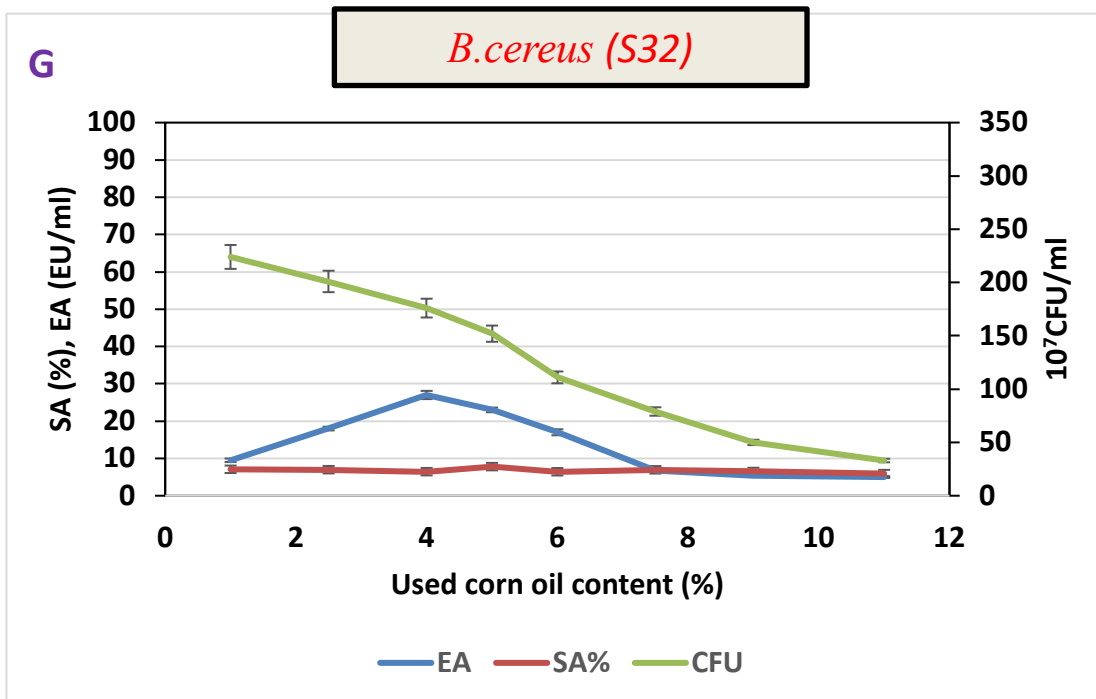
F *B.mojavensis (SA29)*

Figure 12: Effect of used corn oil concentration on growth and emulsification and solubilization production by the selected strain: A: S5 (*B.subtilis*), B: SA6 (*B.subtilis*), C: SA16 (*B.subtilis*), D: S27 (*B.subtilis*), E: SA28 (*B.subtilis*), F: SA29 (*B.mojavensis*), G:S32 (*B.cereus*).

Starting with *B. subtilis* strains, they showed a diversity in their production of biosurfactant activities. Respectively, in S5 the emulsification activities started with 18 EA/ml at 1% of used corn oil and kept increasing until it reached 53 EA/ml then dropped after 7%. On another hand, the solubilization activity remained in constant range. SA16, S27 and SA29 showed a very similar increase in the emulsification activities until 5% of used oil, and then dropped drastically. Although, these 3 strains were collected from different locations but after identification and categorization with PCA, they fell within the same cluster/group, so this can explain their similar performances in degrading hydrocarbons since they have similar protein profiles. *B. mojavensis and B. cereus* recorded higher CFUs in used oil as in the corn oil

compared to the CFUs recorded in diesel. It can be assumed that the carbon catabolic repression mechanism avails carbon needed for bacterial growth more efficiently in the corn oil and used oil substrates than the diesel substrate. Furthermore, repeated heating of cooking oil results in chemical changes that lead to the synthesis of more saturated compounds (Choe & Min, 2007). It can be hypothesized that these high numbers of saturated compounds serve as sources of carbon for the growth of bacteria hence the high CFU was recorded in the used oil. These results are consistent with those reported by Adlin et al. (2020), who observed that the use of waste cooking oil as the sole source of carbon produced greater amounts of biosurfactant compared to fresh cooking oil hence higher bacterial growth. The highest growth of all the seven strains of *Bacillus* was recorded at a 2% concentration of used oil, and the growth continued to decline as the used oil concentration increased. The CCR mechanism availed the most desirable sources of carbon for optimal growth. CCR is less accentuated in the cooked corn oil than fresh corn oil. The complexity of the substrates is responsible of such interesting result.

CONCLUSION

Biosurfactants are produced by microorganisms, especially bacteria, to increase the bioavailability of hydrophobic substrates. If appropriately produced and formulated, they can be applied in different fields ranging from hydrocarbons remediation to the food industry. The surface-active biomolecules have unique properties such as low toxicity and a relatively simple preparation process. Therefore, these characteristics have increased their demand in a wide range of industries of agrochemicals, fertilizers, petrochemicals, pharmaceuticals, cosmetics, and beverages as well as in petroleum and mining industries. At the harsh weather conditions of Qatar, it is anticipated that indigenous microorganisms have adapted to synthesize specific biosurfactants that are effective for these weathered oils, both to mobilize the hydrocarbons and to enhance their bioavailability and biodegradation. Indeed, many failures of bioremediation applications in regions characterized by harsh weather and soils can be attributed to the use of un-acclimated bacteria and their associated biosurfactants. The novelty of this work resides in the ability of the indigenous Qatari strains to produce biosurfactants that have the potential to enhance the biodegradation of weathered hydrocarbons or washing of weathered soil. These biosurfactants may be more active under the harsh physical and chemical conditions with Qatari soils, making them appropriate for use in enhanced oil recovery and bioremediation. However, it was necessary to demonstrate the biodiversity of the producing bacteria and strains, and their adaptation to harsh conditions and weathered hydrocarbons by adapting their biosurfactants structures and activities. A collection of bacteria was formed, and most of the isolates have been identified and differentiated, by MALDI TOF MS and PCA and their potential to produce biosurfactants has been evaluated. The isolates from the highly weathered oily sites would lead to selecting interesting strains more appropriate in applications in areas characterized by harsh weather.

Their adapted biosurfactants were investigated by Fourier-transform infrared spectroscopy (FTIR) and categorized by principal component analysis (PCA).

The ability of the indigenous Qatari strains to produce biosurfactants with great potential to enhance the biodegradation of weathered hydrocarbons was investigated. The obtained findings showed that two types of adaptations occur with hydrocarbons degrading bacteria in the weathered-oily soils, one related to the bacterial cell composition maintaining the biosurfactants composition and one to the biosurfactants which is the primary tool employed by the bacterial cell to interact with the weathered oil. Indeed, the phyloproteomic classification of the studied strains allowed the differentiation and separation of strains belonging to the same *Bacillus* species. High diversity of emulsification and solubilization activities were recorded among biosurfactants produced by the studied strains. Moreover, combining FTIR data with PCA analysis resulted in further classification of the biosurfactants produced by the studied bacterial isolates.

In order to study the limitations and regulations of biosurfactants production, selected *Bacillus* species were compared with *Pseudomonas aeruginosa,* the most reported biosurfactant-producing bacterium. The high diversity of behaviors of strains, even those of the same species, in several hydrocarbons' sources proved that their surfactin-like molecules production is dependent of the cultural media. However, the production of these surfactants depends on the bacterial strain and the origin of the hydrocarbons as well. This supports the biodiversity among our collection.

Then, seven *Bacillus* strains, comprising three belonging to the species *subtilis*, were shown appropriate for the development of production of biosurfactants in Qatar. In vegetative oils, the findings demonstrated that the biosurfactants of all strains of *Bacillus* and *Pseudomonas aeruginosa* are emulsifiers, with low solubilization activities. In diesel, corn and used corn oil, the highest growth of all seven strains of *Bacillus* was mostly recorded at 2% concentration of

oil, and the growth continued to decline as the oil concentration increased. Enhancing other factors such as pH, temperature, and aeration for biodegrading microorganisms could improve the degradation capacity of biodegrading microorganisms. It was demonstrated that the grow of most of the Qatari bacterial isolates were sensitive to hydrocarbons concentration either due to toxicity or carbon catabolite repression. Only one strain of *B. mojavensis* showed regulation of synthesis of biosurfactants by this repression.

Although, the strains of *B. subtilis* were collected from different locations but after identification and categorization with PCA, they fell within the same cluster/group, so this can explain their similar performances in degrading hydrocarbons since they have similar protein profiles. *B. mojavensis and B. cereus* recorded higher CFUs in used oil as in the corn oil compared to the CFUs recorded in diesel. It can be assumed that the carbon catabolic repression mechanism avails carbon needed for bacterial growth more efficiently in the corn oil and used oil substrates than the diesel substrate. Furthermore, repeated heating of cooking oil results in chemical changes that lead to the synthesis of more saturated compounds. It can be hypothesized that these high numbers of saturated compounds serve as sources of carbon for the growth of bacteria hence the high CFU was recorded in the used oil. These results are consistent with those reported by Adlin et al. (2020), who observed that the use of waste cooking oil as the sole source of carbon produced greater amounts of biosurfactant compared to fresh cooking oil hence higher bacterial growth. The highest growth of all the seven strains of *Bacillus* was recorded at a 2% concentration of used oil, and the growth continued to decline as the used oil concentration increased. The CCR mechanism availed the most desirable sources of carbon for optimal growth. CCR is less accentuated in the cooked corn oil than fresh corn oil. The complexity of the substrates is responsible of such interesting result.

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