INDIGENOUS HYDROCARBON DEGRADERS FURTHER EVALUATED FOR THEIR KEROSENE DEGRADATION AND BIOSURFACTANT PRODUCTION POTENTIALS

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ABSTRACT

INDIGENOUS HYDROCARBON DEGRADERS FURTHER EVALUATED FOR THEIR KEROSENE DEGRADATION AND BIOSURFACTANT PRODUCTION POTENTIALS

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Kerosene, known as jet fuel, is one of the most spilled petroleum product causing serious environmental problems due to recalcitrant compounds found in its structure. The only eco-friendly solution for this problem is bioremediation, in which bacteria are used for the degradation and transformation into non or less toxic forms. The efficiency of this process depends not only on biodegradation ability of the bacterial isolates used but also on their biosurfactant production abilities. Therefore, in this study, 22 previously identified bacterial hydrocarbon degraders were further analyzed for their kerosene degradation and biosurfactant production potentials. Out of 22, 19 bacterial isolates were found to utilize kerosene after pre-selection. The degradation abilities of the pre-selected isolates were determined chromatographically and 7 isolates namely; *Pseudomonas plecoglossicida* Ag10, *Staphylococcus aureus* Ba01, *Stenetrophomonas rhizophila* Ba11, *Delftia acidovorans* Cd11, *Acinetobacter calcoaceticus* Fe10, *Pseudomonas koreensis* Hg11 and *Acinetobacter johnsonii* Sb01 were stood out as efficient kerosene degraders with degradation abilities in between 69-84%. All the efficient degraders were showed to harbor the *alk*B gene responsible for kerosene degradation through the polymerase chain reaction (PCR) analyses. Biosurfactant production abilities of 19 kerosene degraders were also tested and *Pseudomonas plecoglossicida* Ag10, *Raoultella planticola* Ag11, *Staphylococcus aureus* Ba01, *Enterococcus faecalis* Cr07, *Acinetobacter johnsonii* Sb01 and *Pantoea agglomerans*

Sn11 were determined as biosurfactant producers through oil spreading activity, emulsification index and microbial adhesion to hydrocarbon tests. Blue agar plate method, thin layer chromatography and fourier transform infrared spectroscopy analysis were used to characterize the biosurfactants. The results revealed that, glycolipid type rhamnolipids were majoring in kerosene degraders. The gene responsible for rhamnolipid biosynthesis, *rhl*AB, was also shown in all the rhamnolipid producers by PCR analysis.

Key words: Kerosene degraders, bioremediation, biosurfactant, rhamnolipid, *alk*B, *rhl*AB

ÖZ

HİDROKARBON PARÇALAYAN LOKAL BAKTERİLERİN KEROSEN PARÇALAMA VE BİYOSÜRFEKTAN ÜRETME POTANSİYELLERİNİN BELİRLENMESİ

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Jet yakıtı olarak bilinen kerosen, doğaya en çok dökülen petrol ürünleri arasında yer almaktadır. Yapısında bulunan inatçı bileşiklerden dolayı, ciddi çevresel sorunlara sebep olmaktadır. Bu problem için en etkili çözüm, çevre dostu bir yöntem olan biyoremediyasyondur. Biyoremediyasyon, mikroorganizmaların kirletici maddeleri metabolizmalarına katarak, onları tamamen zararsız ya da daha az toksik forma dönüştürmesidir. Bu işlemin etkinliği sadece bakteriyel izolatların biyodegradasyon kabiliyetine değil aynı zamanda biyosürfaktan üretim yeteneklerine de bağlıdır. Bu nedenle, bu çalışmada, daha önce hidrokarbon parçalayıcı olarak tanımlanmış 22 bakterinin kerosen degradasyon yetenekleri araştırılmıştır. 22 bakteriyel izolat arasından 19'unun seçici besiyerinde üreyebildikleri saptanmıştır. Bu bakterilerin kerosen degradasyon yetenekleri ise gaz kromatografisi ile belirlenmiş ve 7'sinin (*Pseudomonas plecoglossicida* Ag10, *Staphylococcus aureus* Ba01, *Stenetrophomonas rhizophila* Ba11, *Delftia acidovorans* Cd11, *Acinetobacter calcoaceticus* Fe10, *Pseudomonas koreensis* Hg11 and *Acinetobacter johnsonii* Sb01) keroseni %69-84 aralığında degrede edebildiği tespit edilmiştir. Kerosen degradasyonunda öne çıkan tüm izolatların, kerosen parçalanmasından sorumlu *alk*B

genini barındırdığı, polimeraz zincir reaksiyon (PZR) analizleri ile ortaya çıkartılmıştır. Öte yandan, kerosen kullanma yeteneğine sahip aynı 19 bakterinin biyosürfaktan üretim yetenekleri de araştırılmıştır. 19 izolat arasından, 6'sının (*Pseudomonas plecoglossicida* Ag10, *Raoultella planticola* Ag11, *Staphylococcus aureus* Ba01, *Enterococcus faecalis* Cr07, *Acinetobacter johnsonii* Sb01, *Pantoea agglomerans* Sn11) biyosürfektan üreticileri oldukları yağ yayılma (oil spreading) aktivitesi, emülsifikasyon indeksi ve hidrokarbonlara karşı mikrobiyal adhezyon testleri ile belirlenmiştir. Biyosürfaktanların karakterizasyonu, mavi agar plaka (blue agar plate) metodu, ince tabaka kromatografisi (TLC) testi ve fourier dönüşümlü kızılötesi spektrometresi (FTIR) analizleri ile yapılmıştır. Sonuçlar, biyosürfaktanların glikolipid yapıdaki ramnolipit tipi biyosürfektan olduğunu göstermiştir. Ramnolipit biyosentezinden sorumlu *rhl*AB geninin varlığı, tüm ramnolipit üreticilerinde PZR analizleri ile gösterilmiştir.

Anahtar kelimeler: Kerosen parçalama, biyoremediyasyon, biyosürfektan, ramnolipit, *alk*B, *rhl*AB

To my family

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Last but not least, I would like to thank my family, to whom this thesis is dedicated, for all their love and endless support throughout my life. I am grateful to them for always being there for me.

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ABBREVIATIONS

BAP	Blue Agar Plate
CMC	Critical Micelle Concentration
DNA	Deoxynucleic Acid
EDTA	Ethylene Diamine Tetraacetate
E_{24}	Emulsification Index
FTIR	Fourier Transform Infrared Spectroscopy
GC	Gas Chromatography
MATH	Microbial Adhesion to Hydrocarbon
MSM	Mineral Salt Medium
MTC	Maximum Tolerable Concentration
NA	Nutrient Agar
NB	Nutrient Broth
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
SDS	Sodium Dodecyl Sulfate
TLC	Thin Layer Chromatography

CHAPTER 1

INTRODUCTION

1.1 Environmental contamination with petroleum and its ecological impact

Petroleum products are indispensable chemicals of our daily life (Das and Chandran, 2011). Massive quantity of petrol or oil is required every day to power automobiles, for domestic heating and industrial use. Every year about 35 million barrels of petroleum (Macaulay, 2015) are shipped all around the world. Production of petroleum products and anthropogenic activities such as oil exploration, exploitation, transportation, and their distribution lead to unavoidable oil spillage. Due to their toxic and harmful effects, petroleum contamination is a major environmental problem of today's life (Varjani, 2017).

Figure 1.1 Oil spill from the tanker Exxon Valdez in Alaska's Prince William Sound by Natalie B. Fobes (left), The Deepwater Horizon oil drilling rig burning in the Gulf of Mexico (right) (from REUTERS)

Large-scale oil spill accidents have drawn great attention worldwide. In 1989, the tanker Exxon Valdez spilled about 11 million gallons (Gakpe *et al*., 2007) of crude oil on Alaska's Prince William Sound. The oil spread over a wide area for months resulting by depredation of 28 different types of animals, plants and marine habitats (Peterson *et al.*, 2003).

National Oceanic and Atmospheric Administration (NOAA) reported that, 25 years after the accident 9 species and their habitat are still in recovery period. Scarcely, 3 types of animals (herring, killer whale and pigeon guillemots) haven't recovered yet. Exxon Valdez accident has been the most studied oil spill case in history and was the precursor for today's bioremediation studies (NOAA, 2018).

The largest spill of oil by the time 2010 was Deepwater Horizon oil rig explosion in the Gulf of Mexico (Dave and Ghaly, 2011). For 87 days, 205.8 million gallons of oil was discharged into the gulf. Average of 1.6 km of shorelines were polluted (Dzionek *et al.*, 2016). Thousands of birds, mammals, and sea turtles were contaminated with leaked oil. This accident was recorded as the largest mortality event occurred in the Gulf of Mexico (Dave and Ghaly, 2011). About 1.8 million gallons of dispersants were used for bioremediation studies in order to clean the contaminants up (NOAA, 2018).

Such accidents also happen in Turkey (Erdoğan *et al.*, 2012), where petroleum contamination is an important pollution problem. A total of 461 shipping accidents occurred in the Bosphorus during the 1953–2002 period (Akten, 2006), the majority being collisions. The biggest accident occurred in 1979, a Romanian tanker Independeta collided with a Greek cargo ship Evriali in the Bosphorus of İstanbul. 30 million gallons of crude oil were spilled and caught fire. This was the 11th biggest marine pollution recorded in history (ITOPF, 2009) ending with serious impact to not only the marine environment but also causing significant air pollution due to fire incidents after petrol explosions. More recently, in January 2017, the İzmit gulf was polluted by fuel oil. About 60 ton of fuel oil leaked to the coastline of Yalova and the marine ecosystem of İzmit gulf (TURMEPA, 2017).

Figure 1.2 Independenta ship accident by Cristian Munteanu (left), sea bird covered with oil in the gulf of İzmit (right) (from CNNTurk)

Ecological impact of oil spills is needed to be considered since petroleum components are persistent organic pollutants (Varjani, 2017). After a spillage, oil floats and creates a blanket on the surface of water (Dicks, 1998) and causes damages mostly to marine animals and seabirds. Seabirds whether die from inhaling the toxic fumes or by hypothermia. Their fur is covered with oil, therefore they cannot regulate their body heat (Almeda *et al.*, 2013). Mass mortality is also seen in macroalgae and benthic invertebrates because of chemical toxicity, smothering, and physical displacement from the habitat. Fish embryos exposed to oil lead to indirect effects on growth, deformities and problems with reproduction (Peterson *et al.*, 2003). Another concern is the bioaccumulation of toxic compounds in petroleum (Almeda *et al.*, 2013). These compounds are taken up by aquatic organisms and this leads an accumulation in the food chain (Van der Heul, 2009).

1.2 Petroleum hydrocarbons and their chemical composition

The word Petroleum, comes from Latin, meaning rock oil (Varjani, 2017). It originates from the biosynthetic activity of microorganisms and plants that are buried deep in the earth and heated under great temperature and pressure over prolonged geological periods (Das and Chandran, 2011). Whereas, hydrocarbons are compounds formed by carbon and hydrogen, and may contain some amount of nitrogen, sulfur and oxygen (Abbasian *et al.*, 2015). Petroleum hydrocarbons are mixtures obtained by the distillation of crude oil (Ashraf, 2012). Those mixtures can be categorized into four classes like aliphatic, aromatic, resins and asphaltenes (Olajire and Essien, 2014).

Aliphatics are arranged in a linear or branched chain and usually comprise more than 50% of most crude oils (Rojo, 2009). They can be divided into three classes according to their chemical structures as alkanes, alkenes and cycloalkanes. On the other hand, aromatic hydrocarbons have one or more aromatic rings in their structure with different alkyl groups attached (Figure 1.3) (Ziadabadi and Hassanshahian, 2016). Resins and asphaltenes contain non-hydrocarbon polar compounds having very complex and mostly unknown carbon structures with nitrogen, sulfur and oxygen atoms (Varjani, 2017).

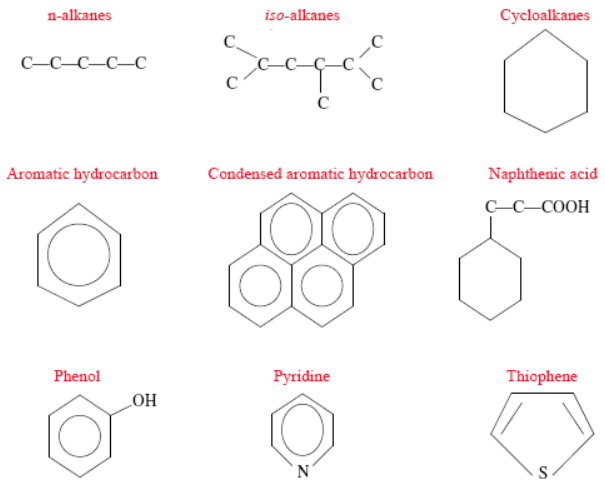


Figure 1.3 Structures of some crude oil components (Hassanshahian and Cappello, 2012)

As mentioned before, hydrocarbon pollutants are one of the most persistent organic pollutants. They are recalcitrant and contains toxic compounds such as benzene, toluene, ethylbenzene, xylene and polycyclic aromatic hydrocarbons (Olajire and Essien, 2014). Toxicity of hydrocarbons depends on chemical properties like volatility, lipid solubility, viscosity and surface tension (Tormoehlen *et al*., 2014).

The toxicity of hydrocarbons increases as their molecular weight decreases *(Singh et al*., 2012). The toxicity increases in the following order; alkanes, alkenes, cycloparaffins, aromatics, and polyaromatics (Varjani, 2017). In aliphatic structures, carbon atoms only share electrons with their adjacent electrons which allows them different conformations, thereby renders aliphatic as non polar or slightly polar. While the polarity increases, their solubility and interaction with water increases. Due to lack of functional groups and low water solubility, serious ecological problems occurs when they are released to the environment (Singh *et al*., 2012). Aromatic hydrocarbons are more water soluble, therefore, they are easily adsorbed into organic matter in water and persist in the ecosystems for extended period of time (Adam, 2001). As the volatility of hydrocarbons increases, the higher absorption occurs during inhalation, which ends up by crossing the blood-brain barrier causing damages in the nervous system. Moreover, hydrocarbons can damage tissues by affecting the lipid part of the cell since compounds are insoluble in water but soluble in most fats (Tormoehlen *et al*., 2014). They can induce malignant tumors since they have a great affinity for nucleophilic center of macromolecules like RNA, protein and DNA (Varjani, 2017).

1.3 Petroleum products and their chromatographic profiles

Crude oil is the unprocessed oil found in reservoirs under the Earth's surface. It contains various components that all have different sizes, weights and boiling points (Bishop, 1997). In the fractional distillation of crude oil, different petroleum compounds are obtained. Since every compound in crude oil have specific boiling temperatures, they are separated easily by a process called fractional distillation (Ashraf, 2012). For example, when crude oil is evaporated, kerosene condenses at a higher temperature than naphtha and as the mixture cools, kerosene is separated from naphtha because it condenses first (Figure 1.4).

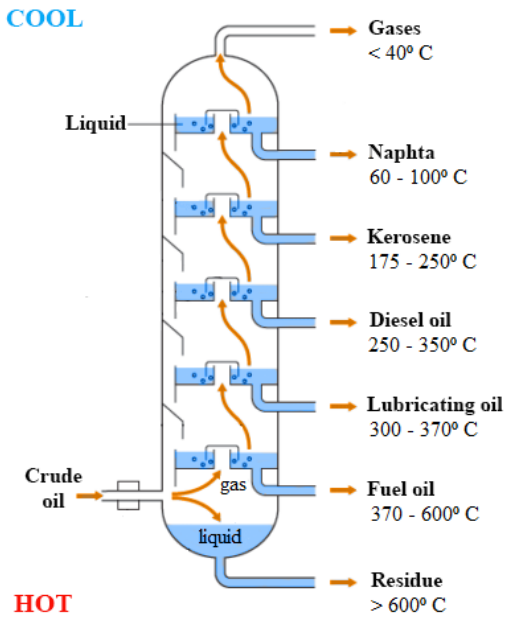


Figure 1.4 Fractional distillation of crude oil (adapted from Ashraf, 2012)

The boiling point distribution of each compound found in crude oil depends on alkane standards ranging from methane (CH_4) to dotricontane ($C_{32}H_{66}$) (Bishop, 1997). Every petrol product has a different range of carbon (Ashraf, 2012). Knowing the distribution ranges of carbons is important for characterization of petroleum products but also gives information about their property. For example, as the carbon chain length increases, the volatility of the product decreases (Varjani, 2017). Petroleum products and their carbon chain length is given in Figure 1.5.

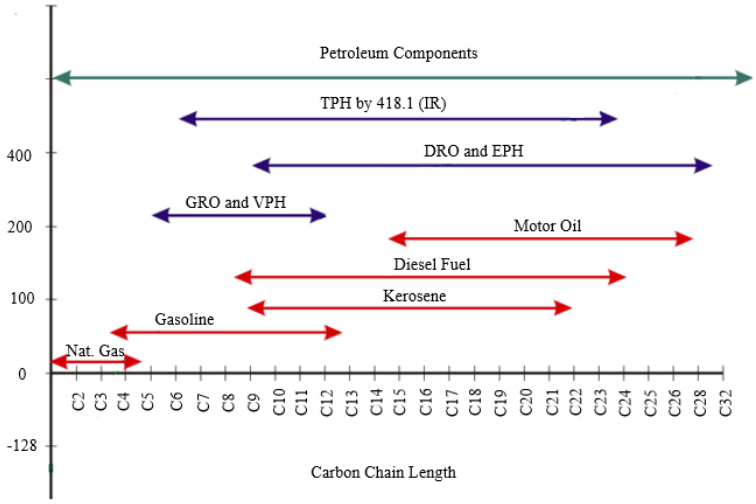


Figure 1.5 Petroleum products and petroleum measurements chart. TPH: Total Petroleum Hydrocarbons obtained from the method 418.1 by Infrared Instrument (IR), DRO: Diesel Range Organics, EPH: Extractable Petroleum Hydrocarbons, GRO: Gasoline Range Organics, VPH: Volatile Petroleum Hydrocarbons (adapted from Bishop, 1997)

In order to characterize the composition of hydrocarbons in petroleum samples, a common laboratory technique called gas chromatography (GC) is used (Ghoreishi *et al.*, 2017). The separation of each compound is based on their vapor pressure and their polarity. Once injected into a gas chromatograph, the product is heated and vaporized, then passes in a gas stream (mobile phase). After injection, the temperature of the column increases slowly and compounds begin to move through the column depending to their various chemical and physical properties. For example, more volatile compounds with lower boiling points starts moving first. Compounds also interacts with specific column filling (stationary phase). At the end, each component exits the column at a different time, named as retention time. While chemicals passes through the column, their detection and identification is electronically done (Bishop, 1997). Figure 1.6 shows the gas chromatogram of gasoline known as motor fuel, a low-weight, high volatile product mostly consisting 5 to 12 carbon atoms (Figure 1.6a) and the chromatogram of diesel, a heavier-weight product containing mostly n-alkanes with carbon atoms greater than 12 (Figure 1.6b).

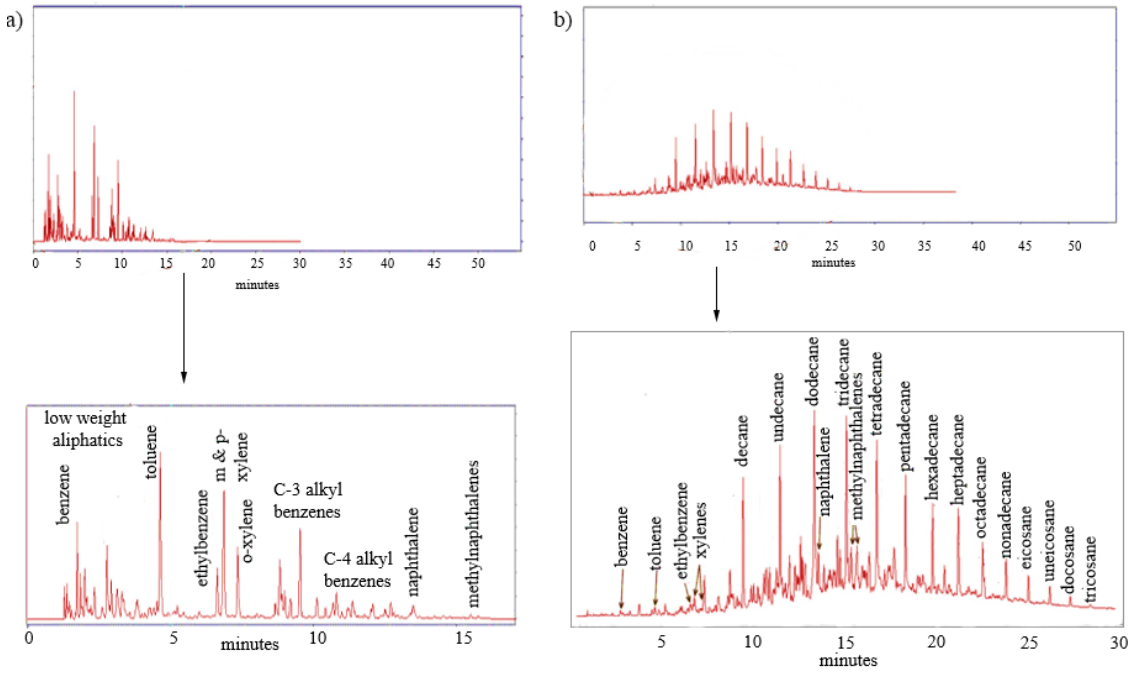


Figure 1.6 GC-based total petroleum hydrocarbon profiles of (a) gasoline and (b) diesel (adapted from Bishop, 1997)

1.3.1 Kerosene

Kerosene is a thin, colorless and odorless liquid oil obtained from the distillation of crude oil between 175°C to 250°C (Gouda *et al.*, 2007). Kerosene is known by several different names including heating oil, boiler juice and paraffin. It has become a major household, commercial, and industrial fuel (Lam *et al.*, 2012). It is used as domestic heating oil or as lamp oil in developing countries when electricity is unavailable. Globally, about 500 million households still uses kerosene (Lam *et al.*, 2012) and 7.6 billion liters is consumed annually (Mills, 2005). Kerosene has other use of area such as spray oil to combat insects (Gouda *et al.*, 2007), solvent in paints and cleaners, also as alcohol denaturant (Nwinyi and Victory, 2014). Scarcely, kerosene is mostly used as aircraft gas turbine and jet fuel, known as Jet A, Jet A-1 or it is largely manufactured for commercial airlines and the military activities named as JP-8 or JP-5 (Gouda *et al.*, 2007). The commercial development of kerosene type fuels started particularly during World War II because of its availability compared to gasoline. In addition, due to its high flash point temperature, kerosene is harder to ignite accidentally (Khan *et al.*, 2015), which makes it much safer and preferable for the aviation industry.

About 70% of kerosene is composed of branched, straight chain alkanes and naphthenes (cycloalkanes) while aromatic hydrocarbons such as alkylbenzenes and alkylnaphthalenes do not exceed 25% by volume of kerosene. Finally, olefins (alkenes) are found less than 5% (Figure 1.7) (Ziadabadi and Hassanshahian, 2016).

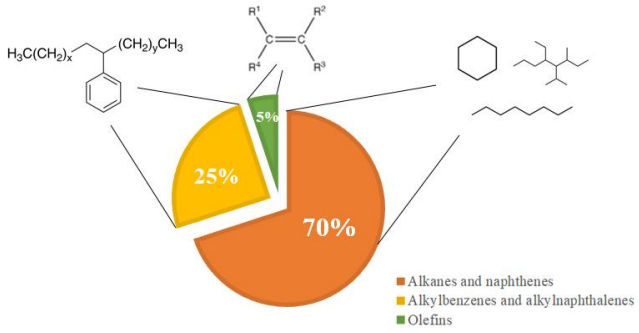


Figure 1.7 Major components of kerosene

This mixture has a density of 0.78–0.81 g/cm^3 and it is immiscible in water with moderate volatility. Kerosene usually contains carbon numbers between C_9 to C_{20}, that can vary due to its distillation process from C_6 to C_{24} (Figure 1.8) (Udoetok *et al.*, 2012).

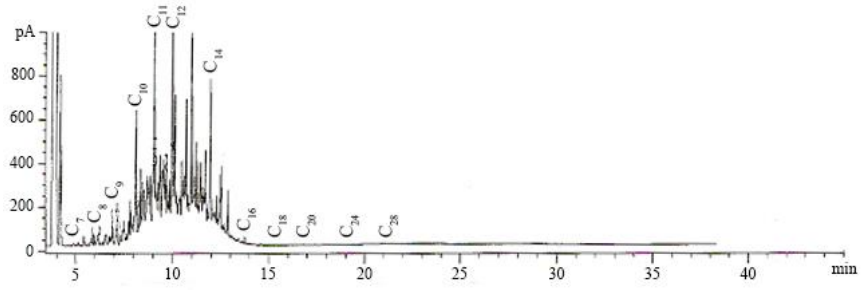


Figure 1.8 Gas chromatogram of kerosene (Udoetok *et al.*, 2012)

The total amount of kerosene consumption throughout the world is about 1.2 million barrels per day (Gouda *et al.*, 2007). Despite the several usefulness of kerosene, it also constitutes a major environmental concern. Because of the aromatic compounds, kerosene is hazardous to living organisms with a toxicity varying from moderate to high (Umanu and Babade, 2013). According to the US Coast Guard Emergency Response Notification System, kerosene is one of the most commonly spilled

petroleum products, causing a global environmental concern (Gouda *et al.*, 2007). Spillage and leakages of kerosene causes potential acute toxicity to both aquatic and terrestrial life as well as inhalation hazards. In humans, kerosene can provoke serious skin irritation and mucous membrane damages, while changes in the liver and harmful effects on the kidney, heart, lungs, and nervous system can be seen in long term (Umanu and Babade, 2013). Furthermore, increased rates of cancer, immunological, reproductive, fetotoxic, genotoxic effects are also associated with lighter, more volatile and water soluble compounds found in kerosene (Irwin, 1997).

1.4 Treatment of petroleum contamination

Petroleum hydrocarbons are classified as priority pollutants and therefore it is necessary to combat this pollution problem. Many conventional engineering based methods are used in order to control and treat petroleum pollutants (Varjani, 2017) such as physical, chemical and biological treatments.

1.4.1 Physical and chemical treatments

Physical treatment is used in order to control oil spills. Therefore, barriers such as booms and devices called skimmers (Figure 1.9) are used along for oil recovery without changing their properties, ending with prevention of oil spillage. Adsorbent materials can also be added for conversion of liquid oil to semisolid phase (Dave and Ghaly, 2011). Other physical methods involves gravity separation, adsorption, membrane separation, reverse osmosis, nano, ultra and microfiltration (Singh *et al.*, 2017). Although physical treatments help to control oil spreading, they also have many limitations. Booms are very sensible to strong winds and high waves while adsorbent materials are whether expensive, non-biodegradable or difficult to operate (Dave and Ghaly, 2011).

Figure 1.9 Application of booms (left), booms and skimmer (middle) and plane dropping oil-dispersing chemical (right) (NOAA, 2015)

Chemical treatment involves precipitation, electrochemical processes and advanced oxidative processes where large amount of chemicals are handled (Figure 1.9) (Singh *et al.*, 2017). Chemicals used with combination to physical treatments are dispersants and solidifiers. Dispersants are efficient but have a high operation and maintenance cost. Furthermore, chemicals can result with extra contamination and cause serious damages to the environment (Dave and Ghaly, 2011).

1.4.2 Biological treatment

The increasing costs and limited efficiency of physico-chemical treatments have driven attention to alternative technologies (Varjani, 2017). Biological treatments involves activated sludges, trickling filters, sequencing batch reactors, chemostat reactors, biological aerated filters and bioremediation (Singh *et al.*, 2017).

1.4.2.1 Bioremediation

Bioremediation is a process that microorganisms and their enzymes are used to degrade or reduce hazardous organic pollutants to less toxic or harmless bio products such as carbon dioxide, water, heat and cell biomass (Varjani, 2017). Various organisms such as archaea, bacteria, algae and fungi are known for their bioremediation capacities. Plants can also be used for removal of contaminants through phytoremediation (Sharma *et al*., 2018).

There are three basic methods of bioremediation: natural attenuation, biostimulation, and bioaugmentation. Natural attenuation is the degradation of contaminants by indigenous microorganisms (Dzionek *et al*., 2016). Although this method is reverting the ecosystem to its original without affecting the habitat, the disadvantage is the slow degradation rate (Sharma *et al*., 2018). In order to increase bioremediation efficiency, the process called bioaugmentation is applied, where specific degraders are added to supplement the existing microbial population. However, this process may not be favorable because of the competition for nutrients between indigenous and exogenous microorganisms (Dzionek *et al*., 2016). Another alternative is adding nutrients or other growth-limiting substrates for accelerating the removal of contaminants, a method known as biostimulation (Das and Chandran, 2011).

Microorganisms are sensitive to growth environment and respond to changes that ends up effecting their biodegradation activity. Biodegradation rates depends on many factors such as physico-chemical properties of the pollutant (availability, volatility, type and length of hydrocarbon), environmental conditions (pH, temperature, nutrition factors, salinity, oxygen etc.) and to microorganisms and their cell metabolic pathways (Varjani, 2017).

1.5 Mechanism of kerosene degradation

Key agents responsible in petroleum hydrocarbon degradation are; bacteria, dominant in marine ecosystems, and fungi crucial in freshwater and terrestrial ecosystems (Olajire and Essien, 2014). They both have a versatile metabolism (Rojo, 2009) that uses petroleum products as a carbon and energy source. Degradability of hydrocarbons depends on their ring number and molecular size that affects their hydrophobicity and sorption capacity (Varjani, 2017). Degradation order of hydrocarbons is given in Figure 1.10 with respect to decreasing susceptibility.

Figure 1.10 Biodegradability of hydrocarbons

As mentioned previously, approximately 70% of kerosene is formed of alkanes and cycloalkanes (Ziadabadi and Hassanshahian, 2016). Therefore, mechanism of kerosene degradation will be explained under alkane degradation.

The metabolic pathways of alkane degradation can be either aerobic where oxygen is utilized as the primary acceptor or anaerobic in which an alternative electron acceptor is utilized, such as nitrate or sulfate (Singh *et al.*, 2017). Compared to anaerobic, aerobic degradation is much faster and more effective due to less free energy for initiation and energy yield per reaction (Olajire and Essien, 2014).

1.5.1 Aerobic degradation

The aerobic degradation of alkane, such as all type of hydrocarbons, starts with the oxidation of the substrate molecules by specific enzymes for alcohol production. Alcohols are further oxidized and broken to smaller molecules that are used in central intermediary metabolism. Finally, produced metabolites leads to biosynthesis of cell biomass as summarized in Figure 1.11 (Olajire and Essien, 2014)

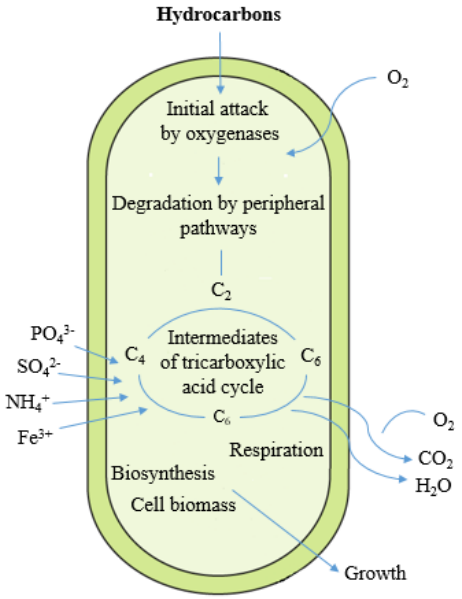


Figure 1.11 Aerobic degradation of hydrocarbons (adapted from Olajire and Essien, 2014)

Alkanes degradation can be classified as terminal and sub-terminal. Terminal methyl group oxidation occurs by alkane hydroxylases and produces primary alcohols. Further, alcohols are oxidized to an aldehyde by alcohol dehydrogenases (Abbasian *et al.*, 2015). Aldehyde dehydrogenases converts aldehyde to a fatty acid, followed by addition of CoA through acyl-CoA synthetize ending up with acetyl-CoA production (Olajire and Essien, 2014) (Figure 1.12).

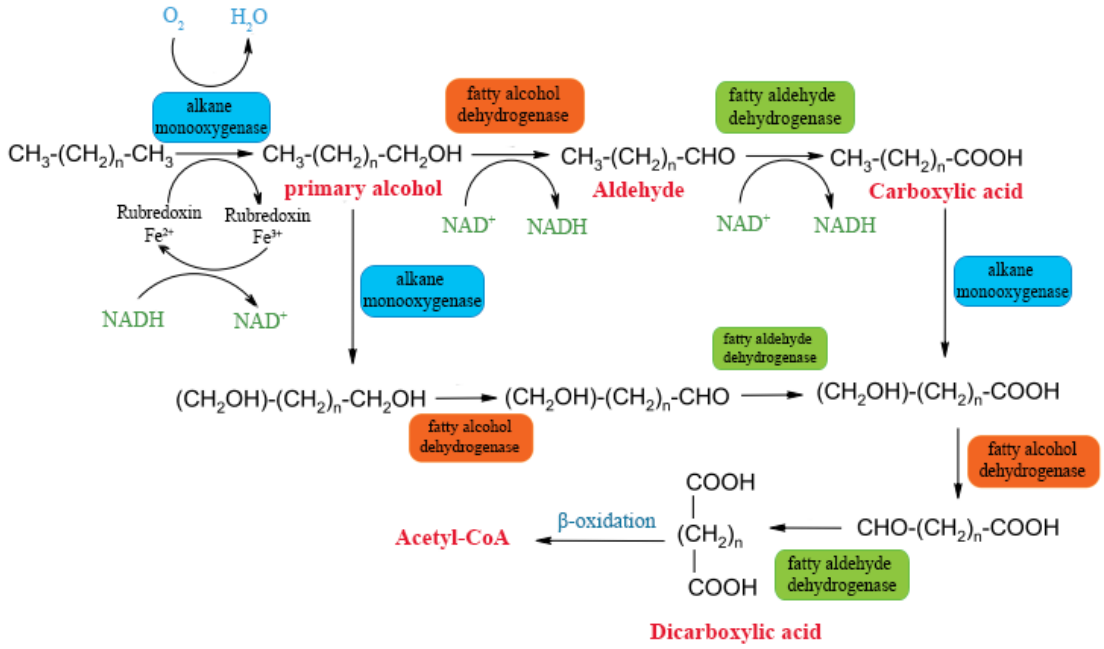


Figure 1.12 The terminal oxidation of n-alkanes to fatty acid catalyzed by bacterial enzymes (adapted from Olajire and Essien, 2014)

In sub-terminal oxidation, a secondary alcohol is transformed to a ketone by a monooxygenase and converted to an ester. Esterase hydrolyses esters to form alcohol and a fatty acid (Figure 1.13). Terminal and sub-terminal oxidation can co-occur in some microorganisms (Rojo, 2009).

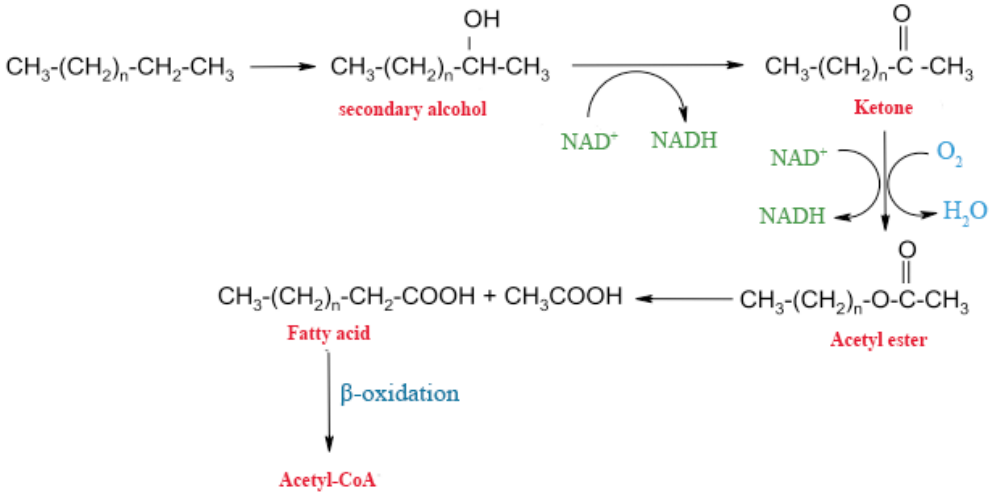


Figure 1.13 Sub-terminal oxidation of n-alkanes (Olajire and Essien, 2014)

1.5.2 Anaerobic degradation

Various organisms are able to use alkanes as carbon source in the absence of O_2. Anaerobic degradation of alkanes occurs in two different ways (Rojo, 2009). First, alkanes are added to the double bond of fumarate, producing alkyl succinate that further enters to β-oxidation (Rojo, 2009), a process performed by denitrifying and sulfate reducing bacteria (Figure 1.14). Secondly, mycobacterium have the ability to degrade multibranched saturated hydrocarbons through putative pathways where squalene is converted to a dionic acid, entering to pristine pathway forming 3,7,11-trimethyldodecandioic acid and further degraded by β-oxidation route (Singh et al., 2012).

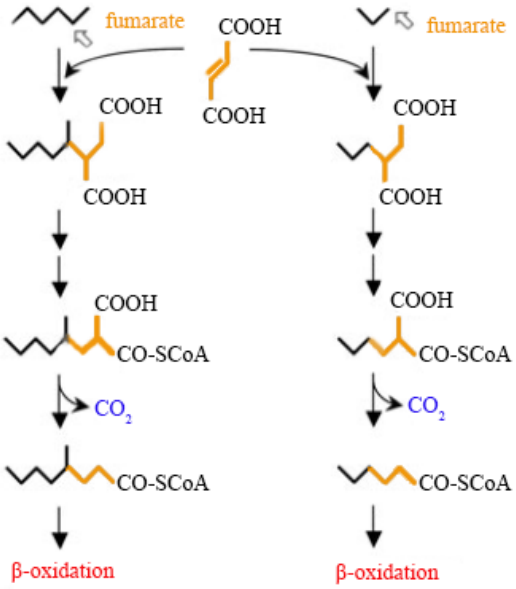


Figure 1.14 Anaerobic degradation of alkanes (Rojo, 2009)

1.6 Microorganisms involved in kerosene degradation

As mentioned above, kerosene is a mixture of hydrocarbon comprised of 75% aliphatic and 25% aromatic hydrocarbons (Bacosa *et al.*, 2010). Therefore, no single microorganism has been found to completely degrade kerosene alone. Some microorganisms have ability to degrade aliphatics, some can degrade aromatics while others degrade resins. Varjani (2017) reported *Achromobacter*, *Acinetobacter*, *Arthrobacter*, *Azoarcus*, *Brevibacterium*, *Cellulomonas*, *Corynebacterium*, *Flavobacterium*, *Marinobacter*, *Micrococcus*, *Nocardia*, *Ochrobactrum*,

Pseudomonas, *Stenotrophomaonas* and *Vibrio* as hydrocarbon degrading bacteria. As shown, many studies are available on the bacterial degradation of hydrocarbons in literature, but studies on kerosene degradation is scarce (Khan *et al.*, 2015). Therefore, a list of studies on kerosene degradation by different bacteria has been revised in Table 1.1

Table 1.1 List of kerosene degrading bacteria

Bacteria	References
Achromobacter	Bacosa, Suto and Inoue, 2010
Acinetobacter	Umanu and Babade, 2013
	Anienye, Ijah and Nnamdi, 2015
Aerobacter	Nwinyi and Victory, 2014
Alcaligenes	Bacosa, Suto and Inoue, 2010
	Umanu and Babade, 2013
	Anienye, Ijah and Nnamdi, 2015
Bacillus	Nwinyi and Victory, 2014
	Anienye, Ijah and Nnamdi, 2015
Bacillus cereus	Borah and Yadav, 2017
Bacillus subtilis	Nwinyi and Victory, 2014
Burkholderia	Bacosa, Suto and Inoue, 2010
Citrobacter sedlakii	Ghoreishi *et al.*, 2017
Cupriavidus	Bacosa, Suto and Inoue, 2010
Enterobacter cloacae	Ghoreishi *et al.*, 2017
Enterobacter hormeachai	Ghoreishi *et al.*, 2017
Gordonia	Gouda *et al.*, 2007
Micrococcus	Umanu and Babade, 2013
	Anienye, Ijah and Nnamdi, 2015
Nocordia hydrocarbonoxydans	Kalme *et al.*, 2008
Pseudomonas	Gouda *et al.*, 2007
	Umanu and Babade, 2013
	Anienye, Ijah and Nnamdi, 2015
Pseudomonas desmolyticum	Kalme *et al.*, 2008
Pseudomonas fluorescens	Nwinyi and Victory, 2014
Rhodococcus	Nwinyi and Victory, 2014
Serratia	Umanu and Babade, 2013

Fungi capable of degrading hydrocarbons has also been studied and can be listed as *Aspergillus*, *Amorphoteca*, *Fusarium*, *Graphium*, *Neosartoria*, *Paecilomyces*, *Penicillium*, *Sporobolomyces*, *Talaromyces* and some yeast of genera *Candida*, *Pichia*, *Pseudozyma Rhodotorula* and *Yarrowia* (Varjani, 2017). Kerosene degrading abilities of some hydrocarbon degrader fungi and yeast has been tested and a summary of studies found in literature is given in Table 1.2.

Table 1.2 List of kerosene degrading yeast and fungi

Yeast and fungi	References
Aspergillus	Umanu and Babade, 2013
	Anienye, Ijah and Nnamdi, 2015
	Khan *et al.*, 2015
Aspergillus niger	Adekunle and Adebambo, 2007
	Hasan, 2014
Candida	Umanu and Babade, 2013
	Khan *et al.*, 2015
Cladosporium	Khan *et al.*, 2015
Fusarium	Anienye, Ijah and Nnamdi, 2015
Mucor	Anienye, Ijah and Nnamdi, 2015
Penicillum	Umanu and Babade, 2013
	Anienye, Ijah and Nnamdi, 2015
Penicillum janthinellum	Khan *et al.*, 2015
Rhizopus	Adekunle and Adebambo, 2007
Rhodotorula	Umanu and Babade, 2013
Trichoderma	Umanu and Babade, 2013

1.7 Genes involved in kerosene degradation

Depending on the alkanes chain-length, different enzyme systems are utilized by microorganisms responsible in oxidation of substrate to initiate biodegradation (Varjani, 2017). There are three major enzymes responsible in alkane degradation. Methane to butane (C_1–C_4) is oxidized by methane monooxygenase-like enzymes. Pentane to hexadecane (C_5-C_{16}) is oxidized by integral membrane non-heme iron (alkane hydroxylases) or cytochrome P450 enzymes, mostly found in fungi and in few bacteria (Van Beilen and Funhoff, 2007). Several bacterial isolates has enzymes responsible in oxidation of alkanes longer than C_{20} but those enzyme systems are still unknown (Rojo, 2009). Because kerosene structure contains hydrocarbons between C_9 to C_{22}, key enzymes involved in kerosene degradation are alkane hydroxylases. This enzyme is composed of a hydroxylase found in the cell membrane and cytoplasmic proteins such as rubredoxin and rubredoxin reductase (Olajire and Essien, 2014). Gene responsible in alkane hydroxylation is encoded by *alk*B. The electrons needed for this process are delivered to alkane monooxygenase by a rubredoxin reductase and two rubredoxins which are encoded by *alk*T and *alk*F, *alk*G respectively (Rojo, 2009). Produced alcohol is further transformed to a fatty acid by alcohol dehydrogenase, an aldehyde dehydrogenase and an acyl-CoA synthetase encoded by genes *alk*J, *alk*H and *alk*K respectively, followed by β-oxidation (Abbasian *et al.*, 2016).

The alkane-degradation gene clusters may be plasmid encoded but mostly they are located in the chromosome (Varjani, 2017). The pathway for alkane degradation has been extensively studied in *Pseudomonas putida* GPo1, which reserves two gene clusters encoding enzymes responsible in conversion of n-alkanes to fatty acids (Rojo, 2009). Genes are organized as *alk*BFGHJKL and *alk*ST, located end to end on a large plasmid named OCT plasmid (Van Beilen *et al.*, 2001). *alk*BFGHJKL genes are regulated by *alk*ST and two loci are transcribed towards each other. Additively, *P. putida* has *alk*L gene providing the importation of n-alkanes into the bacterial cells (Canosa *et al.*, 2000). Position and role of alkane-degrading proteins in *P.putida* is summarized in Figure 1.15.

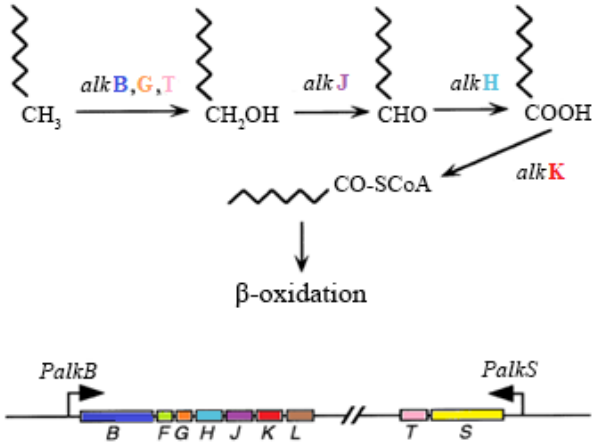


Figure 1.15 Degradation of medium chain length alkanes by genes found in the OCT plasmid (above) and genes clustered in two, *alk*S as transcriptional regular (below) (Canosa *et al*., 2000)

Usually only one *alk*B gene is found in the genome, but several Gram-positive and Gram-negative genera may contain more than one *alk*B genes, as seen in genus *Rhodococcus* and *Acinetobacter* (Viggor *et al.*, 2015). *Acinetobacter* sp. strain M1 has two *alk*B related (*alk*Ma and *alk*Mb) alkane hydroxylases, regulated depending on the alkane present in the medium. Expression of *alk*Ma, is controlled by a regulator *alk*Ra and induced by alkanes having a very long chain length (C_{22}), while *alk*Mb is controlled by *alk*Rb and induced in the presence of C_{16}–C_{22} alkanes (Abbasian *et al.*, 2016).

It is important to show the activity of alkane hydroxylases since *alk*B genes are used as biomarkers for the determination of the abundance and diversity of alkane-degrading bacteria. For that reason, polymerase chain reaction (PCR) is a method used,

where specific primers are designed for detection of marker catabolic genes. As an example, in the study of Jurelevicus *et al.* (2013), combination of *alk*B primers was used to enhance the detection of the *alk*B gene for determination of alkane-degrading bacteria in contaminated environments by use of PCR analysis.

1.8 Biosurfactants and their use in bioremediation

Biosurfactants are surfactants synthesized as secondary metabolites (Varjani and Upasani, 2017) by different microorganisms such as bacteria, yeasts and filamentous fungi. These compounds have amphipathic molecules and are capable of displaying a variety of surface activities that helps solubilizing hydrophobic substrates (Khan and Butt, 2016). Excreted biosurfactants organize their monomers spherically by forming micelles (Satpute *et al.*, 2010). While hydrophobic part is turned to the center, forming a nucleus; hydrophilic part turns to the sphere surface leading to reduction of surface tension and interfacial tension (Souza *et al.*, 2014). Surfactants have other functional properties such as emulsification, wetting, adsorption, foaming, cleansing, and phase separation (Satpute *et al.*, 2010).

Surface activity is an important property for biosurfactants. Water molecules are held together due to cohesive forces (Mnif and Ghribi, 2015). The force per unit length exerted by a liquid in contact with a solid or another liquid is called as surface tension Satpute *et al.*, 2010) while force held within the molecules in a liquid is referred as interfacial tension (Varjani and Upasani, 2017) (Figure 1.16a). An equipment called tensiometer is used to measure both values (Satpute *et al.*, 2010). For example, water has a surface tension value of 72 mN/m that is the highest surface tension value among known liquids. Depending on the efficiency of the surfactant, this value decreases with their addition to the solution (Sáenz-Marta *et al.*, 2015). Another important property of surfactants is their emulsification activity (Banat *et al.*, 2000), dispersion of liquids into each other, allowing emulsion formation of two immiscible liquids such as oil and water (Figure 1.16b). The initial value where surfactant can form micelles is named as critical micelle concentration (CMC) (Figure 1.16c). It is obtained by the measurements of the surfactant solution prepared in several dilutions. Below CMC, surfactants are in monomer form while at CMC, surfactants start to form micelles (Mnif and Ghribi, 2015) and end up with changed physical properties such as conductivity, viscosity, density etc. (Satpute *et al.*, 2010)

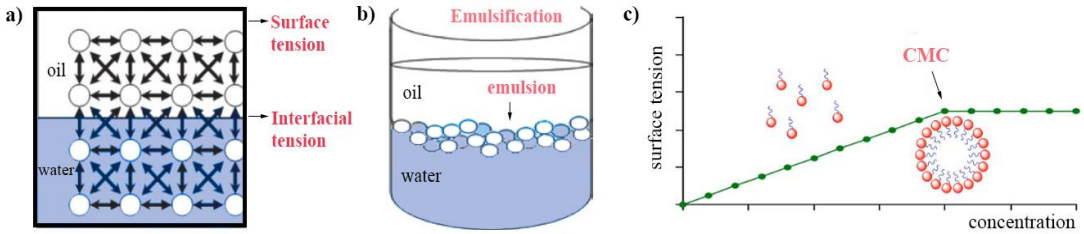


Figure 1.16 Properties of surfactants. (a) Surface tension and interfacial tension, (b) Emulsification (c) Critical micelle concentration (CMC) and micelle formation (modified from Satpute *et al*., 2010)

Biosurfactants can enhance biodegradation rate by two mechanisms (Das and Chandran, 2011). First, they can increase the bioavailability of substrate to microorganisms (Banat *et al.*, 2010). Bacteria growth rate on hydrocarbons can be limited due to interfacial tension between water and oil. When the surface area of microorganisms with hydrophilic solvents like water is limiting, biomass increases arithmetically rather than exponentially (Sáenz-Marta *et al.*, 2015). Biosurfactants are released to the environment and start forming micelles, which end up facilitating the uptake of hydrophobic substrates (Das and Chandran, 2011). As growth on hydrophobic surfaces increases, enhancement in biodegradation activity is observed (Franzetti *et al.*, 2010).

The second mechanism involves biosurfactants affecting the cell surface properties (Souza *et al*., 2014). Produced biosurfactants can bound to cell wall and reduce the lipopolysaccharide index of the wall without damaging the membrane, which leads to a more hydrophobic cell surface. As cell hydrophobicity increases, it is easier for microorganisms to adhere hydrophobic compounds. This way, microorganisms can attach or detach from surfaces depending to their needs, giving them the ability to better degrade hydrophobic compounds (Sáenz-Marta *et al.*, 2015).

1.9 Advantages of biosurfactants over synthetic surfactants

Biosurfactants have a wide range of biotechnological applications (Sobrinho *et al.*, 2013). Currently, the main market is the petroleum industry in which biosurfactants are used for bioremediation process, oil spill up operations, enhanced oil recovery (Banat *et al.*, 2010). In food industry, they are applied as emulsifiers in food products (Shekhar *et al*., 2015).

Biosurfactants can be applied for medical purposes (Khan and Butt, 2016) such as anti-adhesive agents and enzyme inhibitors in pharmaceutics and also in cosmetics (Banat *et al.*, 2010). They are also known to be exploit as washing detergents or as fertilizers for agricultural use (Santos *et al.*, 2016). Compared to synthetic surfactants, biosurfactants are preferred due to their advantages such as being biodegradable and generally having low toxicity (Banat *et al.*, 2010). They are also economic and can be produced by raw materials or industrial wastes that decreases the production cost. Due to their complex structure, biosurfactants are specific in their action and also effective at extreme temperature, pH and salinity conditions (Khan and Butt, 2016).

1.10 Classification of biosurfactants

Biosurfactants can be classified according to their ionic charge on their polar part as anionic, neutral, cationic or amphoteric (Rahman and Gakpe, 2008) or depending on their producer microorganism, their mode of action or their chemical composition (Sáenz-Marta *et al.*, 2015). Their hydrophobic moiety is characterised by long-chain fatty acids and the hydrophilic moiety may be formed by a carbohydrate, amino acid, cyclic peptide, phosphate, carboxyl acid or alcohol (Sobrinho *et al.*, 2013). Depending on their structure, they are gathered into five main groups as glycolipids, lipoproteins and lipopeptides, fatty acids, phospholipids and polymeric compounds (Figure 1.17) (Rahman and Gakpe, 2008).

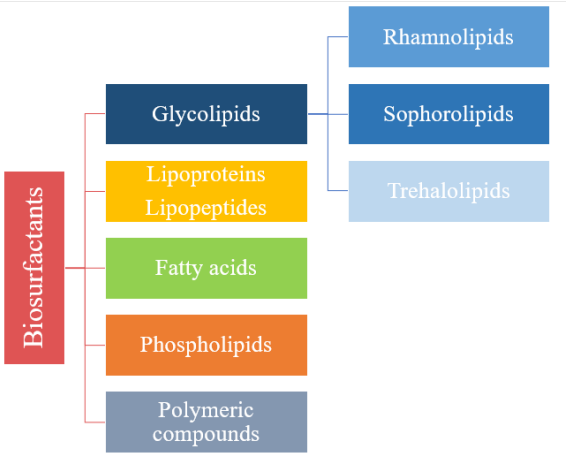


Figure 1.17 Classification of biosurfactants

1.10.1 Glycolipids

The best-studied microbial surfactants are glycolipids composed of mono, di, tri or tetrasaccharides attached to a fatty acid component. They can also consist carbohydrates in combination with aliphatic or hydroxyaliphatic acids (Rahman and Gakpe, 2008). Rhamnolipids, sophorolipids and trehalolipids are the best known glycolipids (Santos *et al.*, 2016).

1.10.1.1 Rhamnolipids

Glycolipid surfactants with one or two rhamnose and 3-hydroxy fatty acid chains are called rhamnolipid (Figure 1.18) (Dobler *et al.*, 2016). Approximately 60 rhamnolipid congeners and homologues have been found so far (Varjani and Upasani, 2017). The most common rhamnolipid producer is *Pseudomonas aeruginosa*. They produce effective surfactants, mostly used in removal of hydrophobic compounds from contaminated soils (Reis *et al.*, 2011) because of their ability to assimilate insoluble substrates. Rhamnolipids are also known in changing the hydrophobicity of cells surface and have different roles including antimicrobial or hemolytic activity in human pathogenesis. Furthermore, in *Pseudomonas*, rhamnolipids work as a quorum sensing molecule and promote swarming motility (Reis *et al.*, 2011).

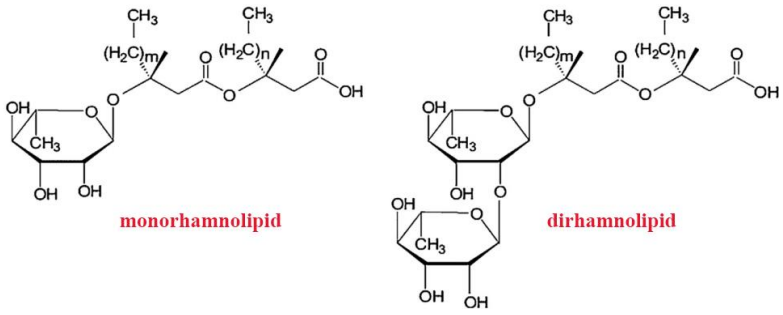


Figure 1.18 Structure of rhamnolipid (Dobler *et al.*, 2016)

Rhamnose is a component of the cell wall lipopolysaccharide and exopolysaccharide in a variety of Gram-negative bacteria, mostly found in *Pseudomonas* strain (Rahim *et al.*, 2000).

For rhamnose production, D-glucose-6-phosphate is converted into D-glucose-1-phosphate by the phosphoglucomutase (AlgC) and is followed by the *rml*BDAC operon gene products (Figure 1.19). Glucose-1-phosphate thymidylyltransferase (RmlA) catalyzes the transfer of a thymidylmonophosphate nucleotide to glucose-1-phosphate with following reactions leading to dTDP-$_L$-rhamnose biosynthesis. Presence of dTDP-$_L$-rhamnose inhibits the activity of RmlA (Dobler *et al.*, 2016).

Rhamnolipid synthesis proceeds by two sequential glycosyl transfer reactions, each catalysed by a different rhamnosyltransferase (Das *et al*., 2008). Rhamnosyltransferase 1 (RhlA and RhlB) are encoded by the *rhl*A and *rhl*B. Both genes are co-expressed from the same promoter (*rhl*AB) and are essential for rhamnolipid synthesis. RhlA catalyses the synthesis of the fatty acid dimer moiety of rhamnolipids and free 3- (3-hydroxyalkanoyloxy) alkanoic acid (Figure 1.19). Sequently, RhlB uses dTDP-$_L$-rhamnose and hydroxyalkanoyloxy alkanoic acid molecule as precursors for production of monorhamnolipid (Varjani and Upasani, 2017). Finally, *rhl*C encodes rhamnosyl transferase 2 (RhlC) that uses monorhamnolipid and dTDP-$_L$-rhamnose as substrate for dirhamnolipid production (Dobler *et al.*, 2016).

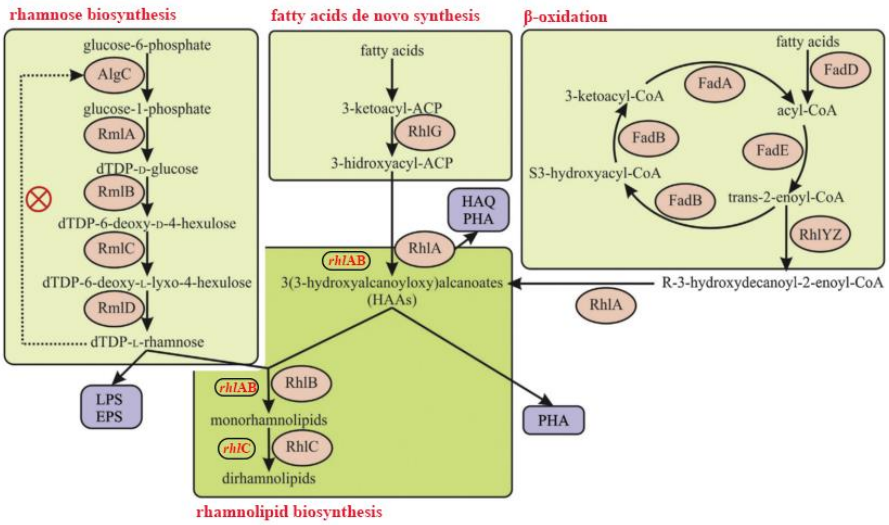


Figure 1.19 Biosynthesis of rhamnolipid (Dobler *et al.*, 2016)

1.10.1.2 Sophorolipids

Sophorolipids are mostly produced by yeasts *Candida* (Santos *et al.*, 2016) and composed of a sophorose disaccharide linked to a long chain hydroxyl fatty acid (Figure 1.20) (de Oliveira *et al.*, 2015). Sophorolipids can be categorized as acidic and lactonic. Acidic forms have a free fatty acid tail and are efficient foaming agents with high water solubility (Gakpe et al., 2007). Therefore, they are mostly applied in food industry, bioremediation and cosmetics. Lactonic forms contains a sophorose head connected to the fatty acid tail. They are more hydrophobic compared to acidic sophorolipids and are known to perform biocide activities (de Oliveira *et al.*, 2015).

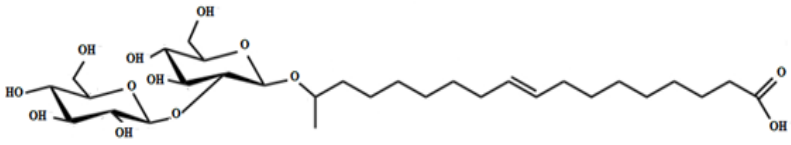


Figure 1.20 General structure of sophorolipids (de Oliveira *et al.*, 2015)

There are five enzymes involved in sophorolipid synthesis; cytochrome P450 monooxygenase, two glycosyltransferases, an acetyltransferase and a transporter (Figure 1.21) (Van Bogaert *et al.*, 2013). CYP52 monooxygenase, a sub family of P450s, is responsible in formation of hydroxyl fatty acids (Huang et al., 2014). Sequently, one of the UDP-glucose dependent transferases, UgtA1, catalyzes the coupling of glucose to hydroxylated fatty acid, forming a glucolipid, while the other transferase UgtB1, uses the glucolipid as an acceptor to form a sophorolipid molecule (Van Bogaert *et al.*, 2013). Acetyltransferase mediates the acetylation of the sophorose. In some cases, lactonization of sophorolipids may occur by the action of a cell wall-bound lactonesterase (de Oliveira *et al.*, 2014). Finally, sophorolipids are excreted by a transporter which is believed to be a multidrug resistance protein encoded by *mdr* gene. Yet, the function of this gene has never been confirmed (Van Bogaert *et al.*, 2013).

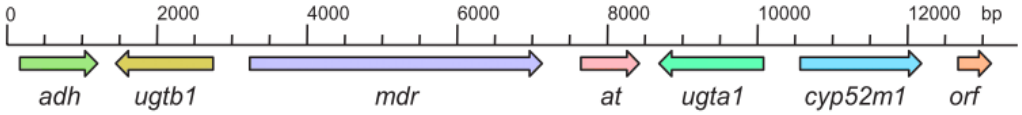


Figure 1.21 Gene clusture of sophorolipid from *Candida bombicola*. *adh*: alcohol dehydrogenase, *ugtB1*: second glucosyltransferase, *mdr*: transporter, *at*: acetyltransferase, *ugtA1*: first glucosyltransferase; *cyp52m1*: cytochrome P450 monooxygenase; *orf*: open reading frame (function unknown)

1.10.1.3 Trehalolipids

Trehalose lipids are made of a carbohydrate group and fatty acids groups (Franzetti *et al.*, 2010). Trehalose is a dissacharide composed of two glucose bond with a glycosidic linkage. It is most commonly produced by *Mycobacterium*, *Norcardia*, *Gordonia* and *Corynebacterium* (Franzetti *et al.*, 2010). Also, different types of trehalose are associated with *Rhodococcus erythropolis* and *Arthrobacter* sp.. They are known to lower significantly surface and interfacial tension of culture broths (Rahman and Gakpe, 2008). The most reported trehalose lipid is trehalose dimycolate (Figure 1.22), a cord factor found in the cell wall of *Mycobacterium tuberculosis* (Franzetti *et al.*, 2010).

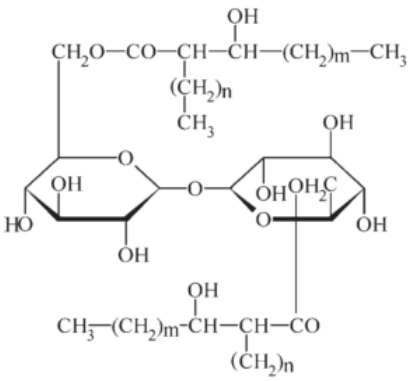


Figure 1.22 Trehalose dimycolate, trehalose esterified to two mycolic acid residues (Franzetti *et al.*, 2010).

In the study of Inaba *et al*. (2013), essential genes for succinoyl trehalose lipids production were determined. The *alk*B gene, encoding alkane monooxygenase converts alkanes to alcohol since alkane oxidation is essential for the initial steps in the succinoyl trehalose lipids biosynthesis.

Second, the gene *fda* works as a gluconeogenesis enzyme and synthesis trehalose from n-alkane. Finally, the gene *tls*A is an acyl-CoA transferase, responsible in transferring fatty acids to trehalose or its derivatives in the final step of the biosynthesis (Figure 1.23).

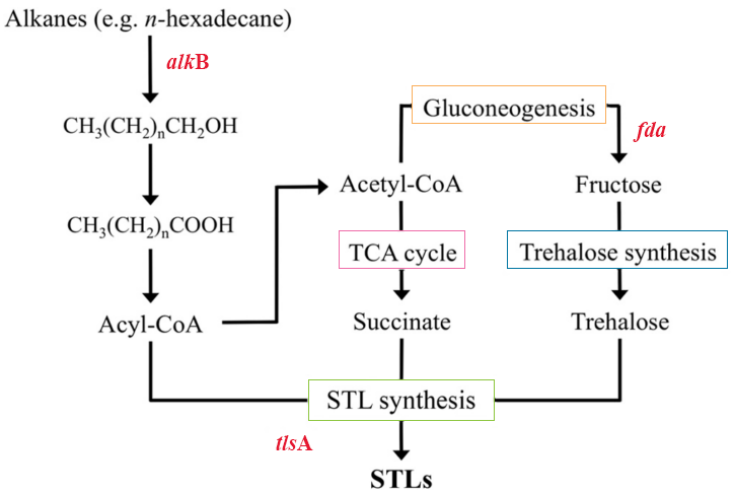


Figure 1.23 Biosynthetic pathway of succinoyl trehalose lipids from *Rhodococcus* spp., TCA**:** tricarboxylic acid, STL: succinoyl trehalose lipids (Inaba *et al.*, 2013)

1.10.2 Lipoproteins and lipopeptides

They are composed of a large number of short linear chains or cyclic amino acids bonded to a fatty acid with ester and/or amide bonds (Shah *et al.*, 2016). Some bacteria are known to produce biosurfactants with antimicrobial action such as surfactin, produced by *Bacillus subtilis* (Khan and Butt, 2016). Besides having high activity in surface tension reduction, they can also lyse mammalian erythrocytes and form spheroplasts (Shekhar *et al.*, 2015). Surfactin contains seven amino acids linked with the number of carbon atoms between 13-16 and forms a ring structure (Figure 1.24) (Banat *et al.*, 2010). Iturin, fengycin and kurstatin are also lipopeptide type biosurfactants produced by the *Bacillus* family, revealing antifungal activities (Mnif and Ghribi, 2015). Other examples of lipopepides are viscosin and lichenysin having similar properties as surfactin (Varjani and Upasani, 2017).

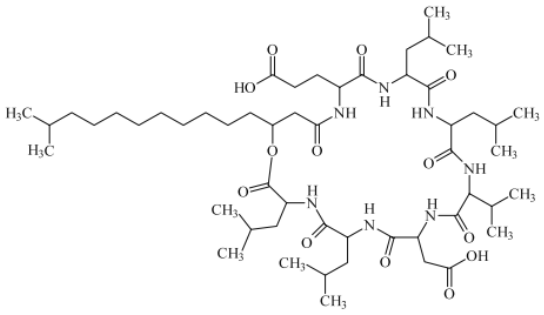


Figure 1.24 Structure of surfactin (Banat *et al.*, 2010)

Surfactin synthetase is responsible in surfactin biosynthesis and composed of three enzymes; SrfA, SrfB and SrfC (Das *et al.*, 2008). The peptide synthetase is necessary for production of amino acid moiety of surfactin. It is encoded by four open reading frame (ORFs) in the *srf*A operon namely SrfAA, SrfAB, SrfAC and SrfA-TE (Jiang *et al.*, 2016). This enzyme links amino acids by ester and amide bonds. Another essential gene is *sfp*, encoding phosphopantetheinyl transferase. It is responsible in the activation of surfactin synthetase, located in the downstream of the *srf*A operon. Finally, an acyl transferase is required to transfer a hydroxy fatty acid to the first amino acid in the peptide (Porob *et al.*, 2013). The surfactin biosynthesis gene cluster is summarized in Figure 1.25.

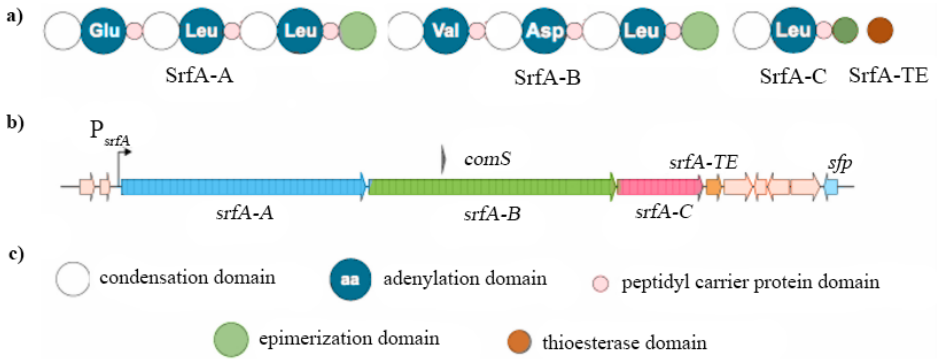


Figure 1.25 The surfactin synthetases A consisting of SrfA-A, SrfA-B, SrfA-C and SrfA-TE (a) modular organization of the surfactin synthetases encoded by the *srf*A operon and *comS* as regulator (b) *srf*A operon is subdivided into five functional domains (c) (Jiang *et al.*, 2016)

1.10.3 Fatty acids and phospholipids

Various bacteria and yeast have the ability to produce large amounts of fatty acids and/or phospholipid surfactants from n-alkanes during microbial oxidation *(Cortés-Sánchez et al*., 2013). Both are major contents of the cell structure, possessing efficient surface activity (Santos *et al.*, 2016). A complex fatty acids containing -OH groups and alkyl branches called Corynomucolic is produced by *Corynebacterium lepus* and *Thiobacillus thiooxidans* (Rahman and Gakpe, 2008). They are known with their phospholipids which are necessary as wetting agent during bacterial growth on sulphur (Silva *et al.*, 2014).

1.10.4 Polymeric compounds

The most studied polymeric biosurfactants are emulsan, liposan, mannoprotein (Rahman and Gakpe, 2008). Emulsan is a powerful bioemulsifier and an efficient emulsion stabilizer, synthesized from *Acinetobacter calcoaceticus*. Liposan is an extracellular product of *Candida lipolytica*. It is composed of 17% protein and 83% carbohydrate including glucose, galactose, galactosamine, galactoronic acid *(*Silva *et al.,* 2014*),* mainly used as emulsifier in food and cosmetic industries (Santos *et al.*, 2016). Mannoprotein from *Saccharomyces cerevisiae* displays significant emulsifying activity towards oils, alkanes and organic solvents. Other polymeric biosurfactants are alasan and biodispersan applied as food and insectides emulsifiers (Khan and Butt, 2016). The *wee* gene cluster is essential for emulsan biosynthesis (Dams-Kozlowska *et al.*, 2008) (Figure 1.26). The ORFs encodes putative enzymes catalyzing the production of nucleotide amino sugar precursors and polymer transport proteins, transglycosylation, transacetylation and polymerization (Nakar and Gutnick, 2001).

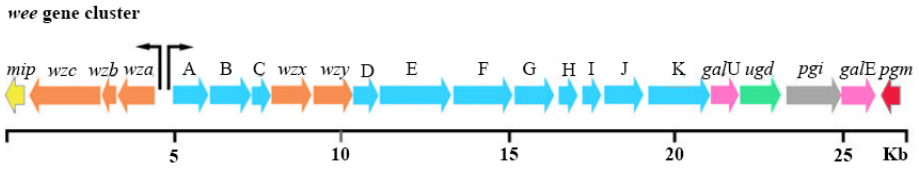


Figure 1.26 Capital letters: putative emulsan specific proteins. *wee*A-K and other genes code proteins as *mip:* macrophage infectivity potentiator, *wzc:* tyrosine kinase, *wzb:* tyrosine phosphatase, *wza:* outer-membrane lipoprotein, *wzx:* flippase, *wzy:* polymerase, *gal*U: UTP-glucose-1- phosphate uridylyltransferase, *ugd*: UDP-glucose dehydrogenase, *pgi*: glucose-6-phosphate isomerase, *gal*E: UDP-glucose 4-epimerase, *pgm*: phosphoglucomutase (Nakar and Gutnick, 2001).

The initial step of emulsan biosynthesis starts with the formation of UDP-N-acetyl-d-glucosamine from l-fructose-6- phosphate by *glm*S, *glm*M and *glm*U. Reaction continuous with GalE, converting UDP-N-acetyl-d-glucosamine to UDP-N-acetyl-d-galactosamin by 4-epimerase in order to obtain one of the nucleotide sugar precursors of emulsan (Nakar and Gutnick, 2001). *wee*A and *wee*B are involved in the production of UDP-N-acetyl-l-galactosaminuronic acid while *wee*H, D and G transfers the three activated nucleotide sugars of emulsan to GDP-galactose to the undecaprenyl phosphate (Dams-Kozlowska *et al.*, 2008). *wee*C and *wee*I are involved in transacylation of polysaccharide backbone. Finally, *wzx* catalyses the translocation while *wzy* is implicated in polymerization of the repeat unit. Biosynthesis ends with the exportation of emulsan through three ORFs (*wza*, *wzb* and *wzc*) (Figure 1.27) (Nakar and Gutnick, 2001).

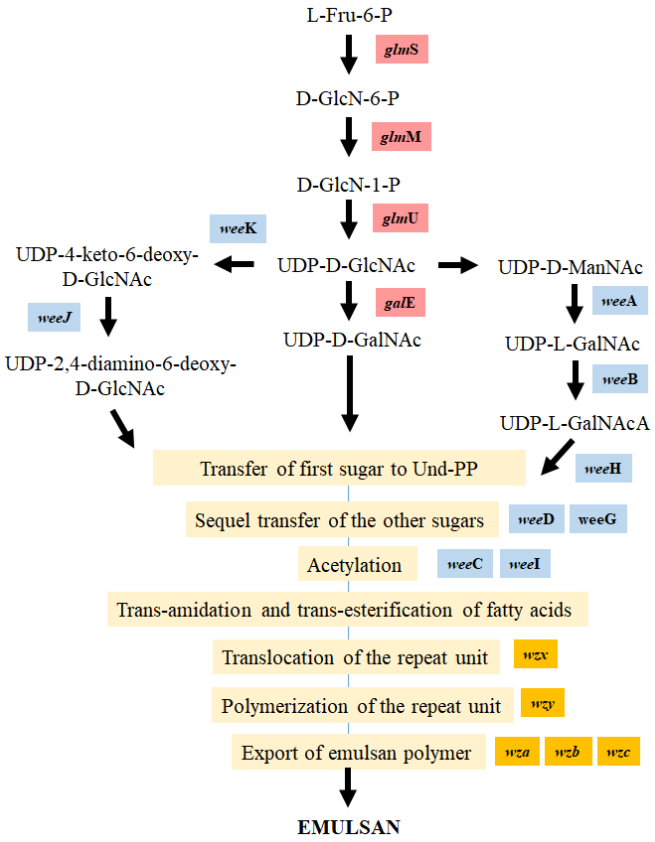


Figure 1.27 Biosynthetic pathway of emulsan. Fru: Fructose, GlcN: glucosamine, GlcNAc: N-acetylglucos- amine, ManNAc: N-acetylmannosamine; GalNAc: N-acetylgalactosamine, GalNAcA: N-acetylgalactosamine uronic acid (Nakar and Gutnick, 2001)

1.11 Biosurfactant producing microorganisms

Interest for biosurfactant production has increased in recent years, especially isolation of microorganisms capable of producing surfactants with low CMC, low toxicity and high emulsifying activity (Santos *et al.*, 2016). Various microorganisms produce biosurfactants with different molecular structures. The literature describes bacteria of the genera *Pseudomonas*, *Acinetobacter*, *Bacillus* and *Rhodococcus* as great biosurfactant producers (Sáenz-Marta *et al.*, 2015) while *Candida bombicola* and *Candida lipolytica* are among the most reported biosurfactant producer yeasts (Santos *et al.*, 2016). Table 1.3 offers a summary of the main classes of biosurfactants and respective producer microorganisms described in the literature.

Table 1.3 Some of the biosurfactants produced by various microorganisms (Sobrinho *et al.*, 2013; Singh *et al.*, 2017)

Type of biosurfactants		**Microorganism**
Glycolipids	Rhamnolipid	*Pseudomonas aeruginosa, Pseudomonas* sp.
	Sophorolipid	*Torulopsis bombicola, Torulopsis apicola, Torulopsis petrophilum, Candida apicola, Candida bambicola, Candida lipolytica, Candida bogoriensis*
	Trehalolipid	*Rhodococcus erythropolis, Nocardia erythropolis, Arthrobacter., Mycobacterium*
Lipopeptide and lipoproteins	Peptide-lipid	*Bacillus licheniformis*
	Serrawettin	*Serratia marcescens*
	Viscosin	*Pseudomonas fluorescens*
	Surfactin	*Bacillus subtilis*
	Lichenysin	*Bacillus licheniformis*
Fatty acids and phospholipids	Neutral lipids	*Nocardia erythropolis*
	Phospholipids	*Thiobacillus thiooxidans*
	Fatty acids	*Candida lepus, Acinetobacter* sp., *Pseudomonas* sp., *Micrococcus* sp., *Mycococcus* sp., *Candida sp.*, *Penicillium* sp., *Aspergillus* sp
Polymeric compounds	Emulsan	*Acinetobacter calcoaceticus*
	Biodispersan	*Acinetobacter calcoaceticus*
	Mannan lipid protein	*Candida tropicalis, Saccharomyces cerevisiae*
	Liposan	*Candida lipolytica*
	Alasan	*Acinetobacter radioresistens*

Some microorganisms may produce various type of biosurfactants since the carbon source used for biosurfactant production affects the structure of the compound (Sáenz-Marta *et al.*, 2015). Nowadays, the main focus is turned for isolating novel biosurfactant producers, capable to grow on low cost substrates (Sobrinho *et al.*, 2013). Today's worldwide biosurfactant production is bigger than three million tonnes per year (about US $4 billion) and is expected to be greater than over four million tonnes by the end of the century (Khan and Butt, 2016).

1.12 Methods for screening and characterization of biosurfactants

There are various methods for detection and characterization of biosurfactants in literature. Oil spreading activity is a common test for determination of surfactant production. For this assay, oil is added to the surface of distilled water in order to form a thin oil layer. When culture is placed on the center of the oil layer, in presence of surfactant a clear halo zone is formed. By measuring the zone diameter, surfactant activity is determined due to the linear correlation between quantity of surfactant and clearing zone diameter (Morikawa *et al.*, 1993). As mentioned previously, emulsification activity is an important characteristic for surfactants. For determination of emulsification activity, culture and oil are mixed and ends up forming a layer of emulsion. By measuring the height of emulsion, the emulsification index (E_{24}) of the surfactant can be obtained (Cooper and Goldenberg, 1987). Biosurfactants have also the ability to change cell structure and enhance cell attachments to hydrocarbons for their cellular uptake (Rosenberg, 2006). Therefore, a photometrical assay for measuring the hydrophobicity of bacteria named as Microbial Adhesion to Hydrocarbons (MATH) assay is used in order to detect the presence of biosurfactants (Sedláčková *et al.*, 2011).

Various methods in literature are applied for biosurfactants characterization after their extraction and purification steps. Broadly these methods involves; thin layer chromatography (TLC), GC and GC coupled with mass spectrometry (GC-MS), Fourier transform infrared spectroscopy (FTIR), electrospray ionization mass spectrometry (ESI-MS), high performance liquid chromatography (HPLC), liquid chromatography–mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) (Varjani and Upasani, 2017).

Thin layer chromatography is a method used both for detection of biosurfactants but also for their characterization (Satpute *et al.*, 2010). A technique that separates mixtures of substances into their components. It contains a mobile phase, which flows through the stationary phase and carries the components of the mixture with it. Different components travel at different rates depending on their properties such as their solubility in solvent or their adsorption to the stationary phase, allowing distinction of the components.

Infra-red spectroscopy (IR) determines the functional groups of gases, liquids and solids samples and gives a structural information of the compound by absorbing different IR frequencies of samples (Satpute *et al.*, 2010). Each compound has an energy that is differentiated by their vibrational and rotational states.

The most reliable method for detection of biosurfactant producers is the investigation of genes involved in their biosynthesis. PCR screening method is a widely used technique employed in the search for biosurfactant-producing isolates (Pacwa-Płociniczak *et al.*, 2014).

1.13 Aim of the study

Kerosene is a petroleum product industrially gathered through by the distillation of crude oil. It is one of the most commonly spilled petroleum product leading to contamination of the surface and ground waters. Kerosene pollution can be overcome through bioremediation, which is an attractive and environmentally friendly approach. One of the most important characteristics of hydrocarbon degrading bacteria is their ability to emulsify hydrocarbons by surface-active agents such as biosurfactants. Addition of biosurfactants increases the availability of kerosene to bacteria and renders them more accessible to bacterial enzyme system. Due to the increasing demand on microbial biosurfactants, the biosurfactants acting on kerosene are of great interest. Therefore, this study aimed at evaluation of kerosene degraders and their biosurfactant producing abilities to use in kerosene bioremediation.

CHAPTER 2

MATERIALS AND METHODS

2.1 Culture media

Culture media and their preparation steps are given below.

2.1.1 Nutrient agar

The amount of 20 g of Nutrient Agar (NA) (Merck, Germany) powder was dissolved in 1 L distilled water by heat and sterilized in autoclave for 15 min at 121°C. Once cooled down, the solution was distributed to petri dishes and left for solidification. For sterility conformation, prepared petri dishes were incubated for an overnight at 37°C before being used for bacterial cultivation. Culture mediums were stored in refrigerator at 4°C.

2.1.2 Nutrient broth

The amount of 8 g Nutrient Broth (NB) (Merck, Germany) was dissolved in 1 L distilled water. The solution was distributed to flasks and autoclaved for 15 min at 121°C for efficient sterilization. NB medium was stored in refrigerator at 4°C and routinely used.

2.1.3 Mineral salt broth

Mineral salt medium (MSM) was prepared by first dissolving 1.0 g $(NH_4)_2SO_4$, 0.02 g $CaCl_2$, 0.2 g $MgSO_4.7H_2O$, 1.0 g K_2HPO_4 and 1.0 g KH_2PO_4 in 1 L distilled water. The amount of 0.05 g $FeCl_3$ was dissolved separately in distilled water. Then, 2 drops of the solution were added to medium. After complete dissolving, pH was adjusted to 7.2 with dilute NaOH (Zhang *et al.*, 2005). The medium was sterilized by autoclaving

for 15 min at 121°C. MSM was used for degradation studies containing kerosene as the sole source of carbon.

2.1.4 Kerosene

Kerosene (purum) used in this study was purchased from Sigma-Aldrich (Germany).

2.2 Study area and sample collection

Previously in our laboratory, water samples were collected from the river Kızılırmak, next to the petrol refinery with the following coordinates; 39°22'16.39"N, 33°26'49.26"E, 890 m to 39°57'22.98"N, 33°25'04.35"E, 679 m. The bacterial isolates used in this study were previously determined as hydrocarbon degraders and identified by 16S rRNA sequencing in our laboratory (Table 2.1).

Table 2.1 List of bacterial isolates used in this study

Bacterial isolates	EMBL accession numbers	References
Pseudomonas plecoglossicida Ag10	KJ395363	Icgen & Yilmaz, 2014
Raoultella planticola Ag11	KJ395359	Koc *et al*., 2013
Staphylococcus aureus Al11	KJ395360	Yilmaz *et al*., 2013
Staphylococcus aureus Ba01	KJ395371	Yilmaz *et al*., 2013
Stenotrophomons rhizophila Ba11	KJ395362	Yilmaz *et al*., 2013
Delftia acidovorans Cd11	KJ209817	Icgen & Yilmaz, 2014
Staphylococcus warneri Co11	KJ395373	Yilmaz *et al*., 2013
Enterococcus faecalis Cr07	KJ395365	Icgen & Yilmaz, 2014
Pseudomonas koreensis Cu12	KJ395364	Icgen & Yilmaz, 2014
Acinetobacter calcoaceticus Fe10	KJ395366	Akbulut *et al*., 2014
Pseudomonas koreensis Hg10	KJ395377	Icgen & Yilmaz, 2014
Pseudomonas koreensis Hg11	KJ395378	Icgen & Yilmaz, 2014
Staphylococcus aureus Li12	KJ395370	Yilmaz *et al*., 2013
Serratia nematodiphila Mn11	KJ395369	Icgen & Yilmaz, 2014
Acinetobacter haemolyticus Mn12	KJ395367	Akbulut *et al*., 2014
Comamonas testosteroni Ni11	KJ395372	Icgen & Yilmaz, 2014
Enterococcus faecalis Pb06	KJ395380	Aktan *et al*., 2013
Acinetobacter johnsonii Sb01	KJ395376	Akbulut *et al*., 2014
Pantoea agglomerans Sn11	KJ395361	Cerit *et al*., 2014
Micrococcus luteus Sr02	KJ395374	Koc *et al*., 2013
Micrococcus luteus Sr11	KJ395375	Koc *et al*., 2013
Acinetobacter haemolyticus Zn01	KJ395368	Akbulut *et al*., 2014

2.3 Selection of kerosene degraders

Selection of kerosene degraders within 22 different bacteria was done as described by John *et al.* (2015). Bacterial cultures from NA were inoculated to NB end left for an overnight in an orbital shaker at 120 rpm and 30°C. Next, bacterial cultures grown in NB were centrifuged for 5 min at 10.000 rpm and obtained cell pellets were washed with MSM in order to remove any traces of NB medium. Cells were suspended in a fresh, sterile MSM. The amount of 100 µL inoculated cells were added to 100 mL MSM containing 1% (v/v) kerosene as a sole source of carbon and incubated by shaking at 120 rpm and 30°C for three weeks. As a control, MSM containing 1% kerosene without inoculum was prepared. After incubation, the turbidity of the mediums was determined spectrophotometrically by using Rayleigh UV-Visible Spectrophotometer (VIS-723, Bejing Beifen-Ruili Analytical Instrument Group Co., Ltd).

2.4 Determining maximum tolerable concentration of kerosene

To determine maximum tolerable concentration (MTC) of kerosene, MSM with bacterial inoculum was prepared as mentioned above and supplemented with increasing concentrations of kerosene in the order of 3, 5, 7, 10, 15 and 20% (v/v) as indicated by Khan *et al.* (2015). Incubation was done for three weeks at 30°C and with 120 rpm shaking conditions. Turbidity was checked at each kerosene concentration separately (Figure 2.1).

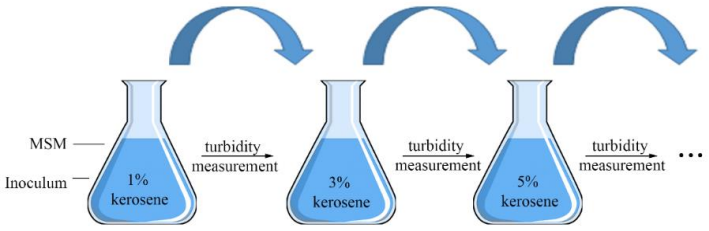


Figure 2.1 Determination of MTC for kerosene

2.5 Analyses of aerobic kerosene degradation rates of bacterial isolates

After selection of potential kerosene degraders, the kerosene degradation rates of the bacterial isolates were investigated by gravimetric and GC analyses. Prior to both tests, the bacteria were grown in NB for an overnight. In order to collect the bacterial cells, the samples were centrifuged and supernatants were decanted.

The cell pellets were washed and suspended in MSM. The amount of 100 µL of bacterial culture was added to 100 mL sterile MSM supplemented with 1% kerosene. The samples then were incubated in 30°C with 120 rpm shaking for 21 days.

2.5.1 Gravimetric analysis

In order to measure the amount of consumed kerosene by the bacterial isolates, gravimetric method was performed (Latha and Kalaivani, 2012). At the end of incubation period, culture medium was centrifuged at 120 rpm for 20 min and the bacterial biomass was decanted (Al-Wasify and Hamed, 2014). For kerosene extraction, culture supernatant was mixed with chloroform (3:1 v/v) and placed in a separating funnel. Mixture was shaken vigorously and allowed to settle down. Formation of two layers was observed through watery layer and chloroform layer with the residual kerosene. Chloroform layer, therefore, was collected and air-dried for 24 h. After evaporation, kerosene degradation rates for each isolate was calculated by using equations 1, 2 and 3. Measurements were done in three repeats.

Weight of residual kerosene = Weight of beaker with extracted kerosene - Weight of empty beaker **(1)**

Amount of kerosene degraded = Weight of kerosene added in the media - Weight of residual kerosene **(2)**

$$\textit{Kerosene degradation (\%)} = \frac{\textit{Amount of kerosene degraded}}{\textit{Amount of kerosene added in the media}} \; x\; 100 \quad \textbf{(3)}$$

2.5.2 Gas chromatographic analysis

Kerosene degradation rates of the isolates were determined by GC analysis at Petroleum Research Center in Middle East Technical University by following the procedure of Al-Wasify and Hamed (2014). The amount of 50 mL hexane was utilized for kerosene extraction. To concentrate the sample, rotary evaporator was used and sample volume was adjusted to 2 mL by nitrogen gas. The samples, later, were analyzed with Agilent Technologies 6850 GC equipped with flame ionization detector (FID) System (Little Falls, California, USA) (Figure 2.2). An amount of 1 µL sample was injected into HP-1 column with 30 m length x 0.32 mm internal diameter and 0.25 µm film thickness in split mode. The column temperature was programmed to rise from 35 to 300°C with a rate of 2°C/min and held for 20 min.

Figure 2.2 Gas chromatography used in this study

2.6 PCR analysis of kerosene degradation

In order to confirm degradation abilities of the bacterial isolates, PCR analysis was performed.

2.6.1 Total DNA extraction

Prior to PCR analysis, total DNA extraction of the bacterial isolates were done in order to obtain genomic DNA samples. Therefore, following protocol of Cheng and Jiang (2006) was carried out. Solutions used in the extraction are given in Table 2.2.

Table 2.2 Solutions and buffers used in DNA extraction of bacterial isolates

Solutions and buffers	Suppliers
Trizma base	>99.9 %, Sigma Aldrich, Germany
EDTA	Sigma Aldrich, Germany
SDS	Merck, Germany
Proteinase K	Sigma-Aldrich, Germany
Lysozyme	BioShop, Canada
RNase	Thermo Fisher, USA
Phenol	Merck, Germany
Chloroform	>99 %, Merck, Germany
Potassium acetate	Merck, Germany
Isopropanol	Merck, Germany
Ethanol	>99.8 %, Sigma-Aldrich, Germany

One mL of overnight grown bacteria cultures were transferred to eppendorf tubes and centrifuged at 13.000 rpm for 5 min. The supernatants were decanted and the cell pellets were suspended in 467 µL TE buffer (50 mM glucose, 25mM Tris-HCl, 10mM EDTA (pH:8)), by pipetting. The amount of 30 µL 10% (w/v) sodium dodecyl sulfate (SDS) and 3 µL of 20 mg/mL proteinase K were added. In this step, bacterial isolates that are Gram-positive were treated with 10 µL lysozyme (10 mg/mL) in 10 mM Tris-Cl (pH:8). Samples were incubated for 2 h at 37°C until complete lysis was observed. Total RNA was removed by adding RNase with a final concentration of 1 mg/mL. The samples were centrifuged for 10 min at 13.000 rpm and the supernatants were transferred to new eppendorf tubes. Equal volume of phenol/chloroform was added and mixed by pipetting. In order to have two phases, centrifugation at 13.000 rpm for 10 min was done. The upper aqueous phases containing DNA was transferred to a fresh new tube. Phenol/chloroform step was repeated twice until white interphase was disappeared. For DNA precipitation 3 M (pH 4.8) potassium acetate 1:10 (v/v) and isopropanol 6:10 (v/v) was added into solution and mixed until observing precipitation. Then, the samples were centrifuged at 13.000 rpm for 10 min and the supernatants were discarded. Remaining pellets were washed with 300 µL 70% ethanol (w/v) and centrifuged at 13.000 rpm for 10 min. Finally, the samples were air-dried and DNA extracts were suspended in 100 µL TE buffer.

To quantify samples and assess their purity, dissolved DNA extracts were measured with Colibri Microvolume Spectrophotometer (Titertek Berthold, Germany) and run on 1% agarose gel electrophoresis (Bio-Rad PowerPack Basic Power Supply, USA) for 1 h at 90V. Finally, the gel was stained with ethidium bromide and visualized under UV light. Samples were stored at -20°C and regularly used as templates for PCR analyses.

2.6.2 Detection of the *alk*B gene

Before performing PCR for each kerosene degrader, optimization experiments were done for determining optimum conditions specific to the target of interest. Optimization experiments were done by changing annealing temperature, $MgCl_2$ and primer concentrations. The primers used for the *alk*B gene detection and PCR conditions are given in Table 2.3.

Table 2.3 The *alk*B primers and the PCR conditions used in this study

Primers	Sequence (5'→ 3')	Amplicon size (bp)	Annealing temperature (°C)	References
*alk*B-F	TGGCCGGCTACTCCGATGATCGGAATCTGG	870	51	Whyte *et al*., 2002
alkB-R	CGCGTGGTGATCCGAGTGCCGCTGAAGGTG			

After optimization, PCR analyses were applied to all of the bacterial isolates. The 25 µL PCR mixture was composed of 1 µL (100 ng) DNA extract as a template, 2.5 µL of 10X PCR buffer, 1.5 µL of $MgCl_2$, 2 mM of each dNTPs, 0.75 µL (10 pmol) of forward and reverse primers and finally 0.125 µL Taq DNA polymerase (NEB, USA). Reactions were performed in Thermal Cycler (Bio-Rad T-100, USA). PCR protocol is summarized in Figure 2.3. After PCR, products were run on 1% agarose gel at 90 V. DNA ladder (NEB, USA) was also loaded to the gel in order to estimate molecular weight of amplicons. Gel was stained with ethidium bromide and visualized under UV light. To determine the sizes of DNA fragments on agarose gel electrophoresis, a standard curve was constructed. The standard curve was calculated by plotting the distances migrated by ladder bands on agarose gel against each band corresponding to different base pairs.

Figure 2.3 PCR temperature/time profile for the *alk*B gene detection

2.7 Screening for biosurfactant production

Biosurfactant production abilities of the bacterial isolates were determined by oil spreading test (Morikawa *et al*., 2000), emulsification index (Peele *et al.*, 2016), microbial adhesion to hydrocarbon test (Zoueki *et al.*, 2010), surface (Chandran and Das, 2010) and interfacial tension measurements (Mendes *et al.*, 2015). For each test, MSM supplemented with 1% kerosene and 100 µL bacterial culture (grown overnight in NB and washed with MSM) was prepared (Thavasi *et al*., 2013). After 7-day incubation, the cultures were centrifuged at 13.000 rpm for 40 min. The supernatants

were used for oil spreading test and measurements of emulsification index while the cells pellets were used for the MATH test.

2.7.1 Oil spreading activity

Oil spreading activity was determined by adding 10 µL kerosene onto the surface of 40 mL distilled water in a petri dish in order to form a thin layer. Then, 10 µL of supernatant was put on the center of kerosene layer. A clear zone was observed under light and the area of the zone was measured as described in Morikawa *et al*. (2000) (Figure 2.4). Measurements were repeated three times and means were calculated.

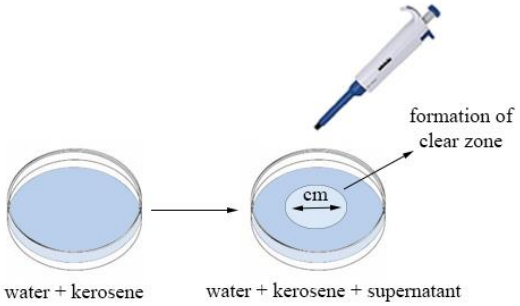


Figure 2.4 Experimental flow of oil spreading test

2.7.2 Measurement of emulsification index (E_{24})

Emulsification ability of the biosurfactants produced by the isolates against kerosene was also studied. The amount of 2 mL kerosene was added to 2 mL culture supernatant and vortexed at a high speed for 2 min (Figure 2.5). The emulsion activity was investigated after 24 h (Peele *et al.*, 2016) and the E_{24} index was measured with equation 4. Measurements were repeated three times and means were calculated.

$$E_{24}\ (\%) = \frac{Height\ of\ the\ emulsion\ layer}{Total\ height\ of\ the\ mixture}\ x\ 100 \quad \textbf{(4)}$$

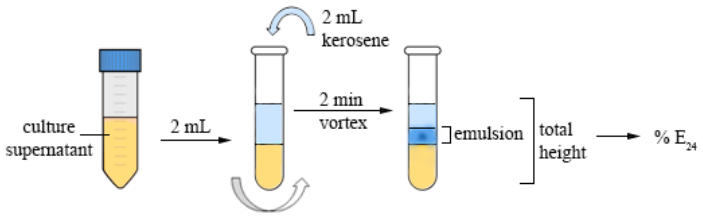


Figure 2.5 Experimental flow of emulsification test

2.7.3 Microbial adhesion to hydrocarbon test

Hydrophobicity of cells towards kerosene was determined as described by Zoueki *et al*. (2010) with microbial adhesion to hydrocarbon (MATH) test. The 7 day-old cultures were centrifuged and the cell pellets were used. The pellets were washed twice with phosphate urea magnesium (PUM) buffer composed of 150 mM phosphate, potassium, urea and magnesium (pH 7.1) (Rosenberg, 2006). The pellets were suspended in 5 mL PUM. The amount of 500 µL of kerosene was added to bacterial suspension and vortexed for 2 min. After 15 min, 1 mL of the bacterial suspension was retrieved while avoiding pipetting the kerosene layer. Measurements were done with a spectrophotometer as indicated previously. The PUM buffer without cells was adjusted to an absorbance of 1.0 at 600 nm. Optical density of the bacterial suspensions for each isolates (A_0) and suspensions treated with kerosene (A_1) was measured (Figure 2.6). Finally, hydrophobicity of the cells was calculated as shown in equation 5. Measurements were repeated three times and means were calculated.

$$Hydrophobicity = 1 - \left(\frac{A_0 - A_1}{A_0}\right) \times 100 \quad \textbf{(5)}$$

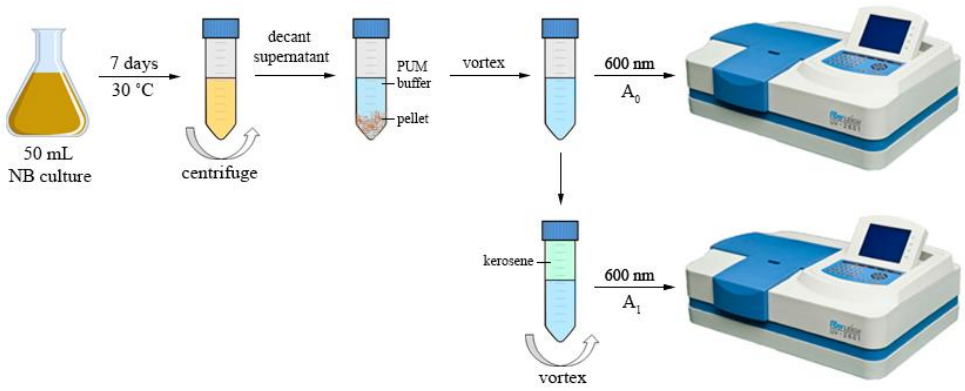


Figure 2.6 Experimental flow of the MATH test

2.8 Interpretation of biosurfactant screening tests

A single method is not sufficient for an effective selection of biosurfactants producers (Satpute *et al*., 2008). To overcome the advantages and disadvantages of each individual method, Walter *et al*. (2010) recommends a combination of different methods for a successful screening. However, as the number of data increases, the

interpretation becomes much more difficult. Therefore, in order to select the most promising biosurfactant producers, statistical analysis was done.

In statistics, a process called standardization is used to facilitate the interpretation of different type of variables (Helms *et al*., 1997). This is a common method used in general science, especially in biology for data analysis (Welham *et al.*, 2014). Briefly, each type of variable was standardized in order to have them on the same scale for proper comparison. To standardize these variables, the mean and standard deviation of the variable were calculated. Then, for each value (x), the mean (μ) was subtracted and divided by the standard deviation (σ). The z score (z) formula used for the interpretation of biosurfactant screening tests was given in equation 6.

$$Z = X\text{-}\mu / \sigma \quad \textbf{(6)}$$

2.9 Preliminary characterization of the biosurfactants produced by the isolates

For preliminary characterization of the biosurfactants, following tests were performed. Phenol sulphuric acid method was done for indication of glycolipids, biuret test for lipopeptides, phosphate test for phospholipids and blue agar plate (BAP) method for rhamnolipids (Kalyani *et al.*, 2014). The 7 day-old cultures grown in MSM were centrifuged at 13.000 rpm for 40 min and the supernatants were used in order to perform characterization tests.

2.9.1 Phenol sulphuric acid method

The amount of 1 mL supernatant was mix with 1 mL 5% phenol in a glass tube. 2 mL of concentrated sulphuric acid was added slowly until development of orange color. Sulfuric acid dehydration reaction separated the resulting carbohydrates into their constituents while the phenol reacted with products causing the color of the mixture to turn dark orange. This color development was accepted as the presence of glycolipids in the mixture (Figure 2.7) (Ellaiah *et al.*, 2002). This test was repeated three times for each isolate.

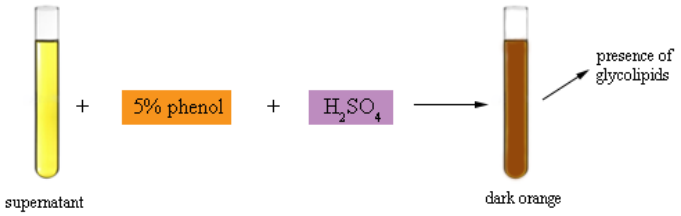


Figure 2.7 Experimental flow of the phenol sulphuric acid method

2.9.2 Biuret test

The presence of lipopeptides was analyzed by the biuret test. First, 2 mL of the supernatant was heated at 70°C for 10 min. Approximately 10 drops of 1 M NaOH was added. Finally, 1% $CuSO_4$ was added slowly. The reaction of the peptide bonds with the copper ions and the alkaline solution led to the formation of a violet-pink ring as indicated by Kalyani *et al.* (2014) (Figure 2.8). This test was repeated three times for each isolate.

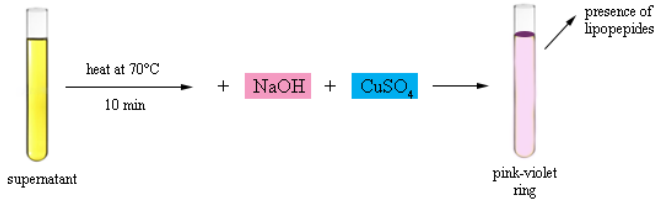


Figure 2.8 Experimental flow of the biuret test

2.9.3 Phosphate test

Almost 10 drops of 6 M HNO_3 was added to 2 mL supernatant and heated at 70°C. Hydrolysis of the phospholipids, exposed to strong acids, releases the phosphate. Then, 5% ammonium molybdate was added to the mixture drop by drop. The free phosphate reacted with ammonium molybdate, bringing a precipitate together with the formation of yellow color as the indicator of phospholipids (Figure 2.9) (Okpokwasili and Ibiene, 2006). This test was repeated three times for each isolate.

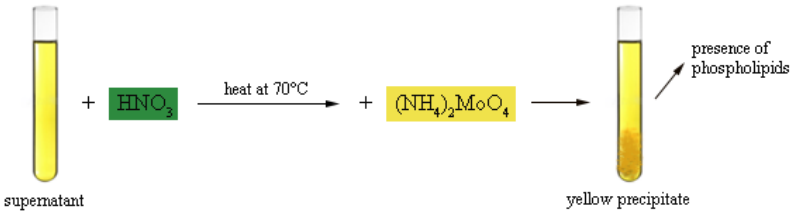


Figure 2.9 Experimental flow of the phosphate test

2.9.4 Blue agar plate method

Anionic surfactants, especially rhamnolipids are identified by blue agar plate (BAP) method (Satpute *et al.*, 2008). Cetyltrimethylammonium bromide (CTAB)-methylene blue agar plates were prepared as described by Siegmund and Wagner (1991). The amount of 0.2 g CTAB, 0.005 g methylene blue and 15 g agar were added to 1 L MSM. The medium was sterilized by autoclaving at 121°C for 15 min. The amount of 10 mL kerosene (1% of the medium) was added right after sterilization. The volume of 20 mL solution was poured to agar plates and incubated at 30°C. A dark blue zone around the culture was considered as positive for rhamnolipid production (Figure 2.10) (Satpute *et al*., 2008). This test was repeated three times for each isolate.

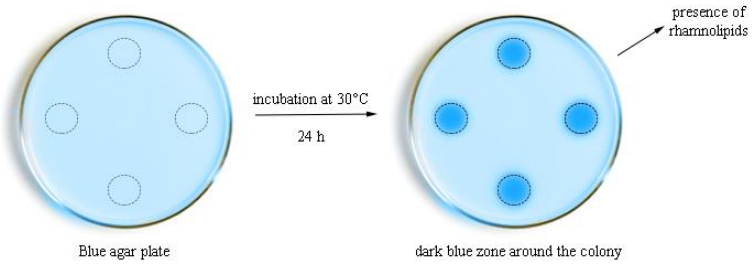


Figure 2.10 Experimental flow of the blue agar plate method

2.10 Surface and interfacial tension measurements

Surface and interfacial tension measurements were performed at Middle East Technical University Central Laboratory. Surface tension was defined with the following procedure of Chandran and Das (2010). MSM with 1% kerosene and 100 µL inoculum was incubated for 21 days. The cultures were centrifuged at 13.000 rpm for 40 min and the supernatants were used for the analyses (Figure 2.11a). Measurements were done with Attension Theta Optical Tensiometer (Biolin Scientific, Sweden).

Calibration of the instrument was done with distilled water prior to the experiment. As positive controls, anionic surfactant SDS and nonionic surfactant Tween 20 was selected. For interfacial tension, the tension between kerosene and distilled water was measured (Mendes *et al*., 2015). The samples were prepared with the equal volume of kerosene and water containing biosurfactants (0.15 g/L) (Figure 2.11b). Positive controls were prepared by adding 10 g/L SDS and Tween 20 to the water. Tension reductions of the samples for both tests were calculated by comparing the tension of MSM without inoculum (γ_m) to the tension of MSM with biosurfactants (γ_c) (Equation 7). The measurements were repeated five times and means were calculated.

$$Tension\ reduction = \left(\frac{\gamma m - \gamma c}{\gamma m} \right) \times 100 \quad \textbf{(7)}$$

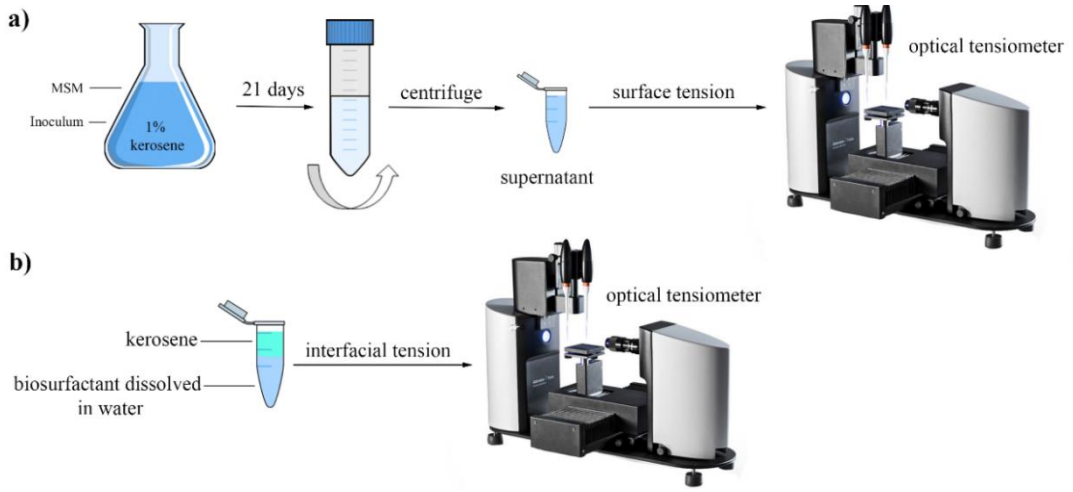


Figure 2.11 Experimental flow of (a) the surface tension and (b) the interfacial tension measurements

2.11 Extraction of biosurfactants

Extraction of biosurfactants was done for the quantification, also for TLC and FTIR analysis (Kumar *et al*., 2014). The amount of 100 µL of overnight grown NB culture was transferred to 50 mL NB media and incubated for 7 days at 30°C with 120 rpm shaking conditions. After incubation period, samples were centrifuged at 13.000 rpm for 20 min to remove the bacterial cells. The culture supernatant, then, was acidified with 6M HCl to attain pH of 2.0. Samples were kept overnight at 4°C in refrigerator in order to obtain a precipitate. An amount of 50 mL of diethyl ether was added to the

sample and shake vigorously for several min. The mixture was poured to a separating funnel and two layers were obtained. The upper organic layer was collected and transferred to pre-weighed empty petri dish. This step was repeated three times for complete purification. The dishes were left in laminar flow cabinet and allowed to evaporate for 24 h. After evaporation, the dishes were kept in an oven at 40°C for 1 h. The brown colored product was obtained and scraped off. The biosurfactant powders were stored in 1.5 mL eppendorf tubes at 4°C (Figure 2.12).

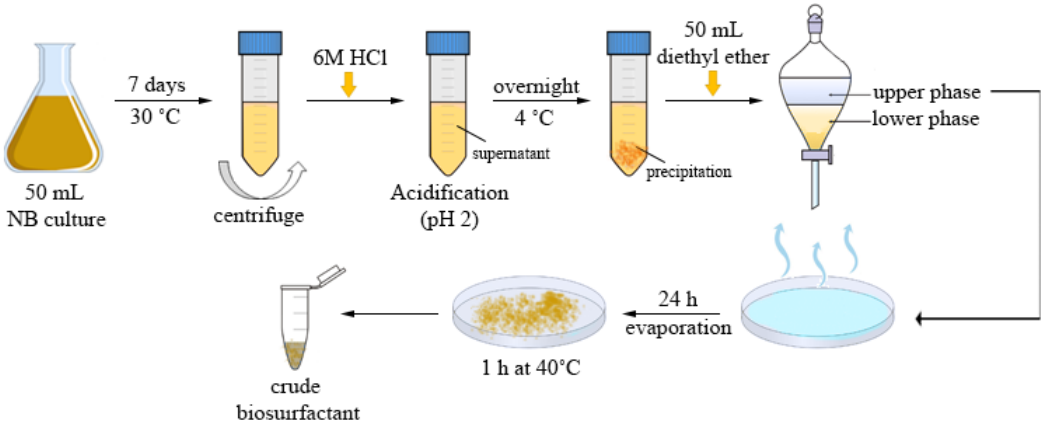


Figure 2.12 Experimental flow of the biosurfactant extraction

2.12 Quantification of produced biosurfactants

Quantification of the biosurfactants was done by gravimetric analysis (Marchant and Banat, 2014). After extraction, evaporated petri dishes containing the biosurfactants were weighted and compared to pre-weighted dishes. Amount of product produced by each bacterial isolate was calculated as shown in equation 8. This test was repeated three times for each isolate.

Amount of biosurfactant = Weight of petri dish with biosurfactant - Weight of empty petri dish **(8)**

2.13 Thin layer chromatography

Approximately, 5 mg of crude biosurfactant was dissolved in 10 mL chloroform. Then, 10 µL sample were applied on silica gel 60 (F_{254} Merck, Germany) and air dried for 10 min (Ibrahim, 2016). The developing agent was prepared as chloroform, methanol and acetic acid (65:15:2, v/v/v).

The gel was, later, placed in a reserved jar for 30 min and let to drain. For the visualization, the dried plate was sprayed with color reagent composed of 0.15 g orcinol, 8.2 mL 60 % sulphuric acid (v/v) and 42 mL deionized water. After 10 min, the gel was heated at 110°C until the detection of the definite spots (Figure 2.13). The retention factor (R_f) of each spot was used to identify compounds. It was calculated by the distance migrated over the total distance covered by the solvent as in equation 8.

$$Rf = \frac{distance\ traveled\ by\ the\ sample}{distance\ traveled\ by\ the\ solvent} \quad \textbf{(8)}$$

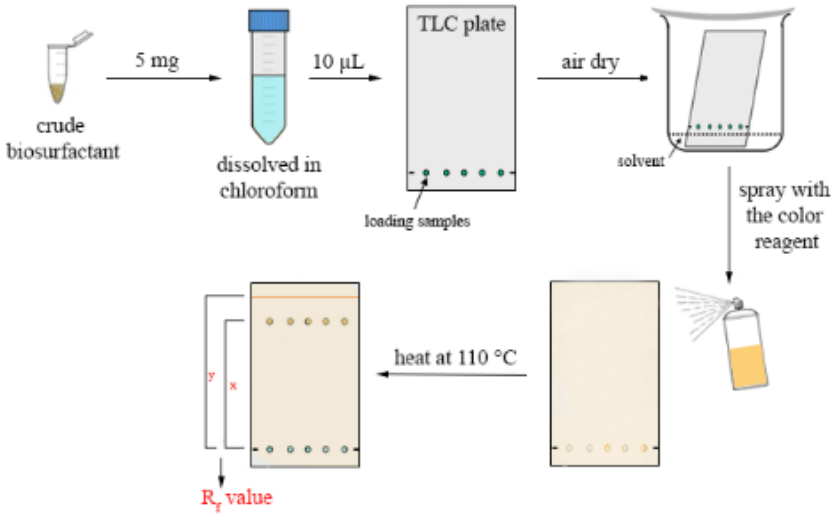


Figure 2.13 Experimental flow of TLC analysis

2.14 Fourier transform infrared spectroscopy

To identify the chemical nature of the extracted biosurfactant, fourier transform infrared spectroscopy (FTIR) analysis were done at Bilkent University, UNAM-National Nanotechnology Research Center. This method (Chandran and Das, 2010) helps to determine the functional groups and the chemical bonds found in the crude extract. One mg of powder biosurfactant was grounded with 100 mg of KBr (Merck, USA) and pressed with 8 kg for 30 sec to obtain translucent pellets. Infrared absorption spectra were recorded on a Bruker Vertex 70 FTIR with microscope (Billerica, Massachusetts, USA) obtaining the spectrum in the range of 450-4000 cm^{-1} at a resolution of 4 cm^{-1}. KBr pellet was used as background reference. All measurements consisted of 500 scans (Figure 2.14).

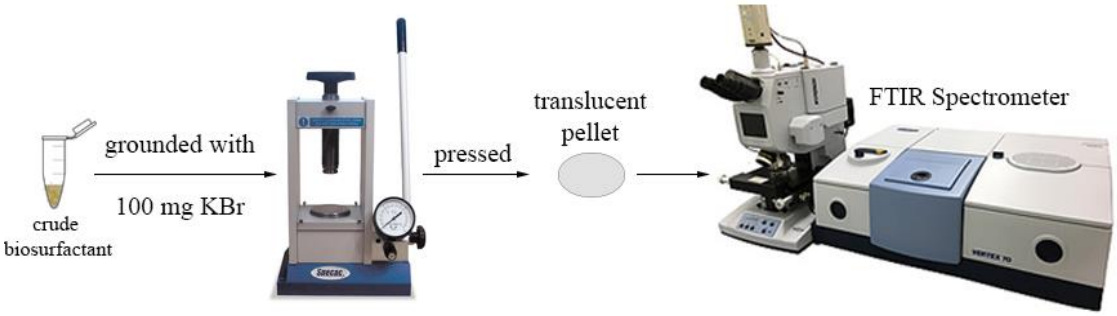


Figure 2.14 Experimental flow of FTIR analysis

2.15 PCR analysis of the *rhl*AB genes

Before performing PCR for each biosurfactant producers, optimization experiments were done by changing annealing temperature, $MgCl_2$ and primer concentrations. The primers used to target the *rhl*AB gene and PCR conditions are given in Table 2.4.

Table 2.4 *rhl*AB primers and the conditions used in this study

Primers	Sequence (5'→ 3')	Amplicon size (bp)	Annealing temperature (°C)	References
*rhl*AB-F *rhl*AB-R	CAGGCCGATGAAGGGAAATA AGGACGACGAGGTGGAAATC	777	50	Pacwa-Płociniczak *et al.*, 2014

After optimization, PCR analyses were run for all of the bacterial isolates. The 25 µL PCR mixture was composed of 1 µL (100 ng) DNA extract as a template, 2.5 µL of 10X PCR buffer, 2 µL of $MgCl_2$, 2 mM of each dNTPs, 0.6 µL (10 pmol) of forward and reverse primers and finally 0.125 µL Taq DNA polymerase. PCR protocol is summarized in Figure 2.15. After PCR, products were run on 1% agarose gel at 90 V. The gel was stained with ethidium bromide and visualized under UV light.

Figure 2.15 PCR temperature/time profile for the *rhl*AB gene detection

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Selection of kerosene degraders

A number of 22 hydrocarbon degrading bacteria were previously isolated by our lab (Icgen and Yilmaz, 2014). In this study, the bacterial isolates were tested for their ability to grow in the presence of kerosene as a sole source of carbon. Out of 22, 19 bacterial isolates namely*; P. plecoglossicida* Ag10, *R. planticola* Ag11, *S. aureus* Ba01, *S. rhizophila* Ba11, *D. acidovorans* Cd11, *S. warneri* Co11, *E. faecalis* Cr07, *A. calcoaceticus* Fe10, *P. koreensis* Hg11, *S. aureus* Li12, *S. nematodiphila* Mn11, *A. haemolyticus* Mn12, *C. testosteroni* Ni11, *E. faecalis* Pb06, *A. johnsonii* Sb01, *P. agglomerans* Sn11, *M. luteus* Sr02, *M. luteus* Sr11 and *A. haemolyticus* Zn01 were found to utilize kerosene (Table 3.1). The isolates *S. aureus* Al11, *P. koreensis* Cu12 and *P. koreensis* Hg10 did not show any growth during 21-day incubation in MSM supplemented with kerosene and were not used for further experiments.

Studies on kerosene are scarce (Khan *et al.*, 2015) and most of the studies are based on fungi or yeasts such as *Aspergillus, Candida* and *Rhizopus*. Some of the kerosene degraders found in this study like *Stenotrophomonas*, *Acinetobacter*, *Pseudomonas, Micrococcus, Staphylococcus* and *Serratia* were also determined by Adetitun *et al*. (2014). Apart from current study, there has been no evidence so far about kerosene degrading *Raoultella planticola*, *Stenotrophomonas rhizophila, Delftia acidovorans, Serratia nematodiphila* and *Comamonas testosteroni*.

Table 3.1 List of kerosene degraders used in this study

Bacterial isolates	EMBL accession numbers	References
Pseudomonas plecoglossicida Ag10	KJ395363	Icgen & Yilmaz, 2014
Raoultella planticola Ag11	KJ395359	Koc *et al*., 2013
Staphylococcus aureus Ba01	KJ395371	Yilmaz *et al*., 2013
Stenotrophomons rhizophila Ba11	KJ395362	Yilmaz *et al*., 2013
Delftia acidovorans Cd11	KJ209817	Icgen & Yilmaz, 2014
Staphylococcus warneri Co11	KJ395373	Yilmaz *et al*., 2013
Enterococcus faecalis Cr07	KJ395365	Icgen & Yilmaz, 2014
Acinetobacter calcoaceticus Fe10	KJ395366	Akbulut *et al*., 2014
Pseudomonas koreensis Hg11	KJ395378	Icgen & Yilmaz, 2014
Staphylococcus aureus Li12	KJ395370	Yilmaz *et al*., 2013
Serratia nematodiphila Mn11	KJ395369	Icgen & Yilmaz, 2014
Acinetobacter haemolyticus Mn12	KJ395367	Akbulut *et al*., 2014
Comamonas testosteroni Ni11	KJ395372	Icgen & Yilmaz, 2014
Enterococcus faecalis Pb06	KJ395380	Aktan *et al*., 2013
Acinetobacter johnsonii Sb01	KJ395376	Akbulut *et al*., 2014
Pantoea agglomerans Sn11	KJ395361	Cerit *et al*., 2014
Micrococcus luteus Sr02	KJ395374	Koc *et al*., 2013
Micrococcus luteus Sr11	KJ395375	Koc *et al*., 2013
Acinetobacter haemolyticus Zn01	KJ395368	Akbulut *et al*., 2014

3.2 Determining maximum tolerable concentration of kerosene

As indicated in the previous section, 19 bacterial isolates were capable to grow in the presence of 1% kerosene. Among them, 9 isolates namely *S. warneri* Co11, *E. faecalis* Cr07, *S. nematodiphila* Mn11, *A. haemolyticus* Mn12, *C. testosteroni* Ni11, *P. agglomerans* Sn11, *M. luteus* Sr02, *M. luteus* Sr11 and *A. haemol*yticus Zn01 showed growth up to 3% kerosene. While the isolate *E. faecalis* Cr07 tolerated 5%, for the isolates *S. warneri* Co11, *C. testosteroni* Ni11 and *M. luteus* Sr11 was 7%. MTC of kerosene for the isolates *S. nematodiphila* Mn11 and *M. luteus* Sr02 were 10% while *A. haemolyticus* Mn12 and *P. agglomerans* Sn11 were found to tolerate the highest MTC used in this study (20%) (Figure 3.1).

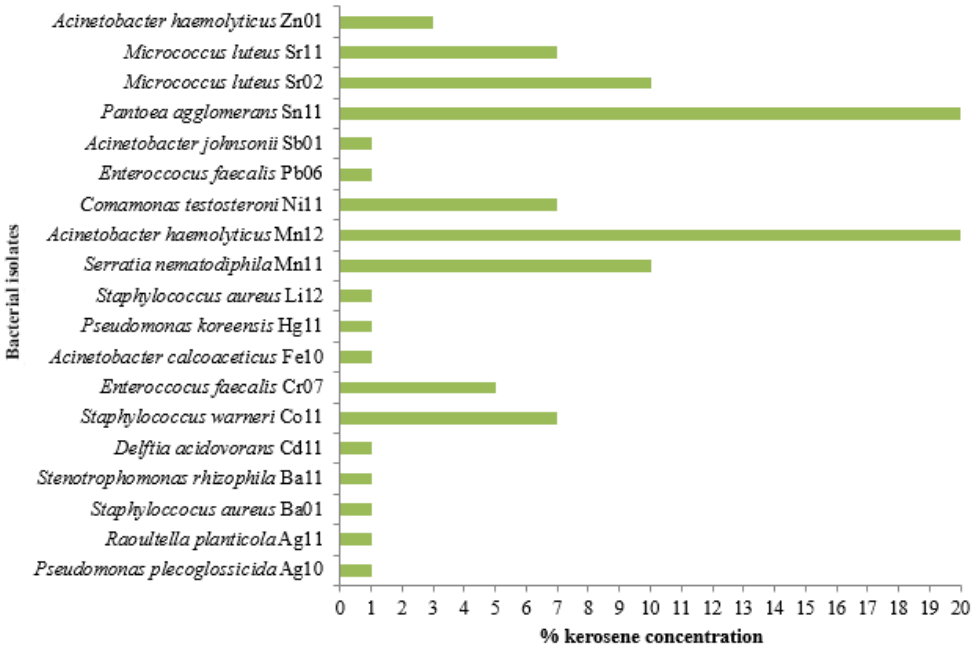


Figure 3.1 MTC values of the kerosene degraders

Shabir *et al.* (2008) observed growth rates of mixed bacterial cultures of *Pseudomonas* up to 5% kerosene concentrations while Agarry *et al.* (2010) performed a treatment strategy with *Pseudomonas* species in 10% kerosene containing soil. Moreover, Islam *et al.* (2013) indicated that *Staphylococcus* sp. were able to tolerate 7% kerosene. Studies above 10% MTC of kerosene seemed to be infrequent except for *Citrobacter koseri/farmer* and *Enterobacter cloacae* with MTC values of 20% for kerosene as determined by Ghoreishi *et al.* (2017).

3.3 Analysis of kerosene degradation

The kerosene utilization rates may vary among microorganisms as indicated by Anienye *et al.* (2015). Therefore, it is necessary to evaluate their biodegradation ability (Ghoreishi *et al.*, 2017). In order to select efficient bacteria for bioremediation, kerosene degradation abilities of the bacterial isolates were measured through gravimetric and chromatographic analyses.

3.3.1 Gravimetric analysis

All of the kerosene degraders were first analyzed gravimetrically. The kerosene degradation abilities of the isolates were calculated (Table 3.2). Eleven bacterial isolates namely; *P. plecoglossicida* Ag10, *R. planticola* Ag11, *S. aureus* Ba01, *S. rhizophila* Ba11, *D. acidovorans* Cd11, *S. warneri* Co11, *E. faecalis* Cr07, *P. koreensis* Hg11, *C. testosteroni* Ni11, *M. luteus* Sr02 and *M. luteus* Sr11 showed more than 70% kerosene degradation ability. Among them, the isolates *E. faecalis* Cr07, *M. luteus* Sr02 and *M. luteus* Sr11 achieved more than 80%. Raw data of gravimetric measurements are given in Appendix A.

Table 3.2 Gravimetric analysis of kerosene degradation capacities of the isolates

Bacterial isolates	Degradation capacity (%)
Pseudomonas plecoglossicida Ag10	70.13±2.83
Raoultella planticola Ag11	77.04±3.59
Staphyloccocus aureus Ba01	75.60±3.85
Stenotrophomonas rhizophila Ba11	77.48±2.36
Delftia acidovorans Cd11	78.05±3.03
Staphylococcus warneri Co11	71.13±2.58
Enteroccocus faecalis Cr07	87.67±2.58
Acinetobacter calcoaceticus Fe10	50.06±5.74
Pseudomonas koreensis Hg11	73.71±5.37
Staphylococcus aureus Li12	46.79±2.82
Serratia nematodiphila Mn11	62.07±3.12
Acinetobacter haemolyticus Mn12	65.16±2.08
Comamonas testosteroni Ni11	75.84±0.98
Enteroccocus faecalis Pb06	60.63±6.91
Acinetobacter johnsonii Sb01	50.19±2.13
Pantoea agglomerans Sn11	46.35±5.03
Micrococcus luteus Sr02	85.22±3.60
Micrococcus luteus Sr11	82.01±2.74
Acinetobacter haemolyticus Zn01	69.94±4.31

±; standard deviations

3.3.2 Gas chromatographic analysis

Although numerous methods have been used for determining biodegradation capacities of hydrocarbon degrading bacteria (Varjani, 2017), gas chromatography (GC) is one of the most reliable (Ghoreishi *et al.*, 2017). Therefore, GC analysis was also performed to measure kerosene degradation abilities of the isolates. Gas chromatograms of all the bacterial isolates were compared with undegraded kerosene and given in Figure 3.2.

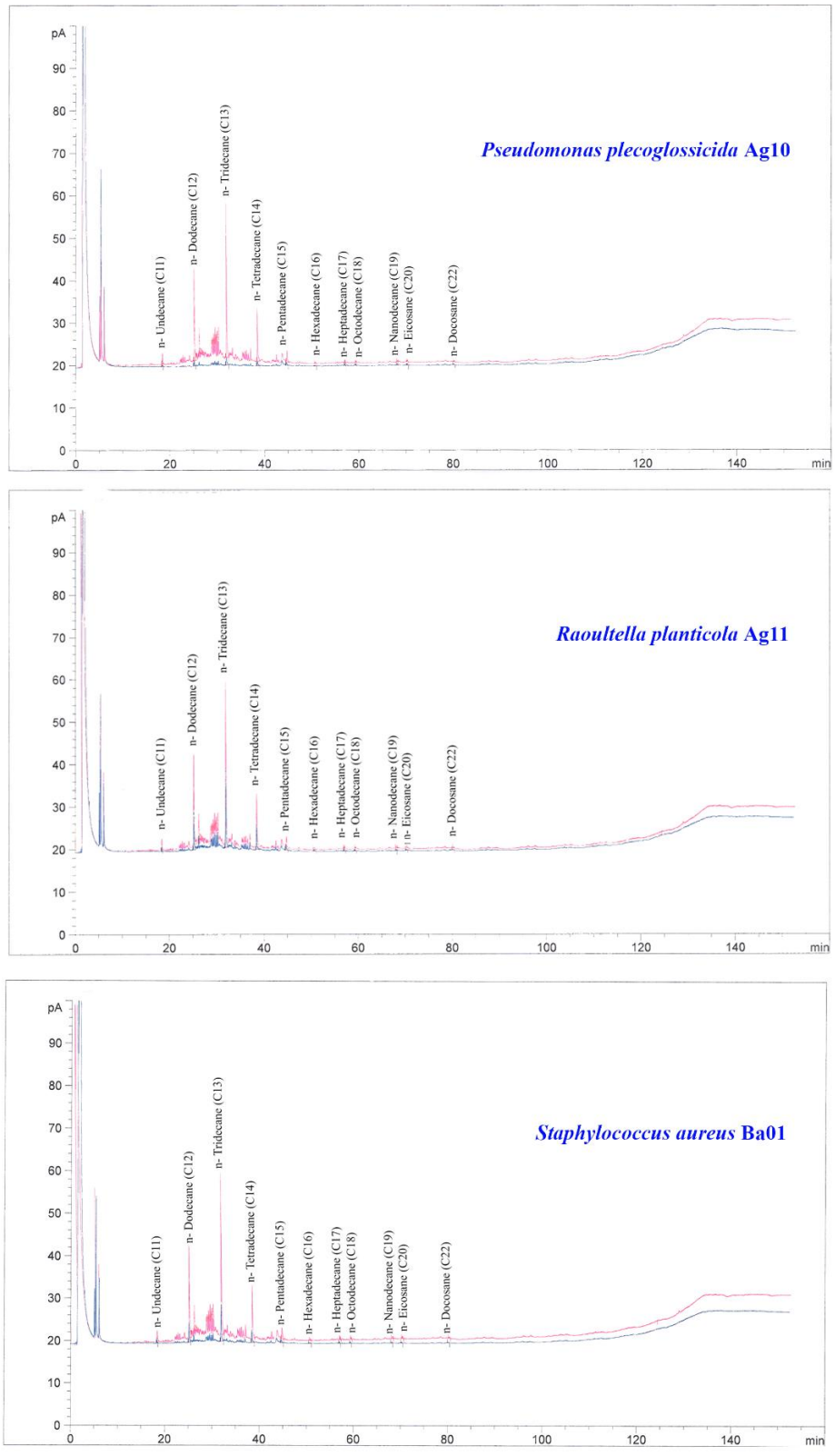


Figure 3.2 GC chromatograms of bacterial isolates used in this study (blue) and control without inoculum (red)

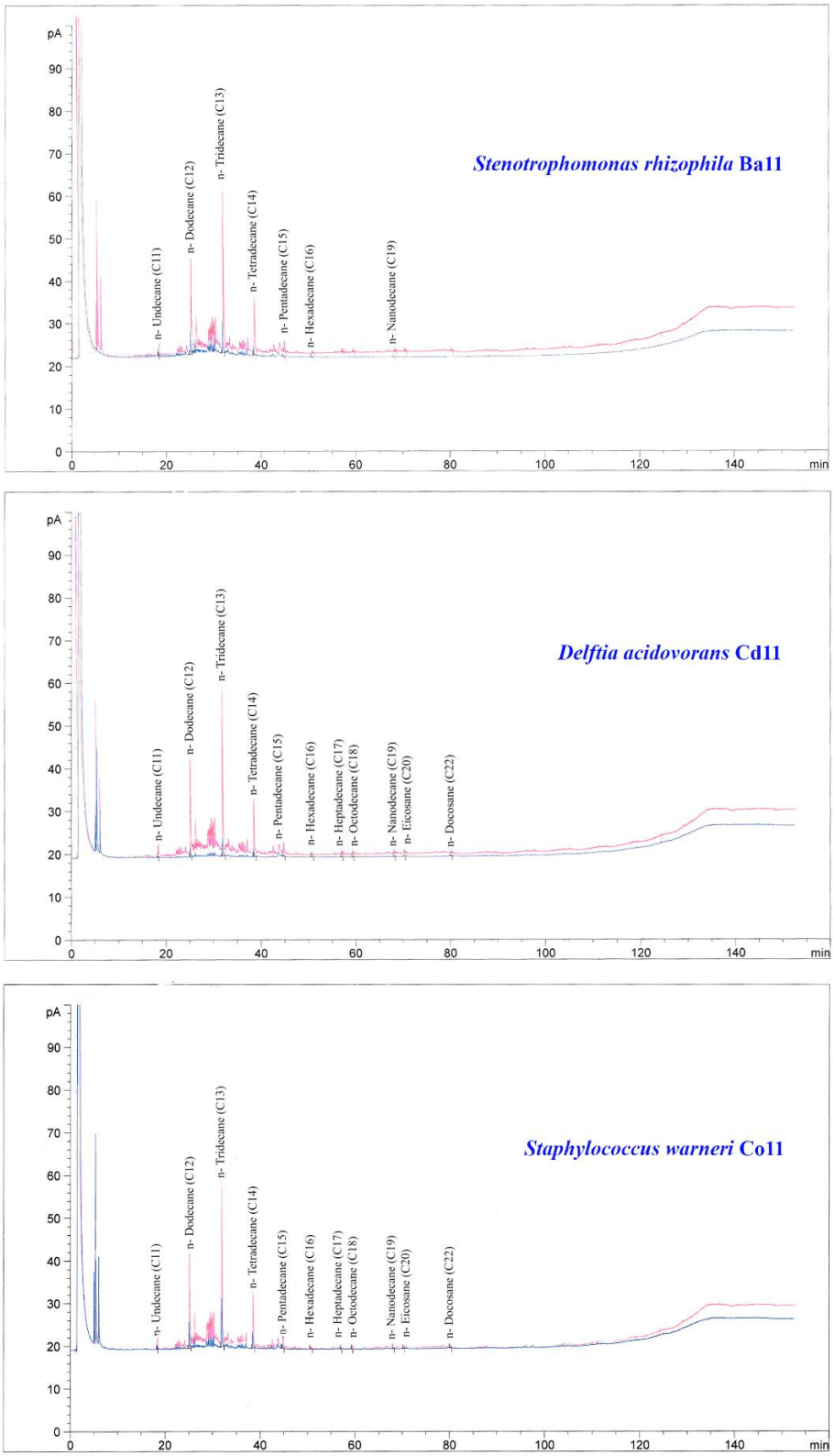


Figure 3.2 cont'd

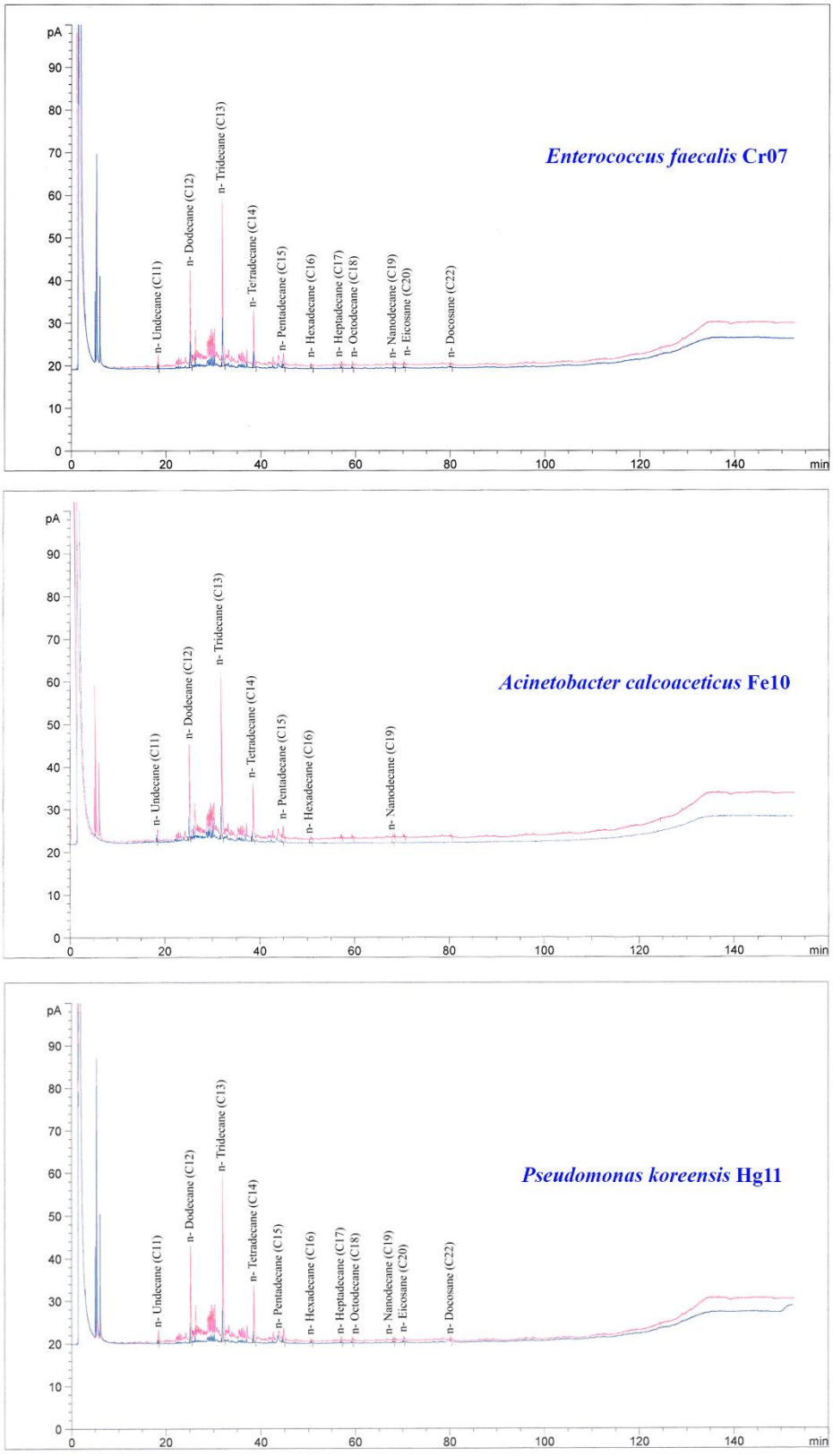


Figure 3.2 cont'd

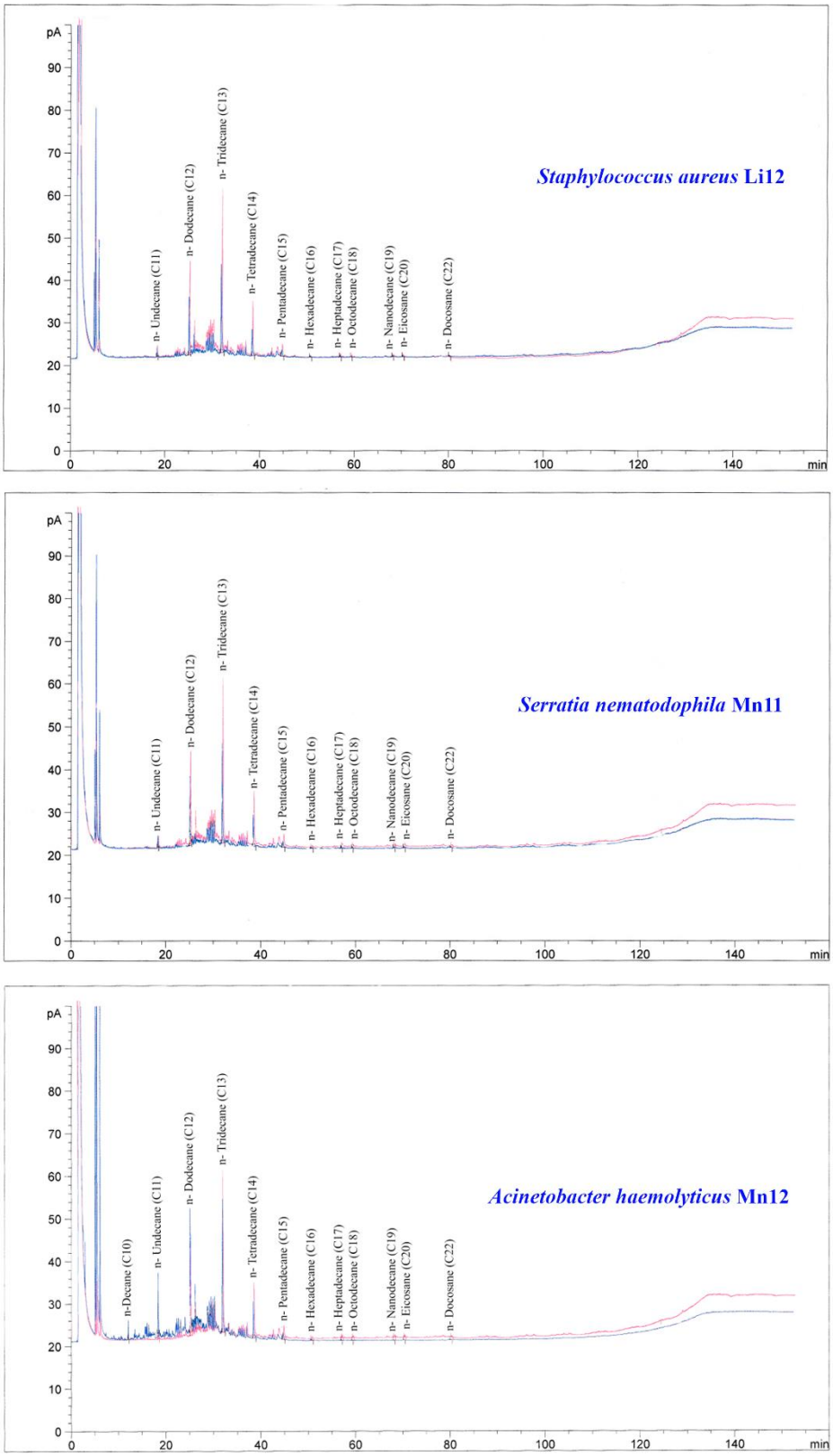


Figure 3.2 cont'd

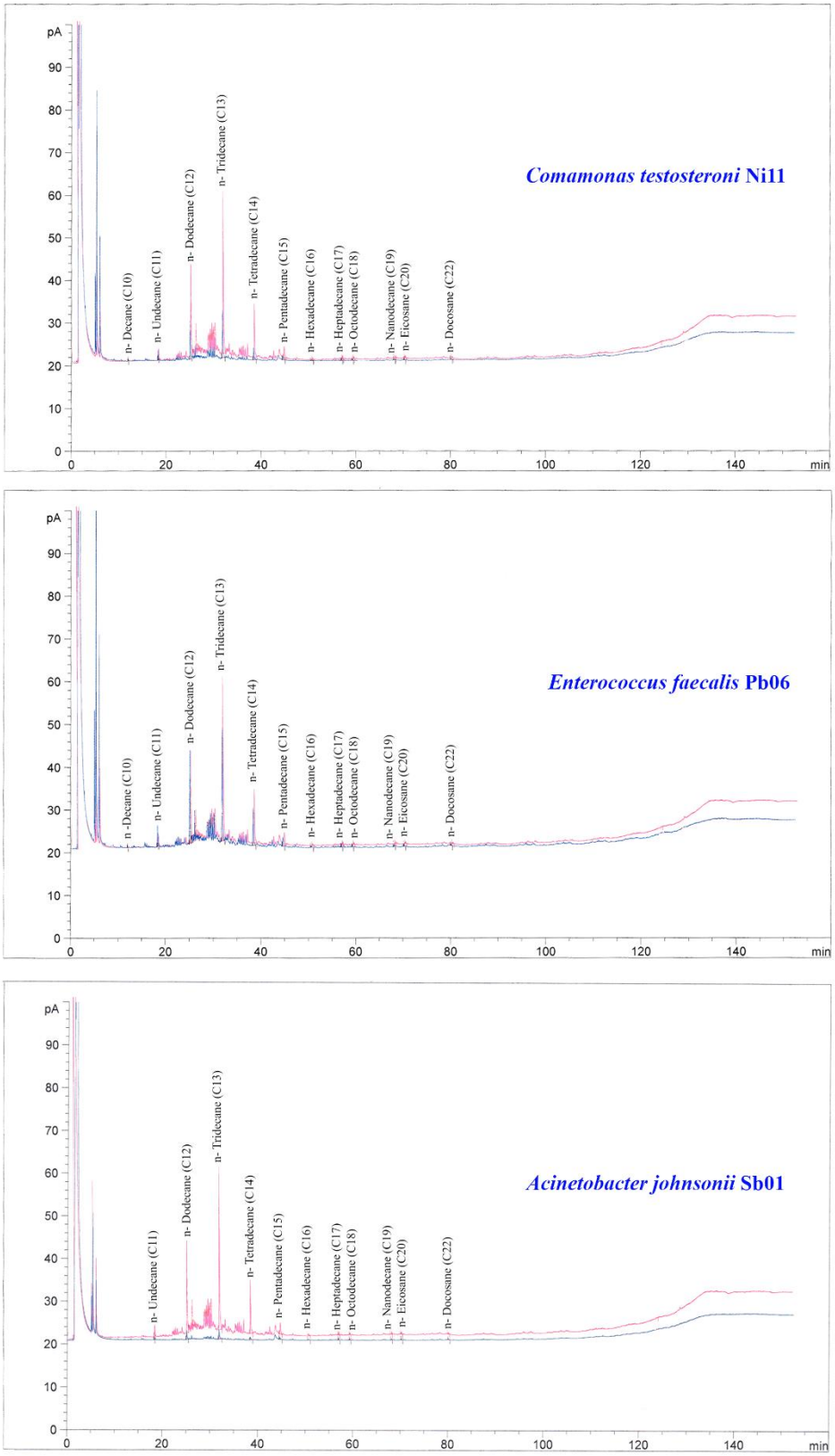


Figure 3.2 cont'd

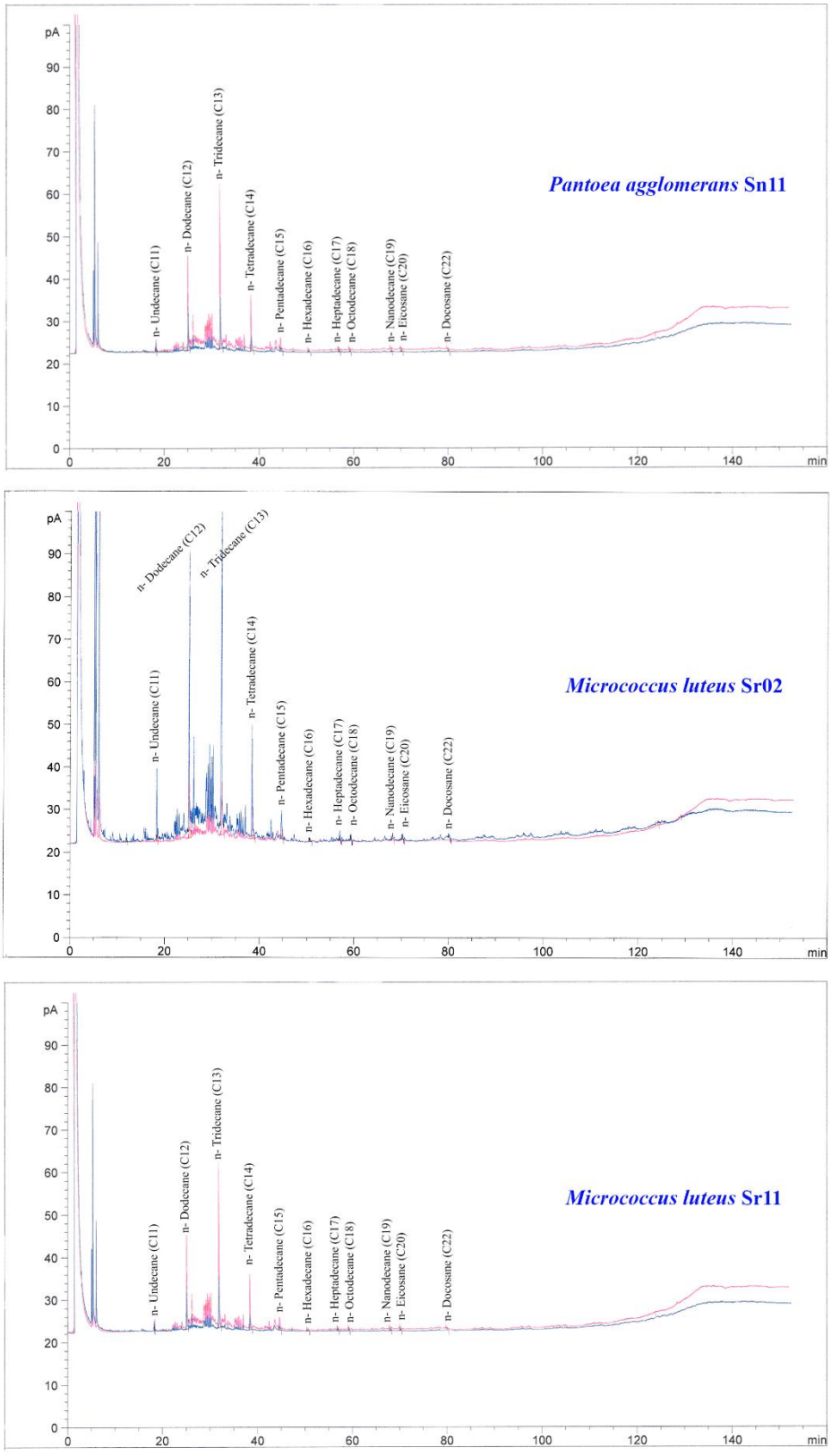


Figure 3.2 cont'd

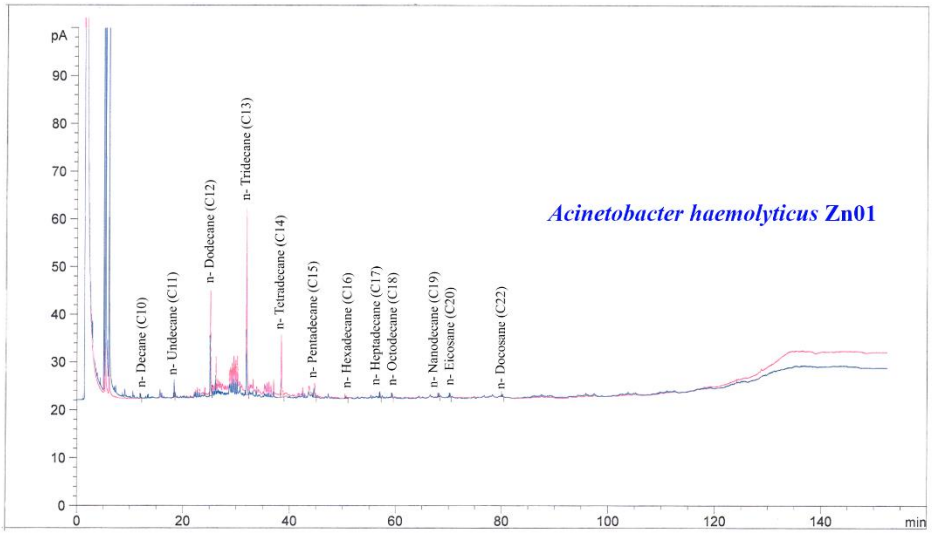


Figure 3.2 cont'd

The initial amount of kerosene in each sample was 1.44 mg/mL before the analysis. After 21 days of incubation, amount of degraded kerosene, degradation percentages and degradation rates in mg/mL per day were calculated for each isolate. The results are tabularized in Table 3.3. Raw data of GC analyses are given in Appendix B.

Table 3.3 Comparison of kerosene degradation capacity and rate of the bacterial isolates used in this study

Bacterial isolates	Residual kerosene (mg/mL)	Degradation ability (%)	Degradation rate (mg/mL/day)
Pseudomonas plecoglossicida Ag10	0.31	78.24	0.05
Raoultella planticola Ag11	0.75	47.71	0.03
Staphyloccocus aureus Ba01	0.44	69.29	0.05
Stenotrophomonas rhizophila Ba11	0.40	72.07	0.05
Delftia acidovorans Cd11	0.28	80.66	0.06
Staphylococcus warneri Co11	0.53	63.25	0.04
Enteroccocus faecalis Cr07	0.96	33.52	0.02
Acinetobacter calcoaceticus Fe10	0.40	72.40	0.05
Pseudomonas koreensis Hg11	0.43	70.10	0.05
Staphylococcus aureus Li12	0.91	36.98	0.03
Serratia nematodiphila Mn11	1.01	29.72	0.02
Acinetobacter haemolyticus Mn12	1.24	13.92	0.01
Comamonas testosteroni Ni11	0.58	59.54	0.04
Enteroccocus faecalis Pb06	1.26	12.61	0.01
Acinetobacter johnsonii Sb01	0.23	83.76	0.06
Pantoea agglomerans Sn11	1.36	5.43	0.00
Micrococcus luteus Sr02	1.44	0.07	0.00
Micrococcus luteus Sr11	0.63	56.43	0.04
Acinetobacter haemolyticus Zn01	0.78	45.70	0.03

Initial amount of kerosene: 1.44 mg/mL

Among all of the kerosene degraders, 7 isolates namely, *P. plecoglossicida* Ag10, *S. aureus* Ba01, *S. rhizophila* Ba11, *D. acidovorans* Cd11, *A. calcoaceticus* Fe10, *P. koreensis* Hg11 and *A. johnsonii* Sb01 stood out with a degradation ability over 70%. The highest degradation was performed by the isolate *A. johnsonii* Sb01 and by *D. acidovorans* Cd11 with 84 and 80%, respectively (Figure 3.3). The GC analysis results were similar to the gravimetrical measurements.

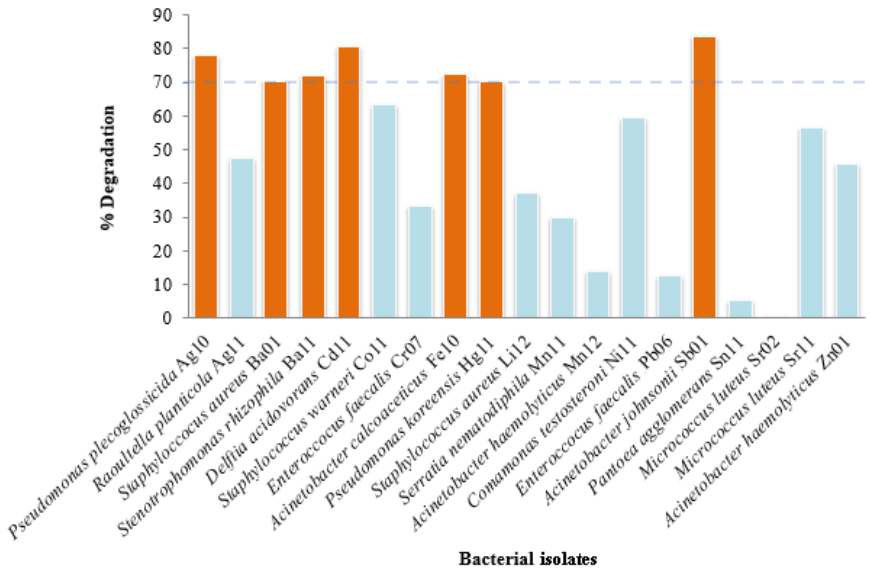


Figure 3.3 Kerosene degradation percentages of all the bacterial isolates used in this study

Gouda *et al.* (2007) reported two *Pseudomona*s sp. and the strain *Gordonia* with degrading ability of 75, 89 and 95% of kerosene, respectively. Similar to their results, in this study, *Pseudomonas plecoglossicida* Ag10 had a degradation rate of 78% and *Pseudomonas koreensis* Hg11 degraded 70% of kerosene. On the other hand, Adebusoye *et al.* (2007) also performed GC and showed that *Acinetobacter iwoffi* could degrade 63% of kerosene. The current study showed that, the isolates *Acinetobacter calcoaceticus* Fe10 and *Acinetobacter johnsonii* Zn01 had 72 and 83% degradation abilities, respectively. Different to the bacterial isolates used in this study, Borah and Yadav (2017) identified a species of *Bacillus cereus* as a kerosene degrader. This isolate was found to degrade 96% of 2% kerosene within 28 days. This result has been reported as the highest degradation rate of kerosene so far.

3.4 Analysis of kerosene degradation gene

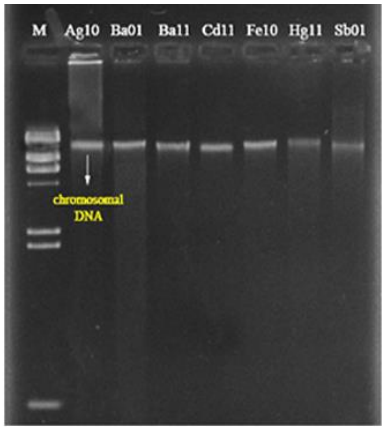
The *alk*B gene is known as a biomarker for determining alkane-degrading bacteria (Jurelevicius *et al.,* 2013). Molecular tools are useful for rapid detection of genes related to petroleum-degrading enzymes (Peixoto *et al.,* 2011). Therefore, by using already-characterized primers, alkane degradation abilities of the bacterial isolates were analyzed in molecular levels by PCR analysis.

3.4.1 Total DNA extraction

The *alk*B gene responsible in alkane degradation might be located on both DNA as shown on the OCT plasmid of *Pseudomonas putida* GPo1 by Belhaj *et al.* (2002) or on chromosomal DNA shown by Viggor *et al.* (2015). For that reason, total DNA extraction was carried out only for the isolates with kerosene degradation rates over 70%. Therefore, 7 isolates namely *P. plecoglossicida* Ag10, *S. aureus* Ba01, *S. rhizophila* Ba11, *D. acidovorans* Cd11, *A. calcoaceticus* Fe10, *P. koreensis* Hg11 and *A. johnsonii* Sb01 were used for the analysis of *alk*B gene through PCR.

To assess the purity of the extracted DNAs, nanodrop measurements were done for each isolate. The protein contamination is measured with a ratio of absorbance between 260 and 280 nm (A_{260}/A_{280}). A ratio of ~1.8 is generally accepted as pure DNA. On the other hand, presence of organic contaminants is measured with the ratio of 260 and 230 nm (A_{260}/A_{230}) and pure sample is expected to be in the range of 2.0-2.2 (Thermo Fisher Scientific, 2009). Taking these information to consideration, agarose gel of the extracted chromosomal DNAs and their corresponding nanodrop results are given in Figure 3.4.

a)



b)

Bacterial isolates	A_{260}/A_{280}	A_{260}/A_{230}	Concentration (ng/µl)
Pseudomonas plecoglossicida Ag10	1.81	1.81	148.97
Staphyloccocus aureus Ba01	1.89	1.92	190.18
Stenotrophomonas rhizophila Ba11	1.84	1.62	228.20
Delftia acidovorans Cd11	1.98	1.94	159.39
Acinetobacter calcoaceticus Fe10	2.00	1.82	394.10
Pseudomonas koreensis Hg11	2.11	2.06	673.00
Acinetobacter johnsonii Sb01	1.95	2.00	485.8

Figure 3.4 Agarose gel electrophoresis of the chromosomal DNA of prominent kerosene degraders; *P. plecoglossicida* Ag10, *S.aureus* Ba01, *S. rhizophila* Ba11, *D. acidovorans* Cd11, *A. calcoaceticus* Fe10, *P. koreensis* Hg11 and *A. johnsonii* Sb01. M, Thermo Fisher Lambda DNA/HindIII DNA ladder; from top to bottom: 23130, 9416, 6557, 4361, 2322, 2027, 564 and 125 bp, respectively (a) and nanodrop results of the total DNA extraction (b)

3.4.2 PCR analysis of the *alk*B gene

The isolate *A. johnsonii* Sb01 was chosen for the PCR optimization experiments. Optimum conditions were investigated by changing annealing temperatures ranging from 48 to 54°C, $MgCl_2$ concentrations in between 1.5 to 2.5 µL and primer concentration ranging from 0.5 to 0.9 µL. As shown in Figure 3.5, optimum results were obtained at an annealing temperature of 51°C with 1.5 µL $MgCl_2$ and 0.75 µL primer concentrations.

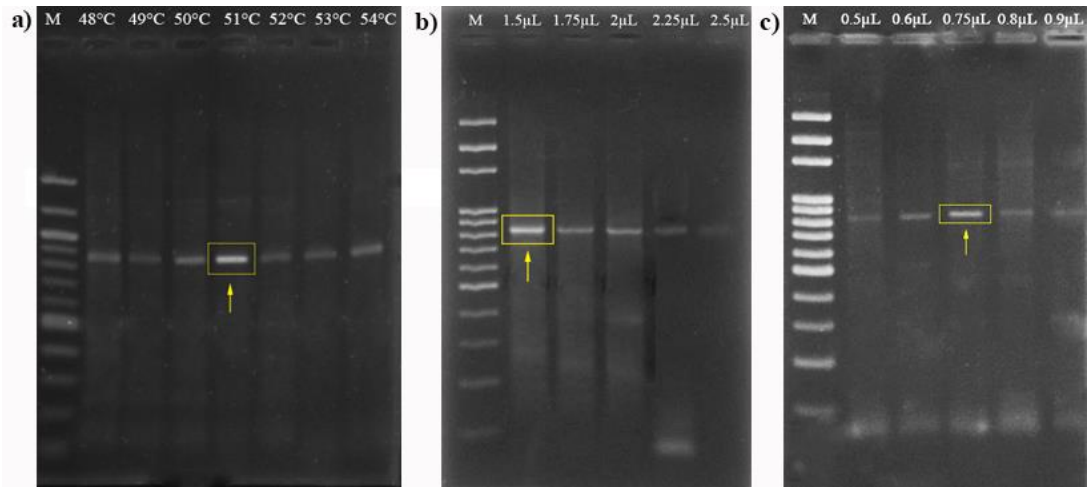


Figure 3.5 PCR optimization for the *alk*B gene in the conditions at different annealing temperatures (a), $MgCl_2$ concentrations (b) and primer concentrations (c) for the isolate *A. johnsonii* Sb01. M, Quick Load 100 bp DNA ladder from top to bottom 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp, respectively

After optimum conditions were determined, PCR was performed under these conditions to analyze the *alk*B gene in all of the isolates. The *alk*B gene was detected in all the bacterial isolates tested; *P. plecoglossicida* Ag10, *S. aureus* Ba01, *S. rhizophila* Ba11, *D. acidovorans* Cd11, *A. calcoaceticus* Fe10, *P. koreensis* Hg11 and *A. johnsonii* Sb01 with an amplification size of 870 bp (Figure 3.6).

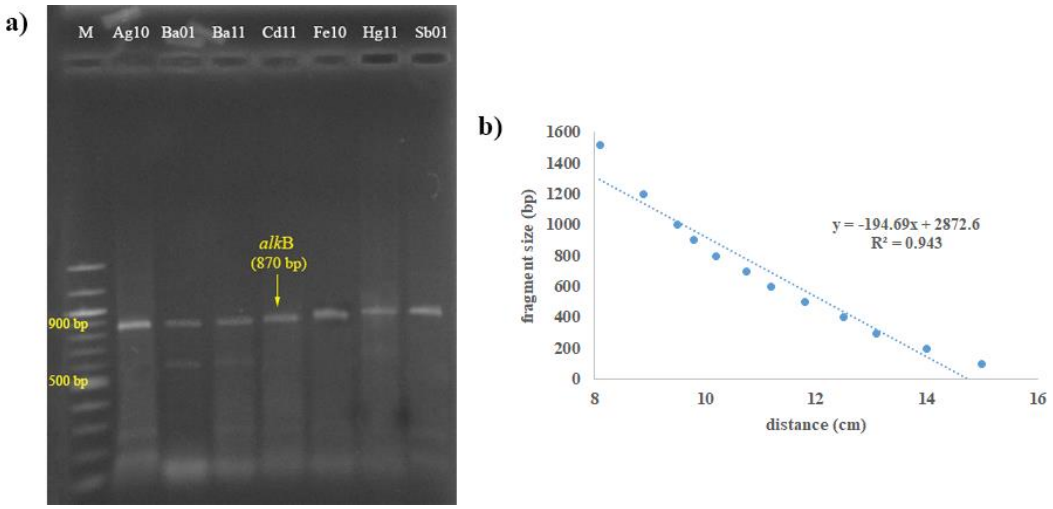


Figure 3.6 PCR analysis results of the *alk*B gene in prominent kerosene degraders*; P. plecoglossicida* Ag10, *S. rhizophila* Ba11, *D. acidovorans* Cd11, *A .calcoaceticus* Fe10, *P. koreensis* Hg11 and *A. johnsonii* Sb01. M, Quick Load 100 bp DNA ladder from top to bottom 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp, respectively (a). Standard curve of agarose gel for molecular weight estimation (b)

Current studies shows that the diversity of the *alk*B gene is still far from being well characterized (Jurelevicius *et al.*, 2013). Even though there are many studies on alkane degraders, molecular studies showing the presence of *alk*B gene in microorganisms are not that prevalent. The *alk*B gene has been detected in *Acinetobacter baumannii*, *Acinetobacter calcoaceticus*, *Acinetobacter oleoverans (Jurelevicius et al., 2013)*, *Acinetobacter haemolyticus* (Onur *et al*., 2015), *Gordonia, Nocardia* (Alvarez *et al.*, 2008), *Pseudomonas aeruginosa*, *Pseudomonas* sp. (Jurelevicius *et al.*, 2010) *Rhodococcus* sp. (Andreoni *et al.*, 2000), *Stenotrophomonas maltophilia* (Jurelevicius *et al.*, 2013), *Stenotrophomonas* sp. (Alvarez *et al.*, 2008). To the best of our knowledge, the presence of the *alk*B gene in *Pseudomonas plecoglossicida*, *Staphylococcus aureus*, *Stenotrophomonas rhizophila*, *Delftia acidovorans*, *Pseudomonas koreensis* and *Acinetobacter johnsonii* was demonstrated for the first time in the current study.

3.5 Screening for biosurfactant production

Many hydrocarbon degraders are known as biosurfactants producers (Patowary *et al.*, 2017) and several reports have been published focusing on the isolation of biosurfactants producing microorganisms (Satpute *et al.*, 2010; Varjani and Upasani, 2017). For that reason, 19 identified kerosene degraders were screened for their biosurfactant producing ability through oil spreading activity, emulsification index measurement, microbial adhesion of hydrocarbon tests.

3.5.1 Oil spreading activity

Areas of the clear zones obtained from oil spreading test indicates indirectly the surface activity of the biosurfactants produced by the hydrocarbon degraders (Rodrigues *et al.*, 2006). Larger zones represent higher surface activity of biosurfactants. Therefore, average of clear zone areas were calculated for all the isolates tested (Table 3.4). The raw data are given in Appendix C. According to the results, highest surface activity was observed in *P. agglomerans* Sn11 with an average area of 1.03 cm^2 (Figure 3.7).

Figure 3.7 Representative picture of clear zone obtained due to the biosurfactant of *P. agglomerans* Sn11

Table 3.4 Oil spreading activity measurements of the biosurfactants produced by the kerosene

Bacterial isolates	Area (cm^2)
Pseudomonas plecoglossicida Ag10	0.09±0.03
Raoultella planticola Ag11	0.09±0.03
Staphyloccocus aureus Ba01	0.06±0.02
Stenotrophomonas rhizophila Ba11	0.15±0.07
Delftia acidovorans Cd11	0.06±0.01
Staphylococcus warneri Co11	0.02±0.01
Enteroccocus faecalis Cr07	0.84±0.10
Acinetobacter calcoaceticus Fe10	0.84±0.10
Pseudomonas koreensis Hg11	0.16±0.04
Staphylococcus aureus Li12	0.06±0.02
Serratia nematodiphila Mn11	0.11±0.04
Acinetobacter haemolyticus Mn12	0.05±0.02
Comamonas testosteroni Ni11	0.03±0.00
Enteroccocus faecalis Pb06	0.79±0.16
Acinetobacter johnsonii Sb01	0.13±0.01
Pantoea agglomerans Sn11	1.03±0.69
Micrococcus luteus Sr02	0.37±0.08
Micrococcus luteus Sr11	0.77±0.16
Acinetobacter haemolyticus Zn01	0.14±0.02

±; standard deviations, yellow color used for biosurfactant producers

Ibrahim *et al.* (2013) reported microorganisms such as *Pseudomonas*, *Serratia*, *Micrococcus* and *Bacillus* having oil spreading activities between 7-20 cm^2. Other studies (Pacwa-Płociniczak *et al.*, 2014; Ibrahim, 2016) also reported surface activities of biosurfactants with areas in between 3 to 38 cm^2. Given these results, the biosurfactants produced by the isolates used in this study were thought to be not powerful towards kerosene.

3.5.2 Measurements of emulsification index (E_{24})

The emulsification index of the bacterial isolates was also measured in the presence of kerosene (Figure 3.8). According to Satpute *et al.* (2010), measurements of E_{24} is a reliable test for identifying biosurfactant producers, since E_{24} stability determines the strength of a surfactant. The E_{24} indices higher than 50% are accepted as promising candidates for biosurfactant production (Rodríguez-Rodríguez, Zúñiga-Chacón and Barboza-Solano, 2012). Out of 19, 9 bacterial isolates (*P. plecoglossicida* Ag10, *R. planticola* Ag11, *S. aureus* Ba01, *E. faecalis* Cr07, *S. nematodophila* Mn11, *A. haemolyticus* Mn12, *E. faecalis* Pb06, *A. johnsonii* Sb01 and *P. agglomerans* Sn11) showed emulsification activity over 50% (Table 3.5).

The highest E_{24} index was observed in *P. plecoglossicida* Ag10 and S. aureus Ba01 with both 67%. Raw data of the E_{24} measurements are given in Appendix C.

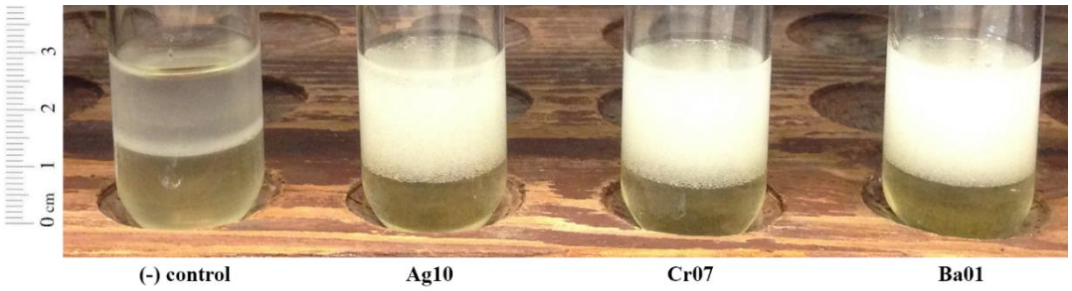


Figure 3.8 Representative picture of the E_{24} indices measurement. MSM without inoculum as a negative control. Emulsion layers formed by *P. plecoglossicida* Ag10, *E. faecalis* Cr07 and *S. aureus* Ba01

Table 3.5 The E_{24} indices measurements of the kerosene degraders

Bacterial isolates	E_{24} index (%)
Pseudomonas plecoglossicida Ag10	67.05±1.14
Raoultella planticola Ag11	62.88±3.47
Staphyloccocus aureus Ba01	67.04±1.14
Stenotrophomonas rhizophila Ba11	27.22±1.68
Delftia acidovorans Cd11	28.52±1.68
Staphylococcus warneri Co11	18.32±3.42
Enteroccocus faecalis Cr07	61.25±1.25
Acinetobacter calcoaceticus Fe10	4.28±0.25
Pseudomonas koreensis Hg11	46.39±4.72
Staphylococcus aureus Li12	48.58±1.63
Serratia nematodiphila Mn11	62.47±0.46
Acinetobacter haemolyticus Mn12	60.72±1.07
Comamonas testosteroni Ni11	30.11±2.68
Enteroccocus faecalis Pb06	52.75±0.58
Acinetobacter johnsonii Sb01	53.27±1.09
Pantoea agglomerans Sn11	56.72±2.65
Micrococcus luteus Sr02	14.86±1.81
Micrococcus luteus Sr11	11.69±0.82
Acinetobacter haemolyticus Zn01	34.57±0.22

±; standard deviations, yellow color used for biosurfactant producers

3.5.3 Microbial adhesion to hydrocarbons test

Measurements of cell surface hydrophobicity is important for the adhesion of bacterial cells to surfaces especially for biodegradation activity (Youssef *et al.*, 2004). According to Pruthi and Cameotra (1997) there is a direct correlation between cell hydrophobicity and biosurfactant production. Sedláčková *et al.* (2011) interpreted their MATH results and categorized the degree of bacterial hydrophobicity such as <10% hydrophilic, 10-29% medium hydrophilic, 30-54% medium hydrophobic and >55%

highly hydrophobic. Therefore, MATH assay was performed for the bacterial isolates. As shown in Table 3.6, 13 isolates showed high hydrophobicity towards kerosene. Highest hydrophobicity was observed in *P. agglomerans* Sn11 and *R. planticola* Ag11 with approximately 93%. The raw data of MATH test can be found in Appendix C.

Table 3.6 Hydrophobicity percentages over kerosene of the bacterial isolates

Bacterial isolates	Hydrophobicity (%)
Pseudomonas plecoglossicida Ag10	66.14±2.10
Raoultella planticola Ag11	92.78±6.74
Staphyloccocus aureus Ba01	77.60±6.07
Stenotrophomonas rhizophila Ba11	81.11±5.51
Delftia acidovorans Cd11	70.12±5.00
Staphylococcus warneri Co11	45.76±1.23
Enteroccocus faecalis Cr07	29.27±7.32
Acinetobacter calcoaceticus Fe10	37.58±1.51
Pseudomonas koreensis Hg11	81.51±4.07
Staphylococcus aureus Li12	24.91±5.26
Serratia nematodiphila Mn11	55.33±4.41
Acinetobacter haemolyticus Mn12	86.63±3.33
Comamonas testosteroni Ni11	91.76±0.51
Enteroccocus faecalis Pb06	35.71±1.70
Acinetobacter johnsonii Sb01	80.16±3.64
Pantoea agglomerans Sn11	92.89±2.54
Micrococcus luteus Sr02	87.96±3.83
Micrococcus luteus Sr11	62.95±0.44
Acinetobacter haemolyticus Zn01	56.92±2.07

±; standard deviations, yellow color for highly hydrophobic bacterial cells

3.5.4 Selection of biosurfactant producers

A single method is not suitable for effective screening of biosurfactant producers, therefore most of the researchers have used two or three screening methods (Satpute *et al.*, 2008). In this study, prominent biosurfactant producers were chosen by performing statistical analysis for the results obtained from oil spreading activity, measurements of E_{24} index, and MATH tests. Standardization of each result was done in order to compare all results in one scale. Total z-score values for each test was given in Table 3.7. The mean of z-score was calculated as zero. Bacteria with a z-score above the mean value were determined as biosurfactant producers as follows; *P. plecoglossicida* Ag10, *R. planticola* Ag11, *S. aureus* Ba01, *E. faecalis* Cr07, *P. koreensis* Hg11, *A. haemolyticus* Mn12, *E. faecalis* Pb06, *A. johnsonii* Sb01 and *P. agglomerans* Sn11.

Table 3.7 Z-score values of each bacterial isolate for biosurfactant screening tests

Bacterial isolates	Oil spreading	E_{24}	MATH	Total z-score
Pseudomonas plecoglossicida Ag10	-0.62	1.19	0.04	0.61
Raoultella planticola Ag11	-0.62	0.99	1.18	1.55
Staphyloccocus aureus Ba01	-0.70	1.19	0.53	1.02
Stenotrophomonas rhizophila Ba11	-0.44	-0.75	0.68	-0.51
Delftia acidovorans Cd11	-0.70	-0.68	0.21	-1.18
Staphylococcus warneri Co11	-0.82	-1.18	-0.84	-2.83
Enteroccocus faecalis Cr07	1.53	0.91	-1.54	0.90
Acinetobacter calcoaceticus Fe10	1.53	-1.87	-1.19	-1.52
Pseudomonas koreensis Hg11	-0.42	0.19	0.69	0.47
Staphylococcus aureus Li12	-0.70	0.29	-1.73	-2.14
Serratia nematodiphila Mn11	-0.56	0.97	-1.13	-0.72
Acinetobacter haemolyticus Mn12	-0.73	0.88	0.91	1.07
Comamonas testosteroni Ni11	-0.79	-0.61	1.13	-0.26
Enteroccocus faecalis Pb06	1.39	0.50	-1.27	0.62
Acinetobacter johnsonii Sb01	-0.50	0.52	0.64	0.66
Pantoea agglomerans Sn11	2.08	0.69	1.18	3.95
Micrococcus luteus Sr02	0.19	-1.35	0.97	-0.19
Micrococcus luteus Sr11	1.33	-1.50	-0.10	-0.27
Acinetobacter haemolyticus Zn01	-0.47	-0.39	-0.36	-1.22

Mean was calculated as 0.00, Biosurfactant producers with a z-score above 0.00 were highlighted in yellow

Among selected biosurfactant producers, there was two *Pseudomonas* (*P. plecoglossicida* Ag10 and *P. koreensis* Hg11), two *Enterococcus* (*E. faecalis* Cr07 and *E. faecalis* Pb06) and two *Acinetobacter* (*A. haemolyticus* Mn12 and *A. johnsonii* Sb01) species. Since the investigation of biosurfactant producers were done in genus level, the degradation abilities of these isolates were also taken in consideration (Table 3.8). Therefore, *P. koreensis* Hg11, *A. haemolyticus* Mn12 and *E. faecalis* Pb06 were not used for biosurfactant characterization tests as their degradation abilities were lower. Consequently, biosurfactants characterization tests were only performed for 6 different genera namely; *Pseudomonas plecoglossicida* Ag10, *Raoultella planticola* Ag11, *Staphylococcus aureus* Ba01, *Enterococcus faecalis* Cr07, *Acinetobacter johnsonii* Sb01 and *Pantoea agglomerans* Sn11.

Table 3.8 Summary of biosurfactant screening tests and degradation abilities of the bacterial isolates

Bacterial isolates	Biosurfactant screening tests			Degradation abilities
	E_{24} (%)	MATH (%)	Oil spreading (cm^2)	Gas chromatography (%)
Pseudomonas plecoglossicida Ag10	67.05±1.14	66.14±2.10	0.09±0.03	78.24
Raoultella planticola Ag11	62.88±3.47	92.78±6.74	0.09±0.03	47.71
Staphyloccocus aureus Ba01	67.04±1.14	77.60±6.07	0.06±0.02	69.29
Stenotrophomonas rhizophila Ba11	27.22±1.68	81.11±5.51	0.15±0.07	72.07
Delftia acidovorans Cd11	28.52±1.68	70.12±5.00	0.06±0.01	80.66
Staphylococcus warneri Co11	18.32±3.42	45.76±1.23	0.02±0.01	63.25
Enteroccocus faecalis Cr07	61.25±1.25	29.27±7.32	0.84±0.10	33.52
Acinetobacter calcoaceticus Fe10	4.28±0.25	37.58±1.51	0.84±0.10	72.40
Pseudomonas koreensis Hg11	46.39±4.72	81.51±4.07	0.16±0.04	70.10
Staphylococcus aureus Li12	48.58±1.63	24.91±5.26	0.06±0.02	36.98
Serratia nematodiphila Mn11	62.47±0.46	55.33±4.41	0.11±0.04	29.72
Acinetobacter haemolyticus Mn12	60.72±1.07	86.63±3.33	0.05±0.02	13.92
Comamonas testosteroni Ni11	30.11±2.68	91.76±0.51	0.03±0.00	59.54
Enteroccocus faecalis Pb06	52.75±0.58	35.71±1.70	0.79±0.16	12.61
Acinetobacter johnsonii Sb01	53.27±1.09	80.16±3.64	0.13±0.01	83.76
Pantoea agglomerans Sn11	56.72±2.65	92.89±2.54	1.03±0.69	5.43
Micrococcus luteus Sr02	14.86±1.81	87.96±3.83	0.37±0.08	0.07
Micrococcus luteus Sr11	11.69±0.82	62.95±0.44	0.77±0.16	56.43
Acinetobacter haemolyticus Zn01	34.57±0.22	56.92±2.07	0.14±0.02	45.70

±; standard deviations, yellow color used for biosurfactant producers, ±; standard deviations

3.6 Surface and interfacial tension measurements

The degradation of hydrocarbons is enhanced by the production of biosurfactant (Parthipan *et al.*, 2017). Therefore, surface and interfacial tension measurements for the prominent kerosene degraders were done to reveal the correlation in between kerosene degradation and biosurfactant production abilities. Tension reductions were also calculated for chemical surfactants like SDS and Tween 20 for the comparison (Table 3.9 and 3.10). Raw data of the analyses are given in Appendix D.

Table 3.9 Surface tension measurements and reduction percentages of prominent kerosene degraders

Bacterial isolates	Surface tension (mN/m)	Surface tension reduction (%)
Control	69.61±0.13	-
Pseudomonas plecoglossicida Ag10	69.54±0.22	0.09
Staphyloccocus aureus Ba01	68.77±0.25	1.20
Stenotrophomonas rhizophila Ba11	63.69±0.75	8.50
Delftia acidovorans Cd11	69.49±0.23	0.17
Acinetobacter calcoaceticus Fe10	68.24±0.11	2.18
Pseudomonas koreensis Hg11	67.59±0.10	2.89
Acinetobacter johnsonii Sb01	69.46±0.28	0.21
SDS	32.77±0.76	52.92
Tween 20	66.81±0.52	4.02

±; standard deviations, yellow color used for biosurfactant producers and blue color represents the chemical surfactants

Walter *et al*. (2010) indicated that, a promising biosurfactant should be able to reduce surface tension of the medium to 40 mN/m or less. As is seen from Table 3.9, biosurfactants from the kerosene degraders were not successful in surface tension reduction. Compared to the control, highest reduction was observed in *S. rhizophila* Ba11 with 8.5% while Tween 20, which is a powerful surfactant, showed only 4% of reduction.

Kaczorek *et al.* (2005) studied the relation between surface tension and biodegradation activity. They reported that the decrease in surface tension is not always related to efficient biodegradation. Many factors are affecting the surface tension like the type and concentration of biosurfactant, the quantity, the bacterial isolates and the properties of hydrocarbons.

The tension between kerosene and culture medium was measured through interfacial tension measurements. Highest reduction was observed in *A. johnsonii* Sb01 with 28%, higher than the chemical surfactant SDS (23%).

Varjani (2017) has reported strong negative correlation between surface tension measurement and interfacial tension reduction. However, biosurfactants produced by the kerosene degraders in this study did not show any significant reduction for both tests. No correlation was found between kerosene degradation and biosurfactant activity.

Table 3.10 Interfacial tension measurements of the kerosene degraders

Bacterial isolates	Interfacial tension (mN/m)	Interfacial tension reduction (%)
Control	22.18±0.19	-
Pseudomonas plecoglossicida Ag10	21.92±0.15	1.17
Staphyloccocus aureus Ba01	21.72±0.35	2.08
Stenotrophomonas rhizophila Ba11	21.83±0.54	1.57
Delftia acidovorans Cd11	21.58±0.43	2.73
Acinetobacter calcoaceticus Fe10	19.21±1.10	13.42
Pseudomonas koreensis Hg11	20.35±0.25	8.27
Acinetobacter johnsonii Sb01	15.96±0.27	28.04
SDS	16.98±0.52	23.44
Tween 20	8.12±0.63	63.41

±; standard deviations, yellow color used for biosurfactant producers and blue color represents the chemical surfactants

3.7 Characterization of the biosurfactants produced by the kerosene degraders

It is important to characterize biosurfactants produced by bacterial isolates since their properties differ depending on their chemical structure (Banat *et al.*, 2010). Regarding their mechanism of action, some compounds are better at decreasing the surface tension while others are able to produce stable emulsions (Sáenz-Marta *et al.*, 2015). For that reason, preliminary characterization of the biosurfactants produced were done by phenol sulphuric acid method, biuret test, phosphate test and blue agar plate (BAP) method. Results of the tests are summarized in Table 3.11.

Table 3.11 Preliminary characterization tests of the biosurfactants produced

Bacterial isolates	Phenol:H_2SO_4	Biuret	Phosphate	BAP
Pseudomonas plecoglossicida Ag10	-	-	-	+
Raoultella planticola Ag11	-	-	-	+
Staphyloccocus aureus Ba01	-	-	-	+
Enteroccocus faecalis Cr07	-	-	-	+
Acinetobacter johnsonii Sb01	-	-	-	+
Pantoea agglomerans Sn11	-	-	-	+

(+); positive, (-); negative, BAP; blue agar plate

As seen, none of the biosurfactant producers did show orange color when phenol and concentrated sulphuric acid were added to supernatants containing biosurfactants. Hence, the results were recorded as negative to indicate that biosurfactants did not contain any carbohydrates (Ellaiah *et al.*, 2002). For biuret test, it was assumed that the samples turned to violet or pink ring due to the reaction of peptide bond proteins (Kalyani *et al.*, 2014). No significant color change or ring was detected when biuret reagent was added to the samples. In the phosphate test, yellow color precipitate was not observed. Therefore, biosurfactants were thought to contain neither carbonhydrate, lipopeptide nor phospholipid (Figure 3.9).

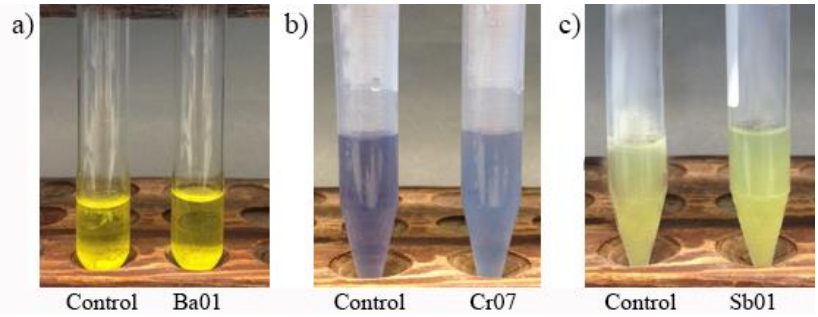


Figure 3.9 Representative pictures of the preliminary characterization tests for the biosurfactants produced. Control and negative result from *S. aureus* Ba01 in phenol sulphuric acid method (a), control and negative result for *E. faecalis* Cr07 in biuret test (b) and control and negative result for *A. johnsonii* Sb01 in phosphate test

Since all results were recorded as negative, the BAP method was also performed as it is suggested as a more specific method for the glycolipid type biosurfactant detection (Youssef *et al.*, 2004). Positive results were obtained with biosurfactant producers by forming blue zones around the colonies (Table 3.11 and Figure 3.10). This colorimetric assay indicated the presence of glycolipid/anionic biosurfactants, mostly rhamnolipids, that reacted with cationic CTAB and methylene blue as explained by Satpute *et al.* (2008).

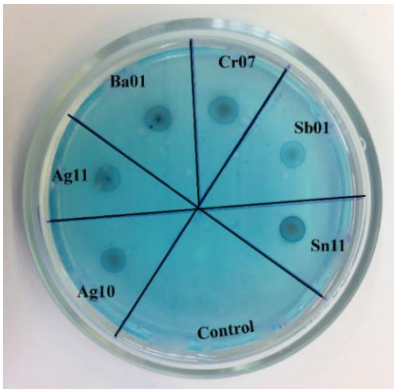


Figure 3.10 Control contains no inoculum. The BAP method applied for biosurfactant producers; *P. plecoglossicida* Ag10, *R. planticola* Ag11, *S. aureus* Ba01, *E. faecalis* Cr07, *A. johnsonii* Sb01, *P. agglomerans* Sn11

3.8 Extraction of biosurfactants

In order to confirm the glycolipid structure, crude biosurfactants were extracted and also quantified. The culture supernatant was acidified and precipitation of biosurfactants were observed as expected since it became less soluble in medium due to protonated form (Satpute *et al.*, 2010). After purification steps, crude biosurfactants were obtained and were further used for TLC and FTIR analysis.

3.9 Quantification of biosurfactants produced by the isolates

All of the screening methods used in this study so far did not give any quantitative information about biosurfactants as indicated by Walter *et al*. (2010). Therefore, the amount of biosurfactant produced in this study, were determined by dry weight measurements. After biosurfactant extraction, crude extracts were weighted and the results were noted in g/L (Table 3.12). The raw data are given in Appendix E.

Table 3.12 Amount of biosurfactants produced by prominent biosurfactant producers

Bacterial isolates	Amount of biosurfactant (g/L)
Pseudomonas plegoclossicida Ag10	3.3±0.07
Raoultella planticola Ag11	3.5±0.03
Staphylococcus aureus Ba01	3.5±0.01
Enterococcus faecalis Cr07	2.8±0.05
Acinetobacter johnsonii Sb01	2.2±0.04
Pantoea agglomerans Sn11	2.3±0.03

±; standard deviations

Most studies have been focused on commercial production of biosurfactants due to their high production cost (Varjani and Upasani, 2017). Commercial production of biosurfactant depends mainly on the carbon sources used (Rodrigues *et al.*, 2006). There are various studies on rhamnolipid production from different carbon sources. Vasileva-Tonkova and Gesheva (2007) obtained rhamnolipid 0.8 to 1.2 g/L by *Pantoea* sp. grown on 2% kerosene in 16 days. In this study, the strain *Pantoea agglomerans* Sn11 produced 2.3 g/L of biosurfactant in NB medium within 7 days. Moreover, Patowary *et al.* (2017) used crude oil as a carbon source and achieved 2.26 g/L of rhamnolipid from *P. aeruginosa* in 7 days. Zhang *et al.* (2005) also reported a 0.45 g/L rhamnolipid production within 3 days in a medium enriched with glucose from *Pseudomonas aeruginosa.*

3.10 Thin layer chromatography

TLC is one of the most commonly used technique to identify the biosurfactants (Satpute *et al.*, 2010). Therefore, spot detections of the samples were done after plates were sprayed with orcinol reagent for the detection of glycolipid type biosurfactant (Bharali *et al.*, 2014). Crude extracts from biosurfactant producers were tested in TLC analysis. All of the tested bacterial isolates showed orcinol-positive spots (Figure 3.11). R_f values for the isolates were between 0.66-0.78 (Table 3.13). Consequently, the TLC results were in consistent with Priya and Usharani (2009) and Bhardwaj *et al.* (2015) who also found out the R_f values in between 0.64 to 0.72 for glycolipid type biosurfactants, respectively.

Figure 3.11 Orcinol-positive spots for prominent biosurfactant producers

Table 3.13 Determining R_f values for the identification of the biosurfactants

Bacterial isolates	Distance of the solvent (cm)	Distance of the sample (cm)	R_f value
Pseudomonas plegoclossicida Ag10	8.60	6.60	0.77
Raoultella planticola Ag11	8.60	6.58	0.77
Staphylococcus aureus Ba01	8.60	6.65	0.77
Enterococcus faecalis Cr07	8.60	6.70	0.78
Acinetobacter johnsonii Sb01	8.30	5.50	0.66
Pantoea agglomerans Sn11	8.30	6.10	0.73

3.11 Fourier transform infrared spectroscopy

FTIR is a useful method for identification of functional groups or chemical bonds (Thavasi *et al.*, 2009). Therefore, the molecular structure of biosurfactants obtained from the bacterial isolates were further analyzed by FTIR spectroscopy (Figure 3.12-27). FTIR spectrums of the extracted biosurfactants were representing a glycolipid structure, mostly similar to rhamnolipids (Table 3.14).

Table 3.14 Characteristic peaks of FTIR spectra for rhamnolipid reported from the literature

Characteristic peaks of rhamnolipid	Wavenumbers (cm^{-1})	References
O-H stretching vibrations of hydroxyl groups	3440-3400	Saikia *et al*., 2013; Singh and Tripathi, 2013
CH aliphatic stretching vibration of $-CH_2$ and $-CH_3$ groups	3000-2700	Rahman *et al.*, 2010; Saikia *et al*., 2013
C=O stretching of the ester linkage	~ 1740	Bharali *et al.*, 2014; Gogoi *et al.*, 2016
C=O stretching frequency of the carbonyl group of -COOH	~ 1650	Bharali *et al.*, 2014; Gogoi *et al.*, 2016
C-H and O-H deformation vibrations	1460-1200	Leitermann *et al*., 2008
C-O stretching bands bonds between carbon atoms and hydroxyl groups in rhamnose ring	1300-1000	Singh and Tripathi, 2013; Gogoi *et al.*, 2016

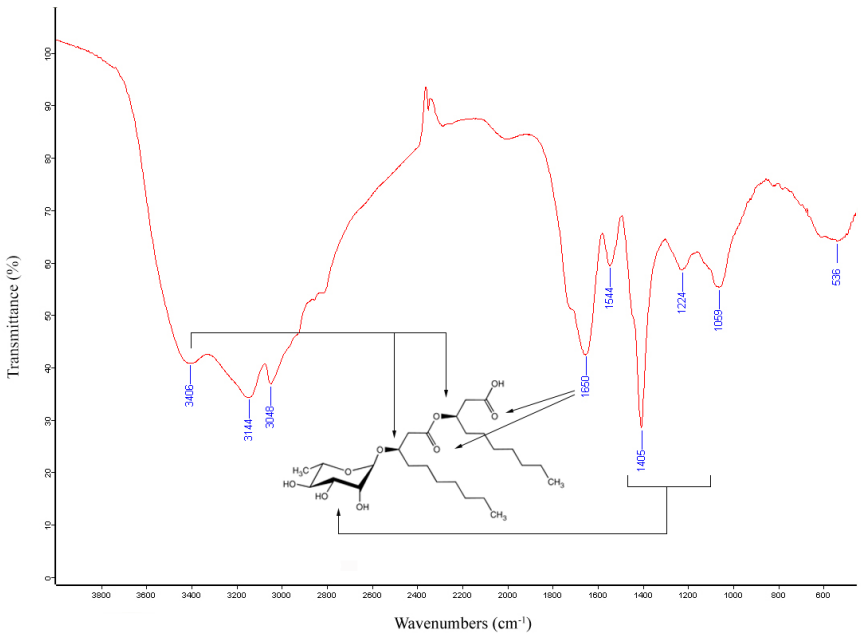


Figure 3.12 FTIR analysis of the biosurfactant produced by the isolate *P. plecoglossicida* Ag10

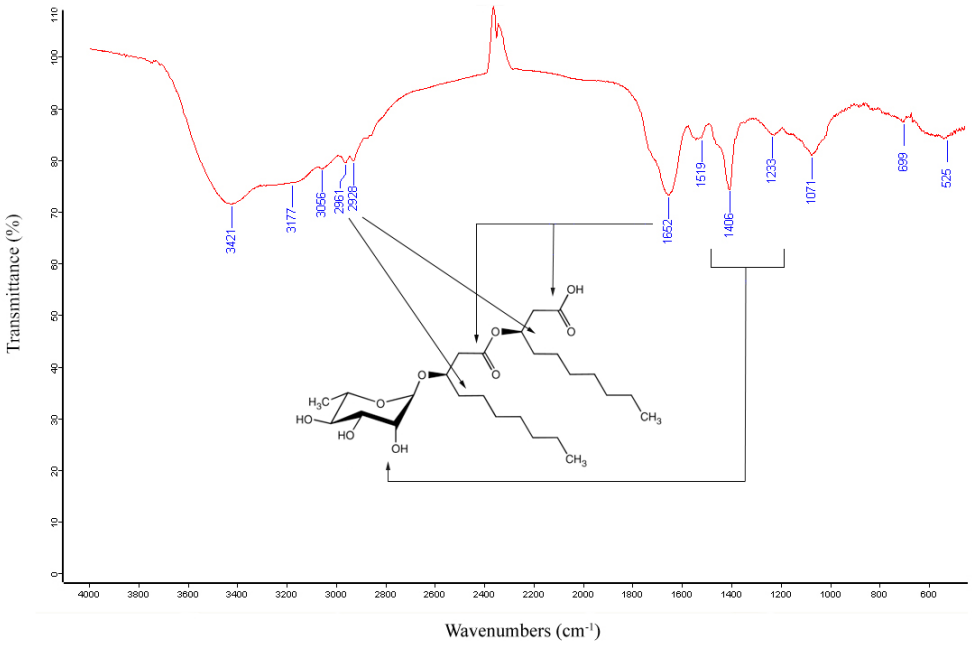


Figure 3.13 FTIR analysis of the biosurfactant produced by the isolate *R. planticola* Ag11

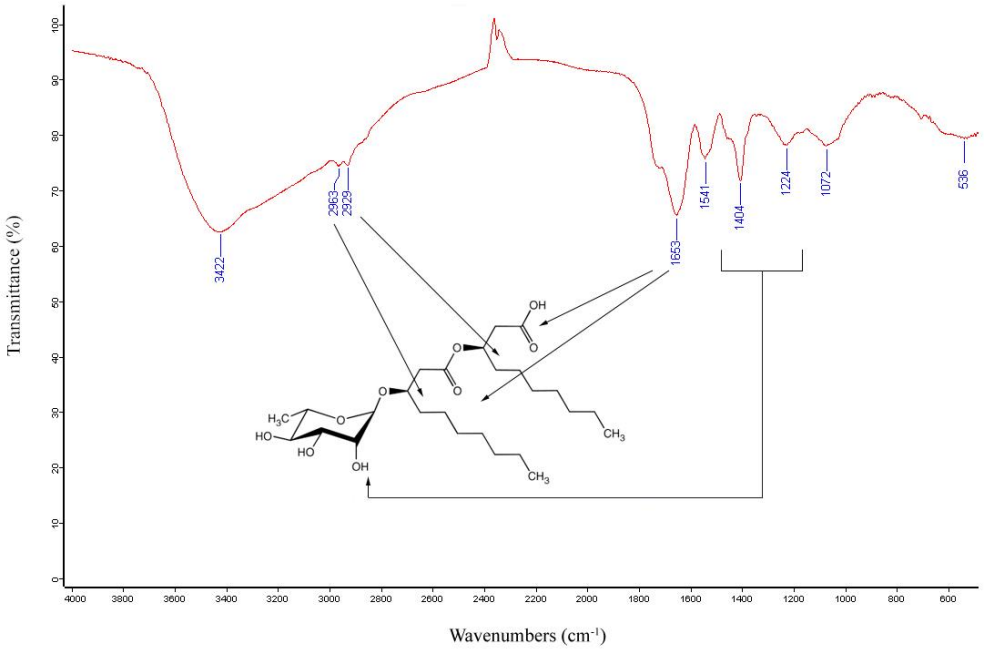


Figure 3.14 FTIR analysis of the biosurfactant produced by the isolate *S. aureus* Ba01

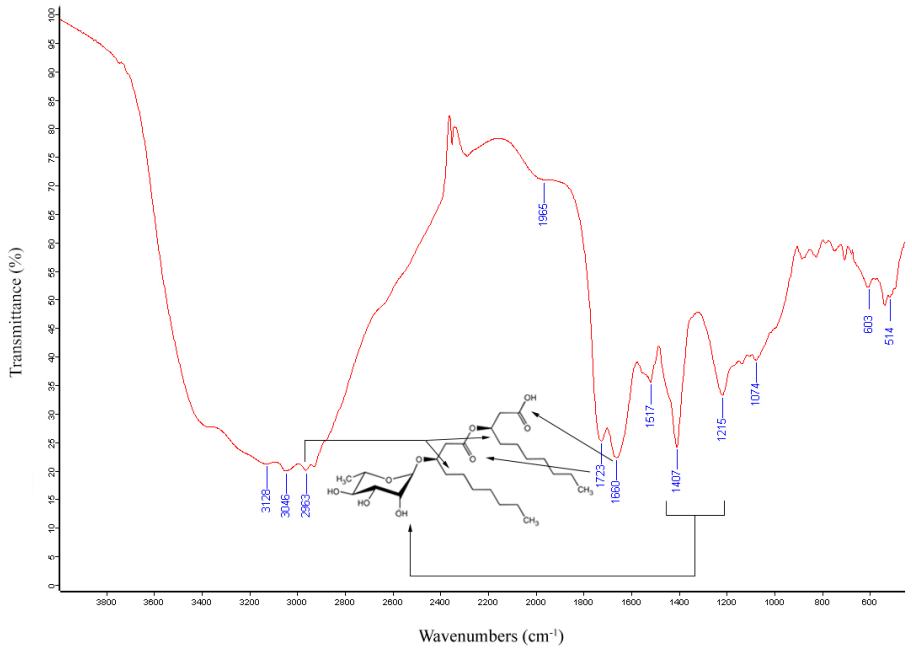


Figure 3.15 FTIR analysis of the biosurfactant produced by the isolate *E. faecalis* Cr07

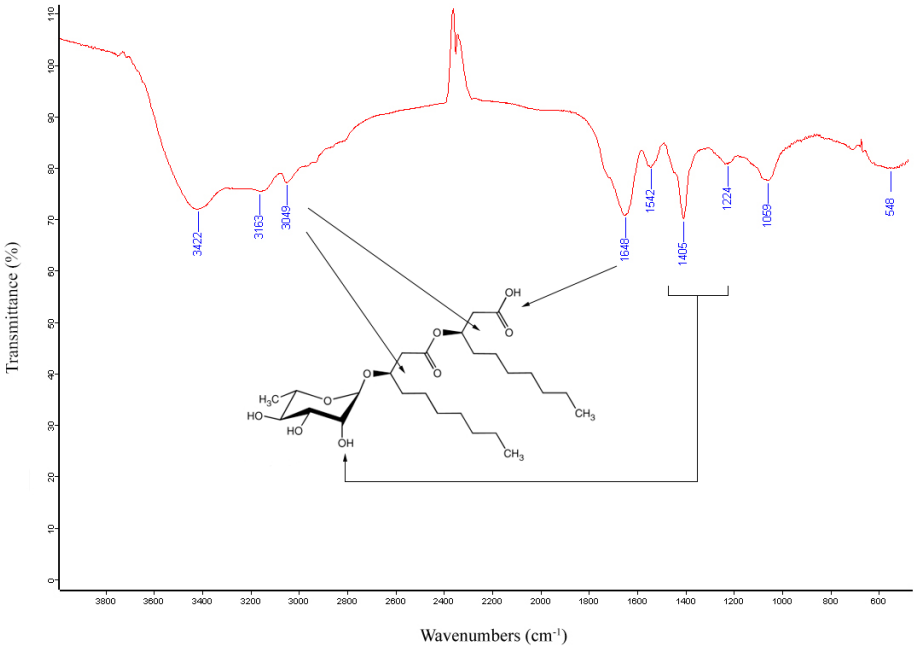


Figure 3.16 FTIR analysis of the biosurfactant produced by the isolate *A. johnsonii* Sb01

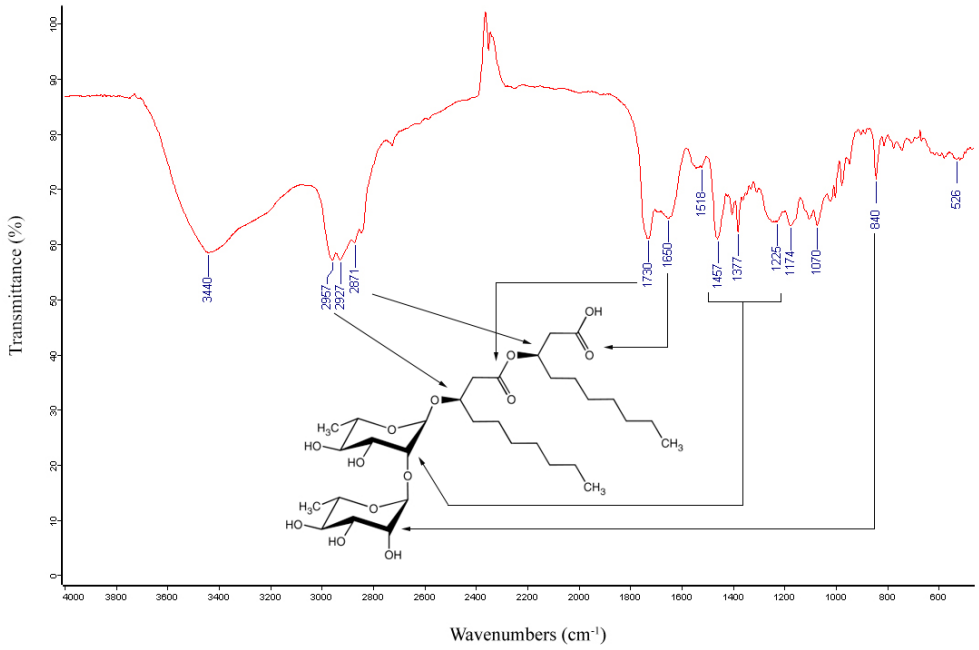


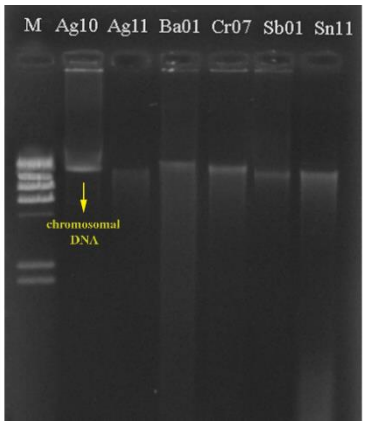
Figure 3.17 FTIR analysis of the biosurfactant produced by the isolate *P. agglomerans* Sn11

The most characteristic peaks for rhamnolipids are between 1720-1680 (Singh and Tripathi, 2013). Among all FTIR results, *E. faecalis* Cr07 and *P. agglomerans* Sn11 showed that characteristic peak representing the C=O stretching of the ester linkage and the C=O stretching frequency of the carboxylate acid groups (Gogoi *et al.*, 2016) Therefore, *E. faecalis* Cr07 and *P. agglomerans* Sn11 were accepted as rhamnolipid producers. Moreover, *P. agglomerans* Sn11 showed a different peak at 840 cm^{-1} (Figure 3.17) that might represent the presence of a di-rhamnolipid structure as indicated by Rahman *et al.* (2010). They reported the presence of di-rhamnolipid with pyranyl I sorption band in region at 918-940 cm^{-1} and α- pyranyl II sorption band in at 838-844 cm^{-1} similar to *P. agglomerans* Sn11. The FTIR spectrums of the bacterial isolates also showed peaks around 1500 cm^{-1}. This peak was not thought to be a characteristic peak for rhamnolipid. Most probably it indicated the presence of protein related compounds as suggested by Bharali *et al.* (2014). The reason of such bands could be the result from the contamination of polypeptides from cell residuals during the extraction process. The extracted biosurfactants were characterized as glycolipid with CTAB, TLC and as rhamnolipid with FTIR analysis. However these methods needs to be supported by other analytical techniques (Irorere *et al.*, 2017). Therefore, *rhl*AB gene responsible in the biosynthesis of rhamnolipid production was investigated with PCR analysis.

3.12 PCR analysis of the *rhl*AB gene

Before starting PCR analysis for the *rhl*AB gene, extraction of total DNA for biosurfactant producers was done. After extraction, the extracts were run in 1% agarose gel (Figure 3.18). The DNA template concentrations were measured with nanodrop and given in Figure 3.18.

a)



b)

Bacterial isolates	A_{260}/A_{280}	A_{260}/A_{230}	Concentration (ng/µl)
Pseudomonas plecoglossicida Ag10	2.01	2.09	760.02
Raoultella planticola Ag11	1.73	2.30	68.34
Staphyloccocus aureus Ba01	1.89	1.92	190.18
Enterococcus faecalis Cr07	1.84	1.62	92.21
Acinetobacter johnsonii Sb01	1.83	1.43	211.38
Pantoea agglomerans Sn11	2.01	2.07	167.27

Figure 3.18 Agarose gel electrophoresis of chromosomal DNA of the biosurfactant producers. M, Thermo Fisher Lambda DNA/HindIII DNA ladder; from top to bottom: 23130, 9416, 6557, 4361, 2322, 2027 and 564, respectively (a), nanodrop results of total DNA extracted (b)

PCR optimization of the *rhl*AB gene was performed with the isolate *A. johnsonii* Sb01. Optimum conditions were investigated by changing annealing temperatures ranging from 48-55°C, $MgCl_2$ concentrations in between 1.25 to 2.5 µL and primer concentrations ranging from 0.4-1 µL. The optimum PCR conditions were obtained at 50°C with 2 µL $MgCl_2$ and 0.6 µL primer concentrations (Figure 3.19).

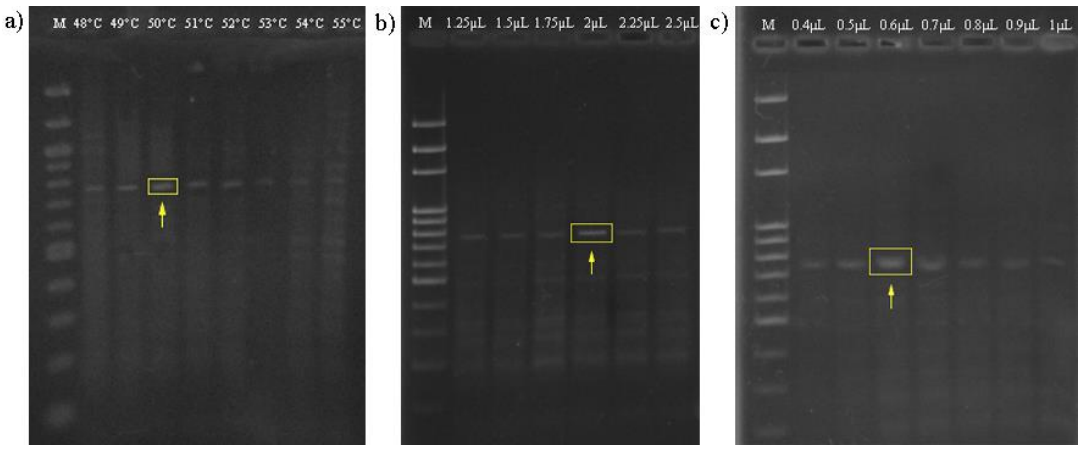


Figure 3.19 Optimization of PCR conditions for the *rhl*AB gene at different annealing temperatures (a), $MgCl_2$ concentrations (b) and primer concentrations (c) of *A. johnsonii* Sb01. M, Quick Load 100 bp DNA ladder from top to bottom 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp, respectively

The presence of the *rhl*AB gene is responsible for the synthesis of rhamnolipid type biosurfactant (Pacwa-Płociniczak *et al.*, 2014). Therefore, single DNA fragment of the expected amplicon size of 777 bp was obtained in all the isolates tested; *P. plecoglossicida* Ag10, *R. planticola* Ag11, *S. aureus* Ba01, *E. faecalis* Cr07, *A. johnsonii* Sb01 and *P.* agglomerans Sn11 (Figure 3.20).

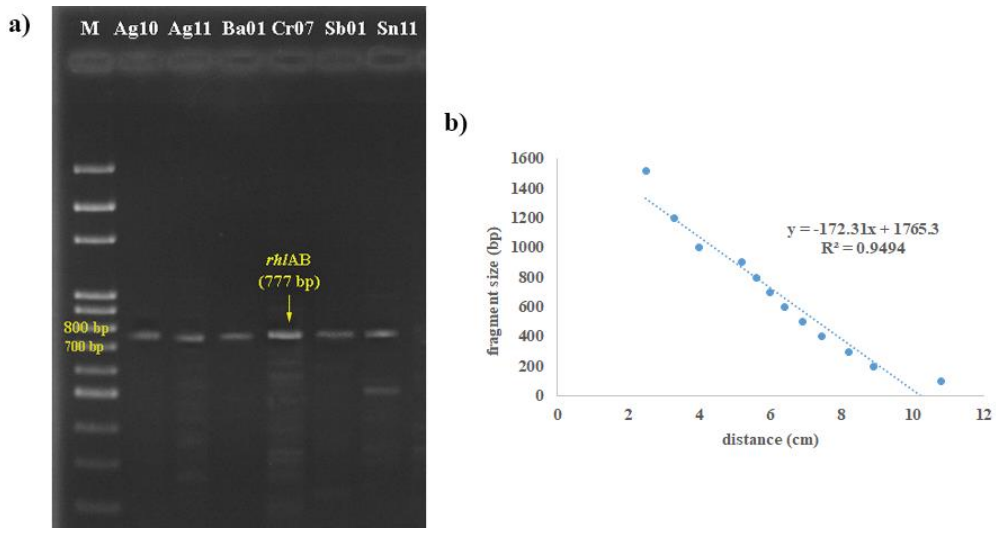


Figure 3.20 Analysis of the *rhl*AB gene in *P. plecoglossicida* Ag10, *R. planticola* Ag11, *S.aureus* Ba01, *E. faecalis* Cr07, *A. johnsonii* Sb01 and *P. agglomerans* Sn11 (a) and standard curve of the agarose gel for molecular weight estimation

Although there are numerous studies reporting rhamnolipid production from different bacterial isolates, the presence of *rhl*AB gene was shown only in few bacteria (Varjani and Upasani, 2017). Shoeb *et al.* (2012) showed the presence of the *rhl*AB gene through PCR in five bacterial isolates from the genus *Pseudomonas*. Other than *Pseudomonas,* it has also been shown in *Burkholderia thailandensis*, *B. pseudomallei* (Dubeau *et al.*, 2009), *Pantoea ananatis* (Smith *et al.*, 2016) and *Serratia rubidaea* (Nalini and Parthasarathi, 2014).

Similar to the current study, rhamnolipid production from *P. plecoglossicida* (Sharma *et al.*, 2015), *Staphylococcus aureus* (Rajesh *et al.*, 2017) and *Acinetobacter calcoaceticus* (Hošková *et al.*, 2015) was also reported. Thus, there is not enough genetic evidence to support rhamnolipid production of these bacteria. In this study, rhamnolipid producers were confirmed by PCR analysis. Even though the *rhl*AB gene was previously detected in the species of *Pseudomonas* and *Pantoea*, the presence the

*rhl*AB gene was reported for the first time in *Pseudomonas plecoglossicida, Raoultella planticola*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Acinetobacter johnsonii* and *Pantoea agglomerans*.

3.13 Conclusion

1. In this study, previously identified 22 hydrocarbon degraders were further evaluated for their ability to degrade kerosene and produce biosurfactant.
2. Out of 22 hydrocarbon degraders, 19 namely *P. plecoglossicida* Ag10, *R. planticola* Ag11, *S. aureus* Ba01, *S. rhizophila* Ba11, *D. acidovorans* Cd11, *S. warneri* Co11, *E. faecalis* Cr07, *A. calcoaceticus* Fe10, *P. koreensis* Hg11, *S. aureus* Li12, *S. nematodiphila* Mn11, *A. haemolyticus* Mn12, *C. testosteroni* Ni11, *E. faecalis* Pb06, *A. johnsonii* Sb01, *P. agglomerans* Sn11, *M. luteus* Sr02, *M. luteus* Sr11, *A. haemolyticus* Zn01 were found to degrade kerosene.
3. GC analyses revealed that *P. plecoglossicida* Ag10, *S. aureus* Ba01, *S. rhizophila* Ba11, *D. acidovorans* Cd11, *A. calcoaceticus* Fe10, *P. koreensis* Hg11 and *A. johnsonii* Sb01 had kerosene degradation abilities in between 69-84%.
4. The *alk*B gene responsible for kerosene degradation was detected in all kerosene degraders studied through PCR analyses.
5. The isolates *P. plecoglossicida* Ag10, *R. planticola* Ag11, *S. aureus* Ba01, *E. faecalis* Cr07, *A. johnsonii* Sb01 and *P. agglomerans* Sn11 were also determined as efficient biosurfactant producers through oil spreading activity, E_{24} index measurement and MATH tests.
6. BAP, TLC and FTIR analyses revealed that kerosene degraders produced glycolipid type rhamnolipids.
7. Presence of the *rhl*AB gene responsible for the synthesis of rhamnolipids was shown to be harbored by all the biosurfactant producers after PCR analysis.
8. The study pointed out that among 19 kerosene degraders, *P. plecoglossicida* Ag10, *S. aureus* Ba01, *S. rhizophila* Ba11, *D. acidovorans* Cd11, *A. calcoaceticus* Fe10, *P. koreensis Hg11* and *A. johnsonii* Sb01 were potential candidates for the remediation of kerosene, while *P. plecoglossicida* Ag10, *R. planticola* Ag11, *S. aureus* Ba01, *E. faecalis* Cr07, *A. johnsonii* Sb01 and *P. agglomerans* Sn11 had potential to produce rhamnolipid type biosurfactants to enhance kerosene bioremediation.

3.14 Future prospects and recommendations

1. The degradation performance of the studied kerosene degraders need to be evaluated at different pH, temperature, oxygen concentration and nutrients under varying conditions before field applications.
2. Mixed culture experiments need to be carried out to enhance kerosene degradation.
3. The effects of biosurfactants on the biodegradation capability of mixed culture is uncertain. For that reason, the effects of the rhamnolipid needs to be investigated on various bacterial consortiums before field investigations.
4. Most studies have been focused on commercial production of biosurfactants due to high production cost. Therefore, optimization studies need to be done by using cheaper, renewable carbon sources for a large-scale rhamnolipid production.

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APPENDIX A

RAW DATA OF GRAVIMETRIC ANALYSIS

Table A.1 Gravimetric measurements for degradation study

Bacterial isolates	Sample no.1				Sample no.2				Sample no.3				Mean
	EB	EBK	WK	Calculation	EB	EBK	WK	Calculation	EB	EBK	WK	Calculation	
P. plecoglossicida Ag10	77.64	77.66	0.05	68.49	75.66	75.67	0.05	73.40	84.89	84.91	0.05	68.49	70.13±2.83
R. planticola Ag11	82.93	82.94	0.05	80.57	80.80	80.81	0.05	77.17	80.67	80.69	0.05	73.40	77.04±3.59
S. aureus Ba01	49.13	49.14	0.05	79.81	87.82	87.83	0.05	74.72	95.75	95.77	0.05	72.26	75.60±3.85
S. rhizophila Ba11	79.97	79.98	0.05	80.19	87.31	87.32	0.05	76.42	79.08	79.09	0.05	75.85	77.48±2.36
D. acidovorans Cd11	29.02	29.03	0.05	75.85	30.32	30.33	0.05	76.79	82.93	82.94	0.05	81.51	78.05±3.03
S. warneri Co11	83.24	83.25	0.05	78.49	89.61	89.63	0.05	69.25	75.02	75.04	0.05	65.66	71.13±6.62
E. faecalis Cr07	77.64	77.65	0.05	84.72	87.82	87.83	0.05	88.87	80.67	80.68	0.05	89.43	87.67±2.58
A. calcoaceticus Fe10	75.02	75.05	0.05	45.85	84.90	84.92	0.05	56.60	80.80	80.83	0.05	47.74	50.06±5.74
P. koreensis Hg11	96.53	96.55	0.05	67.55	82.80	82.82	0.05	76.23	87.12	87.14	0.05	77.36	73.71±5.37
S. aureus Li12	84.77	84.80	0.05	50.00	84.86	84.89	0.05	44.72	89.61	89.64	0.05	45.66	46.79±2.82
S. nematodiphila Mn11	51.57	51.59	0.05	58.87	84.66	84.68	0.05	65.09	78.97	78.99	0.05	62.26	62.08±3.12
A. haemolyticus Mn12	51.57	51.59	0.05	67.55	84.66	84.68	0.05	64.15	78.99	79.01	0.05	63.77	65.16±2.08
C. testosteroni Ni11	77.70	77.72	0.05	75.28	93.59	93.60	0.05	76.98	84.21	84.22	0.05	75.28	75.85±0.98
E. faecalis Pb06	85.05	85.07	0.05	61.32	80.93	80.95	0.05	67.17	96.06	96.09	0.05	53.40	60.63±6.91
A. johnsonii Sb01	75.22	75.25	0.05	51.51	83.51	83.54	0.05	47.74	84.90	84.93	0.05	51.32	50.19±2.13
P. agglomerans Sn11	80.80	80.83	0.05	44.34	96.53	96.56	0.05	42.64	84.86	84.88	0.05	52.08	46.35±5.03
M. luteus Sr02	77.72	77.72	0.05	87.92	84.77	84.78	0.05	81.13	93.60	93.61	0.05	86.60	85.22±3.60
M. luteus Sr11	87.40	87.40	0.05	84.72	49.08	49.10	0.05	79.25	84.90	84.90	0.05	82.08	82.01±2.74
A. haemolyticus Zn01	80.80	80.82	0.05	67.17	88.11	88.12	0.05	74.91	85.13	85.15	0.05	67.74	69.94±4.31

±; standard deviations, EB; weight of empty beaker, EBK; weight of beaker containing extracted kerosene, WK; weight of kerosene added in the media in gram, yellow color used for biosurfactants producers

APPENDIX B

RAW DATA OF GAS CHROMATOGRAPHIC ANALYSIS

Table B.1 Residual amount of hydrocarbons of kerosene in each bacterial culture

Hydrocarbons	Control	Ag10		Ag11		Ba01		Ba11		Cd11		Co11	
	amount (mg/ml)	amount (mg/ml)	%	amount (mg/ml)	%	amount (mg/ml)	%	amount (mg/ml)	%	amount (mg/ml)	%	amount (mg/ml)	%
n-Undecane (C11)	0.04	0.01	84.86	0.01	63.51	0.01	73.04	0.03	32.68	0.01	86.45	0.01	65.97
n-Dodecane (C12)	0.40	0.08	81.33	0.19	52.82	0.12	70.33	0.14	66.04	0.08	81.39	0.14	65.12
n-Tridecane (C13)	0.64	0.09	85.98	0.33	49.19	0.16	74.51	0.15	76.78	0.09	86.59	0.20	68.08
n-Tetradecane (C14)	0.22	0.05	75.72	0.13	42.75	0.07	67.58	0.06	70.89	0.05	77.82	0.09	60.64
n-Pentadecane (C15)	0.06	0.02	67.68	0.03	44.40	0.02	69.40	0.01	84.56	0.01	82.66	0.02	60.27
n-Hexadecane (C16)	0.02	0.02	22.25	0.02	14.00	0.02	22.29	0.02	24.53	0.02	25.17	0.02	19.59
n-Heptadecane (C17)	0.01	0.01	19.30	0.01	34.34	0.00	47.49	0.00	100.00	0.00	64.41	0.00	45.36
n-Octodecane (C18)	0.02	0.02	6.74	0.01	11.97	0.01	16.34	0.00	100.00	0.01	23.69	0.01	14.22
n-Nanodecane (C19)	0.01	0.01	12.27	0.00	34.78	0.00	48.05	0.00	92.64	0.00	60.89	0.00	42.42
n-Eicosane (C20)	0.01	0.01	10.16	0.01	20.11	0.01	23.93	0.00	100.00	0.01	34.01	0.01	23.11
n-Docosane (C22)	0.01	0.01	2.11	0.01	17.22	0.01	20.34	0.00	100.00	0.01	27.93	0.01	20.84
Total	1.44	0.31	78.24	0.75	47.71	0.44	69.29	0.40	72.07	0.28	80.66	0.53	63.25

Table B.1 cont'd

Hydrocarbons	Control	Cr07		Fe10		Hg11		Li12		Mn11		Mn12	
	amount (mg/ml)	amount (mg/ml)	%	amount (mg/ml)	%	amount (mg/ml)	%	amount (mg/ml)	%	amount (mg/ml)	%	amount (mg/ml)	%
n-Undecane (C11)	0.04	0.02	46.81	0.01	83.94	0.01	73.19	0.03	16.57	0.04	2.17	0.04	1.77
n-Dodecane (C12)	0.40	0.27	33.94	0.09	76.70	0.12	71.38	0.27	32.94	0.31	22.41	0.40	0.58
n-Tridecane (C13)	0.64	0.42	34.61	0.14	78.57	0.15	76.78	0.37	41.96	0.42	35.13	0.55	14.81
n-Tetradecane (C14)	0.22	0.15	33.28	0.07	69.41	0.07	67.98	0.13	40.05	0.14	36.68	0.17	24.23
n-Pentadecane (C15)	0.06	0.04	33.90	0.02	62.92	0.02	65.16	0.03	41.15	0.04	37.12	0.03	40.67
n-Hexadecane (C16)	0.02	0.02	16.28	0.02	-4.94	0.02	10.44	0.02	13.92	0.02	14.82	0.02	14.78
n-Heptadecane (C17)	0.01	0.01	28.07	0.01	28.82	0.01	36.59	0.01	25.69	0.01	12.41	0.00	66.04
n-Octodecane (C18)	0.02	0.02	7.72	0.01	10.21	0.01	12.27	0.01	9.42	0.02	6.20	0.01	23.39
n-Nanodecane (C19)	0.01	0.00	30.30	0.00	29.73	0.00	37.81	0.00	33.62	0.01	21.50	0.00	69.99
n-Eicosane (C20)	0.01	0.01	18.27	0.01	19.15	0.01	22.15	0.01	16.16	0.01	13.29	0.01	36.06
n-Docosane (C22)	0.01	0.01	15.02	0.01	14.43	0.01	17.05	0.01	12.57	0.01	9.20	0.01	31.31
Total	1.44	0.96	33.52	0.40	72.40	0.43	70.10	0.91	36.98	1.01	29.72	1.24	13.92

Table B.1 cont'd

Hydrocarbons	Control	Ni11		Pb06		Sb01		Sn11	
	amount (mg/ml)	amount (mg/ml)	%	amount (mg/ml)	%	amount (mg/ml)	%	amount (mg/ml)	%
n-Undecane (C11)	0.04	0.03	21.33	0.06	-68.96	0.01	77.37	0.04	5.13
n-Dodecane (C12)	0.40	0.19	52.83	0.40	-0.31	0.07	82.52	0.40	0.32
n-Tridecane (C13)	0.64	0.20	68.48	0.49	22.84	0.05	92.20	0.55	14.05
n-Tetradecane (C14)	0.22	0.07	65.73	0.16	26.92	0.04	83.82	0.23	-4.23
n-Pentadecane (C15)	0.06	0.02	65.71	0.05	14.96	0.01	83.16	0.06	-1.00
n-Hexadecane (C16)	0.02	0.02	18.26	0.02	9.79	0.02	25.60	0.03	-11.94
n-Heptadecane (C17)	0.01	0.01	28.70	0.01	-33.08	0.00	41.10	0.01	-35.71
n-Octodecane (C18)	0.02	0.01	10.63	0.02	-10.39	0.01	15.31	0.02	-11.60
n-Nanodecane (C19)	0.01	0.00	35.06	0.01	-21.07	0.00	47.76	0.01	20.35
n-Eicosane (C20)	0.01	0.01	19.70	0.02	-3.00	0.01	24.40	0.01	10.29
n-Docosane (C22)	0.01	0.01	16.96	0.01	-7.42	0.01	23.97	0.01	-4.98
Total	1.44	0.58	59.54	1.26	12.61	0.23	83.76	1.36	5.43

Hydrocarbons	Control	Sr02		Sr11		Zn01	
	amount (mg/ml)	amount (mg/ml)	%	amount (mg/ml)	%	amount (mg/ml)	%
n-Undecane (C11)	0.04	0.03	8.20	0.03	21.41	0.05	-39.59
n-Dodecane (C12)	0.40	0.40	0.46	0.20	50.20	0.25	37.98
n-Tridecane (C13)	0.64	0.60	6.78	0.23	64.73	0.25	61.14
n-Tetradecane (C14)	0.22	0.21	4.14	0.09	61.11	0.09	60.20
n-Pentadecane (C15)	0.06	0.07	-23.37	0.02	63.18	0.04	20.27
n-Hexadecane (C16)	0.02	0.03	-19.97	0.02	18.30	0.02	11.47
n-Heptadecane (C17)	0.01	0.02	-199.00	0.01	29.20	0.01	-78.45
n-Octodecane (C18)	0.02	0.02	-30.68	0.01	12.03	0.02	-21.20
n-Nanodecane (C19)	0.01	0.02	-158.44	0.00	34.78	0.01	-47.19
n-Eicosane (C20)	0.01	0.02	-55.62	0.01	23.59	0.02	-22.97
n-Docosane (C22)	0.01	0.01	12.41	0.01	21.86	0.01	-16.88
Total	1.44	1.44	0.07	0.63	56.43	0.78	45.70

APPENDIX C

RAW DATA OF BIOSURFACTANT SCREENING TESTS

OIL SPREADING TEST

Table C.1 Raw data of oil spreading test for each bacterial isolate

Bacterial strains	Diameter (cm)			Zone (cm^2)			Mean
	Sample no.1	Sample no.2	Sample no.3	Calculation 1	Calculation 2	Calculation 3	
P. plecoglossicida Ag10	0.3	0.4	0.3	0.07	0.13	0.07	0.09±0.03
R. planticola Ag11	0.4	0.3	0.3	0.13	0.07	0.07	0.09±0.03
S. aureus Ba01	0.3	0.2	0.3	0.07	0.03	0.07	0.06±0.02
S. rhizophila Ba11	0.5	0.3	0.5	0.20	0.07	0.20	0.15±0.07
D. acidovorans Cd11	0.25	0.3	0.3	0.05	0.07	0.07	0.06±0.01
S. warneri Co11	0.2	0.18	0.12	0.03	0.03	0.01	0.02±0.01
E. faecalis Cr07	1.1	1	1	0.95	0.79	0.79	0.84±0.10
A. calcoaceticus Fe10	1.1	1	1	0.95	0.79	0.79	0.84±0.10
P. koreensis Hg11	0.45	0.5	0.4	0.16	0.20	0.13	0.16±0.04
S. aureus Li12	0.25	0.23	0.31	0.05	0.04	0.08	0.06±0.02
S. nematodiphila Mn11	0.4	0.3	0.42	0.13	0.07	0.14	0.11±0.04
A. haemolyticus Mn12	0.28	0.2	0.28	0.06	0.03	0.06	0.05±0.02
C. testosteroni Ni11	0.19	0.2	0.2	0.03	0.03	0.03	0.03±0.00
E. faecalis Pb06	0.9	1	1.1	0.64	0.79	0.95	0.79±0.16
A. johnsonii Sb01	0.39	0.42	0.4	0.12	0.14	0.13	0.13±0.01
P. agglomerans Sn11	1.1	1.5	0.7	0.95	1.77	0.38	1.03±0.69
M. luteus St02	0.7	0.6	0.75	0.38	0.28	0.44	0.37±0.08
M. luteus Sr11	0.95	1.1	0.9	0.71	0.95	0.64	0.77±0.16
A. haemolyticus Zn01	0.4	0.4	0.45	0.13	0.13	0.16	0.14±0.02

±; standard deviations, yellow color used for biosurfactants producers

Table C.2 Raw data of E_{24} indices for each bacterial isolate

Bacterial isolates	Sample no.1	Sample no.2	Sample no.3	Mean
P. plecoglossicida Ag10	65.91	67.06	68.18	67.05±1.14
R. planticola Ag11	65.91	63.64	59.09	62.88±3.47
S. aureus Ba01	68.18	65.91	67.03	67.04±1.14
S. rhizophila Ba11	27.25	25.53	28.89	27.22±1.68
D. acidovorans Cd11	27.85	27.27	30.43	28.52±1.68
S. warneri Co11	18.34	21.74	14.89	18.32±3.42
E. faecalis Cr07	62.50	60.00	61.25	61.25±1.25
A. calcoaceticus Fe10	4.05	4.55	4.26	4.28±0.25
P. koreensis Hg11	41.67	46.39	51.11	46.39±4.72
S. aureus Li12	46.81	50.00	48.94	48.58±1.63
S. nematodiphila Mn11	62.50	62.92	62.00	62.47±0.46
A. haemolyticus Mn12	61.70	59.57	60.87	60.72±1.07
C. testosteroni Ni11	30.43	32.61	27.27	30.11±2.68
E. faecalis Pb06	53.33	52.74	52.17	52.75±0.58
A. johnsonii Sb01	52.17	53.30	54.35	53.27±1.09
P. agglomerans Sn11	56.25	59.57	54.35	56.72±2.65
M. luteus Sr02	13.04	16.67	14.87	14.86±1.81
M. luteus Sr11	10.87	12.50	11.71	11.69±0.82
A. haemolyticus Zn01	34.78	34.33	34.58	34.57±0.22

±;standard deviations, yellow color used for biosurfactant producers

Table C.3 Raw data of microbial adhesion to hydrocarbon test for each bacterial isolate

Bacterial isolates	Sample no.1			Sample no.2			Sample no.3			Mean
	A0	A1	Hydrophobicity (%)	A0	A1	Hydrophobicity (%)	A0	A1	Hydrophobicity (%)	
P. plecoglossicida Ag10	0.02	0.01	68.42	0.04	0.02	64.29	0.04	0.01	65.71	66.14±2.10
R. planticola Ag11	0.01	0.00	100.00	0.04	0.00	91.67	0.03	0.00	86.67	92.78±6.74
S. aureus Ba01	0.02	0.00	81.25	0.02	0.00	80.95	0.03	0.01	70.59	77.60±6.07
S. rhizophila Ba11	0.09	0.02	82.61	0.07	0.01	85.71	0.10	0.02	75.00	81.11±5.51
D. acidovorans Cd11	0.03	0.01	75.86	0.00	0.00	66.67	0.03	0.01	67.86	70.13±5.00
S. warneri Co11	0.03	0.02	44.44	0.03	0.02	46.88	0.04	0.02	45.95	45.76±1.23
E. faecalis Cr07	0.04	0.03	29.27	0.04	0.03	36.59	0.04	0.03	21.95	29.27±7.32
A. calcoaceticus Fe10	0.03	0.02	37.50	0.02	0.01	39.13	0.04	0.02	36.11	37.58±1.51
P. koreensis Hg11	0.04	0.01	80.56	0.06	0.01	85.96	0.05	0.01	78.00	81.51±4.07
S. aureus Li12	0.06	0.04	27.27	0.06	0.04	28.57	0.09	0.07	18.89	24.91±5.26
S. nematodiphila Mn11	0.01	0.02	60.23	0.01	0.01	56.23	0.02	0.02	49.53	55.33±4.41
A. haemolyticus Mn12	0.06	0.01	90.48	0.09	0.01	84.62	0.08	0.01	84.81	86.63±3.33
C. testosteroni Ni11	0.01	0.00	92.31	0.02	0.00	91.30	0.01	0.00	91.67	91.76±0.51
E. faecalis Pb06	0.08	0.05	33.75	0.08	0.05	36.59	0.09	0.06	36.78	35.71±1.70
A. johnsonii Sb01	0.01	0.00	83.33	0.02	0.01	76.19	0.02	0.00	80.95	80.16±3.64
P. agglomerans Sn11	0.07	0.00	93.94	0.06	0.00	94.74	0.01	0.00	90.00	92.89±2.54
M. luteus Sr02	0.03	0.00	92.00	0.03	0.01	84.38	0.02	0.00	87.50	87.96±3.83
M. luteus Sr11	0.07	0.03	63.38	0.05	0.02	62.96	0.08	0.03	62.50	62.95±0.44
A. haemolyticus Zn01	0.01	0.01	58.33	0.01	0.01	54.55	0.02	0.01	57.89	56.92±2.07

±;standard deviations, A0; Optical density of the bacterial suspensions for each isolates, A1; optical density of the suspensions treated with kerosene , yellow color used for biosurfactant producers

APPENDIX D

RAW DATA OF SURFACE TENSION

Table D.1 Raw data of surface tension measurements

Bacterial isolates	Sample no (mN/m)					Mean
	1	2	3	4	5	
Control	69.51	69.95	69.79	69.70	69.88	69.61±0.13
P. plecoglossicida Ag10	69.47	69.82	69.62	69.11	69.66	69.54±0.22
S. aureus Ba01	68.59	68.79	69.18	68.53	68.77	68.77±0.25
S. rhizophila Ba11	64.08	63.93	62.36	63.92	64.14	63.69±0.75
D. acidovorans Cd11	69.42	69.48	69.05	69.77	69.71	69.49±0.23
A. calcoaceticus Fe10	68.39	68.12	68.14	68.30	68.27	68.24±0.11
P. koreensis Hg11	67.62	67.57	67.75	67.52	67.50	67.59±0.10
A. johnsonii Sb01	69.45	69.88	69.45	69.11	69.40	69.46±0.28
SDS	33.3	32.23	32.61	32.17	31.35	32.77±0.76
Tween 20	66.35	66.24	66.93	67.53	66.98	66.81±0.52

±; standard deviations, yellow color used for biosurfactant producers, blue color represents chemical surfactants

RAW DATA OF INTERFACIAL TENSION

Table D.2 Raw data of interfacial tension measurements

Bacterial isolates	Sample no (mN/m)					Mean
	1	2	3	4	5	
Control	22.29	21.91	22.29	22.35	22.07	22.18±0.19
P. plegoclossicida Ag10	21.96	21.98	21.77	22.13	21.77	21.92±0.15
R. planticola Ag11	21.45	21.24	21.90	21.99	22.02	21.72±0.35
S. aureus Ba01	21.87	21.98	22.08	22.32	20.92	21.83±0.54
E. faecalis Cr07	21.45	21.46	22.27	21.11	21.59	21.58±0.43
A. johnsonii Sb01	18.05	18.52	18.83	19.84	20.79	19.21±1.10
P. agglomerans Sn11	20.03	20.27	20.43	20.29	20.72	20.35±0.25
P. plegoclossicida Ag10	15.52	15.89	16.08	16.13	16.19	15.96±0.27
SDS	16.29	17.17	17.54	16.60	17.31	16.98±0.52
Tween 20	7.70	8.16	8.99	8.37	7.36	8.12±0.63

±; standard deviations, yellow color used for biosurfactant producers, blue color represents chemical surfactants

APPENDIX E

RAW DATA FOR QUANTIFICATION OF BIOSURFACTANT

Table E.1 Raw data of biosurfactant quantification measurements for each bacterial isolate

Bacterial isolates	Sample no.1		Sample no.2		Sample no.3		A.B 1 (mg)	A.B 2 (mg)	A.B 3 (mg)	Mean	Amount (g/L)
	P. (mg)	P. B. (mg)	P. (mg)	P. B (mg)	P. (mg)	P. B. (mg)					
P. plegoclossicida Ag10	83	83	80	81	46	46	0.135	0.105	0.009	0.083±0.07	3.3
R. planticola Ag11	92	92	87	87	92	92	0.054	0.116	0.089	0.086±0.03	3.5
S. aureus Ba01	49	49	92	92	80	80	0.103	0.075	0.086	0.088±0.01	3.5
E. faecalis Cr07	88	88	87	87	51	51	0.100	0.102	0.007	0.070±0.05	2.8
A. johnsonii Sb01	89	89	84	84	86	86	0.104	0.028	0.036	0.056±0.04	2.2
P. agglomerans Sn11	84	84	84	84	96	96	0.070	0.082	0.017	0.056±0.03	2.3

±;standard deviations, P; weight of the petri dish, P.B; weight of the petri dish with biosurfactant; A.B; amount of biosurfactant