SCREENING OF BIOSURFACTANT PRODUCING AND DIESEL OIL DEGRADING BACTERIA FROM PETROLEUM HYDROCARBON CONTAMINATED SURFACE WATERS

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Submitted by **GÖZDE ONUR** in partial fulfillment of the requirements for the degree of **Master of Science in Environmental Engineering Department, Middle East Technical University** by

Prof. Dr. Gülbin Dural Ünver _____

Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. F. Dilek Sanin _____

Head of Department, **Environmental Engineering**

Assoc. Prof. Dr. Bülent İçgen _____

Supervisor, **Environmental Engineering Dept, METU**

Examining Committee Members:

Prof. Dr. Filiz B. Dilek _____

Environmental Engineering Dept, METU

Assoc. Prof. Dr. Bülent İçgen _____

Environmental Engineering Dept, METU

Prof. Dr. Pınar Çalık _____

Chemical Engineering Dept, METU

Assoc. Prof. Dr. Emre ALP _____

Environmental Engineering Dept, METU

Assoc. Prof. Dr. Tuba Hande Ergüder Bayramoğlu _____

Environmental Engineering Dept, METU

Date: 30.01.2015

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last Name: Gözde ONUR

Signature:

ABSTRACT

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ONUR, Gözde

Middle East Technical University

Graduate School of Natural and Applied Sciences

Department of Environmental Engineering, Master's Thesis

Supervisor: Assoc. Prof. Dr. Bülent İçgen

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Hydrocarbon contamination may happen in various ways such as accidents during fuel transportation by trucks and ships, leakage of oil from underground storage tanks, or during extraction and processing of oil. These contaminations can be treated by several methods including physical, chemical and biological treatment. During biological cleaning up, hydrocarbon-degrading bacteria emulsifying hydrocarbons by producing biosurfactants are used. Therefore, isolation and identification of biosurfactant producing and hydrocarbon degrading bacteria are pivotal for effective bioremediation of hydrocarbon contaminated surface waters. Hence, the aim of this study is to isolate and identify efficient biosurfactant producing and diesel oil degrading bacteria to remove spilled diesel oil from surface waters. For this reason, bacteria isolated from the petroleum hydrocarbon-contaminated river water in close vicinity to petrol refinery were screened for their potential to produce biosurfactant and degrade diesel oil. Primary selection of diesel oil degraders was carried out by using conventional enrichment culture technique which was followed by drop-collapse test, oil displacement test and emulsification activity measurement. Primary

determination of diesel oil degradation was done by using the gravimetric analysis. Secondary determination was only carried out with potential isolates by using the gas choromatographic (GC) analysis. The results of GC analysis pointed out two isolates, designated as Zn01 and Fe10, effective in diesel oil degradation with 92 and 61% respectively. The isolates Zn01 and Fe10were identified by using 16S rRNA sequencing as *Acinetobacter haemolyticus* and *Acinetobacter calcoaceticus*, respectively. Both isolates were further characterized for the presence of two novel catabolic genes (*alk*B and *C23*O), responsible for diesel oil degradation, the key enzymes (alkane monooxygenase and catechol 2,3 dioxygenase), encoded by these novel genes, and emulsifying ability of the biosurfactants produced by these two isolates through the use of several methods including DNA extraction, agarose gel electrophoresis, polymerase chain reaction (PCR), protein extraction, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), surface tension measurement, fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM) and zeta potential measurement. The population dynamics of *alk*B and *C23*O harboring bacteria in the polluted river water were also monitored by using DNA probes through the fluorescein *in situ* hybridization (FISH). The study elucidated that *Acinetobacter* species harboring *alk*B and *C23*O seem to have high potential for diesel oil remediation with high emulsifying indices. FISH results also revealed that *alk*B and *C23*O harboring bacteria populate in the polluted surface waters successfully.

Keywords: *Acinetobacter,* petroleum bioremediation, hydrocarbons, E24, *alk*B, *C23*O, FISH

ÖZ

PETROL HİDROKARBONLARI İLE KİRLETİLMİŞ YÜZEY SULARINDA BİYOSÜRFEKTAN ÜRETEN VE DİZEL YAKITINI DEGRADE EDEN BAKTERİLERİN İNCELENMESİ

ONUR, Gözde

Orta Doğu Teknik Üniversitesi

Fen Bilimleri Enstitüsü

Çevre Mühendisliği Anabilim Dalı, Yüksek Lisans Tezi

Danışman: Doç. Dr. Bülent İçgen

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Hidrokarbon kontaminasyonu, gemi veya kanyonlarla yakıt taşınması sırasında oluşan kazalar, yer altı depolama tanklarından sızıntı veya petrolün çıkarılması ve işlenmesi prosesleri gibi çeşitli yollarla olabilir. Bu kontaminasyonlar fiziksel, kimyasal ve biyolojik arıtma olmak üzere çeşitli yöntemlerle arıtılabilir. Biyolojik temizleme sırasında, hidrokarbon degrade eden bakteriler hidrokarbonları biyosürfektanlar kullanılarak emülsifiye edilebilmektedir. Bu nedenle; biyosürfektan üreten ve hidrokarbon degrade eden bakterilerin izolasyonu ve tanımlanması, yüzey sularında oluşan hidrokarbon kirliliğinin etkin bir şekilde biyoremediasyonu açısından önem arz etmektedir. Bu yüzden; bu çalışmanın amacı, yüzey sularına saçılan dizel yakıtın arıtılması için etkin biyosürfektan üreten ve dizel yakıtını degrade edebilen bakterilerin izolasyonu ve tanımlanmasıdır. Bu nedenle, petrol hidrokarbonları ile kirletilmiş nehir suyundan izole edilen bakteriler biyosürfektan üretme ve hidrokarbon degrade etme potansiyelleri bakımından araştırılmıştır. Dizel yakıtı degrade edebilen bakterilerin seçimi ilk olarak zenginleştirme kültürü tekniği ile yapılmış ve “drop-collapse” testi, “oil displacement” testi ve emülsifikasyon

aktivitesi ölçümleri ile biyosürfektan üretme yetenekleri belirlenmiştir. Bakterilerin dizel degradasyon yetenekleri birincil olarak gravimetrik analiz kullanılarak belirlenmiş olup ikincil belirleme ise birincil belirleme ile öne çıkan sadece potansiyel izolatlar için gaz kromatografisi (GC) analiziyle yapılmıştır. GC analizi sonuçları Zn01 ve Fe10 olarak adlandırılan izolatlarının sırasıyla % 92 ve % 61 oranlarında degradasyon yapabildiğini göstermiştir. Her iki izolat 16S rRNA sekans analizi sırasıyla *Acinetobacter haemolyticus* ve *Acinetobacter calcoaceticus* olarak tanımlanmıştır. Tanımlama sonrası izolatlar katabolik genlerin varlığı (*alk*B ve *C23*O), dizel yakıtı degradasyonundan sorumlu anahtar enzimleri (alkan monooksijenaz ve kateşol 2,3 dioksijenaz), ve biyosürfektan üretme potansiyelleri açısından sıralanan yöntemler kullanılarak incelenmiştir: DNA ekstraksiyonu, agaroz jel elektroforezi, polimeraz zincir reaksiyonu (PCR), protein ekstraksiyonu, sodyum dodesil sülfat poliakrilamid jel electroforezi (SDS-PAGE), yüzey gerilimi ölçümü, fourier dönüşümlü kızılötesi spectroskopisi (FTIR), taramalı elktron mikroskobu (SEM) ve zeta potansiyel ölçümü yöntemleriyle daha detaylı karakterize edilmiştir. Kirletilmiş nehir suyundaki *alk*B ve *C23*O içeren bakterilerin populasyon yayılımlarında DNA probları ve fluorescein *in situ* hybridization (FISH) yöntemi kullanılarak gözlemlenmiştir. Yapılan çalışmalar sonucu, *alk*B ve *C23*O genlerini içeren *Acinetobacter* türlerinin yüksek emülsifikasyon indeksleri ile dizel yakıtı biyoremediasyonunda yüksek potansiyele sahip olabileceğini göstermiştir. Ayrıca FISH analizi sonuçları, *alk*B ve *C23*O genlerini içeren bakterilerin kirletilmiş yüzey sularında yaygın olarak bulunabildiğini ortaya çıkarmıştır.

Anahtar kelimeler: *Acinetobacter*, petrol biyoremediasyonu, hidrokarbon, E24, *alk*B, *C23*O, FISH

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Gözde ONUR

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LIST OF ABBREVIATONS

DNA: Deoxyribonucleic acid

E_{24}: Emulsification index

FISH: Fluorescence *in situ* hybridization

GC: Gas chromatography

GC-MS: Gas chromatography-mass spectrometer

MATH: Microbial adhesion to hydrocarbon

PAH: Polycyclic aromatic hydrocarbons

PCR: Polymerase chain reaction

PHC: Petroleum hydrocarbons

RNA: Ribonucleic acid

rRNA: Ribosomal RNA

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

TPH: Total petroleum hydrocarbons

CHAPTER 1

INTRODUCTION

Ecosystems have been changed recently with the growing influence of human activities. Thus, people have become awaken of the need preserve ecosystems and additionally, evaluate the deterioration created by the contamination. (Eman, 2012). Hydrocarbon contamination is one of the most important pollution source around the world. Due to oil spills, hydrocarbon pollution becomes a global problem especially in developing and industrialized countries. Petroleum is used as a conventional energy source even though it has significance as a global environmental pollutant (Ghosh, 2012). Crude oil is converted to gasoline and diesel oil in petroleum refineries (Oliveira et al., 2014). As a petroleum product, diesel oil contains aromatic hydrocarbon compounds as well as normal, branched and cyclic alkanes. Diesel oil can affect both surface and groundwater quality with the toxic compounds in it (Ghosh, 2012). Examples of oil spills such as the ones occurred in Panamanian Coast in 1986, in Exxon Valdez in 1989, and BP oil spill in the Gulf of Mexico in 2010 have attracted attention of the environmentalists, chemists, biotechnologists and engineers because of their effects on the environment and ecosystems (Jackson, 1989; Mazahari Assadi, 2010; Bowyer, 2012).

In July 2013, there was crude oil leakage from Kırıkkale TÜPRAŞ petroleum refinery. Ministry of Environment and Urbanization took some precautions to remove leaked crude oil from soil and to prevent oil from mixing with Kızılırmak River which is located very close to the TÜPRAŞ Refinery (T.C. Çevre ve Şehircilik Bakanlığı, 2013). Therefore, this accident raised concerns about possibility of the pollution and presence of the petroleum hydrocarbons, mainly diesel oil, in Kızılırmak River.

Thus, the primary objectives of this thesis study were mainly to;

- **i.** measure total petroleum hydrocarbon contamination in the river water
- **ii.** isolate diesel oil degrading bacteria from these water,
- **iii.** determine the biosurfactant production ability of the bacteria isolated,
- **iv.** determine the diesel oil degradation ability of the isolates,
- **v.** show isolates for the presence of catabolic genes effective in diesel oil degradation,
- **vi.** search isolates for the key enzymes in diesel oil degradation,
- **vii.** identify effectively diesel oil degrading bacteria,
- **viii.** characterize the biosurfactants of the effective diesel oil degraders.

In order to isolate diesel oil degraders, selective enrichment culture technique was first applied, by using diesel oil as the sole carbon source to the collected water samples. After selective enrichment, biosurfactant production ability of the isolates were tested by using drop-collapse test, oil displacement test and emulsification activity measurement. Diesel oil degradation abilities of the isolates were tested by using both gravimetric and gas chromatographic analyses. Presence of catabolic genes were examined by polymerized chain reaction (PCR) and catabolic enzymes were examined by SDS-PAGE method. Identification of the diesel oil degraders was done by 16S rRNA sequencing. Biosurfactants of the most efficient diesel oil degraders were also characterized for their physicochemical, biochemical and structural properties.

Literature review was done in Chapter 2 of this thesis. Methodology of the experiments performed during this thesis work were described in Chapter 3. In Chapter 4, results of the conducted experiments were given and discussed. In Chapter 5 conclusions of this study and recommendations for future studies were given.

CHAPTER 2

LITERATURE REVIEW

2.1.Hydrocarbon Pollution in Surface Waters

Surface water pollution mainly occurs due to the hazardous substances coming into contact and physically mixing with water or dissolving in the water. Another part of the surface water contamination is considered as contaminated sediments due to their close relationship with water. Contamination of surface waters may occur in various ways such as direct discharge of hazardous materials thorough a pipe or channel, storm water runoff which may get contaminated when rain water contacts with contaminated soil and carries the contaminated soil particles. In addition, surface water can also be polluted due to contaminated groundwaters, sediments and air (EPA, Surface Water Contamination, 2011).

Interests about the possibility for water and soil contamination by oil and oil byproducts gains importance since they are one of the major pollutants in the environment. Contamination can be as a result of various sources such as; accidents during fuel transportation by trucks and ships, leakage of oil from underground storage tanks, extraction and processing of oil. Also, byproducts of oil can serve as source of hydrocarbon contamination. Even though oil refineries and petrochemical plants are in favor of society, a great amount of solid oily waste is produced. This waste is categorized as hazardous since they are toxic, corrosive and flammable. Because of these properties these waste cannot be recycled or reused (Oliveira et al., 2014).

Hydrocarbons are released into the air, water and soil as pollutants through different ways and act as common contaminants. In air, sources of hydrocarbons are mainly

described as anthropogenic emissions to air from combustion of wood and organic matter, vehicle exhaust, production of coal tar, coke and asphalt, cigarette smoke; accidental discharges of petroleum hydrocarbons (PHCs) via gas and oil spills (e.g. U.S. Gulf Oil Spill in 2010) and natural releases such as volcanic eruptions and forest fires. In water, storm water runoff may include hydrocarbons due to asphalt, rubber tire wearing or vehicle exhaust in urban areas. Industrial areas with petroleum refineries, wood treatment plants etc. may also contain hydrocarbons. Source of hydrocarbons in soil is mostly atmospheric deposition. It is mainly caused by the deposition of vehicle exhaust, rubber tire or asphalt emissions. Also, landfilling activities, application of compost or pesticides, disposal of sludge from waste water treatment plants produce hydrocarbon emissions. Therefore, deposition of these emissions causes contamination of soil with hydrocarbons (EPA, 2013).

2.2.Hydrocarbon Types and Importance of Hydrocarbons

Hydrocarbons contain only carbon and hydrogen atoms in different configurations. These configurations are mainly chains, branched chains and rings. Mostly, hydrocarbons can be found as five structural categories which are named as; *n*-alkanes, branched alkanes, unsaturated alicyclic hydrocarbons, alkylated benzenes and polycyclic aromatic hydrocarbons (PAHs) (EPA, 2013). PAHs are derived from anthropogenic processes, when incomplete combustion of organics and emissions of non-combustion related petrogenic processes take place (Ana et al., 2000). PAHs are known as global pollutants which are harmful to the human health and environment. These compounds are persistent in environment due to hydrophobicity, and they become associated with the particulate material (e.g. humics and clays) and deposit in sediments and soils. Another characteristic of PAHs is that, they are lipophilic and they can biomagnify in the food chain (Eman, 2012)

Petroleum hydrocarbons have been considered as priority pollutants due to their biohazard on the living organisms in ecosystems (Ghosh, 2012). Research of World Health Organization (1998) showed that, dermal, inhalation or subcutaneous exposure to PAHs have carcinogenic impacts on animals and humans. Some of the PAHs showed DNA damage, chromosomal effects and mutation on mammalian

cells. Also, epidemiological studies on air pollution revealed that, some of the PAHs, caused tumours in laboratory animals when they are exposed to these substances via several exposure pathways (Ana et al., 2000). Because of these properties of PAHs they are considered as very important pollutants.

2.3.Diesel Oil Pollution

Diesel oil is a mixture of saturated hydrocarbons like paraffin, and aromatic hydrocarbons. (Dussán et al., 2012). It contains both aromatic hydrocarbon compounds and normal, branched and cyclic alkanes. Water solubility of diesel oil is low and it has high stability of the aromatic ring and high adsorption coefficient. Diesel oil is a mixture of low molecular weight compounds, which are usually more toxic than long-chained hydrocarbons, and more bioavailable and more soluble than the long-chained hydrocarbons (Dorn et al., 2000). Diesel oil can affect both surface and groundwater quality with the toxic compounds in it (Ghosh, 2012). The color of diesel fuels changes from colorless to brown, and the water solubility at 20°C is about 5 mg/L (ATSDR, 1995). Diesel fuels are therefore partly soluble in water and possibly accumulative in tissues. Diesel oil has been recognized to cause skin and eye irritation in humans (Muzyka et al.; 2002). It is considered to be potentially carcinogenic to humans due to PAHs in its composition (Kauppi, 2011). Because of its harmful effects, diesel oil should be treated and removed from the environment.

2.4. Treatment Technologies

Currently, a number of different technologies exist for spilled oil removal. Treatment of spilled oil can be done by physical, chemical and biological methods (Al-Zahrani, 2010). Interests for cleaning of oil contaminated sites have been increasing for promotion of environmental methods (Geetha et al., 2013).

2.4.1. Physical Treatment

Physical treatment methods are frequently used for the control of oil spills in water. These methods are primarily used to prevent the spread of the oil, but physical and

chemical properties of the spilled oil are not changed (Dave et al., 2011). Physical collection of spilled oil can be done by using adsorbents, skimmers or booms (Al-Zahrani, 2010).

Booms are a common type of equipment to control oil spreading and act as a barrier and prevent the oil movement. Booms also enhance the recovery of spread oil through skimming or other techniques (Dave et al., 2011). Skimmers are equipments, which can be used in combination with booms, to recover spilled oil from water without changing its properties. Therefore, reprocessing and reuse of the oil can be possible. The thickness and type of the oil spill, amount of debris, weather conditions and the location determine the success of the removal. This method is usually effective in calm waters. (Dave et al., 2011). Hydrophobic sorbents are mainly serve as the final step of cleanup of the remaining oil after skimming. Adsorbent materials include natural organic, natural inorganic and synthetic materials. Vegetable fibers, clay, perlite and polyester sheets are the examples of adsorbents (Dave et al., 2011). For small oil spills, using physical treatment methods like sorption can be a good option (Annunciado et al., 2005). Even though sorption materials are not the main treatment method for the removal of larger oil spills, it can be used as a component of the treatment (Kauppi, 2011). However, the major disadvantage of natural adsorbents are their labor intensive properties during application. Also, collection of adsorbents from the water surface can be very difficult. Also, application of sorbents such as clay in windy weather may pose difficulties and health risks when inhaled. Non-biodegradeability and storage of sytnhetic adsorbents after using are main disadvantages of adsorbents (Dave et al., 2011).

2.4.2. Chemical Treatment

One of the removal method for spilled oil is chemical treatment. Chemical precipitation, adsorption and solvent extraction are the examples of chemical treatment methods (Al-Zahrani, 2010). Generally chemical methods are used in combination with physical methods since physical methods restrict the spreadin of the oil. Generally, chemical treatment is done by using dispersants and solidifiers. Dispersants includes surfactants. Application of dispersants is usually done by

spraying the water with the chemical. Lately, the dispersants available are found out to be less toxic and more effective than previously used ones (Dave et al., 2011). It was proved that capabilities of the dispersants treating spilled oil up to 90% can be less costly than the physical methods (Holakoo, 2001). However, most dispersants are inflammable in nature. This property of the dispersants, may cause human health hazards and harm the marine life. Also, contamination of drinking water sources and fouling of shorelines are caused due to dispersants. Solidifiers are hydrophobic polymers which change its liquid state into solid when reacted with the oil and can be removed by physical methods. However, they were found to have lower removal efficiencies than dispersants (Dave et al., 2011). These methods have expensive equipment and monitoring systems, and high reagent and energy requirements. Also, incomplete oil removal and formation of toxic sludge and other waste products which require disposal are major issues related to chemical treatment of oil (Al-Zahrani, 2010).

2.4.3. Biological Treatment

Bioremediation of petroleum hydrocarbons has been proposed as an effective, economic, and environmentally friendly technology (Liang-Ming Whanga et al. 2007). The rate of microbial growth is influenced by the factors including population diversity, soil moisture, temperature, oxygen supply, pH, and nutrient levels. Microorganisms degrading the components of petroelum hydrocarbons can be isolated from petroleum contaminated sites. Microorganisms can be obtained by enrichment culture techniques, where maximum final cell concentration or maximum specific growth rate can be used as the selection basis (Geetha et al., 2013). It was found that wide range of bacteria and fungi can use n-alkanes as sole carbon and energy source (van Beilen et al., 2003). However, bacteria have the major share in hydrocarbon degradation (Geetha et al., 2013). Bacterial degradation of n-alkanes can be possible under both aerobic and anaerobic conditions (Widdel and Rabus, 2001). However, activation of the chemically inert aliphatics with molecular oxygen increases degradation rates. Even though, several mechanisms of aerobic activation have been described only the terminal oxidation pathway that involves an alkane monooxygenase as key enzyme has been researched in greater detail so far

(Maeng et al., 1997; van Beilen et al., 2003). Aerobic and anaerobic bacterial degradation pathways of hydrocarbon compounds are shown in Figure1.

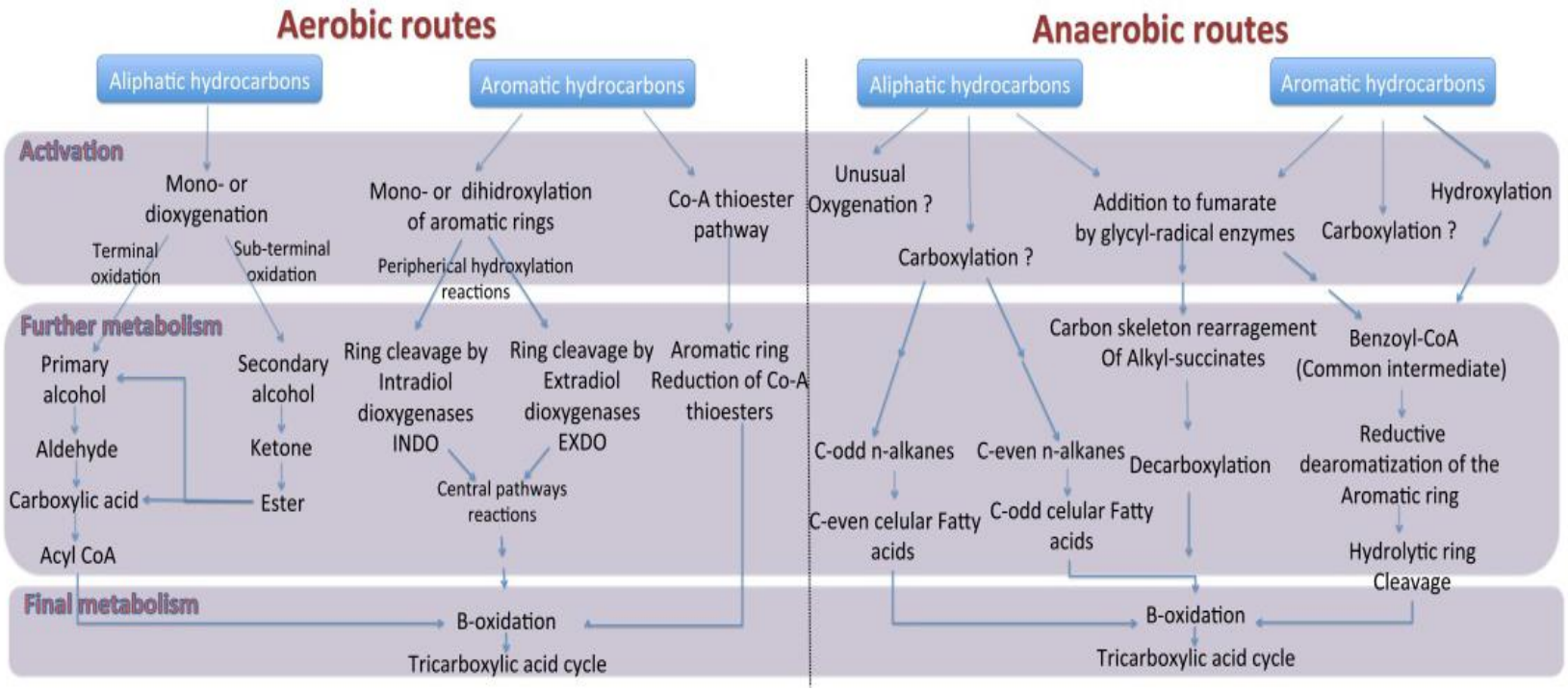

Figure 1. Aerobic and anaerobic bacterial degradation pathways of hydrocarbon compounds (Sierra-Garcia and Oliveira, 2013)

When a new, toxic organic compound is introduced to the environment, it poses stress on indigenous bacteria and it results in the evolution of mutations and adaptation (Vogel, 1996; Sarand et al., 2001; Lynch et al., 2004; Wright 2004). The high initial concentration of spilled oil can possess a negative impact on the biodegradation process and leads to a lag phase of 2-4 weeks (Zahed et al., 2010). Even biostimulation is applied, at least a week is required for microorganisms to accustom to the environment (Atlas, 1995; Zahed et al., 2010).

Biodegradation processes can be improved by bioaugmentation or biostimulation techniques. Bioavailability of a contaminant is the key issue in bacterial degradation therefore, it should be enhanced. With the release of biosurfactants, solubility of the water insoluble pollutants increases. Increasing bioavailability leads to the improvement of accelerated pollutant biodegradation (Kauppi, 2011).

2.4.3.1. Seeding with Microbial Cultures (Bioaugmentation)

Inclusion of microorganisms which are able to degrade hydrocarbons is one approach which is often acknowledged for the remediation of petroleum pollutants after an oil spill biologically. Most microorganisms considered for seeding are obtained by enrichment cultures from previously polluted sites. (Atlas, 1995). Together with the cultivating and non-cultivating methods (Vogel, 1996) and molecular techniques, which give more detailed data about the microorganisms in environmental samples (Romantschuk et al. 2000, Saul et al. 2005, Scow & Hicks 2005), microbes can be investigated broadly. In aquatic environments, microorganisms are mostly bacteria and picoplankton. Also, fungi have potential for the degradation of organic compounds because of their symbiotic association with plants and spreading growth (Kauppi, 2011). As Sarand (2000) stated, bacteria and fungi can have a mutualistic relationship. Table 1 represents the major genera responsible for oil degradation.

Table 1. Major genera of oil degrading bacteria and fungi (Webb, 2005 & Capotorti et al., 2004)

Microorganism	Ability to degrade compounds
Bacteria	
Arthrobacter spp.	phenanthrene, methyl-tert-butyl ether, ethyl-tert-butyl ether and tert-amyl methyl ether
Brevebacterium	Asphaltenes, petroleum oil
Brachybacterium	
Dietzia	n-C12 to n-C38 alkane components
Flavobacterium	chlorophenols
Janibacter	Polycyclic hydrocarbon
Mycobacterium	Polycyclic hydrocarbon, pyrene, phenanthrene, diesel
Nocardia spp	4-chlorobenzoate
Pseudomonas spp.	4-chlorobenzoate
Rhodococcus	Polychlorinated-biphenyl, hexadecane, tricholroethan, polycyclic hydrocarbon
Fungi	
Aspergillus	Pyrene, benzo(a)pyrene
Candida	Toluene
Fusarium	Methyl tert-butyl ether and tert-butyl alcohol
Trichoderma	phenanthrene
Phanerochaete	benzo(a)pyrene, phenanthrene and fluorene
Mortierella	2, 4-Dichlorophenol

2.4.3.2. Environmental Modification (Biostimulation)

Biodegradation of hydrocarbons in water environment is usually limited by abiotic environmental factors like molecular oxygen, phosphate and nitrogen amounts (Atlas, 1995). Other environmental factors being, temperature of the water and dissolved oxygen are important as temperature has impact on the viscosity of crude oil. Also, dissolved oxygen influences the metabolic activity of microorganisms (Yang et al., 2009). However, oxygen is not limiting if the water body is well aerated. In general, marine waters have very low concentrations of nutrients, such as nitrogen, phosphorus and various minerals, which are needed for the incorporation to cellular biomass, and the availability of the nutrients within the environment of hydrocarbon biodegradation is critical (Atlas, 1995).

2.4.3.3. Biosurfactant Application

Due to the hydrophobicity and the low solubility of many hydrocarbon compounds, they are highly unavailable to microorganisms. However, the release of biosurfactants by microorganisms, uptake of hydrophobic compounds is a strategy used by microorganisms. Various microorganisms, bacteria and fungi, utilizing hydrocarbons achieve emulsifying activities due to whole cell or extracellular surface active compounds (Obayori et al., 2009). Applying biosurfactants to polluted soil and water at concentrations higher than their critical micelle concentration (CMC) values, can decrease the tension between the spilled oil and the media, therefore enhance the bioavailability of oil and help their biodegradation. Studies regarding enhanced bioremediation includes addition of surfactants and focused especially on chemical surfactants. However, addition of chemical surfactants can have inhibitory effects on biodegradation. When compared with synthetic surfactants, application of biosurfactants in bioremediation processes can be more agreeable from a social point of view because of biosurfactants are produced naturally (Liang-Ming Whanga et al., 2007).

2.4.3.4. Catabolic Genes

*Alk*B is a catabolic gene responsible for the degradation of hydrocarbons (Dussán et al., 2012). Microorganisms degrading alkanes with 5-11 carbon atoms and long (>C12) alkanes have been generally empathized with the presence of *alk*B genes. Therefore, presence of similar genes is used as the biomarker for the characterization of alkane degrading bacteria in different environmental systems (Sierra-Garcia and Oliveira, 2013)

*C23*O is a catabolic gene responsible for the degradation of hydrocarbon compounds (Jyothi et al., 2012). It plays a key role in the metabolism of aromatic ring cleavage since it is encoding the enzyme *C23*O dioxygenase. Catechol dioxygenase (*C12*O and *C23*O) genes responsible for aliphatic and polyaromatic hydrocarbons degradation (Hesham et al., 2014). Since this gene is responsible for the extradiol cleavage of the aromatics presence of this gene should be detected in the isolates to show the hydrocarbon degradation potential of the isolates (Benedek et al., 2012).

2.4.3.5. Catabolic Enzymes

The initial steps in the biodegradation of hydrocarbons by fungi and bacteria include the oxidation of the substrate by oxygenases, for these steps molecular oxygen is required. Alkanes are later converted to carboxylic acids and further biodegraded by ß-oxidation. Aromatic hydrocarbons generally are hydroxylated and the rings which are then cleaved with the formation of catechols which are subsequently degraded to intermediates of the tricarboxylic acid cycle. However, bacteria and fungi form byproducts with differing stereochemistry. Fungi, similar to the mammalian enzyme systems, form *trans*-diols, bacteria on the other hand almost always form *cis*-diols. The complete mineralization of hydrocarbons produces the non-toxic end products water and carbon dioxide, as well as cell biomass (Atlas, 1995). Figure 2 illustrates the degradation pathway of hydrocarbons.

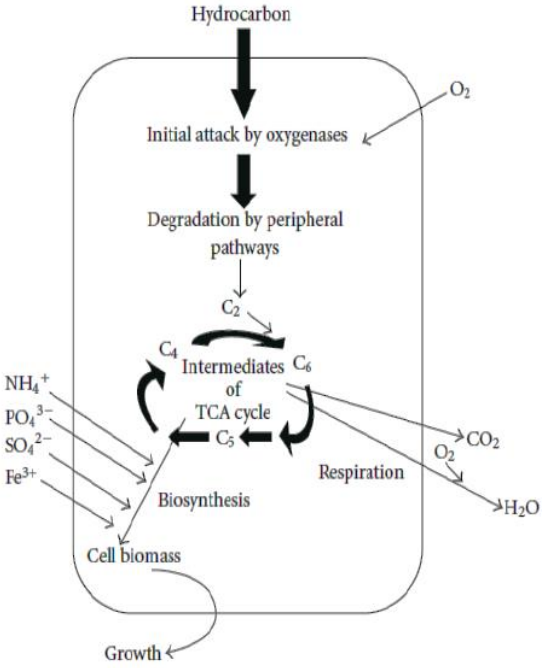

Figure 2. Mechanism of hydrocarbon degradation (Das et al., 2010)

As Luz (2004) stated, catechol 2, 3-dioxygenase is a key enzyme in the lower degradation pathway of the aromatic compounds. Catechol 2, 3-dioxygenase is encoded by *C23*O gene. It is an extradiol-ring fission enzyme. This enzyme is, as a part of the ketoadipate pathway, responsible for conversion of catechol to 2-hydroxymuconic semialdehyde by breaking the C–C bond at the 2, 3-(meta) position. Catechol 2, 3-dioxygenase is a poly peptide, containing 307 amino acid residues with the molecular weight of it is approximately 36 kDa (Yun Jiang, 2004).

Alkane monooxygenase is encoded by *alk*B gene. This enzyme has been studied in diesel oil degrading microorganisms because it is responsible for the conversion of alkanes to acetyl-CoA (Dussán et al., 2012). Alkane monooxygenase contains 383 amino acids with molecular weight of approximately 42 kDa (Liu et al., 2014).

2.5.Importance of Biological Treatment

Contamination of groundwater and soil with petroleum hydrocarbon compounds creates critical problems concerning environment and health. Therefore, attention regarding development of innovative and environmentally sound technologies for the

remediation has increased. Bioremediation of petroleum hydrocarbons has been reccomended as an efficient, economic, and environmentally sound technology, even though the bioavailability of hydrophobic organic compounds to microorganisms could be a inhibitory factor at the time of the biodegradation (Liang-Ming Whanga et al., 2007). By using oil degrading microbes and constituting and maintaining the favored physical, chemical and biological conditions for enhanced oil biodegradation rates, bioremediation offers a less ecologically damaging alternative. Bioremediation methods for oil pollution treatment can offer a cost-effective treatment and overcome the limitations of physical and chemical treatments. Therefore, bioremediation has been considered as a useful tool in treatment of oil residues and removal of spilled oil (Al-Zahrani, 2010). Possible benefits of biosurfactants consist of their structural diversity, which may lead to unique characteristics, the possibility of cost-effective production, and biodegradability. Therefore, biosurfactants are promising for the remediation of enhancing hydrocarbons biologically. (Liang-Ming Whanga et al., 2007).

2.6.Monitoring of Diesel Oil Degrading Bacteria in Surface Waters

2.6.1. Fluorescence *in situ* Hybridization (FISH)

Fluorescence *in situ* hybridization (FISH) is a method for monitoring the bacterial communities in engineering and natural environments (Park et al., 2011). FISH is used to quantify the habitation and relative affluence of microbial populations in a sample. The detection and monitoring of target bacteria, which are directly related to the degradation of pollutants, are also needed for process monitoring and optimization of bioremediation (Rocha et al., 2001). It is a taxonomic method which is mostly used for the observation of whether members of a specific phylogenetic connection are present and also facilitates the quantification of specific microbial groups (Wagner et al., 1993, Wagner et al.1994).

2.6.2. Stable Isotope Probing (SIP)

Before applying bioremediation techniques to a polluted site, potential microorganisms should be determined. Stable isotope probing can be useful for the determination of the organisms which are capable of metabolizing the pollutants present at the environment. SIP tracks the addition of stable isotopes, mostly ^{13}C and ^{15}N or rarely ^{18}O and ^{2}H into biomarkers associated with microbes which assimilate the substrate. Biomarkers are recovered and analysed after the stable isotopes are pulsed to the environment and incorporated to the biomass of metabolically active cells. Biomarkers mostly used in SIP are phospholipid derived fatty acids (PLFA), DNA and rRNA. DNA and, especially, rRNA from these biomarkers are more informative taxonomically even though PLFA-SIP is restricted to the classification of broad taxonomic groups (Uhlik et al., 2012). Among these methods DNA-SIP was developed as a method for the investigation of the metabolic functions of microorganisms in the environment (Chen & Murell, 2010). With SIP, microorganisms from mixed cultures, environmental samples or in situ, can be identified without the need to culture (Cupples, 2011). Figure 3 shows nucleic acid based SIP method.

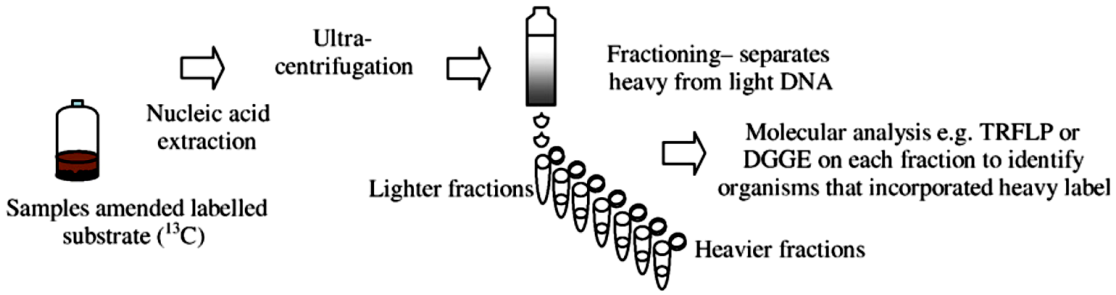

Figure 3. Nucleic acid based stable isotope probing (SIP) method (Cupples, 2011)

CHAPTER 3

MATERIALS AND METHODS

3.1. Collection of Water Samples

Water samples were collected from the river Kızılırmak in close vicinity to petrol refinery. The coordinates were extending from 39º22'16.39''N, 33º26'49.26''E, 890 m to 39º57'22.98''N, 33º25'04.35''E, 679 m of the city Kırıkkale, Turkey. Water samples were put into sterile capped bottles using the aseptic technique and kept in a box which contained ice packs, and transferred urgently to the laboratory. For the FISH analyses, three bottle samples were taken from each station in 2012. Fixation of water samples was done as described in Amann et al. (1995). Water samples were fixed by incubation in paraformaldehyde (4%, w/v, in phosphate-buffered saline) in 2012, at 4°C for 1-2 h, later washed in phosphate-buffered saline and stored in phosphate-buffered saline-ethanol (1:1) at -20°C for *in situ* hybridization.

3.2. Diesel Oil

Diesel oil used in this study was refined diesel oil, which was purchased from local petrol station.

3.3. Measurement of Total Petroleum Hydrocarbons (TPH) in Water Samples

Measurement of TPH in water samples was done by gas chromatographic analysis at Petroleum Research Center in Middle East Technical University. For the measurement of TPH amount in collected water sample by using a gas chromatograph, hexane was used as solvent for extraction. Extraction was repeated three times for 1 L and each time 100 mL of hexane was used. Therefore, for 1 L of

water sample, 300 mL of hexane was used to extract total hydrocarbon in water sample. Rotary evaporator was used to concentrate the sample and the volume was decreased to 10 mL. Then nitrogen gas was used to further concentrate the sample volume to 2 mL. Agilent Technologies 6890N network gas chromatographic (GC) system (Little Falls, California, USA) with electron capture detector (ECD), was used to analyze the water samples. 2 µL of sample volume was injected into DB-XLB column (30 m x 0.25 mm, film thickness 0.25 µm; Little Falls, CA, USA). For the analysis, initial temperature of 35ºC was used and temperature was increased gradually up to 280ºC. The total petroleum hydrocarbons in the water sample were identified according to their retention times. An internal standard, including C_{14}-C_{35} compounds was used.

3.4. Selective Isolation of Diesel Oil Degrading Bacteria

Selective isolation of diesel oil degrading bacteria was done in 2012. Water samples collected from the river Kızılırmak were used to isolate diesel degrading bacteria. Serial dilutions were made from the collected water by taking 1 mL of water and inoculating it into 9 mL sterile distilled water. For the selective isolation, sterile Bushnell-Haas medium was used which contained 1% filter sterilized diesel oil as sole source of carbon. Each dilution was inoculated on Bushnell-Haas medium plates and all plates were incubated for 7 days at 30ºC. After incubation time, isolates appeared in the selective medium were selected for further experiments. Based on the morphological characteristics of the colonies, each bacterial colony were purified by streaking on nutrient agar plates several times and gram stained.

3.5. Biosurfactant Production and Diesel Oil Degradation Ability of the Isolates

Biosurfactant production and diesel degradation capabilities of the isolates were determined by using drop-collapse test, oil displacement test, measurement of emulsification index, microbial adhesion to hydrocarbon, determination of diesel oil degradation ability of the isolates, PCR analysis of the catabolic genes for diesel oil degradation and SDS-PAGE analysis of *C23*O and *alk*B encoded catabolic enzymes.

3.5.1. Drop-Collapse Test

Drop – collapse test was performed by using the protocol as described by Bodour and Maier (2003). All isolates were grown for 7 days in nutrient broth. The microbial cells were separated by centrifugation in 50 mL falcon tubes for 10 min at 10000 rpm. 96 wells well-plate was used. In order to completely cover one well 7 µL of diesel oil was used and well-plate was equilibrated at 37ºC for one h. 5 µL of supernatant was added to the center of the well covered with diesel oil. After 1 min, the shape of the drop was monitored. Distilled water and uninoculated sterile nutrient broth were used as control. The droplets of culture supernatants which made oil drops collapsed was assumed as positive result.

3.5.2. Oil Displacement Test

Method of Morikawa et al. (2000) was used to perform oil displacement test. All isolates were grown for 7 days in nutrient broth. The microbial cells were separated by centrifugation in 50 mL falcon tubes for 10 min at 10000 rpm. 50 mL of distilled water was poured into a petri dish. 20 µL of diesel oil was added to the surface of the water. After that 20 µL of culture supernatant was mixed with 5 µL blue dye in order to observe the cleared zone easily. When culture supernatant was dropped on diesel oil and water interphase, the diameter of the clear zone was measured and area of the clear zones were calculated For each isolate, this test was repeated for three times.

3.5.3. Measurement of Emulsification Activity

This experiment was done as indicated by Desai and Banat (1997). All isolates were grown for 7 days in nutrient broth. The microbial cells were separated by centrifugation in 50 mL falcon tubes for 10 min at 10000 rpm. E_{24} (%) of each isolate were determined for diesel oil, kerosene, paraffin, heptane, benzene mineral oil, xylene and toluene. Emulsification activity was performed by adding 2 mL of each hydrocarbon to 2 mL of the aqueous solution of the biosurfactant, in a test tube, and vortexed at high speed for 2 min. The stability of the emulsion was determined after 24 h by measuring the height of the emulsion layer and total height of the solution. E_{24} was calculated by using the formula given in equation 1.

$$E_{24} = \frac{Heigth\ of\ \ emulsion\ layer}{Total\ height\ of\ solution} x100 \qquad (1)$$

3.5.4. Microbial Adhesion to Hydrocarbon (MATH)

Microbial Adhesion to Hydrocarbon (MATH) was used to determine the cell surface hydrophobicity as stated in Rosenberg et al. (1980). All isolates were grown for 7 days in nutrient broth. The microbial cells were separated by centrifugation in 50 mL falcon tubes for 10 min at 10000 rpm. Bacterial cells of the isolates were obtained then washed two times with 5 mL PUM buffer ($MgSO_4.7\ H_2O$= 0.2g/L, K_2HPO_4=19.7 g/L, KH_2PO_4=7.26 g/L and urea=1.8 g/L (pH 7.0)). 5 mL of PUM buffer was added to each isolate again and cells were re-suspended. Optical densities of suspensions were measured at 600 nm on Rayleigh VIS-723 (Beijing Beifen-Ruili Analytical Instrument Group Co., Ltd.) UV- Visible Spectrophotometer (A_0). 500 µL diesel was added to 5 mL of microbial suspension and vortexed for 2 min. After 10 min optical density of aqueous phases for each isolate were measured (A_1). The degree of hydrophobicity of each sample were calculated by using equation 2.

$$Degree\ of\ hydrophobicity\ = \left(1 - \frac{A_0 - A_1}{A_0}\right) x100 \qquad (2)$$

3.5.5. Determination of Diesel Oil Degradation Ability of the Isolates

Ability of isolates to degrade diesel oil was measured by using both gravimetric and GC analyses.

3.5.5.1. Gravimetric Analysis of Diesel Oil Degradation

Gravimetric analysis of diesel biodegradation was done as conducted in Latha et al. (2012). Amount of diesel oil was measured after extraction of oil from the water sample and evaporated till to dry in liophilizator under reduced pressure. 50 mL of hexane is used to extract remaining diesel oil in 50 mL, 1% diesel containing Bushnell Hass medium. 100 mL of total media was transferred into a separation funnel and the aqueous phase containing the hexane and remaining diesel oil, was

extracted. Each 50 mL medium contains 500 µL of diesel and the weight of diesel of 500 µL was pre-determined. Weight of TPH in the samples was determined by using the formula in equation 3. The weight of extracted diesel oil was deducted from the previously weighed beaker. The formula given in equation 4 was used to calculate the amount of diesel oil degraded. Percent degradation of diesel for each isolate was determined by using the formula given in equation 5.

$$Weight\ of\ TPH\ in\ sample = Weight\ of\ beaker\ containing\ extracted\ TPH - Weight\ of\ empty\ beaker \quad (3)$$

$$Amount\ of\ diesel\ degraded = Weight\ of\ diesel\ added\ in\ the\ media - Weight\ of\ residual\ diesel \quad (4)$$

$$Diesel\ degraded\ (\%) = \frac{weight\ of\ diesel\ degraded}{original\ weight\ of\ diesel\ introduced} x100 \quad (5)$$

3.5.5.2. GC Analysis of Diesel Oil Degradation

Determination of diesel oil degradation by GC analysis was done at Petroleum Research Center in Middle East Technical University. GC analysis of diesel oil degradation was performed as the GC analysis of TPH in water sample. Hexane was used for extraction of residual diesel oil in 1% diesel containing Bushnell Hass medium. 50 mL of hexane was used for extraction. Rotary evaporator was used to concentrate the sample. Then nitrogen gas was used to further concentrate the sample volume to 2 mL. Agilent-Technologies 6890N network GC system (Little Falls, California, USA) with electron capture detector (ECD), was used to analyze the water samples. 2 µL of sample volume was injected into DB-XLB column (30 m x 0.25 mm, film thickness 0.25 µm; Little Falls, CA, USA). For the analysis, initial temperature was 35°C and temperature was increased gradually up to 280°C. Hydrocarbon species in diesel were identified according to their retention times. An internal standard, including C_{14}-C_{35} compounds is used. Residual diesel oil amount was determined by using the formula given in equation 5 as in the gravimetric analysis.

3.5.6. Identification and Characterization of Diesel Oil Degrading Bacteria

3.5.6.1. DNA Extraction

Each isolate were grown in nutrient broth and total DNA of the diesel oil degrading isolates were extracted by using Roche – High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany). Steps for DNA extraction were applied as described in the manual of the kit.

3.5.6.2. Polymerase Chain Reaction (PCR) Analysis of the Catabolic Genes for Diesel Oil Degradation

PCR-based detection of both catabolic genes namely *C23*O and *alk*B were performed. PCR was done by using T100 thermal cycler (Bio-Rad, USA). The PCR mixture in 25 µL contained 1.25 U of AmpliTag Gold, 5 µL of GeneAmp 10 x PCR buffer, 25 mM $MgCl_2$, 2.5 mM dNTP's, 10 pm μL^{-1} of each primer, and 1 µL template.

3.5.6.2.1. PCR Analysis of *C23*O

*C23*O gene fragments were amplified with the primers *C23*Of 5'-CGACCTGATC(AT)(CG)CATGACCGA-3', *C23*Or 5'-T(CT)AGGTCA(GT)(AC)ACGGTCA-3' as stated by Jyothi et al. (2012). The PCR temperature program began with an initial 5-min denaturation step at 94°C, then 35 cycles of 94°C for 45 sec, 55°C for 1 min, and 72°C for 1 min; and a final 10-min extension step at 72°C. Amplification products were then analyzed by electrophoresis on a 1% agarose gel.

3.5.6.2.2. PCR Analysis of *alk*B

*Alk*B gene fragments were amplified with the primers *alk*Bf 5'-AACATAACCGTGGCCATC AC -3', *alk*Br 5'- AACACCACGCTGTACATCCA -3' as stated by Dussán et al. (2012). The PCR temperature program began with an initial 5-min denaturation step at 94°C, then 35 cycles of 94°C for 45 sec, 55°C for 1

min, and 72°C for 1 min; and a final 10-min extension step at 72°C. Amplification products were then analyzed by electrophoresis on a 1% agarose gel.

3.5.6.3. Total Protein Extraction

For SDS-PAGE analysis of catabolic enzymes total protein extraction protocol of Laemmli (1970) was applied to diesel degrading isolates. Each strain were grown in nutrient broth and Bushnell Haas Broth with 1% diesel oil. 50 mL of each culture was centrifuged for 5 min at 10000 rpm and supernatants were discarded. Bacterial pellets were washed twice with 5 mL sterile water. 2 mL of phosphate buffer was added to each sample. Then pellets were kept in ice and sonicated at 50 mHz for 10 min. after centrifuging for 2 min at 5000 rpm the supernatant was discarded. Each 75 µL sample were mixed with 25 µL sample buffer and boiled for 5 min. Extracted protein samples were kept at -20^{o}C.

Content of phosphate buffer (pH = 7) is as below:

6.8 g KH_2PO_4/ L distilled water
8.7 g K_2HPO_4/ L distilled water

Content of sample buffer is as below:

1 M Tris-HCl (pH = 6.8)	0.6 mL
Distilled water	0.9 mL
50% gliserol	5 mL
1% bromphenol blue	1 mL
10% SDS	2 mL
β – mercaptoethanol	0.5 mL

3.5.6.4. SDS-PAGE Analysis of the Catabolic Enzymes

SDS-PAGE analysis was done to observe the presence of the catabolic enzymes responsible for diesel oil degradation. Procedure of the SDS-PAGE analysis was done as described by Laemmli (1970). SDS-PAGE gels were prepared using the following steps. Glass plates, combs, spacers and assemble gel cassettes were cleaned and completely dried. Separating gel and stacking gels were prepared according to the following information. Once the separating gels were applied a small layer of isopropanol was added to top of the separating gel prior to

polymerization to straighten the level of the gel. Isopropanol was removed by using filter paper. Top layer of the gels were washed with distilled water and water was dried as possible by using filter paper. After this step stacking gels were applied above the separating gels and combs were placed. Once the stacking gels were solidified, combs were removed. The gel cassettes were removed from the casting stand and then placed in the electrode assembly with the small plate on the inside. The electrode assembly was pressed down during the time clamping the frame in order to secure the electrode assembly and then clamping frames were put in the electrophoresis tank. 1x electrophoresis running buffer was poured into the opening of the casting frame between the gel cassettes. Buffer was added to fill the wells of the gel. Fill the region outside of the frame with 1x running buffer. While loading the gel; marker, positive control and negative control were loaded to each gel and each isolate were loaded to the gel as isolate grown in nutrient broth and isolate grown in 1% diesel and Bushnell Haas Broth. *Acinetobacter haemolyticus* and *Escherichia coli* were used as positive and negative controls respectively. Comassie blue was used for protein staining. The molecular weights of the proteins were estimated from calibration curves prepared by using Color Plus Prestained Protein Marker (New England BioLabs, UK). The reference marker possessed 8 proteins with the following sizes; 175, 80, 58, 46, 30, 23, 17 and 7 kDa, respectively. A standard curve was constructed for each acrylamide gel using the molecular weights of standard marker and the distances migrated by each in the gel. Then the distance that each of the proteins migrated within the same gel was measured and used to calculate the molecular weight of each protein in the isolates from the standard curve (Laemmli, 1970).

Content of 10% separating gel is as below:

2 mL dH_2O
1.67 mL 30% acrylamide/Bis
1.25 mL 1.5 M Tris, pH 8.8
25 µL 20% SDS
25 µL 10% ammonium persulfate (Make it fresh and store at 4 °C up to a month)
2.5 µL TEMED (before pouring the gel)

Content of 5% stacking gel is as below:

2.088 mL dH2O
0.506 mL 30% acrylamide/Bis
0.375 mL 1 M Tris, pH 6.8
15 µL 20% (w/v) SDS
15 µL 10% ammonium persulfate
1.5 µL TEMED (before pouring the gel)

Content of 10x Running Buffer is as below:

30.3 g Tris-base
144.0 g glycine
10.0 g SDS
Completely dissolve in about 800 mL dH_2O and then more dH_2O up to 1 L.

3.5.6.5. 16S rRNA Sequencing for Identification of Diesel Oil Degraders

Extracted DNA samples were amplified by 16S rRNA primers. *Alk*B and *C23*O genes fragments were amplified by using universal bacterial 16S rRNA primers, 27f (5′-AGAGTT TGATCCTGGCTCAG-3′) and 1492r (5′-GGTGTTTGATTGTTACGACTT-3′) Lane et al. (1985). PCR procedure was performed with a 25 µL reaction mixture containing; 1 µL (10 ng) of DNA extract as template, each primer at a concentration of 5 mM, 25 mM $MgCl_2$ and dNTPs at a concentration of 2 mM, as well as 1.5 U of *Taq* polymerase and PCR buffer used as recommended by the manufacturer (Fermentas, Germany). After the initial denaturation at 94°C for 5 min, there were 35 cycles containing denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 5 min. PCR was carried out in a T100 thermal cycler (Bio-rad, USA). Obtained PCR products were purified, using the GeneJET™ PCR Purification Kit (Fermentas, Germany), according the instruction of the manufacturer and sequenced. Purified PCR products were sent to sequencing for identification. The PCR product was sequenced by 3730 x 1 DNA synthesizer (Applied Biosystems, USA). The 16S rRNA sequences were aligned and compared with other 16S rRNA genes in the GenBank by using the NCBI basic local alignment search tools BLASTn program (Benson, 2002). The 16S rRNA gene sequences were deposited to GenBank using BankIt submission tool and assigned with NCBI accession numbers.

3.6.Characterization of Biosurfactants of Diesel Oil Degraders

Biosurfactants produced by diesel oil degraders were characterized through physicochemical and biochemical methods.

3.6.1. Physicochemical Characterization

For the physicochemical characterization of biosurfactants produced by diesel oil degraders, surface tension measurement was used.

Surface Tension Measurement

Surface tension measurement was done as described in Chandran et al. (2010), to determine the reduction in surface tension in different samples. Surface tension of distilled water, Bushnell Haas Broth, diesel oil and Bushnell Haas Broth were determined and reduction of surface tension with SDS, Tween 20 and biosurfactants were also measured. Triplicates of 1, 1/10 and 1/100 dilutions of SDS, Tween 20 and biosurfactants were used for determination. Surface tension measurement was performed at Middle East Technical University Central Laboratory. The measurements were repeated five times for each sample and average of the measurements was obtained for each. Calibration of the instrument was done by the surface tension of the pure water was measured before each set of experiment. The Critical micelle concentration was later determined from the break point of the surface tension versus its log of bulk concentration curve. Surface tension was calculated by the formula given in equation 6.

$$Tension\ reduction(\%) = \frac{\gamma_m - \gamma_c}{\gamma_m} x100 \quad (6)$$

Where; γ_m is the surface tension of the medium without inoculation and γ_c is the surface tension of the test supernatant.

3.6.2. Biochemical and Structural Characterization

Biosurfactants produced by diesel oil degraders were also characterized by means of biochemical and structural methods. These methods included, Fourier Transform

Infrared Spectroscopy (FTIR), scanning electron microscopy (SEM), zeta potential measurement and gas chromatography-mass spectrometry (GC-MS).

3.6.2.1. Fourier Transform Infrared Spectroscopy (FTIR)

For the FTIR characterization of biosurfactants samples were prepared as described in Chandran et al. (2010). Diesel oil degraders were grown for 7 days and biosurfactants of degraders were extracted. Each 7 day grown culture were centrifuged at 10000 rpm for 10 min and supernatants were collected. Chloroform and methanol mixture (1:1) was added to the biosurfactants at the same volume and left overnight for phase separation. Culture medium and methanol and chloroform were removed and biosurfactants were dried. Dried biosurfactants were analyzed by FTIR analysis at Kırıkkale University Biotechnology Research Center to determine the functional groups and also the chemical bonds in the biologically active part of the biosurfactants by using Bruker Vertex 70V (Billerica, MA USA).

3.6.2.2. Scanning Electron Microscopy (SEM)

Diesel oil degrading isolates were grown in Bushnell Haas Broth with 1% diesel oil for one week and SEM analysis was conducted for determination of biosurfactant production. Samples for SEM analysis were prepared as described in Chandran et al. (2010). 1 cm^2 cover glasses in 7 days grown cultures and glasses were put at 37^oC for 90 min for adhesion. After the adhesion, the cover glasses were washed with sterile phosphate buffer saline (PBS) to remove loosely adherent cells. After washing with PBS, 1 mL of Bushnell Haas Broth with 1% diesel was added to each cover glass and incubated at 37°C for 72 h. Again the cover glasses were washed with sterile PBS, dried and fixed with 3% gluteraldehyde and dehydrated with a series of ethanol solution with concentrations of 50, 75, 95, and 100% then subjected to SEM analysis by using JSM 5600 30kV scanning electron microscope (JEOL Ltd., California, USA) at Kırıkkale University Biotechnology Research Center.

3.6.2.3. Measurement of Zeta Potential

Measurement of zeta potential of the prepared samples was done at Kırıkkale University Biotechnology Research Center. Preparation of the emulsified diesel was done by mixing 97% distilled water and 1% v/v surfactant and 2% diesel for 3 min. The stock emulsions were diluted at a ratio of 1/10 in PBS (0.01 M, pH 7.5) before use. (Chandran, 2010) Zeta potential of a sample is dependent on the pH of the sample. (Clogston, 2009) Therefore, to measure the zeta potential of the bacteria plus diesel, diesel and diesel plus biosurfactant samples were prepared at the pH range in between 3-9. 1 mL of each sample were injected to Malvern Nano – ZS (Malvern Instruments Ltd, Worchestershire, UK) and zeta potential measurement was performed.

3.7.Monitoring of *alk*B and *C23*O-Harboring Population in Surface Water by Fluorescent *in situ* Hybridization (FISH) Method

3.7.1. Collection of Water Samples and Fixation with Paraformaldehyde

Twelve different stations of the river Kızılırmak extending from 39º22’16.39’’N, 33º26’49.26’’E, 890 m to 39º57’22.98’’N, 33º25’04.35’’E, 679 m of the city Kırıkkale-Turkey were chosen as the study location to collect the water samples over the years of 2012. Three sterile screw capped bottles filled with water from the river aseptically and kept in an icebox containing ice packs. For the FISH analyses, paraformaldehyde (4%, w/v, in phosphate-buffered saline) was used to fix the collected water samples in 2012 by Kırıkkale University. The fixed samples were kept at 4°C for 1-2 h first, and then washed in phosphate-buffered saline. The samples were stored in phosphate-buffered saline-ethanol (1/1) at -20°C for in situ hybridization as indicated by Nussbaumer et al. (2006).

3.7.2. Oligonucleotide Probes

16S rRNA-targeted oligonucleotide probe sequences targeting whole bacteria used in the study were selected from probeBase (Loy, 2003). The 16S rRNA-targeted oligonucleotide probes and hybridization conditions given in Table 1 were used in

the study. The probes EUB338, EUB338 II, EUB338 III were used to cover all bacteria. NON338 probe was used as a non-binding negative-control. To determine diesel oil degrading bacteria *alk*B and *C23*O probes were also used in the study. All probes were commercially obtained and labelled with fluorescein isothiocyanate (FITC) at the 5' end (Alpha DNA, Montreal, Canada) and, diluted with TE buffer to 50 ng/μL and stored at –20ºC until use. *alk*B and *C23*O probes were further evaluated for their hybridization stringency and specificity to the targeted *alk*B and *C23*O genes by using paraformaldehyde-fixed pure cultures in order to determine the *alkB* and *C23*O probes with high specificity to alkB and *C23*O genes targeted.

Table 2. Oligonucleotide probes used in the study

Probe Designation	Target gene/ organisms	Sequence of the probe (5'→3')	Length (bp)	GC (%)	T_m (C°)	Reference
*alk*B	*alk*B gene	AAYTAYCTNGARCAYTAYGGNC	22	76.6	84.0	Kloos et al., 2006
*C23*O	*C23*O gene	CGTGAGTAATGCCGTGACTGGTCGGG CCGATATCGA	36	73.2	76.0	Sei et al., 1999
EUB338	Most Bacteria	GCTGCCTCCCGTAGGAGT	18	66.7	55.0	Daims et al., 1999
EUB338 II	Planctomycetales	GCAGCCACCCGTAGGTGT	18	66.7	55.0	Wallner et al.,1993
EUB338 III	Verrucomicrobials	GTCGCCACCCGTAGGTGT	18	66.7	55.0	Wallner et al.,1993
NONEUB	Negative control	ACTCCTACGGGAGGCAGC	18	66.7	55.0	Schleifer et al., 1992

3.7.3. *In Situ* Hybridization

For the optimization of hybridization stringencies for each probe, 5 μL of samples taken from each fixed pure cultures were spotted on glass slides and dried at 45°C for 30 min. Then, the slides were dehydrated sequentially in 50, 80 and 96% ethanol for 3 min each (Amann, 1995; Nielsen, 2009). Hybridization was performed in 9 μL of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl, 5 mM EDTA, 0.01% SDS; pH 7.2) in the presence of variable formamide concentration, 1 μL probe at 46°C for 2 h in a moisture chamber. Optimum hybridization stringency required the addition of formamide to final concentrations specific for each probe. Subsequently the slides were washed in prewarmed washing buffer for 15 min at 48°C, rinsed with deionized water and air dried. The washing buffer contained 20 mM Tris-HCl (pH 7.2), 10 mM EDTA, 0.01% SDS and variable NaCl concentration. The washing buffer was removed by rinsing the slides with double-distilled water. To determine the total bacteria present in the samples, each slide was counter-stained with 10 μL DAPI (4'6-diamidino-2-phenylindole dihydrochloride) solution (1 μg/mL) for 15 min at room temperature, rinsed with deionized water and allowed to air-dry. To avoid losing the signal intensity, the slides were kept in the dark on ice until microscopic observation. For *in situ* application, optimum stringency conditions were adjusted for each FITC-labelled probe performing whole cell hybridizations using targeted and nontargeted cultures (Amann, 1995; Oerther, 2000). The hybridization stringency was gradually enhanced by the addition of formamide to the hybridization buffer in concentration steps of 5% (v/v) according to Wagner et al. (1994). The sodium chloride concentration was adjusted according to the formamide concentration used in the hybridization buffer. The optimum formamide and sodium chloride concentrations for each probe were determined by reference to the target organisms.

3.7.4. Fluorescence Microscopy

The *alk*B and *C23*O harboring isolates were observed at Bilkent University UNAM by using a Leica fluorescence microscope type DM5000B with a mercury lamp (Hg100W; Leica Microsystems, Wetzlar, Germany) and a digital camera type DFC300FX (Leica Microsystems) combined with Leica QWin Plus software. All images were recorded as described above using a fluorescence microscope with two

filters and the following wavelengths: for excitation 450-490 and emission 500-550 nm for (FITC) and excitation 320-400 nm and emission 430-510 nm for DAPI. Slides were mounted in anti-fading solution AF1 (Citifluor Ltd., London, UK) and viewed under oil immersion at *x*100 magnification. Ten images from each triplicate slides for each probe were captured with CCD camera and first processed using the software Adobe Photoshop CS3 *(*Adobe Systems Inc., San Jose, CA, USA*)* in which the blur areas were removed.

3.7.5. Image Analysis to Determine Biomass of *alkB* and *C23O* Harboring Bacteria

The processed images were analyzed using the software Leica QWin Plus. The program calculated the area from which the fluorescence signal was recorded. The final result was presented in terms of the percentage calculated when this area was divided by the total area of the field of observation (Daims, 2005). The pixel areas of green region conferred by FITC-labelled alkB and *C23*O probes chosen and blue region conferred by DAPI were counted separately for each image. Depending on the quantification of pixel areas of total images, the alkB and *C23*O harboring bacterial biomass was determined as shown in equation 7 (Li, 2007).

$$Biomass\ of alkB/C23O\ harboring\ bacteria\ (\%) = \frac{Pixel\ area\ of\ FITC\ image}{Pixel\ area\ of\ DAPI\ image} \quad (7)$$

% of *alk*B and *C23*O harboring biomass was calculated using average values of taken images for pixel areas of FITC probe and DAPI. Before the calculation, the pixel areas were subtracted by the areas of non-binding probe (NON338) to remove auto-fluorescence and background interferences. In this study, the total amount of *alk*B and *C23*O harboring biomass was assumed as the images conferred by oligonucleotide probes alkB and *C23*O and the total amount of biomass was the images conferred by DAPI stained cells. After optimization of hybridization stringency conditions for *alk*B and *C23*O probes with positive and negative pure

culture controls, the river water samples were screened for the alkB and *C23*O harboring isolates by using FISH (Amann, 1995).

3.8.Statistical Analyses

All statistical analyses of intensity measurements obtained from each captured images were performed using Origin Pro 9 software (OriginLab Corporation, Northampton, Massachusetts, USA). During the monitoring of *alk*B and *C23O* harboring bacteria in river water the significance of all parameters in the regression analyses demonstrated were verified ($p<0.05$ significance level) by one-way analysis of variance (ANOVA) and Tukey test.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Measurement of TPH in Water Samples

TPH measurement in collected water samples were performed through gas chromatographic analysis. After the GC analysis of TPH in water, samples the chromatogram obtained was illustrated in Figure 4. The detailed results of GC analysis for measurement of TPH were shown in Table 3.

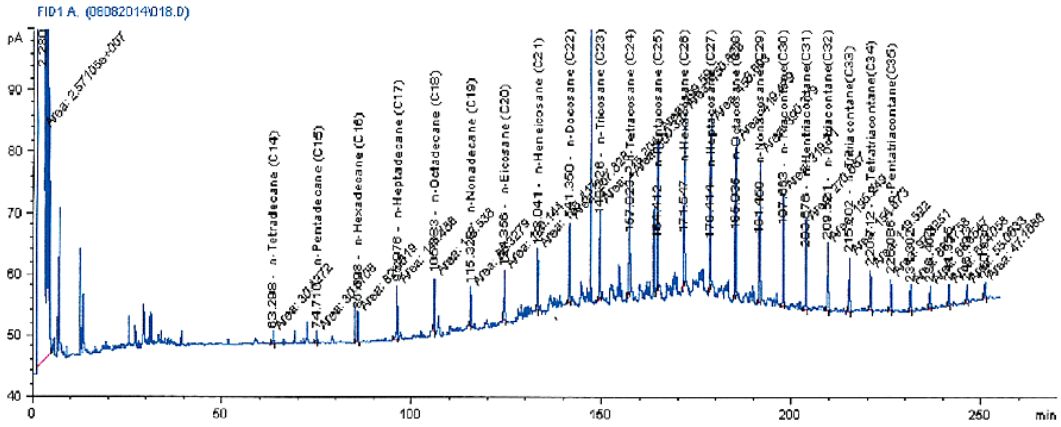

Figure 4. GC chromatogram of TPH in the collected water sample

Figure 4 showed that, there were petroleum hydrocarbons present in the water sample. This chromatogram showed the presence of hydrocarbons with carbon atoms between 14 and 35.

Table 3. GC analysis of TPH in the collected water sample

Hydrocarbon	Retention time (min)	Area (pA*s)	Amount (ppm)
n-Pentane (C5)	1.51	-	-
n-Hexane (C6)	1.94	-	-
n-Heptane (C7)	3.24	-	-
n-Octane (C8)	6.54	-	-
n-Nonane (C9)	14.21	-	-
n-Decane (C10)	17.77	-	-
n-Undecane (C11)	28.03	-	-
n-Dodecane (C12)	39.84	-	-
n-Tridecane (C13)	52.00	-	-
n-Tetradecane (C14)	63.29	30.43	1.74
n-Pentadecane (C15)	74.71	30.87	1.75
n-Hexadecane (C16)	85.59	82.06	4.63
n-Heptadecane (C17)	95.97	145.49	8.32
n-Octadecane (C18)	105.88	118.54	6.74
n-Nonadecane (C19)	115.32	86.33	4.92
n-Eicosane (C20)	124.35	120.14	6.84
n-Heneicosane (C21)	133.04	156.42	9.02
n-Docosane (C22)	141.35	207.83	12.00
n-Tricosane (C23)	149.32	215.20	12.37
n-Tetracosane (C24)	157.02	300.32	17.11
n-Pentacosane (C25)	164.41	399.59	22.82
n-Hexacosane (C26)	171.54	450.82	25.11
n-Heptacosane (C27)	178.41	456.60	26.81
n-Octacosane (C28)	185.03	419.48	23.35
n-Nonacosane (C29)	191.45	390.38	22.36
n-Triacontane (C30)	197.66	319.71	17.63
n-Hentriacontane (C31)	203.67	270.69	14.65
n-Dotriacontane (C32)	209.52	195.25	10.88
n-Tritriacontane (C33)	215.20	154.67	8.35
n-Tetratriacontane (C34)	220.71	119.52	6.41
n-Pentatriacontane (C35)	226.08	97.83	5.51
Totals:			**269.29**

According to Table 2, 269.69 ppm of TPH was determined in the water sample. Highest TPH values were obtained with contained C_{25}-C_{29} hydrocarbons.

4.2. Selective Isolation of Diesel Oil Degrading Bacteria

Water samples were collected from the stations given in Figure 5. After selective isolation, 22 diesel oil degrading bacteria were obtained and purified from the water samples collected from the given locations.

Figure 5. Sampling coordinates (from Google Earth)

4.3. Biosurfactant Production and Diesel Oil Degradation Ability of the Isolates

Twenty two diesel oil degrading bacteria were subjected to determine their biosurfactant production and diesel oil degradation abilities. Biosurfactant production and diesel oil degradation capabilities of the isolates were determined by using drop-collapse test, oil displacement test, and measurement of emulsification index, microbial adhesion to hydrocarbon, determination of diesel oil degradation ability,

PCR analysis of the catabolic genes for diesel oil degradation and SDS-PAGE analysis of *C23O* and *alkB* coded catabolic enzymes.

4.3.1. Drop-Collapse Test

Youssef et al. (2004) stated that drop-collapse test expresses the surface activity and indicates production of biosurfactants.

Drop-collapse test was applied to 22 diesel oil degrading isolates and all 22 isolates achieved collapse in drops in micro well plates as shown in Figure 6. The results of this test were presented in Table 4.

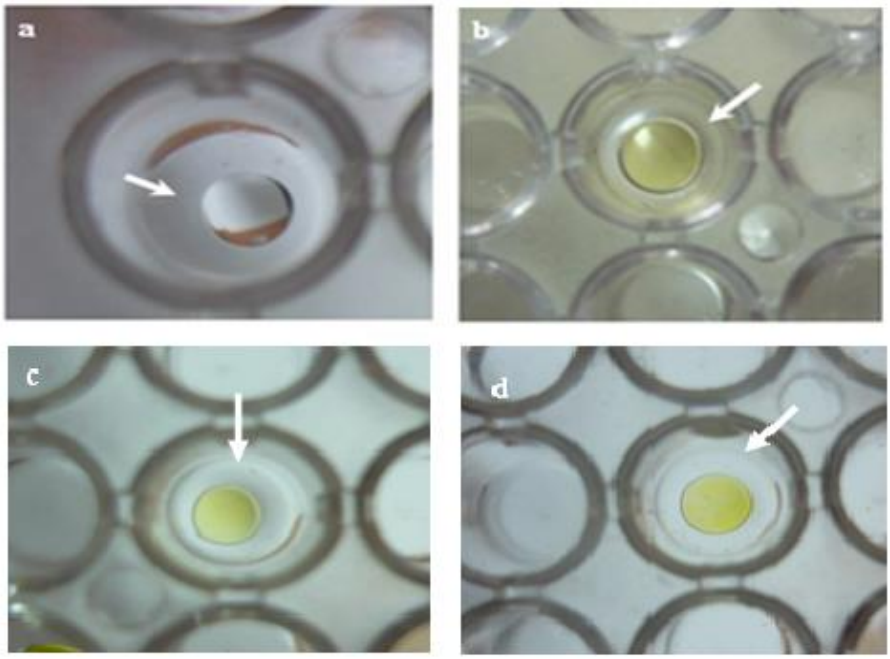

Figure 6. Representative pictures of dispersion of control liquids and supernatants in micro well plate distilled water **(a)** uninoculated nutrient broth **(b)** Ag10 **(c)** Ag11 **(d)**

Table 4. Drop–collapse ability of diesel oil degrading isolates

Strain designations	Drop – collapse abilities
Ag10	+
Ag11	+
Al11	+
Ba01	+
Ba11	+
Cd11	+
Co11	+
Cr07	+
Cu12	+
Fe10	+
Hg10	+
Hg11	+
Li12	+
Mn11	+
Mn12	+
Ni11	+
Pb06	+
Sb01	+
Sn11	+
Sr02	+
Sr11	+
Zn01	+

+, positive
-, negative

Table 3 showed that, all isolates achieved collapse in drops in micro well plates. Drop–collapse technique is dependent on the principle at which a drop of a liquid involving a biosurfactant will collapse and spread over the surface of oil (Youssef et al., 2004). Therefore, achieving collapse in drops was an indicator of biosurfactant production ability of the isolates (Chandran, 2010).

4.3.2. Oil Displacement Test

As Youssef et al. (2004) explained oil displacement test is an indicator of the surface activity. Also this test indicates production of biosurfactants. Figure 7 illustrates oil displacement due to biosurfactant production of bacteria. Table 5 summarizes the data obtained from oil displacement test. Blue zone in Figure 7 shows the displaced diesel oil.

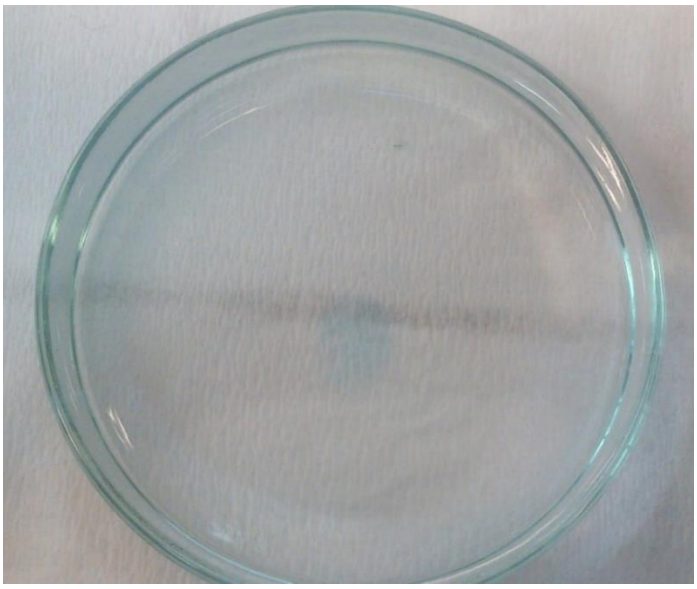

Figure 7. Representative picture of oil displacement test

In Table 5 oil displacement test results was summarized. According to the table, all standard deviations of all isolates were found out to be lower than 1.0.

As reported by Rodrigues et al. (2006), higher diameters of cleared zone means more surface activity of the samples. Therefore, higher areas of oil displacement represented higher activities. Results of oil displacement test indicated that, highest activity was achieved by Ba11 with 3.03 cm^2. Also, Ag10, Al11, Ba01, Cr07, Fe10, Li12, Mn11, Ni11 and Zn01 achieved oil displacement with areas larger than 2 cm^2.

Table 5. Oil displacement test results of the isolates

Strain designations	Oil displacement (cm^2)
Ag10	2.02±0.21
Ag11	1.84±0.11
Al11	2.10±0.23
Ba01	2.85±0.41
Ba11	3.03±0.14
Cd11	1.47±0.26
Co11	1.62±0.11
Cr07	2.47±0.48
Cu12	1.77±0.00
Fe10	2.55±0.23
Hg10	1.20±0.09
Hg11	1.07±0.08
Li12	2.68±0.64
Mn11	2.10±0.12
Mn12	1.69±0.11
Ni11	2.37±0.34
Pb06	1.77±0.19
Sb01	1.27±0.24
Sn11	1.93±0.24
Sr02	1.62±0.11
Sr11	1,62±0.11
Zn01	2.02±0.79

±; standard deviations

4.3.3. Measurement of Emulsification Activity (E_{24})

As Obayori et al. (2009) stated, release of biosurfactants is a strategy used by microorganisms to affect the uptake of hydrocarbon compounds. Therefore, measurement of emulsification activity (E_{24}) experiment was conducted for all of the bacteria which were successful at drop –collapse and oil displacement tests.

E_{24} was calculated using the equation 1.E_{24} (%) of each isolate for diesel oil were summarized in Table 5. The bacteria with emulsification indices higher than 50 % have been defined as potential biosurfactant producers (Rodriguez-Rodriguez et al., 2012). E_{24} (%) of selected isolates, having emulsification indices equal to and higher than 60% for diesel were also marked in Table 6. Graphical expression of emulsification indices of selected isolates, equal to and higher than 60% and for diesel was shown in Figure 8.

Table 6. E_{24} % of the isolates

Strain designations	E_{24} (%)
***Ag10**	80.28±2.64
***Ag11**	77.75±0.82
Al11	57.43±0.91
***Ba01**	62.77±1.23
***Ba11**	62.07±0.00
Cd11	53.85±5.44
***Co11**	65.22±0.00
Cr07	57.88±0.74
Cu12	53.40±0.87
***Fe10**	60.0±1.27
***Hg10**	75.00±0.00
***Hg11**	71.56±0.65
Li12	53.57±1.30
Mn11	55.56±0.71
***Mn12**	61.20±0.46
***Ni11**	72.00±0.00
***Pb06**	65.38±0.00
***Sb01**	63.00±1.50
***Sn11**	70.83±0.00
Sr02	42.02±0.82
***Sr11**	73.68±0.00
***Zn01**	61.10±0.87

±; standard deviations
*selected isolates with E_{24} (% ≥) 60%

Some microorganisms such as *Acinetobacter*, *Pseudomonas* and *Rhodococcus* species produce biosurfactants that efficiently make hydrocarbon compounds more available for microbial usage (Ciric et al., 2009).

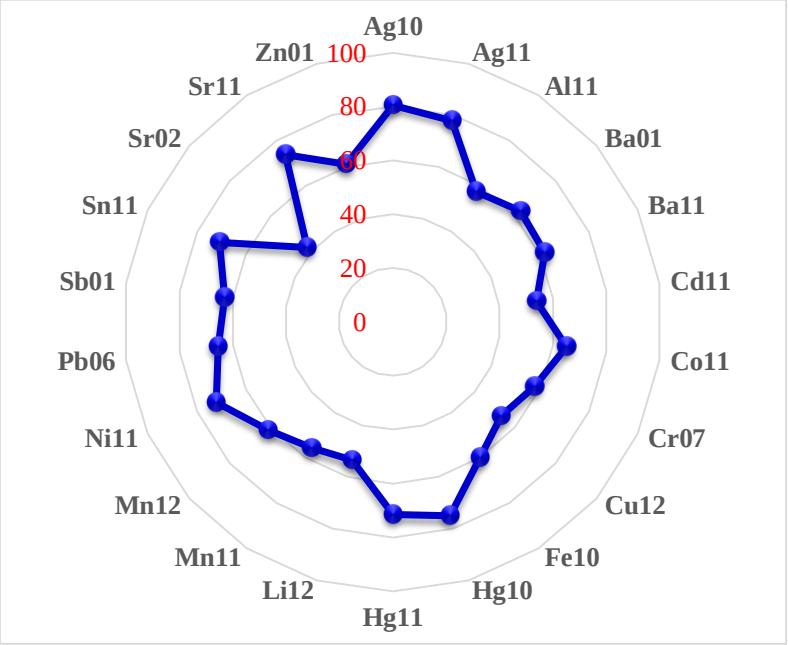

Figure 8. E_{24} % of the isolates for diesel oil

As it can be seen in Figure 8, 15 of the isolates had 60% or higher E_{24} values.

4.3.4. Microbial Adhesion to Hydrocarbon (MATH)

Hydrophobicity was referred to the wettability of a surface. It was shown that hydrophobicity of solid surfaces affects the adhesion of bacteria, eukaryotic cells (Oliveira et al., 2001). Enhancement of bacterial hydrophobicity was found to be beneficial in a number of engineering applications such as biodegradation of organic compounds (Saini, 2010). Therefore, MATH was used to determine the cell surface hydrophobicity of the 15 isolates with high E_{24} (%) values. The degree of hydrophobicity of each sample were calculated by using the formula given in equation 2. Results of MATH experiment are given in Table 7.

Table 7. Degree of hydrophobicity of the selected isolates

Strain designations	Hydrophobicity (%)
Ag10	99.77
Ag11	100
Ba01	89.78
Ba11	100
Co11	69.16
Fe10	100
Hg10	93.05
Hg11	100
Mn12	39.17
Ni11	98.83
Pb06	84.82
Sb01	100
Sn11	97.86
Sr11	100
Zn01	100

Hydrophobic properties of species has a role in growth on hydrophobic substrates, adhesion to host cells. (Rosenberg, 2006) Therefore, higher values of hydrophobicity indicates higher adhesion to diesel oil. According to Table 6, Ag10, Ba11, Fe10, Hg11, Sb01, Sr11 and Zn01 has 100% hydrophobicity which means, these isolates were the most successful isolates adhering to diesel oil.

4.3.5. Determination of Diesel Oil Degradation Ability of the Isolates

Diesel oil degradation ability of the isolates was investigated by using both gravimetric and GC analyses.

4.3.5.1. Gravimetric Analysis of Diesel Oil Degradation

Gravimetric analysis was used to determine the diesel degradation ability of the isolates. Even though the gravimetric analysis is not as sensitive as GC analysis, it is comparatively helpful method for the preliminary determination. Therefore, all 15 isolates which were found out to be successful in previous experiments were subjected to gravimetric analysis of diesel oil degradation. Residual diesel oil

amounts in samples were calculated by using the equations 3, 4 and 5. Diesel biodegradation capacities of the isolates were summarized in Table 8.

Table 8. Gravimetric analysis results of % diesel biodegradation capacities of isolates

Strain designations	Diesel biodegradation
Ag10	68.62±1.98
Ag11	53.06±1.48
Ba01	57.51±8.39
Ba11	61.34±1.85
Co11	76.90±1.35
Fe10	93.70±0.86
Hg10	51.83±0.74
Hg11	53.19±1.36
Mn12	47.75±2.09
Ni11	20.95±1.73
Pb06	39.60±4.57
Sb01	45.16±2.22
Sn11	49.23±1.11
Sr11	32.44±1.61
Zn01	88.88±1.73

±; standard deviations

A graphical expression of diesel degradation abilities of isolates was shown in Figure 9. Both E_{24} (%) and diesel biodegradation abilities of the isolates were compared in Figure 10.

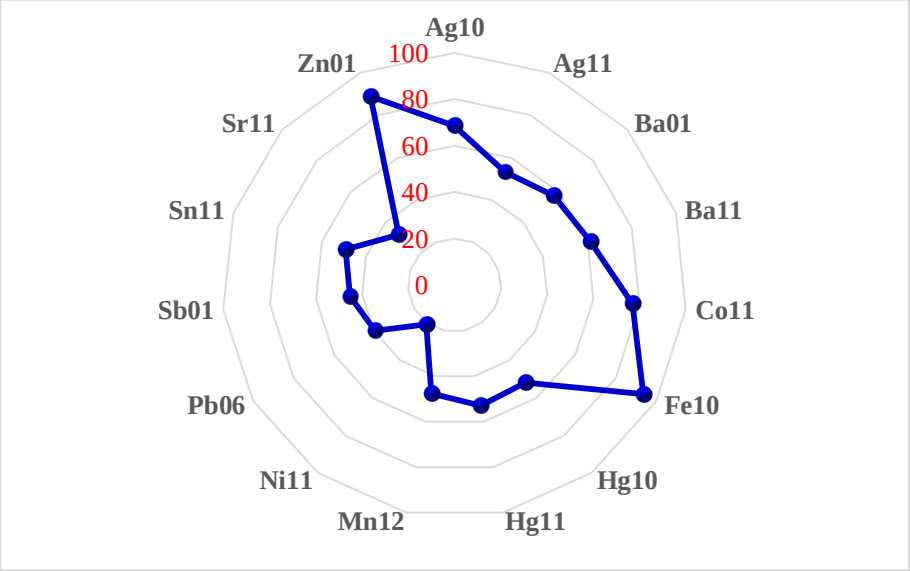

Figure 9. Gravimetric analysis result of % diesel biodegradation of the isolates

According to Figure 9, both Fe10 and Zn01 achieved diesel biodegradation ability over between 85-95% as a result of gravimetric analysis. Co11 and Ag10 achieved diesel biodegradation between 60-80% while other isolates had only around or lower than 50% diesel biodegradation ability.

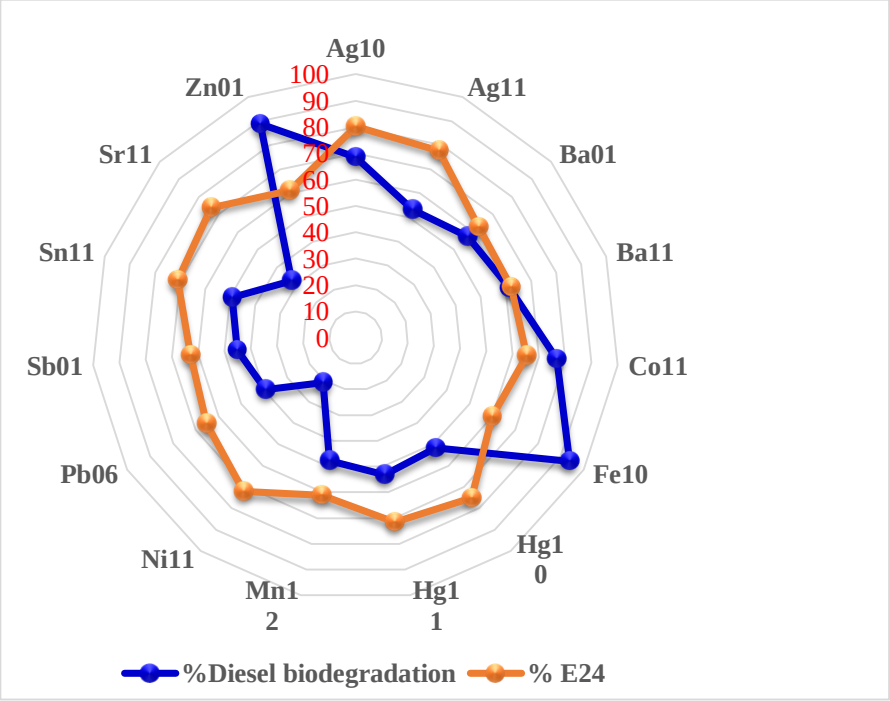

Figure 10. % diesel degradation and E_{24} for the selected isolates

As a result of gravimetric analysis, the isolates Fe10 and Zn01 was shown to have highest diesel biodegradation abilities with 93.7% and 88.9% respectively.

4.3.5.2. GC Analysis of Diesel Oil Degradation

GC analysis was performed for 14 days to observe the biodegradation capacity of the two isolates which were found to have the highest degradation capacities in gravimetric analysis. The dendogram of the GC analysis for both isolates Fe10 and Zn01 were shown in Figure 11 and 12, respectively.

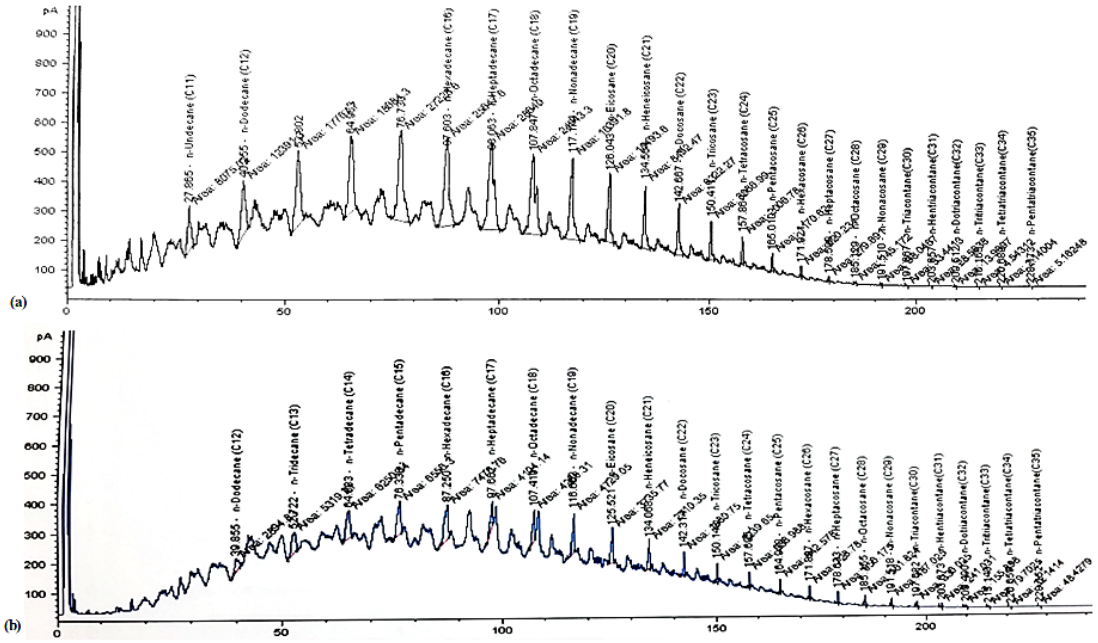

Figure 11. Dendograms of diesel oil biodegradation by the isolate Fe10; without inoculant as control **(a)**, with inoculant after 14 days incubation **(b)**

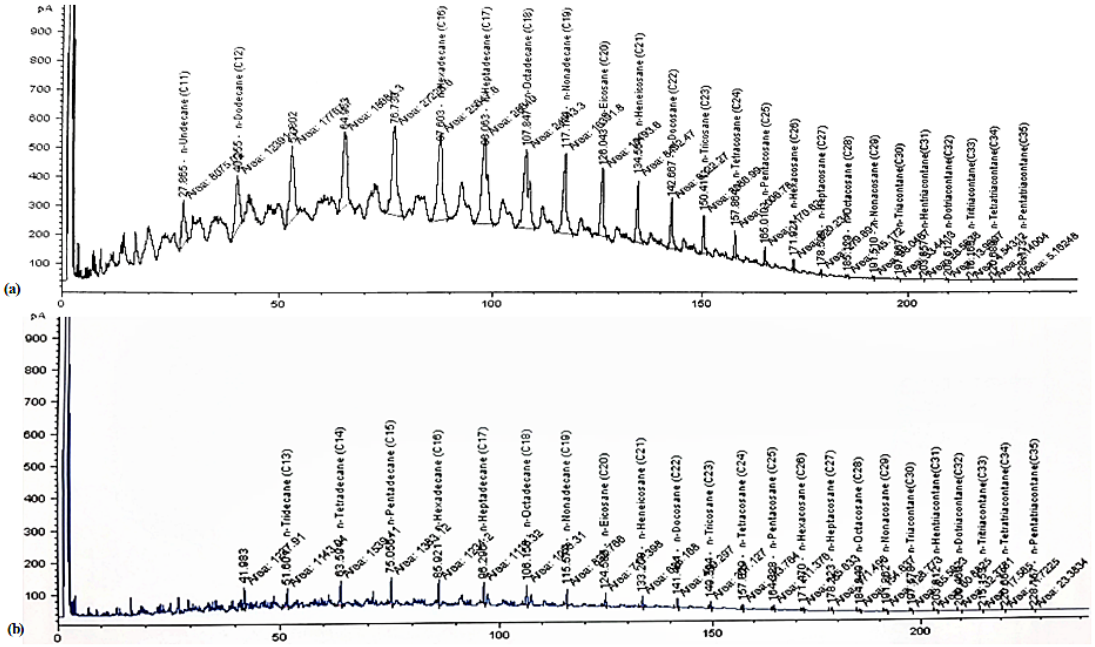

Figure 12. Dendograms of diesel oil biodegradation by the isolate Zn01; without inoculant as control **(a)**, with inoculant after 14 days incubation **(b)**

The details of diesel oil degradation analysis results of the isolates for 14 days was summarized in Table 9.

Table 9. Biodegradation of diesel oil by the isolates Fe10 and Zn01 after 14 days incubation

Hydrocarbons	Control (diesel + medium)			Fe10			Zn01		
	Retention time (min)	Area (pA*s)	Amount (ppm)	Retention time (min)	Area (pA*s)	Amount (ppm)	Retention time (min)	Area (pA*s)	Amount (ppm)
n-Pentane (C5)	1.51	-	-	1.51	-	-	1.51	-	-
n-Hexane (C6)	1.93	-	-	1.93	-	-	1.93	-	-
n-Heptane (C7)	3.24	-	-	3.24	-	-	3.24	-	-
n-Octane (C8)	6.53	-	-	6.53	-	-	6.53	-	-
n-Nonane (C9)	14.21	-	-	14.21	-	-	14.21	-	-
n-Decane (C10)	17.77	-	-	17.77	-	-	17.77	-	-
n-Undecane (C11)	27.86	8075.02	474.58	28.03	-	-	28.03	-	-
n-Dodecane (C12)	40.25	$1.24*10^4$	705.38	39.85	2894.83	164.79	39.84	-	-
n-Tridecane (C13)	52.00	-	-	52.72	5319.21	313.30	56.60	1143.04	67.33
n-Tetradecane (C14)	63.92	-	-	63.88	6250.84	357.48	63.59	1539.41	88.04
n-Pentadecane (C15)	75.39	-	-	76.33	6556.29	372.35	75.06	1383.12	78.55
n-Hexadecane (C16)	87.60	$2.56*10^4$	1446.34	87.25	7476.76	421.64	85.92	1234.20	69.60
n-Heptadecane (C17)	98.06	$2.86*10^4$	1637.09	97.66	4104.14	234.59	96.29	1117.32	63.87
n-Octadecane (C18)	107.84	$2.45*10^4$	1359.06	107.42	4286.31	243.64	106.155	1056.31	60.04
n-Nonadecane (C19)	117.17	$1.64*10^4$	933.25	116.66	4729.05	269.25	115.57	822.77	46.84
n-Eicosane (C20)	126.04	$1.05*10^4$	597.70	125.52	3735.77	212.78	124.57	717.39	40.86
n-Heneicosane (C21)	134.56	8452.47	487.43	134.07	2710.35	156.29	133.21	628.17	36.22
n-Docosane (C22)	142.66	6322.27	365.07	142.31	1980.75	114.38	141.46	489.29	28.25
n-Tricosane (C23)	150.41	3986.99	229.10	150.15	1239.65	71.23	149.39	307.13	17.65
n-Tetracosane (C24)	157.86	2006.78	114.30	157.70	938.99	53.48	157.03	243.76	13.88
n-Pentacosane (C25)	165.01	1170.62	66.84	164.93	832.58	48.11	164.38	211.38	12.07
n-Hexacosane (C26)	171.92	620.23	34.55	171.89	728.78	40.59	171.47	185.63	10.34
n-Heptacosane (C27)	178.59	279.69	16.42	178.63	656.18	38.53	178.32	171.49	10.07
n-Octacosane (C28)	185.12	145.17	8.08	185.16	561.82	31.27	184.95	154.64	8.61
n-Nonacosane (C29)	191.51	98.05	5.61	191.52	487.03	27.89	191.36	128.77	7.37
n-Triacontane (C30)	197.66	53.44	2.95	197.68	336.03	18.52	197.58	85.56	4.72
n-Hentriacontane (C31)	203.65	28.56	1.55	203.67	241.03	13.04	203.61	60.88	3.29
n-Dotriacontane (C32)	209.51	13.99	$7.80*10^{-1}$	209.49	155.89	8.69	209.47	32.38	1.80
n-Tritriacontane (C33)	215.16	4.54	$2.45*10^{-1}$	215.15	79.70	4.30	215.15	17.56	$9.48*10^{-1}$
n-Tetratriacontane (C34)	220.68	2.14	$1.15*10^{-1}$	220.66	32.14	1.72	220.66	11.72	$6.28*10^{-1}$
n-Pentatriacontane (C35)	228.17	5.16	$2.91*10^{-1}$	228.15	48.43	2.73	228.157	23.38	1.32
Totals:			**8522.72**			**3320.61**			**672.31**

Depending on the preliminary results, effectively diesel oil degrading isolates Fe10 and Zn01 were further subjected to GC analysis for 14 days of incubation. Diesel degradation percentages of Fe10 and Zn01 were calculated by using the formula given in equation 5. After 14 days Zn01 achieved 92.11% degradation whereas Fe10 achieved only 61.04%. Even though there were no hydrocarbon species with C_{13}-C_{15} in the control flask, these hydrocarbons were found in both flasks after 14 days incubation. The reason for this is, isolates Fe10 and Zn01 produced these hydrocarbons due to the degradation of hydrocarbons with higher carbon numbers. Also, obtaining higher or lower concentrations can be due to the measurement errors of the GC instrument.

Bihari et al. (2007) explained among different Gram-positive and Gram-negative bacteria which are applied at industrial scale, especially *Acinetobacter* genus are capable of degrading long change n-alkanes. *Aeromonas* sp., *Alcaligenes paradoxus, B. licheniformis* and *P. florescens* were determined as diesel degrading bacteria in the presence of 0.5% (v/v) diesel oil as Kayode-Isola et al. (2008) found. Gopalakrishnan (2014) found that *Acinetobacter* species degraded 77% of the diesel oil after 15 days of incubation. These results were also confirmed by the study done Nilesh (2013) who found that *Acinetobacter* species degraded 50% of the diesel after 20 days.

4.3.6. Identification and Characterization of Diesel Oil Degrading Bacteria

Catabolic genes and catabolic enzymes responsible for diesel oil degradation were identified by using polymerase chain reaction (PCR) analysis and SDS-PAGE analysis respectively. Identification and characterization of diesel oil degrading bacteria was done by 16S rRNA sequencing.

4.3.6.1. Polymerase Chain Reaction (PCR) Analysis of the Catabolic Genes for Diesel Oil Degradation

PCR analysis was conducted to check the presence of the both catabolic genes *C23O* and *alk*B for diesel oil degradation. Results of the analyses for *C23*O and *alk*B were

given in the following parts. PCR analysis was applied to 15 isolates efficient in biosurfactant production. A standard curve was constructed for each gel amplification of *alk*B and *C23*O genes using the molecular weights of standard markers and the distance they migrated in the gel. The standard curve was used to calculate the molecular weight of each gene detected in the surface water isolates. Figure 15 illustrates the standard curve constructed for molecular weight estimation for the amplified *alk*B and *C23*O genes.

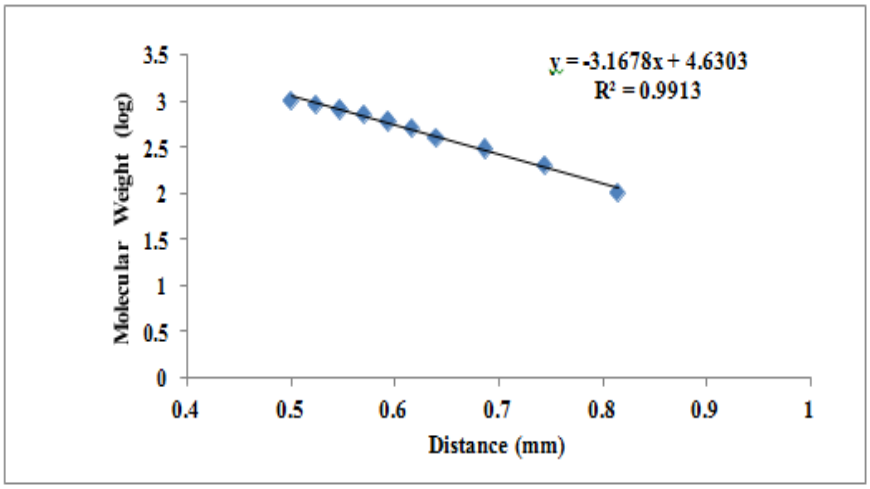

Figure 13. Standard curve for molecular weight estimation of the amplified *alkB* and *C23O* genes

4.3.6.1.1. PCR Analysis of *C23*O

Results of PCR analysis of *C23*O gene is given in Figure 14 and 15.

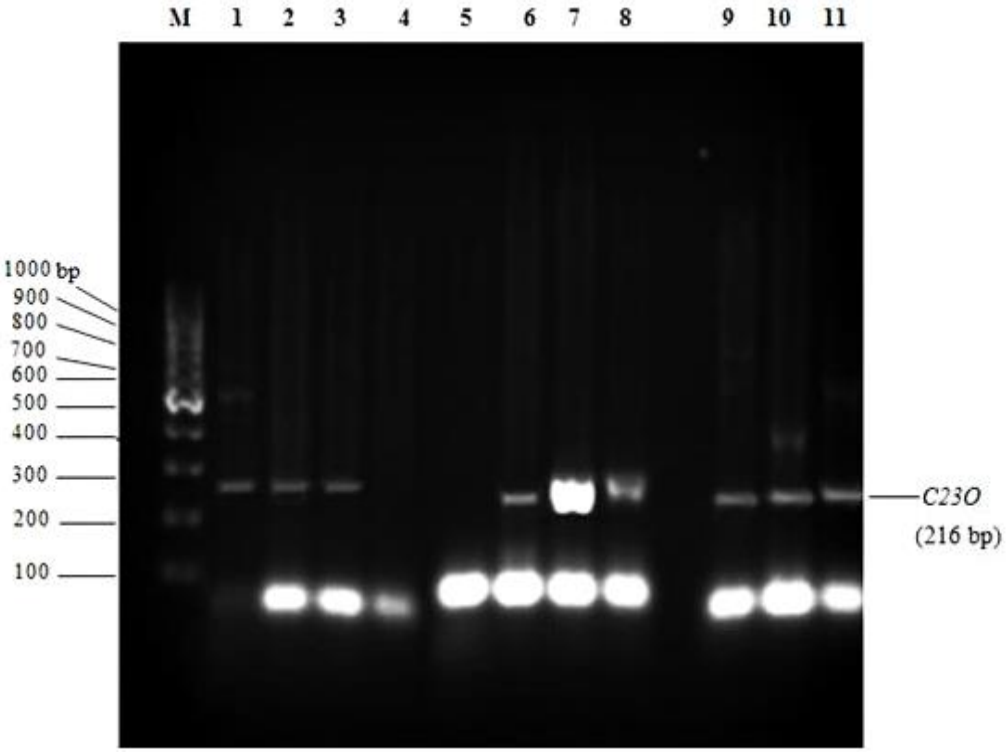

Figure 14. PCR analysis of *C23*O gene for the isolates: Ag10 (1); Ag11 (2); Ba01 (3); Ba11 (4); Co11 (5); Hg10 (6); Hg11 (7); Ni11 (8); Pb06 (9); Sn11 (10); Sr11 (11); M, marker gen ruler 100 bp.

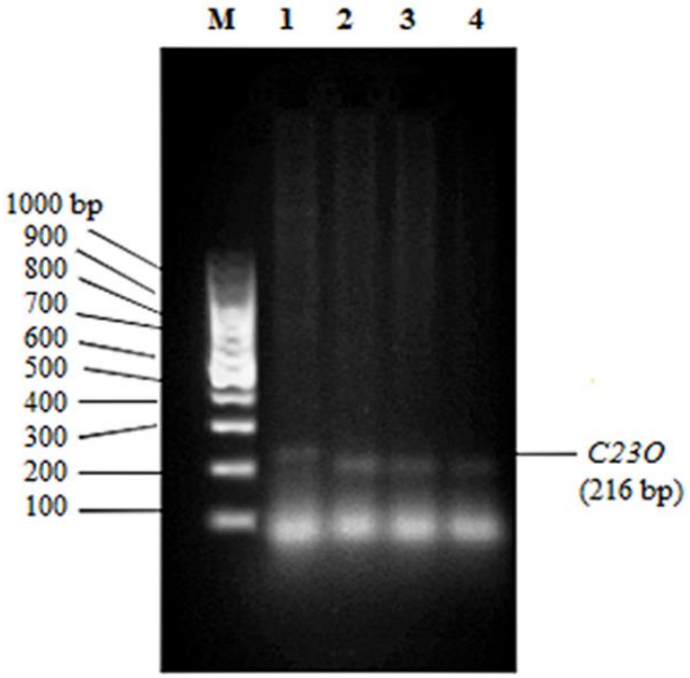

Figure 15. PCR analysis of *C23*O gene for different the isolates: Fe10 (1); Mn12 (2); Sb01 (3); Zn01 (1); M, marker gen ruler 100 bp.

4.3.6.1.2. PCR Analysis of *alk*B

PCR analysis results for alkB gene are given in Figure 16 and 17.

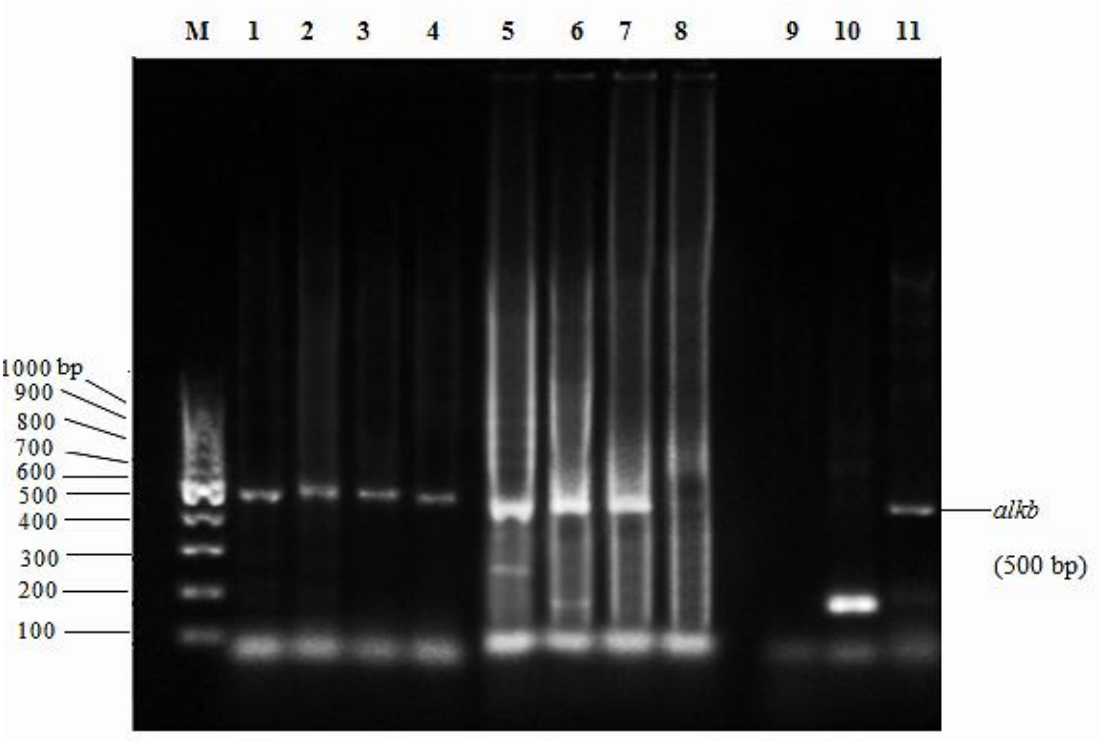

Figure 16. PCR analysis of *alk*B gene for the isolates: Ag10 (1); Ag11 (2); Ba01 (3); Ba11 (4); Co11 (5); Hg10 (6); Hg11 (7); Ni11 (8); Pb06 (9); Sn11 (10); Sr11 (11); M, marker gen ruler 100 bp.

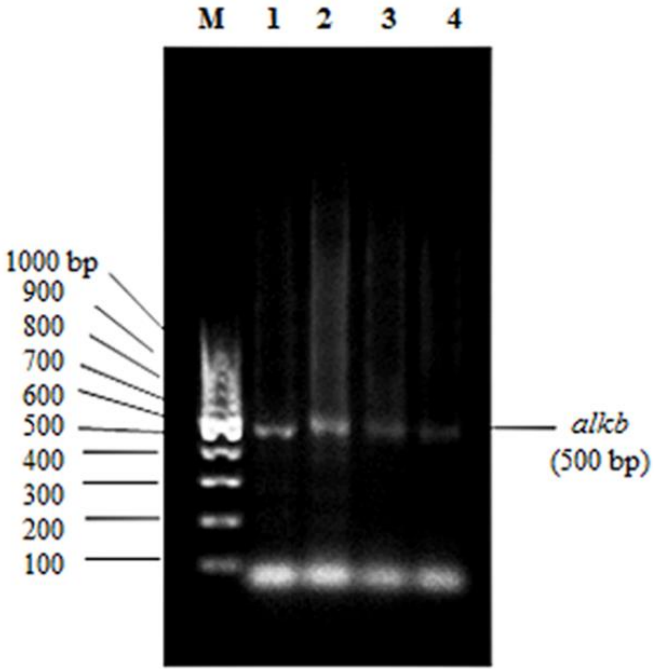

Figure 17. PCR analysis of *alk*B gene for different the isolates: Fe10 (1); Mn12 (2); Sb01 (3); Zn01 (4); M, marker gen ruler 100 bp.

Jyothi (2012) and Dussán (2012) showed that, the genes *C23*O encoding catechol 2, 3-dioxygenase and *alk*B encoded alkane monooxygenase are the catabolic genes responsible for the hydrocarbon degradation. Presence of these genes is an indicator for the ability of the isolates to degrade hydrocarbons. PCR products of *C23*O amplicons were observed between 200-300 bp and PCR products of *alk*B amplicons were observed between 500-600 bp. According to Figure 14 and 15 all isolates except from Ba11 and Co11 were shown to harbor *C23*O gene. Also, Figure 16 and 17 showed that all isolates except from Pb06 and Sn11 were shown to harbor *alk*B gene.

4.3.6.2. SDS-PAGE Analysis of the Catabolic Enzymes

SDS-PAGE analysis was applied to 15 isolates efficient in biosurfactant production. A standard curve was constructed for each acrylamide gel using the molecular weights of standard marker and the distances migrated by each in the gel. Then the distance that each of the proteins migrated within the same gel was measured and

used to calculate the molecular weight of each protein bands on the gel. Figure 18 shows the standard curve for molecular weight estimation of proteins. Results of SDS-PAGE analysis are given in Figure 19, 20, 21, 22 and 23.

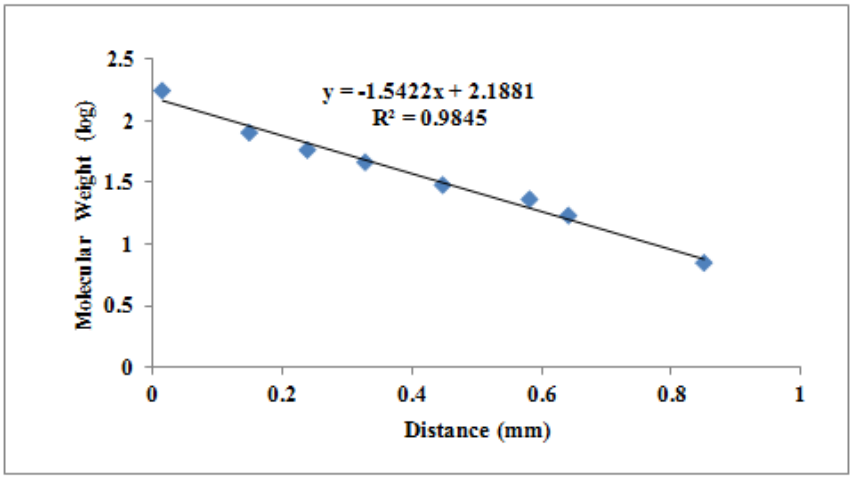

Figure 18. Standard curve for molecular weight estimation of proteins

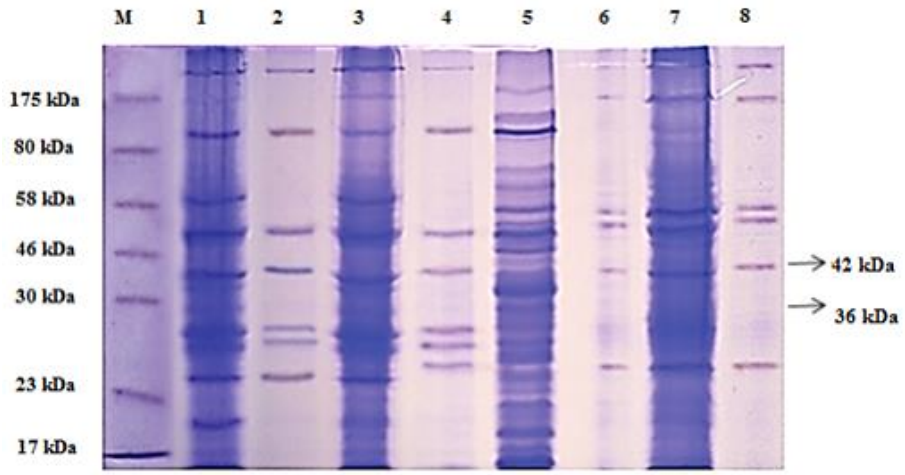

Figure 19. SDS-PAGE analysis of the isolates grown on nutrient broth without diesel (N) and Bushnell-Haas medium contained 1% filter sterilized diesel oil as sole source of carbon (D) positive control (1); negative control (2); Ag10 D (3); Ag10 N (4); Ag11 D (5); Ag11 N (6); Hg10 D (7); Hg10 N (8); M, Color Plus Prestained Protein Marker (New England Biolabs). Arrows indicate enzymes alkane monooxygenase (42 kDa) and catechol 2,3-dioxygenase (36 kDa), respectively.

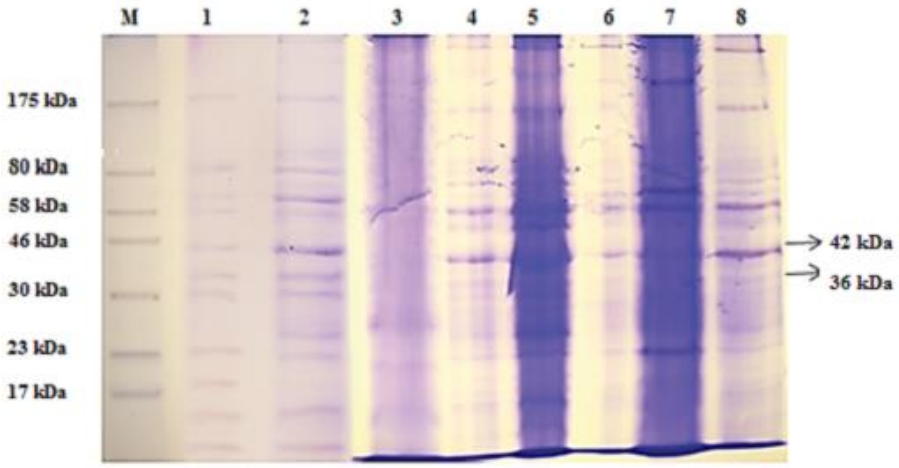

Figure 20. SDS-PAGE analysis of the isolates grown on nutrient broth without diesel (N) and Bushnell-Haas medium contained 1% filter sterilized diesel oil as sole source of carbon (D) positive control (1); negative control (2); Ba01 D (3); Ba01 N (4); Ba11 D (5); Ba11 N (6); Co11 D (7); Co11 N (8); M, Color Plus Prestained Protein Marker (New England Biolabs). Arrows indicate the locations of the enzymes alkane monooxygenase (42 kDa) and catechol 2,3-dioxygenase (36 kDa), respectively.

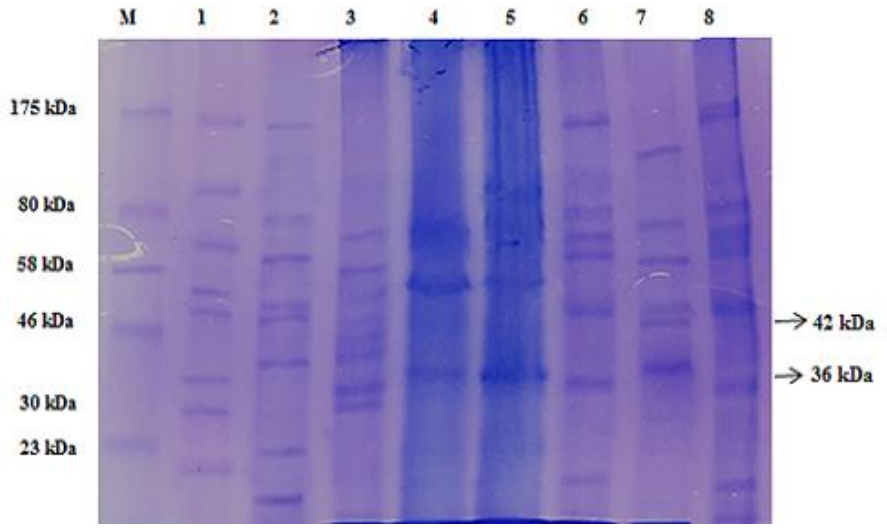

Figure 21. SDS-PAGE analysis of the isolates grown on nutrient broth without diesel (N) and Bushnell-Haas medium contained 1% filter sterilized diesel oil as sole source of carbon (D) positive control (1); negative control (2); Fe10 D (3); Fe10 N (4); Hg11 D (5); Hg11 N (6); Mn12 D (7); Mn12 N (8); M, Color Plus Prestained Protein Marker (New England Biolabs). Arrows indicate the locations of the enzymes alkane monooxygenase (42 kDa) and catechol 2,3-dioxygenase (36 kDa), respectively.

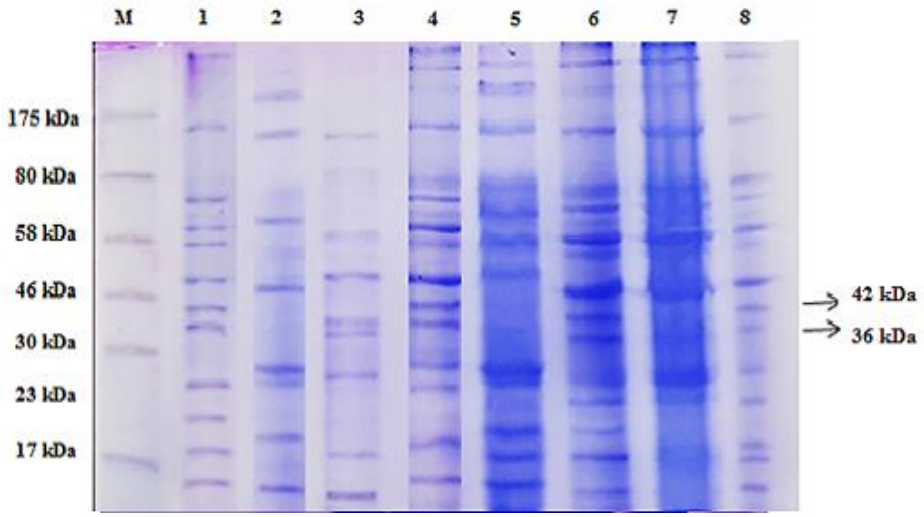

Figure 22. SDS-PAGE analysis of the isolates grown on nutrient broth without diesel (N) and Bushnell-Haas medium contained 1% filter sterilized diesel oil as sole source of carbon (D) positive control (1); negative control (2); Ni11 D (3); Ni11 N (4); Sn11 D (5); Sn11 N (6); Sr11 D (7); Sr11 N (8); M, Color Plus Prestained Protein Marker (New England Biolabs). Arrows indicate the locations of the enzymes alkane monooxygenase (42 kDa) and catechol 2,3-dioxygenase (36 kDa), respectively.

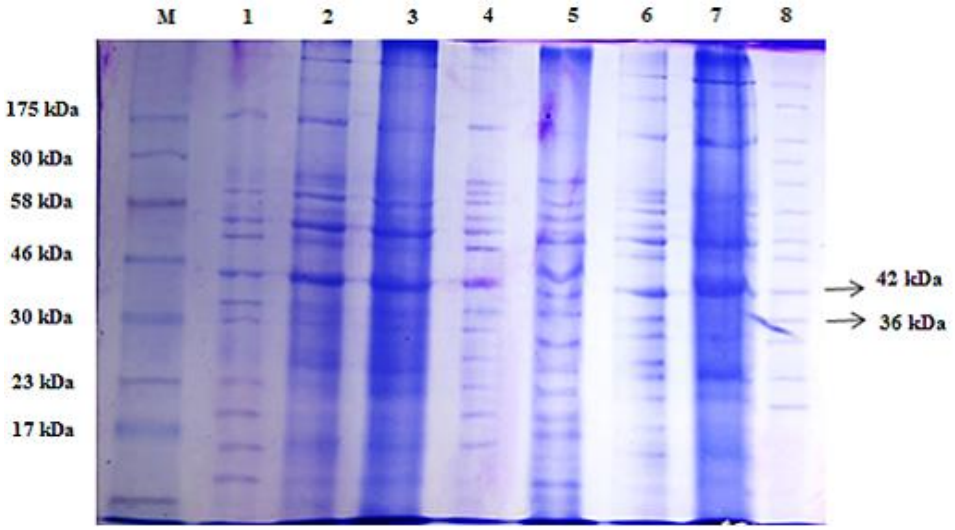

Figure 23. SDS-PAGE analysis of the isolates grown on nutrient broth without diesel (N) and Bushnell-Haas medium contained 1% filter sterilized diesel oil as sole source of carbon (D) positive control (1); negative control (2); Pb06 D (3); Pb06 N (4); Sb01 D (5); Sb01 N (6); Zn01 D (7); Zn01 N (8); M, Color Plus Prestained Protein Marker (New England Biolabs). Arrows indicate the locations of the enzymes alkane monooxygenase (42 kDa) and catechol 2,3-dioxygenase (36 kDa), respectively.

SDS-PAGE analysis of the isolates showed the expression of the enzymes, alkane monooxygenase and catechol 2, 3-dioxygenase in Figure 19, 20, 21, 22 and 23. Catechol 2, 3-dioxygenase is a poly peptide, containing 307 amino acid residues with the molecular weight of it is approximately 36 kDa (Yun Jiang, 2004). Alkane monooxygenase contains 383 amino acids with molecular weight of approximately 42 kDa (Liu et al., 2014). Even though isolates Ba11 and Co11 were not able to harbor the gene *C23*O and Pb06 and Sn11 were not able to harbor the gene *alk*B according to PCR analysis, the proteins encoded by both genes were shown to be expressed in all isolates tested.

4.3.6.3. Identification of Diesel Oil Degraders by 16S rRNA Sequencing

16S rRNA sequencing was used to identify 15 isolates efficient in diesel oil degrading. The identification results are given in Table 10.

Table 10. 16S rRNA sequencing results of the diesel oil degrading isolates

Strain designations	Sampling coordinates	16S rRNA identification (homology %)	EMBL access no	References
Ag10	39º48'38.97''N, 33º29'14.57''E, 684 m	*Pseudomonas plecoglossicida* (99 %)	KJ395363	Akbulut et al., 2014
Ag11	39º50'28.41''N, 33º28'02.13''E, 686 m	*Raoultella planticola* (99 %)	KJ395359	Koc et al., 2013
Ba01	39º22'16.39''N, 33°26'49.26''E, 890 m	*Staphylococcus aureus* subsp. (99 %)	KJ395371	Yilmaz et al., 2013
Ba11	39º50'28.41''N, 33º28'02.13''E, 686 m	*Stenotrophomonas rhizophila* (99 %)	KJ395362	Akbulut et al., 2014
Co11	39º50'28.41''N, 33º28'02.13''E, 686 m	*Staphylococcus warneri* (99 %)	KJ395373	Yilmaz et al., 2013
Fe10	39º48'38.97''N, 33º29'14.57''E, 684 m	*Acinetobacter calcoaceticus* (99 %)	KJ395366	Akbulut et al., 2013
Hg10	39º48'38.97''N, 33º29'14.57''E, 684 m	*Pseudomonas koreensis* (96 %)	KJ395377	Akbulut et al., 2014
Hg11	39º50'28.41''N, 33º28'02.13''E, 686 m	*Pseudomonas koreensis* (98 %)	KJ395378	Akbulut et al., 2014
Mn12	39º57'22.98''N, 33º25'04.35''E, 679 m	*Acinetobacter haemolyticus* (99 %)	KJ395367	Akbulut et al., 2013
Ni11	39º50'28.41''N, 33º28'02.13''E, 686 m	*Comamonas testosteroni* (99 %)	KJ395372	Akbulut et al., 2014
Pb06	39º34'34.39''N, 33º26'11.61''E, 763 m	*Enterococcus faecalis* (98 %)	KJ395380	Aktan et al., 2013
Sb01	39º22'16.39''N, 33°26'49.26''E, 890 m	*Acinetobacter johnsonii* (97 %)	KJ395376	Akbulut et al., 2013
Sn11	39º50'28.41''N, 33º28'02.13''E, 686 m	*Pantoea agglomerans* (98 %)	KJ395361	Cerit et al., 2013
Sr11	39º50'28.41''N, 33º28'02.13''E, 686 m	*Micrococcus luteus* (99 %)	KJ395375	Kabatas et al., 2013
Zn01	39º22'16.39''N, 33°26'49.26''E, 890 m	*Acinetobacter haemolyticus* (99 %)	KJ395368	Akbulut et al., 2013

EMBL, European Molecular Biology

According to the Table 10, Fe10 was identified as *Acinetobacter calcoaceticus* and Zn01 was identified as *Acinetobacter haemolyticus* with 99% homologies.

4.4.Characterization of Biosurfactants Produced by Diesel Oil Degraders

Characterization of biosurfactants produced by efficient diesel oil degraders was done by using physicochemical and biochemical and structural characterization methods.

4.4.1. Physicochemical Characterization

Physicochemical characterization of biosurfactants produced by diesel oil degraders was done by surface tension measurement.

Surface Tension Measurement

Surface tension is defined as a property of the surface of a liquid which causes it to behave as an elastic sheet (Varjani et al., 2014) Anandaraj et al. (2010), defined the ability to reduce surface tension as a major characteristic of surfactant. Surface tension measurement was done to determine the reduction in surface tension in different samples. Surface tension of water was measured as 72.80 mN/m. Surface tension reduction for the samples were calculated by using the equation 6. Results for the surfactants SDS, Tween 20 and biosurfactants were given in Figure 24.

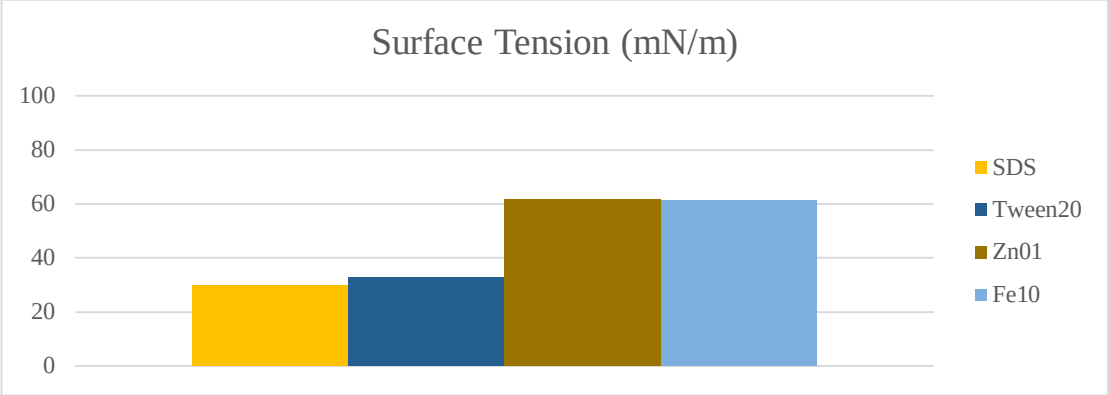

Figure 24. Surface tension measurement of water samples with different surfactants

According to Figure 24 a significant drop in surface tension was observed due to the presence of SDS and Tween20. For the isolates surface tension reductions were calculated by using formula given in equation 6. For the isolate Fe10 and Zn01 reduction values were obtained as 15.8% and 15.1% respectively.

4.4.2. Biochemical and Structural Characterization

As well as being characterized by physicochemical methods, biosurfactants of diesel degraders were also characterized by means of biochemical and structural methods of Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy (SEM), zeta potential measurement and gas chromatography-mass spectrometry (GC-MS).

4.4.2.1. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectroscopy is an approach which is based on the determination of the interaction between a sample and UV irradiation. It measures the frequencies the sample absorbs and the intensities of the absorptions. Frequencies obtained with FTIR analysis are helpful for the identification of the chemical structure of the sample which is due to the chemical functional groups (Simonescu, 2012). Figure 25 shows the FTIR analysis results of biosurfactant produced by Fe10. According to the

peaks in Figure 26, Table 11 was tabulated to summarize the functional groups of the biosurfactant produced by the isolate Fe10.

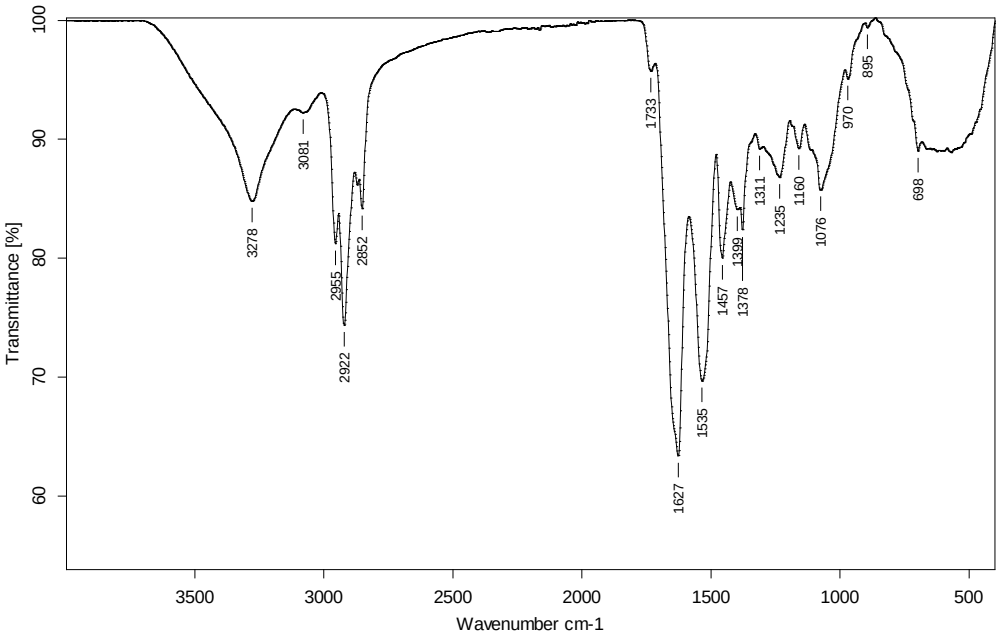

Figure 25. FTIR spectrum of the biosurfactant produced by the isolate Fe10

Table 11. FTIR analysis result of the biosurfactant produced by the isolate Fe10

Wave number (cm^{-1})	Vibration type	Functional groups	Reference
3278	Stretching Bending	NH_3 C-N	El-Shestawy et al., 2013
2955	Stretching	C-H	Jain et al., 2012
2922		C-H	Wijanarko et al., 2012
2852	Stretching	C-H	El-Shestawy et al., 2013
1733		C=O	Zou et al., 2014
1627		C=O	Jain et al., 2012
1535	Stretching Stretching	C=O N-H	Wijanarko et al., 2012 Zou et al., 2014
1457		CH_2	El-Shestawy et al., 2013
1399		$-CH_3$,$-CH_2$	El-Shestawy et al., 2013
1378		$-CH_3$, $-CH_2$	Wijanarko et al., 2012
1076		C-O	Jain et al., 2012

Figure 25 shows the FTIR analysis results of biosurfactant produced by Zn01. According to the peaks in Figure 25, Table 12 was tabulated to summarize the functional groups of the biosurfactant produced by the isolate Zn01.

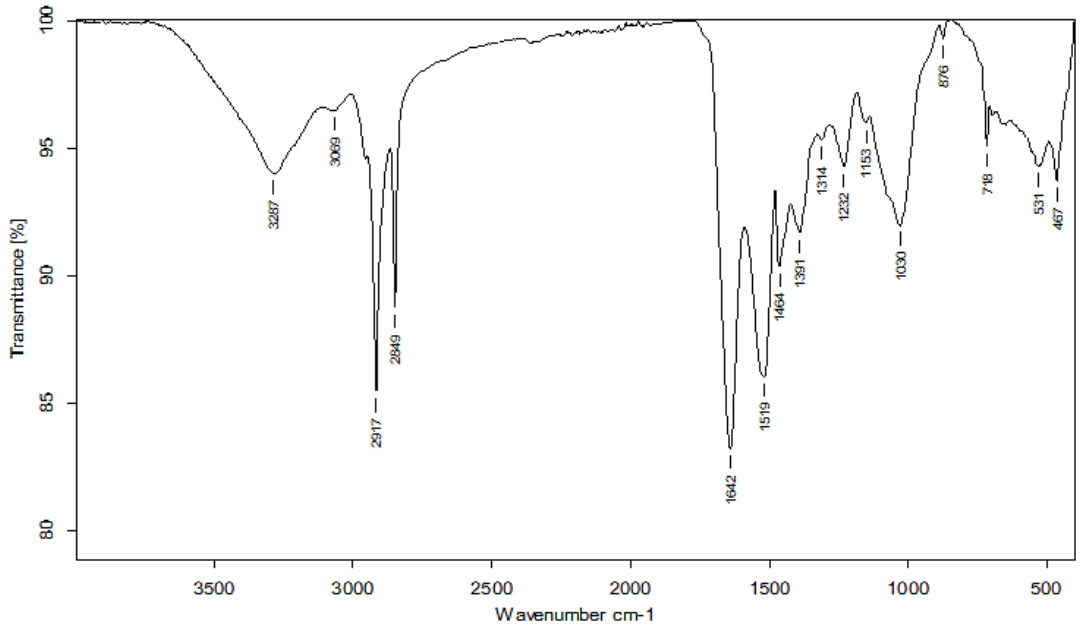

Figure 26. FTIR spectrum of the biosurfactant produced by the isolate Zn01

Table 12. FTIR analysis results of the biosurfactant produced by the isolate Zn01

Wave number (cm^{-1})	Vibration type	Functional groups	Reference
3287	Stretching Bending	NH_3 C-N	El-Shestawy et al., 2013
2917	Stretching	CH	El-Shestawy et al., 2013
2849	Stretching	CH	El-Shestawy et al., 2013
1464	Asymmetric stretching	CH	Mariashobana et al., 2014
1391		CH_3	El-Shestawy et al., 2013
1153		C-O	Jain et al., 2012
1030		C=O	Jain et al., 2012
531		C-I	Jain et al., 2012

FTIR analysis revealed that, the biosurfactant produced by the isolate Fe10 had peptides due to the presence of C-N bonds. Also presence of C=O bonds indicates ester structure. Therefore, it was concluded that Fe10 (*Acinetobacter calcoaceticus*) produced lipopeptide type biosurfactant. The biosurfactant produced by the isolate

Zn01 had peptides due to the presence of C-N bonds. Presence of C=C bonds indicated unsaturated fatty acids. Therefore, it was concluded that Zn01 (*Acinetobacter haemolyticus*) produces lipopeptide type biosurfactant.

As Bao et al. (2014) found, *Acinetobacter* sp. D3-2 produces lipopeptide type biosurfactant. Zou et al. (2014), also found by using FTIR analysis that biosurfactant produced by *Acinetobacter baylyi* is lipopeptide type biosurfactant.

4.4.2.2. Scanning Electron Microscopy (SEM)

Scanning electron microscopy is used to show biosurfactants or exopolymers produced by the microorganisms (Chandran, 2010). Both isolates Fe10 and Zn01 were investigated by using scanning electron microscopy (SEM) under x10000 magnification. SEM results of Fe10 and Zn01 are shown in Figure 27.

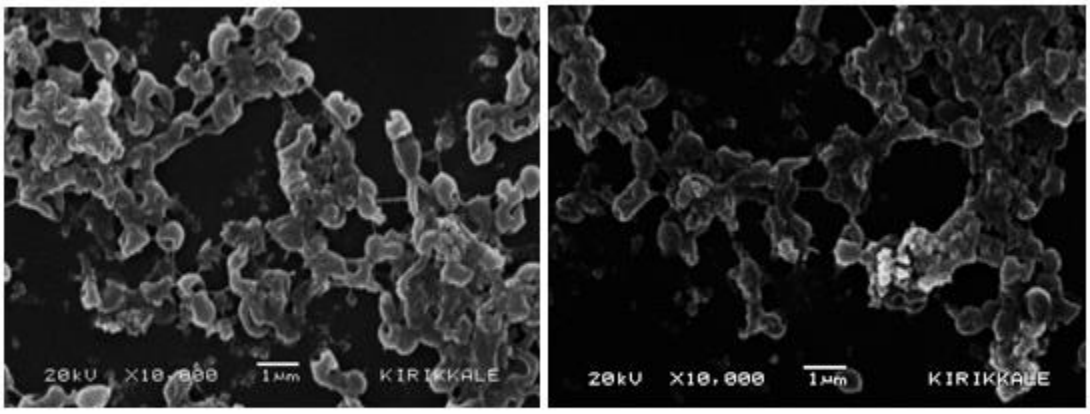

Figure 27. SEM analysis of the isolates Fe10 **(a)** Zn01 **(b)** (x10000 magnification)

Bacterial cells with diesel oil resulted in the formation of sticky colorless structure of exopolymers connecting the individual cells. The biosurfactant production and micelles structure formations by both isolates Fe10 and Zn01 were well observed in SEM pictures (Figure 27).

4.4.2.3. Measurement of Zeta Potential

Zeta potential is described as the electrostatic potential of a particle near its surface (Colloidal Dynamics, 1999). Zeta potential measurements were done by using the inoculants of both isolates Fe10 and Zn01 separately, diesel oil and diesel oil plus biosurfactant from the corresponding isolates. The results were given in Figure 28 and 29 for the isolates Fe10 and Zn01 respectively.

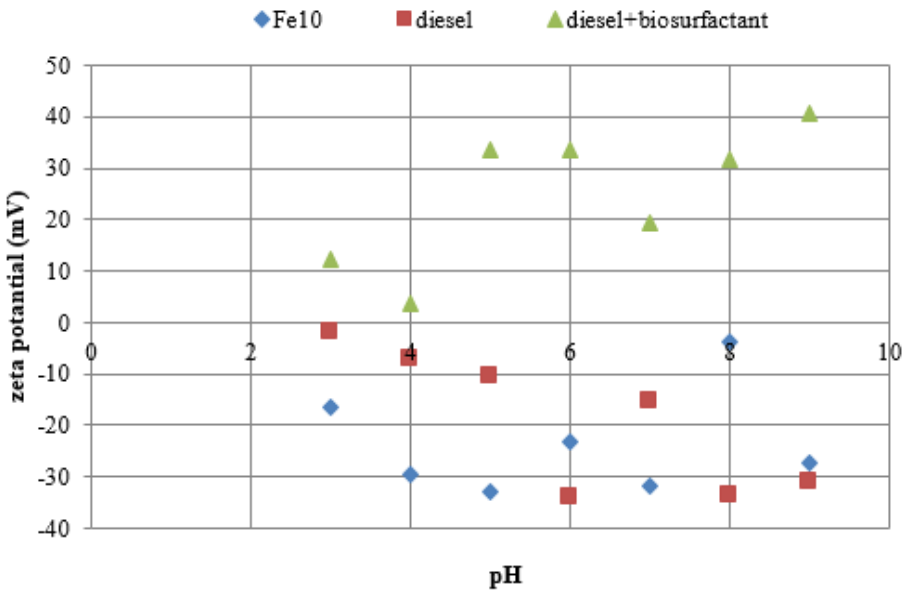

Figure 28. Zeta potential measurement for the isolate Fe10

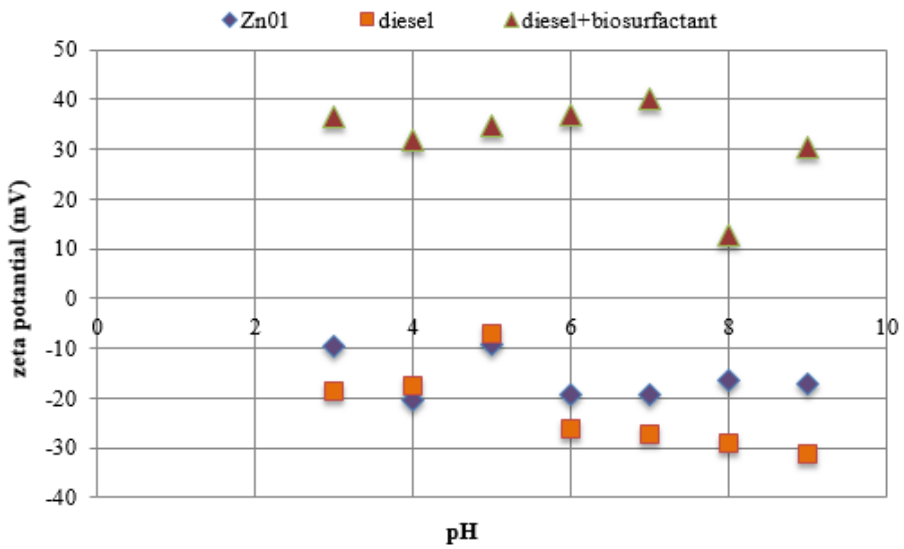

Figure 29. Zeta potential measurement for the isolate Zn01

As Chandran (2010) stated, mostly all of the microbial cells are negatively charged at neutral pH. Here in Figures 28 and 28, both Fe10 and Zn01 showed negative zeta potential values at all pH values in the range. According to the both Figures 28 and 29; diesel oil gave negative zeta potential values in all pH values tested. However, when extracted biosurfactant from each isolate and diesel were mixed positive zeta potential values at all pH values tested were observed due to emulsification of diesel oil by biosurfactant. According to the Figure 28, although the biosurfactant produced by the isolate Fe10 showed better activity around the pH values 5-9, the activity was poorer around the pH values of 3-4. According to the Figure 29, although the biosurfactant produced by the isolate Zn01 showed better activity around the pH values 3-7, the activity was poorer around the pH values of 8. These results demonstrated that biosurfactants are involved in the surface charge modification of diesel and enhance the adsorption of diesel to the bacterial cell surface.

4.5.Monitoring of *alk*B and *C23*O-Harboring Isolates in Surface Waters by Fluorescent *in situ* Hybridization (FISH) Method

The paraformaldehyde-fixed water samples collected from hydrocarbon contaminated river water were also checked for monitoring of prevalence of *alk*B

and *C23*O harboring bacteria over the year 2012. The probes EUB338 (Bact338), EUB338 II (SBACT P 338) and EUB338 III (SBACT V 338) were used to identify the domain Bacteria (Amman et al., 1990; Daims et al., 1999). To detect non-specific binding of EUB338, probe NONEUB (NON338) (non-Bact338) was used as a negative control (Wallner et al., 1993). *E. coli* DH5α strain was also used as a negative control for the stringency check of each species specific probes during all FISH applications in whole FISH procedure. All probes were 5′ end FITC-labeled (Alpha DNA, Montreal, Canada).The probes *alk*B and *C23*O (Kloos et al., 2006 and Sei 1999) were applied to *alk*B and *C23*O harboring pure cultures of *A. haemolyticus* at 45, 50 and 55% formamide concentrations. The visualization results of *alkB* and *C23O* harboring pure cultures of *A. haemolyticus* hybridizing with *alkB* and *C23O* probes were shown in Figure 30 and 31, respectively. As shown in Table 13, the percentages of hybridization intensities for *alk*B and *C23*O harboring pure cultures of *A. haemolyticus* hybridizing with *alk*B and *C23*O probes at 45, 50 and 55% formamide concentration were estimated by calculating the pixel areas (pp^2) of DAPI and FITC images of this strain by using Leica QWin Plus software. After analyses, the highest percentage hybridization intensities for *alk*B and *C23*O probes were obtained as 84.6±1.46 and 81.0±1.97 at 55% formamide and 0.02 M NaCl concentrations, respectively (Table 13). As a negative control, *alkB* and *C23O* probes were also applied to *alk*B and *C23*O-negative pure cultures of *E. coli* DH5α at the same conditions. The visualizations of *alk*B and *C23*O-negative pure cultures of *E. coli* DH5α applied with *alk*B and *C23*O probes at 45, 50, and 55% formamide concentrations were shown in Figure 32 and 33. Percentage of signal intensities of these hybridizations was also calculated (Table 13). The result demonstrated that the percentage of hybridization intensity of both probes with negative controls was the lowest as given in Table 13.

Table 13. Optimization of hybridization stringency conditions for *alk*B and *C23*O probes

Measured % of signal intensities at given hybridization conditions for each probes used						
	45% Formamide + 0.040 M NaCl		**50% Formamide + 0.028 M NaCl**		**55% Formamide + 0.020 M NaCl**	
	***alk*B**	***C23*O**	***alk*B**	***C23*O**	***alk*B**	***C23*O**
A. haemolyticus	60.9±1.76	33.6±1.54	70.3±1.65	73.7±1.16	84.6±1.46	81.0±1.97
***E. coli* DH5α**	1.79±0.28	1.87±0.54	3.37±1.84	2.5%±0.36	3.67±0.75	3.15±0.81

±, calculated standard errors

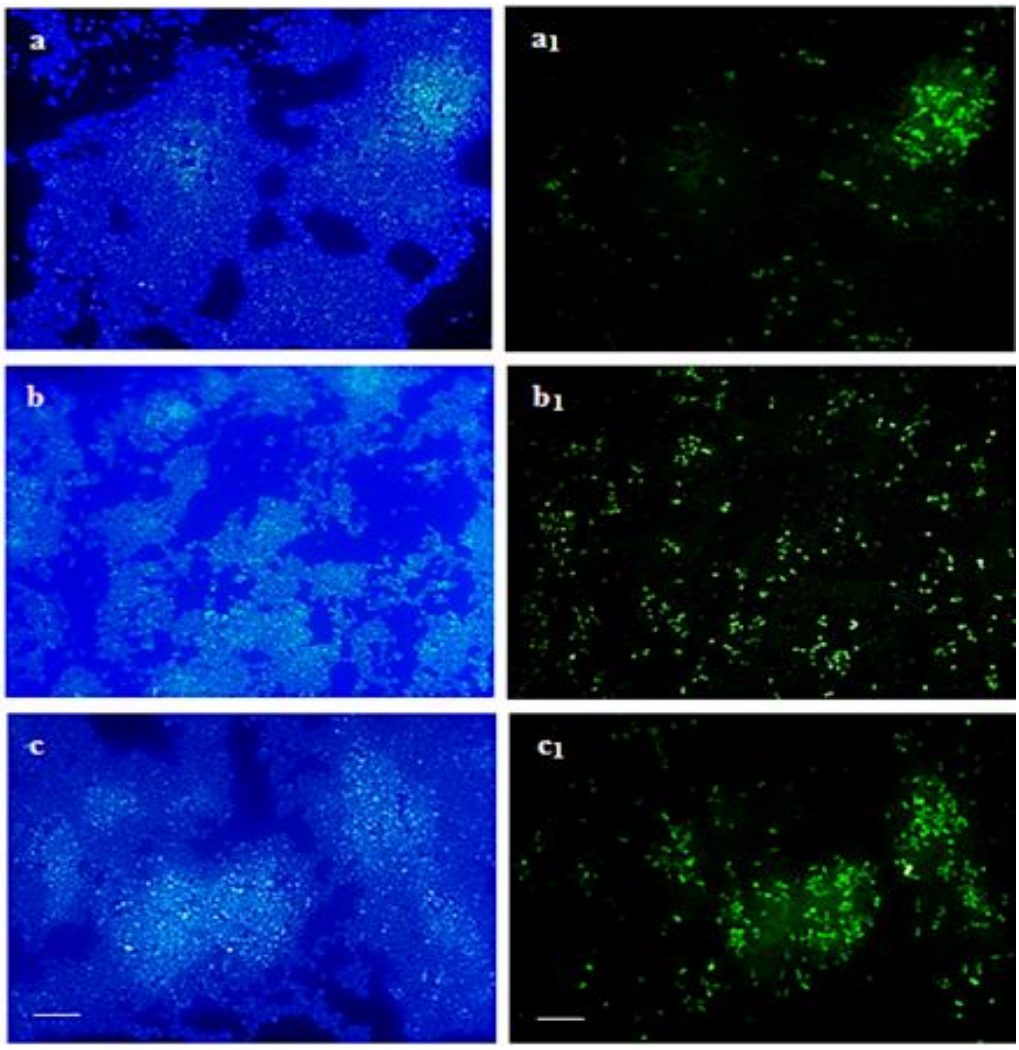

Figure 30. Hybridization stringency for *alk*B harboring pure cultures of *A. haemolyticus* at 45% formamide and 0.040 M NaCl (a-a_1), 50% formamide and 0.028 M NaCl (b-b_1), and 55% formamide and 0.020 M NaCl (c-c_1) concentrations. Total cell populations stained with DAPI (a,b,c) and their corresponding *alk*B probe applied pure cultures (a_1,b_1,c_1). Bar 10 µm and applies to all photomicrographs**.**

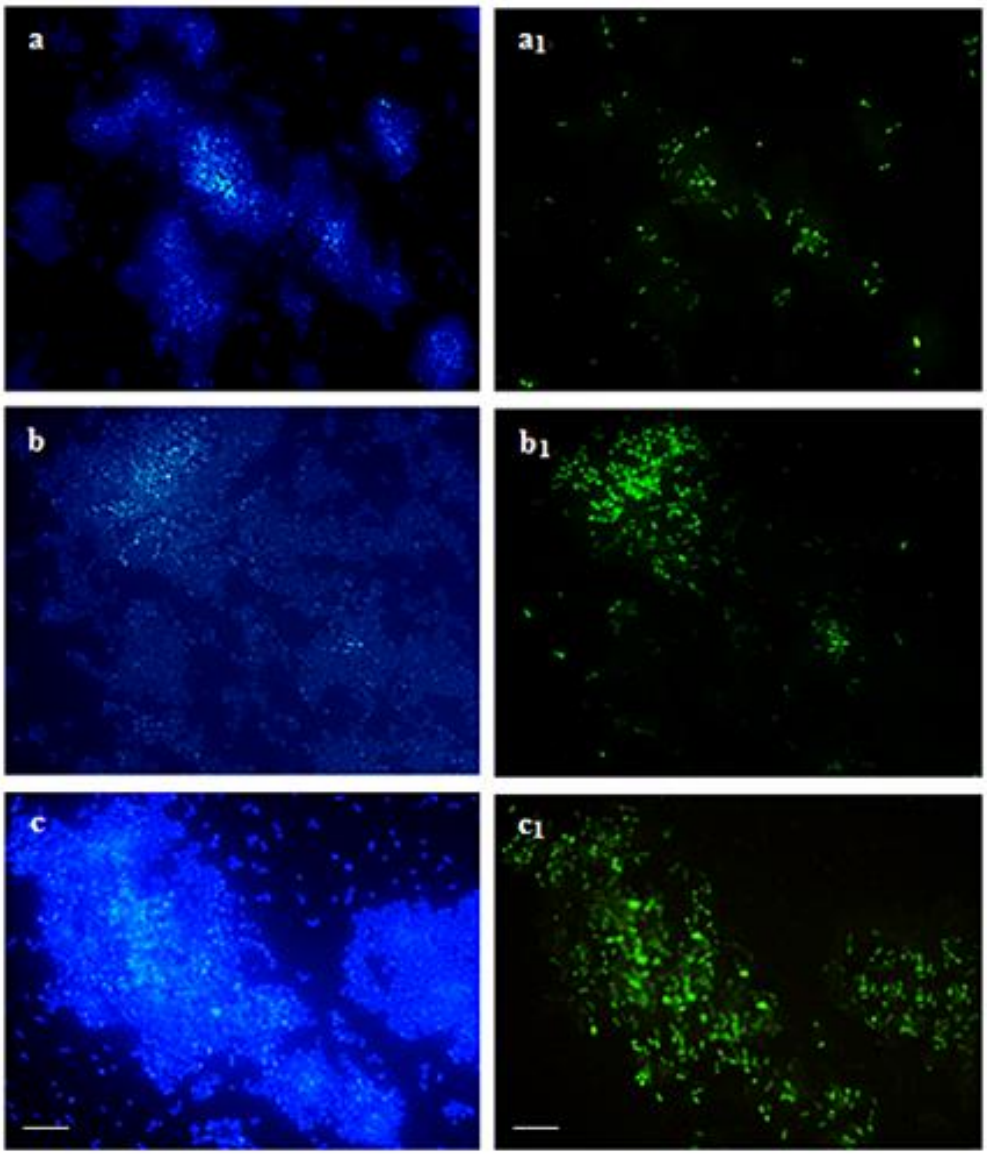

Figure 31. Hybridization stringency for *C23*O harboring pure cultures of *A. haemolyticus* at 45% formamide and 0.040 M NaCl (a-a_1), 50% formamide and 0.028 M NaCl (b-b_1), and 55% formamide and 0.020 M NaCl (c-c_1) concentrations. Total cell populations stained with DAPI (a,b,c) and their corresponding *C23*O probe applied pure cultures (a_1,b_1,c_1). Bar 10 µm and applies to all photomicrographs.

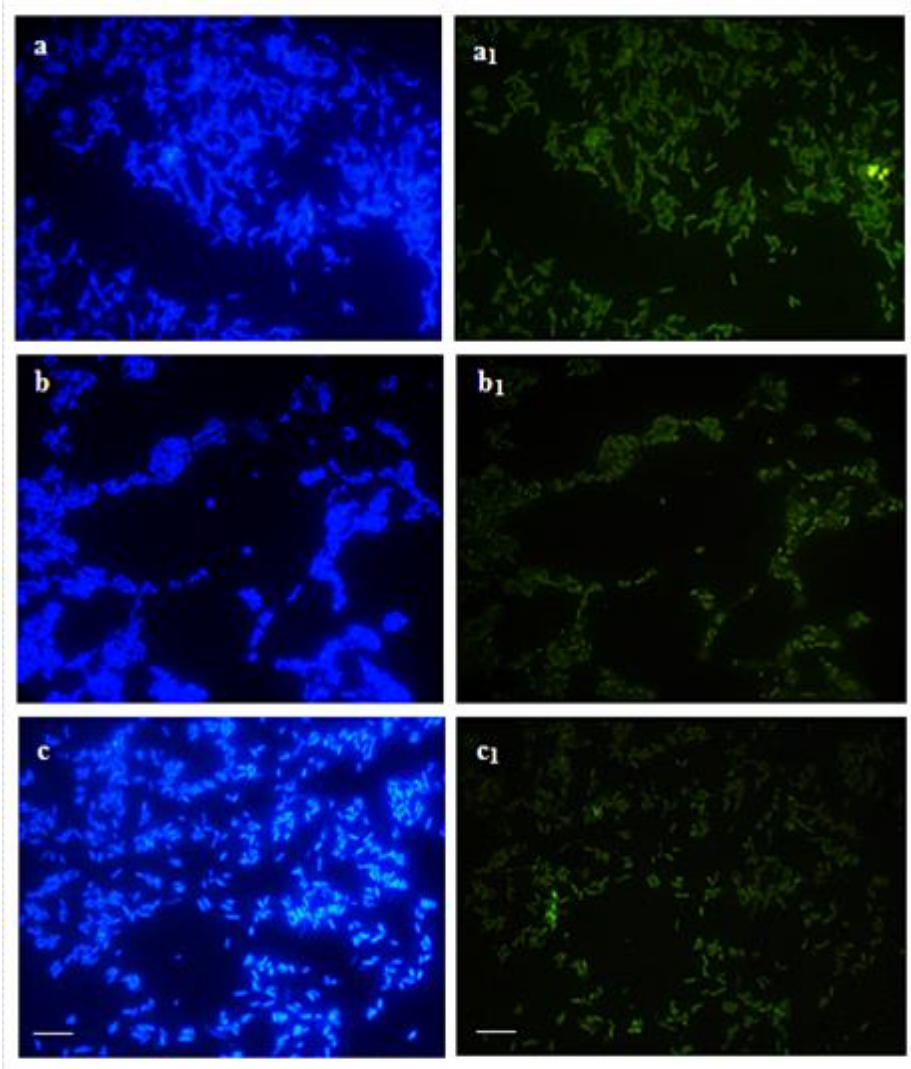

Figure 32. Hybridization results of *alk*B-negative pure cultures of *E. coli* DH5α at 45% formamide and 0.040 M NaCl (a-a_1), 50% formamide and 0.028 M NaCl (b-b_1), and 55% formamide and 0.020 M NaCl (c-c_1) concentrations. Total cell populations stained with DAPI (a, b, c) and their corresponding *alk*B probe applied pure cultures (a_1, b_1, c_1). Bar 10 µm and applies to all photomicrographs.

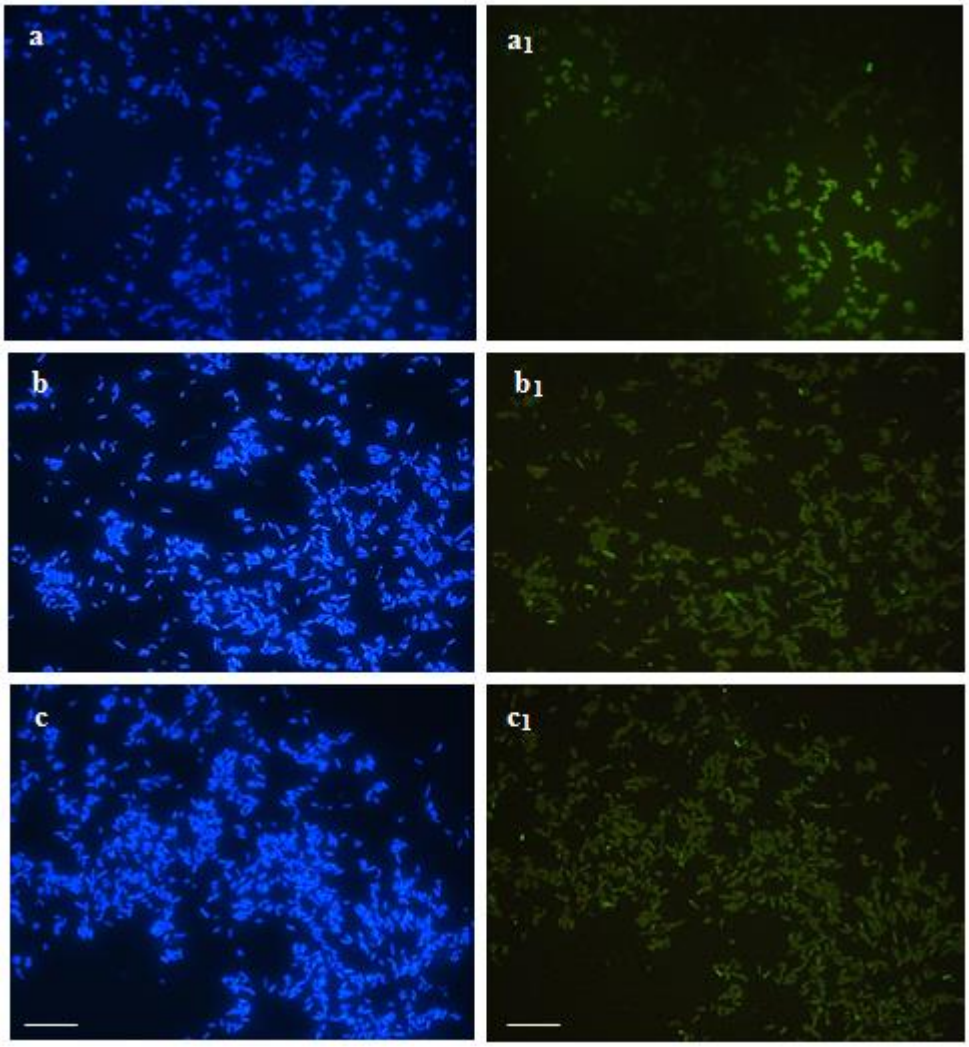

Figure 33. Hybridization results of *C23*O-negative pure cultures of *E. coli* DH5α at 45% formamide and 0.040 M NaCl (a-a_1), 50% formamide and 0.028 M NaCl (b-b_1), and 55% formamide and 0.020 M NaCl (c-c_1) concentrations. Total cell populations stained with DAPI (a, b, c) and their corresponding *C23*O probe applied pure cultures (a_1, b_1, c_1). Bar 10 µm and applies to all photomicrographs.

The optimal hybridization stringency conditions for *alk*B and *C23*O probes were determined as described previously. The fixed water samples collected seasonally over the year of 2012 were treated by *alk*B and *C23*O probes under previously determined optimal hybridization stringency condition. The hybridization results of seasonal water samples over the year of 2012 were shown in Figure 34 and 35.

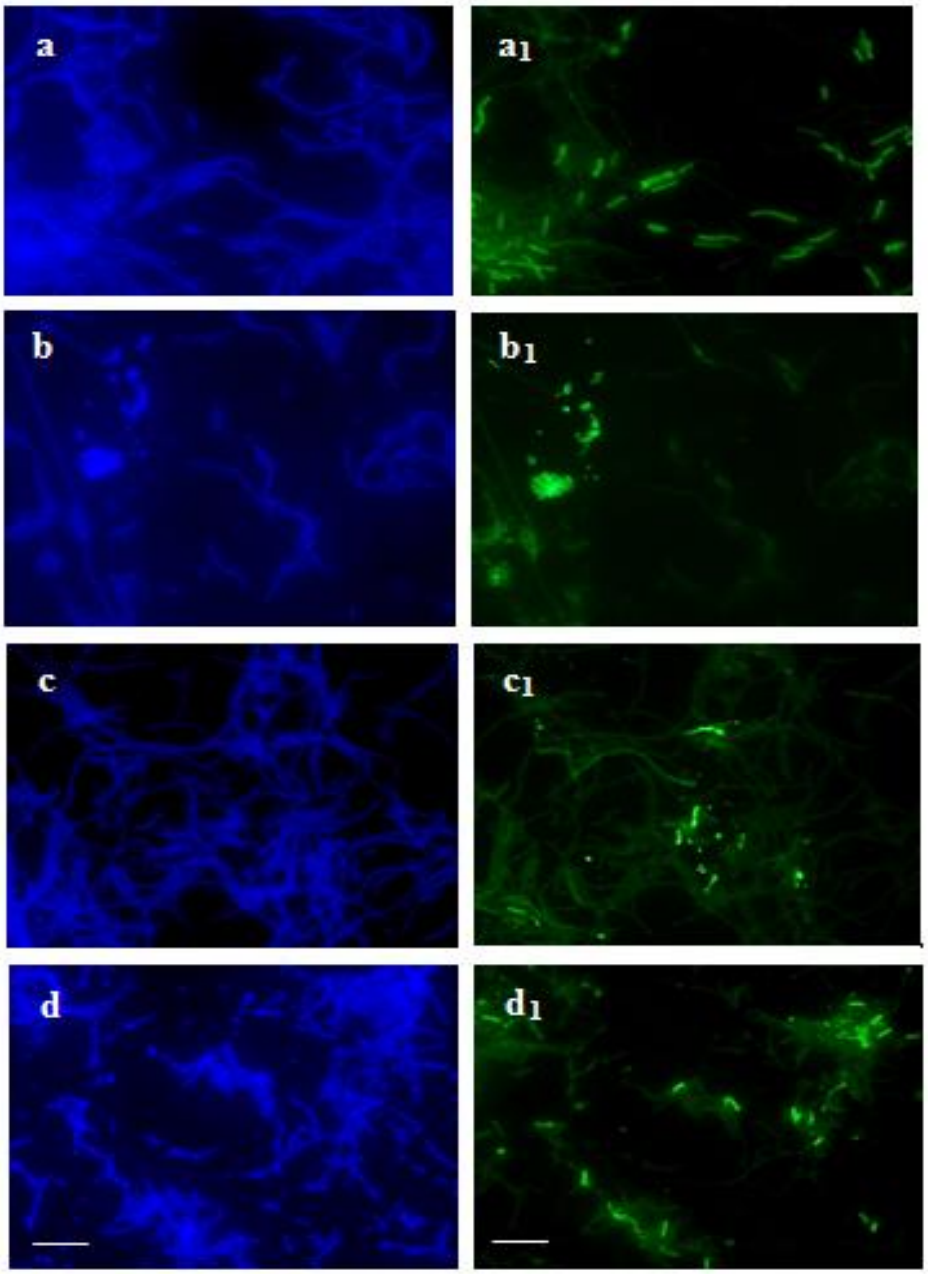

Figure 34. Hybridization results of water samples in January (a-a_1), April (b-b_1), July (c-c_1) and October (d-d_1) of the year 2012 under the optimum hybridization conditions. Total cell populations stained with DAPI (a, b, c, d) and their corresponding *alk*B probe applied mixed cultures (a_1, b_1, c_1, d_1). Bar 10 μm and applies to all photomicrographs.

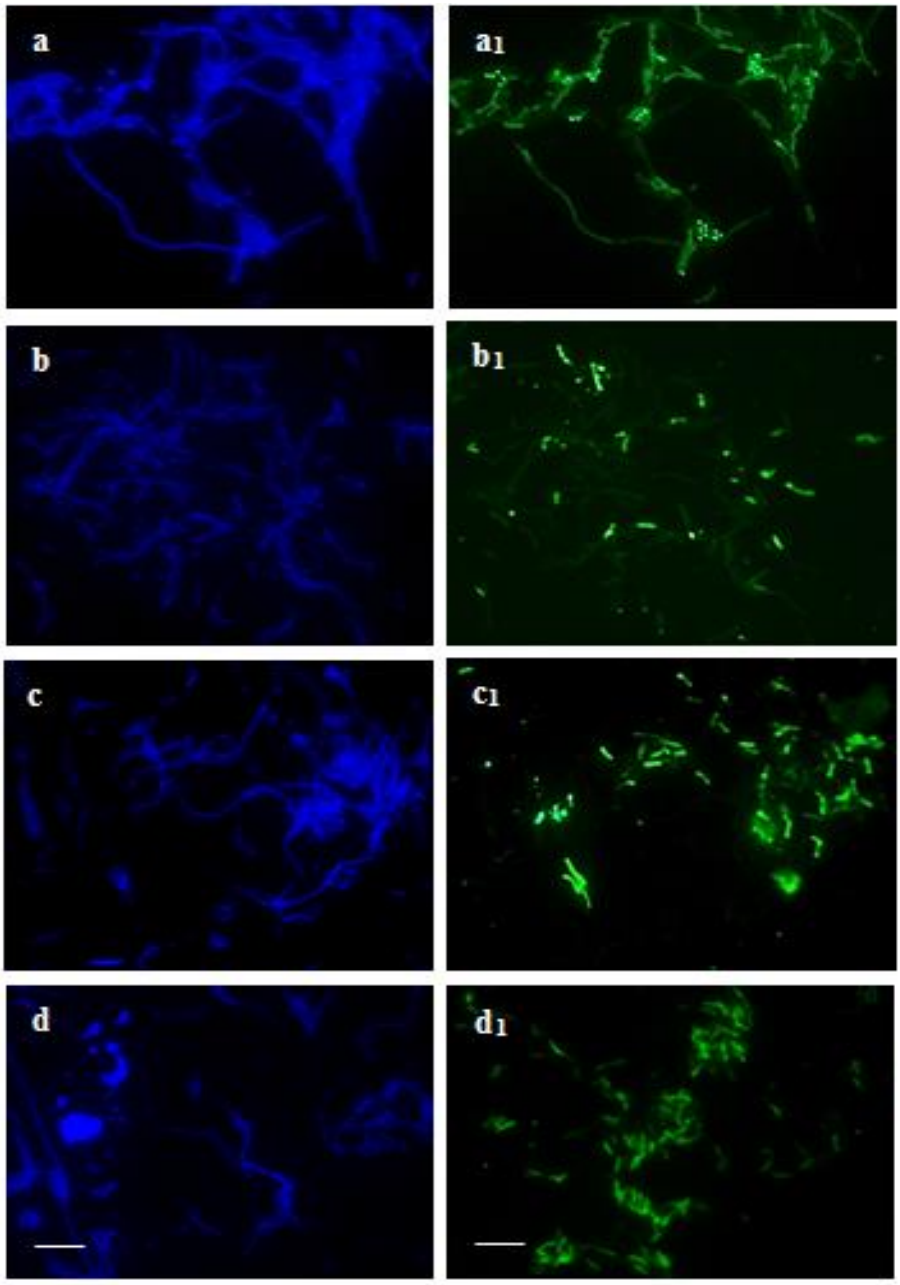

Figure 35. Hybridization results of water samples in January (a-a_1), April (b-b_1), July (c-c_1) and October (d-d_1) of the year 2012 under the optimum hybridization conditions. Total cell populations stained with DAPI (a, b, c, d) and their corresponding *C23*O probe applied mixed cultures (a_1, b_1, c_1, d_1). Bar 10 µm and applies to all photomicrographs.

The population dynamics of *alk*B and *C23*O harboring surface water bacteria was determined by calculating the pixel areas (pp^2) of DAPI and FITC images. Results of *alk*B and *C23*O analysis were shown in Figures 36, 37, 38, 39, 40. Population size of *alk*B and *C23*O harboring surface water bacteria in total biomass in the year of 2012 was also calculated using average values of taken images for pixel areas of FITC-labeled probe and DAPI-stained cells. Pixel areas and respective calculated biomass were represented in Figures 41 and 42.

	A(Y)	B(Y)	C(Y)	D(Y)	E(Y)	F(Y)	G(Y)	H(Y)
Long Name	alkb (pixel area)				DAPI (pixel area)			
Units	January	April	July	October	January	April	July	October
Comments								
1	9776	34140	22396	25418	147881	339335	652100	283833
2	10794	27890	24034	30106	141891	341745	651168	291788
3	15764	22968	52406	19964	141044	350007	654059	297368
4	9750	30838	74748	18250	143194	346139	651063	292814
5	10666	22944	68932	9558	132909	320777	650469	288926
6	6306	29878	36556	22768	141638	343009	653050	296748
7	7290	29782	51112	12926	144761	321772	654381	298031
8	12634	52818	39846	13350	144025	345900	649262	297400
9	6436	33804	25280	23482	139507	360264	653594	244683
10								

Figure 36. Pixel areas of FTIC and DAPI images for probe *alk*B with respect to months

	A(X)	B(Y)	C(Y)	D(Y)
Long Name	BIOMASS VALUE			
Units				
Comments	January	April	July	October
1	6,61072	10,06085	3,43444	8,95527
2	7,60725	8,16106	3,69091	10,31776
3	11,17665	6,56215	8,01243	6,71357
4	6,80894	8,90914	11,48092	6,23263
5	8,02504	7,15263	10,59728	3,30811
6	4,4522	8,71056	5,59773	7,6725
7	5,03589	9,25562	7,81074	4,33713
8	8,77209	15,26973	6,13712	4,4889
9	4,61339	9,38312	3,86784	9,59691
10				

Figure 37. Calculated biomass values harboring *alk*B gene

Notes

Input Data

Descriptive Statistics

	Sample Size	Mean	Standard Deviation	SE of Mean
BIOMASS VALUE	9	7,01135	2,18621	0,72874
B	9	9,27387	2,50309	0,83436
C	9	6,7366	2,96356	0,98785
D	9	6,84698	2,48781	0,82927

One Way ANOVA

Overall ANOVA

	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	3	39,51289	13,17096	2,02499	0,13012
Error	32	208,13474	6,50421		
Total	35	247,64763			

Null Hypothesis: The means of all levels are equal

Alternative Hypothesis: The means of one or more levels are different.

At the 0.05 level, the population means are not significantly different.

Fit Statistics

	R-Square	Coeff Var	Root MSE	Data Mean
	0,15955	0,34154	2,55034	7,4672

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
April January	2,26252	1,20224	2,66144	0,25578	0,05	0	-0,99478	5,51983
July January	-0,27475	1,20224	0,32319	0,99571	0,05	0	-3,53205	2,98255
July April	-2,53727	1,20224	2,98463	0,1715	0,05	0	-5,79458	0,72003
October January	-0,16438	1,20224	0,19336	0,99907	0,05	0	-3,42168	3,09293
October April	-2,4269	1,20224	2,8548	0,20243	0,05	0	-5,6842	0,8304
October July	0,11037	1,20224	0,12984	0,99972	0,05	0	-3,14693	3,36768

Sig equals 1 indicates that the means difference is significant at the 0.05 level.

Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

Figure 38. Results of ANOVA test for probe *alk*B

	A(Y)	B(Y)	C(Y)	D(Y)	E(Y)	F(Y)	G(Y)	H(Y)	I(Y)	J(Y)	K(Y)	L(Y)
Long Name	January 2012		April 2012		July 2012		October 2012		January	April	July	October
Units									biomass			
Comments	c23O	DAPI	c23O	DAPI	c23O	DAPI	c23O	DAPI				
1	10888	424881	39070	539335	31198	652100	22709	483833	2,5626	7,24411	4,78424	4,69356
2	11397	191891	41945	691745	42017	485168	17053	491788	5,93931	6,06365	8,6603	3,46755
3	9882	171044	35484	540007	56203	554059	13982	497368	5,77746	6,57103	10,14387	2,8112
4	7875	443194	26419	612139	27374	580063	19125	492814	1,77687	4,31585	4,71914	3,88077
5	8333	452909	14472	540777	44466	790469	31779	488926	1,83988	2,67615	5,62527	6,49976
6	10153	421638	48939	634009	41278	653050	21384	496748	2,40799	7,71898	6,3208	4,3048
7	8645	344761	44891	581772	25556	455381	23463	498031	2,50753	7,71625	5,612	4,71115
8	10317	302025	16409	565900	42923	649262	15675	497400	3,41594	2,89963	6,61104	3,15139
9	9218	439507	26902	587264	22640	553594	21741	494683	2,09735	4,5809	4,08964	4,39494

Figure 39. Pixel areas and calculated biomass harboring *C23*O gene with respect to months

Notes

Input Data

Descriptive Statistics

	N Analysis	N Missing	Mean	Standard Deviation	SE of Mean
January	9	0	3,14721	1,6114	0,53713
April	9	0	5,53184	1,97852	0,65951
July	9	0	6,28514	1,97017	0,65672
October	9	0	4,21279	1,09018	0,36339

One Way ANOVA

Overall ANOVA

	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	3	52,35862	17,45287	6,02801	0,00225
Error	32	92,64954	2,8953		
Total	35	145,00816			

Null Hypothesis: The means of all levels are equal.
Alternative Hypothesis: The means of one or more levels are different.
At the 0.05 level, the population means are significantly different.

Fit Statistics

R-Square	Coeff Var	Root MSE	Data Mean
0,36107	0,35492	1,70156	4,79425

Means Comparisons

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
April January	2,38462	0,80212	4,20431	0,02711	0,05	1	0,21139	4,55786
July January	3,13793	0,80212	5,53246	0,00241	0,05	1	0,96469	5,31117
July April	0,75331	0,80212	1,32815	0,78414	0,05	0	-1,41993	2,92654
October January	1,06558	0,80212	1,87871	0,55215	0,05	0	-1,10766	3,23882
October April	-1,31905	0,80212	2,3256	0,36907	0,05	0	-3,49229	0,85419
October July	-2,07235	0,80212	3,65375	0,06606	0,05	0	-4,24559	0,10089

Sig equals 1 indicates that the difference of the means is significant at the 0,05 level.
Sig equals 0 indicates that the difference of the means is not significant at the 0,05 level.

Figure 40. Results of ANOVA test for probe *C23*O

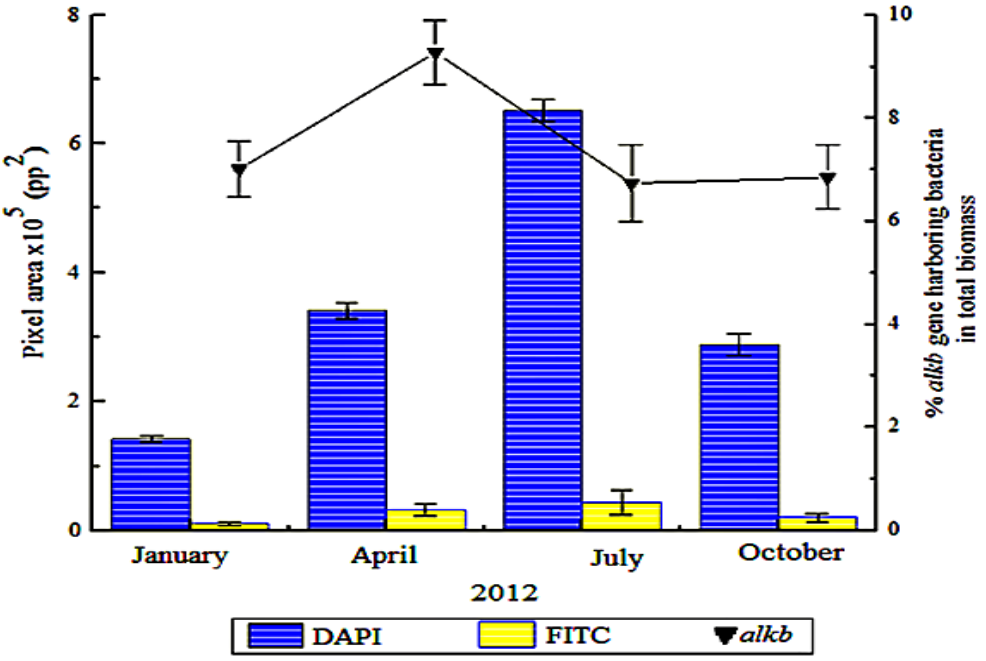

Figure 41. *In situ* monitoring of *alk*B harboring bacterial population in diesel oil contaminated surface water over the year of 2012. The error bars illustrate the calculated standard error.

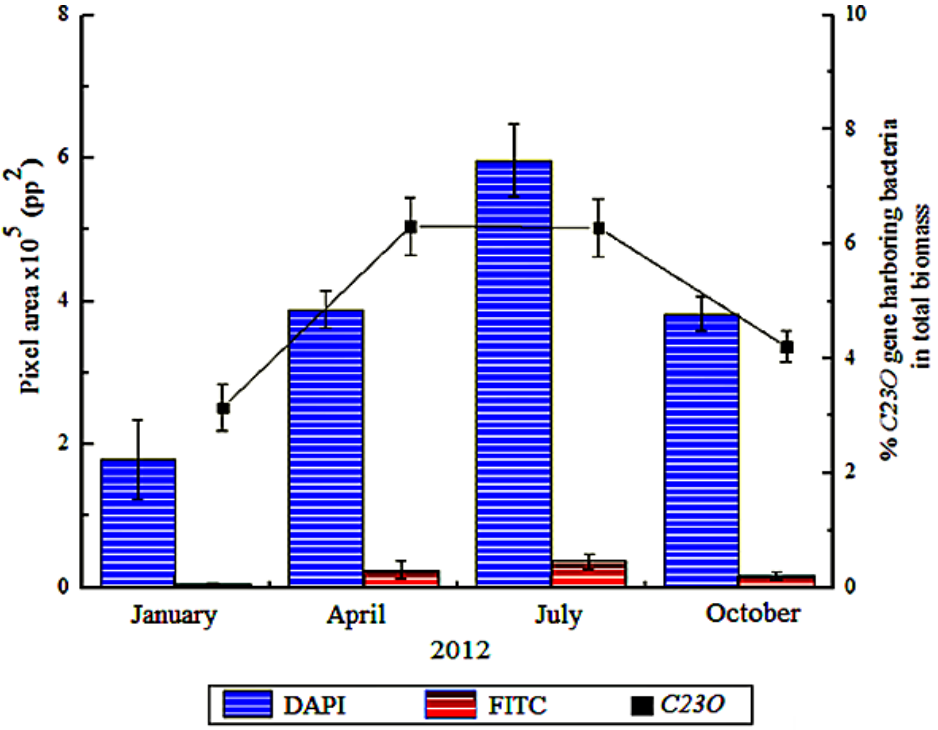

Figure 42. *In situ* monitoring of *C23*O harboring bacterial population in diesel oil contaminated surface water over the year of 2012. The error bars illustrate the calculated standard error.

The result showed that both the population of total bacteria with ($1.42\times10^5\pm0.04\times10^5$ pp^2) and the population of *alk*B harboring bacteria with ($0.10\times10_5\pm0.03\times10^5$ pp^2) were the lowest in January of 2012. The highest population of total bacteria with ($6.52\times10^5\pm0.02\times10^5$ pp^2) and of *alk*B harboring bacteria with ($0.44\times10^5\pm0.19\times10^5$ pp^2) were observed in July of the same year. Similar patterns were also obtained for *C23*O harboring bacteria with ($0.06\times10^5\pm0.01\times10^5$ pp^2) and ($0.37\times10^5\pm0.11\times10^5$ pp^2) in January and July of the year 2012 respectively. However, the percent of *alk*B harboring bacteria in total biomass showed different pattern than the percent of *C23*O harboring bacteria in diesel oil contaminated surface waters. Although the population size of *alk*B harboring bacteria in total biomass was highest in April with 9.27%±0.63 the population size of *C23*O harboring bacteria in total biomass was highest in July with 6.85%±0.62 for the year of 2012. Overall, for the year of 2012, there was no significant difference in the seasonal distribution of *alk*B harboring surface water bacteria within total biomass ($p>0.05$). Unlikely *alk*B harboring

surface water bacteria, the seasonal distribution of *C23*O harboring surface water bacteria was significant ($p<0.05$) ANOVA test revealed that seasonal population shifts of *alkB* and *C23*O harboring surface water bacteria varied during the year of 2012 ($p<0.001$). Shapiro-Wilk test showed that the population of *alk*B and *C23*O harboring bacteria had normal distributions ($p<0.05$) in river during the year tested.

The presence and relative abundance of microbial populations in a sample can be quantified by FISH. For optimization of bioremediation processes, the detection and monitoring of contaminant degrading bacteria are required. In various aquatic environments, bacterial community compositions were successfully characterized by using rRNA-targeted oligonucleotide probes (Rocha, 2001). Therefore, the population shifts in the community structure during the year 2012 was studied by *in situ* hybridization with rRNA-targeted fluorescently labeled *alk*B and *C23*O probes and images were analyzed by using microscopy. As a result, it was shown that the population rate of *alk*B and *C23*O harboring surface water bacteria has been affected the seasonal conditions, as well as environmental factors. For example environmental factors like, the temperature of the water and dissolved oxygen are important as temperature has impact on the reaction and degradation rates. Also, dissolved oxygen has impacts on the metabolic activity of microorganisms (Yang et al., 2009).

FISH is mostly used to examine the presence and quantification of specific microbial groups. Group, species and catabolic gene-specific oligonucleotide probes are also used by Wagner et al. (1993) to define *Acinetobacter* in activated sludge. Oligonucleotides probes specific for group of bacteria can also be designed to use in complex communities like biofilms (Rabuse et al., 1996) and have been demonstrated to represent valuable tools for group and species-specific hybridization studies of bacterial populations in (Ramsing et al.; 1993), marine sediments (Devereux et al., 1994), microbial mats (Ramsing et al.; 1993), anaerobic bioreactors (Icgen et al., 2006), aquifers (Morozova et al.,2011), river water (Yilmaz and Icgen, 2014), and sea water (Bryukhanov et al., 2011) without prior isolation of the target organisms. DNA probes have been used for the detection of catabolic genes in microbial communities after isolation of the bacteria (Whyte et al., 1995; Vomberg and Klinner, 2000). This study also showed that the population dynamics of *alk*B and

*C23*O harboring surface water bacteria can be successfully monitored by using catabolic gene specific DNA probes.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

Toxicity of the petroleum hydrocarbons is one of the primary reasons for remediation work. Diesel oil spills contain a global problem due to the toxic compounds in the oil. Biodegradation by natural populations of bacteria represents one of the primary mechanism by which petroleum hydrocarbons and other hydrocarbon pollutants can be removed from the environments. In this study, 15 of the 22 surface water isolates were found to be efficient biosurfactant producers as a result of the emulsification index (E_{24}%) experiment. Among these 15 isolates, isolates except from Ba11 and Co11 were shown to harbor *C23*O gene and all isolates except from Pb06 and Sn11 were shown to harbor *alk*B gene which are both the responsible genes in diesel oil degradation. Also, SDS-PAGE analysis showed that even though isolates Ba11 and Co11 were not able to harbor the gene *C23*O and Pb06 and Sn11 were not able to harbor the gene *alk*B according to PCR analysis, the proteins encoded by both genes were shown to be expressed in all isolates tested. Gravimetric analysis for degradation of diesel oil revealed that, isolates Fe10 and Zn01 were more efficient diesel oil degraders than the other 13 with the removal efficiencies of 93.7% and 88.9% respectively. Then these isolates were subjected to GC analysis for their diesel oil degradation abilities and after 14 days these isolates Zn01 achieved 92.11% degradation whereas Fe10 achieved 61.04%. These two isolates were identified as *A. calcoaceticus* Fe10, and *A. haemolyticus* Zn01 with 16S rRNA sequencing.

In addition, biosurfactants of these isolates were investigated for their physicochemical and biochemical and structural characteristics. For the isolates Fe10 and Zn01, surface tension reductions were calculated as reduction values were obtained as 15.8% and 15.1% respectively. Also, results of zeta potential

measurement revealed that biosurfactants are involved in the surface charge modification of diesel and enhance the adsorption of diesel to the bacterial cell surface. Moreover, FTIR analysis revealed that, the biosurfactant produced by the isolate Fe10 and Zn01 had peptides due to the presence of C-N bonds. Also presence of C=O bonds and C=C bonds indicated ester structure and unsaturated fatty acids respectively. Therefore, it was concluded that both Fe10 and Zn01 produced lipopeptide type biosurfactant.

This study described the identification of two *Acinetobacter* surface water isolates (*A. calcoaceticus* Fe10, and *A. haemolyticus* Zn01) involved in degrading diesel oil, producing biosurfactants and harboring two novel catabolic genes (*alk*B and *C23*O) of great practical significance in diesel oil degradation. *Acinetobacter* species are widespread in nature, and can be found in water, soil, living organisms, and even the human skin. The comparison of results of this thesis with similar studies revealed that the isolated *Acinetobacter* species can be used for the bioremediation of diesel oil as a potential biodegrading inoculant. *In situ* monitoring of *alk*B and *C23*O harboring bacteria in petroleum hydrocarbon-contaminated surface waters by FISH also revealed that the ubiquitous presence and ecological function of bacteria harboring these two novel genes effective in diesel oil degradation. The results of this study elucidated that *A. calcoaceticus* Fe10, and *A. haemolyticus* Zn01 isolates would have a great application in bioremediation of diesel oil contaminated surface waters.

Natural attenuation and bioremediation are both possible techniques for remediation of diesel oil-contaminated surface waters. To reduce the costs of the remediation, and enable more sites to be cleaned, these sustainable methods could be used more often in the future. *Acinetobacter* isolates seem to have high potential for this clean-up approaches. However, the relative importance of the *Acinetobacter* isolates in soil, sewage, and wastewater treatment plants should be also assessed.

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