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ISOLATION AND INVESTIGATION OF BIOREMEDIATION POTENTIALS OF PETROLEUM- DEGRADING BACTERIA FROM KURDISTAN REGION IN IRAQ

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MASTER THESIS

DEPARTMENT OF BIOLOGY

DIYARBAKIR

January - 2016

T.C.

DİCLE ÜNİVERSİTESİ

FEN BİLİMLERİ ENSTİTÜSÜ

KURDISTAN IRAK BÖLGESİ'NDEN PETROLÜ PARÇALAYAN BAKTERİLERİN İZOLASYONU VE BİOREMEDİASYON POTANSEYİLLERİNİN ARAŞTIRILMASI

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DİYARBAKIR

Ocak– 2016

APPROVAL OF THE THESIS

UNIVERSITY OF DICLE

INSTITUTE OF NATURAL AND APPLIED SCIENCES

DIYARBAKIR

"Isolation and Investigation of Bioremediation Potentials of Petroleum-Degrading Bacteria from Kurdistan region in Iraq", Submitted by **Shelan Shukri KARAM** in partial fulfillment of the requirements for the degree of Master of Science in Molecular Biology.

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ACKNOWLEDGEMENTS

First and foremost, I would like to thank my Merciful Allah for giving me strength and health to do this work. This thesis was prepared to fulfill the Master of Science degree in the Faculty of Sciences of Dicle University in Diyarbakir. I would like to express my sincere gratitude and thankfulness to my supervisor Prof. Dr. Kemal GÜVEN, for his priceless guidance, help, patience, encouragement, excellent guidance from the initial to the final level and enabled me to develop an understanding of this work. I would like to express my deepest gratitude to Dr. Ömer ACER, for excellent guidance from the initial to the final level, enabled me to develop an understanding of this work and for helping me at laboratory work. I would also like to thank Prof. Dr. A. Selçuk ERTEKİN for helping me during Master thesis at Biology Department. I would like to thank Ress. Assist. Dr. Fatma MATPAN BEKLER, Assoc. Prof. Dr. Reyhan GÜL GÜVEN, Prof. Dr. Erhan ÜNLÜ. My special thanks are dedicated to Hawre Ismail ROZHBAYANI and Delsher Mohammed to support and help me to collect samples. Finally, I would like to thank my parents and my husband for their love, support, encouragements, and care. Additionally, this master thesis is dedicated to my son (Seepan) for his unconditional love.

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ABSTRACT

ISOLATION AND INVESTIGATION OF BIOREMEDIATION POTENTIALS OF PETROLEUM- DEGRADING BACTERIA FROM KURDISTAN REGION IN IRAQ

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In our daily life and in industry, petroleum hydrocarbons are important as primary energy resources. Crude oil is used as raw matter in numerous industries, including the refinery-petrochemical industry, where crude oil is refined through different technological processes into usable products. Most of the petroleum goes in the ecosystem via leaks and accidental spills of coastal oil refineries. As a result, it directly or indirectly affects human safety and ecological systems. Toxic organic materials in crude oil can be carcinogenic, mutagenic and potent immunotoxicants and discharged into the soil and groundwater. One of these toxic materials is aliphatic hydrocarbons, particularly alkanes of shorter chain length (C10-C20) are more toxic.

The aims of this study are to isolate and identify the bacterial strains from petroleum contaminated sites as well as determine the ability for the biodegradation of crude oil.

For screening of hydrocarbon degrading microorganisms, different bacterial strains were isolated from different petroleum areas of Iraqi Kurdistan. The final isolates selected as the superiors hydrocarbon degraders were identified by morphological, physiological, biochemical screening tests and 16SrRNA sequence analysis. According to 16S rRNA gene sequencing analysis, five strains designated as 2B, 3B, 4A, 6C and 8F were found to be closely related to *Cronobacter malonaticus* (96.31% similarity), *Cronobacter dublinesis* subsp. *dublinesis* (95.48% similarity), *Serratia marcescens* subsp. *marcescens* (92.94% similarity), *Pseudomonas aeruginosa* (96.23% similarity) and *Acinetobacter calcoaceticus* (99.08% similarity) respectively.

GC-MS analysis of hydrocarbons with chain length (C9- C34) showed that 2B, 4A, 6C and 8F were able to degrade 84.76%, 73.25%, 91.62%, and 41% n-alkanes, respectively, in the crude oil incubated for 10 days. Along with the selected individual strains, a mixed bacterial consortium prepared using the above strains were also used for degradation studies. The mixed bacterial consortium did not lead to enough growth and degradation. The mixed bacterial consortium degraded 69.50% percentage of n-alkanes ranging from C11 to C34.

Key Words: n-Alkanes, crude oil, bacterial identification, biodegradation, 16S rRNA squence analysis, GC-MS analysis.

ÖZET

KURDISTAN IRAK BÖLGESİ'NDEN PETROLÜ PARÇALAYAN BAKTERİLERİN İZOLASYONU VE BİOREMEDİASYON POTANSEYİLLERİNİN ARAŞTIRILMASI

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2016

Günlük yaşamda ve sanayide, petrol hidrokarbonları birincil enerji kaynağı olarak önemli bir yere sahiptir. Hampetrol rafine edildiği yerlerde, kullanılabilir ürünler haline farklı teknolojik süreçler yoluyla Rafineri-Petrokimya endüstrisi dahil, birçok endüstride hammadde olarak kullanılmaktadır. Petrol büyük oranda sızıntı ve kıyı petrol rafinerilerinin kazayla dökülmesi yoluyla ekosistem içerisine girer. Sonuç olarak, doğrudan veya dolaylı olarak insane ve güvenliğini ve ekolojik sistemleri etkiler. Hampetrolün içerisindeki toksik organic maddeler, kanserojen, mutajenik ve güçlü immünotoksik olabilmekte aynı zamanda toprak ve yeraltısularına deşarj olabilmektedirler. Bu toksik maddelerden biri olan alifatik hidrokarbonlar, özellikle alkanların daha kısa zincirli (C10-C20) olanları çok toksiktir.

Bu çalışmanın amacı, petrolle kirlenmiş bölgelerden bakteri izolasyonu ve tanımlanmasına ilaveten petrol parçalama yeteneklerini belirlemektir.

Hidrokarbonları parçalayan bakterilerin taranması için, Irak'ın Kürdistan bölgesindeki farklı petrol alanlarından farklı bakteri türleri izole edilmiştir. Petrolü parçaladığı belirlenen son izolatlar, morfolojik, fizyolojik, biyokimyasal testler ve 16S rRNA dizi analizi yapılarak tanımlanmıştır. 16S rRNA dizi analizine gore, 2B, 3B, 4A, 6C ve 8F şeklinde adlandırılan bakteri suşlarının sırasıyla *Cronobacter malonaticus* (% 96.31 benzerlik), *Cronobacter dublinesis* subsp. *Dublinesis* (%95.48 benzerlik), *Serratia marcescens* subsp. *marcescens* (%92.94 benzerlik), *Pseudomonas aeruginosa* (%96.23 benzerlik) ve *Acinetobacter calcoaceticus* (%99.08 benzerlik) türlerine yakın oldukları bulunmuştur.

%1 Ham petrolde bulunan C9-C34 aralığındaki hidrokarbonların GC-MS analizi, 2B, 4A, 6C ve 8F'in 10 günlük inkübasyon ile %1'lik ham petrolde bulunan *n*-alkanları sırasıyla %84.76, %73.25, %91.62 ve %41 oranlarında parçaladıkları tespit edilmiştir. Seçilen saf türlerin yanında yukarıda belirtilen izolatlardan oluşan karışık bakteriyel konsorsiyumu da degradasyon çalışmaları için hazırlanmıştır. Karışık bakteri konsorsiyumu, yeterli üreme ve degradasyona yol açmamıştır. Karışık bakteriyel konsorsiyumu, C11 den C34'e kadar olan *n*-alkanları %69,50 oranında parçalamıştır.

AnahtarKelimeler: *n*-alkanlar, ham petrol, bakteriyel identifikasyon, biyodegradasyon, 16S rRNA dizi analizi, GC-MS.

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Abbreviations and symbols

BSM	: Basal Salt Medium
L	: Liter
mL	: Milliliter
µL	: Microliter
g	: Gram
g/L	: Grams / liter
mg	: Kilogram
mg/mL	: Milligram / milliliter
min	: Minute
mL/min.	: Milliliter / minute
v/v	: Volume / Volume (volume / volume)
nm	: Nanometer
µm	: Micrometer
N	: Normal
mm	: Millimeter
µg	: Microgram
K_2HPO_4	: Dipotassium phosphate
KH_2PO_4	:Potassium dihydrogen phosphate
$Na_2HPO_4.7H_2O$	: Disodium hydrogen phosphate heptahydrate
NH_4Cl	: Ammonium chloride
$MgSO_4$	: Magnesium sulfate
$CaCl_2$	: Calcium chloride
$FeCl_3$	: Iron (III) chloride
$MnSO_4$	: Manganese (II) sulfate
$ZnSO_4.\ 7H_2O$	: Zinc sulfate heptahydrate
$(NH_4)\ 6\ MO_7O_{24}.4H_2O$	: Ammonium heptamolybdatetetrahydrate
NMR	: Nuclear Magnetic Resonance
GC-MS	: Gas Chromatography / Mass Spectrometry
LC-MS	: Liquid Chromatography / Mass Spectrometry
I_2	: Iodine
KI	: Potassium iodide

H_2O_2	: Hydrogen peroxide
h	: hour
NaCI	: Sodium-chloride
H_2O	: Water
H_2SO_4	: Sulfuric acid
HCI	: Hydrogen chloride
NaOH	: Hydroxide sodium
DNA	: Deoxyribonucleic acid
rRNA	: Ribosomal ribonucleic acid
OD	: Optical Density
min	: Minute
PCR	:Polymarase chain reaction
PPM	: Parts per million
PH	: Potential of hydrogen
RFLP	: Restriction fragment length polymorphism
%	: Percentage
°C	: Centigrade

1. INTRODUCTION

The use of petroleum over the world is very common and sometimes the petroleum accidents take place. Nearly every year five million tons of crude oil and refined oil to enter the environment as a result of anthropogenic sources such as oil spills (Malatova 2005, Mahjoubi et al. 2013). Most of the oil comes from tankers, barges and other vessels as well as from land pipeline spills. Extensive changes in marine and terrestrial ecosystems resulting from the grounding (Malatova 2005).

Most hydrocarbons be falling in crude oil have toxic effects as result of their chemical structure (Al-Wasify and Hamed 2014). Both aliphatic and aromatic compounds are included in these toxic hydrocarbon compositions, such as polycyclic aromatic hydrocarbons (PAHs), whose toxicity expanded proportionally to the number of carbon atoms in the compound and individually in the case of PAHs with higher than four-member rings in its structure.

Toxic organic materials in crude oil can cause sub-lethal chronic toxicity and acute lethal toxicity, or both, depending on the exposure, dosage, and the organism exposed (Gardner et al. 1991, Orisakwe et al. 2004). One of these toxic materials is Polycyclic aromatic hydrocarbons (PAHs), that is the major problem as environmental contaminants (Al-Wasify and Hamed 2014). These polycyclic aromatic hydrocarbons (PAHs) are a group insolubile organic composites in water, composed of two or more fused aromatic rings in their chemical structure. PAHs are of environmental and humanitarian concern, are dangerous pollutants and health jeopardy, because of their toxic, mutagenic and carcinogenic characteristics (Hesham et al. 2014). Another hydrocarbons are toxic aliphatic hydrocarbons. Alkanes may constitute 50% to 95% of crude oil, related on the oil source. Alkanes compound of larger chain length (C20- 40C) are hydrophobic solids difficult to degrade. Also, they have less toxic effect than hydrocarbons of shorter chain length (C10- C20), while those of Longer chain n-alkanes and PAHs in crude oil were commonly considered to be only slightly biodegradable due to their higher hydrophobicity (Xia et al. 2014).

1. INTRODUCTION

Various groups of microorganisms have been found in crude oil contaminated site, despite the toxicity of the chemical synthesis occurring in crude oil (Wolicka and Borkowski 2012). The variety and the number of microorganisms at a given location may help to characterize that site with regard to the toxicity of these hydrocarbons to the microbiota, age of the spill and condensation of the pollutant. For that matter, in a crude oil-polluted soil, the biodiversity and microbial spread of certain microbe(s) may indicate how well the soil is suffering the growth of that microbe(s). Fresh spills and/or high levels of pollutants often kill or inhibit large sectors of the soil microbial population, while soils with lower levels or old pollution show greater numbers and variety of microorganisms (Saadoun et al. 2008). In addition, microbial isolates from the soils that are historically subjected to hydrocarbon pollution exhibit a higher potential of biodegradation than others with no history of such showing.

The improvement of oil removal was studied extensively since the 1970s. One of the methods which are suggested is abiotic method. Physical, mechanical, removal of oil is one types of Abiotic methods, ex: using of the oil sorbents. Those sorbents facilitate in transforming oil into a transportable form for short-run storage. But, most of the used sorbents terminate in the landfills. Also, physiochemical methods often chemical organic solvents with or without surfactants used, especially shoreline cleaners (Mills et al. 2003, Malatova 2005). The shoreline cleaners with surfactants emulsify the adsorbed oil, which transported deeper into the shoreline soil or is entrained beside water. The physicochemical techniques such as washing, surfactant and incineration guidance to the initiation of more toxic compounds in the environment (Oboh et al. 2006). Chemical agents are used in most of the physicochemical methods. For this reason, they cause toxicity killing off aquatic organisms. They produce another source of alloy and also augment the oil recovery cost. Another method is chemical methods. The oil-solvent mixtures usually used in skimming methods.Abiotic losses due to dispersion, evaporation of low molecular hydrocarbons and photooxidation (involve only aromatic compounds), which play a major role in the decontamination of the oil spill environments (Mills et al. 2003).

Also, another method is bioremediation, which is a natural method of removing ptroleum and oil-derived products contamination from the habitats. This is a biotic method or biological procedure that uses living organisms to remove completely or decrease pollutants (organic and inorganic compounds) from polluted zones. It is a natural process

without the addition of chemical materials to the habitats and is cheaper, compared to physiochemical methods. Bioremediation offers a practical alternative to oil spill response. Many soil microorganisms, bacteria and fungi, in aerobic condition, convert crude oil hydrocarbons into composites that are non-toxic or deportment their complete mineralization to compounds that are inorganic. This technology is fount to be effective for the treatment of oil pollution. One reason is that most of the molecules in crude oil and refined products are biodegradable (Malatova 2005, Teramoto et al. 2009, Wolicka and Borkowski 2012). So, bioremediation is an effective method for removing hydrocarbon pollution from different environments.

Stimulation of microorganisms in contaminated areas may solve the polluted problem with a low cost. Methods of conventional remediation commonly move the pollution problem to another location or generate preferable costs. In mutuality, bioremediation is a non-perilous and natural process to the environments. Bioremediation can be performed at the contaminated site without changeable the existing land management plans (Vidali 2001). Use any method, bioremediation has its drawbacks, not all compounds are sensitive to relatively fast and perfect degradation. In some of cases, products of degradation of oil paronymous products have toxic effects on microorganisms. Furthermore, bioremediation requires the usage of complex techniques in the presence of mixtures of compounds that are randomly dispersed in the medium. Bioremediation lasts longer than chemical methods; moreover, its correct duration cannot be precisely determined. In-situ bioremediation ways are not always easy to monitor, whilst ex-situ bioremediation methods are rather expensive due to transportation costs and soil storage (Wolicka and Borkowski 2012). Laboratory and field studies let us the designation of the favorable parameters for the bioremediation of soil contaminated by oil hydrocarbons: biomass content over 105 cells/g dry mass, temperature 20- 30 °C, pH:6.5- 7.5, relative humidity 20- 30%, oxygen content at least, 0.2 mg/l hydrocarbons, Carbon: Nitrogen: Phosporous (C: N: P) = 100:10:1 or 70:7:1 (Wolicka and Borkowski 2012).

Hydrocarbon-degrading microorganisms are generally distributed in aquatic, freshwater, and soil ecosystems. Most of these organisms, the superiority of which are bacteria have been characterized and classified utilizing cultural, biochemical and molecular techniques. Bacteria have developed millions of years ago regulatory systems that guarantee the synthesis of enzymes so that the original attack on these compounds is

produced only when required (Oboh et al. 2006). Some of microorganisms in soil and ground water may be adapted exhibiting selective enrichment and genetic changes using some composition of crude oil as a source of energy. These compounds via microorganisms are transferred to other levels of the food chain (Gardner et al. 1991, Orisakwe et al. 2004). The microbial bioremediation method is better than physical and chemical methods like volatilization, photooxidation, chemical oxidation to removal and cleaning up PAHs, and also these methods are not safe and cost effective (Al-Wasify and Hamed 2014). The most important subject is that these processes are natural and the ultimate products of the microbiological degradation are carbon dioxide, water and inorganic compounds. The effectiveness of hydrocarbon degradation by bacteria does not lead on the number of carbon atoms in the compound (Chikere et al. 2011, Jain and Bajpai 2012). Bacterial application as microbial bioremediation have long been considered as one of the predominant hydrocarbon degrading agents found in the environment, which are available (Al-Saleh et al. 2009). Despite the trouble and expensive in cost, it seems that oil will remain a major origin of energy in the next several decades because a reliable option has not yet been determined (Oboh et al. 2006).

The primary aim of this study was to isolate and identify the most better hydrocarbon-degrading microorganisms in the contaminated soil and aquatic places in Kurdistan region that is located at north of Iraq, to investigate the biodegradation potential of any strain and a mixed bacterial consortium by detailed chemical analysis using GC. MS test, in order to provide a microbial mixture that would be suitable in bioremediation processes with crude oil spills.

Therefore, in this study, we have aimed to isolate and identify the crude oil degrading bacteria from petroleum-contaminated sites in the oil fields (Zakho, Bardarash, Mreba, Atrush, Koya) in Kurdistan region of Iraq. Moreover, we have studied the ability of these isolated bacterial strains to degrade the aliphatic hydrocarbons by looking the total hydrocarbon rates.

2. REVIEW

2.1. History of Bacterial Systematic

There is the tremendous diversity of microorganisms in nature for scientists to study the bacterial systematics. The ultimate goal of biological classification and characterization of the organisms is making a canonical way to regulate them in the group (Schleifer 2009). Systematic and identification plays a central role in microbiology disciplines (Ludwig 2007). The discovery of new microorganisms made continuously changing in bacterial systematics, because they have different characters and put new evidence in the appropriate place (Arda 2011). Classification of the named species of bacteria did not rise easily or fast (Garrity et al. 2001). Western scientific taxonomy started in Grecian some hundred years BC. The most important works are cited and the development of taxonomy (with the focus on botanical taxonomy) are described up to the rotation of the Swedish botanist Carl Linnaeus (1707–1778), who discovered modern taxonomy (Manktelow 2010) and are here separated into pre-Linnaean, most had been botanical taxonomies, and post-Linnaean, other taxonomies after Linnaeus taxonomy. So, the starting point of the novel or modern taxonomy was started by Carl von Linnaeus, who discovered a system known as Linnaean classification for categorization of organisms and binomial nomenclature for naming organisms. In this system, a microorganism is defined by two scientific names. One of these is (genus) and another name is (species) (Arda 2011). In 1773 and 1774 the Danish naturalist Otto Muller was the first to try a systematic arrangement of the animalcules, but he did not create a clear difference between what we now call protozoa and bacteria. For his last performance, however, which was published in 1786, he did produce two form genera, Monas and Vibrio, which included bacteria and accommodated the punctiform and elongated kinds (Logan 1992). The mycologist Link (1809) reported the first bacterium that we know today, named *Polyangium vitellinum* that is now placed by the fruiting myxobacteria (Murray and Holt 2001). Bizio (1823) found *Serratia marcescens* (Garrity et al. 2001). The famous attempt by Ehrenberg (1838) and Cohn (1872, 1875), to gain more than a few names that still survive (e.g. Bacillus, Spirillum and Spirochaeta). Most explanations could hold only on behavior,

habitat and shape since microscopy was the important tool (Garrity et al. 2001). Ferdinand Cohn (1872), by his attempted to classify the known bacteria, six genera of bacteria (Micrococcus, Bacillus, Bacterium, Spirillum, Vibrio, and Spirochaeta) and next (1875) extended the classification to include the cyanobacteria while adding further bacterial genera (Asco Coccus, Sarcina, Leptothrix, Beggiatoa, Crenothrix, Calothrix, Streptococcus [not those recognized today], and Streptothrix) (Schleifer 2009). Buchanan (1925) introduced that Cohn's 1875 classification could be the opportunity date for bacterial nomenclature instead of Linnaeus' Species Plantarum of 1753 and presented the different ideas for the suitable starting date for bacterial nomenclature, the actual change in starting date proposed in the revised Bacteriological Code (Garrity et al. 2001).

During most of the nineteenth century to allow all system, Nomenclature rules have developed during the 19th and 20th century, and during the last decade standard nomenclature has been challenged by supporters of the Phylocode (Manktelow 2010), With the immersion of study fields as phylogenetics, cladistics, and systematics, the Linnaean system has improvements to a system of novel biological classification based on the evolutionary relationships between organisms, both living and extinct. Migula in 1897 has developed a system, taking into account not only microorganisms morphology, but also the color (colony) and some physiological characteristics (such as nitrogen fixation) and the work was published under the name "System of the Bacteria". In the same year, Lehmann and Neumann published their atlas under the name of "Diagnostic Bacteriology" (1886) (Arda 2011). The latter was apparently the superlative success of the systems and was used in sequential editions till 1930, especially in Europe. F.D. Chester constructed reports in 1897 and 1898 of bacteria of interest in horticulture to succeed in 1901 by his "Manual of Determinative Bacteriology". Chester had recognized that the lack of an organized assembly of descriptions and a system of the classification made the identification of separated as known species and the identification of new species an impossible duty (Garrity et al. 2001). Orla-Jensen (1909) was influential because he resolved and explicited of "natural relationships", reflecting further physiological characters. He bounded genera and species on the basis of characteristics such as metabolic products, temperature ranges for development and fermentation of several sugars in addition to morphology.

Bergey' s Manual of Determinative Bacteriology resulted from the interest and efforts of a group of colleagues in the Society of American Bacteriologists (SAB), a committee was formed by scientists such as L.A. Rogers, C. Krumwiede Jr., R.E. Buchanan, and G.H. Smith, which were published in the Journal of Bacteriology in 1917.In this study, bacteria based on phenotypic features, was classified as single-cell plants (Arda 2011). In 1923, a book "Manual of Determinative Bacteriology" was published by a committee from SAB headed by D.H. Bergey. There were two "starters" for the Manual: one of them was R.E. Buchanan and D.H. Bergey. The 7th edition of this book was published in 1957 and the bacteria was still classified as a plant (Schleifer 2009).

Molecular/genetic technology was well established in 1974 when the 8th edition of Bergey' s Manual of Determinative Bacteriology was published, however, was not still widely employed to play a role in broad decisions in taxonomy. The briefer Bergey' s Manual of Determinative Bacteriology in 1977 was published (Garrity et al. 2001). The first of four books of Bergey's Manual of Systematic Bacteriology between 1984-1986 were published, which became the best existing bacterial classifications, that were based on phenotypic characters (observable appearance of the genotype) with a notation for producing identification schemes (Logan 1992). In 1994 9th edition of Determinative Bacteriology was published.

A major start forward in the study of bacteria occurred in 1977 when Carl Woese recognized that Archaea have a separate line of evolutionary relationship from bacteria. This new phylogenetic taxonomy depended on the sequencing of 16S rRNA, and distributed prokaryotes into two evolutionary domains, as section of the three-domain system. The history of classification of bacteria and archea is shown in Table. 2.1 (modified from Schleifer 2009).

Table 2.1. Classification methods: history of bacteria and Archaea

Time Period	Basic Features in classification
Late 19th century	Morphology, reproduction conditions, pathogenic potential
1900-1960	Morphology, physiology, biochemistry
1960-1980	Chemotaxonomy, digital classification, DNA-DNA Hybridization
1977	16S rRNA
1980-Present	Genotypes Analysis, Multilocus series analysis, nucleotide similarity, all Genome Analysis

2.2. Bacterial Identification

2.2.1. Phenotypic Methods

Phenotypical methods are not directly linked to the DNA or RNA tests. It mostly covers classical or traditional phenotypic tests in the identification, which are used in the basic microbiological laboratory. Classic phenotypical features of bacteria are based on morphological, physiological and biochemical characteristics. Many of these features are shown to be unrelated parameters for genetic associations. However, as a whole, they provide descriptive information to identify taxa (Vandamme et al. 1996, Moraes et al. 2013).

Table 2.2. Some of the characteristics and methods are used in classification methods of Bacteria and Archaea modified from (Black 1996, Madigan et al. 2010)

Category	Characteristics
Morphology	Colony morphology, Gram reaction, cell size and shape, pattern of flagellation, the presence of spores, arranged in pairs, clusters or filaments.
Motility	Nonmotile, gliding motility, swimming (flagella) motility, swarming, motile by gas vesicles
Metabolism (Nutrition)	Mechanism of energy conservation (phototroph, chemoorganotroph, chemolithotroph), utilization of individual carbon, nitrogen, or sulfur compounds; fermentation of sugars, nitrogen fixation, growth factor requirements
Physiology	Temperature, pH, and salt ranges for growth; response to oxygen (aerobic, facultative, anaerobic), presence of catalase or oxidase, production of extracellular enzymes
Biochemistry	Fatty acids, RNA molecules, nature of cellular components such as cell wall (Presence or absence of peptidoglycan; amino acid composition of cross-links), pigments, biochemical test
Genetics	Percentage of DNA base, DNA hybridization
Other traits	Luminescence, antibiotic sensitivity, serotype; production of unique compounds, for example, antibiotics

2.2.1.1. The Morphological Identification of Bacteria

The identification and classification of organisms have continually been amongst the pre-eminence of the early scientists. Although botanists and zoologists have a superfluity of morphological traits with that to identify plants and animals, the morphological traits of identifying bacteria are few and limiting. This has not produced a peremptory challenge, but an opening for creativity. Gram staining was as a result of the creating penetration of Hans Christian Joachim Gram (1850-1938) to classify bacteria based on the structural characteristic of their cell walls. It meant that bacteria

could be differently classified by Gram staining as Gram negative or Gram positive, a convenient for identification and classification hand tool that continue useful today. Howsoever there are some morphological traits and few changes in those traits, identity based on morphology Syne has considerable taxonomic value. Another method is growing on the media in order to identify their cultural characteristics since various species can exhibit very different colonies (Christopher and Bruno 2003). Any colony has specifications that may be unique to it and this may be helpful in the primitive identification of a bacterial species. The characteristic of the colonies on solid agar media include theirs shape (round, irregular or rhizoid), size (the width of the colony: small, medium, large), surface (smooth, wavy, rough, granular, papillate or glistening), elevation (convex, concave,umbilicate), margin (entire, wave, created, ciliate or curled), structure/-opacity (opaque, translucent / transparent), color (pigmentation: yellow, green between others), degree of growth (scanty, medium or profuse) and nature (separate or confluent, filiform, spreading or rhizoid). Cell shape has also been employed in the characterization and grouping of bacterial species (Cabeen and Jacobs-Wagner 2005). The most basic shapes of bacteria are cocci (spherical in shape), bacilli (rod-shaped) and spirilla (spiral-shaped). Observations of bacterial morphologies are done by light microscopy, which is helped by the application of stains (Tshikhudo et al. 2013). Gram staining and bacterial morphologies usually are employed as the first steps of identification. Light microscopy was applied for identifying colonies of bacteria and morphologies of individual bacteria (Tshikhudo et al. 2013). An amalgamation of morphological identification with Scanning electron microscopy (SEM) and in situ hybridization (ISH) techniques (SEM-ISH) lead to a better understanding, to achieve the phylogenetic and morphological knowledge about bacterial species to be recognized using in situ hybridization with rRNA-targeted oligonucleotide probes (Kenzata and Tani 2012).

2.2.1.2. Physiological Properties

Physiological characteristics of bacteria show variations depending on bacterial types. Thus, reproduction temperature, incubation time, oxygen requirements, the

composition of the medium and other physiological characteristics needs to be investigated and determined (Gül-Güven 2007).

2.2.1.3. Biochemical Charactersistics

Biochemistry, seldom called biological chemistry, is the study of chemical method within and relating to living organisms. (David 2000*)*. The significance of identifying biochemical activity in the assay of microorganisms is so much as various tests are used for this purpose. These include starch, gelatin, catalase, urea, oxidase, nitrate and nitrite reduction, as well as carbohydrate utilization test, selected and used depending on the type of microorganisms (Gül-Güven 2007).

2.2.2. Genotypic Methods

The traditional methods applied to the finding of either the colony characteristics or morphology of single cells remain reliable for bacterial species identification. However, these common techniques create some disadvantages. Firstly, a variation of culture due to changed environmental conditions may lead to vague results. Secondly, they are time-consuming and difficult. Thirdly, a pure culture is needed to undertake identified, making the identification of particular and unculturable bacteria complex and sometimes impossible. To pass these technical problems, newer and automatic methods which quickly and reliably recognize bacteria have been utilized by many laboratories worldwide. At least one of certain methods, particularly analysis of the 16S rRNA gene, join these traditional methods with the automated systems that produce novel techniques to a higher level of trust for bacterial identification (Tshikhudo et al. 2013). These methods are suitable for studies of unculturable bacteria and fastidious an appropriate tool for the metagenomic analysis of environmental samples (Petrosino et al. 2009, de Oliveira et al. 2011). Metagenomics is defined as unculturable bacteria or culture-independent investigation of the collective set of genomes of mixed microbial crowd in consortia that live in an environment, in plants or in animal hosts (Tshikhudo et al. 2013). Comparative analysis of the genome gives several traits for discriminating

between species of bacteria. Genotypic analysis has particular application in microbial taxonomy because of the insights it affords at the DNA level, with DNA–DNA hybridization and DNA profiling is used in microbial taxonomy (Madigan et al. 2010).

2.2.2.1.GC Ratios

Another method that has been employed to compare and describe bacteria is the GC ratio of their DNA. The GC ratio is the rate of guanine (G) and cytosine (C) in an organism's genomic DNA (Ludwig 2007, Madigan et al. 2010). GC ratio of total nucleic acids containing guanine and cytosine bases in an organism's DNA is expressed as a percentage. This ratio is a measure of DNA melting temperature, or determined by various methods such as chromatography (Madigan et al. 2010). The GC ratio in microorganisms variation over a broad range, with an amount as low as 17% and as high as nearly 80% between species of Bacteria and Archaea, a range that is moderately broader than in eukaryotes (Schleifer 2009, Madigan et al. 2010). However, GC ratios are utilized less generally in bacterial taxonomy than used in the past (Madigan et al. 2010)

2.2.2.2.16S rRNA Analysis

Ribosomal RNA genes are a significant part of the protein synthesis machinery. They are omnipresent and hence classification based on the analysis of ribosomal RNA genes does not leave out each of the known bacteria. 16S rRNA is a component of the small ribosomal subunit of RNA (30S Ribosomal subunit) and sometimes referred as SSU rRNA. 98% of 16s rRNA of all bacteria similar, there for its same species (Woese 1987, Glazer and Nikaido 2007). Carl Woese in the early 1970s used rRNA for comparison, because rRNA is present and performs an identical function in every cellarorganisms, and more importantly, it's sequence has changed extremely slowly during the course of evolution. According to this criterion, the analysis of rRNA genes is a fitting tool for bacterial species identification and systematic categorization. Therewith, ribosomal RNA genes are saved of evolution, but have sufficient variation to

distinguish between taxa (Woese 1987). In prokaryotes, rRNA genes are situated in copies of three or four in a single genome (Glazer and Nikaido 2007, Tshikhudo et al. 2013). The 16S rRNA gene is a suitable strong tool for identifying and classifying bacteria and its length of nearly 1,500 bp is enough for bioinformatic analysis (Janda and Abbott 2007). Analysis and dissociation of the 16S rRNA gene include amplification of the gene by polymerase chain reaction (PCR) and gene sequencing of the resulting PCR products. The gene sequence can be matched with prior collected sequences obtained from several DNA databases of the 16S rRNA gene. It is realized that almost all new sequences trusted have some matches up to some levels, but a 16S rRNA gene copy which does not match that of any known bacterial species to a particular level then it is supposed to be a new species (Tshikhudo et al. 2013). The 16S rRNA gene sequence is applied to identify bacterial species in natural specimens and to find phylogenetic relationships between them (Murat et al. 2010). It is created conceivable by the reality that all bacterial species include the 16S rRNA gene. So, it has a highly conserved area on which to design common primers, as well as hypervariable regions that are useful to identify the species. For bacterial identification, the 16S rRNA gene is regarded as the most broadly accepted gene (Tshikhudo et al. 2013).

2.2.2.3. DNA–DNA Hybridization

DNA-DNA Hybridization useful for comparing species of bacteria that are very closely related, when two organisms contain many identical or extremely similar genes, their DNAs are required to hybridize in approximate relationship to the similarities in their DNA sequences. DNA–DNA hybridization accordingly is helpful for separating between organisms as a complement to small subunit rRNA gene sequence (Glazer and Nikaido 2007, Madigan et al. 2010, de Oliveira et al. 2011). DNA–DNA hybridization is a sensitive technique for revealing delicate variation in the genomes of two organisms and is hence usually useful for differentiating so similar organisms. Although there is no fixed convention as to how much hybridization between two DNAs is important to select two organisms to the same taxonomic rank, hybridization values of 70% or greater are prescribed as evidence that two isolates are the same

species. Rates of at least 25% are expected to show that two organisms are in the same genus (Glazer and Nikaido 2007, Madigan et al. 2010).

Table 2.3. Some genotypic methods utilized in bacterial taxonomy

Method	Description/application
DNA–DNA hybridization	Genome-wide comparison of sequence similarity. Useful for distinguishing species within a genius.
DNA profiling	Ribotyping , AFLP, rep-PCR . Rapid method to distinguish between species and strains within a species
Multilocus sequence	Typing Strain typing using DNA sequences of multiple genes. High resolution, useful for distinguishing even very closely related strains within a species.
GC ratio	Percentage of guanine–cytosine base pairs in the genome. If the GC ratio of two organisms differs by more than about 5%, they cannot be closely related, but organisms with similar or even identical GC ratios may be unrelated. Not much used now in taxonomy because of poor resolution
Multiple-gene or whole genome phylogenetic analysis	Application of cladistic methods to subsets of genes or to whole genomes of the organisms to be compared. Yields better phylogenetic picture than single-gene analysis

2.2.3. Chemotaxonomical Methods

Chemical structure of cellular components in prokaryotes classification is totally useful feature used in the assay. Chemotaxonomic methods are extensively performed particularly where the identification and classification by morphological and physiological analysis is not enough.

2.2.3.1. Fatty Acid and Lipid Analysis

The types and proportions of fatty acids present in cytoplasmic layer lipids and the external membrane lipids of bacteria are important phenotypic traits of interest. The technique for identifying these fatty acids has been nicknamed FAME, fatty acid methyl ester. FAME analyses are generally used in the characterization of new species of bacteria (Black 1996, Schutter and Dick 2000, Madigan et al. 2010).

FAMEs are abundant in the phospholipid bilayer of bacterial membranes (Black 1996, Tshikhudo et al. 2013). The fatty acids presentation of Bacteria differs from species to species in chain length and in the presence or absence of double bonds, circle, branched chains, or hydroxy groups. So, a fatty acid profile can often identify a special bacterial species (Black 1996, Madigan et al. 2010).

Fatty acid defines bacteria based on their physical properties. Profiles of fatty acids are determined by employing gas chromatography (GC), (Nnzeru et al. 2013). The fatty acid analysis for bacterial identification is based on the specific fatty acid structure of the cell wall. Various profile patterns of the extracted fatty acids are compared with pattern identification programs, that exist in microbial databases (Tshikhudo et al. 2013).

2.3. Classification of Bacteria

Currently based on genetic relatedness of rRNA, organisms divided into bacteria, archaea and eukaryotes (Figure 2.1). So great that is believed that these three groups may have diverged from an ancient progenitor rather than evolving from one another (Glazer and Nikaido 2007).

Over 7000 species of Bacteria and Archaea are now known, based principally on 16S rRNA gene sequencing, and thousands more, may be as many as 100 000–1000 000 are thought possible to exist. There are ~1.8 million named species, and most species have yet to be described. Despite decades of effort and thousands of phylogenetic studies on diverse clades, we lack a comprehensive tree of life, or even a summary of our current knowledge. One reason for this shortcoming is lack of data.

GenBank contains DNA sequences for ~411 000 species, only 22% of estimated named species (Madigan et al. 2010, Smith et al. 2014) . Classification is the organization of organisms into progressively further including groups on the basis of either phenotypic similarity or evolutionary relation. A species is made up of one to various strains, and similar species are arranged into genera (singular, genus). Similar genera are classified into families, families into orders, orders into classes, up to the domain, the highest level taxon. Nomenclature is the actual identifying of organisms and follows the binomial system of nomenclature devised by the Swedish medical doctor and botanist, Carl Linnaeus. The names are Latin or Latinized Greek origin, often representative of some key property of the organism, and are written in italics (Black 1996, Madigan et al. 2010, Staton 2015).

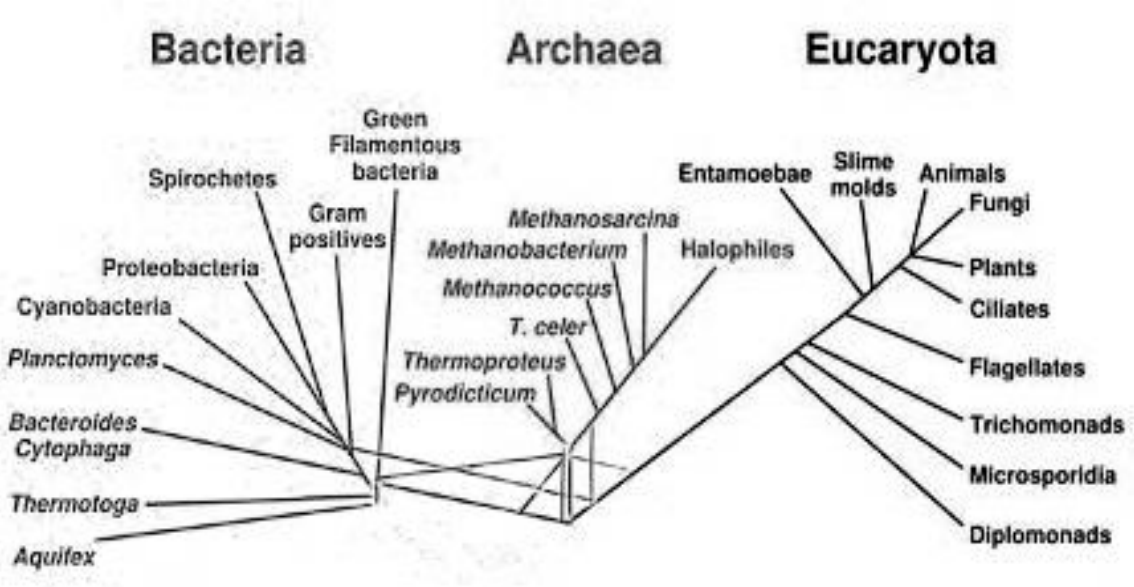

Figure 2.1. That the three branches of life all possess a DNA-based genome (Joseph & Schild 2010).

2.3.1. Phylogenetic Overview of Bacteria

Many major ancestors, called phyla, of Bacteria are known from laboratory cultures study, and several others have been identified from the sequencing and retrieval of ribosomal RNA (rRNA) genes of microbial communities in natural habitats. Figure

2.2 gives a summary of the phylogeny of main phyla of Bacteria for which laboratory cultures have been obtained based on 16S rRNA sequences. The gram-positive bacteria are a large group of essentially chemoorganotrophic Bacteria that can be separated into two subgroups called the Firmicutes plus the Actinobacteria (Manteca et al. 2006, Madigan et al. 2010). The Cyanobacteria are oxygenic phototrophic bacteria, with solstitial roots nearby those of the Gram-positive Bacteria. The remaining phylum of cultured Bacteria, the Proteobacteria (Figure 2.3), is by far the largest and most metabolically diverse of all Bacteria. Proteobacteria constitute the majority of known bacteria of industrial, medical, and agricultural significance. As a group, the Proteobacteria are all gram-negative bacteria (Madigan et al. 2010).

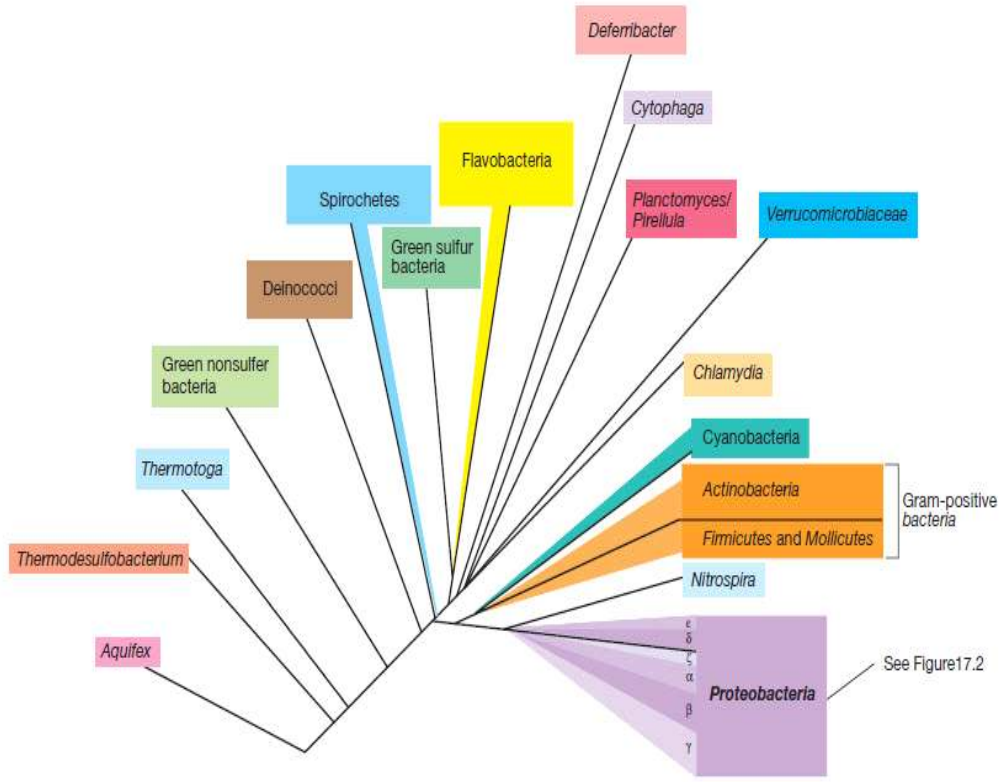

Figure 2.2. Classification of bacteria by 16S rRNA Sequence Analysis

2.4. Proteobacteria

The Proteobacteria is the largest and most metabolically different of all Bacteria. Proteobacteria form the majority and importance of known bacteria for industrial,

medical, and agricultural technology. As a group, the Proteobacteria are all Gram-negative bacteria. They show an exceptionally wide diversity of energy-generating mechanisms, with chemolithotrophic, chemoorganotrophic, and phototrophic species (Figure 2.2). The Proteobacteria are correspondingly diverse in terms of their relationship to oxygen (O_2), with anaerobic, microaerophilic, and facultatively aerobic species known. Morphologically, they also show a wide type of cell shapes, including straight cocci, arched rods, filamentous, spirilla, budding, and appendaged forms.

Based on 16S rRNA gene sequences, the phylum Proteobacteria (Figure 2.3) can be classified into six classes, Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, Epsilonproteobacteria, and Zetaproteobacteria, each containing many genera and species (Madigan 2010). The Proteobacteria are a larger group (phylum) of gram-negative bacteria. They include a wide diversity of pathogens, such as *Escherichia, Salmonella, Helicobacter, Vibrio,* and *Yersinia*, and various other important genera (Madigan 2010). Others are free-living (non parasitic), and include multiple of the bacteria responsible for nitrogen fixation (Woese 1987). Alphaproteobacteria grow at extremely low levels of nutrients and have abnormal morphology such as stalks and buds. They include agriculturally significant bacteria capable of inducing nitrogen fixation in symbiosis with plants. They are as well as motile by flagella and are microaerophilic, but several are non-motile. (http://en.wikipedia.org/wiki/proteobacteria#external links).

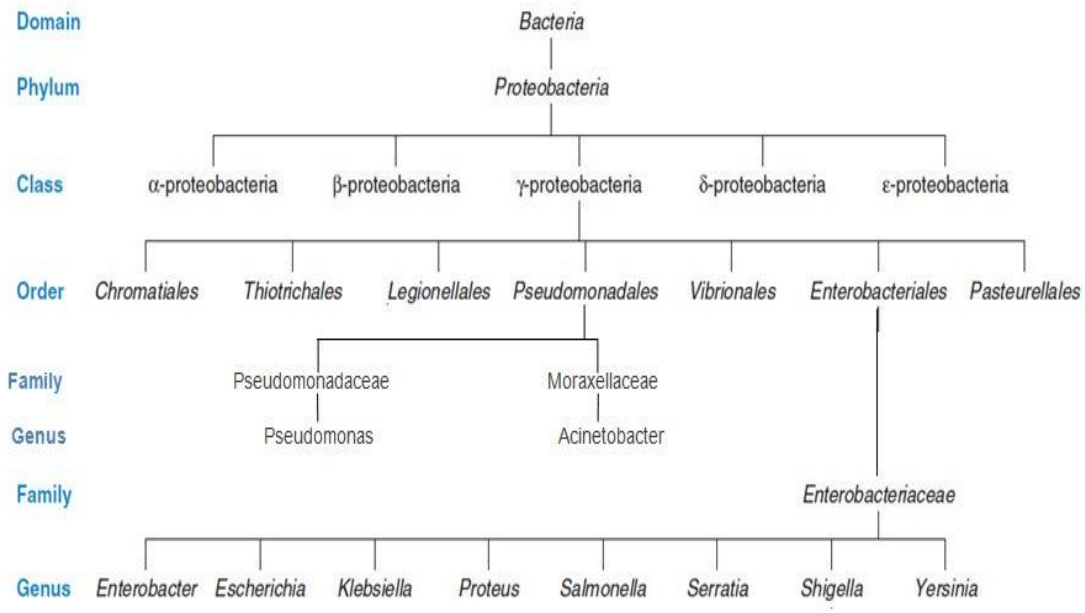

Figure 2.3. Hierarchical Arrangement in Taxonomy modified from (modified from Palleroni et al. 1973, Prescott 2002, Doughari et al. 2011).

2.4.1. *Cronobacter* sp.

The *Cronobacter* genus was recognized first in 2007 by Iversen et al (2006). As a clarification of the taxonomic relationship of the biogroups found among strains of *Enterobacter sakazakii* and revised in 2008. Differentiation among the newly defined *Cronobacter* sp. It is at first based on genotypic (DNA-based) analysis and is largely supported by biochemical traits.

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Gamma Proteobacteria

Order: Enterobacteriales

Family: Enterobacteriaceae

Genus: Cronobacter

(Joseph et al. 2011)

Cronobacter genus was identified as the species *Enterobacter sakazakii* by Farmer et al. in 1980 to honor the Japanese bacteriologist Riichi Sakazaki. They were phenotypically near to *E. cloacae*, so were put in the *Enterobacter* genus (Iversen et al 2007, Forsythe 2010).

The recently designated genus, *Cronobacter* is Gram negative rods that are approximately 3 μm by 1 μm in size, facultative anaerobic. The genus *Cronobacter* comprises oxidase negative, catalase positive. They are generally motile. Previously, G+C ratios of 57% and 56.7% have been reported for strains belonging to the type species (Iversen et al 2007). They were found to include in sections of the Enterobacteriaceae family and closely connected to *Citrobacter* and *Enterobacter*. *Cronobacter* can amplify over a broad temperature range. The lowest is near refrigeration (~5°C) and the maximum replication temperature (44- 47°C) is strain dependent. At normal room temperature (21°C), Cronobacter had a replicated time of 40-94 minutes. Last researches show the organism was extremely thermotolerant, but, subsequent work clarified that the organism was less thermotolerant. The microorganism has achieved with superiority due to its entirety with hard neonatal infections; necrotizing enterocolitis, meningitis and septicaemia , which can be fatal. Moreover, these are rare in infants, and infections arise in all age groups, although fortunately with less intense clinical sequels (Iversen et al 2004, 2006, Forsythe 2010).

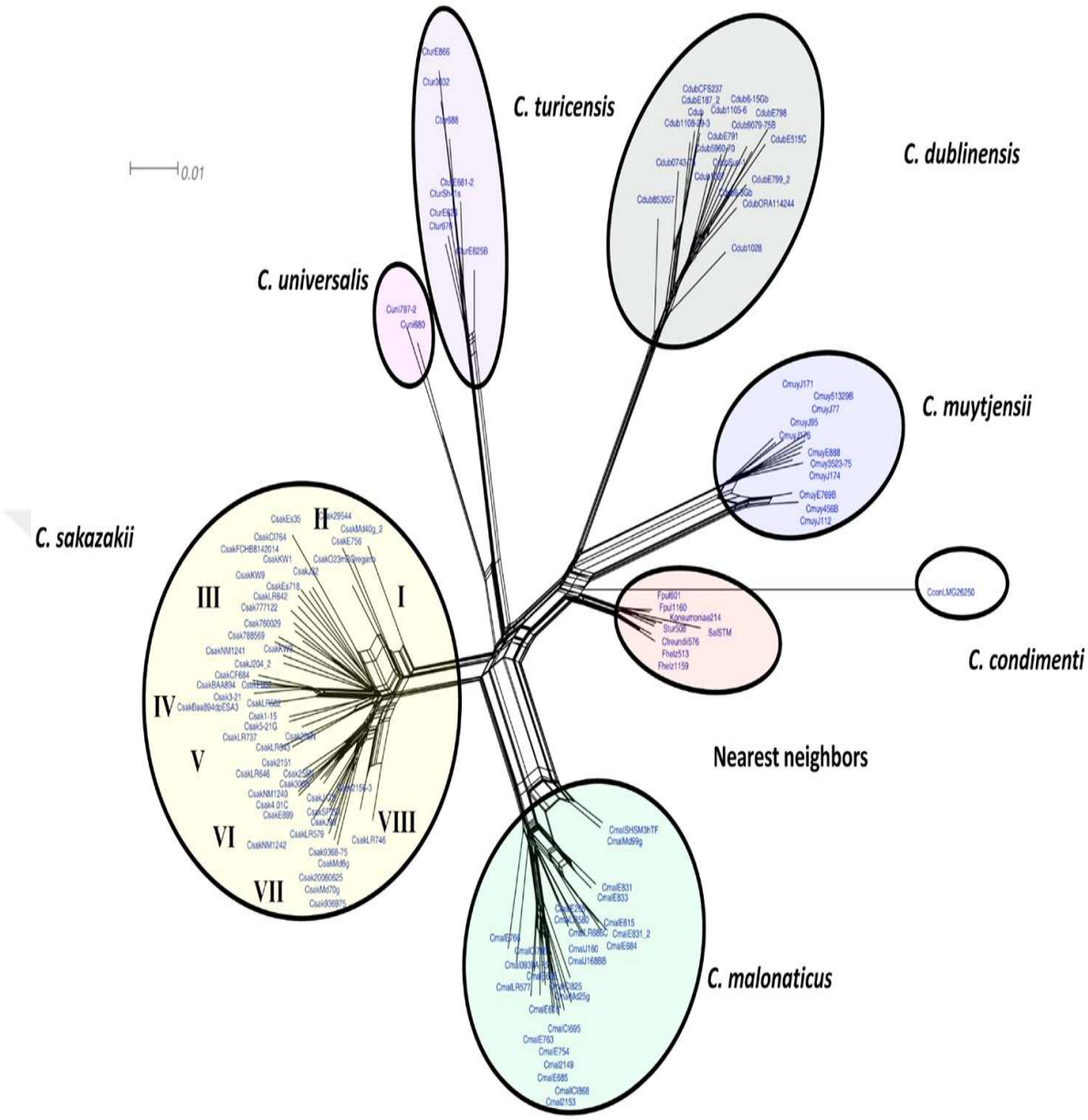

Figure 2.4. 16S rRNA Sequence Analysis of *Cronobacter* species (Tall et al. 2015).

2.4.2. *Serratia sp.*

The generic name *Serratia* used since the 1920s, refers to the physicist Serafino Serrati. It was proved to be responsible for a case of food contamination (Caprette 2009).

Domain: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Enterobacteriales

Family: Enterobacteriaceae

Genus: Serratia

(Bizio 1823)

Serratia was of great interest in medicine due to its antibacterial, antifungal, antiprotozoal, immunosuppressive, antimalarial, and anticancer activities (Juo 2001, Caprette 2009). It is motile, facultatively anaerobic, very small straight rods, between 0.5 and 1 µm in diameter and < 2 µm in length, arranged singly. These characteristics caused to rule out all other genera of Gram negative, rods, facultatively anaerobic, not assigned to one of these three families, placing the isolate in family Enterobacteriaceae. Typical colonies were 1-2 mm in size when not round, convex, crowded, glabrous, and slightly umbonate with entire margins, and opaque. New colonies were red in color. Cultures produced an unpleasant and somewhat sweet odor. strongly catalase positive, oxidase negative. Negative for indole production and mixed acid fermentation of glucose (methyl red test). Liquifies gelatin at 22˚C. All observations were consistent with the description of the genus in Bergey's Manual p. 477. 23 *Serratia* species were listed previously in the first edition book of Bergey's Manual. The only *Serratia* species recognized in the eighth edition of Bergey's Manual was *S. marcescens* (Dworkin and Falkow 2006, Grimont and Grimont 2006).

Serratia marcescens, which belongs to the Enterobacteriaceae family, has been reported to be an important agent of health care-related infections (HCRI), specifically standing out due to its dissemination potential and its high level of intrinsic resistance to the drugs used in Neonatology, and to antiseptic agents. This pathogen persists for long periods in the hospital environment because it colonizes the skin and the gastrointestinal tract of adults and newborns (Grimont and Grimont 2006, Maragakis et al. 2008).

S. marcescens is known for its opportunistic characteristic that is most of their strains are resistant to most antibiotics (Caprette 2009). When food is contaminated with *S.*

marcescens it yields a pink to red discoloration due to the pigment prodigiosin production (Bennett and Bentley 2000).

The presence of R-factors makes most *S. marcescens* strains effectively resistant to used antibiotics, R-factors are a type of plasmid that holds one or more genes that causes the resistance; examples of the resisted antibiotics are ampicillin, first-generation cephalosporins and macrolides (http://en.wikipedia.org/wiki/Serratia_marcescens 2015.11.17).

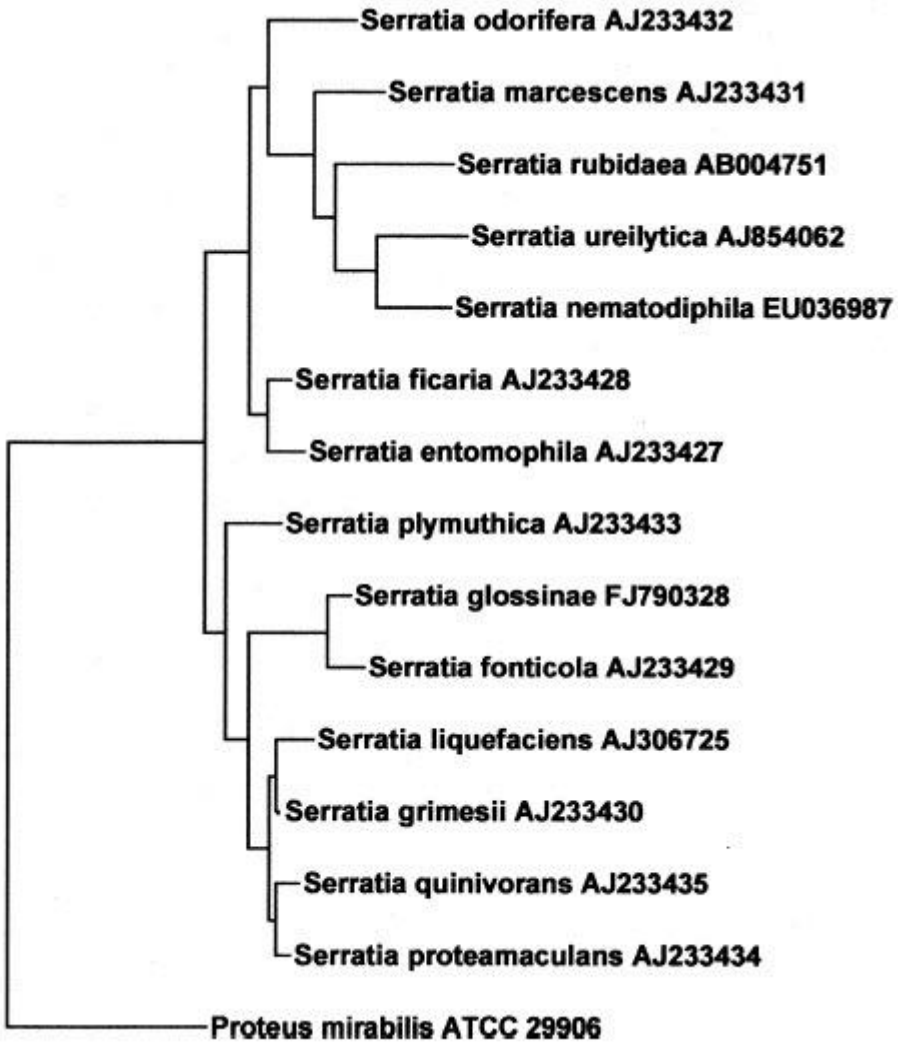

Figure 2.5. 16S rRNA Sequence Analysis of *Serratia* Sp. by relation Between Types (Mahlen 2011).

2.4.3. *Pseudomonas* sp.

The genus *Pseudomonas* includes Genus I of the family Pseudomonadaceae. Five genera (*Pseudomonas, Azomonas, Azotobacter, Azorhizophilus* and *Cellvibrio*) have belonged to the family. Known to all chooser genera are some physiological properties, example aerobic, chemoorganotrophic metabolism, lack of fermentation, lack of photosynthesis, and ability to grow in the consumption of a large variety of organic substrates.

A more universal explanation of the genus carried in 1900 (Migula 1900), studying 75 species and registering *Pseudomonas aeruginosa*, previously identified as "Bacterial aeruginosa", as the type species of the genus (Moore et al. 2006). Palleroni and his colleagues at the University of California at Berkeley were able to recognize different intrageneric groupings among, that time, the species comprising the genus Pseudomonas, by the usage of rRNA similarities it is currently recognized as the class "Gammaproteobacteria" (Palleroni et al. 1973).

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Gamma Proteobacteria

Order: Pseudomonadales

Family: Pseudomonadaceae

Genus: Pseudomonas

The original development of the genus *Pseudomonas* established a taxon based only on characteristics of cell morphology (Moore et al. 2006), with straight or slightly bent rods, 0.5–1.0 micrometer in diameter by 1.5–5.0 micrometer in length. They are Gram negative, motile by one or more than one polar flagella; rarely nonmotile. Endospore formation is seen in several species, but few; e.g., *Pseudomonas violacea* (Migula 1900, Moore et al. 2006).

Pseudomonas species are rich in the environment, available from different soil and water environments to animal and plant tissues (Moore et al. 2006, Silby et al. 2011). Particularly each habitat has temperatures between about 4 to 42° C, mesophilic,

a pH of 4 - 8, neutral PH condition. Oxidase-positive or negative, catalase-positive, chemoorganotrophic using organic compounds (simple or complex) as a potential habitat (Migula 1900, Moore et al. 2006, Franzetti and Scarpellini 2007, Raaijmakers et al. 2010). They are aerobic, in some cases anaerobic. They were not found in extreme thermophilic and acidophilic habitats (Moore et al. 2006).

Pseudomonas represents a genus able of using a large margin of organic and inorganic compounds and capable of living in different environmental conditions (Palleroni 2005, Moore et al. 2006, Franzetti and Scarpellini 2007, Raaijmakers et al. 2010). They may possess various strategies to tolerate periods of "starvation", therefore they are present in water and soil ecosystems and are significant as human, plant and animal pathogens. In nature, *Pseudomonas* species are present as saprophytes and parasites (Moore et al. 2006, Silby et al. 2011).

The genus *Pseudomonas* is famous for its metabolic diversity and genetic flexibility. The species of *Pseudomonas*, in general, grow quickly and are solely regenerateed for their ability to metabolize a vast number of substrates. *Pseudomonas* is a genus of directly agreeable organisms, which looks to be a result of their simple nutritional requirements and their genetic and metabolic compatibility, the range of carbon compounds their usage, including toxic organic chemicals, like aliphaticand aromatic hydrocarbons. Strains of *Pseudomonas* species are frequently resisting antibiotics, disinfecting agents, deterging agents, metals, and organic solvents. Many of of the species are not accumulating poly-β-hydroxybutyrate granules, however, accumulating polyhydroxyalkanoates of monomer lengths higher of C4 can happen when growing on alkanes or gluconate (Migula 1900, Moore et al. 2006, Franzetti and Scarpellini 2007, Raaijmakers et al. 2010).

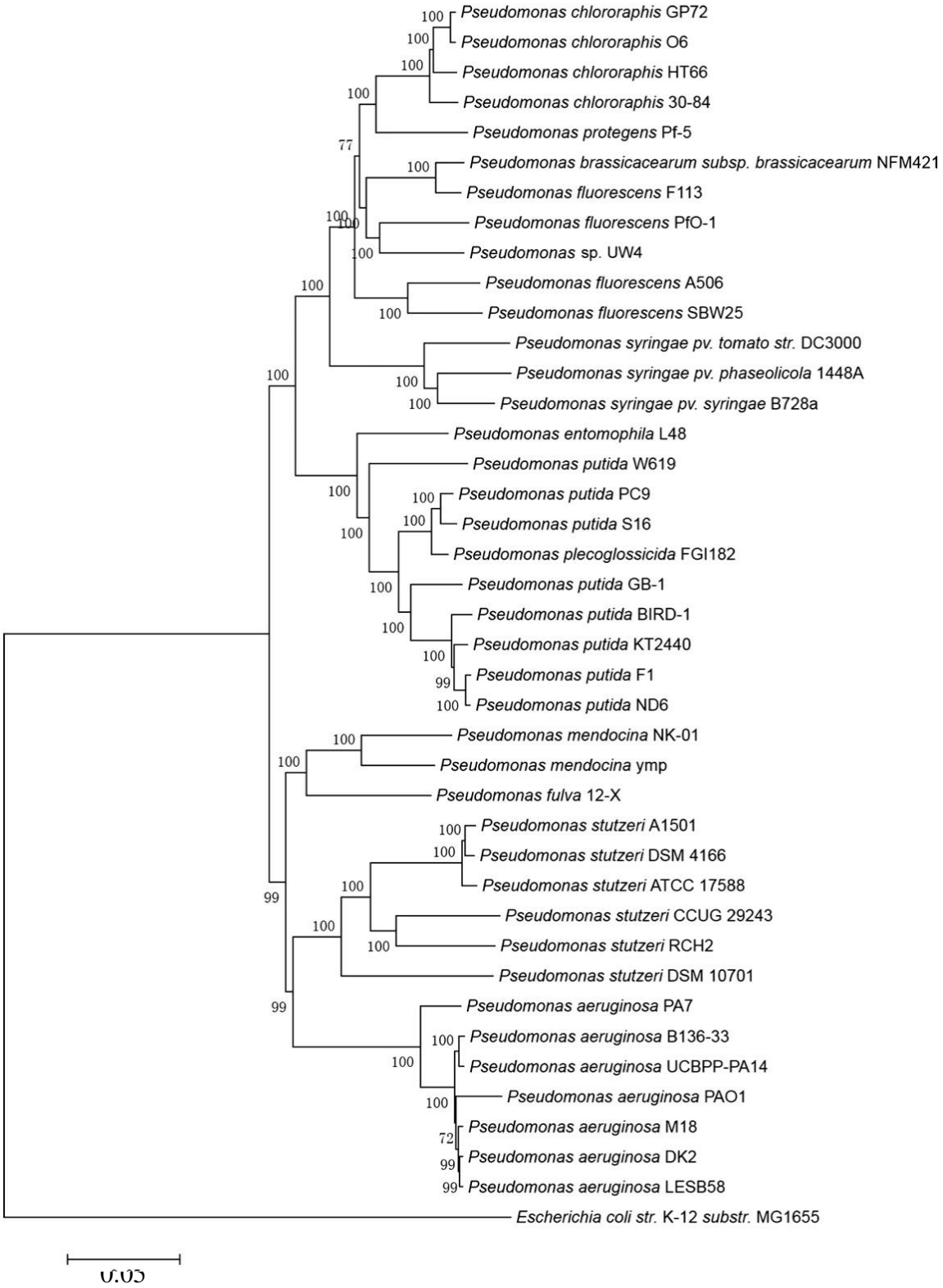

Figure 2.6. Phylogenetic tree of some *Pseudomonas* species drawn by using 16S rRNA Sequence Analysis (Chen et al. 2015).

2.4.4. *Acinetobacter* sp.

The name "*Acinetobacter*" originates from the Greek word "akinetos" indicate "unable to move", since these bacteria are not motile but they represent a twitching kind of motility (Doughari et al. 2011). The first strain of *Acinetobacter* sp. was separated from soil and classified as *Micrococcus calcoaceticus* by Beijerinck in 1911. *Acinetobacter* groups were incompletely known for long time classified as many different genera (*Achromobacter, Alcaligenes, Cytophaga, Diplococcal, Bacterium, Herellea, Mima, Lingolsheim, Micrococcus, Moraxella and Neisseria*). In fact, the Bergey's Manual of Bacteriology placed these bacteria, *A. calcoaceticus*, in the family Neisseriaceae with just A. calcoaceticus as a species and the pair subspecies *A. anitratum* and *Acinetobacter lwoffii*. The genus *Acinetobacter* was first discovered in 1954 by Brisou and Prevot to isolate the non motile forms from the motile one of the family "Achromobactereae" (Barbe et al. 2004). *Acinetobacter* species are comprehensive in nature and the earth, the water surface, sewerage waters also It can be isolated from human skin. For example, *A. calcoaceticus*, *A. pittii*, *A. johnsonii* and *A. guillouiae* have been isolated from soil and water. Furthermore, *Acinetobacter* sp. can also be commensals of the human skin (Doughari et al. 2011).

Modern classifications which seem to have gained deep recognition amongst bacterial taxonomists have recognized this group of heterogeneous bacteria as gamma Proteobacteria classified in the order Pseudomonadales and the family Moraxellaceae (Doughari et al. 2011). So the taxonomical classification is given as;

Domain; Bacteria,

Phylum; Proteobacteria,

Class; Gammaproteobacteria,

Order; Pseudomonadales,

Family; Moraxellaceae,

Genus —Acinetobacter (DNA G+C content 39–47%).

New classifications using cell form, absence of flagella, G+C content of DNA and nutritional characteristic, placed these organisms (*A. baumannii, A. haemolyticus* and *A. calcoaceticus* also other Acinetobacters) in the genus *Moraxell*a, now known as

Acinetobacter (Barbe et al. 2004), depending on DNA-DNA hybridization studies. Thirty two species of *Acinetobacter* have now been distinguished, with 22 specific reliable names and the rest assigned numbers and referential to as a 'genomic group delineated by DNA-DNA hybridization (Prashanth and Badrinath 2005) between the named species.

The genus *Acinetobacter* is composed of aerobic, nonmotile, catalase-positive, oxidase-negative, indole negative, Gram negative, non fermentative encapsulated coccobacilli rods (Garrity et al. 2005, Doughari et al. 2011). In the exponential stage of growth, they are bacilli 0.9 to 1.6 μm in diameter plus 1.5 to 2.5 micrometers in length, often in pairs or group into longer chains of alternate length. Many strains are weak to reduce nitrates to nitrites (Doughari et al. 2011). *Acinetobacter* cell wall is generic of that of Gram negative bacteria, but destaining is hard due to an orientation to hold crystal violet and this causes a wrong identification as Gram positive cocci. *Acinetobacter* generally makes smooth and seldom mucoid colonies on solid media, ranging in color between white to pale yellow or grayish white.

Acinetobacter is a major concern because of their accelerated growth and resistance to antimicrobials of an extensive range. Also, it has rapid profundity in transforming, surviving desiccation and durability in the environment for a very long time (Doughari 2011).

The most important problem with *Acinetobacter* sp. is their resistance to antibiotics (Savov et al. 2000, Doughari et al. 2011). these organisms are commonly resistant to ampicillin, gentamicin, amikacin, cephalothin, carbenicillin, chloramphenicol, tetracycline, co-trimoxazole, ciprofloxacin and cefoperazone. Previously ampicillin, second generation cephalosporins, colistin, amynoglycosides, impenim, quinolones, minocyline, sulbactam and gentamicin were used to treat *Acinetobacter* infections. Resistance to these antibiotics leaded to the control healing management, creating increasing concern over the world (Doughari et al. 2011).

Table 2.4. Applications for *Acinetobacter* sp. and their products (Doughari et al.2011).

Bioremediation of waste waters and effluents	Bioremediation of soils and effluents contaminated with heavy metals	Production of biopolymers and biosurfactant	Biomass production
i) Phosphate removal	i) Textile or tannery industrial effluent containing heavy metals	i) For prevention of dental plaque	i) Protein production
ii) Degradation of petrochemicals	ii) Lead from digested sewage sludge	ii) For use in paper making and other industries	ii) Manganese leaching from ores
iii) Breakdown of organic pollutants	iii) Chromium-contaminated activated sludge or wastewater	iii) For efficient emulsification of oil waste pollutants	iii) Production of immune adjuvants
	iv) Silver contaminated photographic wastewater	iv) For incorporation in cosmetics, detergents and shampoos	

The members of the *Acinetobacter* group are nutritionally versatile chemoheterotrophs (Doughari et al. 2011, Peleg et al. 2008). Recently, *Acinetobacter* sp. have demonstrated a hydrocarbon-degrading capacity (Margesin et al. 2003, Mandri and Lin 2007), that is of interest for soil bioremediation and a specific strain *Acinetobacter baylyi* ADP1 has shown an exceptional capability for natural transformation, thus making it a potentially significant tool for biotechnology (Doughari et al. 2011).

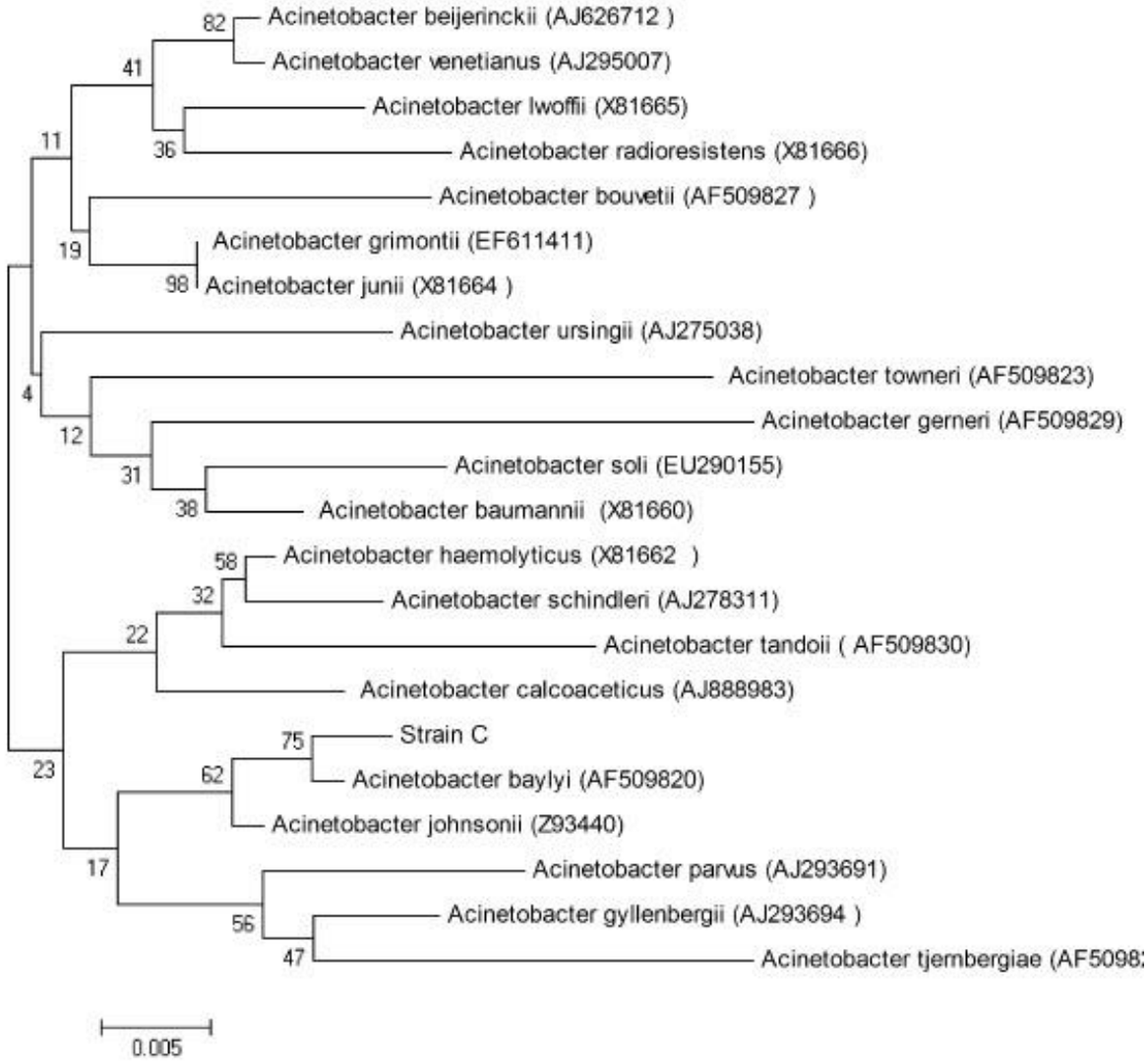

Figure 2.7. Phylogenetic tree of some *Acinetobacter* species drawn by using 16S rRNA Sequence Analysis (Zou et al. 2014).

A wide range of molecular methods and semi-automated systems have been described to explain *Acinetobacter* isolates to the species level. These include, for example, DNA-DNA hybridization, amplified fragment length polymorphism (AFLP), amplified ribosomal DNA restriction analysis (ARDRA), etc. (Garrity et al. 2005, Doughari 2011, Zou et al. 2014).

2.5. Oil Spill

Whitehead (1983) describes crude oil as a complex mixture of many compounds comprising mainly compounds of hydrogen and carbon. Hydrocarbons (aliphatic and aromatic) are the source of carbon in crude oil, as well as organic compounds which results from the degradation of crude oil such as formic, propanoic, acetic, benzoic, butyric, and naphthenic acids (Dandie et al. 2004).

Crude oil is formed of thousand of different organic and inorganic compounds. They have a very complex chemical composition. So, hyrocarbon compounds, include aliphatics maximally; i.e. methane, paraffin, and polycyclic aromatic; i.e. resins and asphalts, non-hydrocarbons composition, heteroatoms; i.e. N, S, O and trace elements; i.e. Ti, Zn, Ca, and Mg. They include in average up to 80-87% C, 10-14% H, sulphur compounds (0.01–8%) and 0.01–2% N and 14% heteroatoms; in which 1-2% composed of metal-organic compounds. Hydrocarbons diverse in their solubility, from very low solvability non-polar compounds, like high molecular weight polynuclear aromatic hydrocarbons to polar composition, like methanol (Malatova 2005, Wolicka and Borkowski 2012).

Chemical composition of hydrocarbons affects its biodegradation in two ways. First, the molecule can have groups or exchanges that don't react with existing or persuadable enzymes. Second, the structure can define the composition to be in a physical condition wherever microbial degradation does not easily happen. More often, when the structure is larger and more complex, it will be slower to oxidize.

Crude oil is broad in compositional and physical properties. Particular petroleum product ranges from the very low molecular weight hydrocarbons to the very highest. Saturated hydrocarbons containing without double bonds. According to their chemical structure, these are categorized into alkanes (paraffin) and cycloalkanes. The highest percentage constituent of crude oil represents the saturates. Usually, aromatic hydrocarbons with one or more aromatic rings, like resin and asphaltenes, is embodied with alkyl groups (Harayama et al. 2004, Korenblum et al. 2012, Wolicka and Borkowski 2012). Porphyrins often happen in crude oil. Crude oil maturity is highly correlated inversely with porphyrins concentration (Wolicka and Borkowski 2012).

Crude oil is used as primary material in several industries, such as the refinery-petrochemical industry, where crude oil is refined through different technological processes into other products such as paraffin oils, gasoline, asphalt, oils, domestic fuel oil, lubricants, vaseline, and polymers. Oil-derived products are generally employed in many other chemical processes. In our daily life and industrial used petroleum hydrocarbons as important and primary energy resources (Kvenvolden and Cooper 2003, Wolicka and Borkowski 2012, Mahjoubi et al 2013).

The use of petroleum over the world is very common and sometimes the accidental spills occur regularly during the discovery, production, refining, transportation, and storage of petroleum and petroleum products. Giant amount crude oil and refined oil to enter the environment each year as a result of anthropogenic sources such as oil spills (Kvenvolden and Cooper 2003, Malatova 2005, Mahjoubi et al. 2013). Oil spills showed that most of the oil comes from tankers, barges and other vessels as well from land pipeline spills. Extensive changes in marine, as well as terrestrial ecosystems resulting from the grounding (Malatova 2005).

Figure 2.8. Zakho region of Kurdistan is polluted through crude oil, as well as hazard from H_2S gas.

Most of the crude oil spreads in the ecosystem via leak of coastal oil refineries, which cause a risk to the environment and harm to the global economy (Malatova 2005, Mahjoubi et al 2013). The discharge of petroleum products in the environment results in environmental damages that can directly or indirectly affect human safety and ecological systems (marine and continental) (Jiang et al. 2010). Owing to petroleum has complex composition, it can elicit multiple types of toxic effects. Toxic organic

materials in crude oil, which through oil spillage and oil pollution in water environment have been a major threat to ecosystems food chain. Most hydrocarbons in crude oil exerts toxic effects resulting mainly from their chemical structure (Al-Wasify and Hamed 2014). Toxic organic materials in crude oil can cause acute lethal toxicity, sub-lethal chronic toxicity, or both, depending on the exposure, dosage, and the organism exposed (Gardner et al. 1991, Orisakwe et al. 2004). Bacteria can only be degraded low rate of them, because they accumulate in and disrupt cell membranes, and consequently stay often as persistent pollutants in the environment. Therefore, toxic effects of hydrocarbons on microorganisms can cause problems in bioremediation, waste gas and wastewater treatment. On the other hand, not all bacteria display the sensibility towards a certain organic compound. Very recently, a first systematic interrogation of the toxicity of hydrocarbons belonging to the classes of (chlorinated) phenols, BTEX and n-alkanols were carried out for anaerobic bacteria (Heipieper and Martinez 2010.) Stimulation of the growth of indigenous microorganisms, biostimulation and inoculation with foreign oil-degrading bacteria are helpful means of accelerating the detoxification of a polluted site with minimal impact on the ecological systems (Cappello et al. 2007).

These toxic hydrocarbon compositions include both aliphatic and aromatic compounds, such as polycyclic aromatic hydrocarbons (PAHs). Polycyclic aromatic hydrocarbons (PAHs), which are of environmental and humanitarian concern, are dangerous pollutants and health jeopardy, due to their toxic, carcinogenic, and mutagenic characteristic (Hesham et al. 2014).

Aliphatic hydrocarbons are another hydrocarbons that cause toxicity. n-Alkanes compounds are the most biodegradable of the petroleum hydrocarbons, and many microbial species attack them rather than naphthenic and aromatic compounds. However, n-alkanes in the range of C5–C10 have inhibitory effects on the many hydrocarbon-degrading microorganisms at high concentrations because lipid membranes are disrupted by acting as dissolvents (Acer et al. 2014). Alkanes of shorter chain length (C10-C20) are more toxic (Xia et al. 2014). Due to their very low solubility in water, long-chain alkanes (>C12) are generally not considered as toxic. However, at high concentrations, relatively short-chain alkanes (C5-C12) and cyclic alkanes have negative effects on biological membranes (Ali Akbari et al. 2014). There are many oil-

polluted sites over the World, such as the site of the 1991 Haven tanker shipwreck (HAV) in Genoa, Italy; Northwest of Athens, Greece; the Bizerte Lagoon, located in Northern Tunisia; www.nature.com/scientificreports/ Scientific Reports | 5:11651 | DOI: 10.1038/srep11651, as well as the coast west of Alexandria, Egypt; and the Gulf of Aqaba (AQ), along the Jordanian coast at the northern end of the Red Sea (Bargiela et al. 2015).

Shipping accidents have an important effect on the aquatic environment. The consequences include widespread and long-term damage to marine ecosystems, terrestrial life, human health and natural resources.

It is thus necessary to select a befitting cleanup method (Malavota 2005). Natural oil reserves may serve either as substrates for specific microorganisms or alternatively can be extremely toxic to many microbes or other organisms. It is therefore important to assess the influence of crude oil contamination on bacterial communities and to evaluate their potential to degrade petroleum hydrocarbons (Gerdes et al. 2005).

2.6. Microbial Treatment of Crude Oil

2.6.1. Microbial Improvement of Crude Oil

The life activity of microorganisms taking place in crude oil has a notable character of its composition, trace elements and physico-chemical properties; temperature, pH, and as a result regularly alters its exploitation conditions or price. This efficacy could be positive, e.g. reduces viscosity of heavy crude oil promotes and increase of its API (American Petroleum Institute) grade, however, could be negative, e.g. drilling equipment corrosion due to bacterial generation of hydrogen sulfide (Atlas 1981, Mokhatab and Giangiacomo 2006, Wolicka et al. 2009, Wolicka and Borkowski 2012, Korenblum 2012).

MEOR (Microbes-enhance-oil-recovery) include the demulsification of crude oil. In crude oil processing there are two types of emulsions, e.g. oil/water and water/oil. Those emulsions are produced at different stages of crude oil extraction, production, and processing, causing significant operational problems in the industry (Singh et al.

2007). The separation of crude oil from these emulsions is known as demulsification. Usually demulsification methods are: heating, centrifuging, electrical current usage, and the addition of chemical compositions to break up the emulsions. However, the usage of microbiological demulsification techniques during drilling process have been successful, so saving on transportation and on equipment expenses (Lepp Chen et.al. 2006). There are many microorganisms with desirable demulsification attributes including *Acinetobacter calcoaceticus*, *Aeromonas* sp., *Alteromonas* sp., *Bacillus subtilis, Pseudomonas aeruginosa*, *Corynebacterium petrophilium, Rhodococcus aurantiacus, Micrococcus* sp., *Sphingobacterium thalpophilum.* The microorganisms profit of the both hydrophobic-hydrophilic nature of surfactants or the hydrophobic cell surface to dispel emulsifiers from the interfacial surface between water and oil (Singh et al. 2007).

2.6.2. Microbial Degradation of Crude Oil

Crude oil may be discharged onto the lithosphere surface and leads to major danger to the environment. So cases are often related to spilled oil and oil byproducts during oil exploration, processing, and transportation. This type of pollution is generally removed by microorganisms in nature; these microorganisms have the talent to decompose crude oil and oil-derived products (Mokhatab 2006, Wolicka et al. 2009, Wolicka and Borkowski 2012, Korenblum et al. 2012). A certified advantage of biodegradation is the low outlay (difficult and expensive technology is not needed). Additionally, techniques used in bioremediation do not use of chemical compounds that may negatively affect the soil and water. The most important subject is that these techniques are natural and the final products of the microbiological treatment are CO_2 and H_2O. The effectiveness of hydrocarbon degradation by bacteria is not dependent on the number of carbon atoms (Chikere et al. 2011, Jain and Bajpai 2012). Hydrocarbon degradation by the microbial population in environment is influenced by many factors; e.g. content, temperature, organic matter, reaction, humidity, chemical; concentration and toxicity of hydrocarbons and biological factors; microbiological soil potential (enzyme activity, biomass condensation, population variability) that contribute to the degradation of petroleum and individual hydrocarbons.

The rate of biodegradation depends greatly on the composition, state, and the concentration of the oil or hydrocarbons. Salinity and pressure may also influence biodegradation rates in some water environments while moisture and pH may limit biodegradation in soils (John and Okpokwasili 2012, Wolicka and Borkowski 2012, Hesham et al. 2014).

Microorganisms use the crude oil for their growth. The concentration of compounds in petroleum will influence the number of organisms found. It was shown that the higher condensation of gasoline in polluted water was dependent on higher amount of microorganisms (Malavota 2005, Hesham et al. 2014). In many ecosystems, there is beforehand a sufficient domestic microbial community capable of vast oil biodegradation (Capelli at al. 2001, Capello at al. 2007). There are many advantages of using the indigenous microorganisms and not adding microorganisms to degrade hydrocarbons e.g. natural populations have been developed via many years. Microorganisms are adapted to survive and duplicate in this environment. Second, the strength uses hydrocarbons is distributed among a different microbial population. This population occurs in normal ecosystems and either freely or in combination to metabolize multiple hydrocarbons (Chaineau et al. 2005, Coulon et al. 2007).

A generalized arrange of crude oil in order of decreasing biodegradability is represented as follows: n-alkanes > branched alkenes > low-molecular-weight n-alkyl aromatics > monoaromatics > cycloalkanes > polynuclear aromatics >> asphaltenes (Ulrici 2000, van Hamme et al. 2003). Very recently, many microbial ecologists have identified multiple microbial species (Bacteria, Fungi, Algae and Archaea), that are lucrative and highly specialized degraders of hydrocarbons in natural environments. They are called hydrocarbonoclastic and have an important function in the removal of hydrocarbons from polluted habitats. A new review showed out that there are near 80 bacterial genera while 9 are cyanobacterial genera, 103 fungal genera, and 14 algal genera are found also comprise members able to degrade or transform hydrocarbons. In the same way, 56 yeasts species are able to utilize hydrocarbons. They can use hydrocarbons as only source of carbon and energy (Head et al. 2006, Aliakbari et al. 2014).

Bacteria are the most powerful and plentiful in such ecological niches. Some examples of hydrocarbonoclastic microorganisms are *Actinomyces, Pseudomonas, Spirillum, Acinetobacter, Stenotrophomonas, Achromobacter, mycobacterium Nocardia, Bacillus, Burkholderia, Exiguobacterium, Klebsiella, Streptomyces, Flavobacterium, Streptococcus, Alcaligenes, Roseomonas, Capnocytophaga, Corynebacterium, Providencia, Sphingobacterium, and Moraxella* (Bundy et al. 2004, Malavota 2005, Mandri and Lin 2007, Wang and Shao 2013, Mahjoubi et al. 2013).

Hydrocarbonoclastic alkane-degrading bacteria are of the genera *Oleiphilus* (Golyshin et al. 2002), *Oleispira* (Yakimov et al. 2003), *Thalassolituus* (Yakimov et al. 2004), *Acenetobacter* sp. and *Alcanivorax* sp. Participating in the biodegradation of oil spills in several environments (Yakimov et al. 2007, Coulon et al., 2007). A typical soil, gravel or ocean sediment contains 104–106 hydrocarbon degrading microbes per gram. These values can increase considerably in oil-polluted sites (Harayama et al. 2004)

Small molecule (two or three rings) of aromatic hydrocarbons are degraded by many soil bacteria, in aerobic condition, also multiple fungi genera, e.g. *Rhizopus*, *Candida, Psilocybe, Penicillium, Aspergillus* and *Smittum*. The capability to degrade large-molecule (4 or more rings) of PAHs is rather uncommon between bacteria (e.g. *Flavobacterium sp., Pseudomonas putida, P. aeruginosa, P. saccharophila, Rhodococcus sp., Burkholderia cepacia, Stenotrophomonas sp.* and *Mycobacterium* sp.). These compounds are rather degraded by ligninolytic fungi, such as: *Phanerochaete chrysosporium, Bjerkandera* sp., *Pleurotus ostreatu*s, *Trametes versicolor,* and by those tats are non ligninolytic fungi, such as *Cunninghamella elegans, Penicillium janthinellum*, and *Syncephalastrum* sp. (Wolicka et al. 2009). Biodegradation of compounds with five or more aromatic rings depends largely on the activity of the population microorganism.

The chemical reactions involved in metabolism of crude oil are interposed by enzymes (Figure 2.9). Many plasmids contain genes which code for the enzymes required for the isolated pathways significant to bioremediation. Enzymes involved in the degradation of toluene, salicylate, naphthalene, octane etc. have been shown to be plasmid encoded (Romanus et al. 2013). Enzymes involved in the degradation of

petroleum hydrocarbons shown in Table 2.5. (modified from Rojo 2010, Das and Chandran 2010).

Table 2.5. Enzymes involved in biodegradation of petroleum hydrocarbons

Enzyme class	Substrate
Soluble Methane Monooxygenases	C1–C8 alkanes alkenes and cycloalkanes
Particulate Methane Monooxygenases	C1–C5 (halogenated) alkanes and cycloalkanes
AlkB related Alkane Hydroxylases	C5–C16 alkanes, fatty acids, alkyl benzenes, cycloalkanes and so forth
Eukaryotic P450	C10–C16 alkanes, fatty acids
Bacterial P450 oxygenase system	C5–C16 alkanes, cycloalkanes
Dioxygenases	C10–C30 alkanes

The substrate range is approximate; upper and lower limits may vary in different strains.

Metabolic plasmids type carries some genes that help in cell metabolism. Antibiotic resistance, heavy metal resistance, virulence, environmental adaptability and persistence, and metabolic functions that allow utilization of different nutrients are some of the known functions coded by plasmids. Other feature such as plasmid-mediated biodegradation include various toxic substances like toluene and other organic hydrocarbons, salicylate, naphthalene, camphor, xylene, herbicides, octane, and pesticides (Leahy and Colwell 1990, Glazer and Nikaido 2007, Borah and RNS 2015, Weber et al. 2015). Some plasmids have an important role in the conformity of natural microbial populations to the oil and other hydrocarbon degradation. Some degradation,

comprising the breakdown of C5 to C12 n-alkanes, toluene and naphthalene pathways have been extensively distinguished and are usually placed on large catabolic plasmids (John and Okpokwasili 2012, Borah and RNS 2015). Catabolic pathways, which encoded various aromatic hydrocarbon degradation ways, are generally located on plasmids, in spite degradative genes can be determined on either chromosome or plasmid (Coral and Karagoz 2005).

Plasmids are also mainly important in the transformation of new organisms with the enhanced degradative ability. Using molecular biology techniques, it is conceivable to incision pieces of DNA containing genes for particular degradative pathways into plasmids. These plasmids can then be famous into a host organism resulting in a recombinant or genetically engineered microorganisms (GEM) with novel degradative capabilities (Madigan and Martinko 2010).

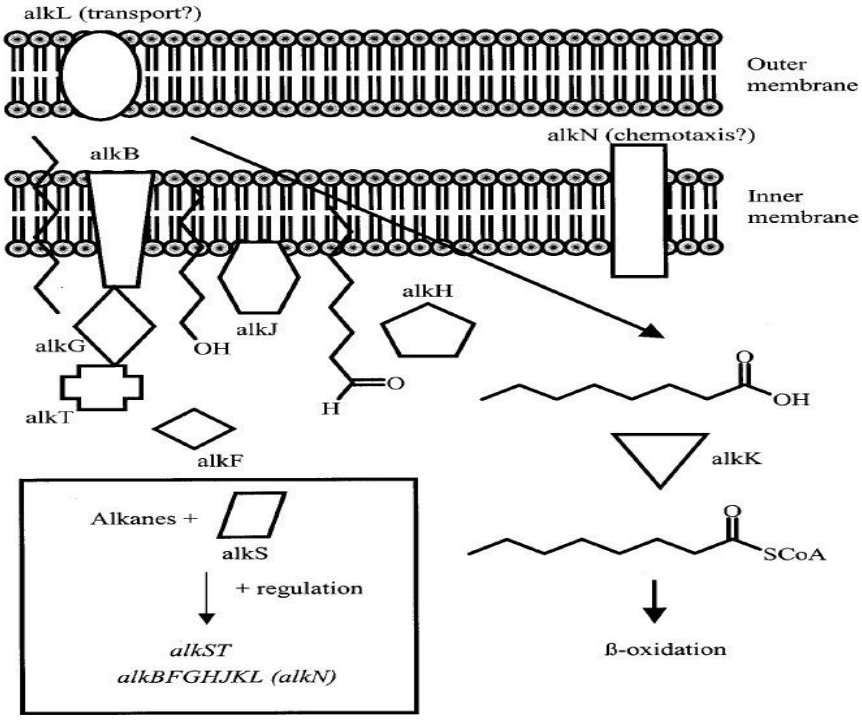

Figure.2.9. Schematic of alkane degradation in gram-negative bacteria, showing the locations and functions of the *alk* gene products. The products include AlkB (alkane hydroxylase), AlkF and AlkG (rubredoxins), AlkH (aldehyde dehydrogenase), AlkJ (alcohol dehydrogenase), AlkK (acyl-CoA synthetase), AlkL (outer membrane protein that may be involved in uptake), AlkN (a methyl-accepting transducer protein that may be involved in chemotaxis), AlkT (rubredoxin reductase), and AlkS (positive regulator of the *alkBFGHIJKL* operon and *alkST* genes) (van Hamme et al. 2003).

2.6.2.1. Metabolic Machinery

Microorganisms are supplied with the metabolic system to use petroleum products as energy origin and a carbon source. The metabolic pathways that hydrocarbon-degrading heterotrophs consumption can be either aerobic (i.e. they employ oxygen as the initial electron acceptor) or anaerobic (i.e. they utilize a serial electron acceptor such as nitrate or sulfate). The quickest, more effective and complete degradation of the majority of organic pollutants is afforded about under aerobic conditions (Kvenvolden and Cooper 2003, Malatova 2005). (Figure 2.10) shows the principle of aerobic degradation of hydrocarbons (Rojo 2010). Alkanes can be metabolized aerobically or anaerobically (Malatova 2005).

Many bacteria are able to degrade n-alkanes product and release surfactants of various chemical nature which let emulsification of the hydrocarbons. On the other hand, alkane metabolism is not straightaway. The exact way in which alkanes arrive at the cell is vagus, while the mechanism (shown in Fig.2.10) may vary depending on the bacterial species selected, the molecular weight of the alkane and the physico-chemical characteristics of the surrounding (Rojo 2010). However, many alkanes are hydrophobic and their water solubility is very low. This is a problem for their attraction. The alkanes hydrophobicity and multiples of the different compounds generated during their metabolism simplify their agglomeration in the cytoplasmic membrane, which can be harmful because it changed membrane fluidity. Furthermore, alkanes are chemically rather static and must be activated forward they can be metabolized; so for activation needs energy-costly process. However, many microorganisms (bacteria, filamentous fungi and yeasts) have acquired the capability to degrade alkanes and utilize them as a carbon source (Fig.2.10) (Rojo 2010, Bundy et al. 2004).

2.6.2.2. Pathways for Degradation of n-Alkanes

Alkanes are saturated hydrocarbons. They can be linear (n-alkanes), branched (Iso-alkanes) or cyclic (Cyclo-alkanes). Alkanes are an important organizer of crude oil, and also organisms such as plants, green algae, bacteria and animals also produce it, where they use as chemoattractants or take part in defending against dehydration.

Accordingly, alkanes are existing in low quantities in most soils and aquatic habitats, but concentrations are low and constant, so it means that biosynthesis and biodegradation processes are present (van Hamme et al. 2003). Alkanes are very reduced molecules containing much carbon and energy source, so act as a good carbon and energy sources to microorganisms which are able to metabolize them. (Prince 1997, Capelli et al. 2001, Rojo 2010).

The complete and very quickly biodegradation of most organic pollutants is conducted under aerobic circumstances. (Figure 2.10) explains the basic principle of hydrocarbons aerobic degradation (Das 2010). The starting intracellular attack of organic pollutants is an oxidation reaction (activation), also the involvement of oxygen, is the enzymatic key reaction catalyzed by oxygenases and peroxidases. The enzymes that primarily oxidize these alkanes are related to methane monooxygenases (Hamamura et al. 1999). Two different forms of methane monooxygenases. All methanotrophs create a membrane-bound particulate form of methane monooxygenase (pMMO) that oxidizes few of alkanes, on the other hand some methanotrophs produce additionally a soluble methane monooxygenase (sMMO) which is active on a larger view of substrates and oxidizes C1–C7 alkanes to the corresponding alcohols (Das 2010, Rojo 2010).

In peripheral degradation pathways transform organic pollutants gradually into central intermediary metabolism intermediates. Aerobic degradation of n-alkanes start up by the terminal methyl group oxidation to form a primary alcohol, that oxidized to the corresponding aldehyde, then finally into a fatty acid. Fatty acids are conjugated to CoA and subsequently processed by β-oxidation to generate acetyl-CoA. Biosynthesis of cell biomass occurs from the central precursor metabolites, for example, acetyl-CoA, succinate, pyruvate. Sugars required for various biosynthesis and growth are synthesized by gluconeogenesis. The degradation of petroleum hydrocarbons can be mediated by specific enzyme system (van Hamme et al. 2003, Wentzel et al. 2007, Das 2010, Rojo 2010).

Microorganisms degrading short-chain-longitude alkanes (C2–C4) include enzymes relevant to methane monooxygenases. Strains breaking medium-chain-length alkanes (C5–C11), or long-chain-length alkanes (>C12), often contain integral membrane, non-heme iron monooxygenases relative to the well-characterized

Pseudomonas putida GPo1. AlkB alkane hydroxylase. Some strains contain alkane hydroxylating enzymes that belong to a family of soluble cytochrome P-450s and that against C5-C11 alkanes . In additions, several bacterial strains have been reported to assimilate alkanes larger than C20. However the enzymes responsible for the oxidation of long-chain alkanes are still poorly characterized and only recently have started to be characterized (Rojo 2010, Xia et al. 2014). Cytochrome P450 alkane hydroxylases represents a super family of ubiquitous Heme-thiolate Monooxygenases that play a major role in oil microbial degradation, chlorinated hydrocarbons, fuel additives, and many other compounds. Depending on the chain length, enzyme systems are required to introduce oxygen in the substrate to initiate biodegradation (Das 2010).

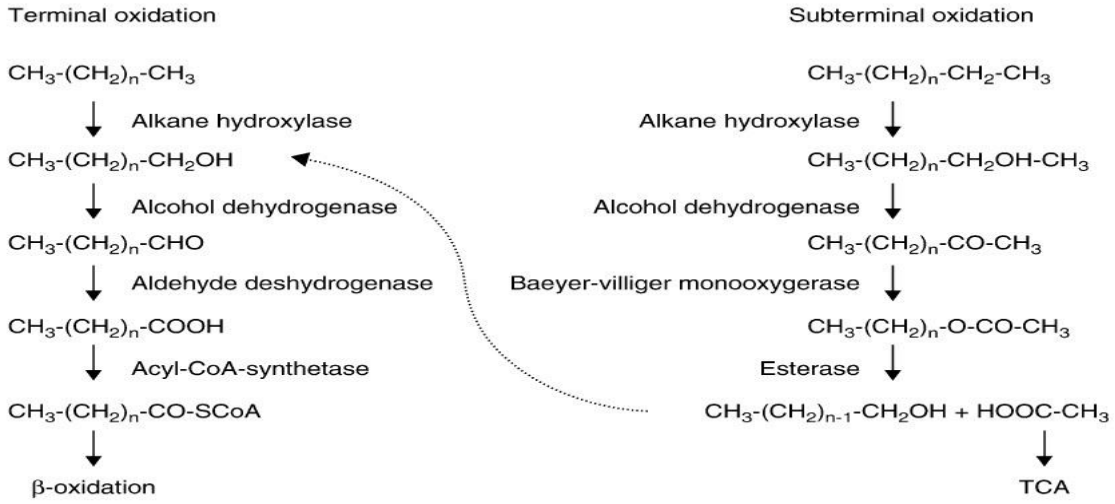

Figure 2. 10. Most frequent pathways for the degradation of n-alkanes by terminal and subterminal oxidation.

2.7. Summary of Relevant Literature by Subject Coverage

Iversen et al. (2007, 2008) reported a collection of 210 *E. sakazakii* isolates applying 16S rRNA gene sequencing in combination with DNA–DNA hybridizations, ribotyping and amplified fragment length polymorphisms (AFLP). Their results not only form the basis for the classification of this pathogen into the new *Cronobacter* genus but also clarify species designations. Based on the new nomenclature they have

characterized a collection of isolates, reclassifying as novel species and subspecies within a proposed novel genus, *Cronobacter* gen. nov.

Caprette (2009) isolated Motile, facultatively anaerobic. Oxidase negative, strongly catalase positive. Negative for indole production and mixed acid fermentation of glucose (methyl red test). Liquifies gelatin at 22 °C, etc. All studies were consistent with the description of the genus in Bergey's Manual p. 477 (Holt, 1984) and characteristics noted for *S. marcescens*

Wittaker (2011) determined the cellular fatty acid (CFA) profiles of six species in the new genus *Cronobacter (Enterobacter sakazakii)*. The six various species are *C. sakazakii, C. dublinensis, C. malonaticus, C. turicensis, C. muytjensii,* and C. *genomo*. Analysis of FAMEs from *Cronobacter* strains grown on BHI agar by a rapid GC-FID method is a delicate process for the identification of these organisms, and this analytical method provides a procedure for the differentiation of strains from closely related *Cronobacter* species.

Franzetti and Scarpellini (2007) isolated *Pseudomonas* sp. from different foods. Strains identified by 16S rRNA gene sequense, then grouped the isolates into 6 clusters: *Pseudomonas fluorescens* (cluster I), *Pseudomonas fragi* (cluster II and V) *Pseudomonas migulae* (cluster III), *Pseudomonas aeruginosa* (cluster IV) and *Pseudomonas chicorii* (cluster VI).

Ali (2015)recorded that bacteriological investigation were carried out for forty patients presented with diabetic foot wounds, during Jan. 2004 – Jan. 2005. All isolates that include nine genera. *Serratia marcescens* and *Serratia plymuthca* were one of the isolates which reported for the first time to be involved in diabetic foot infection. *Serratia marcescens* was found to tolerate 4% glucose. They were resistant to penicillin, 83% were resistant to chloramphenicol and only 42.3% of the isolates were found sensitive to gentamycin.

Khosravi et al. (2015) isolated *Acinetobacter* sp. Based on 16S rRNA and *rpoB* genes analysis separately. So, the majority of *Acinetobacter* spp. isolates, were identified as: *A. baumannii* (131 isolates, 66%), *A. calcoaceticus* (9 isolates, 4.5%), and *A. genomosp* 16 (8 isolates, 4%). The rest of identifying species showed the lower frequencies. In susceptibility test, more isolates, presented high antibiotic resistance of

90% to ceftriaxone, piperacillin, piperacillin tazobactam, amikacin, and 81% to ciprofloxacin.

Cormack and Fraile (1997) analyzed antarctic soil chronically exposed to gas-oil. Gram-negative, non-motile, non-spore forming isolated bacteria belonging to the genus *Acinetobacter* sp. *Acinetobacter ADH-1* had essential hydrocarbon degrading characteristics that indicated no difference at a primary pH of 7.0, 7.5 and 8.0. The optimum temperature was between 25-30 °C, but the strain was able to grow on n-dodecane at 4 °C. Hexadecane and dodecane were used as a carbon source. They also used shorter chain hexane, heptane, octane which are not found in environment.

Twenty-five bacterial strains isolated by Di Cello et al. (1997) at Venice lagoon were used for the biodegradation of n-alkanes, n-alkanols, n-alkanals and n-alkanoates. The isolates were grouped into 7 spieces by amplified ribosomal DNA restriction analysis (ARDRA) procedure, and identification by 16S rDNA sequencing process. Genetic variability among strains was seen by random amplified polymorphic DNA (RAPD). Only strains of new species *Acinetobacter venetianus* showed growth in n-alkanes (C10, C14 and C20) and their respective oxidation products as the only carbon sources. The other three species identified were thrived on n-alkane oxidation products (n-alkanols, n-alkanals, n-alkanoates). Plasmid constituent analysis showed that only *A. venetianus* strains harboured plasmids. These plasmids contained sequences homologous to the *Pseudomonas oleovorans alkBFGH* genes.

Microorganisms that use hydrocarbons; *Aeromonas, Serratia, Micrococcus*, *Pseudomonas, Bacillus, Aspergillus, Candida, Proteus, Penicillium,* and *Geotrichum* were isolated using enrichment techniques from water and sediment samples collected of oil spill locations in the Niger Delta (Benka-Coker and Ekundayo 1997). Similarly, the ability of two bacterial isolates *Acinetobacter calcoaceticus* COU-27 and *Serratia marcescens* OCS-21 and yeast isolate; *Candida tropicalis* PFS-95 degrading crude oil isolated from pipeline crude oil in Niger were also reported by Ijah (1998).

Huy et al. (1999) isolated the four petroleum-degrading bacterial strains. They were isolated from various oil-contaminated sites in Vietnam. Determination of the nucleotide sequence of the gene encoding 16S rRNA allowed to be identified with one of them as *Acinetobacter* sp. and three other stains as *Pseudomonas* sp. Among the four

isolates, *Acinetobacter* sp. was most effective in degrading crude oil: in 1 day, it degraded 95% of the crude oil in the culture medium (5%, v/v).

The *Acinetobacter* sp. E11, isolated from Port Dickson Beach, Malaysia, was capable of growing in media containing crude oil as the sole carbon and energy origin It used aliphatic hydrocarbons only in the long-chain alkanes tested (pentadecane, dodecane and hexadecane) for growth, while aromatic and cyclic hydrocarbons inhibited their growth. Gas chromatography analysis showed that the bacterium was able to degrade more than 60% of the hydrocarbons in the crude oil in 15 days at 37°C compared to the uninoculated media (Razak et al. 1999).

Yuste et al. (2000) isolated and characterized seven bacterial strains that can use a residue from crude oil processing as a carbon and energy source. The residue was a complex mixture of high molecular mass compounds, such as saturated, aromatic and polycyclic aromatic hydrocarbons (PAHs). The isolates were Gram-negative cocci / rod-shaped bacteria from oil-contaminated soil. According to fatty acid profile and 16S rRNA sequence analysis the identified bacteria *Acinetobacter calcoaceticus* broke down alkanes straight-chain in C10-C34 range.

Capelli et al. (2001) examined the bioremediation of the hydrocarbons from crude oil extraction waste. Bacteria (indigenous) to test biodegradation was inoculated for one month and half. There was a total reduction of the hydrocarbon content by ~70% of its initial value at the end of the experiments. Saturated and aromatic hydrocarbons were the most readily degraded fractions with, respectively, ~70% and ~60%. Among isolates from several areas of Hokkaido, Japan, 2 strains were identified as *Pseudomonas aeruginosa* and *Serratia marcescens* species, respectively. They showed a relatively high capacity and wide spectrum to degrade the hydrocarbons in gasoline, kerosene, diesel, and lubricating oil.

Bacterial strains isolated from Western New York State capable of degrading hydrocarbons belonged to the genera *Serratia marcescens, Acinetobacter baumannii* and *Pseudomonas* sp. Carbon dioxide evolution experiments were used as the major indicator of microbial degradation of oil in biometric flasks. The chemical composition of the residual oil was determined by gas-chromatographic techniques. *Pseudomonas emerges* was responsible for biodegrading maximum of the aromatics in gasoline,

however the yield in degrading aromatics hydrocarbons can vary amid strains. The most extensively characterized polycyclic aromatic hydrocarbons degradation pathway was encoded by the NAH7 plasmid from *Pseudomonas putida* (Malatova 2005).

Oboh et al. (2006) isolated fifteen hydrocarbon degrading bacteria and fungal species to use in bioremediation of petroleum contaminated systems in Nigeria. The predominant species belonged to the genera *Pseudomonas* and *Aspergillus*. The ability of *Pseudomomnas stutzeri*, *Pseudomonas mullei* and *Alcaligenes* sp. Were found to degrade naphthalene, kerosene and diesel. The results show a maximal increase in optical densities and total viable counts concomitant with a decrease in pH of the culture media. All the isolates utilized the hydrocarbons as sole carbon and energy sources equally well.

Three bacterial isolates able of using engine-oil as a carbon source were isolated from contaminated soils using the enrichment technique by Mandri and Lin (2007). The isolates were found to be *Flavobacterium* sp., *Acinetobacterium calcoaceticum* and *Pseudomonas aeruginosa* determined by biochemical tests and 16S rRNA sequencing. The geometric analysis showed that *A. calcoaceticum* and a consortium of the isolates were able of using 80% and 90% of used engine oil, respectively, at laboratory conditions at 30°C and 160 rpm with Bushnell-Haas media in a 4 week period. An increasing in oil biodegradation was related to an increasing in cell number showing that the bacterial isolates were responsible for the oil degradation. All isolates were able of biodegrading the n-paraffin up to 80% in a 2 week period. The optimum temperatures at which biodegradation occurred was at 30–37°C.

Moreover, in another study, Microbial populations' inhabitants in crude petroleum contaminated soils were found to belong to the genus *Pseudomonas* which was represented by the following species: *P. maltophilia, P. putidae* and *P. mallaie*. Other bacterial genera were also identified namely: *Enterobacter, Acinetobacter, Bacillus, Micrococcus, Corynebacterium* and *Listeria*. Also, the prevalence of members of the genus *Pseudomonas* in the hydrocarbon-contaminated soils with the occurrence of other genera, *Enterobacter* and *Acinetobacter* were reported (Saadoun 2008).

Kim and Loessner (2008) collected a non motile and rod shaped bacterium, designated strain B1T from forest soil at Mt. Baekwoon, Republic of Korea. Cells were Gram-negative, catalase-positive, and oxidase-negative. A phylogenetic tree based on 16S rRNA gene sequences revealed that strain B1T constituted a lineage within the genus *Acinetobacter* and was related to *A. baylyi* DSM 14961T (98.6% sequence similarity), followed by *A. baumannii* DSM 30007T (97.4%), *A. calcoaceticus* DSM 30006T (97.0%) and 3 genomic species (96.8~7.6%). Phenotypic characteristics, gyrB gene sequence analysis and DNA-DNA relatedness data distinguished strain B1T from type strains of *A. baylyi, A. baumannii, and A. calcoaceticus*.

Al-saleh et al. (2009) isolated total of 272 crude oil-biodegrading bacteria from 7 locations along the coast of Kuwait. The analysis of the 16S rDNA sequences of isolated bacteria revealed the predominance of six bacterial genera: *Pseudomonas, Bacillus, Staphylococcus, Acinetobacter, Kocuria* and *Micrococcus*. Using 16S-RFLP analyses to analyze the diversity of the dominant crude oil-degrading bacterial genera, four phylotypes of *Pseudomonas* sp. and 7 phylotypes of *Bacillus* sp. were determined. This suggested a wide diversity of crude oil-degrading bacterial population at the strain level, but low diversity at the genus level.

Crude oil samples with high- and low-water content from two offshore platforms (PA and PB) in Campos Basin, Brazil, were assessed for bacterial communities by 16S rRNA gene-based clone libraries. RDP Classifier was utilized to assess 156 clones within 4 libraries obtained from two platforms. The clone sequences were greatly affiliated with *Gammaproteobacteria* (78.2% of the total clones); however, clones associated with *Betaproteobacteria* (10.9%), *Alphaproteobacteria* (9%), and *Firmicutes* (1.9%) were also identified. *Pseudomonadaceae* was the most common family related with these clone sequences (Kurenblum et al. 2012).

John and Okpokwasili (2012) indicated that certain plasmids played a major role in the adaptation of natural microbial populations to oil and other hydrocarbons. Some degradation, such as breakdown of C5 to C12 n-alkanes, naphthalene and toluene pathways were extensively characterized and were generally located on large catabolic plasmids. Similarly, various environmental isolates *Arthrobacter* sp. *Acinetobacter* sp.

and *Alcaligenes* sp. were found to degrade both alkanes and naphthalene, although the genes and catabolic pathways responsible were not described

Romanus et al. (2013) determined the role of plasmid-borne genes in the biodegradation of Chevron Escravos Crude Oil in bacteria by plasmid extraction and curing, transformation experiments and biodegradation. Plasmid extraction studies showed that two of the six selected crude oil degrading bacterial isolates had two plasmids each. The isolates were *Klebsiella pneumoniae* from ripe pawpaw fruit and *Serratia marscencens* from oil palm mill effluent. The plasmids were of small (300bp) and large (>1.5kbp) sizes. The results also showed that the isolates were successfully cured of plasmids using 1% Sodium Dodecyl Sulphate (SDS). The transformation experiment using the extracted plasmid DNA and competent *Escherichia coli* K12 DH1 cells was successful. The percentage degradation of crude oil at 37 ^{o}C by *E. coli* K12 DH1 transformed with the plasmid DNA from *Klebsiella pneumoniae* was 93.03%, while that transformed with the plasmid DNA from *Serratia marscencens* degraded 76.97% of the crude oil. It was observed that loss of plasmids by *Klebsiella pneumoniae* and *Serratia marscencens* did not lead to complete loss of their degradative abilities. It only resulted in reduction in their degradation potential.

A study conducted by Mhjoubi et al. (2013), 125 strains, adapted to grow on minimal medium supplemented with crude oil, was obtained from contaminated sediments and seawater from a refinery harbor of the Bizerte coast in Tunisia. The variability of the bacterial collection was analyzed by amplification of the internal transcribed spacers between the 16S and the 23S rRNA genes (ITS-PCR) and by 16S rRNA sequencing. Thirty six distinct ITS haplotypes were discovered on agarose matrix. Partial 16S rRNA gene sequencing performed on 50 isolates showed high level of identity with known sequences. Strains were related to *Acinetobacter, Achromobacter, Agrobacterium, Bacilllus, Ochrabactrum, Novosphingobium, Sphingobium, Microbacterium, Brevundimonas, Stenotrophomonas, Luteibacter, Gordonia, Rhodococcus, Kocuria* and *Pseudomonas* genera. *Stenotrophomons and Acinetobacter* were found to be the most abundant species characterized by a marked microdiversity.

Pseudomonas aeruginosa, Bacillus subtilis, and *Acinetobacter lwoffi* isolated from petroleum contaminated water and soil samples to degrade crude oil, separately and in a mixed bacterial consortium. Capillary gas chromatography was used for testing the effect of those bacterial species on the biodegradation of crude oil. Individual bacterial cultures showed less growth and degradation than did the mixed bacterial consortium. At temperature 22 ^{o}C, the mixed bacterial consortium degraded a maximum of 88.5% of Egyptian crude oil after 28 days of incubation. This was followed by 77.8% by *Pseudomonas aeruginosa*, 76.7% by *Bacillus subtilis*, and 74.3% by *Acinetobacter lwoffi*. The results showed that the those bacterial isolates could be effective in oil spills biodegradation individually and had better biodegradation ability when they were used as mixed bacterial consortia (Al-Wasify et al. 2014).

Hydrocarbon degrading bacteria from the petrol contaminated soil of Karachi were isolated by Mujahid et al. (2015) to study their biodegradation abilities of aromatic hydrocarbons. One dozen of bacterial strains were isolated by culture enrichment technique in Bushnell Hass medium in the presence of petroleum. The isolated bacterial strains were capable of degradeing aromatic hydrocarbons, especially *Pseudomonas* sp. SA044, biodegrading all the tested 5 aromatic hydrocarbons while *Burkholderia* sp., *Ralstonia* sp., *Stenotrophomonas* sp., *Micrococcus* sp. and *Staphylococcus* sp. degraded 3 or more aromatic hydrocarbons. Naphthalene and phenanthrene by these isolates were the most degraded aromatic hydrocarbons.

Faidah et al. (2015) studied *Serratia marcescans strains* by current phenotypic procedure and antibiotic susceptibility techniques. The isolated strains showed resistance to ampicillin (97.5%), Cefoxitin (90%), Tetracycline (86%), Piperacillin/Tazobactam (63%), Moxifloxacin (60%), Ceftazidime (59%), Fosfomycin (58%) and Tigecycline (53%). Isolates remained susceptible to Imipenem (85%), Meropenem (73%), Gentamycin (69%), Amikacin (67%), Cefepime (67%), Trimethoprim/Sulfamethoxazole (59%), Ertapenem (57%), Ciprofloxacin (51%) and Cefotaxime (41%). The most active antibiotic being Imipenem and Meropenem. Resistance to other tested antibiotics was variable with significantly higher resistance to ampicillin, cefoxitin and Tetracycline.

3. MATERIAL AND METHODS

3.1.1. Collection of Samples

In this study, the samples were collected from oil-contaminated soils at 8 different areas of Northern Iraq (Kurdistan). The samples were taken from the oil-contaminated sites around the petrol wells (1. Zakho T2; 2. Zakho T20; 3. Bardarash B4; 4. Bardarash B1; 5 and 6. Mreba; 7. Koye and 8. Atrush) in March 2014 (See Figure 3A and 3B for samples sites in Zakho). First, about 2 cm of topsoil was removed at contaminated locations and then a certain amount of contaminated soil was collected by sterile spatula, placed in sterile Falcon and added 10 ml of distilled water for each sample. Sludge samples were maintained in cold conditions at 4 °C until they brought to the laboratory. In total, 17 varieties of bacteria isolated, 5 of these varieties were selected due to utilizing hydrocarbons for growth. Methods was done according to the Acer (2014) methods.

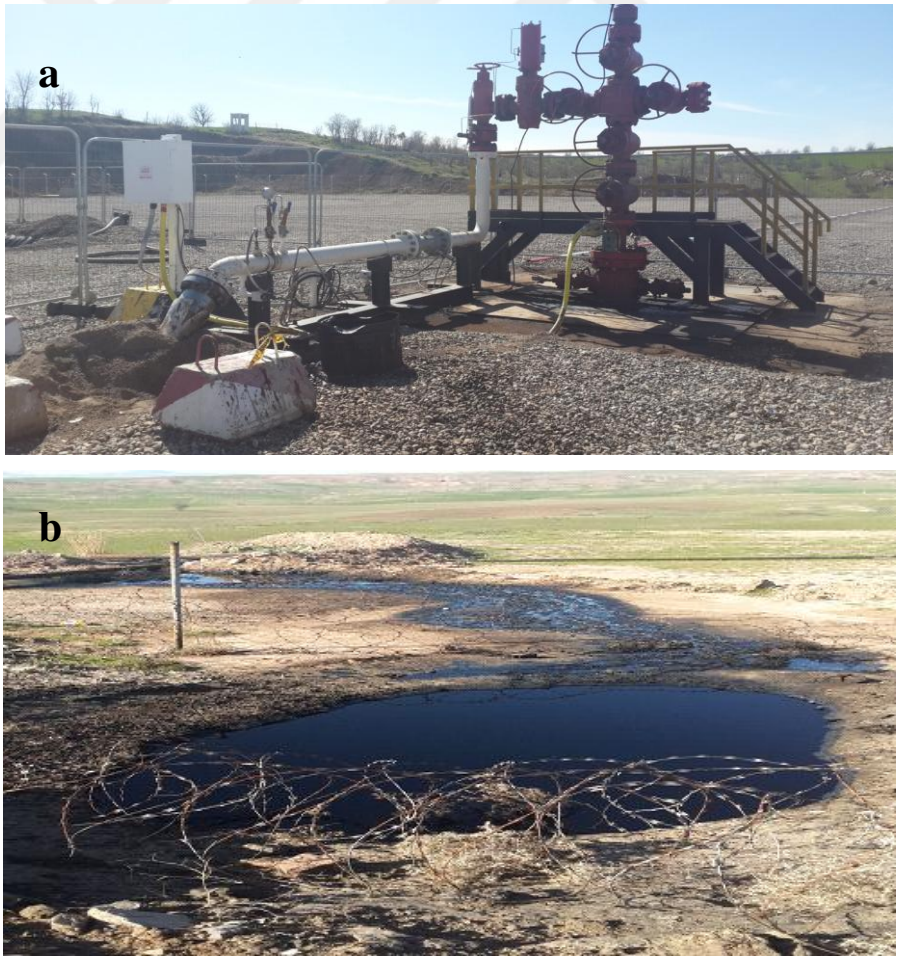

Figure 3.1. Samples were taken from Oil wells (a and b) in Zakho region.

3.1.2. Media Compositions Used

BSM:

5.0 ml of Sodium phosphate buffer (pH 7.0)

19.7 g /L K_2HPO_4, 8.7 g /L KH_2PO_4, 3,744 g /L Na_2HPO_4 .$7H_2O$, 5.0 g /L NH_4Cl

1.0 ml of Calcium chloride solution (36.4 g /L $CaCl_2$)

3.0 ml Magnesium sulfate solution (22.5 g /L $MgSO_4$)

1.0 ml of Ferric chloride solution (0.25 g /L $FeCl_3$)

1.0 ml of trace mineral elements solution ($MgSO_4$ (39.9 mg /L), $ZnSO_4$. $7H_2O$ (42.8mg /L) and

$(NH_4)_6mO_7O_{24}$. $4H_2O$ (34.7 mg /L))

Nutrient broth (Merck): 13 g

Purified water: 1000 ml

Solid media:

Nutrient Agar (NA):

Agar (Sigma): 15 g

Nutrient Broth (NB): 8g

Distilled water: 1000 ml

Mineral salt media (MSM) with the following composition:

(g/L): 0.2 $MgSO_4$, 0.02 $CaCl_2$, 1.0 KH_2PO_4, 1.0 K_2HPO_4, 1.0

NH_4NO_3, and 0.05 $FeCl_3$

3.2. Methods

3.2.1. Isolation of Microorganisms

Samples remained at cold and sterile ambient at the laboratory. One gram of sample weighed and added to BSM medium. Medium (BSM) was prepared for the cultivation of these samples, containing crude oil. Before adding crude oil to the medium, crude oil was sterilized for 21 min at 121°C in an autoclave twice, after which it was passed through a sterile filter with a diameter of 0.2 µm. 1% of the crude oil was added to the medium as the sole source of carbon. One gram of sample was inoculated into the medium containing 1% crude oil. It was incubated for 2 days at 30 °C at 120 rpm. After 2 days, 1 mL of the sample was taken from the culture, transferred to the new medium containing crude oil. This process was repeated twice. Then, 1 mL of culture were inoculated into NB medium for 24 h at 30 °C at 120 rpm. After 24 h of incubation serial dilution-agar plating procedure was applied to get a single colony (0.1 ml of a series of dilutions: 10-1, 10-2, 10-3 and 10-4 was spread on nutrient agar plates and incubated at 30 °C for 24 h). After incubation, the grown bacteria from single colonies on petri dishes were taken and streaked on the new NBA to obtain pure cultures. After 24 h incubation, pure colonies were isolated by using one colony isolation method. Isolated colonies were stored at 4 °C. Bacterial isolates not used in biodegradable experiments were mixed with 50% glycerol and stored at -7 °C for future use.

3.2.2. Determination of Optimum Growth Conditions

25 ml NB in 250 mL conical flask was used to determine the optimal growth temperature, pH and course- time. The isolated bacteria were incubated in the medium at temperatures ranging from 5-55 ºC for 24 hours. After the incubation period, 1 mL of cultures were taken and the optical density was measured by spectrophotometer at 600 nm. The optimum pH for growth was detected by using pH ranges 4.0-12.0, in NB medium of 25 mL. After the incubation, the OD values were measured at 600 nm.

Following the determination of optimum temperature and pH, the time course experiments of growth carried out. At each several hours intervals (at 3-96 hours), 1mL of each sample was taken and the growth rate was measured on spectrophotometer at 600 nm.

3.3. Morphological, Physiological and Biochemical Tests

3.3.1. Gram Staining

Some loop full of water was added to the slide. A loop of a colony from the Petri dish culture with an inoculation loop transferred and spread on the slide. Bacteria gently was spread to obtain a thin layer over a circle of 1 to 1.5 cm in diameter. The culture was fixed through a gentle fire. Following addition of Crystal violet stain on the culture, waiting for 60 sec; the stain was removed off and lightly was rinsed the extra stain with a plenty of distilled water from a faucet or a plastic water bottle. The lugol solution was added on the spot, just to cover the fixed culture, allowed to remain for 2 min. The lugol solution was cleaned on the slide with stream water. The extra water was bounced off from the surface. Then, a some drops of decolorizer were added, so, the solution drops down on the slide. It was washed off with water after 5 sec. The exact time to stop was when the solvent was no longer colored as it flows on the slide. Further delay will cause wealth decolorization in the gram-positive cells and the purpose of staining will be craven. After that, fuchsine solution was added on the slide for 40 to 60 sec. The solution rinsed off with water. The smear was covered with Absorbent paper to remove the excess water on it.

3.3.2. Spore Painting

Bacteria were developed for 24 h at 30 °C on the NA. At the end of the incubation period, the bacterial culture was kept by 4 °C for 3 nights. Then, the stain film was fixed with powerful carbol fuchsin for 3-5 min, the stain was removed off and lightly was rinsed the extra stain with a current of water from a faucet or a plastic water

bottle. The culture was fixed through a gentle fire. Then 10% nitric acid was used for 10 sec again, it was washed with water. Counterstain did with 1% hydrous methylene blue for 2 min. Then it was rinsed and dried smear. The slide was checked under the oil immersion lens (1 000X) to see endospores.

3.3.3. Mobility and Oxygen Requirement

This test was applied to determine the capability of the actions of microorganisms. Bacterial isolates inoculated from the fresh culture and incubated one night. 0.4% semi-solid agarose medium was used in capillary tubes. Vertical single stab of bacteria prepared with straight needle and allowed to incubate for 5 days at optimum temperature. Results motility was recognized visually by diffuse growth that spread from the line of inoculation, if bacteria were reproduced along the line of inoculation medium and micro-organisms spreading at straight or left branch or go to the bottom this is accepted as a positive result.

3.3.4. Starch Hydrolysis Test

The medium employed 2% starch in nutrient agar. One loop of inoculum from a pure culture was streaked on a sterile plate of starch agar. The inoculated plates were incubated at 35 °C for 24 h, then Iodine reagent was added to flood the development. Clear halos surrounding colonies were showing, so it was positive for their capability to digest the starch and thus mean the presence of alpha-amylase.

3.3.5. Catalase Test

Three ml of culture incubated for 24 hours was transferred to a clean glass tube, then 2-3 drops of 3% H_2O_2 were placed into the tubes. A positive result is the quick release of oxygen (within 5-10 sec.) seen as bubbles. No bubbles means a negative result or just a few separated bubbles. Also, 15% H_2O_2 solution is used for the identification of anaerobic bacteria.

3.3.6. Gelatin Hydrolysis Test

The standard and most commonly used method is the nutrient gelatin stab method. This method designs an easy and perfect interpretation of results (medium liquefaction). In this method, an inoculum of an 18- to 24-hour-old bacteria strains were stab-inoculated into tubes including nutrient gelatin. The inoculated tubes and a non-inoculated control tube are incubated at the bacteria's optimal growth temperature, for up to 1 week, and checked daily for gelatin liquefaction. Gelatin normally liquefies at 28°C and above, so to confirm that liquefaction due to gelatinase activity, the tubes were placed in an ice bath for 30 min, afterwards tubes are tilted to observe if the gelatin has been hydrolyzed. Hydrolyzed gelatin will result in liquefaction when exposed to cold temperature (ice bath), although the uninoculated control medium will stay solid. The hydrolysis of gelatin means the secretion of gelatinase by the microorganism into the medium.

3.3.7. Oxidase Activity Test

A filter paper soaked with the substrate tetramethyl-p-phenylenediamine dihydrochloride which dissolved in distilled water is used for this test. The colony to be tested is picked with a platinum loop and smear on the filter paper. The inoculated area of paper turns into deep blue or purple colour within 10-30 sec, if positive.

3.3.8. Degradation of Tyrosine

L-tyrosine (0.5 g) solved in 10 ml of distill water. 90 mL Agarose NB medium was prepared. After that both of them separately sterilized for 20 minutes at 121 °C. L-Tyrosine and NB medium were mixed at sterile conditions. 10ml of NB-Tyrosine Medium were added to falcon tubes 50 mL for each strain. The tubes were inoculated with a culture of 18- to 24-h old. The tubes were kept in the incubator for 10 days and evaluated by comparing with the control tube. The test is accepted as positive when a dark color, forms compared to the control.

3.3.9. Urease Test

In this test, microorganisms synthesized urease enzyme to the hydrolysis of urea. Urease hydrolysis test applied to determine the genus and species of bacteria. 2 g pure urea and 2.4 g of urea agar was dissolved in 80 mL and 20 mL, respectively, in distilled water. Urea agar autoclaved, but 80 ml pure urea was passed through a sterile filter of 0.2 µm pore diameter. Then it was added to the autoclaved urea agar. 5 mL of mixed pure urea and urea agar was added to the capillary test tubes. The tubes were allowed to solidify in an inclined position. The surface of a urea agar slant was streaked with a part of well-isolated colony. The tubes were incubated at the 35 ^{o}C for 48 hours to 7 days. If organism provides urease enzyme, the color of the slant changes from light orange to magenta or bright pink. If organisms do not produce urease, the agar slant remains light orange (no color change). An increase in pH due to the production of ammonia results in a color conversion from yellow (pH 6.8) to bright pink (pH 8.2)

3.3.10. Citrate Test

The medium citrate participating as the carbon origin for microorganisms, it applied in the identification, capability to detect genera and species of bacteria. For this test, 2.25 g Simmons citrate agar was dissolved in 100 mL of distilled water and it was autoclaved. 5 mL of it was added to the capillary test tube. The tubes were permitted to solidify in an inclined situation. Simmons Citrate Agar was Inoculated on the slant by touching the tip of a needle to a colony that was 18 to 24 h old. They were Incubated at 35 to 37 ^{o}C for 18 to 24 h. Some organisms were required for 2-5 days of incubation due to their limited rate of development of citrate medium. The development of blue color saw; denoting alkalinization.

3.3.11. Sodium Azide Sensitivity

Sodium azide (0.02%) is dissolved in a NB liquid medium (25 mL in a flask). Then, it is autoclaved for 20 min at 121 ^{o}C. 1 ml of culture inoculum was added and incubated for 1-2 days. The results of sensitivity to sodium azide were obtained by

measuring the growth with spectrophotometer at 600 nm, and finally compared to controls in the absence of sodium azide.

3.3.12. NaCl Tolerance

To determine bacterial tolerance 25 mL of NB medium, including various NaCl concentrations: 0.5%, 1%, 2%, 5%, 7% and 10% were prepared and autoclaved for 20 min at 121 ^{o}C. 1 ml of culture inoculum was added and incubated for one day. The results of NaCl tolerance were obtained by measuring the growth with spectrophotometer at 600 nm, and finally compared to controls in the absence of NaCl.

3.3.13. Lysozyme Sensitivity

Lysozyme (0.001%) is dissolved in a NB liquid medium (25ml in flask) and autoclaved for 20 min at 121 ^{o}C. 1 ml of culture inoculum was added and incubated for 1-2 days. The lysozyme sensitivity was determined by measuring the growth with spectrophotometer at 600 nm, and finally compared to controls in the lysozyme.

3.3.14. Antibiotic Sensitivity Test

Antibiotic disk diffusion method was used to determine the antimicrobial susceptibility profiles of the bacterial isolates. Antibiotic multi-disks used were consisted of Fucidic acid (10 µg), Chloramphenicol (30 µg), Kanamycin (5 µg), Novobiocin (5 µg), Penicillin (2, 10 units), Nystatin (100 units), Gentamicin (10 µg), Streotomycin (10 µg), Tilmecocin (15 µg), Bacitracin (10 unit). Ampicillin (10 µg), Lincomycin (15 µg), Tetracyclin (30 µg), Neomycin (10 µg). The NB agar was inoculated by streaking bacterial strains. The antibiotic disks were put on nutrient agar in petri dishes, using sterile forceps and sufficiently separated from each other in order to prevent overlapping of the inhibition zones. The agar plates were left on the bench for 30 min to allow for diffusion of the antibiotics and the plates were incubated as inverted at 30 ^{o}C for 48 h. Results were recorded by measuring the zone of inhibition.

3.4. 16S rRNA Gene Analysis

The final isolates selected as the hydrocarbon degraders were identified by 16s rRNA gene sequence analysis. Partial 16S rRNA analyses of the isolates obtained were conducted by Ref-Gen (METU Technocity/Ankara). The sequences consisting of about 1000 nucleotides of the 16S rRNA gene were determined (2B: 976, 3B: 805, 8F: 797, 4A: 856, 6C: 796 bp). These sequences were compared with those available in the GenBank database using a BLAST search (http://www.ncbi.nlm.nih.gov/blast/). The 16S rRNA gene sequences of the species most closely related to our strain were retrieved from the database. Phylogenetic tree was constructed using the software package CLCu. Sequence Viewer 6.0(http://www.clcbio.com/products/clcsequence-viewer/).

3.5. Degradation Test and GC Analyses of Petroleum Products

GC-MS (gas chromatography-mass spectrometry) was used to determine which isolates exhibited n-alkane degrading activity and n-alkane degrading and oil-emulsifying activities.

Pre-cultured bacterial cells were incubated overnight at 30 ^{o}C in NB medium. They were transferred to a 250 ml Erlenmeyer flask which containing 25 ml of BSM plus with 1% crude oil. Cells were grown for 10 days at 30 ^{o}C with shaking at 120 rpm, and petroleum hydrocarbons remaining in the culture medium were determined the ratio of degradation of crude oil.

Following biodegradation, gas chromatography (GC) using a flame ionization detector (FID; HP 6850; Hewlett Packard, xx, xx) and equipped with a PONA quartz capillary column (100 m £ 0.250 mm [inner diameter, i.d.] £0.50 m) was used in order to 185 analyze the n-alkane fraction of crude oil. Helium was used as a carrier gas in order to perform Split injections. The column temperature increased from 60 ^{o}C to 320 ^{o}C at a rate of 3 ^{o}C per minute. An injector temperature of 300 190 ^{o}C and a detector temperature of 350 ^{o}C were used. In order to detect individual components of the n-alkane fraction, matching the retention time with authentic standards was utilized.

Analysis conditions are as follows.

Injector temperature: 300 ° C Detector temperature: 350°C

Total analysis time: 163 min.

Table 3.1. Oven Temperature Program of Gas Chromatography

	The rate of increase (°C /min)	**Temperature (°C)**	**Wait time (min)**
Beginning	0	35	8
Plus 1	3	320	60

Total analysis time: 163 min.

4. RESEARCH FINDINGS

4.1. Isolation of Bacteria

One of the objectives of this study is to isolate as many cultivable strains as possible in order to determinate their hydrocarbon biodegradation potential in standardized culture conditions. So, seventeen bacterial strains from seven different petrol stations in Iraq Kurdistan were isolated. According to16S rRNA sequence analysis and petroleum degradation properties, some of these bacterial strains which may possibly are new species were selected. Five of these isolated bacteria were named as 2B, 3B, 4A, 6C and 8F. The locations isolated were 2B (Figure 4.1) from Zakho (T20) petrol station, 3B (Figure 4.2) from Bardarash (B4) petrol station, 4A (Figure 4.3) from Bardarash (B1) petrol station, 5D from the Mreba petrol station, 6B from the Mreba petrol station, 6C (Figure 4.4) from the Mreba petrol station, 8F (Figure 4.5) from the Atrush petrol station.

4.2. Morphological Analysis

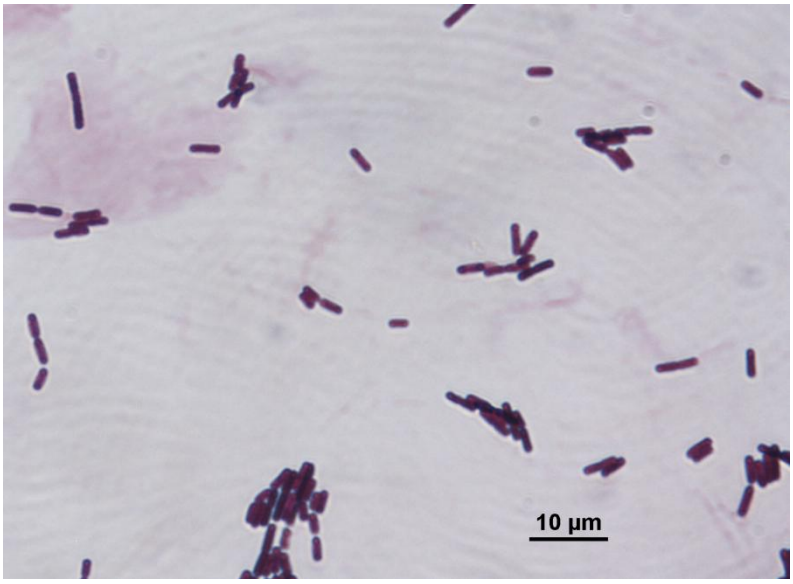

Figure 4.1. Gram staining of 2B

Figure 4.1 shows the Gram-negative 2B. Cells of isolated 2B were rod-shaped, from the family Enterobacteriaceae. This was motile and facultative anaerobic, non-spore formingat 37 °C for 24 h. Cells were found in couple, diplobacilli, or single.

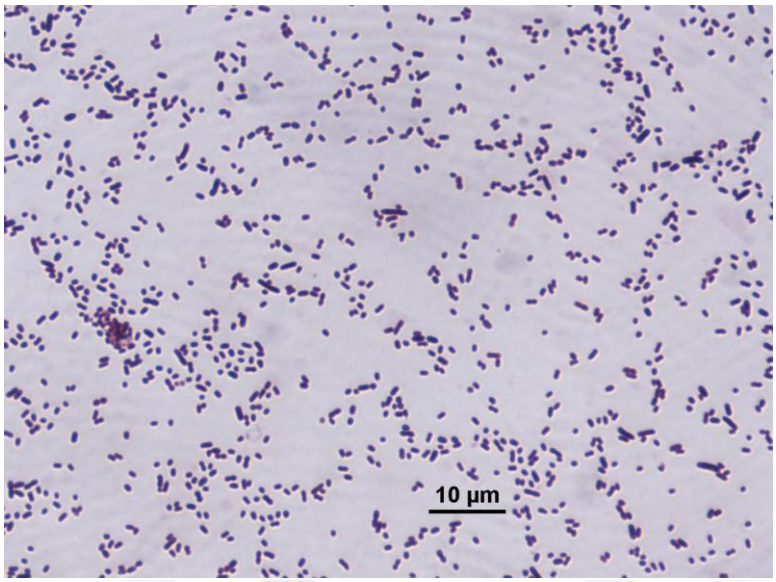

Figure 4.2. Gram staining of 3B

As can be seen in Figure 4.2 3B is gram negative, short rod-shape bacterium. Cells were found in couple or small clusters.

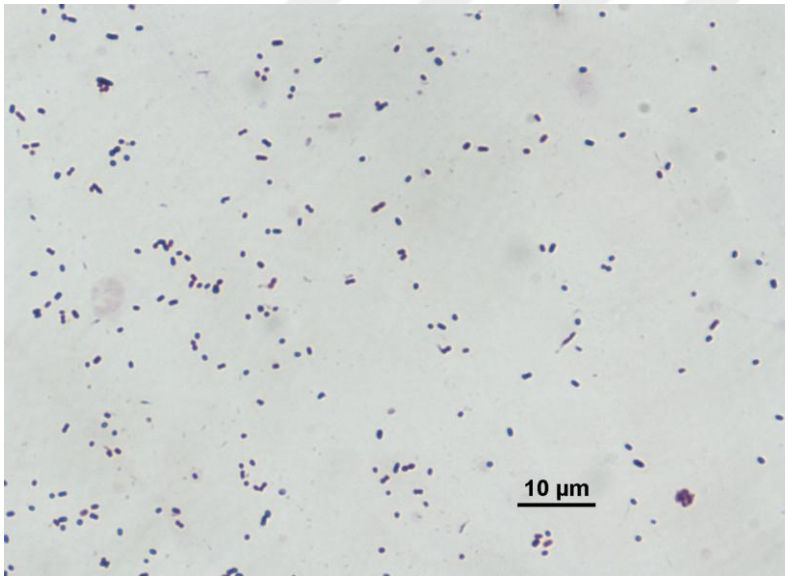

Figure 4.3. Gram staining of 4A

Figure 4.3 shows that 4A is the gram negative. The cells occur in pair or single and motile. It is very small straight rod-shaped, facultative anaerobe bacterium and non-spore forming.

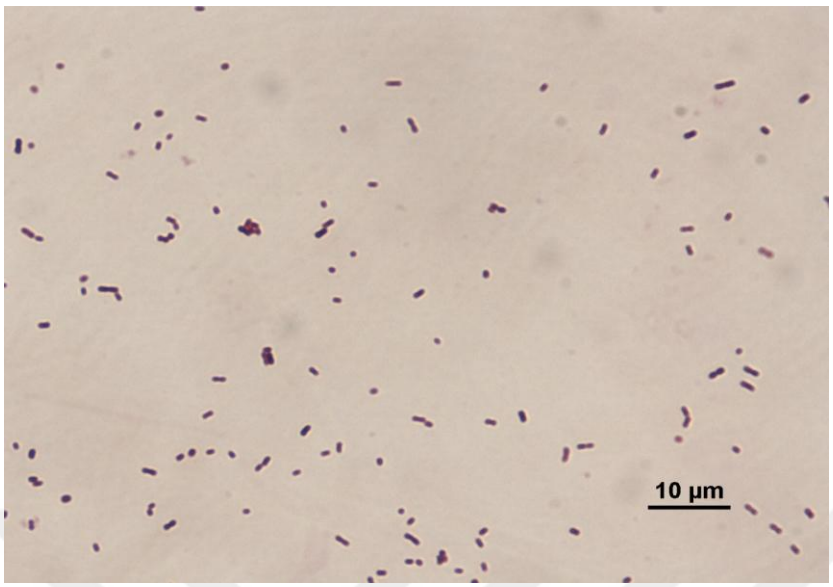

Figure 4.4. Gram staining of 6C

Figure 4.4 displays the cells of isolating 6C. It is Gram negative, facultative anaerobic, rod shape, motile, non-spore forming. Cells were found in couple,diplobacilli or single.

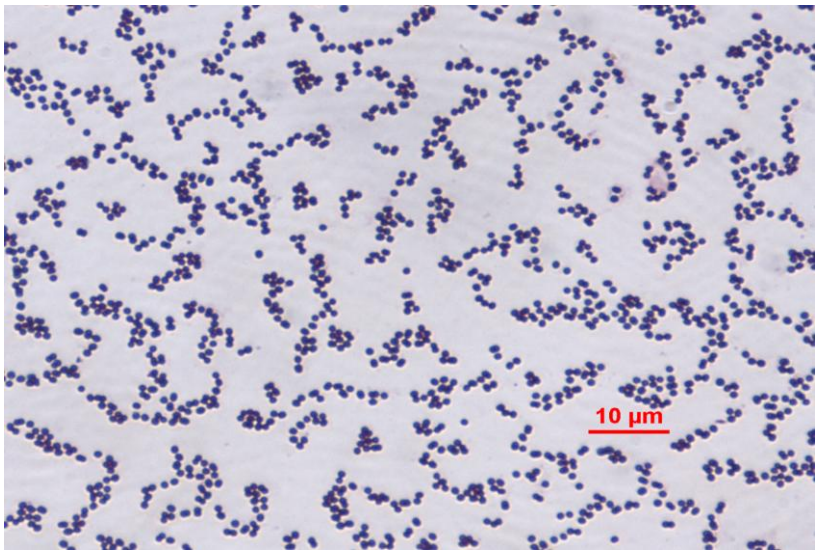

Figure 4.5. Gram staining of 8F

As seen in Figure4.5 the cells of isolated 8F were Gram-negative, strictly aerobe, non-motile, non-spore forming and coccobacilli. Cells were found in couple or small clusters.

4.3. Determination of The Optimal Growth Conditions

4.3.1. Determination of the Optimal Growth Temperature

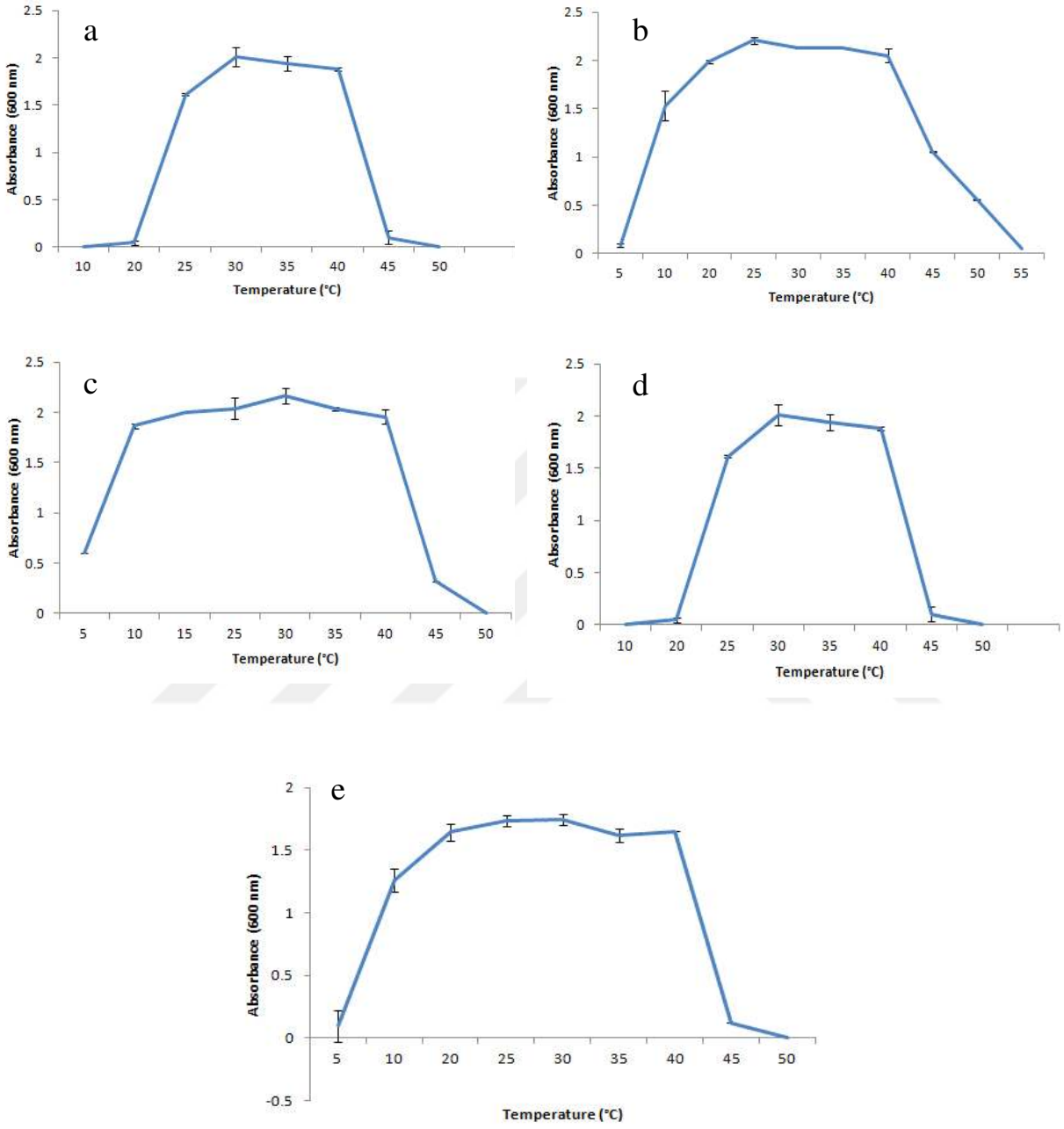

Figure4.6. Effect of temperature on the growth of isolated bacterial strains.

(**a**: 2B; **b**: 3B; **c**: 4A;**d**: 6C and **e:** 8f)

Figure4.6 shows that the effect of temperature on the growth of isolates. As can be seen in Figure 4.6a-4.6e The growth was observed between 25- 40 °C (optimum 30 °C), 10- 50 °C (optimum 25 °C), 5-45°C (optimum 30 °C), 25 to 40 °C (optimum 30 °C) and 10-40 °C (25-30 °C) for 2B, 3B, 4A, 6C, 8F, respectively.

4.3.2. Determination of the optimal GrowthpH

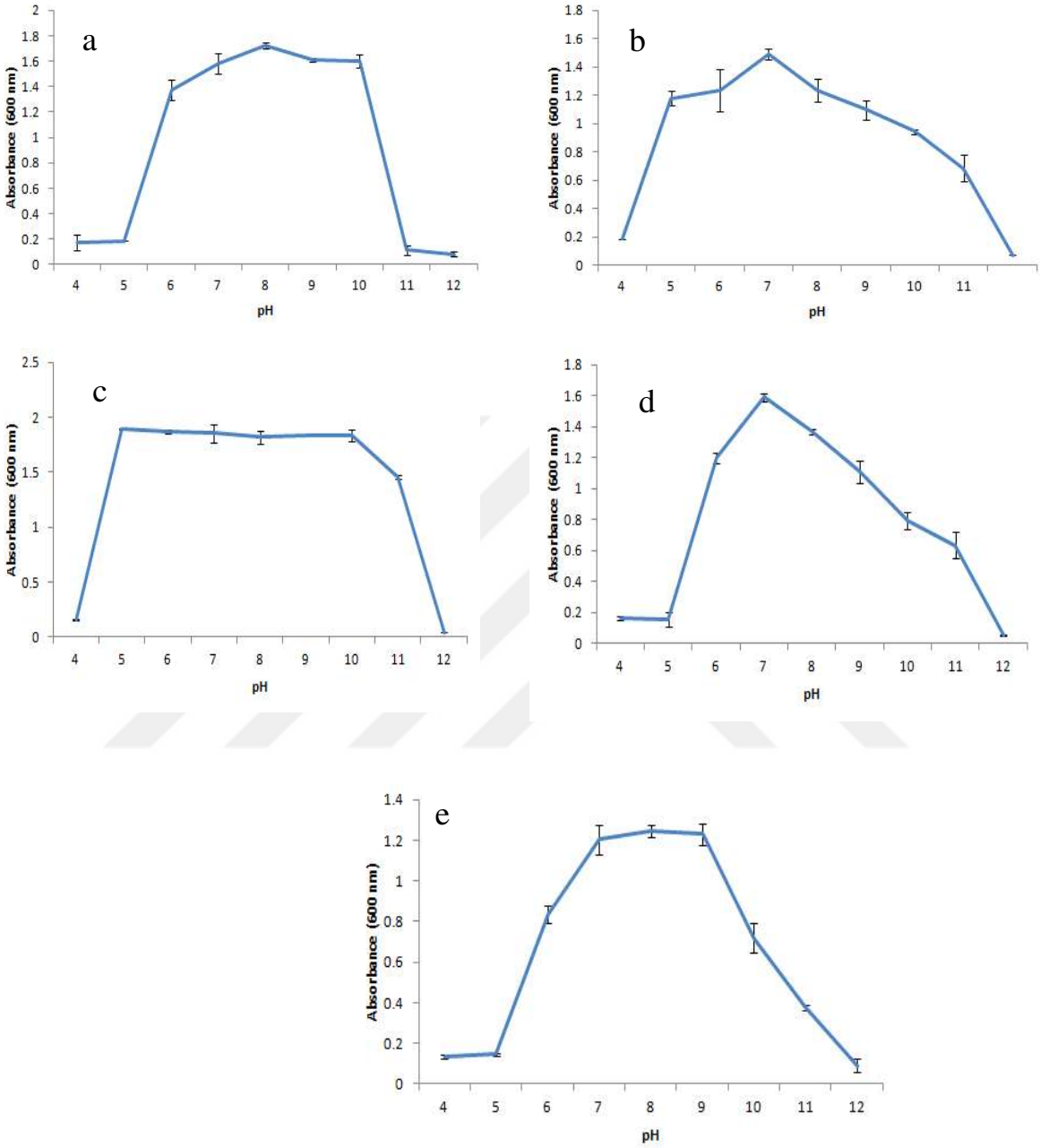

Figure 4.7. Effect of pH on thevisolated bacterial strains

(**a**: 2B; **b**: 3B; **c**: 4A; **d**: 6C and **e:** 8f)

Figure 4.7 shows that the effect of temperature on the growth of isolates. As can be seen in (Figure 4.7a- 4.7e)the growth were observed between 6.0 -10.0 (optimum at pH 8.0), 5.0-11.0 (optimum at pH 7), 5.0 - 11.0 (optimum pH 5.0), 6.0-11.0 (optimum at pH 7) and 6.0- 10.0 (pH7.0- 9.0) for 2B, 3B, 4A, 6C, 8F, respectively.

4.3.3. Determination Of the Optimum Growth Time

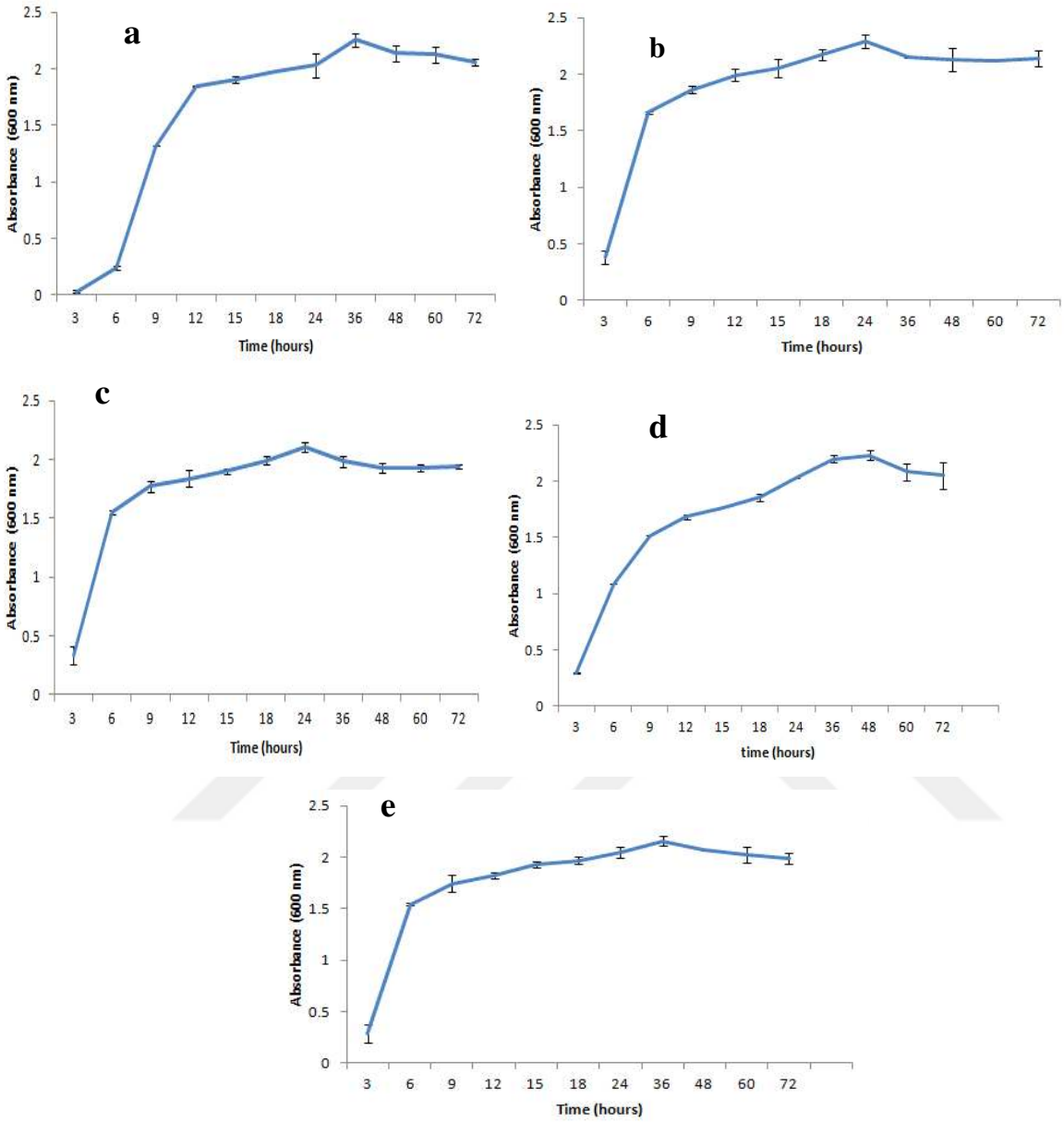

Figure 4.8.Effect of time-course on bacterial growth

(**a**: 2B; **b**: 3B; **c**: 4A; **d**: 6C and **e:** 8f)

Time-course of bacterial growth is shown inFigure 4.8a-8e. As can be seeninFigure 4.8a.growth was observed from 9th h to 72th hand the maximum growth was obtained at 36th h for 2B. The growth time graph of 3B is shownin Figure 4.8b.Growth was detected between 6to 72 h and the maximum growth was detected at 24 h for 3B. Figure 4.8c. shows the effect of time on the growth of 4A. As shown in the figure, growth was detected between 6h to 72 h, and the maximum growth was

determined at 24 h.As can be seen in Figure 4.8d. growth was detected from 6 h to 72 h. The maximum growth was obtained between 36th h- 42th h for 6C. Effect of incubation time on the growth of 8F is seen in Figure 4.8e. growth was observed between 6h to 72 h. The maximum growth was obtained at 36 h of incubation for 8F.

4.4. Biochemical and Physiological Tests

4.4.1. Mobility and Oxygen Requirement

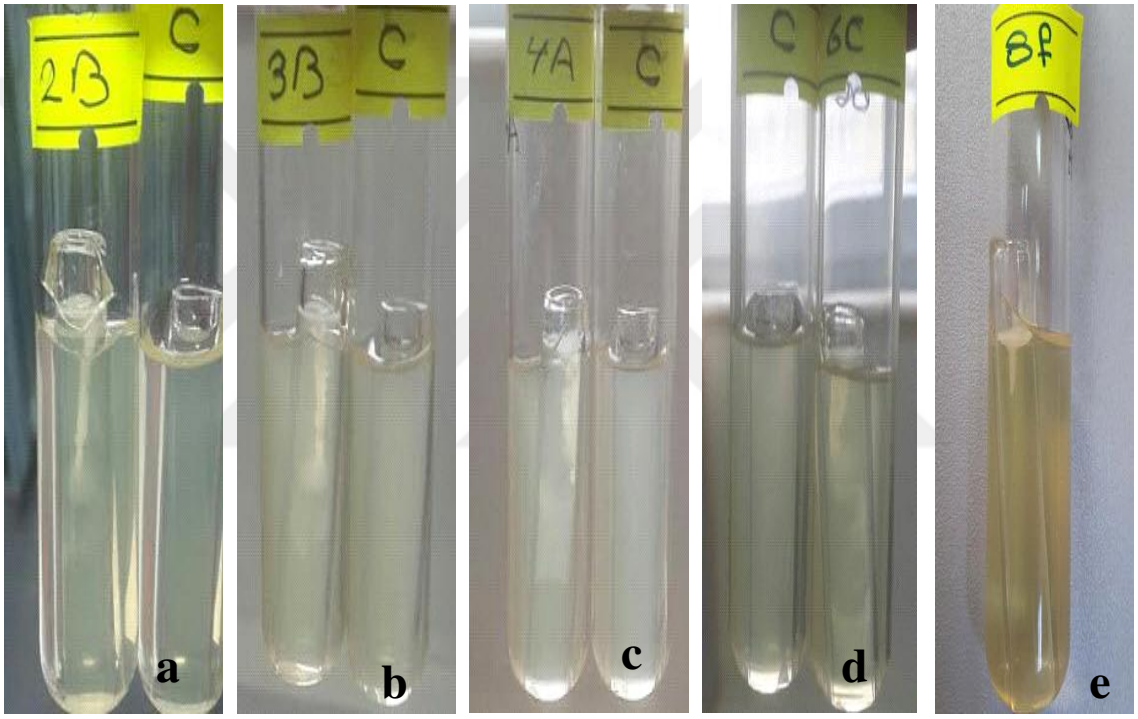

Figure 4.9.Motility and oxygen requirement test on the isolates after 24 hour incubation at 30°C. (**a**: 2B; **b**: 3B; **c**: 4A; **d**: 6C and**e**: 8f)

2B in Figure 4.9a shows growth spreading from the line of inoculation, motility was indicated by turbidity of the medium or growth extending from the inoculating stab line, the organism was appeared as a fog beyond the line of inoculation. The growth of 2B cells appeared at the top and middle of the capillary tube which means that it is facultative anaerobic bacterium.

(Figure 4.9b) shows the motility of 3B cells which are visualized as a diffused zone ofgrowth flaring out from the line of inoculation. Also, it showed the growth at top and middle of the test tube. So, 3B seems to be facultative anaerobic bacterium.

4A and 6C in Figure 4.9c and Figure 4.9d respectively werealso found to be motile and facultative anaerobic bacterium as they wer away from the line of incubation. Inoculation growth were around and bottom of inoculation line and upward direction of the oxygen existence.

The motility test for 8F in Figure 4.9e shows that the surrounding medium from inoculation line is clearly transparent which means 8F is non- motile. Reproductive inoculation growth was in the upward direction of the oxygen requirement, growth only at the top, so it is strictly aerobic.

4.4.2. Starch Hydrolysis Test

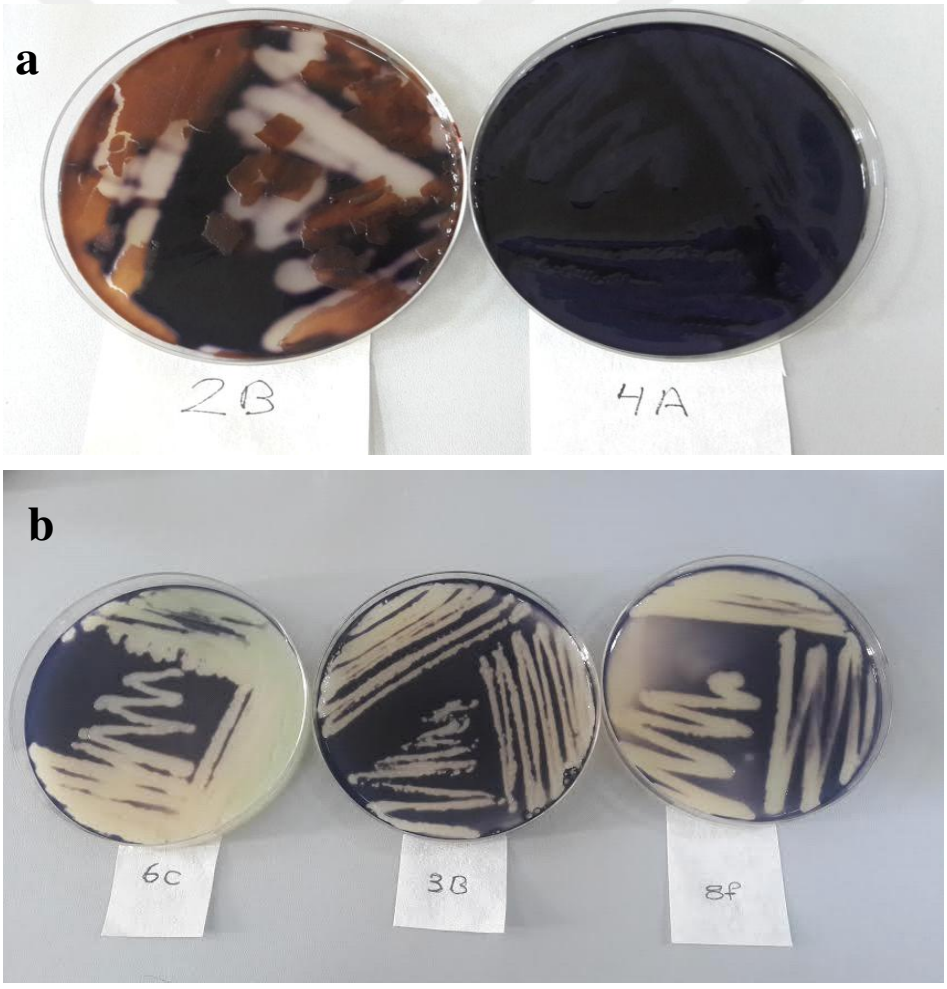

Figure 4:10. Isolated bacteria showing the zone of clearance with iodine solution on starch plates

(**a:**2B and 4A; **b:** 6C, 3B and 8F starch hydrolysis test)

The results in Fig 4.10. indicate the reducing sugar production from the hydrolysis of the starch at 30°C by the enzyme from isolated bacteria. So, the principle of this test is to discover if the organism had capable of breaking down starch into maltose by the activity of the extracellular α-amylase enzyme. All bacteria, except 4A,had positive result ("+"): The field surrounding isolated colonies where starch has been hydrolyzed by amylase was clear. 4A was negative for this test ("-"): The medium was stained dark, right up to the edge of the colonies.

4.4.3. Catalase Test

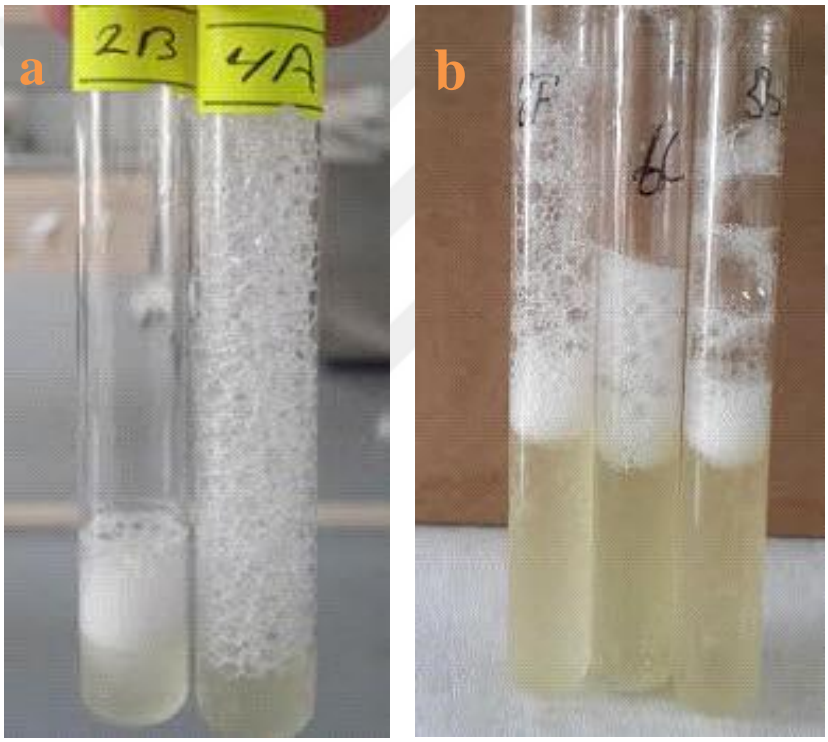

Figure4.11.Catalase test on isolated bacteria.
(Results showing the bubbles after adding 3% H_2O_2s olution .**a:**2B and 4A; **b:** 8F, 6C and3B.)

Figure 4.11. shows the catalase test. After adding 3% H_2O_2 to the test tubes,all of them produced bubbles. It means that they allhad ability to use oxygen as a terminal electron acceptor,means that these isolated bacteria are able to produce the enzyme catalase that degrades H_2O_2 to H_2O and O_2. Also means thatthese isolated bacteria are facultative anaerobe. Catalase-negative bacteria may be anaerobes, or they may be facultative anaerobic bacteria that only ferment and do not respire by using oxygen as a last electron acceptor.

4.4.4. Gelatin Hydrolysis Test

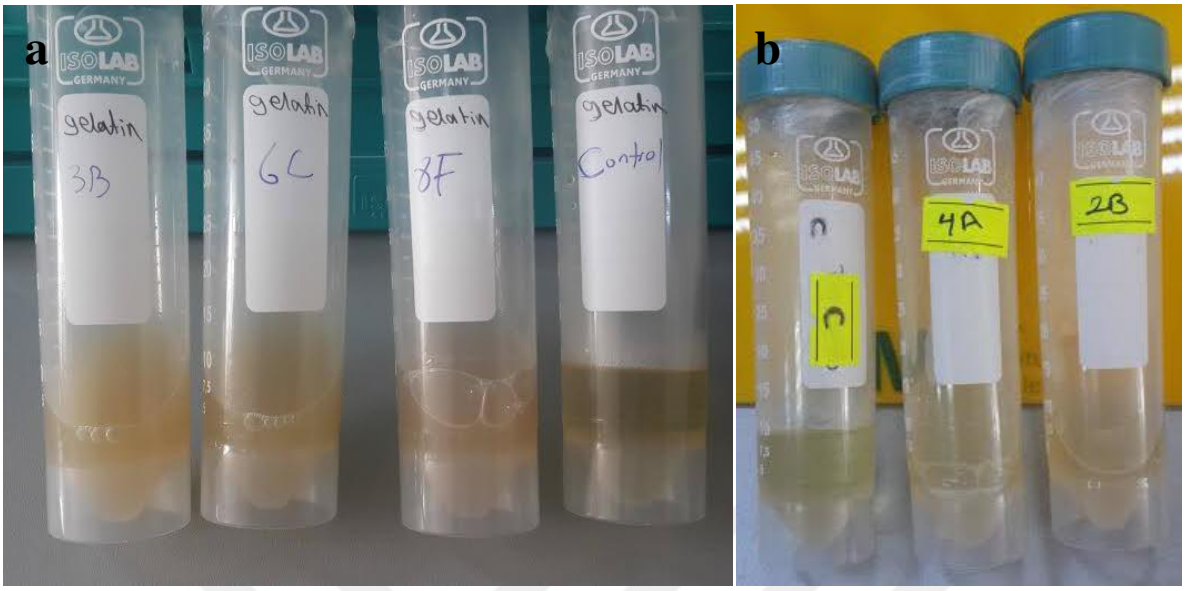

Figure 4. 12. The gelatin hydrolysis test results after 24 h incubation at 30°C.
(**a:**6C; 3B and 8F; **b:**2B and 4A)

Fig 4.12 demonstrates the gelatin hydrolysis test which is used to detect the enzyme gelatinase digestinggelatin present in the medium after which solid medium is changed to liquid medium. All of the isolated bacteria at Fig 4.12a and Fig 4.12b had positive result about gelatin hydrolysis test. The hydrolysis of gelatin means the secretion of gelatinase by the organism into the medium.

4.4.5. Oxidase Test

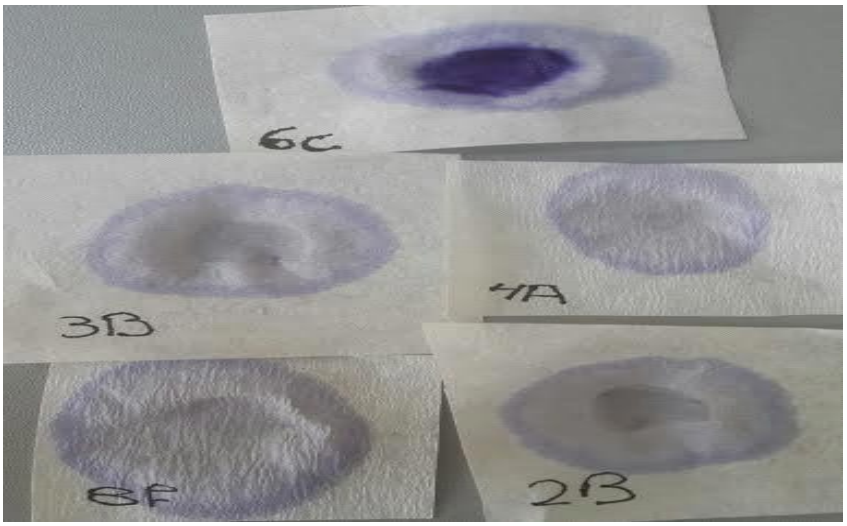

Fig 4:13. Oxidase test on the isolated bacteria after 24 h incubation at 30 °C.

Fig 4.13 shows oxidase test results on the isolated bacteria. 8F, 3B, 4A and 2B had negative results, but 6C had a positive resultwhich is used to determine the presence of bacterial cytochrome oxidase enzyme using the oxidization of the substrate "tetramethyl-p-phenylenediaminedihydrochloride" to indophenol a dark purple colored end product.

4.4.6. L-tyrosine Test

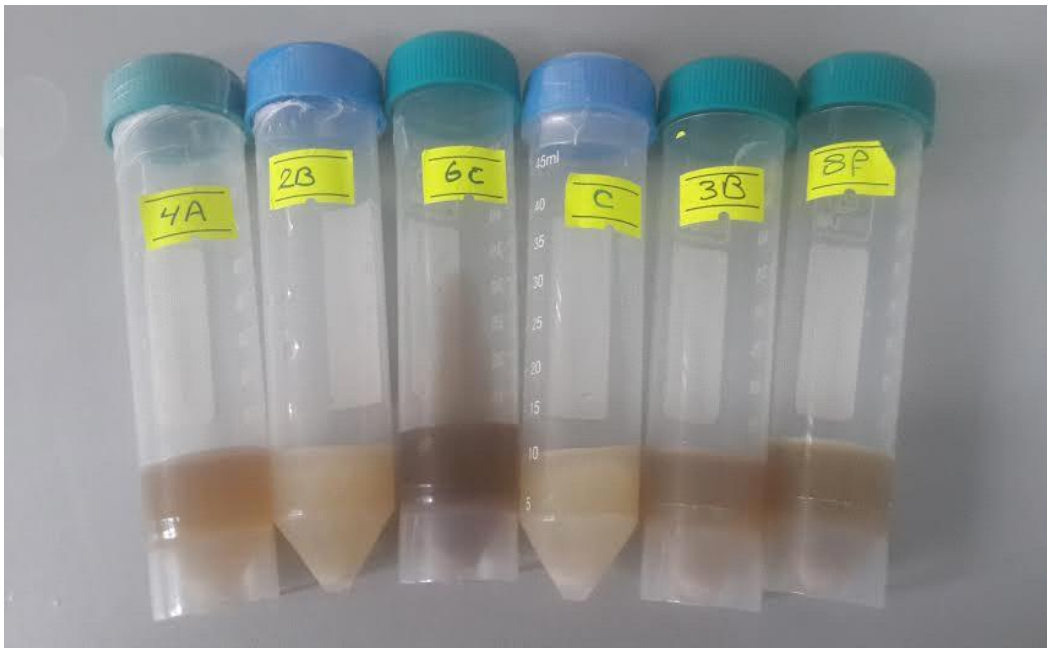

Fig 4.14. The degradation of L- tyrosine after 24 h incubation at 30°C.

Fig 4.14 demonstrates the degradation of L-tyrosine test: Color in all of the samples, except 2B, was changed to brown comparedto control. These bacteria break down this amino acid by the tyrosine hydroxylase enzyme to synthesizeproteins.Thecolor of 6C was darker than another isolated bacteria which means that this bacteria produced the tyrosine hydroxylase enzyme more than others.

4.7. Urease Test

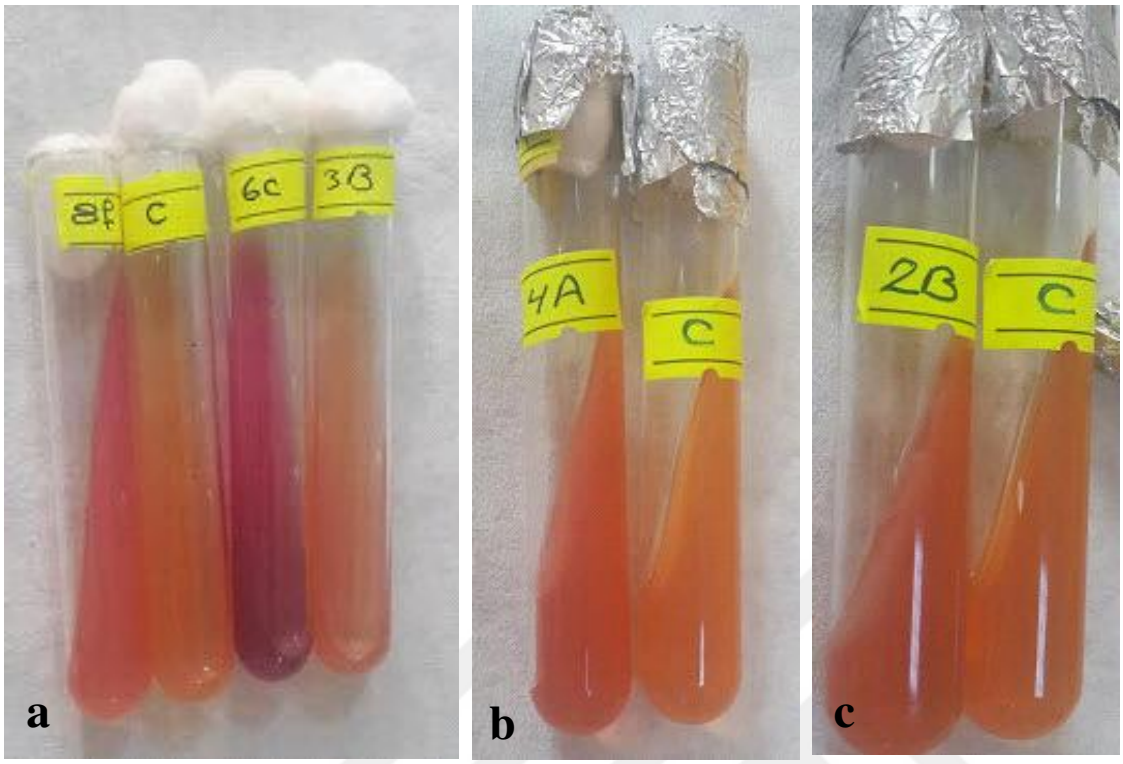

Fig 4.15. Urease test after 24 h incubation at 30 °C
(**a**:6C, 3B, 8F; **b:** 4A;**c**: 2B.)

It can seen in Fig 4.15 that 6C isurease-positive organism that turned the entire medium into pink within 24 h. Also, 8F and 4A were positive;turningthe entire medium pink **s**lowly within 48 hours. So, these organisms were capable of hydrolyzing urea to produce ammonia and carbon dioxide, which means that they produced urease enzyme for degradation of urea. An increase in pH due to the production of ammonia results in a color changed from yellow (pH 6.8) to bright pink (pH 8.2).But, 2B and 3B had negative results.

4.4.8. Sodium Citrate Test

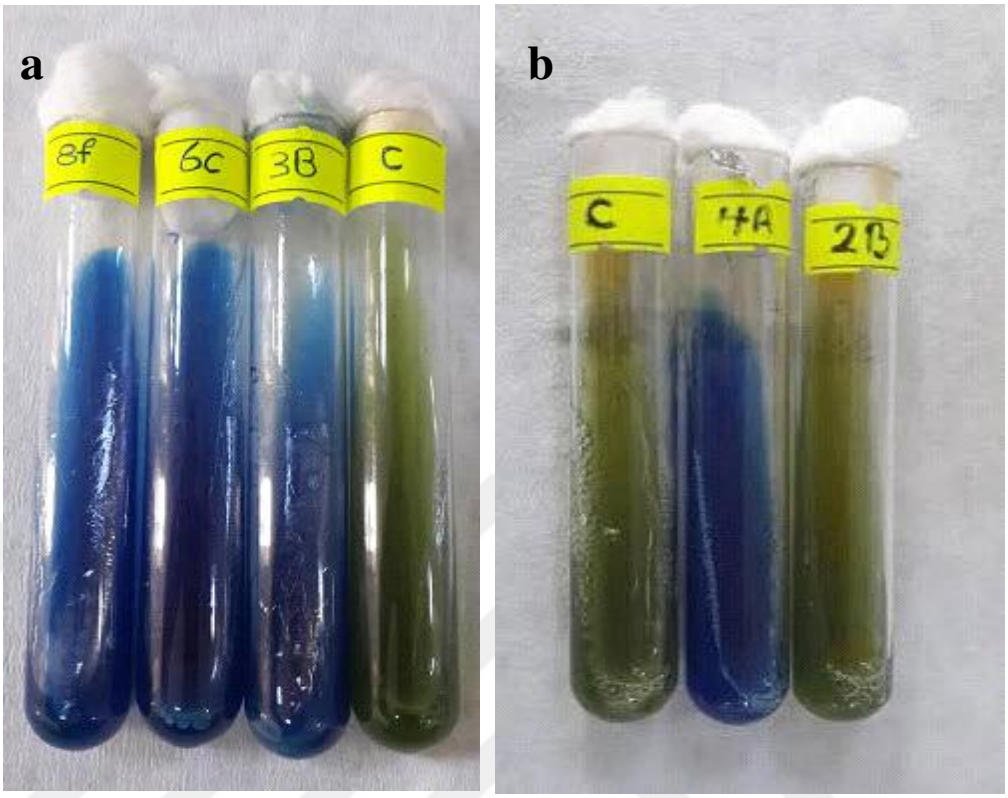

Figure 4.16.Ability of isolated bacteria utilizing citrate.
(**a:** 8F, 6C; **b:** 4A, 2B)

Fig 4.16 shows the sodium citrate test. The color of all strains, except 2B changed from green to blue, so they are positive. There was Bromothymol blue indicator in medium for detecting ability of isolated bacteria containing citrate permeaseto use citrate as sole carbon source.

4.4.9. Sodium Azide Sensitivity

As shown in Table 4.1 after an entire end of a day incubation period isolates were found to be sensitive to sodium azide.

Table 4.1. Sodium azide sensitivity (OD at 600 nm) . The results shows the average of two replicates.

The strains	Control	% 0.02 Sodium Azide
2B	1.8860	0.0685
3B	1.8777	0.2012
4A	1.8370	0.1882
6C	1.5817	0.1894
8F	1.7845	0.0585

In (Table 4.1.)the effect of sodium azide on the reproduction ofisolated bacterial strains shows thatall of them were sensitive to sodium azide (0.02%), by comparing with control.

4.4.10. Tolerance to NaCl

The growth of isolates incubated for 24 h at different NaCl concentrations was assessed in comparison with control. The growth was inhibited in liquid broth at different concentrations of salt. All of the isolates have the ability to grow at 10% (2B, 4A, 6C) and 7% (3B and 8F). Bacterial growth atdifferent concentrations of salt weremeasured at 600nm (OD600)

Table 4.2. Tolerance of isolated bacteria on different NaCl concentration (OD600).The results indicate the average of two repeats.

Thestrains / NaCl(%)	2B	3B	4A	6C	8F
Control	1.9025	1.9606	1.7906	1.6187	1.829
0.5%	1.77	2.314	1.9857	1.9336	1.9804
1%	1.6742	2.012	1.8018	1.8723	1.9566
2%	1.6867	1.9996	1.9859	1.8533	1.9459
5%	1.7512	1.9434	2.0474	1.5958	1.718
7%	1.486	1.7748	1.9614	1.5868	1.43
10%	1.5806	0.2786	1.8925	0.5019	0.2786
15%	0.1002	-----	0.1417	----	-----
20%	0.084	-----	0.0669	-----	------

4.4.11. Lysosome Hydrolysis Test

Table 4.3.Tolerance of isolated bacteria on % 0.001 lysosome concentration (OD600)

Isolatoted bacteria	Control	% 0.001 lysosome
2B	1.8921	1.7001
3B	1.8505	1.7388
4A	1.8370	0.1882
6C	1.7795	1.6919
8F	1.7455	1.6919

After incubation at 0.001% lysosome for 24 h, the results were assessed in comparison with control. It can be clearly seen that only 4A is sensitive to 0.001% lysosome. But,2B, 3B, 6C and 8F strains had atolerance to 0.001% lysosome.

4.4.12. Antibiogram (Disc Diffusion Method) Test

For antibiotic susceptibility test, the isolates were prepared for overnight culture in nutrient broth and incubated at 30^{o}C. The medium used was nutrient agar (NB). The NB agar was inoculated by streaking using sterile cotton swab of each cultures. The antibiotic disks were applied using sterile forceps and sufficiently separated from each other in order to prevent overlapping of the zones of inhibition. Antibiotic multidisc used consisted of 1.P2 (Penicillin 2 units/disc), 2.MY15 (Lincomycin 15 µg/disc), 3.TIL15 (Tilmicosin 15 µg), 4.AMP10 (Ampicillin 10 µg), 5.K5 (Kanamycin 5 µg), 6.FD10 (Fusidic acid10 µg), 7.TE30 (Tetracycline 30 µg), 8.N10 (Neomycin 10 µg), 9.S10 (Streptomycin10 µg), 10.NV 5 (Novobiocin 5 µg), 11.CN10 (Gentamicin 10 µg), 12.NS100 (Nystatin 100 unit), 13.C30 (Chloramphenicol 30 µg), 14.B10 (Bacitracin 10 unit), 15.P10 (Penicillin 10 unit).

The agar plates were left on the bench for 30minutes to allow for diffusion of the antibiotics and after that the plates were incubated inverted at 30^{o}C for 48 hours. Results were recorded by measuring the zone of inhibition. In the event of an empty zone around the disk was evaluated susceptibility and result is positive. Tests were performed twice. Summary of the results is presented in Table 4.4.

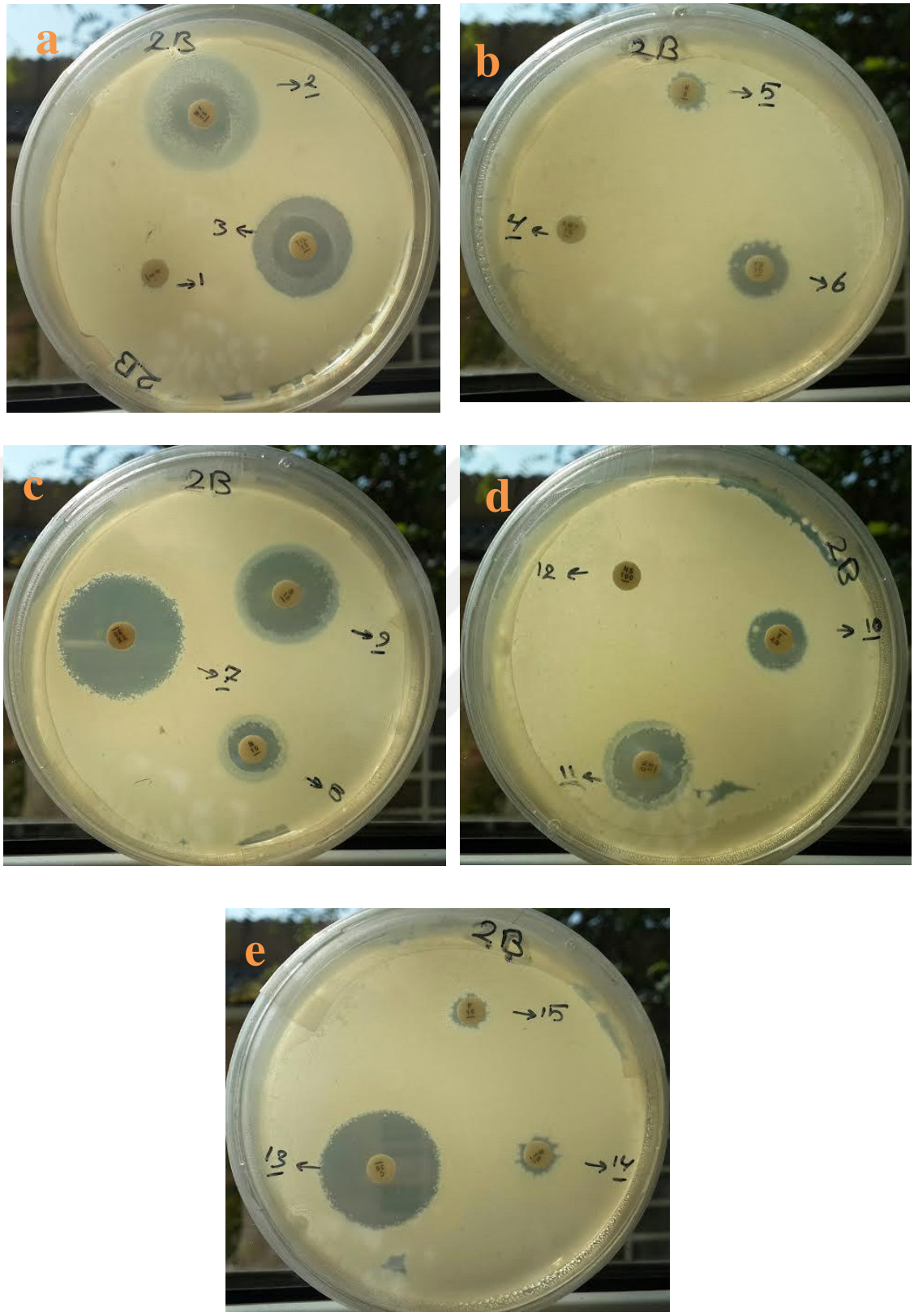

Figure 4.17.Antibiotics disc diffusion test of 2B

(**a:** 1. P2, 2. MY15, 3. TIL15; **b:**4. AMP10, 5.K5, 6.FD10; **c:** 7. TE30, 8. N10, 9. S10; **d:**10. NV5, 11.CN10, 12. NS100; **e:** 13. C30, 14.B10, 15. P10.)

2B was found to be sensitive to 2. MY 15 (28 mm), 3. TIL 15 (26 mm), 7. TE 30 (30 mm), 9.S10 (26 mm), 11.CN10 (22 mm), 13.C30 (28 mm). Also, it was semi-sensitive to 6. FD10 (16mm), 8.N10 (16 mm), 10.NV5(14 mm). Very small zone of inhibition was formed around 5. K5 (8 mm), 14.B10(6 mm). 15. P10 (4 mm)and but no zone formation, it was resistance, around other antibiotics like1. P2(Resistance), 4. Amp10(R), 12.NS 100(R).

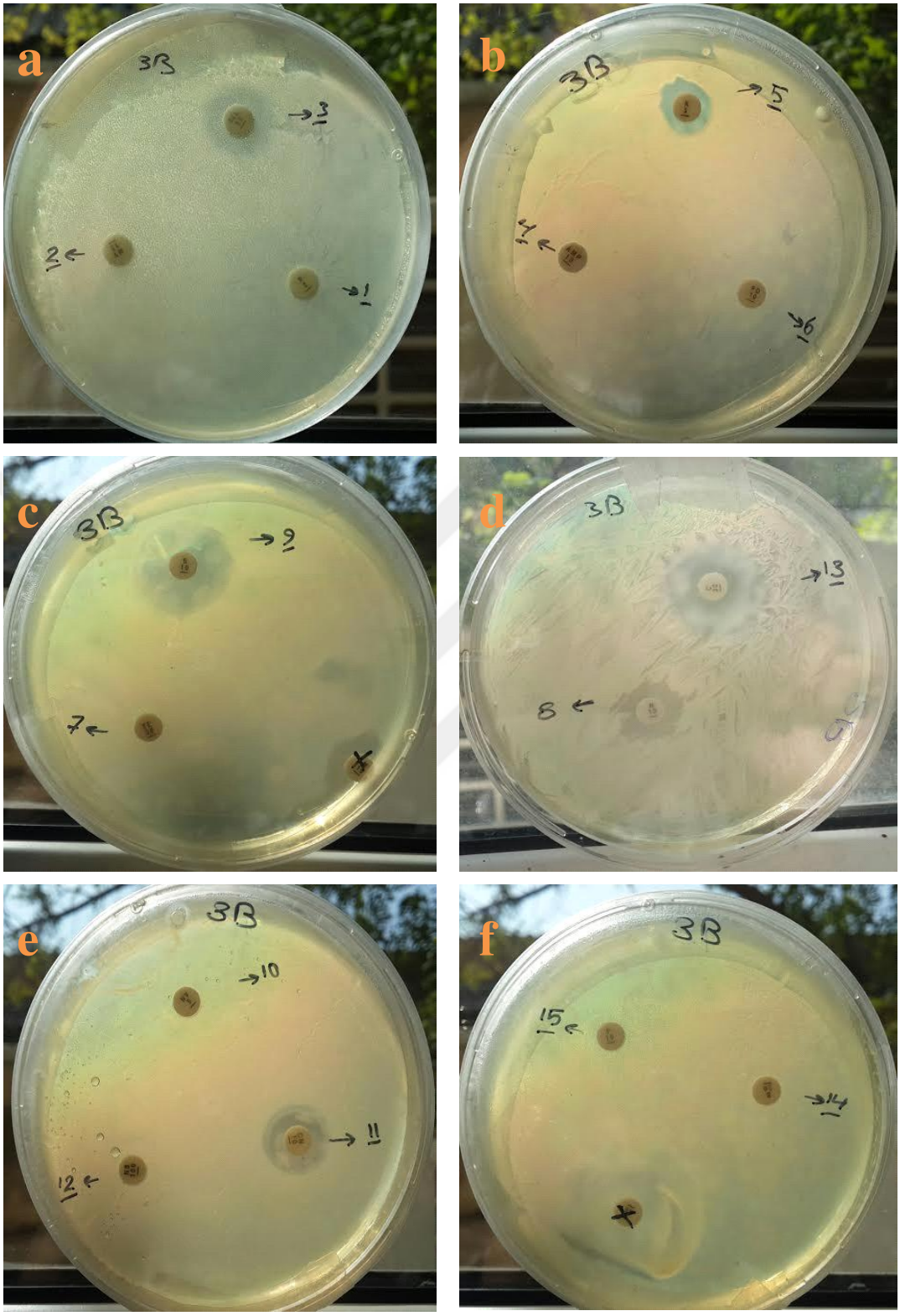

Figure 4.18. 3B Antibiotics disc diffusion test

(**a:**1. P2, 2. MY15 and 3.TIL15; **b:** 4. AMP10, 5. K5 and 6. FD10; **c:**7.TE 30, 9. S 10 on 3B**; d:** 8. N10 and 13. C30;**e:** 10. NV5, 11. CN10 and 12. NS100; **f:**14. B10 and 15. P10.

3B shows sensitive towards some antibiotic like 9. S10 (20 mm). It was semi-sensitive to 3.TIL15 (16 mm), 5. K5 (10 mm), 8.N10(14 mm), 11.CN10 (14 mm), 13. C30 (10 mm), Very small zone of inhibition, it was resistance, was formed around 12.NS100 (6 mm). But no zone formation around other antibiotics such as: 1. P2 (R), 2. MY15 (R), 4.Amp10 (R), 6.FD10 (R), 7.TE30 (R), 10.NV5 (R), 14. B10 (R). 15. P10(R).

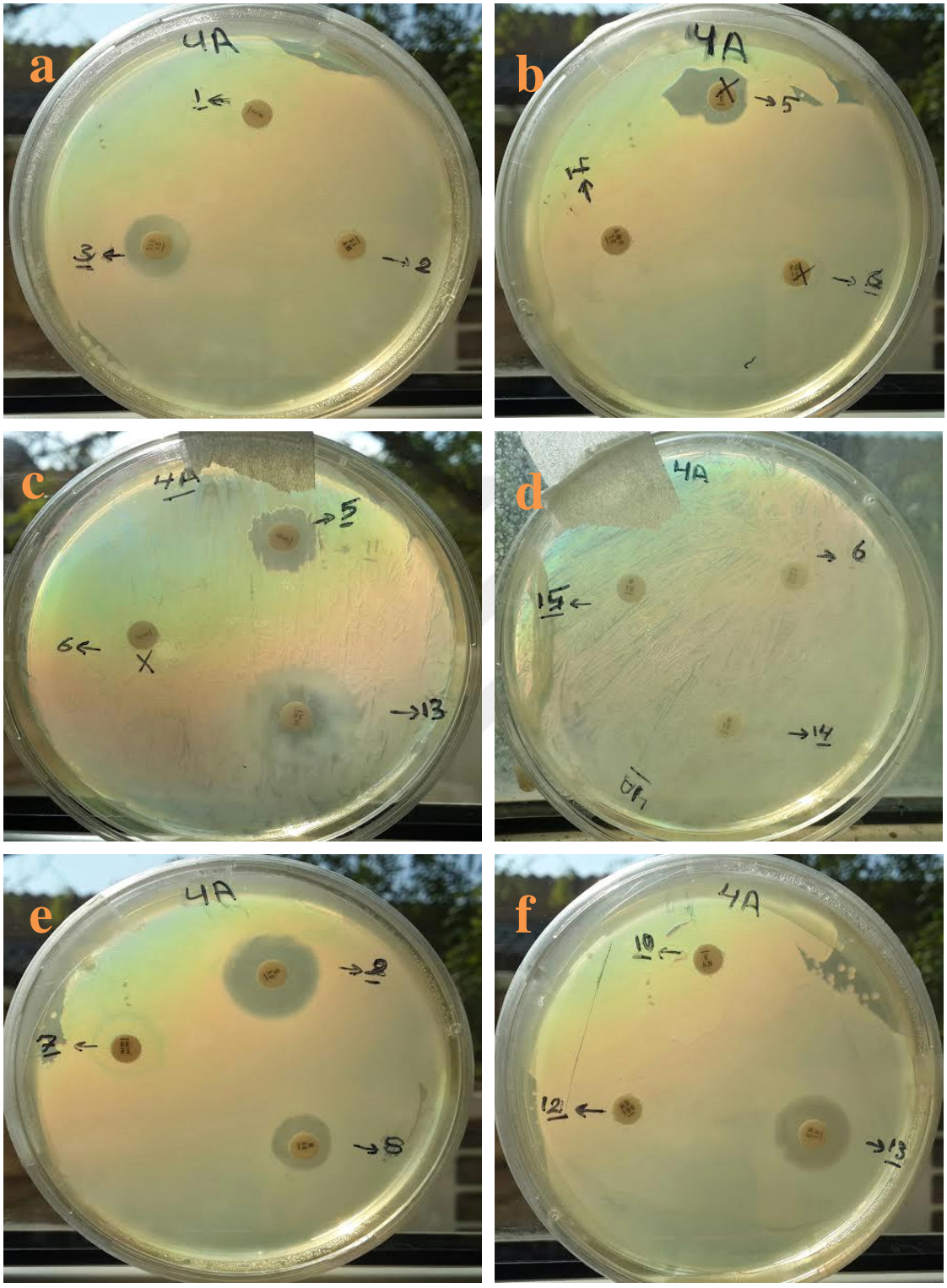

Figure 4.19. Disc diffusion susceptibility test on 4A

(**a:** 1. P2, 2.MY 15, 3.TIL 15; **b:** 4. AMP 10;**c:** K 5, 13. C30; **d:**6. FD10 ,14. B 10, 15. P10; **e:** 7. TE 30, 8. N 10, 9. S10; **f:**10. NV 5, 11.CN 10, 12. NS 100.)

4Awas found to be sensitive to 9. S 10 (22 mm), 11. CN 10 (18 mm).A small zone of inhibition was formed around to 5.K 5 (16 mm), 8. N 10 (14 mm), which means that It was semi-sensible. But it had resistance to 1. P2 (R), 2.MY 15 (R), 3.TIL 15 (12 mm), 4.AMP 10 (R), 6. FD 10 (R), 7. TE 30 (R), 10.NV 5 (R), 12.NS 100 (Nystatin 4 mm), 13.C30 (11 mm), 14.B 10 (R), 15.P 10 (R).

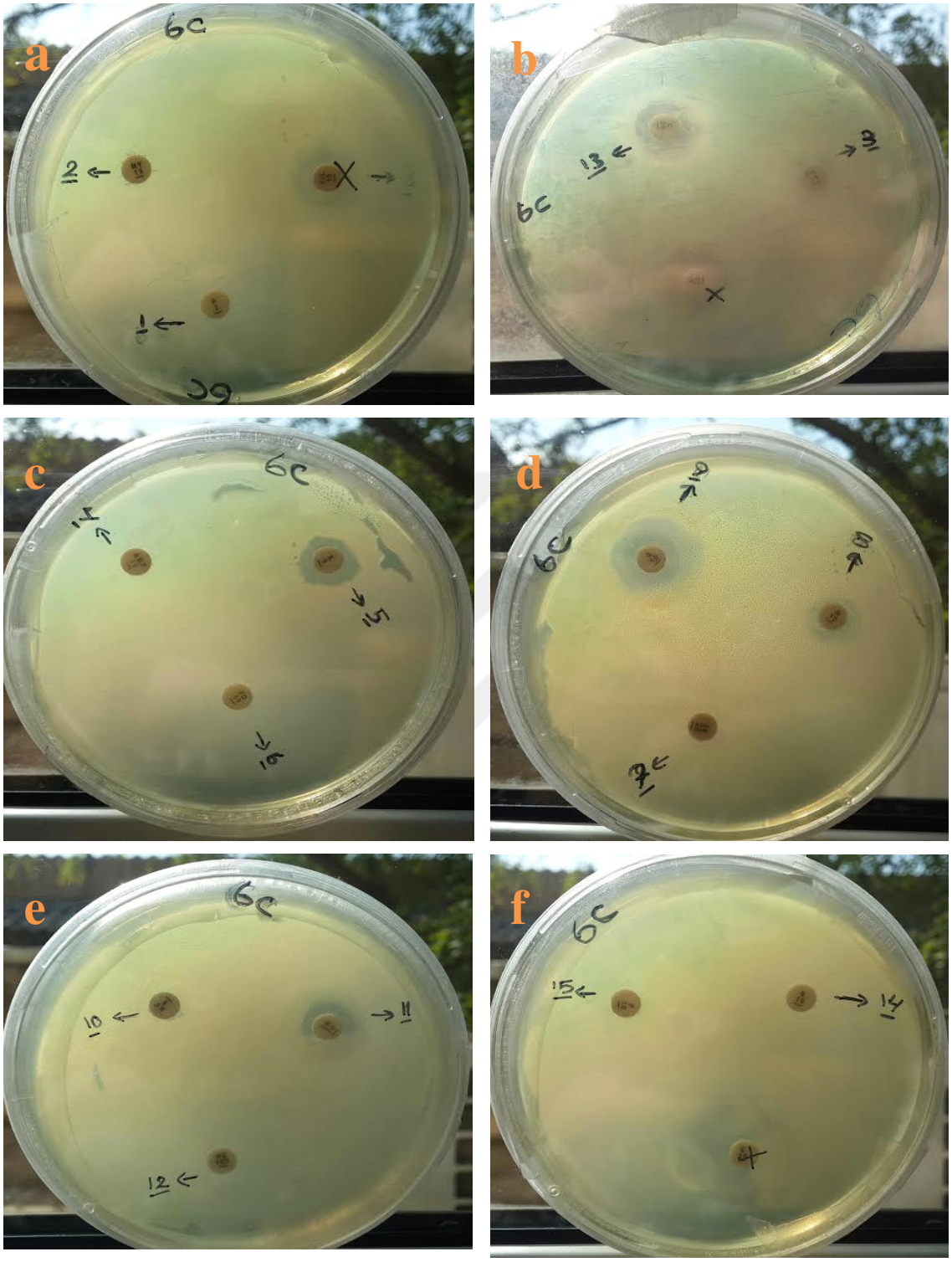

Figure 4.20. Disc diffusion susceptibility test on 6C

(**a:**1. P 2, 2. MY 15; **b:** 3.TIL 15, 13. C 30; **c:** 4. AMP 10, 5.K 5, 6. FD 10; **d:** 7. TE 30, 8. N 10 , 9. S 10; **e:** 10. NV 5, 11.CN 10, 12. NS 100; **f:** 14. B 10, 15.P 10.)

The 6C shows sensitive towards some antibiotic like 9. S 10 (18 mm).Very small zone, it was resistance, of inhibition was formed around 8. N 10 (8 mm).5.K 5 (12 mm), 13. C 30 (12 mm), 11. CN 10 (10 mm),1.P 2 (R), 2.MY 15 (R), 3.TIL 15 (R),4. AMP 10 (R), 6.FD 10 (R), 7.TE 30 (R), 10.NV 5 (R), 12.NS 100 (R), 14.B 10 (R), 15.P 10 (R).

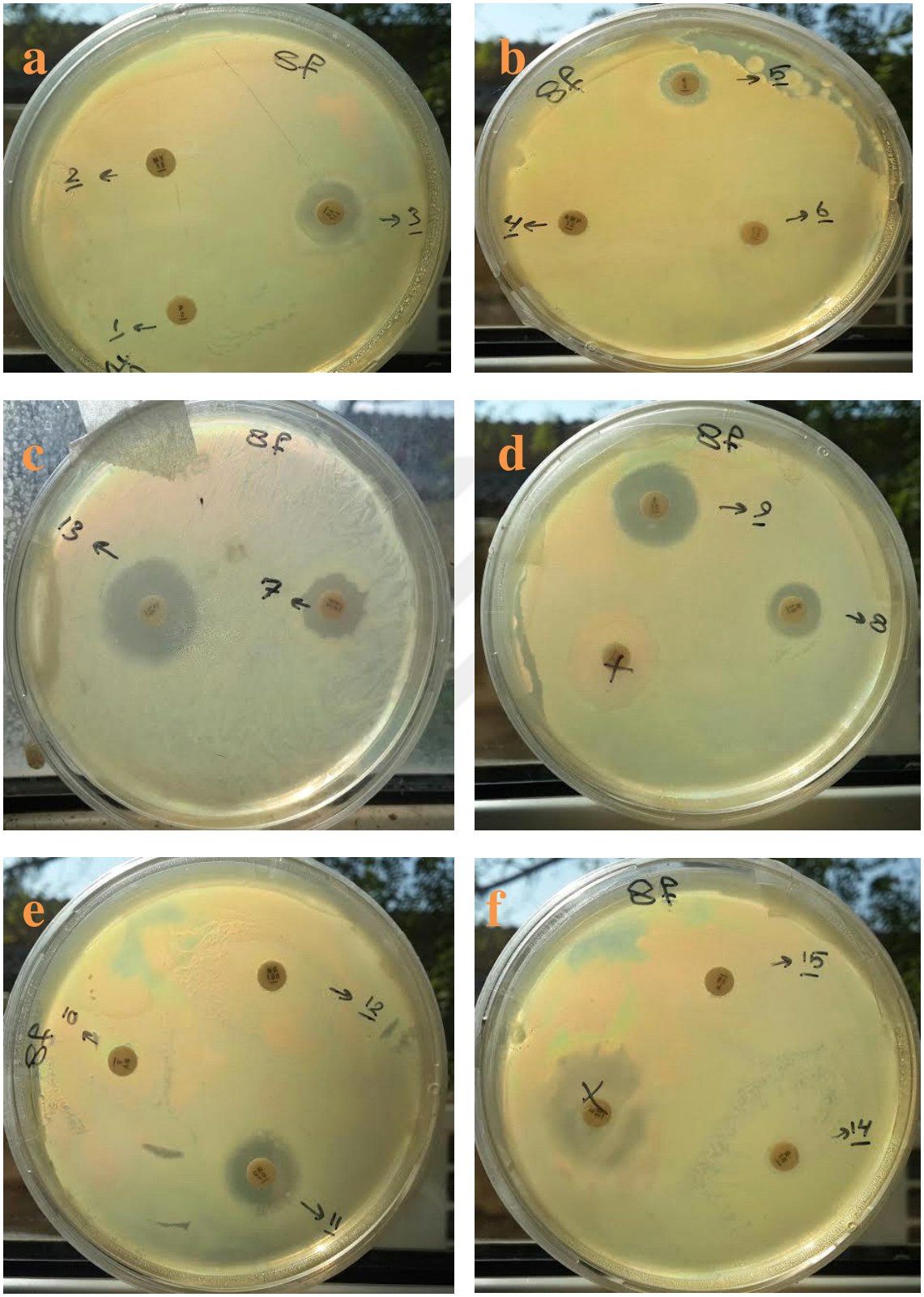

Fig 4.21. Antibiotics disc diffusion test on 8F

(**a** 1. P 2, 2.MY 15, 3.TIL 15; **b:** 4. AMP 10, 5.K 5, 6. FD 10; **c:** 7. TE 30, 13. C 30; **d:** 8. N 10, 9. S 10; **e:** 10. NV 5, 11.CN 10, 12. NS 100; **f**: 14. B 10, 15.P 10.)

8F was found to be sensitive to 3.TIL 15 (18 mm), 9. S 10 (18 mm), 11.CN 10 (18 mm), 13.C 30 (22 mm). Also,small zone of inhibition was formed around, semi sensitive,7. TE 30 (14 mm), but no zone formation, resistance, around other antibiotics such as: 1.P 2 (R), 2.MY 15 (R), 4. AMP 10 (R), 5. K 5 (10 mm), 6. FD 10 (R), 8.N 10 (10 mm).10. NV 5 (R), 12. NS 100 (R), 14.B 10 (R), 15.P 10 (R).

Table 4.4. Effects of antibiotics on the isolated bacteria

The strains / Antibiotic	**2B**	**3B**	**6C**	**4A**	**8F**
1.P2	R	R	R	R	R
2.MY	S	R	R	R	R
3.TIL	S	S	R	SM	S
4.AMP	R	R	R	R	R
5.K	R	R	R	SM	R
6.FD	SM	R	R	R	R
7.TE	S	R	R	R	SM
8.N	SM	SM	R	SM	R
9.S	S	S	S	S	S
10.NV	SM	R	R	R	R
11.CN	S	SM	R	S	S
12.NS	R	R	R	R	R
13.C	S	R	R	SM	S
14.B	R	R	R	R	R
15.P10	R	R	R	R	R

(R: resistance (0-13 mm), SM:semisensitve (13-18 mm), S:sensitive(>18 mm)

1. P2 (Penicillin 2unit), 2. MY15 (Lincomycin 15 µg), 3.TIL15 (Tilmicosin 15 µg), 4.AMP10 (Ampicillin 10 µg), 5.K5 (Kanamycin 5 µg), 6.FD10 (Fusidic acid10 µg), 7.TE30 (Tetracycline 30 µg), 8.N10 (Neomycin 10 µg), 9.S10 (Streptomycin10 µg), 10.NV 5 (Novobiocin 5 µg), 11.CN10 (Gentamicin 10 µg), 12.NS100 (Nystatin 100 unit), 13.C30 (Chloramphenicol 30 µg), 14.B10 (Bacitracin 10 unit), 15.P10 (Penicillin 10 unit)).

4.5. 16S rRNA Sequence Analysis

16S rRNA base analysis sequence is shown in Table (4.5.-4.9.). The isolate was identified using the EzTaxonserver (http://www.ezbiocloud.net/eztaxon; Kim et al. 2012)on the basis of 16S rRNA sequence data. The resultsof 16S rRNA gene sequence analysis showed thatthe strain2B has 96.31% sequence similarity to *Cronobacter malonaticus* LMG 23826(Table 4.5.),3B has 95.48% sequence similarity to *Cronobacter dublinesis* subsp. *dublinesis* DES187 (Table 4.6.), 4A has 92.94% sequence similarity to *Serratia marcescens* subsp. *Marcescens* ATCC13880 (Table 4.7.), 6C has 96.23% sequence similarityto *Pseudomonas aeruginosa* JCM 5962 (Table 4.8.), 8F has 99.08% sequence similarity to *Acinetobacter calcoaceticus* DSM 30006e (Table 4.9.).Figure 4.37 shows the phylogenetic tree drawn from the partial 16S rRNA sequence analysis of all strains, showing the phylogenetic relationship between strains 2B, 3B, 4A, 6C, 8F and relative to other strains of the genus of *Cronobacter, Serratia, Pseudomonas* and *Acinetobacter*.

Table 4.5. 16S rRNA sequence analysis about 976 base pairs in length of *Cronobacter* sp. strain 2B

CCAGCTCTGGGCTCCTCTCTAGGGAGCGCCCTCCCGAGGGTTAAGCTACCTACTTCTTTTGCG
ACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTGGCAT
TCTGATCCACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGA
CTACGACGCACTTTATGAGGTCCGCTTGGTCTCGGGAGGTCGCTTCTCTTTGTATGCGCCATT
GTAGCACGTGTGTAGCCCTGGTCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCT
CCGGTTTATCACCGGCAGTCTCCTTTGAGTTCCCGGCCGAACCGCTGGCAACAAAGGATAAG
GGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCA
GCACCTGTCTCAGAGTTCCCGAAGGCACTCCCGCATCTCTGCAGGATTCTCTGGATGTCAAG
ACCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCC
CCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGACTTAACGCGT
TAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTCGACATCGTTTACGCGTGGACT
ACCAGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTCGTCAAGG
GGCGCTTCGCCACCGGTATTCTCAGATCTCTACGCATTTCACGCTACAGCTGGAATTCTACCC
CCTCCTACGAGACTCAGCTGCAGTTTCAAATGCAGTTCCCAAGTGAGCAGGATTTCCATCTG
ACTAACGACCGCTGCTGGCCTTTACGCCAGTATTCGATTAGCTGACCTCCGGATTACCCGCG
CTGACGGAATAGCGGTCCTCCTCGGTAACGTCAATGCGTGG

Table 4.6. 16S Rrna sequence analysis about 805 base pairs in length of *Cronobacter* sp. strain 3B

CAATCGCTCCGTGTGAGTGTGAGTTTGATCTGGCTCAGGAATCCTAACATGGCTGACCCTTA
AGATTTGATCTGGCACAGGTGACAATGTCTGGGACACTGACTGATGGAGGGGGATAACTAC
TGGAAACGGTAGCTAATACCGCATAACGTCCCAAGACCAAAGAGGGGGACCTTCGGGCCTC
TTGCCATCAGATGTGCCCAGATGGGATTAGCTAGGAGGTGGGGTAATGGCTCACCTAGGCG
ACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGAC
TCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGC
CGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAGGTGGTGAG
CTTAATACGCTCATCAATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCA
GCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAG
GCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTTGAAACTGG
CAAGCTAGAGTCTCGTAGAGGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAAGAT
CTGGAGGAATACCGGTGGCGGAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAA
GCGTGGGGAGCCAAACAGGGATTAGATACCCTGGGTAGTCCACCGCTGTAAACGATGTCGA
TTTCGGAGG

Table 4.7. 16S rRNA sequence analysis about 856 base pairs in length of *Serratia* sp. strain 4A

TCCGACGCCGTGACCTCGCAGTCGAGCGGTAGCACAGGGGGAGTTGCTCCCGCGTGACAGA
GCGTCGGACGGGTGAGTATGGCTGGGAGCTGCCTGATGGAGGGGGATACTGACTGGGCCGG
TAGCTAATACCGCATATCGTGGCAAGAGCAGAGATGGGGACCTTCGAGGCCTCTTGCCAGC
AGATGTGCCCAGATGGGATGAGCTAGTAGGGGGGGGAACGGCTCACCTAGGCGACGATCCC
TAGCTGTGCTGGAGAGGATGACCAGCCACGCTGAAAACTGAGCATGATCAAACTACTCTGA
GGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGT
GTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAGGTGGTGAGCTTAA
TACGCTCATCAATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGC
AGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGT
TTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGTGTGTGAAACTGGGCAAG
CTAGAGTCTCGGTAGGAGGGGGGGTAGAATTTCCAGGTGTAGCGCGTGAAATGCGTAGAGA
TCTGGAGGGAATACCGGTGGCGAAGGCGGGCCCCTGGACGAAGACTGACGCTCAGTGCGAA
AGCGTGGGGAGCAAACAGGGATAGATACCCTGGTAGTCCACCGCTGTAACGATGTCGATTT
GGAGGTGTGCCCTTGAGGCGTGGCTTCGGAGCTACGCGTTAAAATCGACGCTGGGGAGTAC

Table 4.8. 16S rRNA sequence analysis about 796 base pairs in length of *Pseudomonas* sp. strain 6C

CAAGCCGCTGACGTGGCCGTTGAGTTTGATCTGGCTCAGGAATCCTAACTTGGTACCCGAAA
CATTTCAGACGGGCTAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGAA
ACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCT
ATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGGGGGGTAAAGGCCTACCAAGGCGACGAT
CCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTAC
GGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTG
TGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAAT
ACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCG
GTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTT
CAGCAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCCAAACTACTGAGCT
AGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAG
GAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAGGCGTGG
GGAGCAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCACTAACCCTTGG

Table 4.9. 16S rRNA sequence analysis about 797 base pairs in length of *Acinetobacter* sp. strain 8F

TGTTTAAAAAGGCTGACGCGCTTAACACATGCAGTCGAGCGGAGTGATGGTGCTTGCACTAT
CACTTAGCGGCGGACGGGTGAGTAATGCTTAGGAATCTGCCTATTAGTGGGGGACAACATTT
CGAAAGGAATGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGATCTTCGGACCTTG
CGCTAATAGATGAGCCTAAGTCGGATTAGCTAGTTGGGGGGGTAAAGGCCTACCAAGGCGA
CCATCTGTAGCGGGTCTGAGAGGATGATCCGCCACACTGGGACTGAGACACGGCCCAGACT
CCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCC
GCGTGTGTGAAGAAGGCCTTATGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTACTGAAG
TTAATACCTTCAGATAGTGGACGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCA
GCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGATTTACTGGGCGTAAAGCGCGCGTAG
GCGGCTAATTAAGTCAAATGTGAAATCCCCGAGCTTAACTTGGGAATTGCATTCGATACTGG
TTAGCTAGAGTGTGGGAGAGGATGGTAGAATTCCAGTGTAGCCGGTGAAATGCGTAGAGAT
CTGGAGGAATACCCGATGGCGAAGGCAGCCATCCTGGGCTAACACTGACGCTGAGGTGCGA
AAGCCTGGGGAGCCAACAGGATTAGATACCCTGGTAGTCCATGCCCGAAACGATGTCTACT

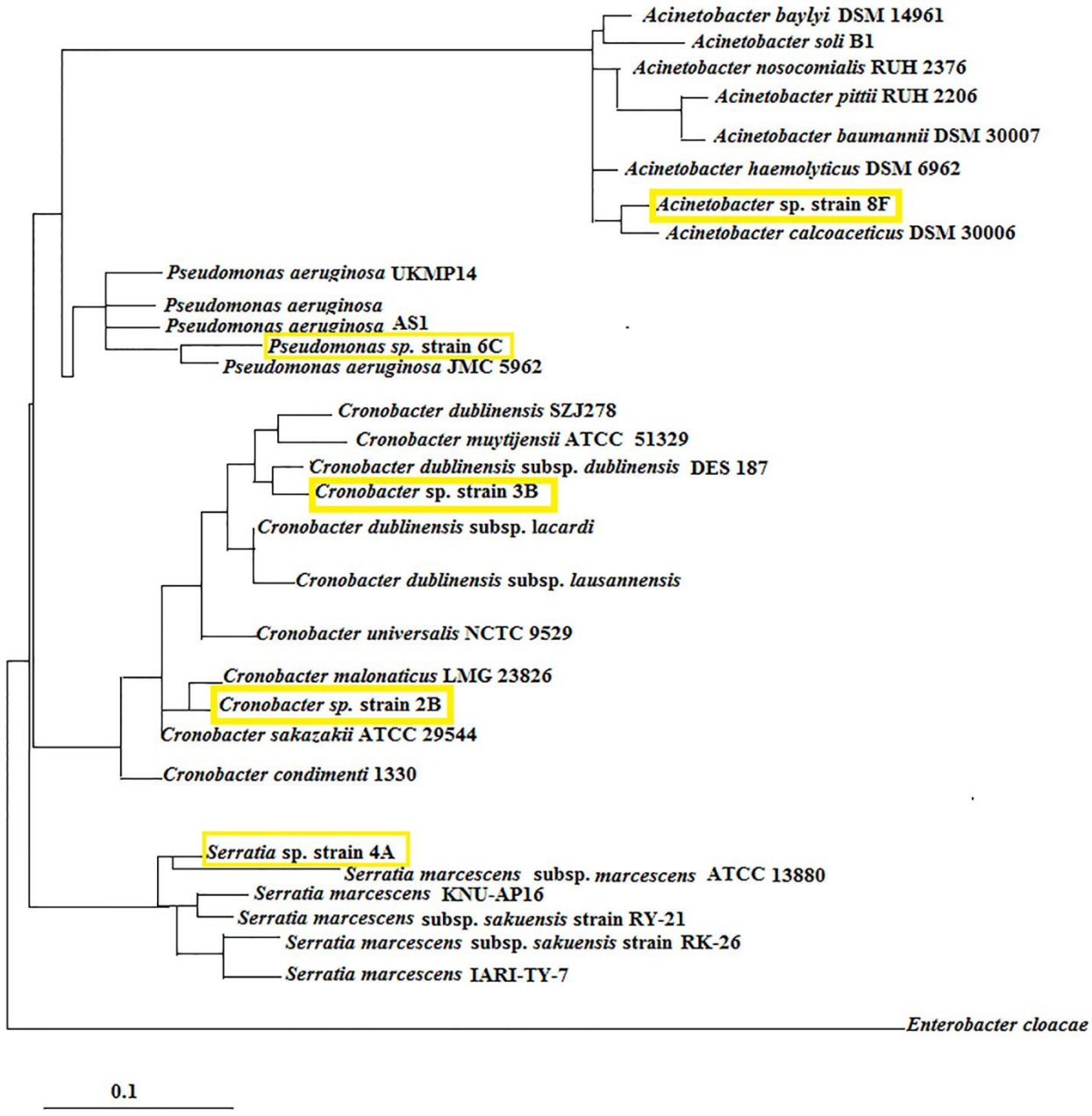

Fig. 4.22. Partial 16S rRNA sequence-based phylogenetic neighbour-joining tree showing the phylogenetic relationship between strains 2B, 3B, 4A, 6C, 8F and relative to other strains of the genus of *Cronobacter, Serratia, Pseudomonas, Acinetobacter*. *Enterobacter cloacae* is used as the out-group. Bar indicates 0.01.nucleotide substitutions per position.

4.6. Degradation of Crude Petroleum by Bacterial Strains Analysed by GC-MS

GC-MS (gas chromatography-mass spectrometry) was used to determine which isolates exhibited n-alkane degrading activity and oil-emulsifying activities.

Bacterialcells precultured overnight at 30 °C in NB medium were transferred to a 100-mL Erlenmeyer flaskswhich containing 25 mL of BSM plus with 1% crude oil. Cells were grown for 10 days at 30 °C with shaking at 120 rpm, and petroleum hydrocarbons remaining in theculture medium were determinedfor the ratio of degraded crude oil.

Following biodegradation, gas chromatography (GC) using a flame ionization detector (FID; HP 6850; Hewlett Packard, xx, xx) and equipped with a PONA quartz capillary column (100 m £ 0.250 mm). capillary column ([i.d; inner diameter] £0.50 m) was used in order to analyze the n-alkane fraction of crude oil. Helium was used as a carrier gas in order to perform Split injections. The column temperature increased from 60 °C to 320 °Cat a rate of 3 °C per minute. An injector temperature of 190-300 °C and a detector temperature of 350 °C were used. In order to detect individual components of the n-alkane fraction, matching the retention time with authentic standards was utilized. The values so obtained were normalized against the internal standard.

The percentage of total hydrocarbons remaining after incubation period was calculated by comparing area of their peaks with that of the corresponding peaks shown by a control that had been subjected to the same experimental conditions as the samples, except for the absence of a bacterial culture.

For calculating the degradation percentage of n-alkanes present in the crude oil, the gas chromatograms of the undegraded control and the degraded sample in the absence and the presence ofthe strains 2B, 3B, 4A, 6C and 8F, respectively, were compared (Figures 4.38-43). The results of the degradation were presented in Table 4.10.Gas chromatographic analysis revealed that the strain 2B degraded 84.76% of n-alkanes ranging from C9 to C34 in the 1% crude oil after incubation of 10 days. The strain 3B seems not to degrade the crude oil. The strain 4A degraded 73.25% of n-alkanes ranging from C9 to C34. The strain 6C was found to be the best degrader with 91.62% n-alkanes degrading ability ranging from C9 to C34. The strain 8F degraded

41% percentage of n-alkanes ranging from C12 to C34. Mixure of strains degraded 69.50% percentage of n-alkanes ranging from C11 to C34.

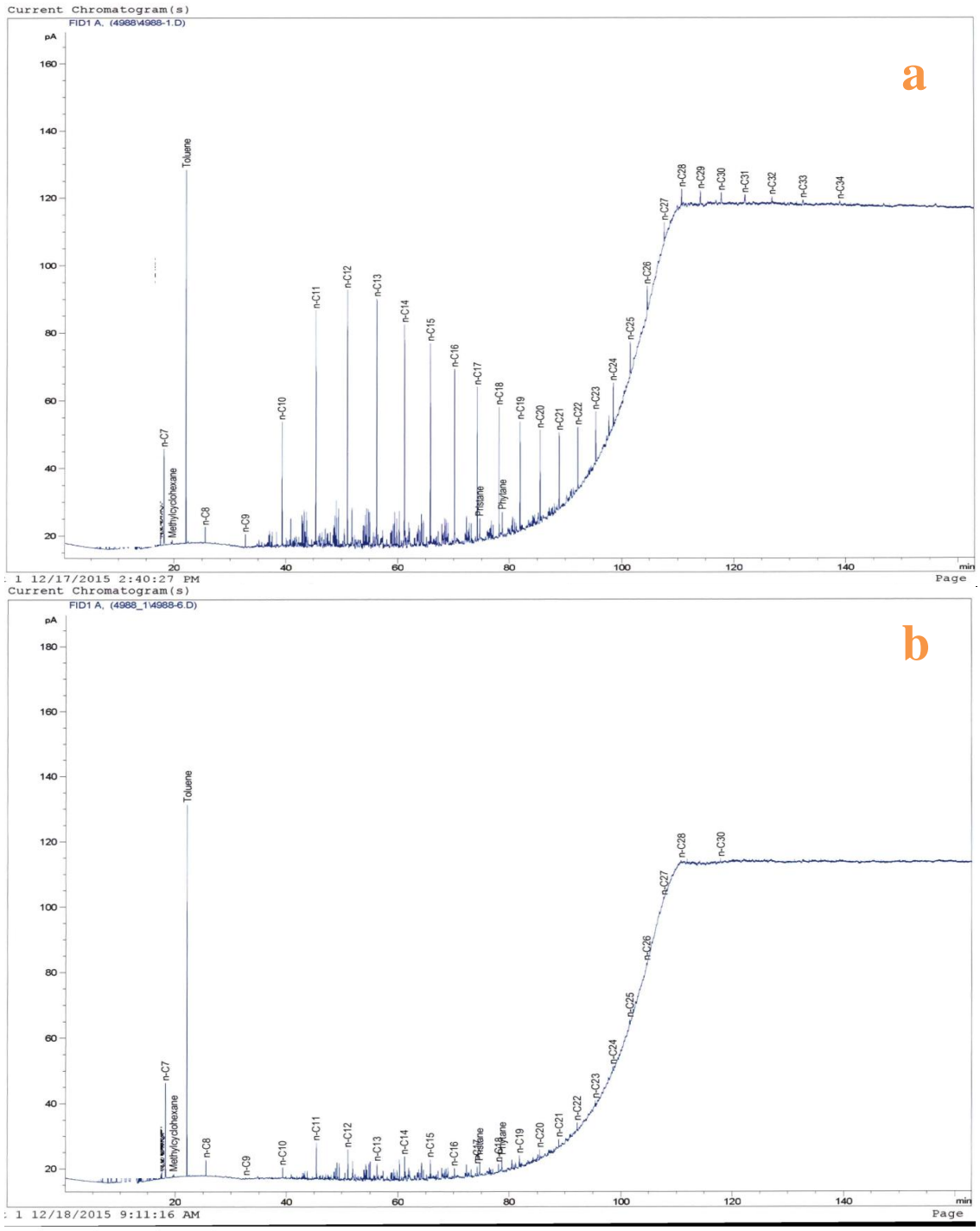

Figure. 4.23. GC profiles of crude oil remaining in the BSM after incubation without (A) and with (B) strain 2B at 30°C for 10 days. Toluene, pristane and phytane were used as the internal standards.

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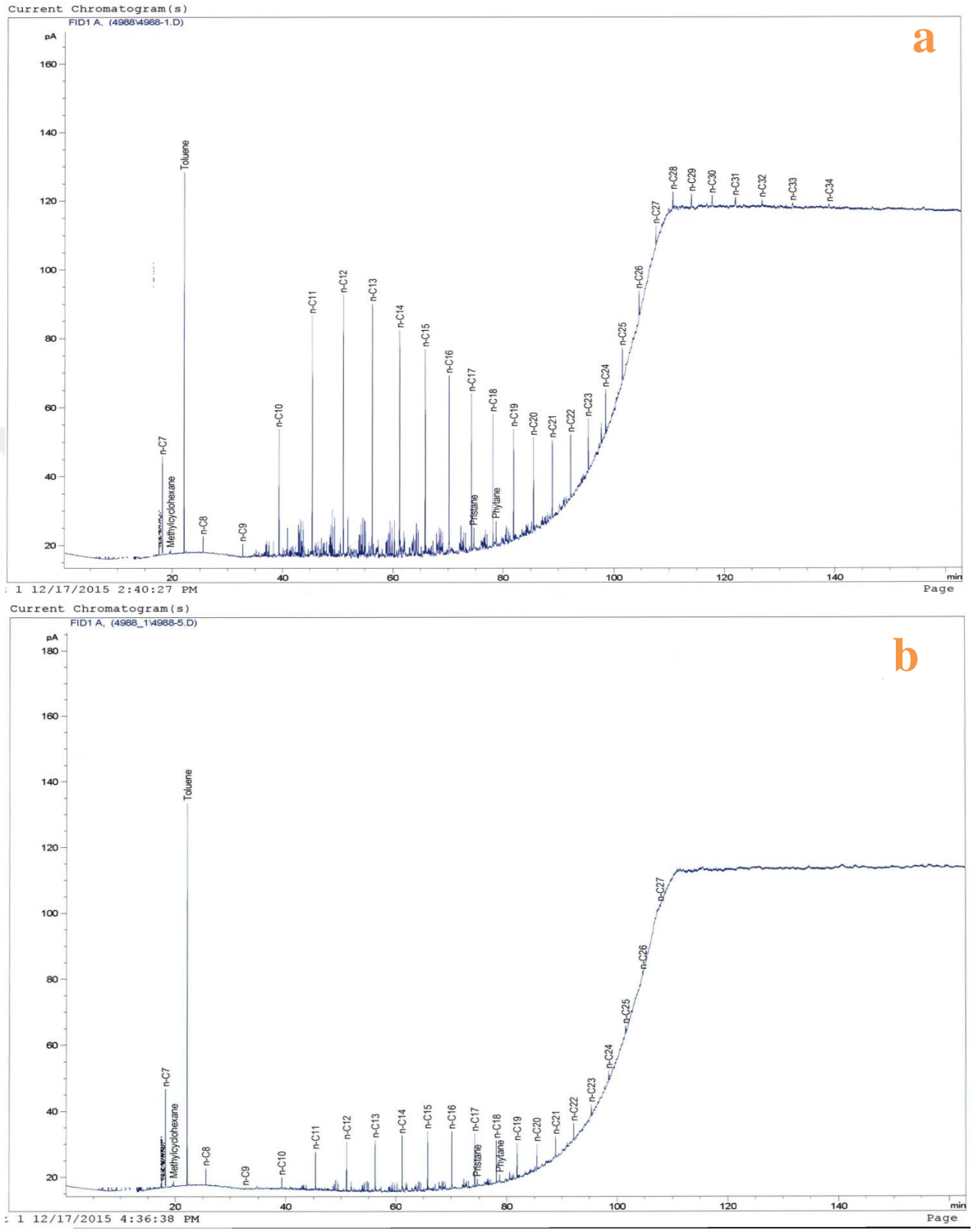

Figure. 4.24.GC profiles of crude oil remaining in the BSM after incubation without (A) and with (B) strain 4A at 30 °C for 10 days. Toluene, pristane and phytane were used as the internal standards.

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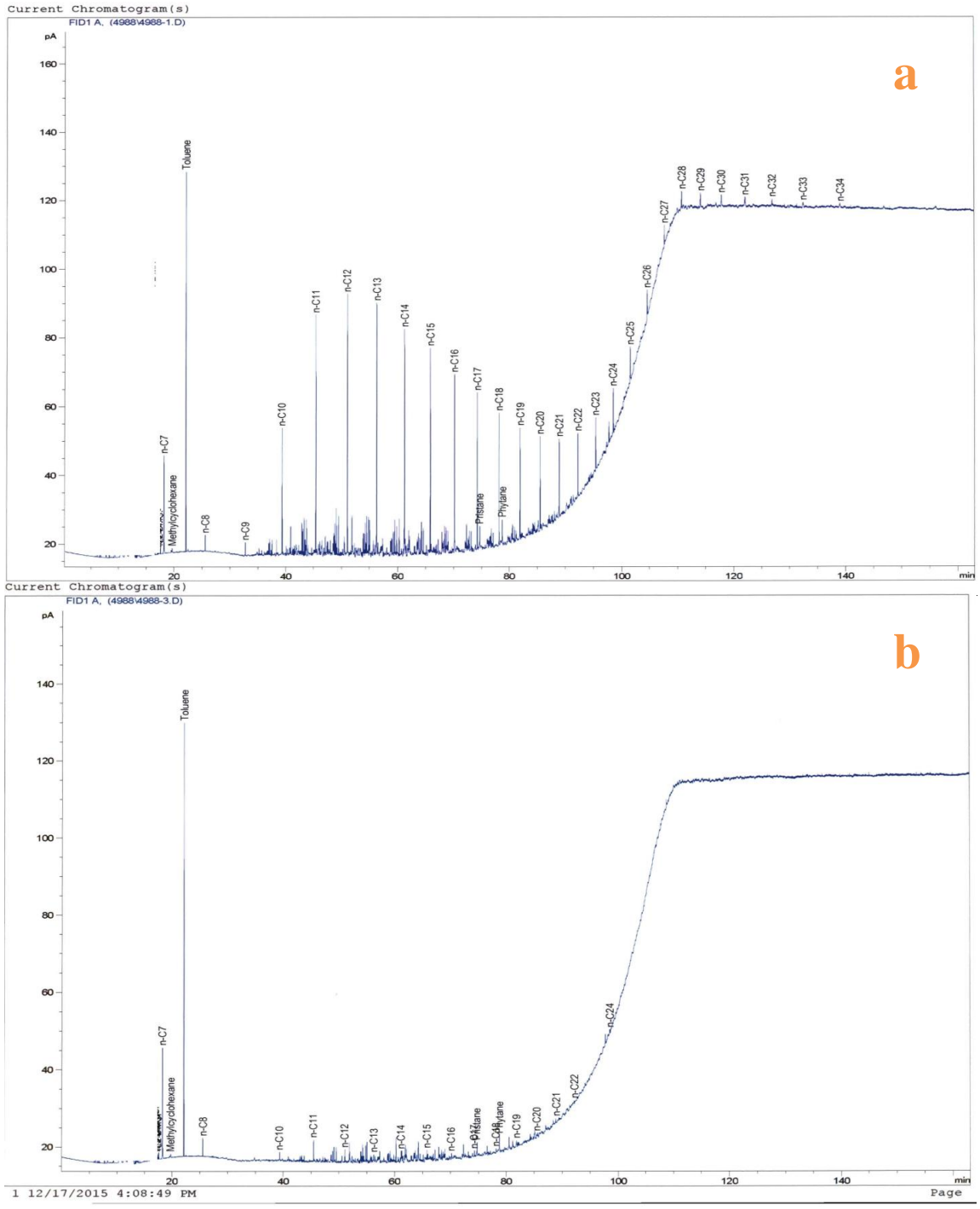

Figure. 4.25. GC profiles of crude oil remaining in the BSM after incubation without (A) and with (B) strain 6C at 30 °C for 10 days. Toluene pristine and phytane were used as the internal standards.

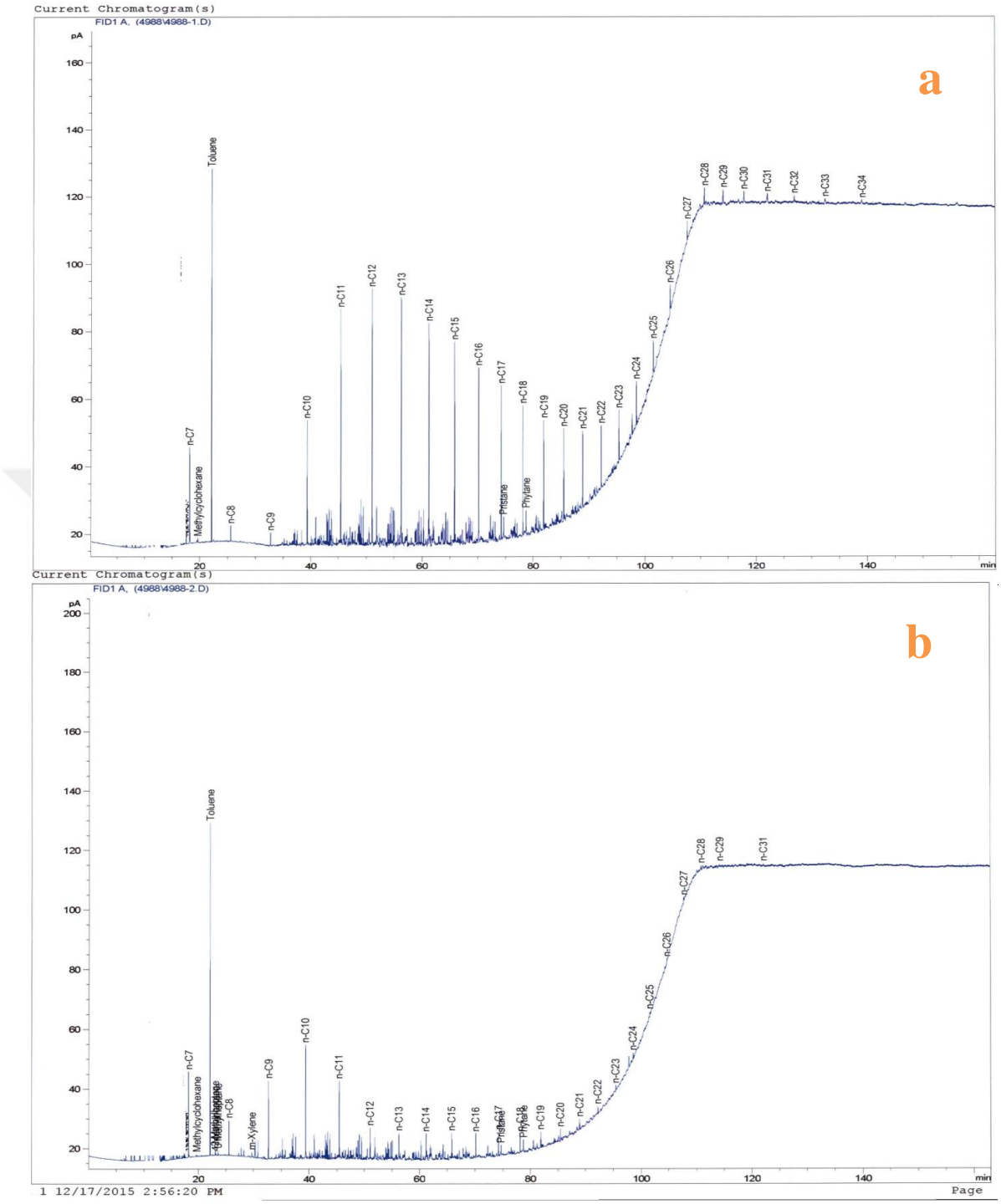

Figure. 4.26. GC profiles of crude oilremaining in the BSM after incubation without (A) and with (B) strain 8F at 30 °C for 10 days. Toluene, pristine and phytane were used as the internal standards.

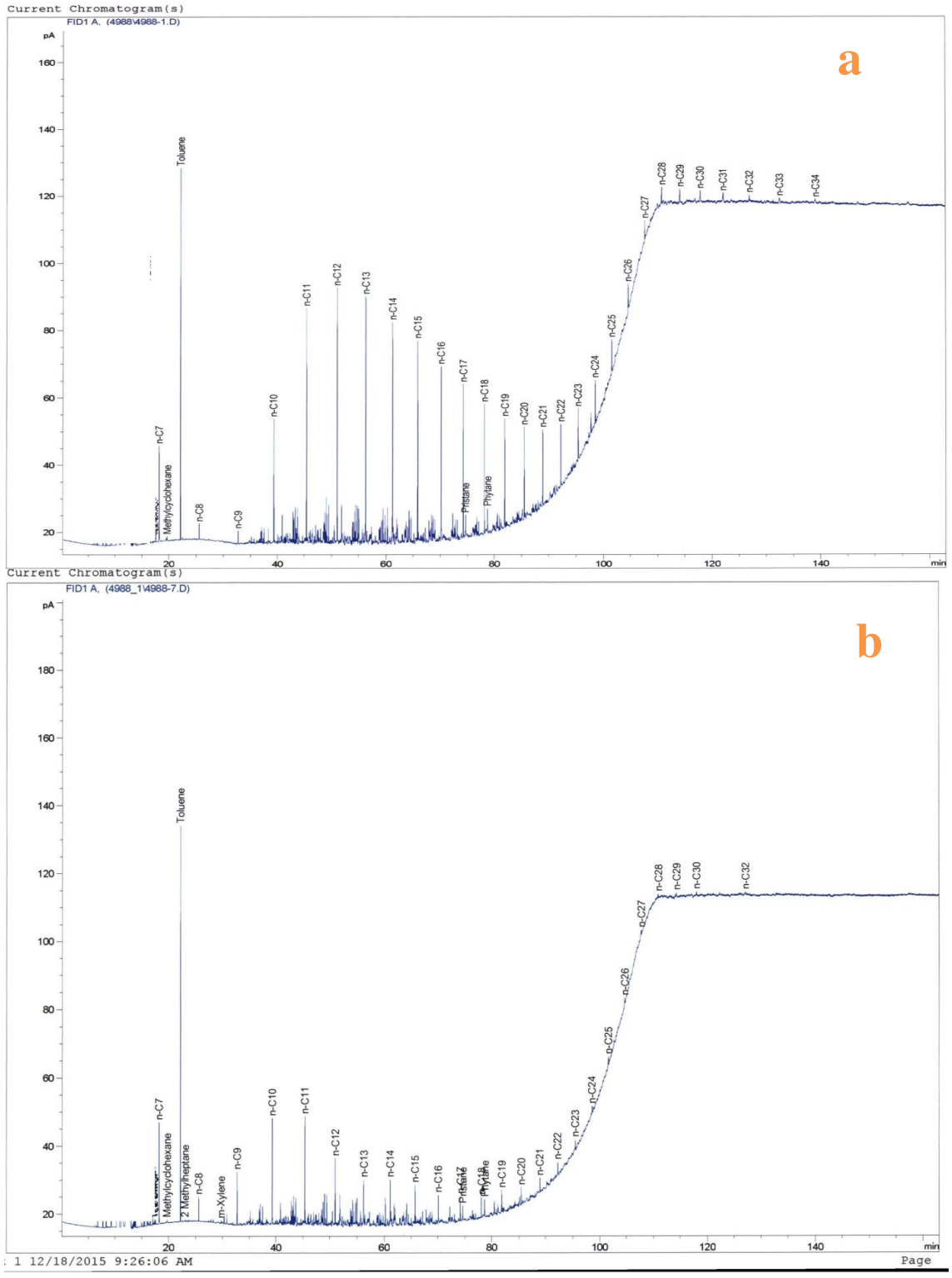

Figure. 4.27. GC profiles of crude oilremaining in the BSM after incubation without (A) and with (B) strains mix at 30 °C for 10 days. Toluene ,pristane and phytane were used as the internal standard. nC10 to nC34 indicate n-alkanes with the number of carbon atoms from 10 to 34.

Table 4.10.The utilization range and degradation percentage of n- alkanes in crude oil by isolated strains.

Hydrocarbons	**Control**	**6C**	**2B**	**4A**	**8F**	**Mix**
	Area%	**Area%**	**Area%**	**Area%**	**Area%**	**Area%**
***n*-C7**	0.00465	0.00452	0.00449	0.00476	0.00413	0.00415
***n*-C8**	0.00076	0.00073	0.00074	0.00080	0.00186	0.00107
***n*-C9**	0.00068	0	0.00012	0.00014	0.00427	0.00249
***n*-C10**	0.00622	0.00036	0.00060	0.00064	0.00646	0.00505
***n*-C11**	0.01170	0.00102	0.00192	0.00196	0.0455	0.00525
***n*-C12**	0.01273	0.00058	0.00157	0.00243	0.00171	0.00318
***n*-C13**	0.01271	0.00038	0.00103	0,00256	0.00174	0.00249
***n*-C14**	0.01191	0.00071	0.00161	0.00307	0.00190	0.00263
***n*-C15**	0.01032	0.00046	0.00104	0.00306	0.00149	0.00195
***n*-C16**	0.00937	0.00043	0.00076	0.00308	0.00164	0.00171
***n*-C17**	0.00829	0.00038	0.00056	0.00283	0.00153	0.0014
***n*-C18**	0.00687	0.00023	0.00040	0.00223	0.00123	0.00096
***n*-C19**	0.00635	0.00053	0.001	0.00197	0.00117	0.00137
***n*-C20**	0.00488	0.00024	0.00058	0.00135	0.00067	0.00097
***n*-C21**	0.00447	0.00028	0.00044	0.00119	0,00077	0.00071
***n*-C22**	0.00338	0.00015	0.00068	0.00104	0,00045	0.00099
***n*-C23**	0.00300	0	0.00048	0.00082	0,00032	0.00071
***n*-C24**	0.00274	0.00028	0.00052	0.00072	0.00055	0.00043
***n*-C25**	0.00214	0	0.00056	0.00048	0.00028	0.00086
***n*-C26**	0.00200	0	0.00027	0.00048	0.00026	0.00054
***n*-C27**	0.00150	0	0.00017	0.00023	0.00043	0.00033
***n*-C28**	0.00124	0	0.00027	0	0.00043	0.0004
***n*-C29**	0.00141	0	0	0	0.00039	0.0005
***n*-C30**	0.00127	0	0.0007	0	0	0.00052
***n*-C31**	0.00146	0	0	0	0.00039	0
***n*-C32**	0.00086	0	0	0	0	0.00039
***n*-C33**	0.00096	0	0	0	0	0
***n*-C34**	0.00068	0	0	0	0	0
Total oilhydrocarbonsbyAreapercentage	**0.13455**	**0.01128**	**0.02051**	**0.036**	**0.07957**	**0.04105**
DegradationPercentage (%)	**100%**	**91.62%**	**84.76%**	**73.25%**	**41%**	**69.50%**

5. RESULTS AND DISCUSSION

Petroleum is employed as raw matter in numerous industries, such as the refinery-petrochemical industry, where crude oil is refined through different technological processes into user products that are used for daily usage such as oils, gasoline, asphalt, domestic fuel oil, paraffin oils, lubricants, vaseline and polymers. Also, oil-derived products are generally employed in many other chemical processes. In our daily life and industrial used petroleum hydrocarbons as important and primary energy resources (Wolicka and Borkowski. 2012, Mahjoubi et al. 2013), for its low energy consumption, low environmental impact, and cost-effectiveness (Tang et al. 2012).

As a result, most of the crude oil goes in the ecosystem via leak of coastal oil refineries. It causes a risk to the environment and central in the global economy (Mahjoubi et al. 2013). The discharge of petroleum products in the environment results in environmental damages that can directly or indirectly affect human safety and ecological systems (marine and continental) (Jiang et al. 2010). Owing to petroleum has complex composition, it can elicit multiple types of toxic effects (Gardner et al. 1991). Toxic organic materials in crude oil released through oil spillage and oil pollution in water environment have been a major threat to ecosystem food chain (Al-Saleh et al. 2009). So, most of people decide to retain ecosystems as well as to appraise the damage caused by contamination. During the last years, pollution in habitats has led to extensive research. To prevent this pollution, physical and chemical methods and more microbial bioremediation have been used to degeradate these materials (Zhao et al. 2008, Prince 1997).

Bioremediation devices an environmentally friendly choice technology that has been established and implemented. Microorganism activity serves to remove pollution from crude oil and oil-derived products from groundwater, soil and seawater occurring nearby extraction sites, leaking pipelines, and in dispersed locations such as petrol stations (Wolicka and Borkowski 2012). It aggregates the primary mechanism for the resolving of hydrocarbons from contaminated sites by microorganisms. Through a set of complicated biochemical reactions, microorganisms degrade the molecular structure of pollutants into

inactive compounds, without raising the waste volume, neither noxious emissions discharged into the atmosphere and especially empowering the treatment of contaminated soil in situ (Nyyssönen et al. 2008).

Recently, extensive research has been conducted on oil-contaminated areas by crude oil to isolate the degradation bacteria. A recent compilation of microdegradation published includes the hydrocarbon degrading microorganisms, 79 bacterial genera, 103 fungal genera, nine cyanobacterial genera and 14 algal genera (Head et al. 2006, Hassanshahi et al. 2013, Aliakbari et al. 2014).

The important aim of this study is to isolate and identify the petroleum degrading bacteria from petroleum-contaminated sites in the oil fields. Moreover, we have studied the ability of these isolated bacterial strains to degrade the aliphatic hydrocarbons by evaluating the total hydrocarbon rates.

The strains 2B, 3B, 4A, 6C and 8F were isolated from the Zakho (T20), Baradarash (B4) Baradarash (B1), Mreba and from the Atrush petrol stations, respectively. All results were consistent with the description of the genus in Bergey's Manual of Systematic Bacteriology, as all of which were found to belong to Gammaproteobacteria (Garrity et al. 2007). The morphological, physiological and phylogenetic characteristics of this isolate were analyzed by the prenciples described in Bergey's Manual of Systemic Bacteriology and by the method of 16S rRNA sequencing (as described in the previous section). In our study, 2 strains namely 2B and 3B were identified as members of *Cronobacter* genus isolated from contaminated soil by crude oil in Zakho and Baradarash petrol stations.

The present study showed that the cells of the petroleum- degrading strain 2B were Gram-negative, rod-shaped, non-spore forming from the family Enterobacteriaceae. The 2B strain was also motile, facultative anaerobic that grew in Nutrient Broth Medium. The temperature growth was ranging from 25 to 40 ° C with an optimum growth at 30 ° C. The pH growth was ranging from 6.0 to10. 0 with an optimum at pH 8.0. The time-dependent growth was observed up to 72^{th} h. The isolate 2B was positive for starch hydrolysis, Catalase, and gelatin hydrolysis, whereas negative for oxidase, urease and sodium azide.

The isolate 2B didn't utilize citrate and L-tyrosine as carbon source, and tolerated up to 10% NaCl (w/v). 2B strain had also tolerated 0.001% lysosome.

3B that was not good at degrading petroleum was Gram-negative, rod-shaped bacteria, facultative anaerobic, motile and non- spore forming, The temperature growth was ranging from 10 to 50 ° C. Optimum growth temperature was designated as 25 ° C. The pH growth was ranging from 5.0 to11.0 with an optimum at pH 7.0. The time-dependent growth was observed up to 72th h. The isolate 3B was positive for starch hydrolysis, Catalase and gelatin hydrolysis, whereas negative for oxidase, urease, and sodium azide. The isolate 3B utilized citrate and L-tyrosine as carbone source, and tolerated up to 7% NaCl (w/v) and to 0.001% lysosome.

The bacteria were identified as *Cronobacter* sp. as previously named as *Enterobacter sakazakii* (Iversen et al. 2007, 2008). *Cronobacter* is Gram-negative, facultative anaerobic, in general, reported as motile. Oxidase test is negative, catalase and urea hydrolysis are reported as positive. *Cronobacter* species utilizes citrate. This genus was found to be able to reproduce in the pH range 5.0-10.0 and 6-45 °C and in NaCl concentration of 7%. Kandhai et al. 2006) reported the temperature range of *Cronobacter* as 5.5- 45 °C and an optimum temperature of 39.4 °C. Lambert and Bidlas (2007) also reported an optimum pH of 5-9, and growing in a salt concentration of 9.1%. L-tyrosine is not utilized as a sole source of carbon.

The phenotypic characters differentiating *Cronobacter* species are summarized in the Table 5.1.

The present study compared with other studies researched by Iversen et al. (2007, 2008), Lambert and Bidlas (2007) shows that *Cronobacter* is able to grow in high concentration of salt and generally all of them are mesophilic bacteria.

Comparative analysis conducted between 2B , 3B and other *Cronobacter* species studied by (Iversen et al. (2007, 2008)), showed that they were different in growth temperature and for some of biochemical tests, for example: 2B and 3B were negative for urea hydrolysis and 2B didn't utilize citrate. The 2B temperature growth ranged from 25 to 40 ° C with an

optimum growth at 30 ° C while 3B ranged from 10 to 50 ° C with an optimum growth temperature of 25 ° C.

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Table 5.1. Morphological, physiological and biochemical tests on selected *Coronobacter* sp.

Bacteria / Features	1	2	3	4	5
Cell shape	Rod	Short Rod	ND	Rod	Rod
Gram feature	-	-	-	-	-
Mobility	+	+	+	-	-
Oxygen requirement	Facultative anerobic	Facultative anerobic	Facultative anerobic	ND	ND
Growth temperature (°C)	25-40	10-40	6-45	ND	ND
Optimum growth temperature (°C)	30	25	37-43	45	37
Growth pH	6.0-10.0	5.0-8.0	5.0-10.0	ND	ND
Optimum growth pH	8.0	7.0	ND	ND	ND
Starch hydrolysis	++	++	ND	ND	ND
Gelatin hydrolysis	+	+	ND	+	-
Oxidase	-	-	-	-	-
Catalase	+	+	+	+	+
L-tyrosin	-	+	ND	ND	ND
Sodium citrate	-	+	+	+	+
Urea hyrolysis	-	-	+	-	-
Sodium azide	-	-	ND	ND	ND
Lysosome	-	-	ND	ND	ND
NaCl tolerance	10%	7%	7%	ND	ND

+: Positive, ++:strong positive, -: negative, ND: no detected

1:2B, **2:**3B, **3:** *Cronobacter* spp (Iversen et al. (2007, 2008)), **4:** *Cronobacter condimenti,* **5:** *Cronobacter universalis* (Joseph et al. 2012).

The strain 2B was found to be sensitive to Lincomycin (15 µg), Tilmicosin (15 µg), Tetracycline (30 µg), Streptomycin (10 µg), Gentamicin (10 µg), Chloramphenicol (30 µg). It was found to be semi-sensitive to Fusidic acid (10 µg), Neomycin (10 µg), Novobiocin (5 µg), but resistant to Kanamycin (5 µg), Bacitracin (10 unit), Penicillin (10 unit), Penicillin (2unit), Ampicillin (10 µg) and Nystatin (100 unit).

3B was sensitive to Streptomycin (10 µg). It was found to be semi-sensitive to Tilmicosin (15 µg), Kanamycin (5 µg), Neomycin (10 µg), Gentamicin (10 µg), Chloramphenicol (30 µg) and was resistant to Nystatin (100 unit), Penicillin (2 unit), Lincomycin (15 µg), Ampicillin (10 µg), Fusidic acid (10 µg), Tetracycline (30 µg), Novobiocin (5 µg), Bacitracin (10 unit) and Penicillin (10 unit). Antimicrobial susceptibility testing indicated that 2B and 3B shared resistantancy to Nystatin (100 unit), Penicillin (2 unit), Ampicillin (10 µg), Bacitracin (10 unit) and Penicillin (10 unit). Both were found to be sensitive to Streptomycin (10 µg).

Cronobacter sp. tends to be more sensitive to most antibiotics than other Enterobacteriaceae, although resistance to ampicillin has developed. In 1980, all strains tested were susceptible to ampicillin, whereas in 2001, Lai described five cases of *Cronobacter* infection in which one or more of the isolates were resistant to ampicillin (Farmer et al. 1980, Lai 2001).

Nevertheless, Nazarowec-White and Farber (1997) found that two *Cronobacter* strains out of eight were resistant to tetracycline and chloramphenicol. Al-Nabulsi et al. (2011) showed that streptomycin, gentamicin and kanamycin are effective against both stressed and unstressed *C. sakazakii* cells. El-Sharoud et al. (2009) reported that *C. malonaticus* CFS-FSMP 1500 and *C. malonaticus* CFS-FSMP 1510 were found to be resistant to neomycin. However, 2B and 3B were semi sentitive to neomycin. Hunter and Bean (2013), reported *C. sakazakii*, typical of most Enterobacteriacae, has natural resistance to fusidic acids. Al-Sharoud et al (2009) recorded that two strains of *Cronobacter* species, *C. sakazakii and C. malonaticus* were susceptible to streptomycin, ampicillin and gentamicin. Strydom et al. (2012) showed that the strain of *Cronobacter* species were penicillin-resistant, also some species sensitive to chloramphenicol and ampicillin. Acer et al. (2014)

recorded that *C. malonaticus* bt1b was sensitive to gentamicin, ampicillin 10, streptomycin 10 and chloramphenicol 30. It was determined to be resistant to neomycin, penicillin and penicillin 2 and 10 antibiotics.

The isolates selected as the hydrocarbon degraders were also identified by 16s rRNA squence analysis. The resulting sequence was entered into the BLAST nucleotide search program of the National Center for Biotechnology Information to obtain closely related phylogenetic sequences indicating similarity of the isolates.

2B has 96.31% sequence similarity *to Cronobacter malonaticus* LMG 23826. The results were presented as a phylogenetic dendrogram in Figure 3. The strain 2B was identified and named as *Cronobacter* sp. 2B. 3B has 95.48% sequence similarity *to Cronobacter dublinesis subsp. dublinesis* DES187. The results were presented as a phylogenetic dendrogram in Figure 3. The strain 3B was identified and named as *Cronobacter sp.* 3B.

Iversen et al. (2007, 2008) reported with applied ribotyping, amplified fragment length polymorphisms (AFLP) and 16S rRNA gene sequencing in combination with DNA–DNA hybridizations to a collection of 210 *E. sakazakii* isolates. Their results not only formed the basis for the classification of this pathogen into the new *Cronobacter* genus but also clarified species designations. Iversen et al (2007) reported by 16S rRNA gene sequencing and ribotyping identifying a new species and named as *Cronobacter malonaticus.*

Moreover, the proposed species *Cronobacter dublinensis* sp. nov. comprises strains was identified previously (Farmer et al. 1980). But Iversen at 2008 reported the species and designated as *Cronobacter dublinensis* subsp. *dublinensis* subsp. Nov by DNA–DNA hybridizations.

Turcovský et al. (2011) utilized 16S rRNA gene sequencing for identification of 27 *Cronobacter* strains. 5 strains had 100% sequence similarity to *C. dublinensis.*6 strains had sequence similarity to *C. malonaticus*, but could not be distinguished by partial 16S rRNA sequencing and formed one common cluster with 98.3% similarity.

Acer et al. (2014), revealed that by 16S rRNA gene analysis, strain BT1B was detected 99.7% similarity with *C.malonaticus* DSM 18702 and 99.6% similarity to *C.sakazaki*ATCC 29544.

Our results also demonstrate that not only 16S rRNA gene sequencing but also DNA-DNA hybridisations should be carried out for identifications of *Cronobacter* strains, due to recent taxonomic revisions.

Our study shows that the cells of isolating 4A were Gram-negative, very small straight rod-shaped, coccobacilli, non-spore forming from the family Enterobacteriaceae. The 4A strain was motile, facultative anaerobic that grew in Nutrient Broth Medium. The temperature growth ranged from 5 to 45 ° C, with an optimum growth of 30 ° C. The pH growth ranged from 5.0 to10.0 with an optimum of pH 5.0. The time-dependent growth was observed up to 72 th h.

The isolate 4Awas positive for catalase, urease and gelatin hydrolysis, whereas negative for starch hydrolysis, oxidase and sodium azide tests. The isolate 4A utilized citrate and L-tyrosine as carbon source. 4A tolerated up to 10% NaCl (w/v), but sensitive to % 0.001 lysosome.

Caprette (2009) found similar results about morphological characters of *Serratia marcescens.* Wongsa et al. (2004) discovered same results about starch hydrolysis, catalase, oxidase and citrate in *Serratia marcescens.* HokM. But, they found different result about the temperature growth as it ranged from (0-50) ˚C. Dhahi et al. (2011) reported optimum growth of all strains of *Serratia* at pH 7 and at a temperature from 20 to 37 ˚C. Alariya et al. (2013) found different results of optimum growth condition for *Serratia marcescens*, with a optimum temperature of 35-40 ° C. The optimum pH was found to be 7. Caprette (2009) recorded about *S. marcescens* as oxidase negative, catalase strongly positive and liquifies gelatin at 22 °C. Kaira and Pandey (2015) discovered same result of optimum growth temperature between 4 to 45 °C (opt. 25 °C). But, he obtained different result of optimum growth pH. The bacteria was grew pH 3 to 14 (opt. 5 pH).

The phenotypic characters differentiating *Serratia* species are collected in the Table 5.2

Table 5.2. Morphological, physiological and biochemical tests on isolated *Serratia* sp.

Bacteria / **Features**	**1**	**2**	**3**	**4**	**5**	**6**
Cell shape	coccobacilli	Very small straight rods	Very small straight rods	Short rod-shape	ND	rod- shape
Gram feature	**-**	ND	ND	**-**	**-**	**-**
Mobility	**+**	**+**	**+**	ND	ND	**-**
Oxygen requirement	Facultative anerobic	Facultative anerobic	Facultative anerobic	ND	ND	ND
growth tempreture (°C)	5-44	20-37	ND	ND	ND	ND
Optimum growth temperature (oC)	30	30	ND	ND	ND	ND
growth pH	5.0-10.0	8.0-8.5	ND	ND	ND	ND
Optimum growth pH	5.0	7	ND	ND	ND	ND
Starch hydrolysis	**-**	ND	ND	ND	-	ND
Gelatin hydrolysis	**+**	ND	+	ND	+	ND
Oxidase	**-**	ND	**-**	**-**	ND	**+**
Catalase	**++**	ND	**+**	**+**	+	**-**
L-tyrosin	**+**	ND	ND	ND	ND	ND
citrate	**+**	ND	ND	ND	+	ND
Urea hyrolysis	**W**	ND	ND	ND	-	ND.
Sodium azide	**-**	ND	ND	ND	ND	ND
Lysosome	**-**	ND	ND	ND	ND	ND
NaCl tolerance	15%	ND	ND	ND	ND	ND

+: Positive, ++: strong positive, -: negative, ND: no detected, w: weak

1: 4A, **2:***Serratia* sp. (Dhahi et al (2011)) **3:***Serratia marcescens* (Caprette (2009)), **4:***Serratia* sp. strain WPRA3 (JX020764); *Serratia* sp. strain SM11-3j; *Serratia* sp. srtain SC-G18 (Jafarzade et al. (2012)), **5:** *Serratia rubidaea (Abd- Alla et al. (2011)),* ***6:*** *S. plymuthica* RVH1(Van Houdt et al. 2005)

In this study, 4A was sensitive to Streptomycin (10 µg), Gentamicin (10 µg). It was semi-sensitivity to Kanamycin (5 µg), Neomycin (10 µg), but it was resistant to Penicillin (2unit), Tilmicosin (15 µg), Lincomycin (15 µg), Chloramphenicol (30 µg), Ampicillin 1 (0 µg), Fusidic acid (10 µg), Tetracycline (30 µg), Novobiocin (5 µg), Nystatin (100 unit), Bacitracin (10 unit), Penicillin (10 unit).

Singlton and Sainsbury (2001) and Caprette (2009) reported *Serratia* was of great interest in medicine due to its antifungal, antibacterial, antiprotozoal, antimalarial, immunosuppressive, and anticancer activities.

Both the present study and study done by Faidah (2015) showed that the strains of *Serratia marcescans* were resistant to Ampicillin, Tetracycline. Isolates remained susceptible to Gentamycin. Wilkowske et al. (1970) said that the only antibiotics to which strains of *S. marcescens* were consistently susceptible in vitro were kanamycin and gentamicin. It was found to be resistant to Ampicillin, Tetracycline, Streptomycin.

16S rRNA squence analysis of the strain 4A showed that closely related phylogenetic sequences indicated similarity to *Serratia marcescens* subsp. *marcescens* ATCC13880 with 92.94%. There have been several studies on identification of *Serratia marcescens* by 16S rRNA sequence analysis carried out by Wongsa et al. (2004), Van Houdt et al. (2005), Whitehouse et al. (2007), and Rajasekar et al. (2007).

The petroleum- degrading strain 6C was Gram-negative, rod-shaped, facultative anaerobic, motile and non- spore forming, the temperature growth ranged from 25 to 40 ° C. Optimum growth temperature was found as 30 ° C. The pH growth ranged from 6.0 to11.0, with an optimum of pH 7.0. The time-dependent growth is carried out up to 72 th h.

The isolate 6C was positive for starch hydrolysis, oxidase, catalase, urease, and gelatin hydrolysis, whereas negative for sodium azide test. The isolate 6C utilized citrate and L-tyrosine as carbone source, and tolerated up to 10% NaCl (w/v) and to 0.001% lysosome.

Schroeter (1872) and Migula (1900) reported that *Pseudomonas* sp. was Gram-negative,straight or slightly bent rods but not helical, motile by one or multiple polar flagella; sometime nonmotile.

Moore (2006) reported that it was Aerobic, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor; allowing development to happen anaerobically. In general, they are not eminent anaerobic bacteria, and they do not live in extreme thermophilic and acidophilic habitats. In previous studies, the optimum temperature range for *Pseudomonas* sp. was found 4–42°C as mesophilic and pH between 4- 8. Oxidase test was positive or negative and catalase was positive (Schroeter (1872), Migula (1900), moore (2006), Franzetti and Scarpellini (2007), Raaijmakers et al. (2010).

In comparison with the present study, Wongsa et al. (2004) reported that *Pseudomonas* aerogenusa WatG was oxidase, catalase, gelatin hydrolysis positive, whereas starch hydrolysis was negative.

Xio et al. (2014) reported that Strain WJ6 was one of the 86 strains isolated from the heavy oil-contaminated soil. The microorganism was gram-negative, facultative anaerobic, oval to rod-shaped (0.3-0.5μm×0.5-1.0μm), and motile with single flagellum. Colonies (0.5-2 mm) growing on LB agar for 48 hours at 37ºC were smooth, circular convex, wet and yellowish-brown in color. The organism was grown at 5-50 ºC, in 0-15% (w/v) NaCl and pH 4-10, with the optimum growth occurring at pH 6.5-7.5, 37 ºC and 0-2% (w/v) NaCl. It was positive for catalase, urease, oxidase, as well as for the hydrolysis of starch and gelatin.

The phenotypic characters differentiating *Pseudomonas* species are summarized in the Table 5.3.

Table 5.3. Morphological, physiological and biochemical tests on isolated *Pseudomonas* sp.

The strains / Features	1	2	3	4	5	6
Cell shape	Rod	Rod	N.D.	N.D.	N.D.	N.D.
Gram feature	-	-	-	-	-	-
Mobility	+	+	+	+	+	+
Oxygen requirement	Facultative anerobic	ND	N.D.	N.D.	ND	N.D.
The growth tempreture (°C)	25-40	ND	N.D.	N.D.	ND	N.D.
Optimum growth temperature (oC)	30	ND	N.D.	N.D.	N.D.	N.D.
The pH growth	6.0-10.0	4.0-8.0	N.D.	N.D.	ND	N.D.
The optimum pH for growth	7.0	ND	N.D.	N.D.	N.D.	N.D.
Starch hydrolysis	++	+	+	+	+	+
Gelatin hydrolysis	+	-	+	+	-	-
Oxidase	+	+	-	-	-	-
Catalase	++	+	+	+	+	+
L-tyrosin	++	ND	N.D.	N.D.	N.D.	N.D.
Sodium citrate	+	-	+	+	+	+
Urea hyrolysis	++	-	+	+	+	ND.
Sodium azide	-	ND	ND	ND	ND	ND
Lysosome	+	ND	ND	ND	ND	ND
NaCl tolerance	7%	1%	7%	ND	ND	7%

+: Positive, ++:strong positive, -: negative, ND:no detected, w: weak

1: 6C**, 2:** *Pseudomonas* SUK 1207 (Satarupa and Paul (2013)), **3:***P. fluourescens,* **4:** *P.aeruginosa* **5:***P. pudia,* ***6:****P. stutzeri*(Abdelzaher, et al.(2004)).

6C was sensitive to Streptomycin (10 µg). It was resistance to Kanamycin (5 µg), Nystatin (100 unit), Chloramphenicol (30 µg), Neomycin (10 µg), Penicillin (2 unit), Lincomycin (15 µg), Tilmicosin (15 µg), Ampicillin (10 µg), Fusidic acid (10 µg), Tetracycline (30 µg), Novobiocin (5 µg), Gentamicin (10 µg), Bacitracin (10 unit), Penicillin (10 unit).

The isolate 6C was found to have 96.23% 16S rRNA sequence similarity *to Pseudomonas aeruginosa* JCM 5962. Morphological, physiological and phylogenetic properties also indicated that strain 6C was a member of the genus *Pseudomonas.*

Huy et al. (1999) recorded that the 16S rDNA sequence similarity of the strains 6TBX-CL and XCK isolated from oil-contaminated area to *Pseudomanas aeruginosa* as 96.8 % and 96.4 %, respectively.

Wongsa et al. (2004) also studied 16S rDNA sequence alignment and phylogenetic tree analysis, revealing 16S rRNA sequence of strain WatG to be 100% identical to that of *P. aeruginosa*. And designating the strain WatG as a novel strain of this species.

Xia et al. (2014) reported that strain WJ6 was one of the 86 strains isolated from the heavy oil-contaminated soil. The nearly-complete 16S rRNA gene sequence (1498 bp) of strain WJ6 was obtained and phylogenetic analysis indicated that it belonged to *Proteobacteria*, displaying the highest16S rRNA sequence similarity of 99.358% with *Pseudomonas sp.*

The petroleum- degrading strain 8F was Gram-negative, short rods (coccobacilli), bacteria strictly aerobic, non-motile, non-spore forming, the temperature growth ranged from 20 to 40 ° C. Optimum growth temperature was designated as 25-30 ° C. The pH growth ranged from 7.0 to9.0 with an optimum of pH 7.0. The time-dependent growth is carried out up to 72 th h. The isolate 8F was positive for starch hydrolysis, catalase, urease, and gelatin hydrolysis, whereas negative for oxidase, sodium azide test. The isolate 8F utilized citrate and L-tyrosine, as carbon source. It tolerated up to 7% NaCl (w/v) and to 0.001% lysosome.

The genus *Acinetobacter* was first discovered in 1954 by Brisou and Prevot to isolate

the non-motile from the motile members of the family “Achromobactereae”. Since then , these bacteria are regarded as non-motile, but it was reported by Doughari (2011) and Garrity et al. (2007), representing a twitching kind of motility.

Abbott et al. (1973) discovered a soil microorganism, identified as *Acinetobacter calcoaceticus*.The pH optimum for growth was between 6.5 and 7.5, and the temperature optimum was between 32 and 35 ºC.

In another study, Cormack et al. (1997) analysed Antarctic soil chronically exposed to gas-oil isolating *Acinetobacter* and *Acinetobacter* ADH-1. They were Gram-negative, non-motile, non-spore forming. The optimum temperature ranged between 25-30°C and growth was observed in the presence of 3.5% NaCl.

Patil et al. (2001) reported that *A.calcoaceticus* had negative result against gelatin test.

Table 5.4. Morphological, physiological and biochemical tests on selected *Acinetobacter* sp.

Bacteria / Features	1	2	3	4	5	6	7	8
Cell shape	coccobacilli	coccobacilli	coccobacilli	coccobacilli	N.D.	Rod	coccobacilli	coccobacilli
Gram feature	-	-	-	-	-	-	-	-
Mobility	-	+	-	+	-	-	ND	ND
Oxygen requirement	Aerobic	Aerobic	Aerobic	Aerobic	ND	Aerobic	ND	Aerobic
The growth tempreture (°C)	20-40	10-40	10-35	10-45	ND	10-40	ND	25-37
Optimum growth temperature (oC)	25-30	35	25-30	30	25-30	30	ND	ND
The growth pH	7.0-9.0	5.0-12.0	6.0-11.0	5.0-11.0	ND	5.0-10.0	ND	ND
The optimum pH for growth	7.0	5.0	6.5-10.0	5.0	7.0-8.0	6.0-8.0	ND	ND
Starch hydrolysis	+	+	+	+	ND	-	ND	ND
Gelatin hydrolysis	+	-	-	+	ND	ND	ND	+
Oxidase	-	-	-	-	-	-	ND	-
Catalase	++	+	+	+	+	+	ND	+
L-tyrosin	+	+	-	-	ND	+	ND	ND
Sodium citrate	+	+	-	+	+	+	ND	+
Urea hyrolysis	+	+	+	+	+	ND.	ND	ND
Lysosome	-	ND	ND	ND	ND	ND	ND	ND
Sodium azide	-	-	-	-	-	ND	ND	ND
NaCl tolerance	7%	5%	3%	4%	5%	5%	ND	ND

+: Positive, ++: strong positive, -: negative, ND: no detected, w: weak

1:8F **2:** *Acinetobacter* sp**.** ST5, **3:** *Acinetobacter* sp. GC2, **4:***Acinetobacter* sp. BT1A, (Acer et al. (2014)) **5:***Acinetobacter* sp. ADH1(*Acinetobacter lwoffii)* (Walter et al. (1997), **6:***Acinetobacter baumanii* (Chaineau et al. (1999), **7:** *Acinetobacter calcoaceticus* strain RR8 (Yuste et al. (2000). **8:** *A.beijerinckii* sp. nov. NIPH 838^{T} (Nemec et al. (2009).

8F was sensitive to Tilmicosin (15 µg), Streptomycin (10 µg), Gentamicin (10 µg), Chloramphenicol (30 µg). Also, it was semi-sensivity to Tetracycline (30 µg). But it had resistance to Kanamycin (5 µg), Neomycin (10 µg), Penicillin (2unit), Lincomycin (15 µg), Ampicillin (10 µg), Fusidic acid (10 µg), Novobiocin (5 µg), Nystatin (100 unit), Bacitracin (10 unit), Penicillin (10 unit).

Savov et al. (2000) and Doughari et al.(2011) stated an important problem of *Acinetobacter* sp. about resistance to antibiotics. Doughari et al. (2011) summarized that these organisms are commonly resistant to ampicillin, gentamicin, chloramphenicol, tetracycline. Previously ampicillin, second generation cephalosporins, colistin, amynoglycosides, impenim, quinolones, minocyline, sulbactam and gentamicin were used to treat *Acinetobacter* infections. Moreover, Acer et al. (2014) recorded that the strain *Acinetobacter* sp BT1A was found to be resistant to ampicillin, penicillin, and chloramphenicol, but sensitive to gentamicin.

The isolate 8F had 99.08% sequence similarity to *Acinetobacter calcoaceticus* DSM 30006. Acer et al, (2014) recorded that the similarity of 16S rRNA sequence between strain ST5 and *A. calcoaceticus* DSM 30006, *A. pittii* RUH DSM 2206 was %99.5. The homology between strain GC2 and *A.beijerinckii* and *A. lwoffii was* 99.9 % and % 98.2, respectively. The similarity between strain BT1A and *Acinetobacter baumannii* DSM 30007 was found as %100. In another study, Huy et al. (1999) showed the homology of 16S rDNA sequence between ZTN-NB and *Acinetobacter* sp. 1227 as 92.0 %.

GC-MS (gas chromatography-mass spectrometry) was used to determine which isolates exhibited n-alkane degrading activity and oil-emulsifying activities. The percentage of total hydrocarbons remaining after incubation time was calculated by comparing area of their peaks with that of the corresponding peaks shown by a control that had been subjected to the same experimental conditions as the samples, except for the absence of a bacterial culture.

For calculation of the percentage degradation of n-alkanes present in the crude oil, the gas chromatograms of the undegraded control and the degraded sample in the absence

and the presence of the strains 2B, 3B, 4A, 6C and 8F, respectively, were compared (Figure 4. 38-43). The results of the degradation were presented in Table 4.10.

The most common bacterial hydrocarbon–degraders reported in the literature by (Rusansky et al., 1987; Kiyohara et al., 1992; Johnson et al., 1996; Barathi and Vasudevan, 2001; Bhattacharya et al. 2002; Pokethitiyook et al., 2003; Van Hamme et al., 2003). Gas chromatographic analysis revealed that the *Cronobacter* sp. strain 2B degraded 84.76% of n-alkanes ranging from C9 to C34 in the 1% crude oil after incubation of 10 days. The strain *Cronobacter* sp. 3B didn't degrade n-alkanes good enough. Acer et al. (2014) reported that a strain designated as BT1B was found to be closely related to *Cronobacter malonaticus*, but similarly degradation was not recorded.

The *Serratia* sp. strain 4A degraded 73.25% of n-alkanes ranging from C9 to C34.

Wongsa et al. (2004) found a strain, which was identified as new strains of *Serratia marcescens* species, showed a relatively high capacity and wide spectrum to degrade the hydrocarbons in gasoline, kerosene, diesel, and lubricating oil. This strain could also degrade kerosene, diesel, and lubricating oil from C10 to C35 with a capacity of 50–60%. In another study, Rajasekar et al. (2007) recorded that *Serratia marcescens* ACE2 degraded almost all the n-alkanes (C10–C20) and many of the branched alkanes. Moderately degraded branched alkanes and naphthalene derivatives could be found in the degraded products, which is a common feature for many other alkane-degrading microorganisms. The GC–MS analysis suggested that ACE2 degraded almost all the hydrocarbon present in diesel (C10 and C22), showing 69% degradation efficiency. So, *Serratia marcescens* strains reported in literatures indicated that strain 4A could also use a broader range of crude oil components as the sole carbon source.

The strain *Pseudomonas* sp. strain 6C degraded 91.62% of n-alkanes ranging from C9 to C34, which was the best strain studied in the present study. Wongsa et al. (2004) identified strain WatG as a new strain of *Pseudomonas aeruginosa*, with relatively high capacity and wide spectrum to degrade the hydrocarbons in gasoline, kerosene, diesel, and lubricating oil (C10 to C35) with a capacity of 30–57%.

Xia et al. (2014) reported that the growth of *Pseudomonas* spWJ6 was observed in the presence of n-alkanes (C12, C22, C32, C40) as the sole carbon sources. It grew obviously and rapidly with n-alkanes (C12, C22, and C32), while it grew a bit slower when utilizing C40. Specifically, n-dodecane (C12) was degraded by 46.65% in the 20-days. Comparatively, only 42.62%, 31.69% and 23.62% of C22, C32 and C40 were degraded.

Al-Wasify et al. (2014) recorded 77.8% biodegradation of (n-C_{17}/Pr and n-C_{18}/Ph) from oil spills by *Pseudomonas aeruginosa*. Erdogan et al. (2014), in order to establish an experimental basis for bioremediation of soil contaminated with crude oil, 33 strains of bacteria with hydrocarbon-degrading ability were isolated from the contaminated soil in Adana, Batman and Adiyaman, Turkey. Strains were identified as *Pseudomons* spp.*, Pseudomons aeruginosa, Pseudomonas putida.*

Comparisons with *Pseudomonas* strains reported in the literatures indicate that strain 6C has a great potential for use in bioremediation of a broader range of crude oil components.

The strain *Acinetobacter* sp. strain 8F degraded only 41% of n-alkanes ranging from C12 to C34.

The *Acinetobacter* sp. were most common bacterial hydrocarbon–degraders reported in the literature by (Rusansky et al. (1987), Kiyohara et al.(1992), Johnson et al. (1996) , Barathi and Vasudevan (2001), Bhattacharya et al.(2002), Pokethitiyook et al. (2003), Van Hamme et al. (2003).

Comparative analysis of crude oil degradation by strain *Acinetobacter* sp. strain 8F and another strains of *Acinetobacter* sp. studied by Acer et al. (2014) showed that almost all bacterial strains were found to use crude petroleum as carbon and energy sources in order to grow. With the aliphatic hydrocarbons, growth was seen only in the long-chain alkanes tested (tridecane, pentadecane and hexadecane). No growth was recorded in the short-chain alkanes tested (hexane). Among the long-chain alkanes tested, generally hexadecane was the most preferred. GC-MS analysis showed that strains *Acinetobacter* sp. BT1A, ST5 and GC2 were able to degrade 83%, 41% and 6% n-alkanes in the crude oil in 7 days, respectively.

Al-Wasify et al. (2014) reported 74.3% degradation of (n-C_{17}/Pr and n-C_{18}/Ph) from oil spills by *Acinetobacter lwoffii*. Erdogan et al. (2014), in order to establish an experimental basis for bioremediation of soil contaminated with crude oil, isolated 33 strains of bacteria with hydrocarbon-degrading ability from the contaminated soils in Adana, Batman and Adiyaman, Turkey. One of them was *Acinetobacter genomospecies*.

Cormack et al. (1997) isolated bacterial strains *Acinetobacter and Acinetobacter* ADH-1, using hexadecane and dodecane as a carbon source.

Huy et al. (1999) in Vietnam isolated from oil-contaminated area the 4 units bacteria that break down oil. One of them was *Acinetobacter* sp. ZTN-NB, most effective in degrading crude oil: in 1 d, it degraded 95% of the crude oil in the culture medium (5% , v/v). The strain could degrade alkanes and alkenes in crude oil with carbon chain lengths from 6 to 30.

Yuste et al. (2000) isolated and characterized seven bacterial strains that can use a residue from crude oil processing as a source of carbon and energy. According to fatty acid profile and 16S rRNA sequence analysis one was identified as *Acinetobacter calcoaceticus,* breaking down alkanes straight-chain in C10-C34 range. Sarma and Sarma (2010) also recorded that *Acinetobacter lwoffii* degradated 93.78% of crude oil. Comparisons with *Acinetobacter* strains reported in literatures indicated that strain 8F was not a good candidate for degradation of a broader range of crude oil components.

Mixure of strains(2B. 3B, 4A, 6C, 8F) degraded 69.50% percentage of n-alkanes ranging from C11 to C34. The results demonstrated that the selected bacterial isolates could be effective in biodegradation of oil spills individually and showed better biodegradation abilities when they are used individual than together in mixed consortium.

Al-Saleh et al. (2009) isolated a strains mixure of 272 crude oil-degrading bacteria from seven locations along the coast of Kuwait. The analysis of the 16S rDNA sequences of isolated bacteria revealed the predominance of six bacterial genera: *Pseudomonas, Bacillus, Staphylococcus, Acinetobacter, Kocuria and Micrococcus*. Investigation of the factors

associated with bacterial predominance revealed that, predominant culturable bacteria constituted 54.2–89.7% of the total crude oil-degrading bacterial communities.

Al-Wasify et al. (2014) also isolated *Pseudomonas aeruginosa, Bacillus subtilis*, and *Acinetobacter iwoffi* from petroleum contaminated water and soil samples to use in degrading crude oil, separately and in a mixed bacterial consortium. Capillary gas chromatography was used for testing the effect of those bacterial species on the biodegradation of crude oil. Individual bacterial cultures showed less growth and degradation than did the mixed bacterial consortium. At temperature 22°C, the mixed bacterial consortium degraded a maximum of 88.5% of Egyptian crude oil after 28 days of incubation. This was followed by 77.8% by *Pseudomonas aeruginosa*, 76.7% by *Bacillus subtilis*, and 74.3% by *Acinetobacter iwoffi*.

Sarma and Sarma (2010) studied co-culture with three strains (*Acinetobacter lwoffii, Staphylococcus sp. Enterobacter agglomerans*) reporting a maximum of 96.63 crude oil degradation .

Several workers (Vasudevan and Rajaram (2001) and Rahman et al. (2002) described the ability of mixed bacterial consortia to degrade 28–51% of saturates and 0–18% of aromatics present in crude oil or up to 60% crude oil, while in a study by Rahman et al. a mixed bacterial consortium from *Micrococcus* sp., *Bacillus* sp., *Corynebacterium* sp., *Flavobacterium* sp., and *Pseudomonas s*p. showed a maximum of 78% of degradation of crude oil after 20 days of incubation while the maximum percentage of degradation was by *Bacillus* sp. and *Micrococcus* sp. was 59% and 49%, respectively. The mixed bacterial culture gave the maximum degradation percentage because there is no single strain of bacteria with the metabolic capacity to degrade all the components found within crude oil (Venosa et al. (2001). This agrees with Friello et al. (2002) who reported that a wide variety of metabolic and physiological factors are required for the degradation of different compounds in diesel oil. All of such properties are not found in one organism (Bento et al.2005).

6. CONCLUSIONS AND FUTURE PROSPECTS

In this study, oil-degrading bacteria from petroleum contaminated soils near petroleum wells in Iraqi Kurdistan were isolated and identified, and their crude oil degrading potential was evaluated. Bacteria play an important role in the removal of crude oil in crude oil fields or along transportation routes. These bacteria have expanded the capability to degrade hydrocarbons due to increased exposure to oil contamination.

Bacterial strains related to different bacterial genus, with a predominance of *Cronobacter, Serratia, Pseudomonas* and *Acinetobacter*, which were isolated from crude oil-contaminated soil samples were identified by morphological, physiological and biochemical tests and 16S rRNA gene sequences analysis.

In this study, isolated bacteria were selected based on the efficiency of crude oil utilization. It was found that bacteria isolated can degrade many of the n- alkanes contained in crude oils. At 1% crude oil concentration, among the long-chain alkanes tested, generally chain length (C9- C34) were the most preferred by the bacterial strains. GC-MS analysis showed that 2B, 4A, 6C and 8F were able to degrade 84.76%, 73.25%, 91.62%, and 41% n-alkanes in the crude oil in 10 days, respectively, but the strain 3B did not degrade enough. Major understanding of the metabolic process of these organisms on the hydrocarbons will develop possibilities of expanding models and strategies for removing hydrocarbon pollutants from the oil impacted environment.

Along with the selected individual strains, a mixed bacterial consortium prepared using the above strains were also used for degradation studies, which did not lead to enough growth and degradation. The mixed bacterial consortium degraded 69.50% percentage of n-alkanes ranging from C11 to C34. This study provides insights into the awesome variety of microbial populations for degradation of crude oil. All these results showed that the selected bacteria could be impressive in biodegradation of oil spills individually and showed better biodegradation abilities when they are used individually than together in mixed consortium.

Research Needs :

1- Determination of plasmids containing genes which code for the enzymes required for crude oil degradation, and of pathways significant to bioremediation.

2- Using gene cloning and gene recombination techniques, improvement of the systems for biodegradation of crude oil components can be commercialized with environmental advantages and cost effective.

3- Purification and characterization of enzymes degrading crude oil

4- Metabolic research will, in turn, prprovide a greater insight into novel biocatalytic mechanisms. Subsequent researches in this area will undoubtedly lead to exciting novel findings and add an important dimension to the overall scientific quest to better understanding of all cellular mechanisms.

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EDUCATION

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- Teacher (High school) in Ministry Education(from2010 to 2014)
- Education Faculty, Department of Biology Education, Salahadden University, Erbil, Iraqi Kurdistan (from 2006 to 2010)
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T.C.
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TEZ KONUSU	ISOLATION AND INVESTIGATION OF BIOREMEDIATION POTENTIALS OF PETROLEUM-DEGRADING BACTERIA FROM KURDISTAN REGION IN IRAQ
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BENZERLİK ORANI	%21
RAPORLAMA TARİHİ	23/03/ 2016

Yukarıda başlığı/konusu gösterilen tez çalışmamın kapak sayfası, giriş, ana bölümler, sonuç ve tartışma kısımlarından oluşan toplam 138 sayfalık kısmına ilişkin, 23/03/2016 tarihinde şahsım/tez danışmanım tarafından *Turnitin* adlı intihal tespit programından aşağıda belirtilen filtrelemeler uygulanarak alınmış olan intihal raporuna göre, tezimin benzerlik oranı %21 'dir.

Uygulanan filtrelemeler:

☒ Kabul/Onay sayfaları hariç,
☒ Kaynakça hariç
☐ Alıntılar hariç/dâhil
☒ Diğer

Dicle Üniversitesi Fen Bilimleri Enstitüsü Lisansüstü Programlarda Tez Çalışması İntihal Raporu Uygulama Esasları'nı inceledim ve bu Uygulama Esasları'nda belirtilen azami benzerlik oranlarına göre tez çalışmamın herhangi bir intihal içermediğini; aksinin tespit edilmesi durumunda doğabilecek her türlü hukuki sorumluluğu kabul ettiğimi ve vermiş olduğum bilgilerin doğru olduğunu beyan ederim.

Gereğini saygılarımla arz ederim.

Shelan Shukri KARAM

(İmza)

(Öğrencinin Adı Soyadı)

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