Republic of Iraq

Ministry of Higher Education and Scientific Research

University of Basrah/ College of Science

Department of Biology

Enhancing the Performance of Methanotrophs Bacteria by using CRISPR/Cas9 Gene Editing System

A Thesis

Submitted to the College of Science/ University of Basrah

in Partial Fulfillment of the Requirements for the Degree of

Ph.D. in Biology/Microbiology

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بسم الله الرحمن الرحيم

ومن يتق الله يجعل له مخرجا * ويرزقه من حيث لا يحتسب ومن يتوكل على الله فهو

حسبه ان الله بالغ امره قد جعل الله بكل شيء قدرا*

صدق الله العلي العظيم

(سورة الطلاق: 2-3)

Certification

We certify that this study has been prepared under our supervision at Department of Biology, College of Science, University of Basrah, as a partial requirement for the degree of Doctor of Philosophy in Biology/Microbiology.

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Recommendation of the Head of the Department

In view of the available recommendation, I forward this study for the debate by the examining committee**.**

Signature:

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Date:

Dedication

This modest effort is dedicated to:

My two kids (Ali and Zahraa), who are the motivation behind my successes, to my brothers and all of my loved ones.

Anwar

Acknowledgments

I thank Almighty God for creating microorganisms for us that we are unable to see with our unaided eyes and that protect us and our surroundings from threats that may otherwise take our lives. And I thank God very much, who gave me strength and patience to accomplish this work.

I extend my gratitude and heartfelt thanks to the distinguished prof. Dr. Asaad Mohammed Ridha Al-Taee (Department of Biological Development, Marine Science Center, University of Basrah) and Prof. Dr. Zeenah Weheed Atwan (Department of Microbiology, College of Medicine, University of Basrah) for suggesting a research topic and supervising the thesis and for all the directions, observations, and support they provided me for the duration of the study and their constant encouragement, which helped me a lot in overcoming the difficulties I went through during the research.

I want to thank the Deanship of the College of Science, especially the Dean, Prof. Dr. Jalal J. Hassan and the Head of the Department of Biology, Prof. Dr. Saad S. Mahdi. I'm grateful to Asst. Prof. Dr. Abdulameer Raheem Jasim, the head of the biotechnology laboratory at the Marine Science Center. I would also like to express my appreciation to Prof. Dr. Wesal Fakhary for mapping the locations of sample collection.

My thanks to colleagues, Asist. Prof. Dr. Zuhair Ali and Ph.D. students Suhyila Fadhil, Salah Mahdi, and Zeena Hashim for their help and advice.

Anwar

Summary

Pollution with petroleum hydrocarbons is a critical problem that threatens the ecosystem around the world. Removing such waste needs many expensive and time-consuming procedures, and biological tactics are an effective example.

The study is designed to develop new genetic modification approach in methylotrophic bacteria through an effective expression system to enhance the ability of bioremediating bacteria to consume oil in polluted areas. This objective is achieved by isolating and identifying methylotrophic bacteria from three oil-contaminated stations connected to the petroleum production industries. The methylotrophic bacteria are isolated by utilizing the enrichment culture technique and a basal methanol medium supported with 2% methanol as the only source of carbon. The identification and genotypic characterization of bacterial isolates have been based on biochemical tests and the gene encoding *16S rRNA.* All analyzed sequences had an expected (E) value of 0, and more than 99% of the reported valid genera are very closely related to the BLAST identity percentage and identified as *Methylorubrum extorquens* and *Pseudomonas balearica*.

Regarding the bioremediation ability and the enzymatic system that is responsible for, a specific primer is used to amplify the *MMO* gene (encoding the methane monooxygenase enzyme) and the *MxaF* gene (encoding the methanol dehydrogenase enzyme) of the identified methylotrophic bacteria. Bands of 439- and 550-bp size from the chromosomal *MMO* gene and the DNA bound *MxaF* gene are detected. Comparison of MDH (methanol dehydrogenase enzyme) expression across identified species showed a 44-fold increase in *M. extorquens* culture compared to the control (*P. balearica*). The expression of the *pMMO* gene in *P. balearica* is ten times higher than that is quantified in the control (*M. extorquens*).

P. balearica showed growth on NMS broth medium in the presence of methane as the sole carbon and energy source, and the test for naphthalene were positive when the color of the colonies turns purple meanwhile *M. extorquens* did not grow on NMS broth medium and is negative for naphthalene. On MSM broth medium supplemented with methanol at different concentrations, *M. extorquens* grew at highest at 1% and 2% concentrations, where the optical density reached 1.9 and 1.8, respectively, whereas the growth of *P. balearica* increase at 1% and 3% and the optical density reached 0.1. Both exhibited the slowest growth at concentration 6%. The MDH expression of *M. extorquens* was 12 folds at concentration 1- 4% of methanol and reduced to 1 fold at concentration 6%.

Both genera displayed the capability to grow in mineral medium with 1% of crude oil. The *P. balearica* showed higher growth than *M. extorquens* after 7 days of agitation in an incubator at 120 rpm and 30 °C. The optical densities were 0.17 and 0.1, respectively. The antibiotic sensitivity test showed that the minimum inhibitory concentrations of *M. extorquens* for kanamycin, ampicillin, and spiramycin were (20, 200 and 500) μgmL^{-1} respectively. Meanwhile for *P. balearica*, the minimum inhibitory concentration was (50, 200 and 100) μgmL^{-1} respectively.

The plasmid-treated CRISPR-Cas9 system was inserted into bacterial chromosomal DNA confirmed by gene expression of the CRISPR-Cas9 insert system using qPCR technology. In CRISPR-MDH treated *M. extorquens*, MDH gene expression increased 6 folds. While in CRISPR-treated *P. balearica*, *pMMO* gene expression raised 25 folds. The CRISPR-Cas9 technology was chosen to study and utilize in the laboratory to improve the biodegradation efficiency of hydrocarbons (n-alkane and PAH).

Within 7 days of an incubation period, the biodegradation rate of n-alkane in the modified *M. extorquens* increased from 61.14% to 74.35% compared to the control, and in the modified *P. balearica*, the biodegradation rate increased from 90.32% to 92.90%. As for the polycyclic aromatic hydrocarbons in the modified *M. extorquens*, the percentage increased from 65.69% to 78.23%. As for the cured *P. balearica*, the percentage increased from 83.16% to 94.07%. To our knowledge in this study, methylotrophic bacteria are isolated for the first time in Iraq, and in addition to that, globally this the first time used, a CRISPR-Cas9 system to increase the effectiveness of these bacteria in the biodegradation of hydrocarbon molecules.

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LIST OF ABBREVIATIONS

Symbols	Abbreviation
%	Percentage value
$^{+}$ve	Positive
°C	Degree centigrade
µg	Micro Gram
µL	Micro litter
µm	Micro meter
ATP	Adenosine Tri Phosphate
bp	Base pair
cm	Centimeter
D.W	Distill Water
Da	Dalton
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra acetic acid
g /L	Gram per liter
G+C	Guanine and Cytosine content
GC	Gas Chromatography
gm	Gram
gRNA	Guide Ribonucleic Acid
mg/L	Milligram per liter
Mg/ml	Milligram per/ millimeter
min	Minute
ml	Milliliter
MLSA	Multilocus sequence analysis
mm	Millimeter
mM	Mill molar
NAD+	Nicotinamide adenine dinucleotide
ng	Nano gram
nm	Nano meter
nov.	Novel
PAHs	Poly Aromatic Hydrocarbons
PCR	Polymerase chain reaction
PH	Power of hydrogen
Pmol	Pico Molar
RNA	Ribonucleic acid
rpm	Round per minute
rRNA	Ribosomal Ribonucleic acid
Sec.	Second
sgRNA	Single guide

UK	United Kingdom
USA	United State of America
UV	Ultra Violet
v/v	Volume per volume
¯ve	Negtive
α	Alpha
β	Beta
γ	Gamma
λ	Lambda

Chapter one

Introduction

1. Introduction

Petroleum hydrocarbon pollution of soil and groundwater is a severe and pervasive problem all over the world. Petrol, kerosene, diesel, lubricating oil, paraffin, and asphalt are all petroleum hydrocarbons, which introduce a complex collection of organic molecules. Petroleum products have a high risk of contaminating soil and groundwater due to their widespread use (Li *et al.*, 2020). Due to the destructive influence of these chemicals on human health and environment, petroleum pollution is serious to water and soil ecosystems which may be outcome from leaking storage tanks above ground and down ground, gas sites obsolete, diverse processes of industrial and spillage during convey products of petroleum and treatment these site is high costly(Geetha *et al.*, 2013).

Total petroleum hydrocarbon compounds affect the human body in several ways particularly the smaller compounds like toluene, benzene and xylene, causing effect on human central nervous system (Zhang *et al.*, 2019). Most petroleum hydrocarbon products, especially the initial saturated and unsaturated alkanes, and low molecular weight polycyclic hydrocarbons (PAHs), can be degraded or changed by microorganisms (Onibiyo, 2016).

Hydrocarbons molecules are attacked by enzymes of microbes and causing degradation during the metabolic pathways. Luckily, several microbes in the nature evolved to do this chemical process. Microbes such as *Alcaligenes* sp., *Flavobacterium* sp., *Corynebacterium* sp., *Bacillus* sp., *Bacillus subtilis*, *Micrococcus roseus*, *Pseudomonas aeruginosa*, *P. fluorescents*, and *Acinetobacter lwoffi* are known to breakdown hydrocarbons (Onibiyo, 2016; Zeyaullah *et al.*, 2009).

Microorganisms have enzymes that allow them to catalyze environmental toxins, hence this method is suitable for removal of contaminants (Endeshaw *et al*., 2017). Bioremediation is a low-cost, ecologically friendly method that depends on microorganisms' capacity to lower pollutant concentrations and/or toxicity (Dell'Anno *et al.*, 2021).

Bioremediation is an excellent method for removing petroleum hydrocarbons from a polluted environment while simultaneously reducing or eliminating toxic components and/or hazardous chemicals (Sayed *et al*., 2021; Abdel-Shafy and Mansour, 2018; Muangchinda *et al*., 2018). Development in molecular techniques led to use gene editing tools in bioremediation of petroleum, acid drainage, xenobiotic, heavy metal, persistent organic pollutants (Boudh and Singh, 2019; Basu *et al.*, 2018; Dai *et al*., 2018; Gaur *et al*., 2018; Malla *et al*., 2018) .

Methylotrophs are mainly strictly aerobic organisms, and according to the way that they use carbon substrate, they are divided into two taxonomic groups obligate and facultative methylotrophs. Obligate methylotrophic bacteria use one carbon compounds whereas, facultative methylotrophs are ubiquitous and use either one-carbon compounds or multi-carbon sources (Chistoserdova, 2018; Mosin and Ignatov, 2014; De Marco *et al*., 2004).

Methylotrophs are a type of microorganism that breakdown reduced carbon substrates that do not include carbon-carbon bonds. Methylotrophic bacteria include bacteria that can assimilate methane (a subset of methylotrophs known as methanotrophs), as well as methylotrophs that can assimilate carbon substrates other than methane, such as methylamine, dimethylamine, methanol, formate, and formaldehyde, even if methane is not the sole source of carbon and energy (Kumar *et al.*, 2018).

Methylotrophic bacteria have enzymes that seem suitable for their employment in processes of bioremediation and biodegradation due to oxidize methanol to formaldehyde and formic acid and non-forming of unwanted products (Giri *et al.*, 2021). They can be lived in vary extreme habitats of water, soil, sediment as well as grow well on plants and animals because they have large plasticity in phenotypic (Dourado *et al.*, 2015; Fedorov *et al.*, 2011). Methods of bioremediation could be development by choosing methylotrophs from these habitats that adapted to the challenges specific environmental by regulating their metabolic processes (Chistoserdova, 2018).

Genome editing is the gold standard for scientists looking into the genetic foundation of physiological and metabolic processes in any organism, especially bacteria of scientific and industrial importance (Arroyo-Olarte *et al.*, 2021). Gene editing using engineering endonucleases known as "molecular scissors" is distinctive technique to manipulate DNA. The technique was evolved to be used in many applications in various fields associated to animals, plants and microorganisms (Butt *et al.*, 2018).

The gene editing procedure begins with the creation of a complementary guide sequence to the target gene sequence and engineering nuclease to break at site and repair by recombination according to the base pairing rule, manipulation is done deletion or insertion of wanted sequence piece (Bier *et al.*, 2018). Bioremediation processes could be enhanced performance by using gene editing like pesticide degradation to another simple compounds, transformation toxic compounds to less toxicity and disposal of xenobiotic (Basu *et al.*, 2018; Hussain *et al.*, 2018). Transcription Activator-Like Effector Nucleases (TALEN), Zinc Finger Nucleases (ZFN), clustered regularly interspaced short palindromic repeats associated protein

(CRISPR-Cas) are all gene editing approaches (Singh and Ali, 2021; Tavakoli *et al.*, 2021).

To repair double stranded breaks (DSB) in the target gene sequence, the non-homologous end joining (NHEJ) pathway and homology-directed repair (HDR) are utilized. This serves as a focus point for researchers to modify genes at specific locations (Loureiro and da Silva, 2019; Arazoe *et al.*, 2018). Approach of gene editing supply the best microbe with complex genes, resulting in a microorganism that is more distinct (Dangi *et al.*, 2019; Basu *et al.*, 2018). As a result, it is possible to obtain changed genetic sequences of distinct wild types (Dai *et al.*, 2018; Stein *et al.*, 2018).

Adaptive immunological response to invading foreign nucleic acids is accomplished by CRISPR-Cas discovered on a range of archaea and bacteria. It works by recombinant storing genetic sequences from invaders as spacers when bacteriophages and dangerous plasmids attack bacteria. Then transcribed into crRNAs that bind to Cas proteins and target specific gene sequence (Arroyo-Olarte *et al.*, 2021; Horodecka and Düchler, 2021). Researchers used this approach to develop an efficient and reliable method that confers precise and focused gene editing in organisms (Lone *et al.*, 2018).

In recent years, The CRISPR system proved to be a successful method for genome editing in humans, plants, animals and microbes (Liang *et al.*, 2020). The CRISPR-Cas systems consist of two parts: Cas proteins and CRISPR RNA (crRNA). The crRNA complements the target gene sequence by directing Cas proteins to the right gene sequence for cleavage. This breaking of target DNA opens up new possibilities for engineering genomic DNA through HDR, which allows for precise genomic editing, or NHEJ, which is more error-prone (Perisse *et al.*, 2020).

Aim of the study

Increased soil hydrocarbon contamination and a sluggish biodegradation process involving bacteria, is the main reason for this current study which aims to genetically modify the biodegrading bacteria to become potent biodegradable adopted machinery to eliminate such heavy pollution. The approaches are:

1. Isolation and purification of methylotrophic bacteria from petroleum-contaminated soil through biochemical and genetic tools.
2. Detection of the methane monooxygenase (*MMO*) and methanol dehydrogenase (*MxaF*) as functional marker genes for methylotrophs.
3. Preliminary evaluation of the ability of bacteria to breakdown hydrocarbon compounds through biodegradation.
4. Modifying the genomes of bacteria with CRISPR-Cas9.
5. Analyzing the effect of delivering CRISPR- Cas9 on biodegradation capabilities of the target bacteria.

Chapter Two
Literature Review

2. Literature Review

2.1. Bioremediation of hydrocarbons

Bioremediation has an imperative role in reducing the impacts of hydrocarbon pollution in soil and water. This process depends on exploiting the metabolic capabilities of microorganisms that can consume hydrocarbons as a source of carbon and energy or modify them through co-metabolism. Removing rate and efficiency of these compounds depends on their bioavailability (concentration, toxicity, mobility, and access), and the physicochemical conditions in the environment. It is the process through which microorganisms (bacteria) convert pollutants (hydrocarbons) into energy (Abdulla *et al.*, 2019).

Bacteria that degrade hydrocarbons and other microorganisms are widely distributed in their native soil. However, studies suggest that microorganisms obtained from contaminated soil can help with hydrocarbon breakdown. Many factors are implicated in developing efficient biodegradation, microbes must enhance their catabolism. So they follow different strategies: the accessibility of microorganisms to the targeted pollutants that they can degrade, the pollutant's molecular structure, the active action of those microbes and the amount of bacteria that can degrade these pollutants (Abdel-Shafy and Mansour, 2018).

Methanotrophs can breakdown a variety of toxic chemical compounds, including chlorinated ethane through its enzymatic machinery (Shahid *et al.*, 2020). Bioremediation pollutants and waste are slowly degraded by natural and wild microbial strains. As a result, bioaugmentation was developed, in which genetically modified microbial strains were put into polluted areas for efficient and rapid degradation (Pant *et al.*, 2020).

Genetically Engineered Microorganisms (GEMs) are microorganisms that have been genetically modified (bacteria, fungi, and yeasts, for example). Studies employed molecular techniques to modify them, and they can be used effectively for bioremediation (Liu *et al.*, 2019; Peeters *et al.*, 2019).

Jechorek *et al.* (2003) analyzed the ability of *Methylocystis* sp. GB 14 DSM 12955 to degrade high amounts of chlorobenzene, meanwhile, Doronina *et al.* (2000) evaluated the bioremediation capacity of *Methylopila helvetica* and *Methylobacterium dichloromethanicum* using a variety of polycarbonic compounds as a carbon and nitrogen sources.Ventorino *et al.* (2014) isolated *Methylobacterium populi* from severely contaminated soil and revealed its ability to degrade polyaromatic hydrocarbons. Van Aken *et al.* (2004) discovered that *Methylobacterium* sp. can degrade hazardous explosives TNT (2,4,6-trinitrotoluene); RDX (hexahydro-1,3,5-trinitro-1,3,5-triazene); HMX (octahydro-1,3,5,7-tetranitro-1,3,5-tetrazocine).

2.2. Methylotrophic bacteria

Aerobic methylotrophic bacteria require certain metabolic pathways for carbon metabolism and energy to grow on reduced C1 molecules. Formaldehyde as intermediate is a main trait of aerobic methylotrophs. Methanotrophs either oxidize the formaldehyde to CO_2 for energy or integrated into cell carbon by: serine cycle or the ribulose monophosphate cycle (Fig. 2-1) (Schrader *et al.*, 2009; Lidstrom, 2006).

According to the easiness of carbon use, aerobic methylotrophic bacteria are categorized into three classes. First, obligate methylotrophs using only one carbon compounds include (methanotrophs type I, methanotrophs type Π). Second, restricted facultative methylotrophs that use only a few types of complex carbon molecules, such as *Methylophilus* and *Methylobacillus* of β-proteobacteria. Finally, facultative methylotrophs consume variety of

carbon molecules, including *Methylobacterium* genus of α-proteobacteria (Table 2-1) as well as Gram-positive methylotrophs (Giri *et al.*, 2021; Lidstrom, 2006). However, there are other methylotrophs called autotrophic methylotrophs oxidize C1 molecules to CO_2, then use the Calvin Benson-Bassham cycle to assimilate CO_2 (Anthony, 1982).

The serine route consumes reduced C1-substrates and fixes formaldehyde to the carrier molecule tetra hydromethanopterine in presence of formaldehyde activating enzyme Fae (H_4MPT). The C1 moiety is released once it is oxidized to formate. After that, formate can be further oxidized to CO_2, or it anchors on tetrahydrofolate (H_4T) while consuming ATP.

Carbon metabolism begins when it fixed to glycine to produce serine, which eventually transformed to 2-phospho-D-glycerate by three enzymes. Enzymes implicated in this process are serine-glyoxylate aminotransferase (SgaA), hydroxypyruvate reductase (HprA) and glycerate- 2-kinase (GckA). 2-phospho-D-glycerate is then metabolized to oxaloacetate, the TCA cycle metabolite. Malate dehydrogenase converts oxaloacetate to malate (Mdh). Malate thiokinase (MTK) interacts with malate to generate malyl-CoA, which is then divided into acetyl-CoA and glyoxylate (Belkhelfa, 2019).

The RuMP route binds formaldehyde to D-ribulose-5-phosphate (Ru5P) to generate hexulose-6-phosphate, which is isomerized from D-fructose-6-phosphate (F6P), to use reduced C1 substrates. These pathways are catalyzed by 6-phospho-3-hexulose isomerase (PHI) and 3-hexulose-6-phosphate synthase (HPS). F6P is phosphorylated to fructose 1,6-bisphosphate. Glyceraldehyde-3-phosphate and F6P are used to regenerate the formaldehyde acceptor Ru5P (Belkhelfa, 2019).

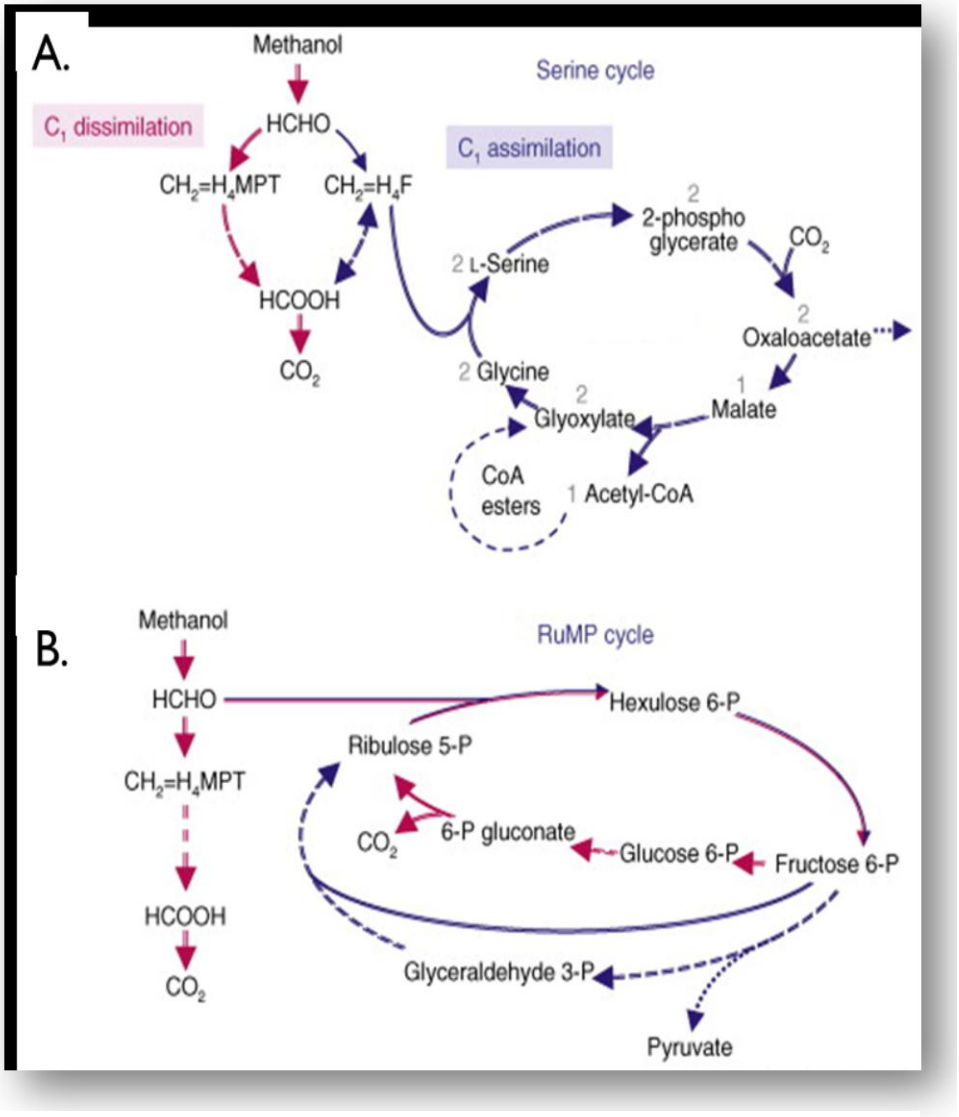

Figure 2-1: Metabolisms pathways of methanol in several types of methylotrophic bacteria. A. serine cycle, B. RuMP cycle (Schrader *et al.*, 2009)**.**

Table 2-1: Diversity in aerobic methylotrophic bacteria (Lidstrom, 2006).

Group	Major assimilation pathway	N2 fixing	Phylogenetic position
Obligate methylotrophs			
Type I methanotrophs			
Methylobacter	RuMP pathway	YES	γ -Proteobacteria
Methylocaldum		NO	
Methylococcus		YES	
Methylomicrobium		NO	
Methylomonas		YES	
Methylosphaera		NO	
Type II methanotrophs			
Methylocella	Serine pathway	YES	α -Proteobacteria
Methylocystis			
Methylosinus			
Restricted facultative methylotrophs			
Methanol utilizers			
Hyphomicrobium	Serine pathway	NO	α -Proteobacteria
Methylobacillus	RuMP pathway	NO	β -Proteobacteria
Methylophaga			γ -Proteobacteria
Methylophilus			β-Proteobacteria
Facultative methylotrophs			
Aminobacter	Serine pathway	NO	α -Proteobacteria
Marinosulfonomonas			
***Methylobacterium* (*Pseudomonas*)**			
Methylorhabdus			
Methylopila			
Methylosulfonomonas			
***Ancylobacter (Microcyclus*)**	CBB Cycle	YES	α-Proteobacteria
Paracoccus		NO	
Rhodobacter		NO	
Rhodopseudomonas		NO	
Thiobacillus		NO	
Xanthobacter		YES	
Acetobacter	RuMP pathway	ND	γ -Proteobacteria
Amycolatopsis (*Nocardia*)	RuMP pathway	ND	Gram-positive (high G +C)
Arthrobacter			
Mycobacterium			
Bacillus	RuMP pathway	ND	Gram-positive (low G +C)

2.2.1. Methanol oxidation

In gram-negative methylotrophs, a periplasmic methanol dehydrogenase (MDH) converts methanol to formaldehyde (Fig. 2-2). MDH is a quinoprotein consists of two large comprises α2β2 tetramer 66kDa (MxaF) and two small subunit 8.5-kDa (MxaI) (Nakagawa *et al.*, 2012; Hanson and Hanson, 1996). Each tetramer includes 2 mol pyrroloquinoline quinine (PQQ) and 1 mol calcium. MDH transfers electrons to cytochrome cL, a common cytochrome that acts as MDH's electron acceptor. Cytochrome cL is then oxidized by cytochrome cH. It is also specific for methanol oxidation (Hanson and Hanson, 1996; Barta and Hanson, 1993).

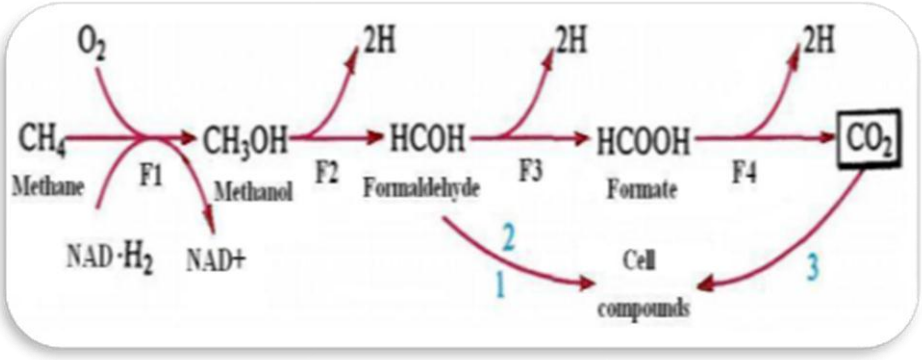

Figure 2-2: oxidation of enzyme to methane: F1 – methane monooxygenase, F2 – methanol dehydrogenase, F3 – formaldehyde dehydrogenase, F4 – formate dehydrogenase (Mosin and Ignatov, 2014).

Many methylotrophs genomes were used as templates for gene cloning to encode the MDH's large component (*MxaF*). *MxaF* gene sequence are substantially conserved among facultative methylotrophs (*Methylobacterium extorquens* AM1, *Paracoccus denitrificans*, and *Methylobacterium organophilum* XX), hence it can be employed to detect methylotrophy in the environment (Chistoserdova *et al.*, 2009; Murrell, 1994; Harms *et al.*, 1993; Lidstrom, 1992; Murrell, 1992; Harms and Van Spanning, 1991).

Complete genome sequencing of *M. extorquens* AM1 has uncovered many genes encoding anticipated paralogs of the major subunit of methanol dehydrogenase (*MxaF*, META1 4538), including *XoxF* with a calculated mass of 65 kDa and *XoxF2* with a predicted mass of 73 kDa. The amino acid sequences of *XoxF* and *XoxF2* are 90% similar to one another and have a 50% similarity to *MxaF* (Schmidt, 2010).

2.2.2. Methane Monooxygenase (*MMO*)

Methane monooxygenases found in methanotrophic bacteria come in three formulates: soluble methane monooxygenase (*sMMO*), particulate methane monooxygenase (*pMMO)*, and *pXMO*, a third divergent *pMMO* variant. Hydrocarbons, which have serious effects on both human health and the environment, can be degraded by both *sMMO* and *pMMO* (Scott and Chiu, 2006; Bolt, 2005).

While other microbes break down halogenated hydrocarbons in reductive ways, the biodegradation of chlorinated hydrocarbons by the methanotropic route occurs in an oxidative manner (Maymo-Gatell *et al.*, 1999). Although *sMMO* and *pMMO* have different amino acid sequences in their quaternary structures and metal types, they both use methane and turn it into methanol. While *pMMO* is found in the vicinity of the membrane, *sMMO* is soluble in the cytoplasm (Dalton, 2005).

2.2.2.1. Particulate methane monooxygenase (*pMMO*)

Two elements make up the active *pMMO* complex: the hydroxylase is the first (pMMOH). There are three polypeptides or subunits α, β and γ and with molecular weights of roughly (45,000, 26,000, and 23,000) Da respectively, as well as a putative reductase (pMMOR) made up of (63,000, 8,000) Da proteins. These are all encoded by the pmoBAC genes (Semrau *et al.*, 2010; Basu *et al.*, 2003).

A study using X-rays revealed that the *pMMO* has α, β and γ structure in the cell. The *pMMO* may be complex with MDH, which serves as the *pMMO*'s electron donor (Fig. 2-3) (Fig. 2-3) (Smith and Murrell, 2009; Myronova *et al.*, 2006).

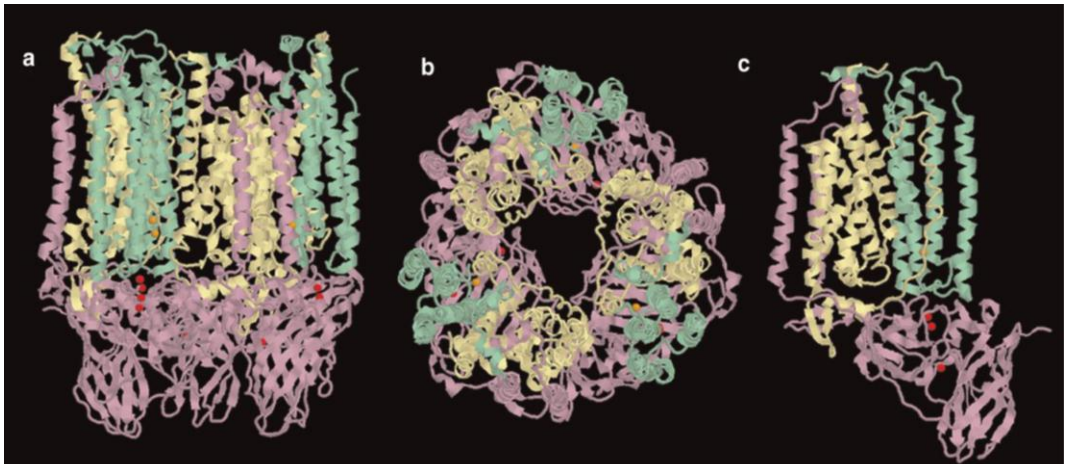

Figure 2-3: The *pMMO*s structure the colors for the (49, 27 and 22) KDa subunits are, respectively, lilac, yellow, and green. Copper red and zinc- orange metal atoms are shown as spheres. (a) The enzyme α, β and ɣ ; (b) a view from above looking down on (a); (c) a promoter displaying mono and di-nuclear copper and zinc (Enbaia, 2019).

2.2.2.2. Soluble methane monooxygenase (*sMMO*)

Three different proteins make up *sMMO*: a hydroxylase protein A (MmoH), a regulatory protein B (MmoB), and a reductase protein C (MmoR). For *sMMO* to operate properly during the conversion of methane to methanol, these three proteins are necessary (Semrau *et al.*, 2010).

The MmoH is the primary component of the *sMMO* complex (Fig. 2-4). Three polypeptide subunits make up the complex: (α2, β2 and γ2) complex, with molecular weights of roughly 60,000 Da, 40,000 Da, and 25,000 Da, respectively (Murrell *et al.*, 2000).

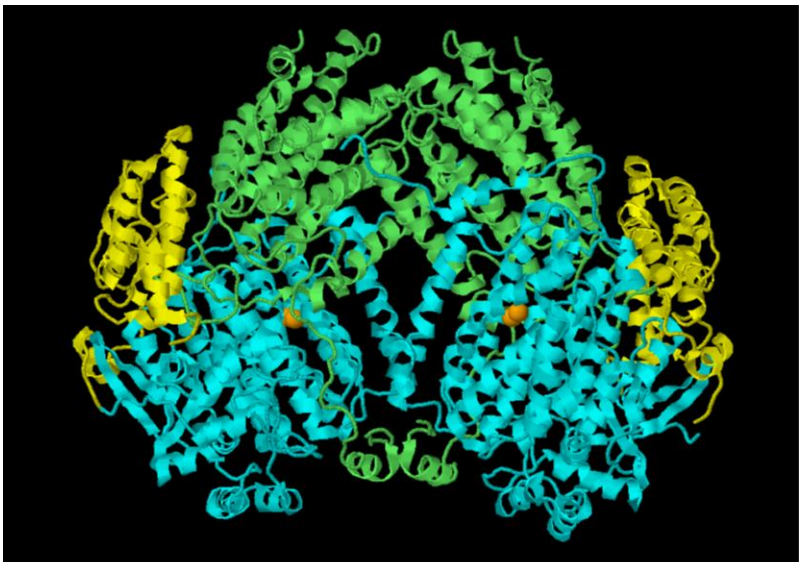

Figure 2-4: The hydroxylase in *sMMO* and its composition Orange balls are the center of the diiron, whereas the colors blue, green, and yellow represent subunits (Enbaia, 2019).

2.2.2. 3. Methanol dehydrogenase (MDH)

MDHs in methylotrophs are divided into three classes based on the electron acceptors: PQQ (pyrrolo-quinoline quinone)-dependent *MDH*, NAD^+-dependent *MDH*, and O_2-dependent AOX (alcohol oxidase) (Fig. 2-5).

The PQQ is responsible for methanol oxidation. MDH catalyzes the synthesis of formaldehyde, which is an intermediary in both assimilative and dissimilative metabolisms in methylotrophs, and hence plays an important role in bacterial one-carbon (C1) metabolism. MDH is the second enzyme in the methane oxidation pathway in methane-oxidizing bacteria (methanotrophs), and it oxidizes the methanol produced by methane monooxygenase's oxidation of methane (McDonald and Murrell, 1997).

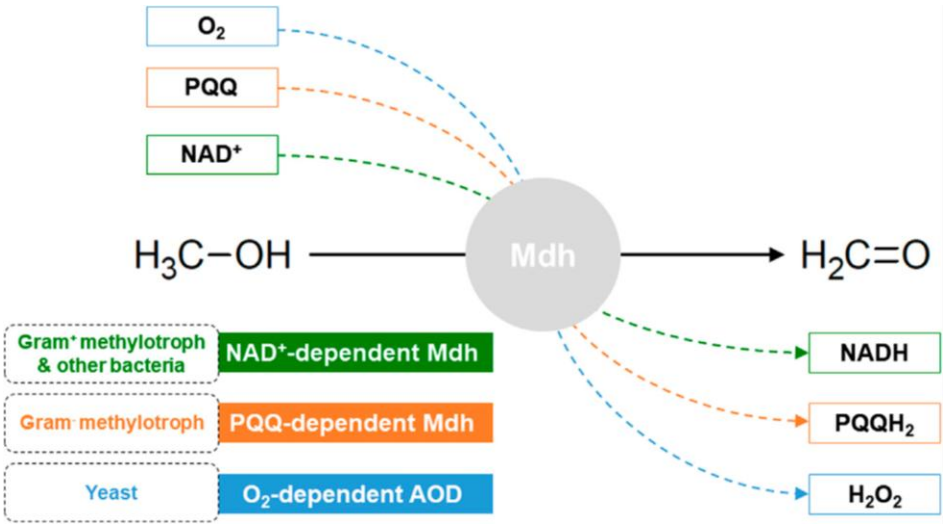

Figure 2-5: Three different types of methanol dehydrogenases (Le *et al.*, 2021).

The β subunit of MDH is folded around the α subunit and is not reversible. The large α subunit made up of eight radially oriented β-sheets in a super barrel (the "propeller blades") (Fig. 2-6). The PQQ is positioned between a co-planar tryptophan residue's indole ring and an odd eight-membered disulfide ring structure made up of nearby cysteine residues and connected by an unconventional non-planar peptide bond. After site-directed mutagenesis of either or both cysteine residues (Cys^{103}, Cys^{104}) to serine, only tiny amounts of MDH were generated in *M. extorquens*, and this MDH had no activity (Afolabi *et al.*, 2001).

The disulfide link is reduced quite quickly, resulting in a loss of activity; however, re-oxidation in air or carboxyl-methylation of the free thiols restores activity (Avezoux *et al.*, 1995). The disulfide ring is absent from the quinoprotein glucose dehydrogenase, which transfers electrons from the quinol $PQQH_2$ to membrane ubiquinone and is unlikely to involve the semiquinone free radical as a stable intermediary. This unusual structure is thought to help with the stability of the free radical PQQ semiquinone or its protection from solvent at the MDH active site's entrance (Avezoux *et al.*, 1995; Blake *et al.*, 1994).

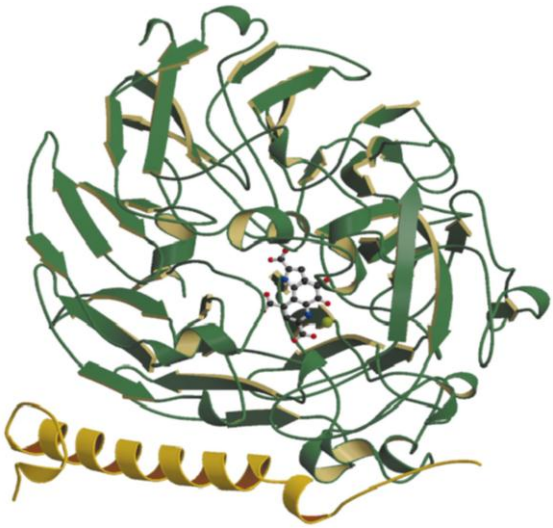

Figure 2-6: αβ unit of methanol dehydrogenase. The propeller-structured subunit (green, *MxaF*) contains PQQ and a calcium ion (green sphere), while the tiny subunit (yellow, *MxaI*) wraps around the side. (Williams *et al.*, 2005).

2.2.3. *Methylobacterium* sp.

The genus *Methylobacterium* has 45 species that can be found in a variety of environments, including the atmosphere, water, soil, and sediments (Bijlani *et al.*, 2021). Patt *et al.* (1976) proposed the first *Methylobacterium* genus and strain, *Methylobacterium organophilum*, which are facultative methylotrophs capable of growing on reduced C1 compounds as a source of carbon and energy, as well as multi-carbon compounds. This genus belongs to phylum Proteobacteria, class Alphaproteobacteria, order Rhizobiales, and family Methylobacteriaceae.

According to *16S rRNA* gene sequence, multi-locus sequence analysis (MLSA), genomic, and phenotypic data, 11 species from the genus *Methylobacterium* were renamed into a new genus is called *Methylorubrum* (Green and Ardley, 2018).

Methylorubrum gen. nov. strains are pink-pigmented facultative methylotrophs (PPFMs) are Gram-negative asporogenous rods, able to grow on methanol as sole carbon and energy source, cell diameter range from (0.5-

1.5;1.0-10.0) µm. Most species are motile with single polar, sub-polar or lateral flagellum; Colonies appears dark pink/red to orange red and generally between 0.5–1.0 mm in diameter when grow on methanol salts agar and GP agar (Green and Ardley, 2018).

It exhibits an excellent growth after seven days' incubation at 30°C, the pigment is carotenoid kind not spreadable showing a pink surface ring or pellicle when grow in liquid media. It grows at optimal temperature (25–30) °C and optimal pH (7.0–7.7), contain on phosphatidylethanolamine, phosphatidylcholine and cardiolipin. The G+C content of DNA is (65.8-71.8) mol%. *Methylorubrum* gen. nov. and *Methylobacterium* genus have common traits, *Methylorubrum extorquens* is type species of *Methylorubrum* (Kelly *et al.*, 2014).

Dourado *et al.* (2012) identified *Methylobacterium* strains from mangrove samples. *Methylobacterium* strains were identified and their tolerance to various heavy metals was tested by exposing them to various concentrations of cadmium, lead, and arsenic.

2.2.4. *Methylorubrum extorquens*

Protomonas extorquens Basonym. Bacillus extorquens, Vibrio extorquens, Pseudomonas extorquens, Flavobacterium extorquens, Methylobacterium extorquens represented Synonyms. *Methylobacterium chloromethanicum* (McDonald *et al.*, 2001) and *Methylobacterium dichloromethanicum* (Doronina *et al.*, 2000) is an earlier heterotypic synonym (Green and Ardley, 2018).

Bousfield and Green (1985) , Urakami and Komagata (1984) provided descriptions of the species. Cells are rod-shaped, polarly flagellated, gram-negative, non-spore-forming cells that can be found individually or in pairs. After 5 days at 30°C, shiny, smooth, raised, entire, pink, or red colonies with a diameter of 1–3 mm grow on glucose–yeast extract–peptone agar.

Colonies that are lighter pink are more common, while colorless colonies are uncommon. In the cells, carotenoids and bacteriochlorophyll are produced, having absorption maxima of 465, 495, and 525 nm. Nitrate cannot be decomposed into nitrite, negative for methyl red and Voges-Proskauer. No evidence of producing indole or hydrogen sulfide, gelatin or starch hydrolysis and ammonia production.

The species is aerobic, positive for oxidase, catalase and urease. The serine route uses methanol to incorporate formaldehyde, and the tricarboxylic acid cycle is functioning. As single carbon sources for energy and growth, ethanol, acetic acid, succinic acid, betaine, methanol, and methylamine can be employed. However, D-xylose, L-arabinose, D-glucose, D-fructose, D-mannose, galactose, maltose, lactose, trehalose, sucrose, D-sorbitol, inositol, D-mannitol, citrate, soluble starch and methane are not employed (Green and Ardley, 2018).

2.2.5. *Pseudomonas sp.*

Genera like *Pseudomonas*, *Arthrobacter*, *Mycobacterium*, *Bacillus*, *Acetobacter*, *Achromobacter*, *Nocardia*, *Hyphomicrobium*, *Brevibacterium* and others are all included as facultative methylotrophs. Although the obligate methylotrophs can only reproduce by assimilating methanol as the sole carbon source, the use of substrates in cumulative cultures enriches them with another type of microorganism, the so-called facultative methylotrophs (Mosin and Ignatov, 2014).

The genus of *Pseudomonas* has 272 species (http: //www.bacterio.net/pseudomonas.html) that have been isolated from a wide range of hosts including various animals, plants, fungi, and algae, as well as environment such as water, air, soil, and sediment. It is a very varied and widespread genus. gram-negative, spore-free, rod-shaped bacteria (Nikolaidis *et al.*, 2020; Hesse *et al.*, 2018).

Pseudomonas sp. are widespread microorganisms that are frequently recognized as vital members of bacterial communities and, as a result, perform crucial ecological roles in the environment (Girard *et al.*, 2021). *Pseudomonas* sp. can survive in harsh environmental circumstances such extreme heat and cold, high salt concentrations and a shortage of water, oxygen, and nutrients (Riveros-Rosas *et al.*, 2019).

In general, *Pseudomonas* sp. are known for their capacity to break down a wide range of substrates, including hazardous organic compounds like aliphatic and aromatic hydrocarbons, which are known to be particularly poisonous (Moore *et al.*, 2006). According to Isaac *et al.* (2013), after 48 hours of incubation, different isolates of *Pseudomonas* sp. showed signs of naphtalene breakdown at rates close to 100%. The *P. monteilii* and *P. xanthomarina* strains were also able to break down the phenanthrene added to the culture medium to 58 and 54%, respectively.

Pseudomonas sp. and *pseudomanase putida* were discovered by Zouari *et al.* (2019) following 28 days, were able to degrade diesel at rates of 55 and 68%, respectively. Biosurfactants produced by *Pseudomonas* are widespread and may be detected in soil and the rhizosphere, especially in contaminated soils containing hydrocarbons, where they facilitate hydrocarbon decomposition (Zouari *et al.*, 2019).

2.2.6. *Pseudomonas balearica*

Pseudomonas balearica is a gram-negative, rod-shaped, no fluorescent, mobile, strictly oxidative and denitrifying bacteria. These bacteria have been identified primarily in contaminated environments and thrive there. Many of the isolates have proven their ability to annihilate a range of chemicals. Some of the strains decompose naphthalene (Bennasar *et al.*, 1996).

Nejad *et al.* (2020) found that *Pseudomonas balearica* strain Z8 was most effective at removing petroleum hydrocarbons overall, with a reported removal rate of 35% for an NH_4Cl nitrogen source at 40 °C. According to Yateem and Al-Sharrah (2011) , *Pseudomonas balearica* and *Bacillus subtilis* both displayed increased hydrocarbon compounds breakdown along with an increase in the growth percentage as measured by optical density. Nkem *et al.* (2022) identified in Malaysia a group of bacteria that can degrade Diesel oil at rate 93.6% in 30 days and these bacteria including *Pseudomonas stutzeri*, *Cellulosimicrobium cellulans*, *Acinetobacter baumannii*, and *Pseudomonas balearica*.

2.3. Genome editing

Genome editing technology, which is cutting-edge and unlike prior biological technologies, it may be used to read genetic code as well as comprehend gene function. It is a form of disruptive biotechnology that rewrites genetic code, resulting in the efficient and precise insertion, deletion and replacement of nucleic acid sequences for a given genome's DNA or RNA, thereby altering an organism's features. Genome editing's goal is to replace a targeted DNA sequence in the native context of a cell's genome with a new, desired DNA sequence (Anzalone *et al.*, 2020; Zhu *et al.*, 2019).

The genome editing tool is made up of two parts that work together. The first, like a navigator, can quickly find the desired nucleic acid sequence among billions of bases, while the second, like a micro scalpel, can precisely edit nucleic acids (Anzalone *et al.*, 2020).

2.3.1. Techniques of Gene Editing

At the moment, there are three distinct gene editing technologies: Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases

(TALENs), and Clustered Regularly Interspaced Palindromic Repeats (CRISPRs) with CRISPR-associated Cas nucleases (Perisse *et al.*, 2020).

2.3.1.1. Zinc Finger Nucleases (ZFNs)

Zinc finger nucleases were derived in mammalian transcription factors proteins in mammalian cells that bind to DNA to activate genes in 2005 were first reported (Archard *et al.*, 2016). Zinc fingers are 20–30 amino acid protein motifs governed by a zinc ion that binds to DNA and recognizes a three-base pair (bp) pattern (Fig. 2-7a). To create a programmable nuclease that can recognize target sequence sites, the motifs were combined with the genetically engineered restriction enzyme FokI.

The ZFNs are effective when two zinc finger modules bind to DNA in two opposing sites with the FokI enzyme in the middle, generating a homo- dimer complex. Once the homo-dimerization is formed, the nuclease breaks both DNA strands and randomly inserts mutations (Adli, 2018). By when residues in the single zinc finger are modified, the specificity of the target site will not be recognized by DNA. As a result, the finger patterns can be tweaked to recognize a wide variety of DNA triplet nucleotides (Carroll, 2017).

2.3.1.2. Transcription Activator-Like Effector Nucleases (TALENs)

A more effective gene editing tools, a novel class of nucleases known as transcription activator-like effector nucleases was introduced in 2009. Originally discovered in *Xanthomonas* genus of plant pathogenic bacteria, TALEs are DNA-binding domains with repeat motifs of 33–35 amino acids that distinguish each bps (Fig. 2-7b). Two highly variable amino acids known as repeat-variable di-residues dictate its site-specificity (Gaj *et al.*, 2013). The number of nucleotides recognized by the protein domains differs

between ZFNs and TALENs, which are 3-bp versus 1-bp, resulting in TALENs being more site-specific and less sensitive to cause off-target cleavage (Khan, 2019) .

2.3.1.3. CRISPR-associated Cas

2.4. History of the CRISPR-Cas

A remarkable repeating pattern in the *Escherichia coli* genome was discovered in 1987, Five 29-bp lengths, including a 14-bp dyad symmetry, are interspersed by 32-bp spacer sequences (Ishino *et al.*, 1987). A few years later, similar repeating patterns were revealed in the genome of the halophilic Archaea *Haloferax mediterranei*, with 14 totally conserved 30bp regions repeated at regular intervals (Mojica *et al.*, 1993).

In prokaryotes these repeats were discovered to be dominant, and they all had a short, a partially palindromic element that arranged in clusters and was separated by unique intervening sequences of consistent length, signifying a common ancestor and biological significance (Fig. 2-7c) (Mojica *et al.*, 2000).

CRISPR-associated Cas genes, a group of genes found only in CRISPR-containing prokaryotes. The Cas genes encode proteins with helicase and nuclease motifs, implying that they are involved in DNA digestion or gene expression (Jansen *et al.*, 2002). Later on, a number of Cas protein subfamilies were discovered (Makarova *et al.*, 2006). Until 2005, studies revealed that these unique CRISPR sequences were similar to sequences in viruses, plasmids and bacteriophages. Afterward, the functional importance of these sequences was displayed as an adaptive immune system in prokaryotic. So, they prevent viruses from infecting bacteria harboring them (Mojica *et al.*, 2005).

In 2007, It was confirmed that CRISPR-Cas systems can confer resistance to invading foreign DNA (Barrangou *et al.*, 2007). In 2011, Makarova and colleagues revised their research on the links between CRISPR and Cas proteins (Makarova *et al.*, 2011). To cleave any double-stranded DNA sequence, the Cas9 endonuclease can be programmed with guide RNA generated as a single transcript, according to Charpentier and Doudna, who published their findings in 2012 (Jinek *et al.*, 2012). As a result of their discovery, the CRISPR-Cas9 system has become widely employed as a potent and versatile tool for genome editing.

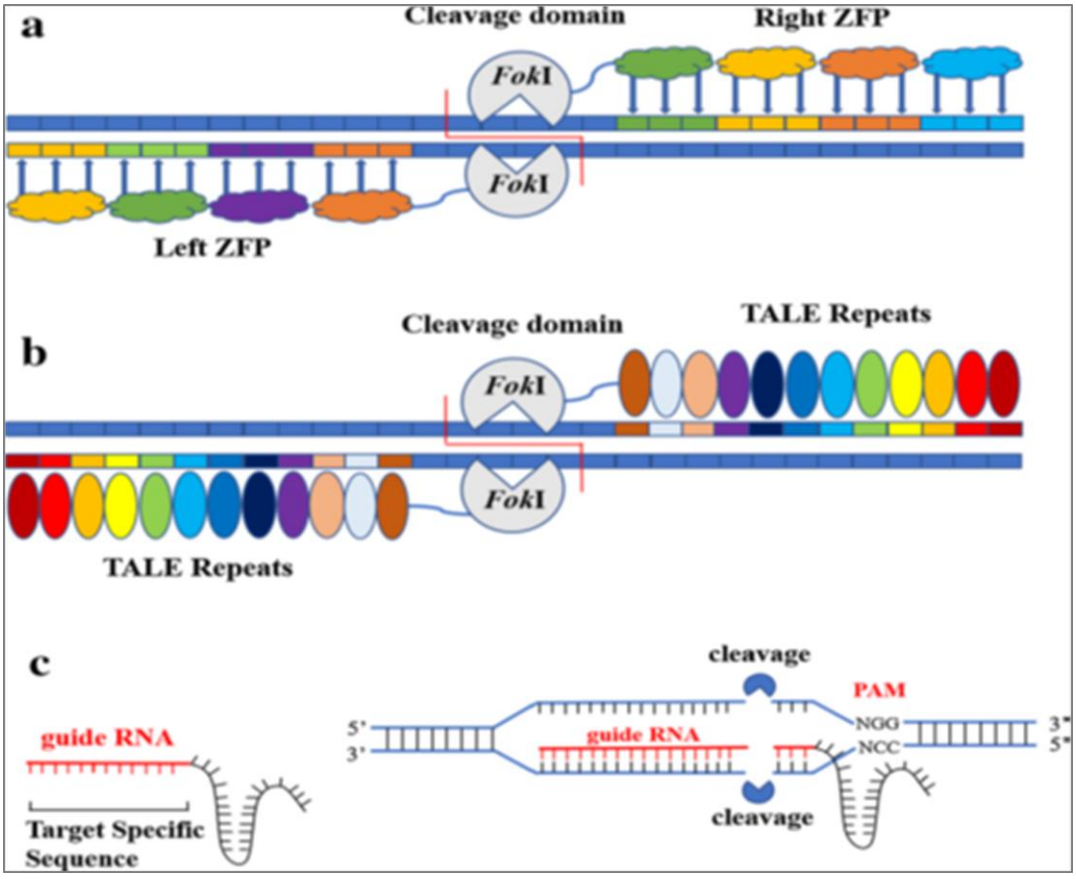

Figure 2-7: a- The FokI cleavage domain is connected to numerous zinc fingers on each ZFN, which can be customized to detect the cleavage site's flanks. b- A spacer sequence of variable lengths separates the two TALE binding sites in TALEN target sites. c- The CRISPR–Cas9 system is made up of two parts: Cas9 and gRNA. A double break occurs three base pairs upstream of the PAM site if the gRNA attaches to the DNA after Cas9 recognizes a PAM site. (Dai *et al.*, 2018).

2.5. CRISPR Loci Structure

CRISPR is found in several different species, has unique sequence and Cas protein, but they all have the same DNA-encoded, RNA-mediated activity. The CRISPR area is made up of small repeated sequences that are typically between (28 –37) bp long. Each has a distinct, similar-length sequence separated by spacers. Each repeat is palindromic, which means that the repeat sequence on one side of the strand is identical to the sequence on the opposing strand when both strands are read in their respective 5́ to 3́ orientations. Spacer sequences are crucial for CRISPR's defense systems which contain genetic sequence originally obtained either from phages or plasmids, are essential components of the uniqueness of CRISPR's defense mechanisms (Barrangou and Marraffini, 2014).

These spacers act as an immunological memory bank, storing sequences from previous interactions with invading organisms. Depending on the harboring species, the number of spacers in a CRISPR array might range from one to hundreds (Grissa *et al.*, 2007).

Each CRISPR loci is preceded with the leader sequence, which is high in the adenine and thymine. These 500-bp leader sequences contain promoter elements and signals for CRISPR system adaption, which are essential for the synthesis of crRNA (CRISPR RNA) and the effective incorporation of foreign genetic material into CRISPR sequences (Yosef *et al.*, 2012; Pul *et al.*, 2010) .

Cas genes are structurally and functionally well connected to CRISPR sequences and are located upstream to it or its leader sequences. Cas proteins that are encoded by Cas genes, form CRISPR-Cas effector complexes. which combine with crRNA transcribed from CRISPR loci to mediate the silencing and cleavage of unfamiliar nucleic acids (Fig. 2-8) (Chylinski *et al.*, 2014).

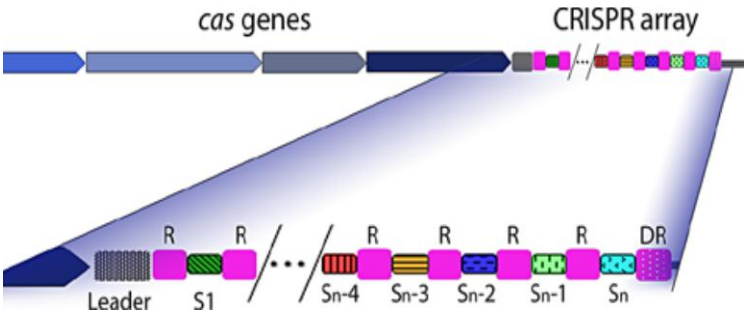

Figure 2-8: A CRISPR-Cas system's structure. The array's total number of spacers is n. At least one spacer, flanked by two repetitions, can be found in a CRISPR array. The last repeat, which is prone to mutations, is referred to as a degraded repeat (DR) (Butiuc-Keul *et al.*, 2021).

2.6. Advantages of CRISPR's technique

Compared to ZFNs and TALENs CRISPR-Cas introduced important advantages in gene editing applications. Both ZFNs and TALENS rely on custom-made proteins to lead the enzyme to its target. While, Cas9 systems rely solely on the engineering of short gRNA molecules, which saves time and cost in validating and designing of proteins (Table 2-2). Multiplex gene editing is difficult for ZFNs and TALENs due to the necessity for specialized proteins individually designed for each gene, but Cas9 systems can target many genes with ease. The cells are given many gRNAs (Jao *et al.*, 2013; Wang *et al.*, 2013).

CRISPR-Cas swept the scientific community in 2013, and its widespread use was justified by its high efficacy (Cong *et al.*, 2013; Ding *et al.*, 2013; Mali *et al.*, 2013a; Pennisi, 2013).

Table 2-2: The primary distinctions between the three genome editing techniques (Loureiro and da Silva, 2019)

Feature	ZFN	TALEN	CRISPR/Cas9
Basis of tying	Protein/DNA	Protein/DNA	RNA/DNA
Design easiness	Midst	Simple	Very simple
Grouping	Hard	Simple	Very Simple
Time of constructing	7 days	7 days	3 days
Price	High -priced	Modest-priced	Low-priced
proficiency	changing	Higher	Higher
Effects off-target	Changing but high	Lower	Higher
one or two units	Two-unit	Two-unit	One- unit

2.7. Proteins of the Cas

CRISPR systems are made up of spacers and repetitive sequences on CRISPR arrays, as well as CRISPR-associated Cas genes that encode for proteins involved in DNA repair and immunity (Brouns *et al.*, 2008; Makarova *et al.*, 2002). Initially, In genomes harboring the CRISPR-Cas system, four Cas genes have been found (Jansen *et al.*, 2002). However, as more genome sequences accumulated, 93 Cas genes were discovered, which were divided into 45 families of genes according to the similarity of the encoded proteins' sequences (Makarova *et al.*, 2015; Haft *et al.*, 2005).

Six of these Cas genes (Cas1–Cas6) are widely distributed, with Cas1 and Cas2 serving as a distinguishing feature in genomes with CRISPR loci. The examination of Cas1 sequences demonstrates that the CRISPR-Cas systems are available in a variety of forms (Haft *et al.*, 2005). All case genes bind to CRISPR loci although they differ in structure and arrangement (Table 2-3) (Makarova *et al.*, 2006).

Subtypes of Cas proteins have been divided and named after representative species with only one CRISPR-Cas locus. Such as, Cse1 was given to Cas proteins from *Escherichia coli* (CRISPR system of *E. coli* gene1), another subtypes: *Yersinia* (Csy), *Neisseria* (Csn), *Aeropyrum* (Csa), *Thermotoga* (Cst), *Desulfovibrio* (Csd), *Haloarcula* (Csh), and *Mycobacterium* (Csm) (Haft *et al.*, 2005).

Considering the evolutionary relationships between Cas operon, the CRISPR-Cas system can be classified into two systems: The effector system that process crRNA interferes and even damaging the invading DNA.

The adapting system needs the spacer acquisition proteins (Cas1, Cas2). Within the CRISPR subtypes, crRNA-processing Cas proteins may differ, they either implicate a number of Cas proteins or rely on one multifunctional protein.

In CRISPR-Cas system (Class 1), in which the Type I and Type III effector complexes are ribonucleoproteins that are phylogenetically linked (Makarova *et al.*, 2015; Brouns *et al.*, 2008).

In contrast, the Class 2, as seen in Types II, V, and VI, only includes one interference protein (Shmakov *et al.*, 2017). Pre-crRNA processing appears to include a number of other proteins with a minimum of one RNA recognition motif with unknown function (Makarova *et al.*, 2011).

2.8. The CRISPR-Cas System's Classification

Due to the horizontal gene transfer, most prokaryotes have numerous CRISPR loci and CRISPR-Cas systems have a wide range of Cas proteins and as a consequently, it is difficult to classify them within one modest system. Despite this, two classes and six types of CRISPR-Cas systems were identified, each with several subtypes. Multi subunit crRNA-effector complexes are seen in class 1 systems, but in class 2 systems, a single protein,

such as Cas9, fulfills all effector complex activities (Table 2-3). Types (IV, V) belong to classes (1, 2) (Nidhi *et al.*, 2021; Koonin and Makarova, 2019).

In terms of expression, interference, and adaption modules, the CRISPR-Cas systems differ in a number of aspects (Makarova *et al.*, 2015). Despite most prokaryote only have one kind of CRISPR-Cas, different types of systems could be coexisted. CRISPR-Cas systems in the class 1 are thought to be evolutionary related. Class 2 systems evolved from class 1 by the introduction of transposable elements encoding various nucleases, and are today used as genome editing tools (Mohanraju *et al.*, 2016). All type I systems have the Cas3 protein as a common feature (Sinkunas *et al.*, 2011).

Cas genes come in various numbers, it divides the type I system into six subcategories (type I-A to type I-F). Cas1, Cas2, and Cas3 proteins are encoded as a Cascade-like complex in all type I systems. Target localization, spacer acquisition, and crRNA processing are all aided by the Cascade complex. The type (I-A) system exhibit a particular Cascade complex with Cas3 (Rath *et al.*, 2015) .

Cas1, Cas2, Cas9, and occasionally Csn2 or Cas4 proteins are encoded by type II CRISPR Cas system. Cas9 is implicated in the cleavage of target DNA, the processing of crRNA, and adaptability (Heler *et al.*, 2015; Wei *et al.*, 2015; Barrangou *et al.*, 2007). Subtypes (II-A, II-B, II-C) were created from type II systems. The Csn2 or Cas4 is absent in type II-C (Chylinski *et al.*, 2013; Koonin and Makarova, 2013). In type III CRISPR Cas systems, the protein Cas10 was discovered, although its function is unknown. The majority of Cas proteins are found in Csm (Type III-A) or Cmr (Type III-B) complexes, which are similar to Cascade (Rouillon *et al.*, 2013; Staals *et al.*, 2013).

The target DNA in both Type I and II CRISPR Cas systems differs as well as (DNA, RNA) in Type III CRISPR Cas system. Only bacteria have Type II systems, although type (I , III) systems have been discovered in both bacteria and archaea (Makarova *et al.*, 2011). To provide effective protection against DNA and RNA intruders, only the Type III CRISPR Cas system uses three nuclease activities (specific DNA split, specific DNA/RNA split, and nonspecific SS-DNA split). The Cas7 protein may act as a switch to limit Cas10's DNase activity once the cleaved target RNA has been released from the complex (Samai *et al.*, 2015).

Table 2-3: The function of the major Cas proteins (Nidhi *et al.*, 2021)

Proteins	**Type/Subtype Association**	**Role**
Cas-1	Universal	DNA nuclease
Cas-2	Universal	RNA nuclease
Cas-3	Type I (a, b, c, d, e, f)	DNA nuclease , helicase
Cas-4	Type I (a, b, c, d), II (b), V	DNA nuclease
Cas-5	I (a, b, c, e), III, IV	pre-crRNA processing
Cas-6	I (a, b, d, e, f), III (a, b)	pre-crRNA processing
Cas-7	Type II (a, b) I (a, b, c, d, e, f), III, IV	RNA recognition, crRNA binding
Cas-8	I (a, b, c), IV	Cascade complex's big subunit
Cas-9	Type II signature (a, b)	DNA nuclease
Cas-10	I (D), type III signature (a, b)	Csm or Cmr complex's big subunit
Cas-11	I, III, IV	effector complexes' smallest subunit
Cas-12	V	DNA nuclease, processing of crRNA
Cas-13	VI	DNA nuclease, processing of crRNA

2.9. CRISPR-Cas9 Adaptive Immunity Mechanism

Immunity mechanisms in Type (I, III) CRISPR-Cas system are complicated and are not used for genome editing. Type II CRISPR-Cas system is the simplest of the CRISPR-Cas systems, as it only requires a single multi-functional Cas9 protein to interfere with invading genetic material (Makarova *et al.*, 2011). In the endogenous CRISPR-Cas9 system, three elements are required for target cutting: CRISPR RNA (crRNA), Cas9 protein and trans activating crRNA (tracrRNA) or what is so called the scaffold. That aids the formation of the Cas9 complex in addition to crRNA maturation. There are three phases in the type II CRISPR-Cas system: CRISPRs are first acquired, followed by crRNA synthesis, and finally, invading DNA to interfere with (Fig.2-9).

Three main steps summarize the bacterial adaptive immune response to the invading viruses:

1- Acquisition of protospacers: During the acquisition step, a Cas nuclease breaks down the invading phage DNA into short DNA fragments called protospacer sequences, it is subsequently inserted as a new spacer into the bacterial genome's CRISPR locus (Wiedenheft *et al.*, 2012).

Acquired spacers separated by repeat sequences are encoded in each CRISPR array. Protospacer selection is aided by the detection of protospacer adjacent motifs (PAMs) located within the viral genome. However, PAM sites are not present in protospacer sequences incorporated into the CRISPR locus (Mojica *et al.*, 2009). The PAM sequence's identity is determined by the Cas9 protein's species (for instance, 5́-NNNNGATT-3́ PAM from *Neisseria meningitides*, 5́-NGGNG-3́ PAM in *Streptococcus thermophiles* and 5́-NGG-3́ PAM in *Streptococcus pyogenes*) so it is species-dependent sequences (Cho *et al.*, 2013; Hou *et al.*, 2013; Karvelis *et al.*, 2013).

2- Biogenesis: CRISPR RNA is transcribed from the CRISPR locus (pre-crRNA). TracrRNA binds to the pre-repeat crRNA's sequences, which is then cleaved by endogenous RNase III, resulting in mature crRNAs with a partial repetition sequence and single spacer (Deltcheva *et al.*, 2011; Pougach *et al.*, 2010).

3- Interference: At the final stage, mature crRNA leads Cas9 protein to complementary invading nucleic acids, degrading invading phage DNA sequences (Garneau *et al.*, 2010; Marraffini and Sontheimer, 2008).

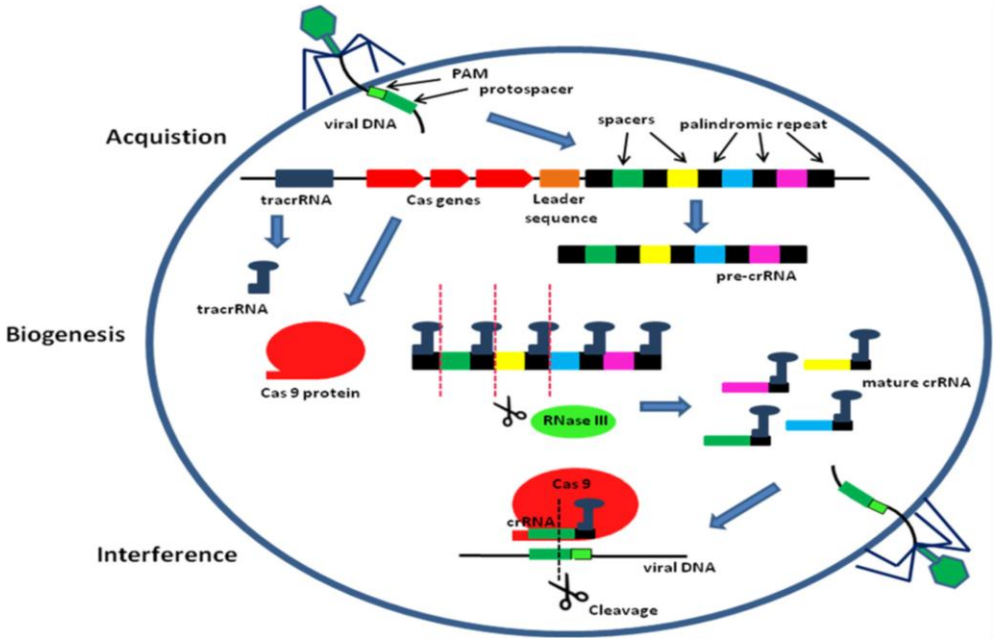

Figure 2-9: Acquisition, crRNA biogenesis, and viral DNA interference are the three steps of the CRISPR-Cas bacterial adaptive immune system. (Hryhorowicz *et al.*, 2017).

2.10. Exploitation of the CRISPR-Cas9 System for Genomic Engineering

The CRISPR-Cas9 system is derived from prokaryotes and used as a novel technology that allows for precise and targeted genome editing in live cells (Mali *et al.*, 2013b). When the Cas9 nuclease binds to the crRNA-tracrRNA duplex, it causes a double-strand break in the target DNA, which is required for genome editing (Cong *et al.*, 2013; Jinek *et al.*, 2012).

The complementary target DNA sequence and the PAM sequence in the target DNA, as well as RNA–DNA complementarity base pairing between the 20-nt guide RNA sequence, are both required for Cas9 target recognition (Jinek *et al.*, 2012).

In gRNA molecules, there is a scaffold sequence that links to Cas9 and a targeting region that guides the system to the target locus (Anders *et al.*, 2014). If a PAM site is found, the Cas9-gRNA targeting sequence's first (8–12) PAM-proximal bases sometimes referred to as the seed sequence. Once the Cas9-gRNA has identified a likely target, it will start coupling with the target DNA in the 3′-5′ direction (Semenova *et al.*, 2011). Cas9 cannot couple and split because of mismatches in the seed sequence, while mismatches at the 5′ PAM-distal end do not always prohibit Cas9 from cleaving (Liu *et al.*, 2016).

A double-strand break (DSB) occurs in the DNA when gRNA and the target area are homologous, Both the Cas9, HNH, and RUVCE catalytic domains catalyze this reaction (Loureiro and da Silva, 2019).

2.11. Mechanisms of DNA repair

Engineered nucleases can easily knock off genes out by causing mutations in the targeted gene through double stranded breaks (DSB). Following any break event, cells use one of two main repair mechanisms to repair that damage: The NHEJ or HDR (Riordan *et al.*, 2015). When DNA DSB occurs,

the NHEJ pathway is the major DNA repair process. Using the protein Ku and a new ligase, LigD, it entails ligation of the blunt ends formed by the symmetric DNA break (Fig. 2-10) (Shuman and Glickman, 2007).

During the repair of blunt DNA, end resection causes various deletions and insertions. NHEJ is known as the homology-independent method since only one to a few complementary bases are aligned to re-ligate two ends. It's an error-prone repair technique that commonly leads to indels (out-of-frame mutations) in the corrected region. Furthermore, even after effective DNA repair, CRISPR-Cas9 continues to bind to and modify the DNA sequence, increasing the risk of other mutations. Frame shift codon alterations are typically caused by indels, causing a disturbance in the protein-coding sequence and, in certain cases, a precocious stop codon (Dow, 2015).

The HDR pathway is the second DSB repair process, which reconstructs the original sequence using DNA from the sister chromatid's allelic gene as a template (Johnson and Jasin, 2000). The DNA template contains instructions for precisely repairing the chromosomes that have been broken (Yeh *et al.*, 2019). To repair (single-stranded DNA, dsDNA damage) raised by Cas driven split, DNA repair pathways may result in significant mutation. HR in bacteria is a more well-studied and universal DNA repair pathway (Wigley, 2013).

In *Escherichia coli*, the Rec F and Rec BCD proteins are involved in two HR-mediated DSB repair processes. Rec B, in addition to working as an exonuclease, elicits helicase activity in response to the induction of a DSB. When a chi site is recognized, the complex comes to a halt and undergoes

conformational changes, Rec A proteins can then be placed onto the single-stranded overhang that remains (Dixon and Kowalczykowski, 1993).

The RecA–DNA complex then subjected to conformational proofreading and strand attack of DNA sequences that are analogous. Reciprocal or nonreciprocal recombination occurs when the DNA hetero-duplex is resolved. In bacteria, Rec F has partial redundancy for Rec BCD's ability to repair DSBs (Morimatsu and Kowalczykowski, 2003).

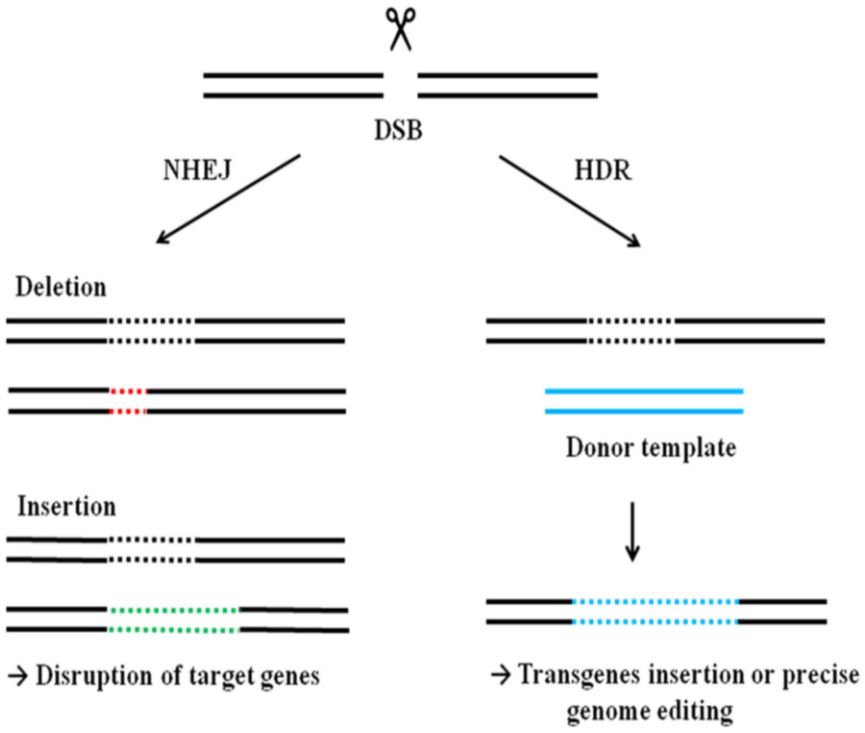

Figure 2-10: Genome editing using an engineered nuclease. A double-stranded break (DSB) in the targeted sequence can be repaired using non-homologous end joining (NHEJ) and homology-directed repair (HDR) ways. (Hryhorowicz *et al.*, 2017).

2.12. Application of CRISPR-Cas9 biotechnology in bacteria

Genetic manipulation became easier due to the development in CRISPR-Cas9 based biotechnologies that allowed for better editing capacity and specificity. In bacteria, CRISPR-Cas9 has been heavily exploited for a

variety of uses, metabolism engineering and synthetic biology are the most frequent two examples. Bacteria as cell factories can use simple and inexpensive resources, such as renewable biomass and even wastes, for fundamental cell metabolism and critical chemical biosynthesis. Genetic alteration of the cell machinery components is required to improve its performance. Traditional recombination-based methods exist, but they are expensive and take longer time (Yao *et al.*, 2018).

CRISPR-Cas systems provide several advantages that have led to their usage in a variety of implementations. The CRISPR-Cas system excels at cleaving the target sequence initially; as a result, it can be combined with λ-Red recombineering to edit with precision and efficiency without the need for marks. Jiang *et al.* (2015) in *Escherichia coli* genome, devised a two-plasmid system that allowed iterative genome editing of a number of targets with the ability to remove the plasmids after they do the mission. These technologies eliminate the need for selection markers and allow for the simultaneous introduction of many alterations, considerably increasing engineering throughput.

Li *et al.* (2015) took advantage of this ability to incorporate a beta-carotene synthesis route into the *E. coli* genome. Researchers evaluated 33 genomic modifications in search of clones with increased beta-carotene production via combinatorial modulations, with the best producer generating 2.0 gL^{-1} in fed-batch fermentation.

CRISPR-Cas systems are also adaptable, allowing them to be used to repress or increase transcriptional gene expression.Wu *et al.* (2015) used the CRISPRi system in *E. coli* to change the expression of several genes in the major metabolic pathway, resulting in a 223 percent increase in malonyl-CoA levels.

Employed Cas9 assisted genome editing to develop a high-performing succinate-producing *Synechococcus* sp. (titer succinate of 0.44 mgL^{-1}, an 11-

fold raise over the starting). In a different investigation, Cobb *et al.* (2015) compared the expression of a dual tracr/crRNA expression cassette to that of a sgRNA expression cassette. According to the findings, utilizing sgRNA in the CRISPR method for multiplex engineering improved performance, resulting in genomic deletions of 20bp to 30kb and spCas9 acceptance in industrial *Streptomyces*.

Lactobacillus reuteri was exposed to high-efficiency targeted mutagenesis (90–100%) using single-strand DNA recombineering with the aid of spCas9 (Oh and van Pijkeren, 2014). Song *et al.* (2017) created a very efficient nCas9 system in *Lactobacillus casei* for chromosomal insertion of foreign genes and in-frame gene deletions with an efficiency augmented from 25% to 62%.

With the aim of detecting infrequent homologous recombination events in *Clostridium* species, CRISPR-Cas tools have been frequently employed as a counter-selection technique (Wang *et al.*, 2016; Wang *et al.*, 2015) .Using spCas9,Wang *et al.* (2015) demonstrated effective and marker-free deletion of chromosomal genes in *Clostridium beijerinckii* NCIMB 8052.

In *Bacillus licheniformis*, Li *et al.* (2018) created a new nCas9 genome modifying method for gene integration and single, double, and large-fragment gene deletions with an efficiency of (100, 11.6, 79.5, and 76.5)% respectively. Another study conducted by Tapscott *et al.* (2019) in *Methylococcus capsulatus* used Cas9 and Cas9D10A nickases to change green fluorescent protein (GFP)-expressing cells to blue fluorescent protein (BFP)-expressing cells with 71 % efficiency in vivo editing of plasmid DNA. The Cas9D10A nickase was also employed to successfully induce a premature stop codon into the hydroxylase component-encoding mmoX gene, affecting soluble methane monooxygenase function.

Mo *et al*. (2020) used CRISRR system to disrupt the essential metabolism of glyA in that is a dynamic repression efficacy of cell growth in *Methylorubrum extorquens* from (41.9 to 96.6) % depending on the sgRNA targeting sites. The optimized CRISPRi system mitigated the abundance of the exogenous fluorescent protein gene mCherry by more than 50% and the expression of the phytoene desaturase gene crtI by 97.7%.

Zhang *et al*. (2022) using the CRISPR-Cas system, a flexible gene-editing tool with a two-plasmid method was created for a native *Pseudomonas fulva* strain obtained from the model organism silkworm. An antibiotic-related gene, oqxB, was knocked out, resulting in poor growth of the *P. fulva* deletion mutant in chloramphenicol-containing LB.

Chapter Three
Material & Methods

3. Material & Methods

3.1. Materials

3.1.1. Chemicals

All chemicals and molecular reagents which are used in this study are analar and listed in Tables 3-1, 3-2 and 3-3.

Table 3-1: Chemicals, providers and origins.

Chemical	Provider	origin
Agar	Hi Media	India
Benzene	Merck	Germany
$CaCl_2$. $2H_2O$	Hi Media	India
$CaCl_2$. $6H_2O$	Hi Media	India
Chloroform	Merck	Germany
$CoCl_2$. $6H_2O$	Hi Media	India
Crystal Violet	Hi Media	India
$CuSO_4.5H_2O$	Hi Media	India
D-Biotin	Hi Media	India
EDTA-Na_2	Hi Media	India
$FeCl_3$	Hi Media	India
$FeSO_4.7H_2O$	Hi Media	India
Fluconazole	Brawn	India
Glycerol	J. T. Baker	USA
HCl	Scharlau	Spain
H_3BO_3	Hi Media	India
Iodine Crystals	Hi Media	India
Kanamycin	Solarbio	China
KH_2PO_4	Hi Media	India
K_2HPO_4	Hi Media	India
KNO_3	BDH	UK
Methanol	BDH	UK
$MgSO_4.7H_2O$	Hi Media	India
$MnCl_2$. $4H_2O$	Hi Media	India
$MnSO_4.5H_2O$	BDH	UK
Na_2HPO_4	Hi Media	India
Na_2HPO_4. $12H_2O$	Hi Media	India
NaCl	Hi Media	India
$Na_2MoO_4.2H_2O$	Hi Media	India
Naphthalene	Hi Media	India

n-hexane	Merck	Germany
$(NH4)_2SO_4$	Hi Media	India
NiCl. $6H_2O$	Hi Media	India
Nutrient broth	Hi Media	India
O-dianisidine	Hi Media	India
Peptone	Hi Media	India
Safranin	Hi Media	India
Spiramycin	Solarbio	China
Thiamine. HCL	Hi Media	India
Yeast extract	Hi Media	India
$ZnSO_4$-$7H_2O$	Hi Media	India

Table 3-2: Molecular reagents, providers and origins.

Molecular reagents	**Provider**	**origin**
100bp DNA Ladder	Promega	USA
10X TBE buffer	Bioneer	Korea
Agarose	BioBasic	Canada
Bromo phenol blue	Geneaid	Taiwan
DNA extraction kit	Geneaid	Taiwan
Ethanol (absolute)	BDH	UK
Ethidium Bromide	BioBasic	Canada
Gene Pulser Cuvette	Bio Rad	UK
Go Taq®G2 Green Master Mix	Promega	USA
GoScript™ReverseTranscription System	Promega	USA
Go Taq®qPCR Master Mix	Promega	USA
Nuclease free water	Bioneer	Korea
PureYield™Plasmid Miniprep System	Promega	USA
RNA extraction kit	Geneaid	Taiwan

Table 3-3: Primers, sequence, product and references.

	Primer	Gene name	Sequence	Reference
1	F27	*16S rDNA*	Forward: 5́-AGAGTTTGATCCTGGCTCAG-3́	(Miyoshi *et al.*, 2005)
	R1492	*16S rDNA*	Reverse: 5́-GGTTACCTTGTTACGACTT-3́	
2	mmoX1	*sMMO*	Forward: 5́-CGGTCCGCTGTGGAAGGGCATGAAGCGCGT-3́	(Miguez *et al.*, 1997)
	mmoX2	*sMMO*	Reverse: 5́-GGCTCGACCTTGAACTTGGAGCCATACTCG-3́	
3	F1003degen	*MxaF*	Forward: 5́-GGNCANACYTGGGGNTGGT-3́	(Lau *et al.*, 2013)
	R1561degen	*MxaF*	Reverse: 5́-GGGARCCNTTYATGCTNCCN-3́	
4	1004F	*MxaF*	Forward: 5́-CCWGGYGAYAAYAARTGGTC-3́	(Ramachandran and Walsh, 2015)
	1232R	*MxaF*	Reverse: 5́-GCCCAGTTNAYRAAKGGRTG-3́	
5	PmoC374	*pMMO*	Forward: 5′-AGCARGACGGYACNTGGC-3′	(Ghashghavi *et al.*, 2017)
	PmoA344	*pMMO*	Reverse: 5′-ANGTCCAHCCCCAGAAGT-3′	
6	16S-Fw	*16S rRNA*	Forward: 5′- TGAGATGTTGGGTTAAGTCCCGCA-3′	(Zhang *et al.*, 2016)
	16S-Rv	*16S rRNA*	Reverse: 5′- CGGTTTCGCTGCCCTTTGTATTGT-3′	

***Y=C or T; D=G, A, or T; R=A or G; S=G or C; N=A, G, C or T; W=A or T; K=G or T.**

3.1.2. Equipment

The equipment's used in this study are listed in Table 3-4.

Table 3-4: The equipment's, providers and origins.

Equipment	Provider	origin
Autoclave	Hirayama HG-80	Japan
Gene Pulser II	Bio-Rad	UK
Centrifuge	Eppendorf	Germany
Cooling Centrifuge	Eppendorf	Germany
Deep freeze (-80°C)	Artiko	Germany
Electrophoresis system	Fisher Scientific	USA
Eppendorf centrifuge	Eppendorf	Germany
Eppendorf tube	Citotest	China
Fine tips 10,100,1000 µl	Citotest	China
Gas Chromatography spectroscopy	Agilent	UK
Glass slides	Citotest	China
Hot plate and magnetic stirrer	Heidolph	Germany
Ice maker	Lab-kits	Hong Kong
Incubator	Binder	Germany
Light microscope	Zeiss	Germany
Micropipettes 0.5-10 µl, 20-100 µl, 100-1000 µl	Eppendorf	Germany
Mini vortex minifuge	Eppendorf	Germany
Nano drop	Optizen	Korea
Oven	Binder	Germany
PCR tube (microcentrifuge tubes)	Citotest	China
pH meter	Fisher Scientific	USA
Platinum wire loop	Hi Media	India
Real Time PCR	Bioneer	Korea
Shaker incubator	Sartorius	Germany
Spectrophotometer (UV/Vis)	Beckman coulte DU530	UK
Thermal cycler for PCR	Eppendorf	Germany
Vortex	Fisher Scientific	USA
UV trans-illuminator	ATTA	Korea
Water Bath	GFL	Germany
Water distiller	GFL	Germany

3.2. Methods

3.2.1. Sampling

To isolate hydrocarbon-degrading bacteria, roughly 250 g of oil-contaminated soil was collected aseptically in sterile plastic bags from five regions of three oil sites in Basra city, southern Iraq, in December 2020, from a depth of 5–15 cm, transferred to the lab, and kept at room temperature (Fig. 3–1). The coordination was indicated in the Table 3-5.

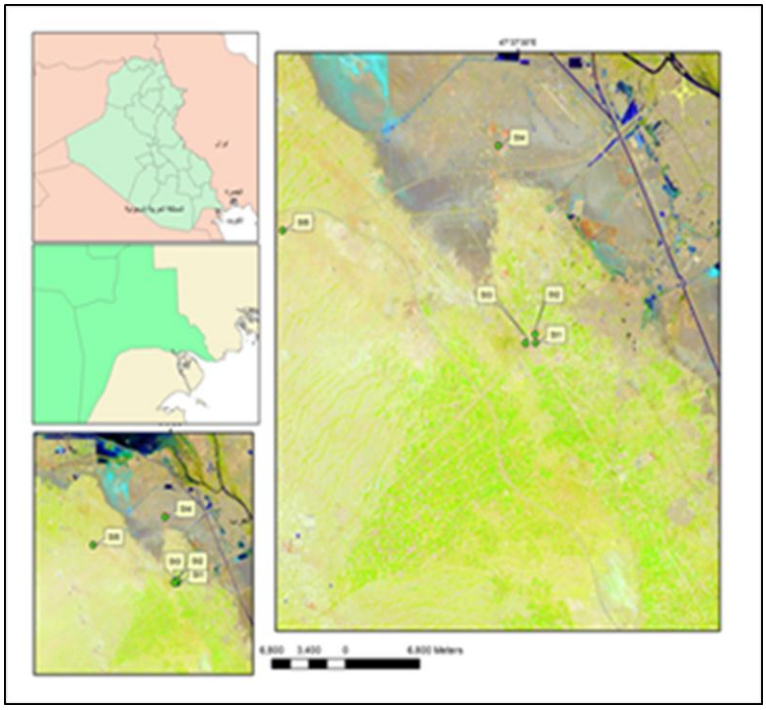

Figure 3-1: Stations for sampling: 1, 2, 3/ Al-Zubair 4/ Hammar Mushrif gas station /5/ Nahran Omar.

Table 3-5: Sampling stations' coordination.

No.	Latitude	Longitude
1	30.36449	47.63551
2	30.36475	47.63612
3	30.36587	47.63696
4	30.51714	47.60423
5	30.45591	47.39529

3.2.2. Preparation of culture media

3.2.2.1 Basal Methanol Medium (BMM)

Basal methanol medium for isolation of Kouno *et al.* (1973) has been modified according to Fujii *et al.* (1982) by increasing thiamine and biotin (Table 3-6). The BMM powder was dissolved in 1000 mL of distilled water (pH 6.8-7.0), sterilized by autoclaving at 15 lbs/in^2 and 121 °C for 15 min. After sterilization, thiamine, biotin, and 20 mL of methanol were added to the medium in addition to that fluconazole was added as antifungal. To make solid medium, 15 gL^{-1} of agar was added.

Table 3-6: Basal methanol medium.

Component	Kouno *et al*	Current study
Nitrogen and Sulfur source		
Yeast extract	0.1 g	1.0 g
$(NH_4)_2SO_4$	3.0 g	3.0 g
$MgSO_4.7H_2O$	0.5 g	0.5 g
$MnSO_4.5H_2O$	2.0 mg	2.0 mg
$FeSO_4.7H_2O$	2.0 mg	2.0 mg
Phosphorous source and pH buffering		
KH_2PO_4	2.0 g	2.0 g
K_2HPO_4	7.0 g	7.0 g
Amino acid		
Thiamine. HCl	100 µg	0.2 mg
Vitamin		
Biotin	10 µg	0.1 mg
Antifungal		
Fluconazole	--	0.05 mg

3.2.2.2 Methanol Salt Medium (MSM)

Methanol salt medium of Kouno *et al.* (1973) was also modified by increasing the weights of some ingredients and adding fluconazole as antifungal (Table 3-7). The medium components were dissolved in 1000 mL

distilled water (pH 6.8). Fluconazole and methanol (10 mL) were added after sterilization. 15 gL^{-1} of agar was added to make solid medium.

Table 3-7: Methanol Salt medium.

Component	Kouno *et al*	This study
$NaNO_3$	2 g	-
KNO_3	-	2 g
$MgSO_4.7H_2O$	0.2 gm	0.2 g
$FeSO_4.7H_2O$	1 mg	10 mg
Na_2HPO_4	0.21 g	0.21 g
KH_2PO_4	0.09 g	0.09 g
$CuSO_4.5H_2O$	50 µg	0.5 mg
H_3BO_3	10 µg	30 mg
$MnSO_4.5H_2O$	10 µg	2 mg
$ZnSO_4.7H_2O$	70 µg	10 mg
$Na_2MoO_4.2H_2O$	10 µg	3 mg
Fluconazole	-	0.05 mg

3.2.2.3 ATCC medium: 1306 Nitrate mineral salts medium (NMS)

Nitrate mineral salts medium was used for isolation and identification of methanotrophs bacteria (Dianou and Adachi, 1999) (Table 3-8; 3-9 and 3-10).

Table 3-8: Composition of standard Nitrate mineral salts medium.

Component	Amount
$MgSO_4.7H_2O$	1 g
$CaCl_2.6H_2O$	0.20 g
KNO_3	1 g
KH_2PO_4	0.272 g
$Na_2HPO_4.12H_2O$	0.717 g
Chelated Iron Solution	2 mL
Trace Element Solution	0.5 mL
Distilled water	1000 mL

15 g of agar was added to prepare solid media. Adjust pH to 6.8, and sterilized at 121°C for 15 min.

Table 3-9: Chelated Iron Solution.

Component	Amount
$FeCl_3$	0.05 g
EDTA, sodium salt	0.2 g
HCl	0.3 mL
D.W	100 mL

Table 3-10: Trace Element Solution.

Component	Amount
EDTA	0.5 g
$FeSO_4. 7H_2O$	0.2 g
$ZnSO_4. 7H_2O$	0.01 g
$MnCl_2. 4H_2O$	0.003 g
H_3BO_3	0.03 g
$CoCl_2. 6H_2O$	0.02 g
$CaCl_2. 2H_2O$	0.001 g
$NiCl. 6H_2O$	0.002 g
$Na_2MoO_4. 2H_2O$	0.003 g
D.W.	1000 mL

3.2.3. Isolation and identification of bacteria

Methanol-utilizing bacteria were obtained from various sources by placing 0.5 g of soil in Erlenmeyer flasks containing 100 mL of basal methanol medium and incubated for 7 days at 30°C in a shaking incubator at 180 rpm. The bacteria were plated on a basal methanol agar plate after several subcultures and picked up after 5–7 days of incubation at 30°C. Single colonies were streaked onto a petri dish containing methanol salt medium for identification and incubated at 30°C for 5–7 days. Following incubation, the colonies are stained with gram's stain and examined under a microscope (Kouno *et al.*, 1973).

3.2.4. Morphological and biochemical assay

Morphological and biochemical assays were carried out which including gram stain, cell shape, colony morphology, pigments, catalase and oxidase. After that, the isolates were identified by sequencing the *16S rDNA* gene (Fig. 3-2).

3.2.4.1 Gram's staining and the bacterial morphology

Gram's stain was used to discriminate between gram^{+ve} and gram^{-ve} bacteria, as well as to identify the strains' morphology under the microscope (Barrow and Feltham, 2004).

3.2.4.2. Catalase assay

The catalase assay was performed according to MacFaddin (2000). Using a wooden stick, a single colony was placed in a clean slide, and a few drops of catalase reagent (3% H_2O_2) were applied, resulting in the formation of gas bubbles, indicating a positive outcome.

3.2.4.3. Oxidase assay

To carry out the oxidase test a piece of filter paper was soaked in the reagent solution in a sterile petri dish, and then new growth was scraped from the culture plate using a disposable loop and smeared over the treated filter paper till the dark purple color appeared after 10 sec. (Brown and Smith, 2014).

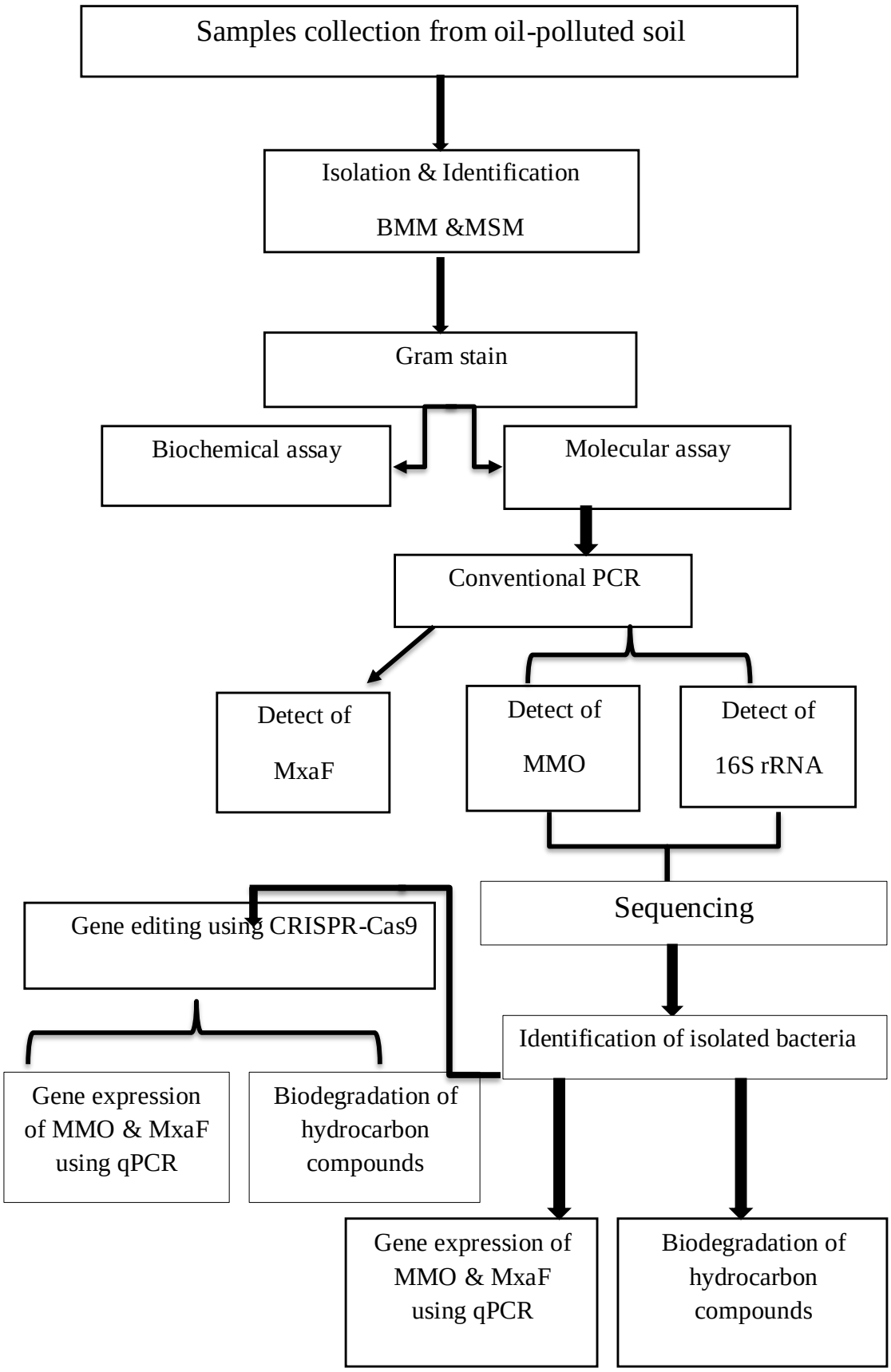

Figure 3-2: Experimental design

3.2.5. Molecular Techniques

3.2.5.1. Genomic DNA extraction

Bacterial DNA was extracted according to the procedure of Geneaid company using Presto™ Mini gDNA bacteria kit (Cat. No. GBB100) as the following:

1- Each bacterial isolate was refreshed by suspending a single colony in 250 mL Erlenmeyer flasks containing 50 mL MSM broth and incubated for 72 h. at 30°C.

2- The cells were collected by transferring 10 mL of the growth to falcon tubes and centrifuged at 2000 rpm for 2 min. and the supernatants were discarded. The pellet was moved to 1.5 µL microcentrifuge tube and spun down for 1 min. at 14000 rpm, the supernatant was discarded. The cellular pellet was lysed with 180 µL of GT buffer and vortexed for few seconds. The cell lysate was mixed with 20 µL of proteinase K and is incubated for at least 10 min. at 60 °C. The tubes were inverted every 3 min. during the incubation period.

3- The cell lysate was mixed with 200 µL of GB buffer was added to the sample and mixed by vortex for 10 sec. and incubated at 70 °C for 10 min. to ensure the sample lysate is clear, the tubes were inverted every 3 min. during the incubation.

4- The sample lysate was immediately mixed with 200 µL of absolute ethanol and mixed immediately by shaking vigorously with pipette to break precipitate; the mixture was transferred to the GD column which placed in 2 mL collecting tube, at 14000 rpm, centrifuged for 2 min. The precipitate-filled 2 mL collecting tube was discarded, and the GD column was put in a new 2 mL collecting tube.

5- Each sample was washed by adding 400 µL of W1 Buffer to the GD column and centrifuged at 14000 rpm for 30 sec. The supernatant was discarded, and the GD column was reinserted into the 2 mL collection tube, 600 µL of the washing buffer was poured to the GD column,

which was centrifuged for 30 sec. at 12000 rpm. To dry the column matrix, the flow-through was discarded and the GD column was placed back in the 2 mL collection tube and centrifuged for 3 min. at 14000 rpm.

6- The DNA was eluted by transferring the dried GD column to clean 1.5 mL microcentrifuge tube by micropipette. The elution buffer was pre-warmed at 70 °C and 100 µL of it the center of the column matrix and was left for 3 min. The columns were centrifuged at 12000 rpm for 30 sec. to elute the purified DNA; this step was repeated one time to ensure that all DNA was absorbed. The purified DNA was stored at -20 °C.

3.2.5.2. Detection of genomic DNA by agarose gel electrophoresis

The agarose gel was prepared according to Sambrook and Russell (2001). 0.25 g of agarose powder was mixed with 25 mL of 1X TBE buffer to make a 1 % agarose gel (Prepared by adding 1 part of 10XTBE to 9 parts of distilled water) and heated to the boiling by hot plate to be a clear mixture and allowed to cool down to 50-60º C, then 0.2 µL of 10 $mgmL^{-1}$ ethidium bromide was added and mixed well.

3.2.5.2.1. The casting of the horizontal agarose gel using an agarose gel to load and run DNA

Horizontal electrophoresis tank with gel casting tray and combs were arranged, the gel was poured above the tray at 45 °C and allowed to solidify, the comb was removed carefully to form wells and tank was filled with 1xTBE to cover the gel. DNA samples were mixed with bromophenol blue at a ratio of 4µL: 2µL and wee carefully loaded into wells by disposable micropipette fine tip. The samples were

electrophoresed at 60 volts. The gel is examined by UV light to detect and photograph the DNA bands.

3.2.5.3. Amplifying of bacterial *16S rDNA* gene

The *16S rDNA* gene was used to identify the bacterial genomes using universal *16S rDNA* primers (Table 3-3).

The *16S rDNA* gene amplification reaction conditions were set according to Go Taq^{R} G2 Green Master Mix (Cat. No. M7822), 2X from Promega kit instructions were used and the reaction mixture was prepared as Table 3-11. The thermal cycler program is described in Table 3-12.

Table 3-11: Reagent 50 µL for amplification of *16S rDNA* gene.

Reagent	Volume (µL)
GoTaq green Master Mix	25
DNA template	2
Forward Primer	2
Reverse Primer	2
Nuclease-free Water	19
Total volume	50

Table 3-12: Thermal cycler program for *16S rDNA* gene amplification.

Stage	Temperature(°C)	Time	Cycles
Initial denaturation	95	5 min.	1
Denaturation	95	30 sec.	35
Annealing	55	30 sec.	
Extension	72	1min.	
Final extension	72	5 min.	1
Hold time	4		

The amplified DNA was loaded and run as described in section 3.2.5.2. In 25 mL of 1XTBE buffer, to prepare 2% agarose gel. The well was filled with 6 µL of PCR product. The first well of the gel was loaded with 4 µL of 100 bp DNA ladder.

The reaction was performed for 1 h., the bands of 1500 bp were detected and photographed under UV light, and the PCR products were stored at -20 °C for further analysis.

3.2.5.4. Detection of the *sMMO* gene by cPCR

Amplification of the *sMMO* gene was carried out using PCR (Miguez *et al.*, 1997). The sequence as well as the length of the *sMMO* gene primers that were utilized and amplified can be found in Table 3-3. The conditions for the *sMMO* gene amplification reaction were established in accordance with the instructions that came with the Go Taq^{R} G2 Green Master Mix (Cat. No. M7822), 2X from the Promega kit (Table 3-13). The annealing temperature was determined using gradient PCR, whereas the thermal cycle of PCR is presented in Table 3-14.

Table 3-13: Reagent 25µL for amplification of *sMMO* gene.

Reagent	Volume (µL)
GoTaq green Master Mix	12.5
DNA template	3
Forward Primer	1
Reverse Primer	1
Nuclease-free Water	7.5
Total volume	25

Table 3-14: PCR program for detecting the *sMMO* gene.

Stage	Temperature (°C)	Time	Cycles
Denaturation	95	2 min.	1
Denaturation	94	1 min.	30
Annealing	59	1 min.	
Extension	72	1 min.	
Hold time	4		

3.2.5.5. Identification of bacteria species for *16S rDNA* and *sMMO* gene sequencing

Each sample 20 µL of *16S rDNA* or *sMMO* PCR product was transferred to a PCR tube and labeled with the same number in an excel file provided by Macrogen firm "http://dna.macrogen.com" according to the Macrogen biotechnology company protocol. Samples were sent to Macrogen Company, South Korea, for sequencing. Each sample's query sequences were aligned to the Basic Local Alignment Search Tool (BLAST) website https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch. The software was used to identify the bacterial species through the National Center for Biotechnology Information (NCBI) http://www.ncbi.nlm.nih.gov.

3.2.5.6. Detection of the *MxaF* gene by cPCR

PCR was performed to amplify the *MxaF* gene (Lau *et al.*, 2013). The sequence and the size of the *MxaF* gene primers were used and amplified at Table 3-3. The *MxaF* gene amplification reaction conditions were set according to Go Taq^{R} G2 Green Master Mix (Cat. No. M7822), 2X from Promega kit instructions (Table 3-15). The PCR thermal cycle as in Table 3-16, while the annealing temperature was detected by gradient PCR.

Table 3-15: Reagent 25 µL for amplification of *MxaF* gene.

Reagent	Volume (µL)
GoTaq green Master Mix	12.5
DNA template	3
Forward Primer	1
Reverse Primer	1
Nuclease-free Water	7.5
Total volume	25

Table 3-16: PCR program for detecting the *MxaF* gene.

Stage	Temperature(°C)	Time	Cycles
Denaturation	94	45 sec.	30
Annealing	59	1 min.	
Extension	72	1.5 min.	
Final extension	72	10 min.	1
Hold time	4		

All conditions and reaction preparation were applied as in (3.2.5.3).

3.2.5.7. Total RNA extraction with TRIzol

Total RNA was extracted from all samples using the Geneaid GENEzol™ TriRNA Pure Kit (Cat. No. GZXD100) according to the manufacturer's protocol:

- **Sample Homogenization**

1- Log-phase cultures were prepared and transferred to a 1.5 mL micro-centrifuge tube (RNase-free).

2- Cells were collected by spinning the cultures down at 13000 rpm for 2 min. then the supernatants were removed.

3- The pellet was mixed with 700 µL of GENEzol™ Reagent by a pipette, and incubated for 5 min. on ice.

- **RNA Binding**

1- Each sample was centrifuged at 13000 rpm at 4℃, for 1 min. to remove cell debris then the clear supernatant was transferred to a new 1.5 mL microcentrifuge tube (RNase-free).

2- An absolute ethanol was added at a ratio of 1:1 to the sample, the mixture was vortexed thoroughly and then each RB Column was placed in a fresh 2 mL collection tube.

3- The mixture was moved to the RB Column at a volume of 700 each time. The columns were centrifuged for 1 min. at 13000 rpm at 4°C, and then flow-through was discarded.

4- The remaining sample mixture was transferred to the RB column and RNA binding step was repeated.

5- The columns were spun down at 4°C for 1 min. at 13000 rpm, and then the flow-through was removed. The RB columns were moved to new 2 mL collection tube.

- **Washing step**

1- The RB column were washed with 400 µL of pre-wash buffer and centrifuged for 30 sec. at 13000 rpm at 4°C.

2- The flow-through was removed and RB column was placed back to the 2 mL collection tube.

3- The RB column was washed with 600 µL of washing buffer. The RB column was spun down for 30 sec. at 13000 rpm at 4°C, and then the flow-through was discarded. RB column was returned to the 2 mL collection tube.

4- Step 3 was repeated for one more time.

5- The columns were dried by centrifuge at 13000 rpm for 3 min. at 4°C.

- **RNA Elution**

1. The dry column was placed in a clean 1.5 mL microcentrifuge tube, (RNase-free). The RNA was eluted by adding 30 µL RNase free water at the middle of each column.
2. The column was left for 3 min. at room temperature. The column was centrifuged at 13000 rpm for 1 min. at 4°C. A Spectrophotometer Nano-Drop device was used to determine the RNA concentration and quality.

3.2.5.8. Reverse transcription

Promega's GoScript reverse transcription system kit (A5000) was used to convert the isolated RNA to cDNA. The reaction components and RNA (20 ng) were mixed, and the reaction was carried out as directed by the manufacturer:

1- Random primers were added to the template RNA at a 1:4 ratios to generate a final volume of 5 µL. Before preparing for the reaction, each component was individually mixed and spun for a few seconds.

2- The RNA tube was firmly closed and incubated for 5 min. at 70°C in a water bath, then immediately chilled on ice for 5 min. Each sample was centrifuged for 10 sec. to regain its original volume, and the tube was maintained on ice until the reverse transcription reaction was prepared.

3- For each cDNA synthesis reaction, the GoScript™ reverse transcription components were combined on ice in a microcentrifuge tube to a final volume of 15 µL (Table 3-17).

4- To make a final volume of 20 µL, the revers transcription reaction components were mixed with the extracted template RNA.

5- The thermal cycle was used to finish the synthesis of the first cDNA strand (Table 3-18).

Table 3-17: Reverse transcription reaction.

Reagent	Volume (µL)
GoScript 5X reaction buffer	4
$MgCl_2$	2
PCR nucleotide mix	1
GoScript™ reverse transcriptase	1
Nuclease free water	7
Total Volume	15

Table 3-18: Thermal cycler program for cDNA synthesis.

Step	Temperature (°C)	Time
Primer annealing (Random primer)	25	5 min.
cDNA Synthesis	42	60 min.
Heat inactivation	70	15 min

3.2.5.9. qPCR for detection and gene expression assay

The qPCR primers used to detect gene and gene expression the *MxaF, pMMO* gene and *16S rRNA* as an internal control is listed in Table 3-3.

The real-time PCR reaction was prepared according to Promega's instructions. The reaction mix was prepared by mixing 10 µL of SYBR green with 5 µL of cDNA template, 1 µL (100 Pmol) of each forward and reverse primers of *MxaF, pMMO* and *16S rRNA* with 3 µL of free nuclease water to make a total volume of 20 µL.

The real time PCR program to *MxaF* and *pMMO* primer was optimized as follow:

An initial denaturation was achieved at 96 °C for 5 min., followed by 40 cycles of denaturation at 96 °C for 30 seconds, annealing at 56 °C or 55°C

(for *pMMO*) for 60 sec. and the extension at 72 °C for 30 sec. The reaction was submitted to melting curve analysis to ensure the primer specificity.

While the real time PCR program to *16S rRNA* primer was optimized as follow:

An initial denaturation was achieved at 94 °C for 3 min, followed by the second step of 45 cycles for each denaturation at 94 °C for 30 sec., annealing at 52 °C for 30 sec. and the extension at 72 °C for 40 sec. The reaction was submitted to melting curve analysis to ensure the primer specificity.

3.2.6. Growth in methane medium

One mL of liquid bacteria culture was inoculated into 50 mL of NMS medium broth in bottles (100 mL) provided with rubber stoppers were used to seal the bottles' tops. 20 mL of methane were injected into each bottle using a 0.22 µm filter syringe, giving about 18% methane in a head gas-phase. Additionally, control tubes without methane injection were created. The tubes were incubated at 30°C in the dark for 3–4 weeks while being checked every 3 days or 1 week. 0.1 mL of cultures that had grown on NMS broth were transferred to NMS agar and incubated for one to four weeks (Dianou and Adachi, 1999).

3.2.7. Monooxygenase activity assay

The protocol relied on turning naphthalene into 1-naphthol, which is then colorimetrically monitored by adding the aromatic diazo compound o-dianisidine to the reaction mixture. This technique was used to determine soluble monooxygenase activity among microorganisms (Graham *et al.*, 1992). Liquid culture were grown 100 mL glass bottle at 30°C in copper – free NSM medium with 20% methane, and agitated in rotary incubator at 240 rpm for 7 days. 50 µL of liquid culture was spread plate. Plates were incubated in a jar at 30 °C under a 25% methane- air for 7 days to allow development of colonies of different age and size.

Following incubation, naphthalene crystals, ranging from 300 to 400 mg, were spread on the lids of the plates, and the plates were incubated at 30 °C for 60 min. to enable naphthalene conversion to naphthol. Freshly prepared 5 $mgmL^{-1}$ o-ianisidine was added (20 µL) and directly applied to the bacteria biomass. The lid was replaced. It was then incubated at 30 °C for 15 min. to permit color development.

3.2.8. Growth in different concentration of methanol (1-10) %

On the MSM agar plates, a single colony of pure culture was cultured, and its growth until the log phase was monitored by measuring its optical density (OD) at 600 nm. Larger culture was prepared by mixing 1 mL of growth with 100 mL of sterile media. The media contained (1-10) % methanol to assess the ability to grow at different concentrations, and it was cultured for 10 days at 30°C in a shaking incubator and measurement of growth rate was daily monitored by spectrophotometer.

3.2.9. Growth in crude oil

Pure bacterial culture was inoculated at a ratio of 1:100 mL of sterilized MSM broth containing 1% crude oil as the sole carbon source. The growth rates of bacteria were assessed by OD_{600} and compared daily with flasks containing MSM medium supplemented with 1% crude oil without bacteria as a negative control (Godini *et al.*, 2018).

3.2.10. Gene Editing Experiment

3.2.10.1. Extraction of DNA from Plasmid

Plasmid DNA is purified from the plasmid-transformed bacteria according to the manufacturer instructions Promega, using the PureYield™ Plasmid Miniprep System kit (A1223) as follows:

1. The pellet was collected by transferring 1.5 mL of bacterial biomass 1.5 mL microcentrifuge tube and spun-down at 14000 rpm for 1 min. The supernatant was discarded.
2. The cell pellet was re-suspended in 600 µL of TE buffer. The cells were lysed with 100 µL of cell lysis buffer, and mixed by inverting the tube 6 times.
3. The cell lysate was mixed with 350 µL of cold (4–8°C) neutralization solution, and mix thoroughly by inverting the tube till a yellow precipitate appears. The mixture was spun down at 14000 rpm for 3 min.
4. The supernatant was transferred to a Pure Yield™ mini-column (~900 µL). The mini-column was placed in a pure Yield™ collection tube, and centrifuged for 15 sec. at 14000 rpm. The flow-through was discarded and then the column was replaced in the same collection tube.
5. The endotoxins were removed by adding 200 µL of endotoxin removal wash and centrifuged at 14000 rpm for 15 sec.
6. The mini-column were washed with 400 µL of column wash solution and spun down at 14000 rpm for 30 sec. The flow-through was discarded and the column was moved back to the collection tube.
7. The DNA was eluted with 30 µL of elution buffer and was left for 1 min. at room temperature. The column was centrifuges for 15 sec. at 14000 rpm. Eluted plasmid DNA was stored at –20 °C. The concentration of DNA plasmids was determined using a spectrophotometer Nano-drop device.

3.2.10.2. Antibiotic Sensitivity Assays

The sensitivity of the bacterial isolates to specific antibiotics (ampicillin, kanamycin, spiramycin) was determined using disc diffusion assays, as follows:

A stock solution is prepared (100 $mgmL^{-1}$), 1 g of antibiotic powder was dissolved in 10 mL of sterile distilled water. The temperature of the solution was maintained at 4 °C.

1. A bacterial suspension of 100 µL was disseminated on agar plates containing MSM supplemented with 1% methanol or LB agar.
2. Sterile filter paper discs with a diameter of 0.25 inches were put into the petri dish. Then, 20 µL of an antibiotic solution was put into the center of the disc and left for about 30 min. to dry.
3. Filter paper discs containing antibiotics are placed on the bacterial lawn.
4. The plates were incubated for 3- 5 days at 30°C.
5. The diameter of the zone surrounding the disc where no growth can be seen was used to measure antibiotic sensitivity or resistance.

The antibiotics' minimal inhibitory concentrations (MICs) were measured for *M. extorquens* and *P. balearica* by plating 100 µL of bacterial culture on solid MSM supplemented with 1% methanol and LB agar respectively, using different concentrations of antibiotics and incubated the plates for 3–5 days at 30 °C. The appearance of colonies indicated resistance to the antibiotic concentration, while the absence of colonies showed sensitivity. In addition to plate experiments, antibiotic sensitivity was also measured in liquid growth media.

3.2.10.3. Preparation of electrocompetent (*M. extorquens*)

Primary culture was prepared and 5mL of *M. extorquens* of the growth was seeded at 30°C with shaking for 3 days. 50 mL of MSM supplemented with 1% methanol in 250 mL flasks was inoculated with 500 µL of the 3days culture. The fresh culture was incubated for 24-30 h. at 30°C with shaking at 250 rpm, the OD_{600} reached to 0.6-0.65. The cultures were incubated on ice

for 30 min, and then poured into 50 mL Falcon tubes, and then centrifuged at 5000 rpm for 10 min at 4°C. The pellet was re-suspended with 50 mL of 10% ($^{v}/_{v}$) ice-cold glycerol and washed 3 times for 10 min each. After the last washing step, the bacterial pellet was re-suspended with 1000 µL of 10% ($^{v}/_{v}$) ice-cold glycerol. Aliquots (100 µL) of the cell suspensions were dispensed and flash-frozen and stored in liquid nitrogen.

3.2.10.4. Preparation of electrocompetent (*P. balearica*)

Primary cultures of 5 mL of *P. balearica* were grown at 37 °C. 50 mL of nutrient broth in 250 mL flasks and inoculated with 500 µL of overnight culture. The fresh culture was incubated for 4-5 h at 37°C with shaking at 250 rpm, the OD_{600} reached to 0.55-0.6. The cultures were incubated on ice for 30 min, and then moved to 50 mL Falcon tubes, and then centrifuged at 5000 rpm for 10 min at 4°C. The pellet was re-suspended with 50 mL of 10% ($^{v}/_{v}$) ice-cold glycerol and washed 3 times for 10 min each. After the last washing step, the bacterial pellet was re-suspended with 1000 µL of 10% ($^{v}/_{v}$) ice-cold glycerol. Aliquots (100 µL) of the cell suspensions were dispensed and flash-frozen and stored in liquid nitrogen.

3.2.10.5. Electroporation of *M. extorquens*

The Bio-Rad Gene Pulser II is utilized in order to carry out the electroporation procedure.

1. Using electrocompetent cells, 500 ngμL^{-1} of plasmid DNA is added to 100 µL of thawed cells on ice, and then transferred to a sterile, ice-cold Bio-Rad cuvette with a 0.1cm gap.
2. Then the cuvette was inserted in the pulsing chamber and subjected to 1.8 kV using the electroporator. When the pulsing was finished, 1 mL

of MSM broth containing 1% methanol (without antibiotics) was added to the cells.

3. Bacterial suspensions were agitated and incubated at the 30 °C for 6 to 8 h. Next, the bacteria were centrifuged at 4000 rpm for 1 min. 900 µL of the supernatant were discarded.
4. The cells were pelleted and re-suspended in the remaining 100 µL of media before being disseminated on agar plates with MSM supplemented with 1% methanol and the necessary antibiotic.
5. The plates were kept in an incubator for 5–7 days at 30 °C which was determined to be the ideal temperature for the bacterial strain.

3.2.10.6. Electroporation of *P. balearica*

The electroporation method was utilized in order to deliver each plasmid into electrocompetent cells.

- After thawing electrocompetent cells on ice, 50 µL of cells were mixed with 500 ngµL^{-1} of plasmid DNA. After that, the mixture was transferred to a pre-cooled electroporation cuvette on ice.

- Placing the cuvette within the pulsing chamber and applying a pulse at a fixed voltage of 1.8 kV using the electroporator. The sample was then re-suspended in 1 mL of LB broth (without antibiotics).

- The modified strains were incubated at 37 °C with agitation for 1 h. After incubation, the cells were then collected for 1 min. at 4000 rpm in a centrifuge

- The supernatant was discarded, and the pellet was re-suspended in 100 µL of broth medium and spread on nutrient agar plates containing the antibiotics and incubated at 37 °C for 24 h.

3.2.10.7. Detection of plasmid DNA by agarose gel electrophoresis

The plasmid DNA was loaded and run as described in section 3.2.5.2. in 0.5% agarose gel. The first well of the gel was loaded with 5 µL of 1k bp DNA ladder. DNA samples were mixed with bromophenol blue at a ratio of 20µl: 10µl and were carefully loaded into wells by disposable micropipette fine tip. The samples were electrophoresed at 80 volts. The run continued for 1 h., the bands of plasmid DNA were detected and photographed under UV light.

3.2.10.8. Plasmids used for Gene Editing

PHSPO2 with size 14.102 bp resistances to kanamycin antibiotic contain Cas9 and gRNA ordered from Add gene (Fig.3-3).

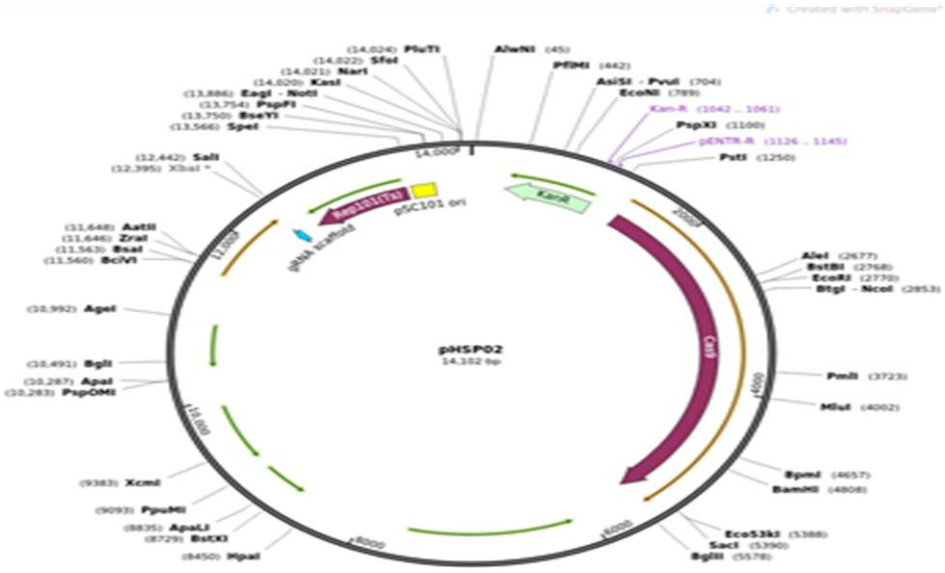

Figure 3-3: Plasmid map of pET22.

pCas9D10A

pCas9D10A with size 9270 bp resistance to spiramycin antibiotic contain Cas9 was ordered from Add gene (Fig 3-4).

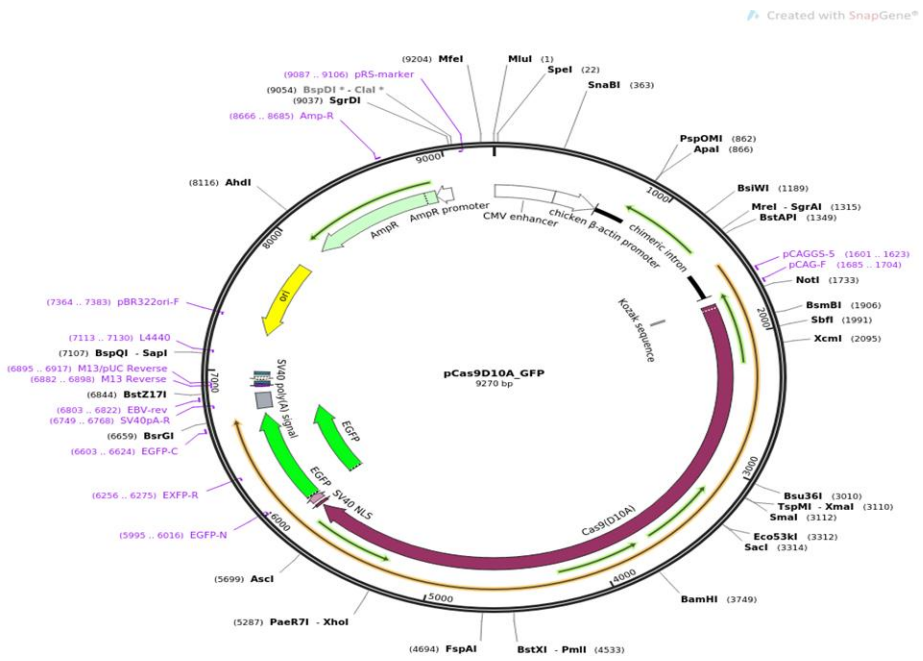

Figure 3-4: Plasmid map of Cas9.

pgRNA plasmid

PHSPO2 with size 2584 bp resistance to kanamycin antibiotic contain gRNA was ordered from Add gene (Fig. 3-5).

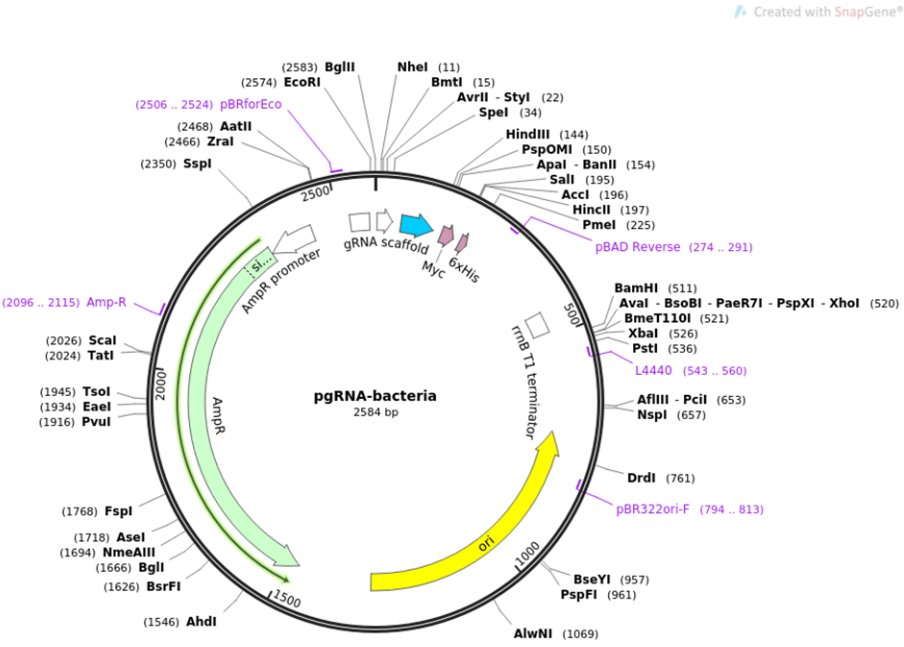

Figure 3-5: Plasmid map of pgRNA.

3.2.11. Biodegradation of crude oil by the bacteria

The bacteria were incubated at 30 °C in a shaker incubator at 120 rpm when they reached the log phase by measuring the optical density (OD_{600}). 1 mL of bacteria was inoculated in the conical flask size 250 mL containing 100 mL of MSM broth with crude oil (Al-Shua'aba Refinery- Basra city) at a concentration of 0.5% ($^{v}/_{v}$). Three set-ups were used in the final experiment:

- Cured bacteria with CRISPR-Cas9.
- Uncured bacteria.
- MSM medium with crude oil without bacteria as a control.

The residual crude oil was extracted and separated into aliphatic and aromatic fractions after 7 days of incubation. A liquid-liquid extraction technique was used to extract the leftover crude oil using a separating funnel. Add 50 mL of carbon tetrachloride (CCl_4) solvent to the bacterial culture. The funnel was forcefully agitated, and then the contents were allowed to settle to separate the stages. The aqueous phase was discarded, and the residual oil was dried at 40 °C to remove CCl_4 (Adebusoye *et al.*, 2007).

The aliphatic fraction was separated, and the residual oil was diluted in 25 mL of n-hexane. The aromatic fraction was extracted, and the remaining crude oil was dissolved in 25 mL of benzene. The aromatic and aliphatic fractions were analyzed by gas chromatography (GC). The proportion of biodegradation was estimated using GC charts (Alkanany *et al.*, 2017; Al-Taee *et al.*, 2017) .

Chapter Four

Results

4. Results

4.1. Isolation and identification of bioremediating bacteria

In order to isolate the methanol utilizer's bacteria, a modified basal salt medium, supplemented with 2% methanol as the only source of organic carbon is inoculated with 0.5 g of polluted soil. The bacterial growth had a pink color. A loopful of the broth is spread onto methanol salt agar plates. After 3-5 days of incubation at 30 °C, tiny spherical colonies with pink color were appeared. These bacteria are gram-negative, bacilli shape, showing singly, in pairs or in mass, non-spore-forming, catalase and oxidase positive (Fig. 4-1).

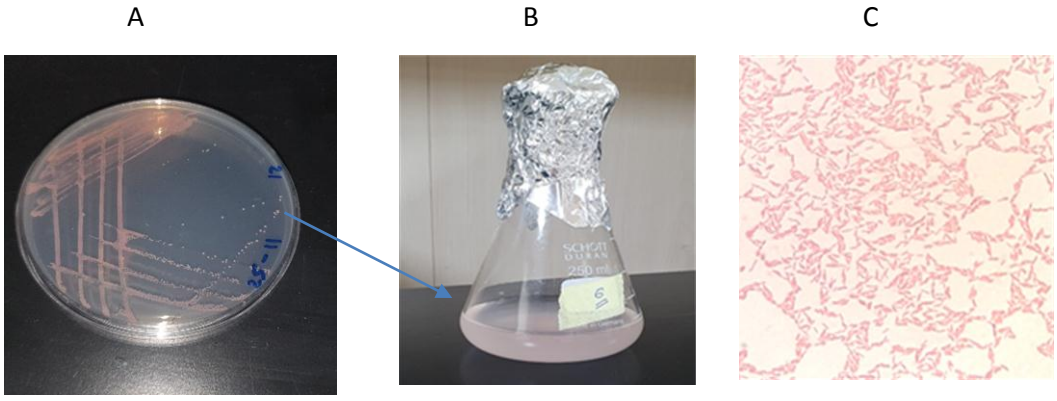

Figure 4-1: A/ *M. extorquens* in MSM agar; B/ *M. extorquens* in MSM broth; C/ cells of *M. extorquens* under microscope x 100.

Furthermore, some of these bacteria are white, medium in size, gram negative, rod-shaped, non-fluorescent, and positive for catalase and oxidase.

4.2. Morphological and biochemical characteristics

The results of the essential morphological and biochemical of 15 isolates are shown in Table 4-1.

Table 4-1: morphological and biochemical tests.

Numbers of bacteria	Gram's stain	Cell shape	colony size	pigment	catalase	oxidase
1	-ve	bacilli	medium	white	+	+
2	-ve	bacilli	medium	white	+	+
3	-ve	bacilli	medium	white	+	+

4	-ve	bacilli	medium	white	+	+
5	-ve	bacilli	small	pink	+	+
6	-ve	bacilli	small	pink	+	+
7	-ve	bacilli	small	pink	+	+
8	-ve	bacilli	small	pink	+	+
9	-ve	bacilli	small	yellow	+	+
10	-ve	bacilli	small	yellow	+	+
11	-ve	bacilli	small	white	+	-
12	-ve	bacilli	small	white	+	-
13	-ve	bacilli	small	white	+	-
14	-ve	bacilli	medium	white	+	-
15	-ve	bacilli	medium	yellow	+	+

4.3. Molecular Techniques

4.3.1. Detection of genomic DNA

Pure bacterial cultures were used to obtain total DNA, which will be used later in the molecular detection of methylotrophic bacteria (Fig. 4-2). The purity and the concentration were determined using an agarose gel electrophoresis and nano drop (Appendix1).

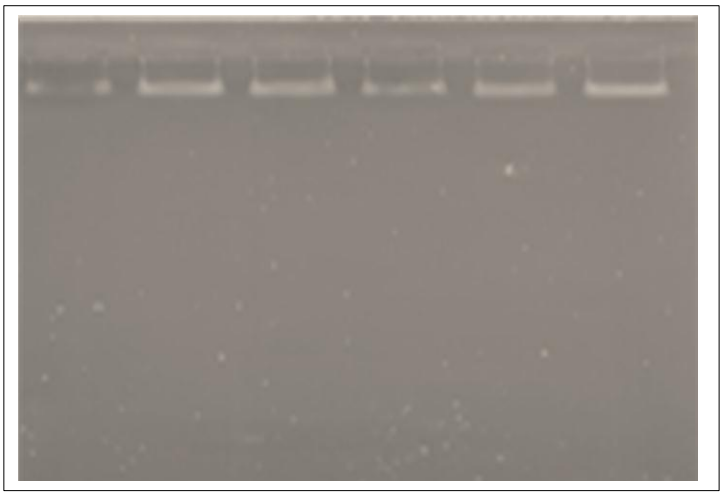

Figure 4-2: Genomic DNA bands after 20 min. of electrophoresis on an agarose gel.

4.3.2. Amplification of the *16S rDNA* gene in bacterial isolates

An agarose gel electrophoresis revealed that each of the 15 bacterial isolates had a *16S rDNA* gene that manifested itself as a single band. Its

position is around 1500bp away, as measured against a typical DNA ladder (Fig. 4-3).

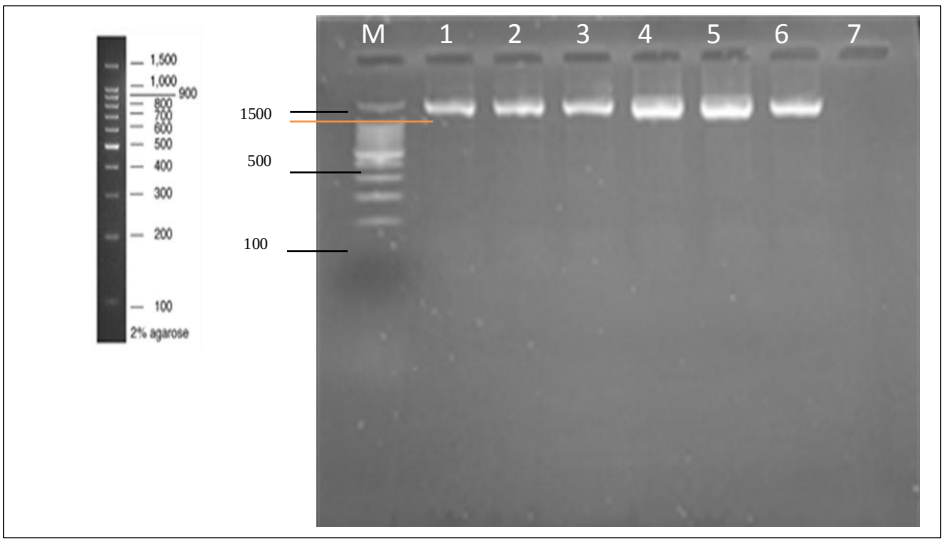

Figure 4-3: Amplification of the *16S rDNA* gene (~1500bp) on an agarose gel electrophoresis. Lane M: 100bp Marker, Lane 1-6: *16S rDNA* gene bands for isolates No. 1 to 6, Lane 7: Negative control.

4.3.3. Detection of the *sMMO* gene by cPCR

The amplification of *Pseudomonas balearica sMMO* gene by gene-specific primers resulted in a 369bp band in an agarose gel, which representing the targeted area of the *sMMO* gene from the *Pseudomonas* genome (Fig. 4-4).

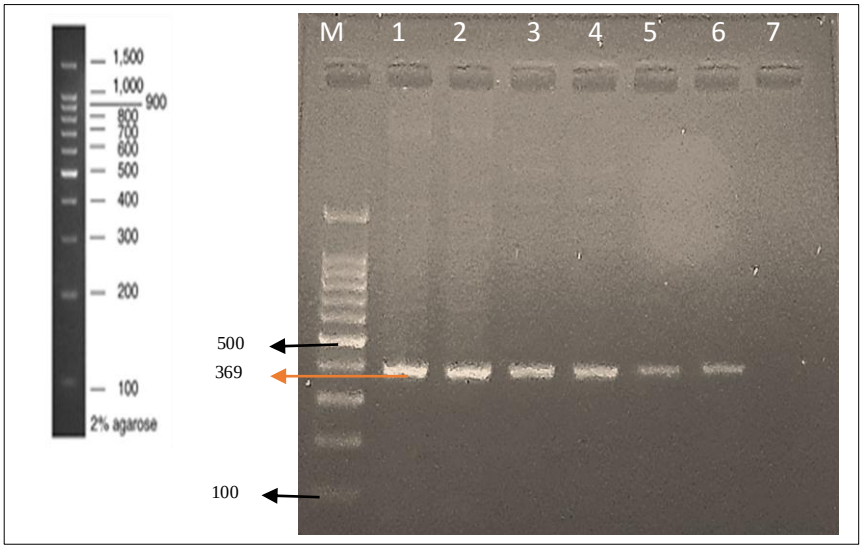

Figure 4-4: Amplification of the *sMMO* gene (369bp) on an agarose gel electrophoresis. Lane M: 100bp Marker, Lane 1-6: *sMMO* gene bands for isolates No. 1 to 6 are *Pseudomonas balearica*, Lane 7: Negative control.

4.3.4. Identification of bacterial species using the *16S rDNA* and *sMMO* gene sequence

Fifteen isolates as methylotrophic bacteria are selected and sent for *16S rDNA* sequences. The percentage of matching to NCBI data base was greater than 99% as *Methylorubrum extorquens* and *Pseudomonas balearica* (Appendix 2). Also, *sMMO* PCR products were delivered to Macrogen Co., South Korea, for sequencing. The results of the sequence analysis were then used to search the NCBI database for sequences with similar characteristics using the Basic Local Alignment Search Tool (BLAST). (Appendix 3)

4.3.5. Detection of the *MxaF* gene by cPCR

Amplification of the methylotrophic bacteria *MxaF* gene was performed using a *MxaF* gene-specific primer pair, and the resulting 550-bp band from the *MxaF* gene of *Methylorubrum* genomic DNA was observed on a 2% agarose gel (Fig. 4-5).

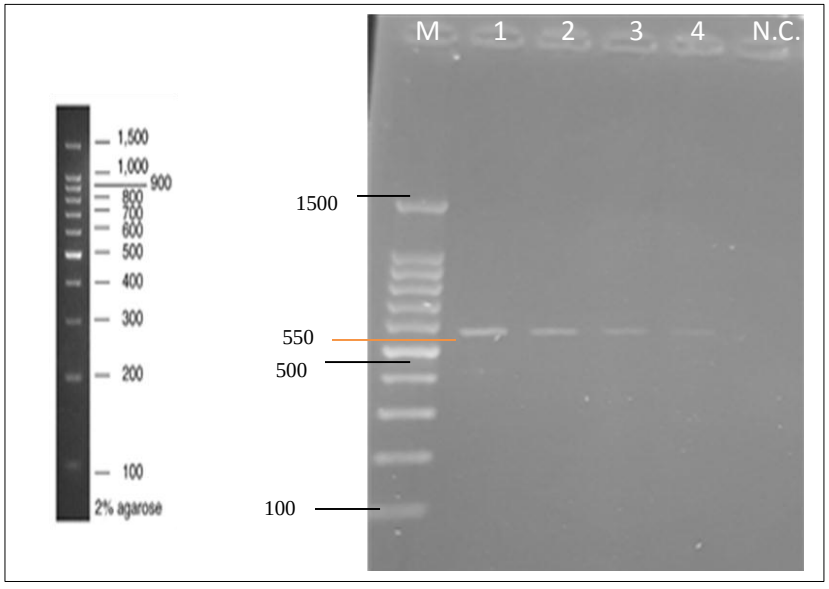

Figure 4-5: The amplified *MxaF* gene (550bp) on an agarose gel electrophoresis Lane L is a 100-bp marker, while Lanes 1-4 are *Methylorubrum* sp, *MxaF* gene bands.

4.4. Quantitative PCR (qPCR) detection

4.4.1. Detection *pMMO* gene

The *pMMO* gene was detected using the isolated RNA as a template for the reverse transcription of the separated RNA into cDNA. This process allowed for the gene's detection. In order to initiate the reaction, the cDNA, a primer that is specific for *pMMO* and SYBR green dye were mixed together (Fig. 4-6 and 4-7).

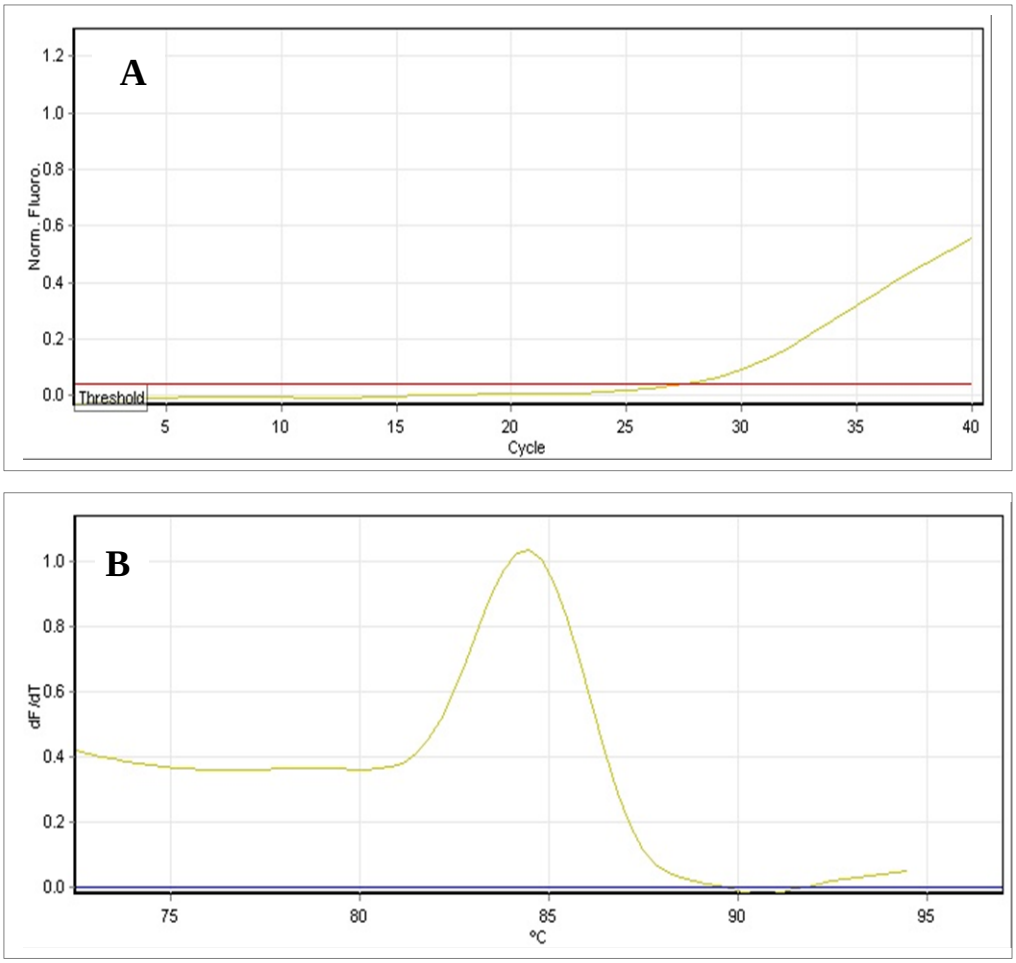

Figure 4-6: A/Amplification curve analysis of *pMMO* gene expression for 1 replicating of *M. extorquens*, the signal was detected at the CTs range. B/ Melting curve analysis of the *pMMO* gene expression revealed the degree of plot specificity in one peak at (84 ℃).

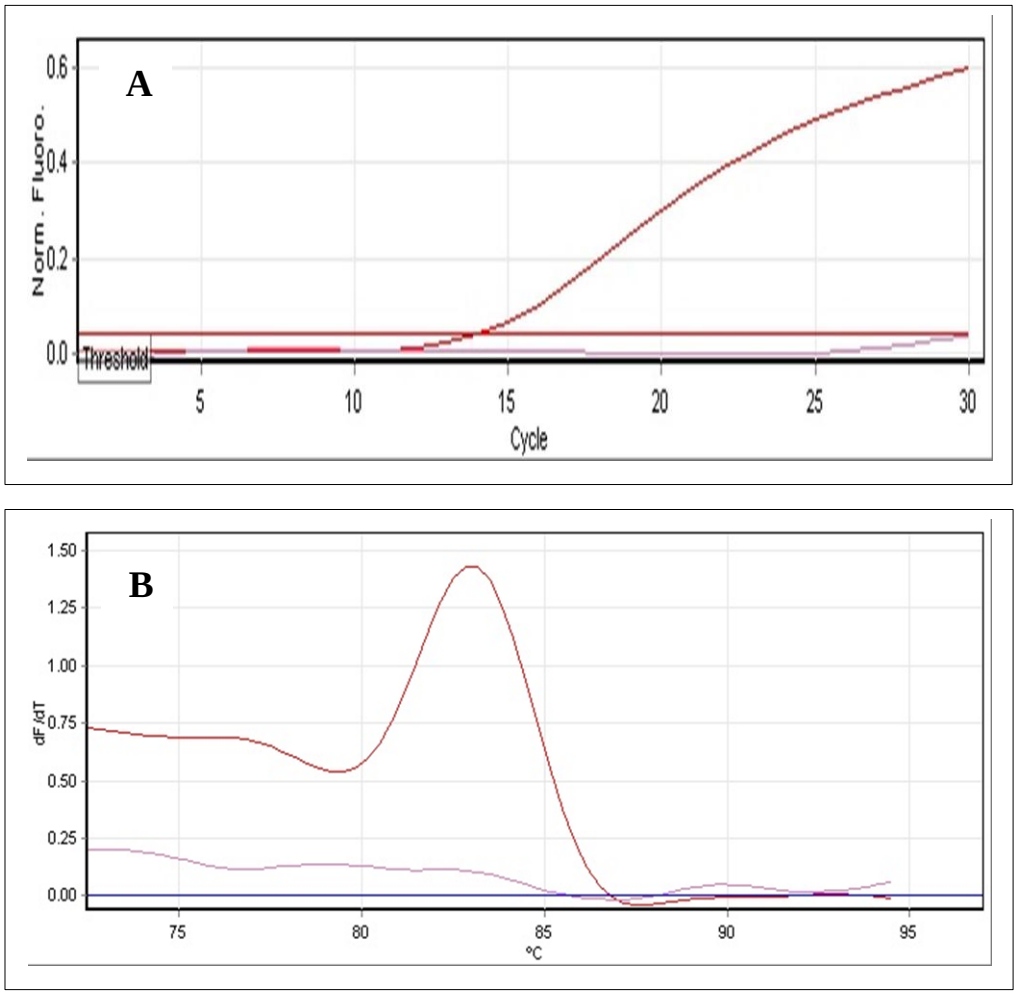

Figure 4-7: A/Amplification curve analysis of *pMMO* gene expression for 1 replicating of *P. balearica*, the signal was detected at the CTs range. B/ Melting curve analysis of the *pMMO* gene expression revealed the degree of plot specificity in one peak at (83°C).

4.4.2. Detection *MxaF* gene

In order to evaluate the level of *MxaF* gene expression, the isolated RNA was subjected to reverse transcription to produce cDNA, and the resulting cDNA sequences were used as templates. The cDNA, a *MxaF* specific primer, and SYBR green dye were mixed together to create the reaction, which was then used to further applications and confirming conventional PCR findings (Fig. 4-8 and 4-9).

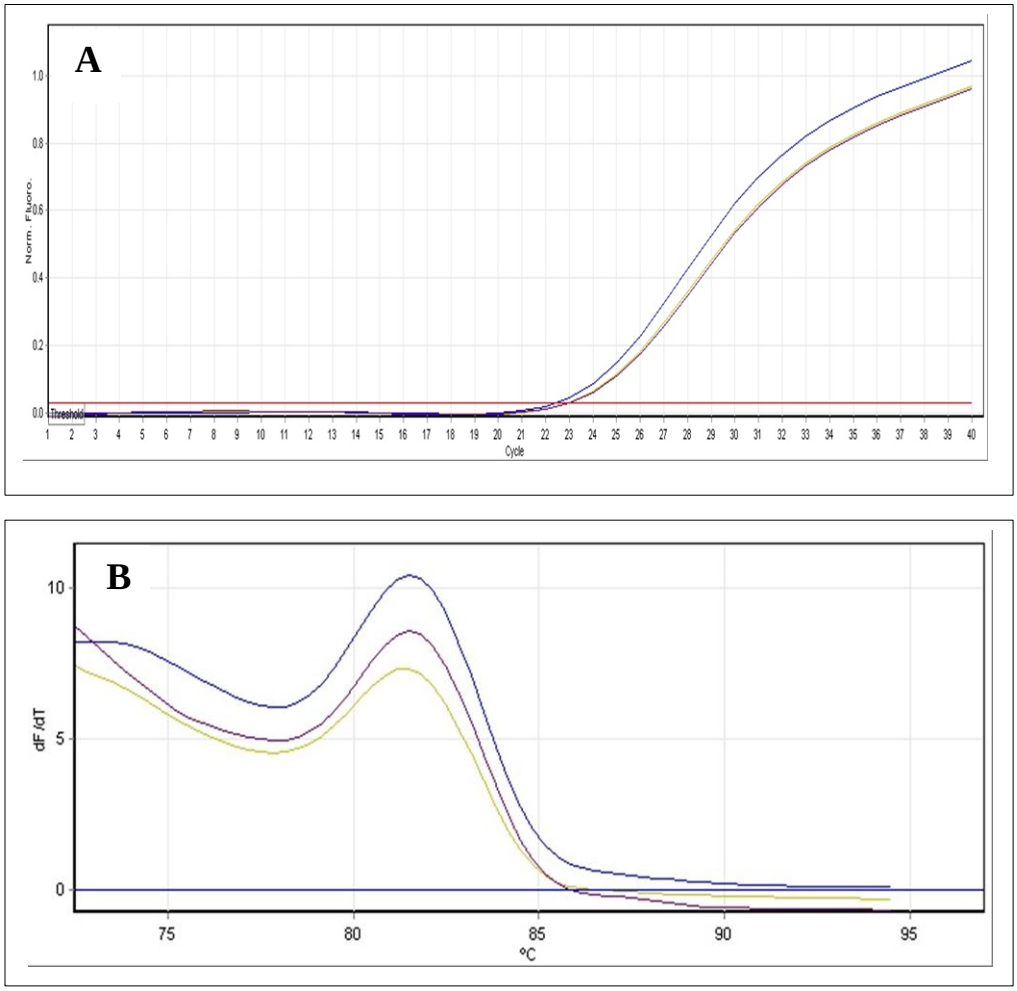

Figure 4-8: A/Amplification curve analysis of *MxaF* gene expression for 3 replicating of *M. extorquens*, the signal was detected at the CTs range. B/ Melting curve analysis of the *MxaF* gene expression revealed the degree of plot specificity in one peak at (82 ℃).

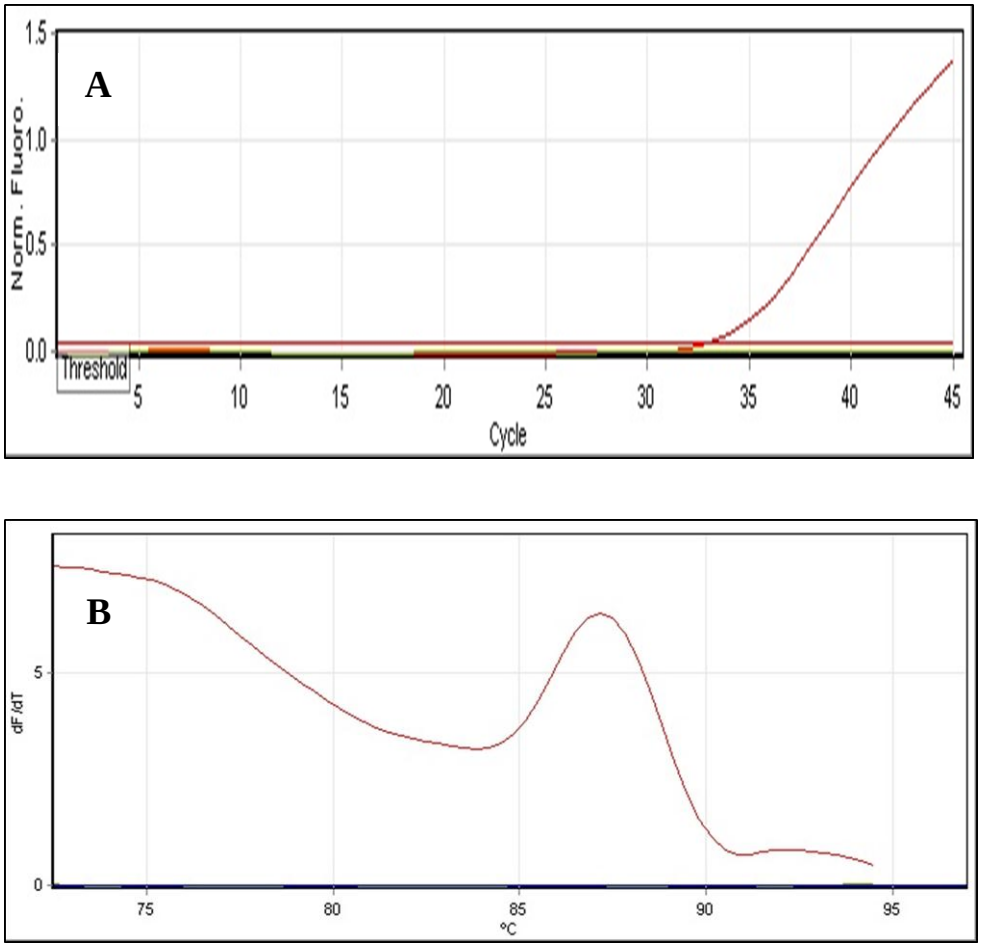

Figure 4-9: A/Amplification curve analysis of *MxaF* gene expression for 1 replicating of *P. balearica*, the signal was detected at the CTs range. B/ Melting curve analysis of the *MxaF* gene expression revealed the degree of plot specificity in one peak at (87 ℃).

4.4.3. Detection housekeeping gene *16S rRNA*

The gene expression of *16S rRNA* was analyzed by employing quantitative PCR, SYBR green dye, and specific primer mixtures for the housekeeping gene *16s rRNA* (Fig. 4-10 and 4-11).

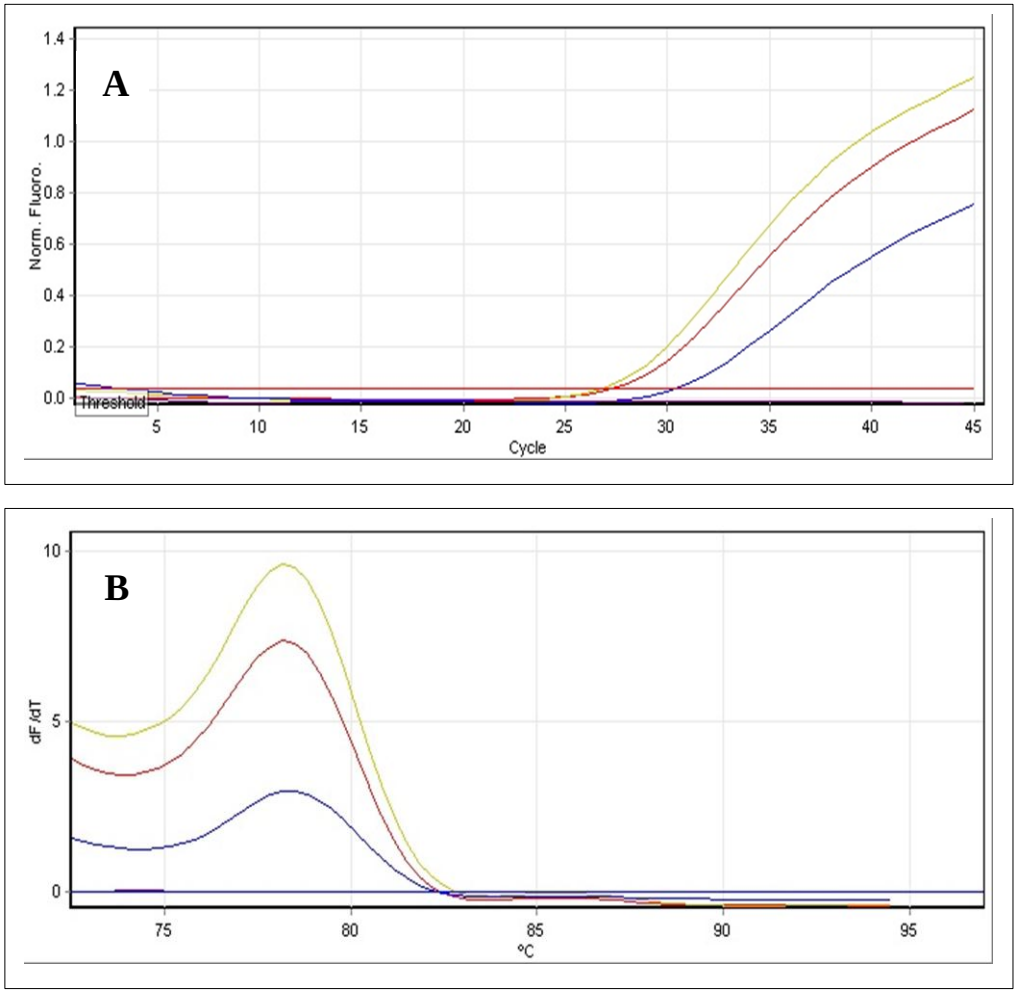

Figure 4-10: A/Amplification curve analysis of *16S rRNA* gene expression for 3 replicating of *M. extorquens*, the signal was detected at the CTs range. B/Melting curve analysis of *16S rRNA* gene expression, the degree of plot specificity was displayed in one peak at (78℃).

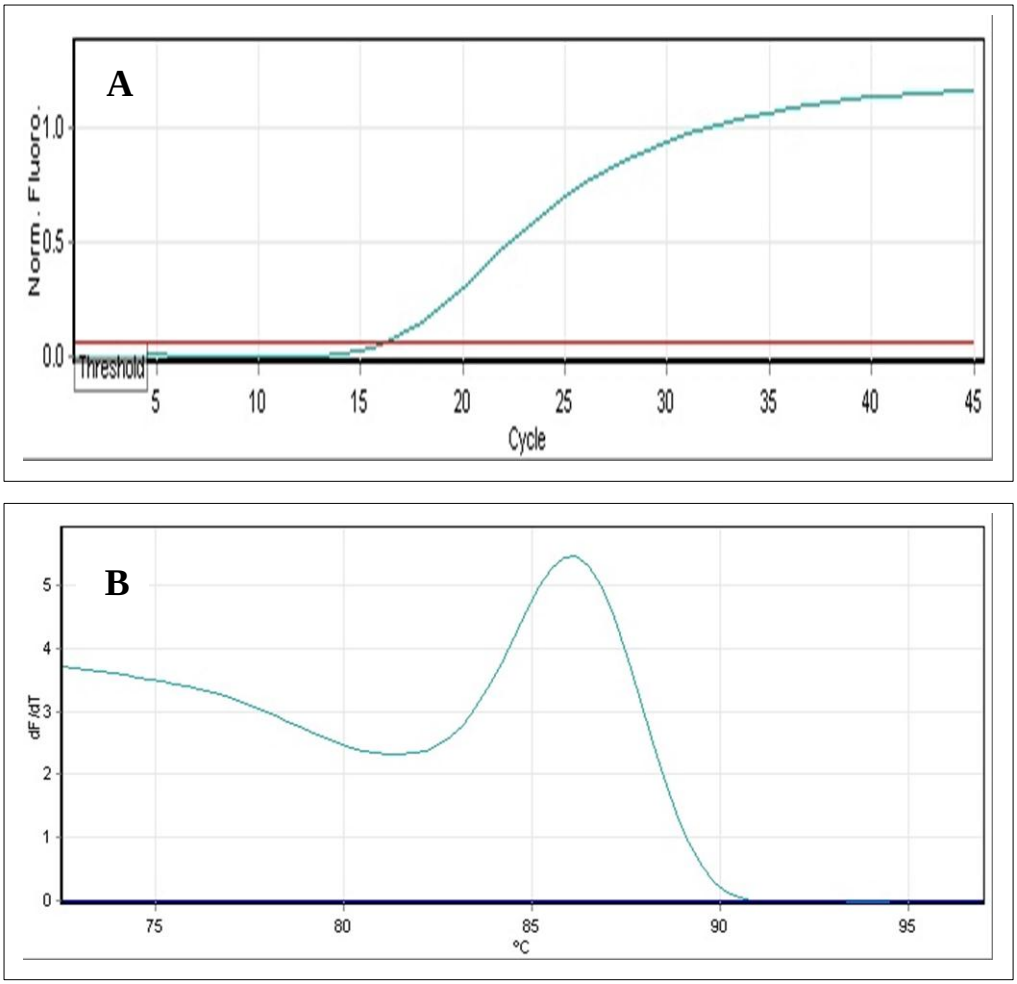

Figure 4-11: A/Amplification curve analysis of *16S rRNA* gene expression for 1 replicating of *P. balearica*, the signal was detected at the CTs range. B/Melting curve analysis of *16S rRNA* gene expression, the degree of plot specificity was displayed in one peak at (87°C).

4.5. Gene Expression analysis of MDH in *M. extorquens*

Analysis of MDH gene expression in *M. extorquens*, it's compared with other species isolated in the same study (control). For such purposes, Real Time PCR using SYBR green dye and *MxaF* gene-specific primers was employed. MDH expression was upregulated to 44-fold compared to the control sample, which was normalized to 1. ΔΔ CT analysis was applied to conclude the MDH gene expression. The *16S rRNA* value was subtracted as the housekeeping gene (Fig. 4-12 and 4-13).

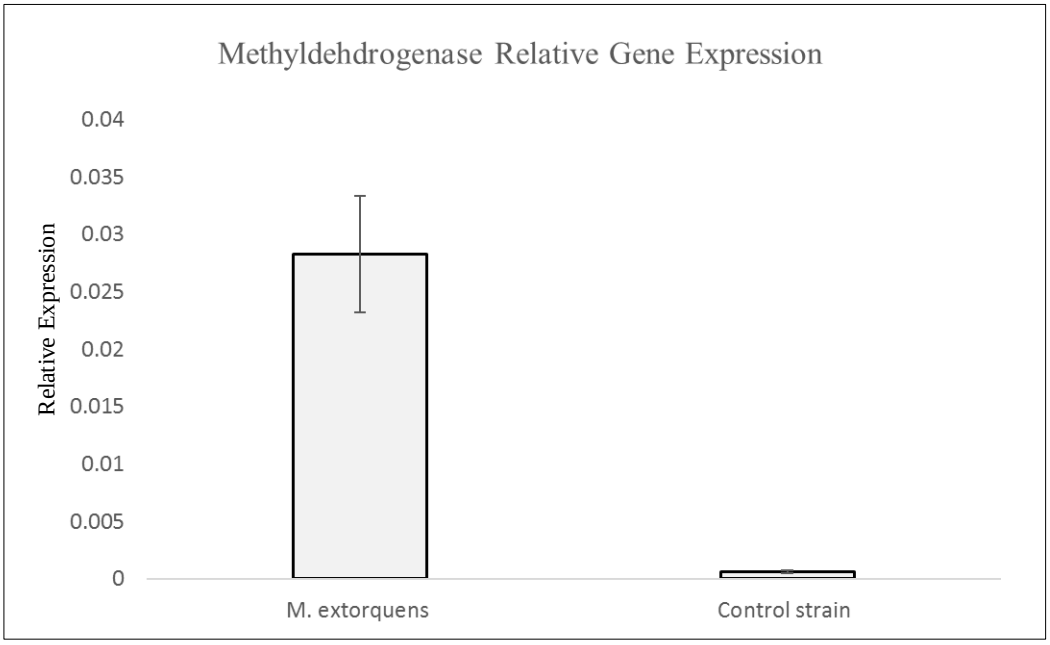

Figure 4-12: Relative Methyl Dehydrogenase gene expression in *M. extorquens* and control strain (*P. balearica*). Total RNA was extracted from fresh cultures of both species, reverse transcription of the synthesized DNA was performed, and the synthesized DNA was employed as a template in a qPCR relative expression experiment using SYBR green master mix. Analyses were performed using ΔΔ CTs, and results were normalized using *16S rRNA* as the housekeeping gene.

Figure 4-13: Methyl Dehydrogenase fold change analysis in *M. extorquens* and control strain (*P. balearica*). Total RNA was extracted from fresh cultures of both species, reverse transcription of the synthesized DNA was performed, and the synthesized DNA was employed as a template in a qPCR relative expression experiment using SYBR green master mix. Analyses were performed using ΔΔ CTs, and results were normalized using *16S rRNA* as houses keeping gene and control sample was normalized to 1.

4.6. Gene Expression analysis of *pMMO* in *P. balearica*

The expression of the *pMMO* gene was examined in *P. balearica* and compared to that of other species isolated during the same experiment (control). SYBR green dye and *pMMO* gene-specific primers were used in Real Time PCR for this purpose. In comparison to the control sample, this was normalized to 1. ΔΔ CT analysis was applied to determine the *pMMO* gene expression; the expression of *pMMO* was increased 10-fold. The *16S rRNA* value was subtracted as the housekeeping gene (Fig. 4-14 and 4-15).

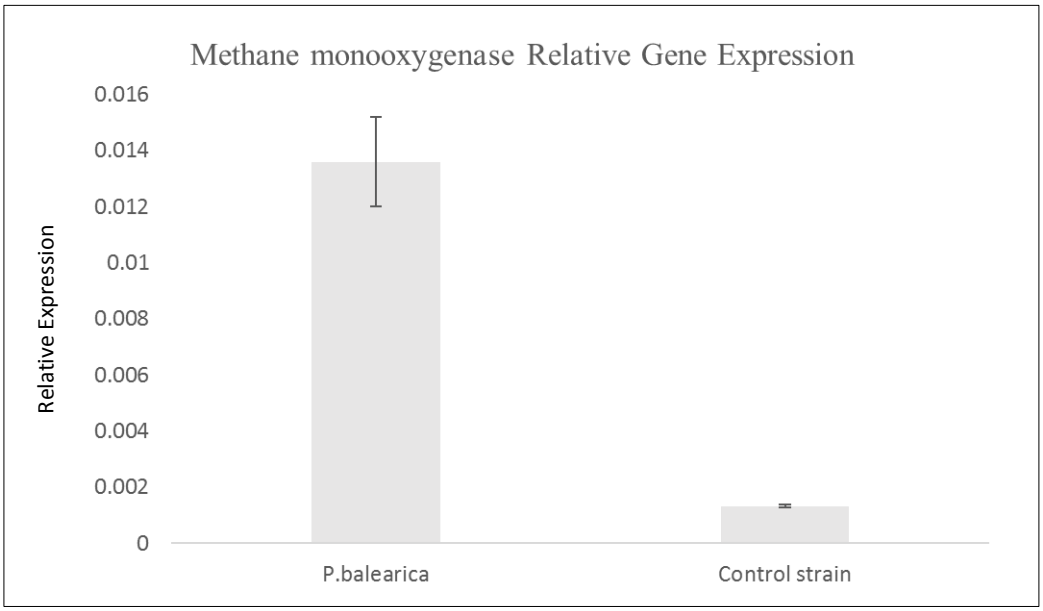

Figure 4-14: Relative Methane monooxygenase gene expression in *P. balearica* and control strain (*M. extorquens*). Total RNA was extracted from fresh cultures of both species, reverse transcription of the synthesized DNA was performed, and the synthesized DNA was employed as a template in a qPCR relative expression experiment using SYBR green master mix. Analyses were performed using ΔΔ CTs, and results were normalized using *16S rRNA* as the housekeeping gene.

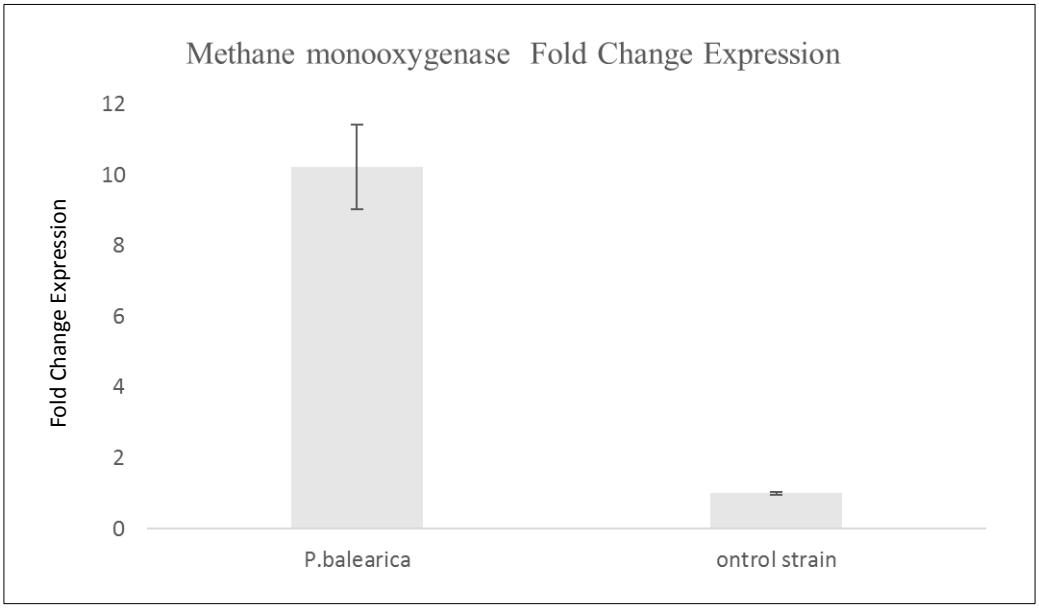

Figure 4-15: Methane monooxygenase fold change analysis in *P. balearica* and control strain (*M. extorquens*). Total RNA was extracted from fresh cultures of both species, reverse transcription of the synthesized DNA was performed, and the synthesized DNA was employed as a template in a qPCR relative expression experiment using SYBR green master mix. Analyses were performed using ΔΔ CTs, and results were normalized using *16S rRNA* as houses keeping gene and control sample was normalized to 1.

4.7. Growth in methane

Bacterial isolates were cultured in NMS broth for up to 30 days, in which *P. balearica* flourished. Meanwhile NMS broth was ineffective as a growth medium for *M. extorquens*. However, when transferred to NMS agar, a small number of colonies began to form.

4.8. Methane Monooxygenase assay

Following the addition of naphthalene crystals and o-dianisidine to the identified species, *M. extorquens* gave a negative result. While that *P. balearica* give a positive reaction, and the color changed from white to purple (Fig. 4-16).

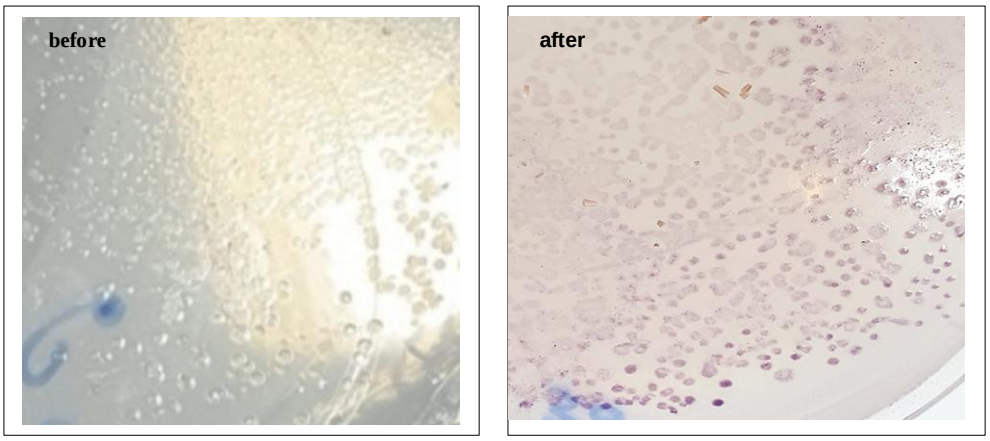

Figure 4-16: Use of the naphthalene-o-dianisidine assay to detect *sMMO*-bearing colonies of *P. balearica* cultivated in a copper-free solid medium on NSM.

4.9. Growth at different concentration of methanol (1-10) %

Methylotrophic bacteria are able to get necessary nutrients, energy and carbon, from methanol. *M. extorquens* and *P. balearica* have the ability to grow in methanol at varying concentrations for several days. *M. extorquens* grew at its greatest at 1% and 2% concentrations, when the optical density reached 1.9 and 1.8, respectively, while *P. balearica* thrived at 1% and 3% with an optical density of 0.1. Both genera failed to grow at concentrations above 6% (Fig. 4-17 and 4-18).

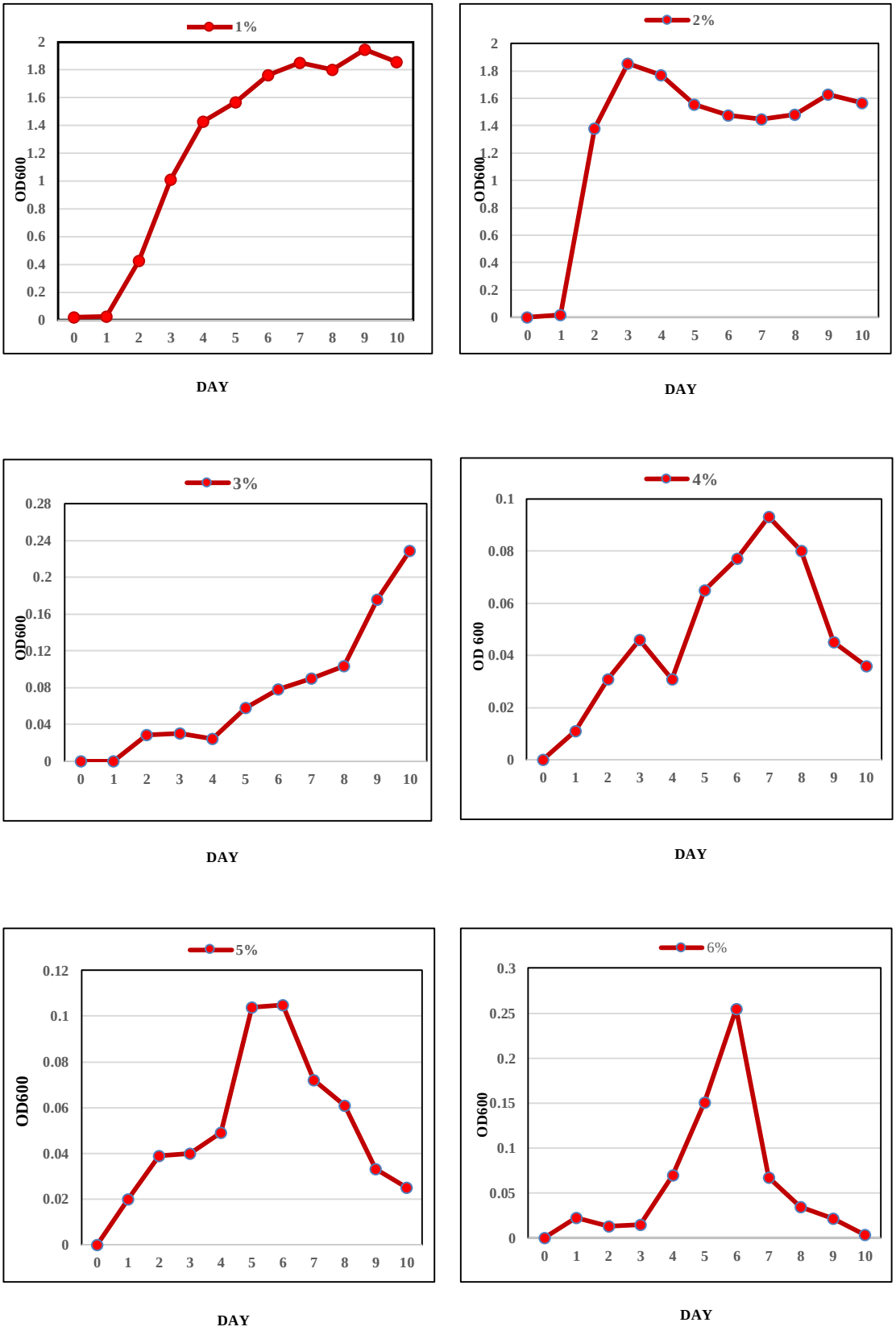

Figure 4-17: Growth of *M. extorquens* on methanol salt medium supplemented with 1–6% of methanol after measuring by spectrophotometer at an optical density of 600 nm during 1–10 days of incubation at 30 ℃.

Figure 4-18: Growth of *P. balearica* on methanol salt medium supplemented with 1–6% of methanol after measuring by spectrophotometer at an optical density of 600 nm during 1–10 days of incubation at 30 ℃.

4.10. Gene Expression analysis of MDH in *M. extorquens* at different concentrations of Methanol

In order to evaluate the MDH gene expression in *M. extorquens* at different concentrations of methanol, RNA templates were converted to cDNA and used as templates for gene expression analysis. Quantitative PCR using SYBR green and *MxaF* gene specific primers was employed to assess the relative and fold change expression. MDH expression roughly expressed at the same level from 1 to 4%. At 6% of methanol, the MDH expression declined to its lowest level 1 compared to 12 at 1% methanol. ΔΔ CT analysis was applied to conclude the MDH gene expression. *16S RNA* value was subtracted as a housekeeping gene (Fig. 4-19 and 4-20).

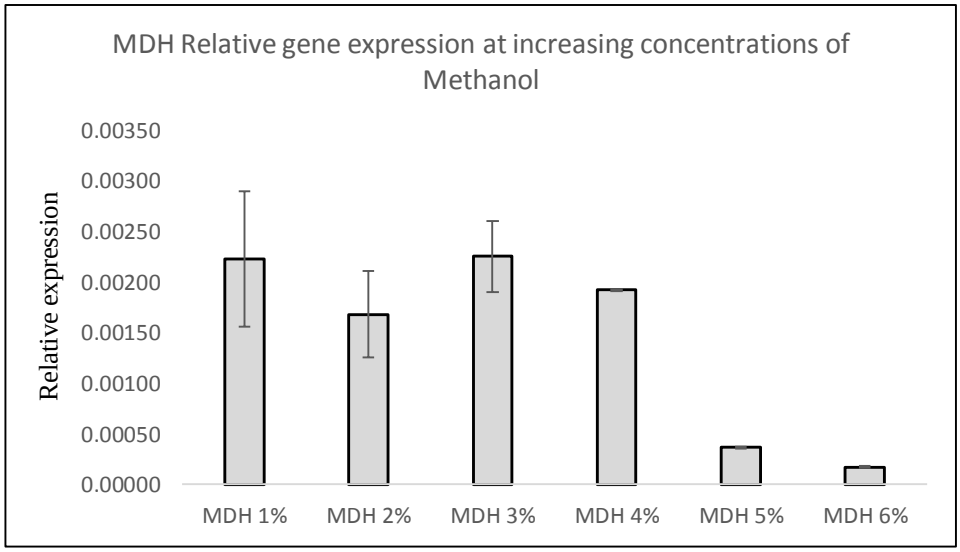

Figure 4-19: Methyl Dehydrogenase relative gene expression analysis in *M. extorquens* at different concentrations of methanol. Total RNA was extracted from fresh cultures supplemented with 1% to 6% concentration of methanol, reverse transcribed and the synthesized DNA was used as a template for qPCR relative expression assay using SYBR green master mix. Data were analyzed by ΔΔ CTs and normalized to *16S rRNA* as houses keeping gene and control sample was normalized to 1.

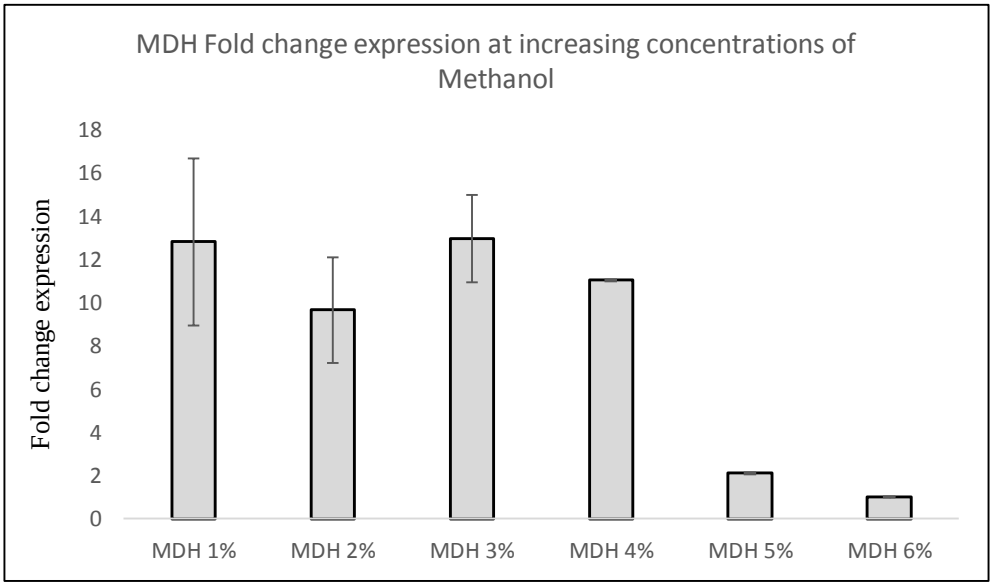

Figure 4-20: Methyl Dehydrogenase fold change analysis in *M. extorquens* at different concentrations of methanol. Total RNA was extracted from fresh cultures supplemented with 1% to 6% concentration of methanol, reverse transcribed and the synthesized DNA was used as a template for qPCR relative expression assay using SYBR green master mix. Data were analyzed by ΔΔ CTs and normalized to *16S rRNA* as houses keeping gene and control sample was normalized to 1.

4.11. Growth in crude oil

The use of crude oil as the only source of carbon and energy for seven days resulted in observable increases in cell density as well as concomitant decreases in a number of the component elements of crude oil. In order to evaluate the bacteria's ability to break down hydrocarbon molecules, 1% of crude oil was added to MSM medium (Fig. 4-21).

After 7 days the optical density of culture medium of *M. extorquens* was 0.1, meanwhile was 0.17 for *P. balearica* (Fig. 4-22 and 4-23).

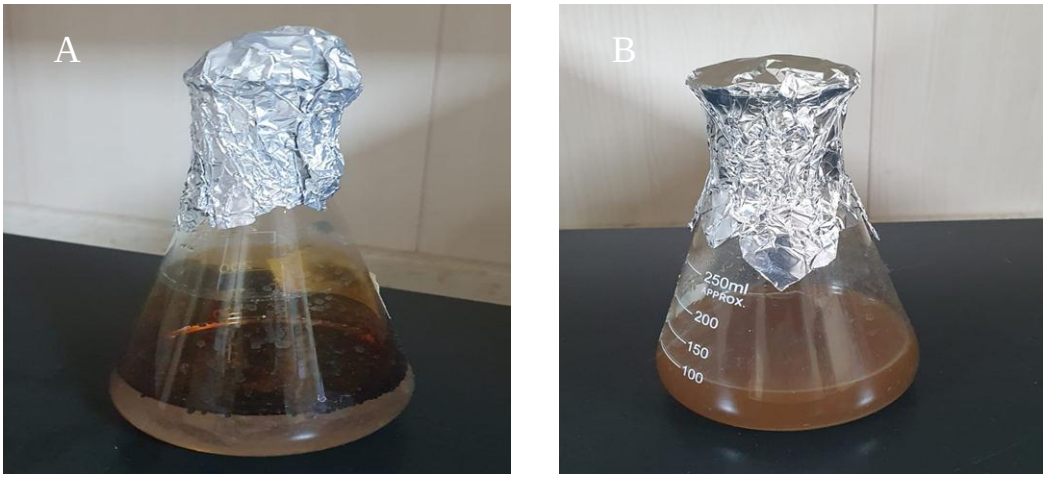

Figure 4-21: A/ control has crude oil. B/ bacteria with 1% crude oil after 7 days' incubation.

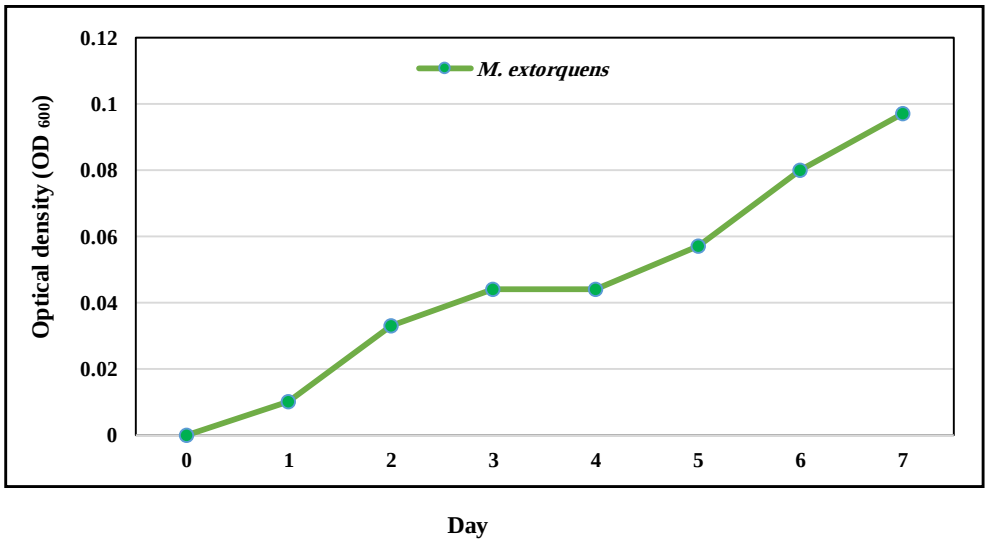

Figure 4- 22: The optical density of *M. extorquens* growth with 1% crude oil after 7 days of incubation.

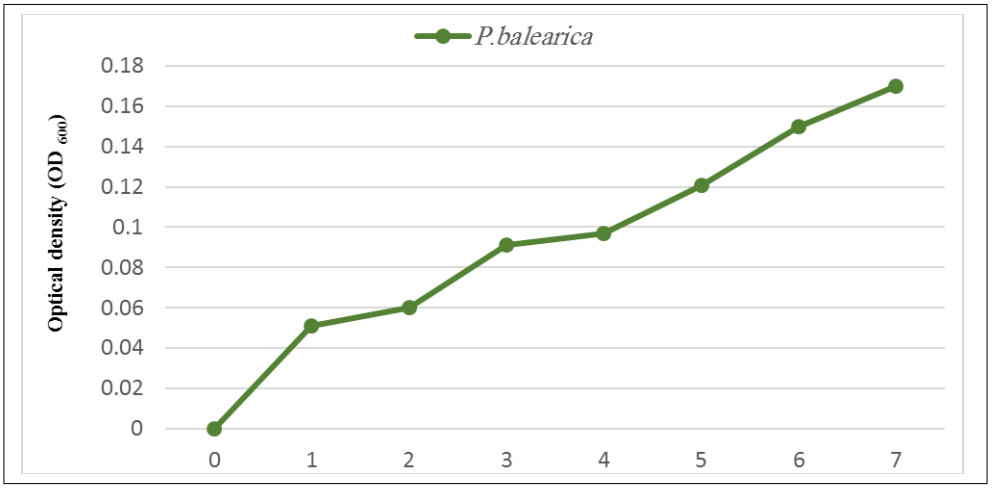

Figure 4- 23: The optical density of *P. balearica* growth with 1% crude oil after 7days of incubation.

4.12. Antibiotic resistance assay

Methylorubrum extorquens was examined for their ability to resistant ampicillin, kanamycin and spiramycine that are frequently used as markers in genetic modification. As in section 3-2-6-3, a preliminary assessment was carried out in order to elicit a reaction of high quality by utilizing filter paper on methanol salt medium agar.

After an incubation period of 3-5 days on plates, experiments using a range of dosages for each antibiotic indicated minimal inhibitory concentrations (Table 4-2 and 4-3).

Table 4-2: Antibiotic resistance assay of *M. extorquens* on MSM agar.

	Ampicillin		**Kanamycin**		**Spiramycin**	
Concentration µgmL^{-1}	3 days	5 days	3 days	5 days	3 days	5 days
20	+	+	-	-	+	+
50	+	+	-	-	+	+
100	+	+	-	-	+	+
200	-	-	-	-	+	+
500	-	-	-	-	-	-

***Growth (+); Inhibition (-); green color (MIC)**

Table 4-3: Antibiotic resistance assay of *p. balearica* on LB agar.

	Ampicillin	Kanamycin	Spiramycin
Concentration µgmL^{-1}	3 days	3 days	3 days
20	+	+	+
50	+	-	+
100	+	-	-
200	-	-	-
500	-	-	-

***Growth (+); Inhibition (-); green color (MIC)**

4.13. Transformation of Bacteria

The electroporation approach was used in order to convert *M. extorquens* and *p. balearica* bacteria to competent harboring CRISPR-Cas9 encoding plasmids. The technique resulted in the creation of individual colonies of transformed cells (Fig. 4-24).

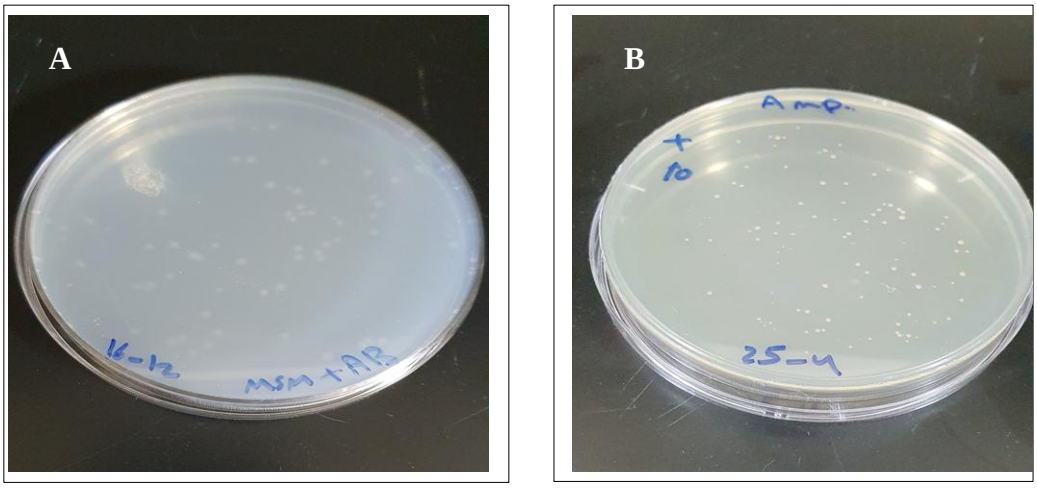

Figure 4-24: A, Transformed *M. extorquens* resistant to Kanamycin on MSM selected medium. B, Transformed *p. balearica* resistant to Ampicillin on LB medium.

4.13.1. Gel electrophoresis of transforming bacteria

The presence of the recombinant plasmids was verified by agarose electrophoresis after extracted from the transformed bacteria. The results showed bands of the same size as the control plasmid inserted in compared to the control (Fig. 4-25). The bands were visualized by UV light, the large one (14.1Kb) was purified from transformed *M. extorquens*. While the smaller on (9.2Kb) was extracted from *P. balearica*.

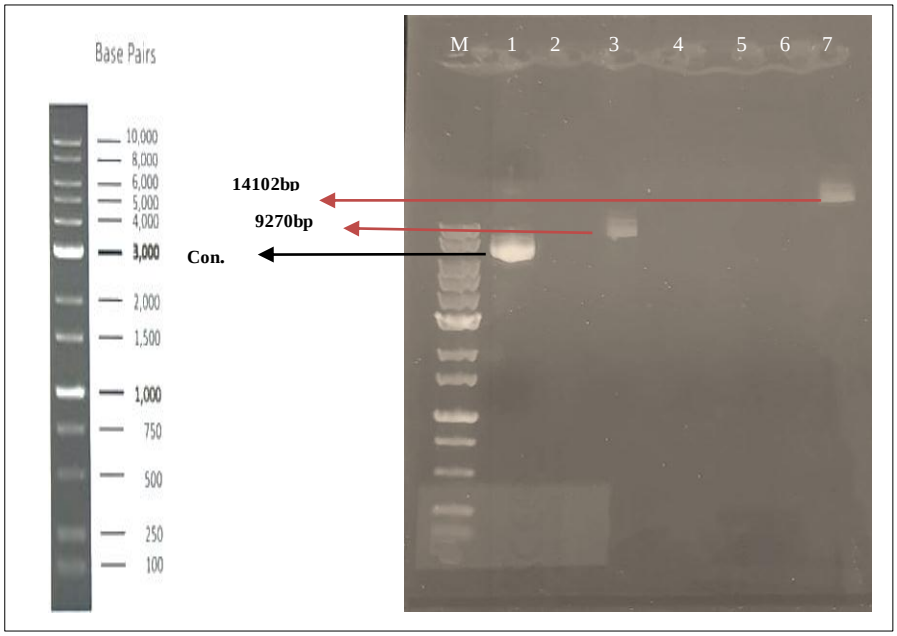

Figure 4-25: Agarose gel electrophoresis of the intact (control) and recombinant containing colonies. Lane M:1Kbp Marker, Lane 1: control positive, Lane 3: CRISPR plasmid extracted from positive selected clones of *P. balearica*, Lane 7: CRISPR MDH guided plasmid extracted from *M. extorquens* positive selected clones.

4.14. Gene Expression Analysis

4.14.1. Gene Expression Analysis of CRISPR-Cas9 system in *M. extorquens*

After treating cells with the CRISPR-Cas9 system, RNA was collected and reverse-transcribed to generate the first strand of DNA that would be utilized as templates in determining whether or not the *MxaF* gene is expressed in *M. extorquens* and transformed *M. extorquens*. MDH

expression after treating with the CRISPR-Cas9 system was upregulated to 6-fold compared to the *M. extorquens* before editing, which was normalized to 1. ΔΔ CT analysis was applied to conclude the MDH gene expression. The qPCR and *MxaF* amplification primers are added to cDNA templates. Expression levels of genes may be measured using SYBR green. *16SRNA* value was subtracted as a housekeeping gene. (Fig. 4-26 and 4-27).

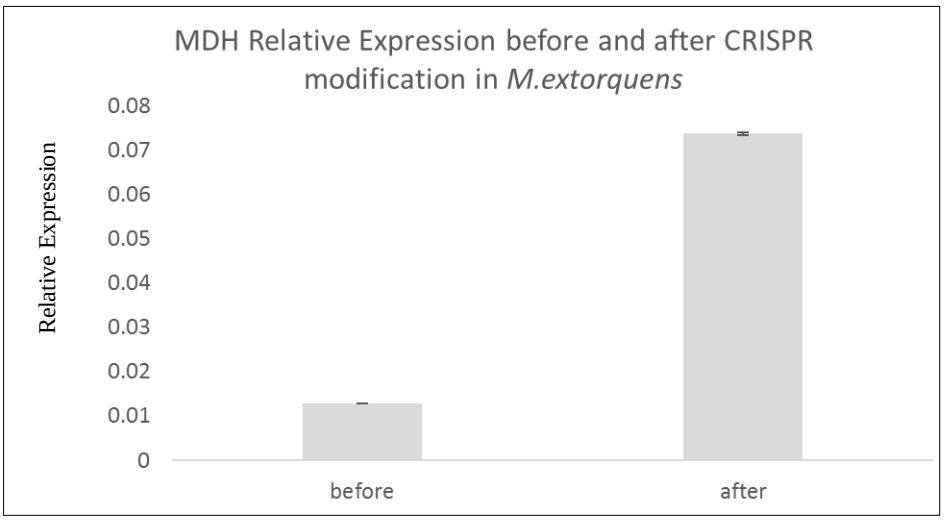

Figure 4-26: Relative Methyl Dehydrogenase gene expression in *M. extorquens* before and after modification with CRISPR-Cas9 system. Total RNA was extracted from fresh cultures of both species, reverse transcription of the synthesized DNA was performed, and the synthesized DNA was employed as a template in a qPCR relative expression experiment using SYBR green master mix. Analyses were performed using ΔΔ CTs, and results were normalized using *16S rRNA* as the housekeeping gene.

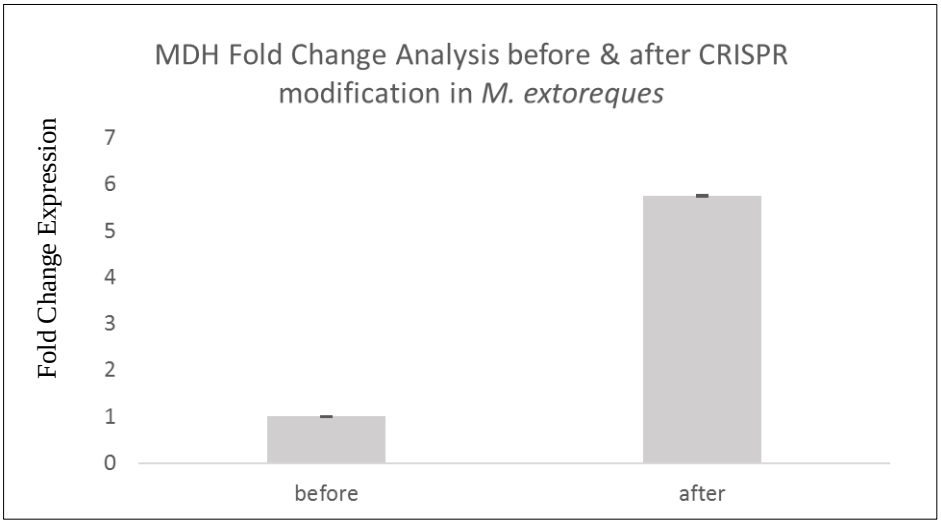

Figure 4-27: Methyl Dehydrogenase fold change analysis in *M. extorquens* before and after modification with CRISPR-Cas9 system. Total RNA was extracted from fresh cultures of both species, reverse transcription of the synthesized DNA was performed, and the synthesized DNA was employed as a template in a qPCR relative expression experiment using SYBR green master mix. Analyses were performed using ΔΔ CTs, and results were normalized using *16S rRNA* as houses keeping gene and control sample was normalized to 1.

4.14.2. Gene Expression Analysis of Cas9 in *P. balearica*

To investigate if the *pMMO* gene is expressed in *P. balearica* and transformed *P. balearica*, RNA samples were collected from cells treated with the Cas9D10A method and reverse transcribed to create the first strand of DNA used as templates. *pMMO* expression after treating with the CRISPR-Cas9 system was upregulated to 25-fold compared to the *P. balearica* before editing, which was normalized to 1. ΔΔ CT analysis was applied to conclude the *pMMO* gene expression. The qPCR and *PMMO* amplification primers are added to cDNA templates. Expression levels of genes may be measured using SYBR green.*16S rRNA* value was subtracted as a housekeeping gene (Fig. 4-28 and 4-29).

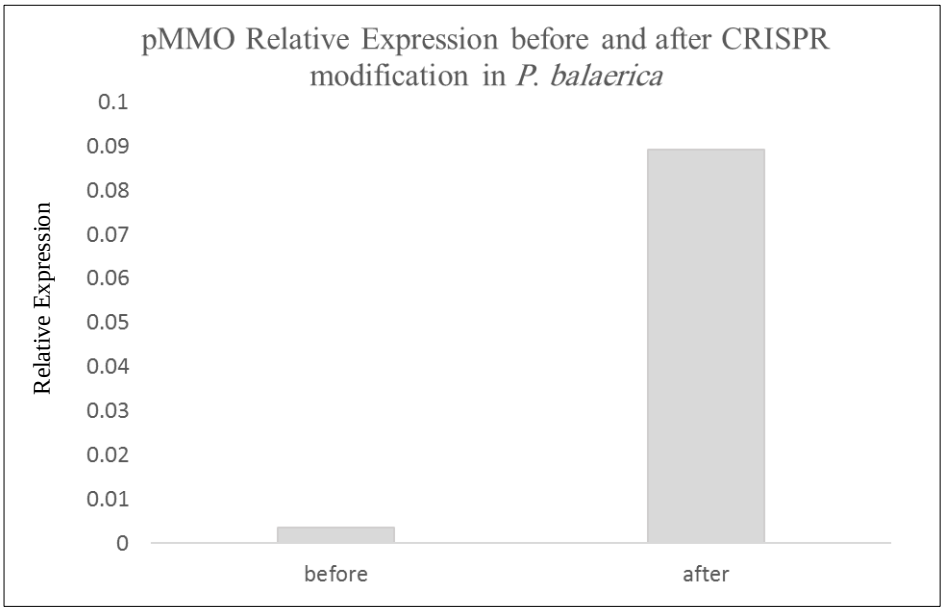

Figure 4-28: Relative methane monooxygenase gene expression in *P. balearica* before and after modification with Cas9D10A. Total RNA was extracted from fresh cultures of both species, reverse transcription of the synthesized DNA was performed, and the synthesized DNA was employed as a template in a qPCR relative expression experiment using SYBR green master mix. Analyses were performed using ΔΔ CTs, and results were normalized using *16S rRNA* as the housekeeping gene.

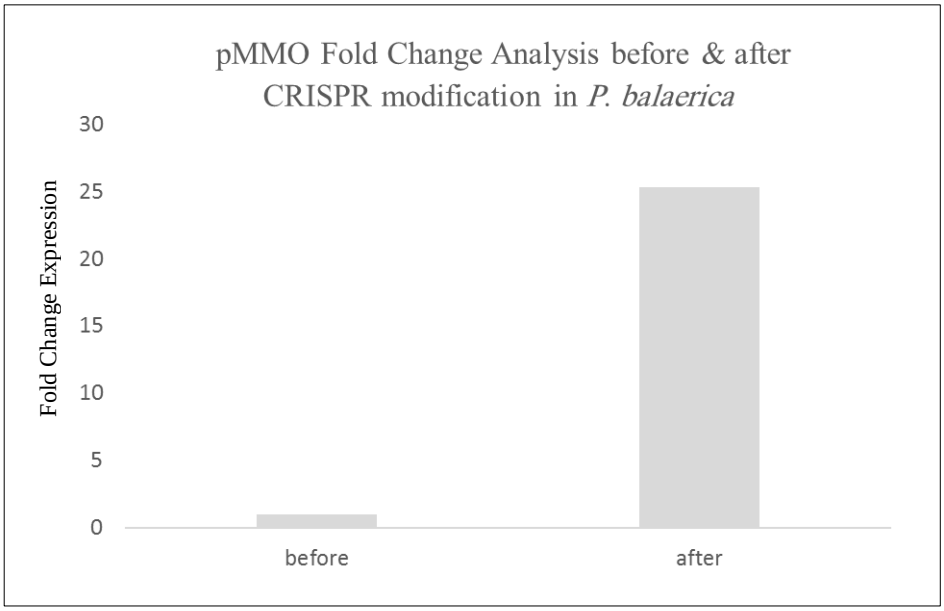

Figure 4-29: Methane monooxygenase fold change analysis in *P. balearica* before and after Cas9D10A transformation. Total RNA was extracted from fresh cultures of both species, reverse transcription of the synthesized DNA was performed, and the synthesized DNA was employed as a template in a qPCR relative expression experiment using SYBR green master mix. Analyses were performed using ΔΔ CTs, and results were normalized using *16S rRNA* as houses keeping gene and control sample was normalized to 1.

4.15. Biodegradation of Crude Oil Using Bacteria before and after CRISPR-Cas9

4.15.1. Estimation of Aliphatic portion biodegradation in *M. extorquens*

The aliphatic components of the crude oil were analyzed by GC, and the results showed many peaks over the hump. These peaks represent n-alkane. The hump is a representation of the heavier chemicals that were not eluted. After 7 days of incubation without bacterial inoculation, the sample of crude oil that was being analyzed had carbon levels that ranged from C_{12} to C_{37}. These values served as a control for the experiment (Fig. 4-30).

Time is plotted along the horizontal axis of the GC charts, while PA is plotted along the vertical axis (Pico-ampere). During the incubation phase, major changes occurred in a number of the component elements that made

up the utilized crude oil. According to the report generated by the GC, on the seventh day, the bulk of the peaks had drastically dropped. *M. extorquens* was responsible for the efficient destruction of short-chain alkanes (nC_{12}-nC_{15}). While longer-chain alkanes (nC_{16}-nC_{37}) broke down at a slower rate.

The total amount of crude oil that was degraded by the aliphatic component residues of uncured *M. extorquens* was 61.14% (0.5% $^{v}/_{v}$), (Fig. 4-31).

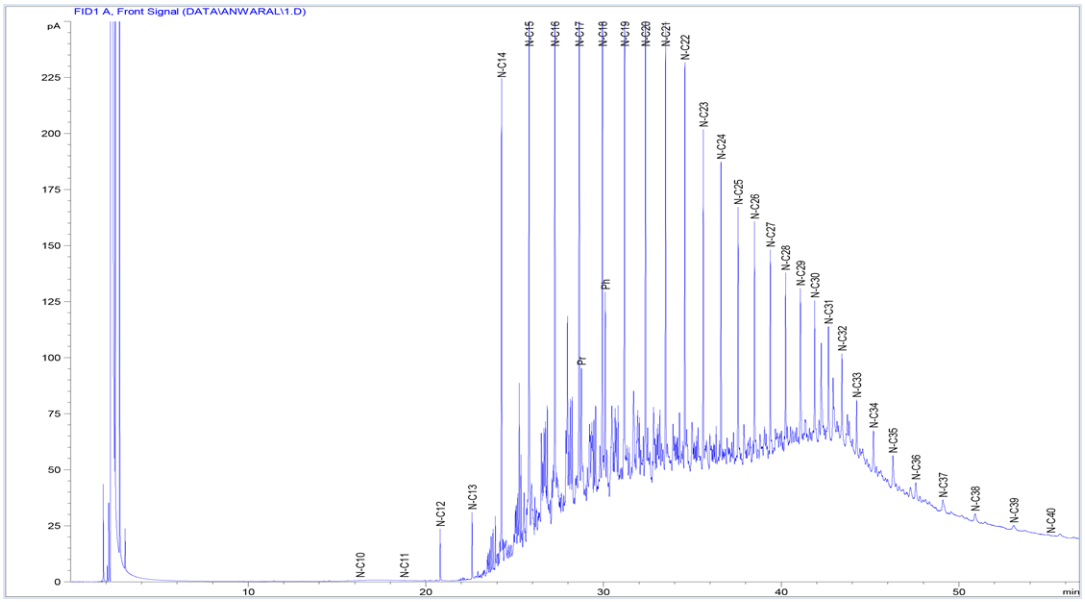

Figure 4-30: GC of residual control crude oil (aliphatic portion) 0.5% after 7 days of incubation.

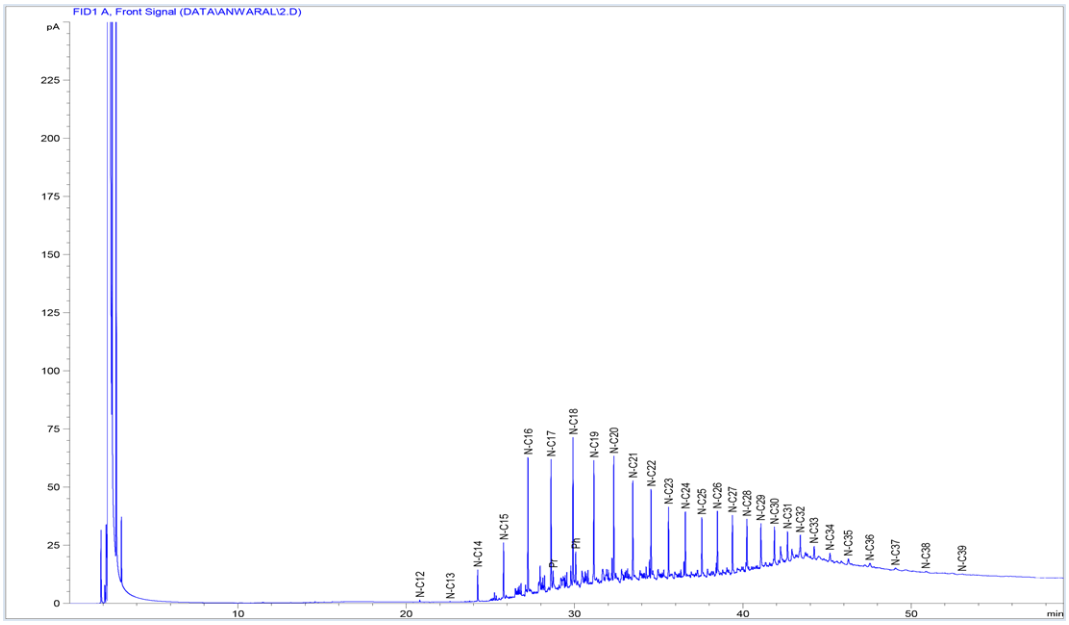

Figure 4-31: GC of residual crude oil (aliphatic portion) of 0.5% after 7 days of uncured *M. extorquens* incubation.

The majority of the constituent components of the utilized crude oil went through significant transformations while the incubation process was taking place. According to the results of the GC analysis, the vast majority of the peaks had a noticeably decreased concentration of n-alkanes (nC_{12}-nC_{37}), which is evidence that these compounds had been successfully eliminated.

Using the GC report, the percentage of degradation was determined, and it was found to be 74.35% of aliphatic component residues after 7 days of incubation with 0.5% crude oil of cured *M. extorquens* with CRISPR-Cas9 (Fig. 4-32).

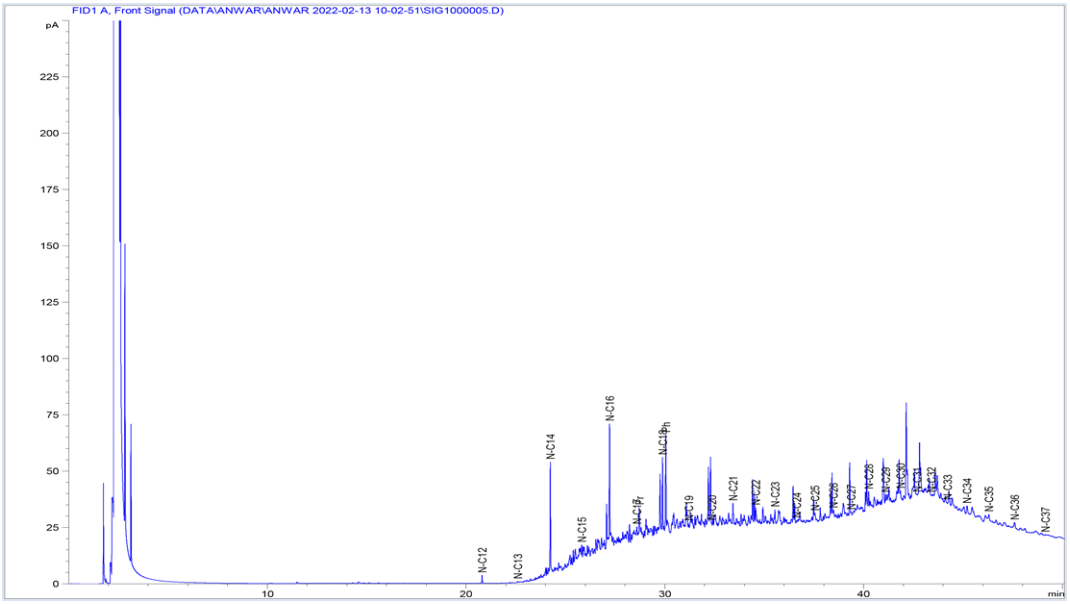

Figure 4-32: GC of residual crude oil (aliphatic portion) of 0.5% after 7 days of incubation of cured *M. extorquens* with CRISPR-Cas9.

4.15.2. Estimation of Aliphatic portion biodegradation in *P. balearica*

During the incubation time, a number of the component constituents that made up the used crude oil underwent significant modifications. According to GC report, *P. balearica* was the only suggested agent that was responsible for the effective elimination of alkanes on the seventh day (nC_{12}–nC_{37}).

Aliphatic component residues of non-transformed *P. balearica* were responsible for the degradation of 90.32 % (0.5 $^{v}/_{v}$) of the crude oil (Fig. 4-33).

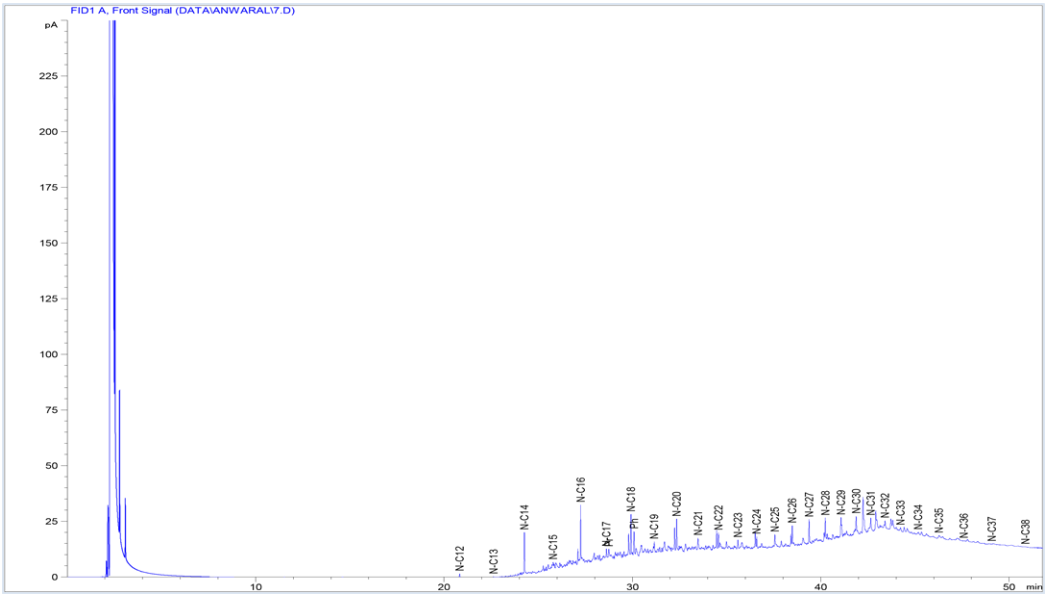

Figure 4-33: GC of residual crude oil (aliphatic portion) of 0.5% after 7 days of non-transformed *P. balearica* incubation.

Degradation was calculated using GC data, and it was discovered that after 7 days of incubation with 0.5% crude oil of cured *P. balearica* with Cas9, 92.90% of aliphatic component residues were gone (Fig. 4-34).

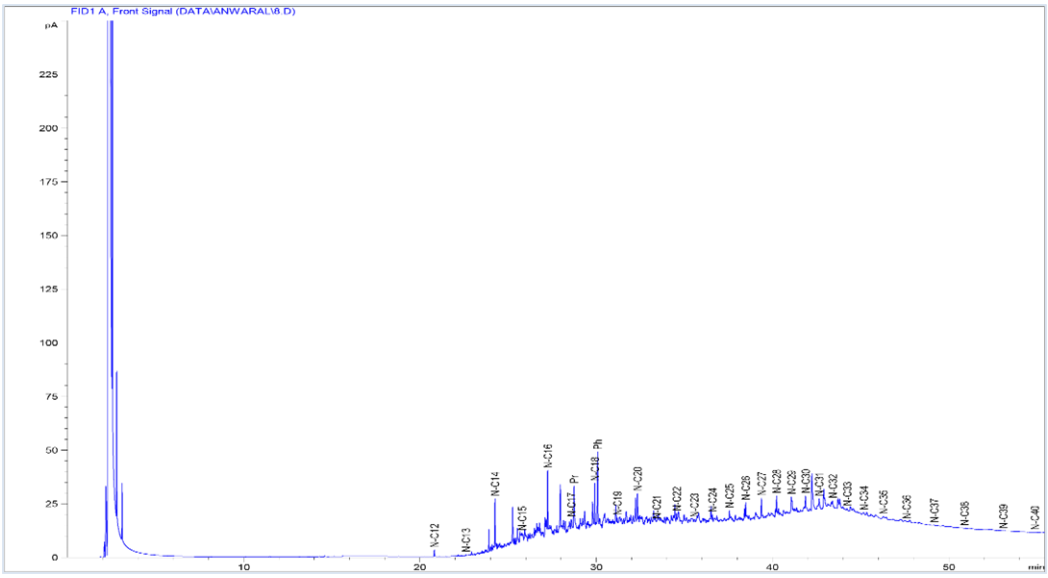

Figure 4-34: GC of residual crude oil (aliphatic portion) of 0.5% after 7 days of cured *P. balearica* with Cas9.

4.15.3. Estimation of Aromatic part biodegradation by *M. extorquens*

After 7 days of incubation without bacteria, the aromatic component residues of crude oil that had been retrieved from the conical flask were utilized as a control (Fig. 4-35).

The GC results showed that the aromatic component residues were degraded at a ratio of 65.69% following 7 days of incubation of plasmid-uncured *M. extorquens* with 0.5% crude oil. Where the degradation percentages for phenanthrene, Benzo(A) Anthrac, Chrysene, Fluoranthene, Benzo(B) Fluora, and Benzo(A) Pyrene were 81.81%,75.35%, 71.71%, 70.13%, 69.09%, and 60.01%, respectively. Meanwhile, the 2-methylnaphtha and Acenaphthnen fractions were completely degraded (Fig. 4-36).

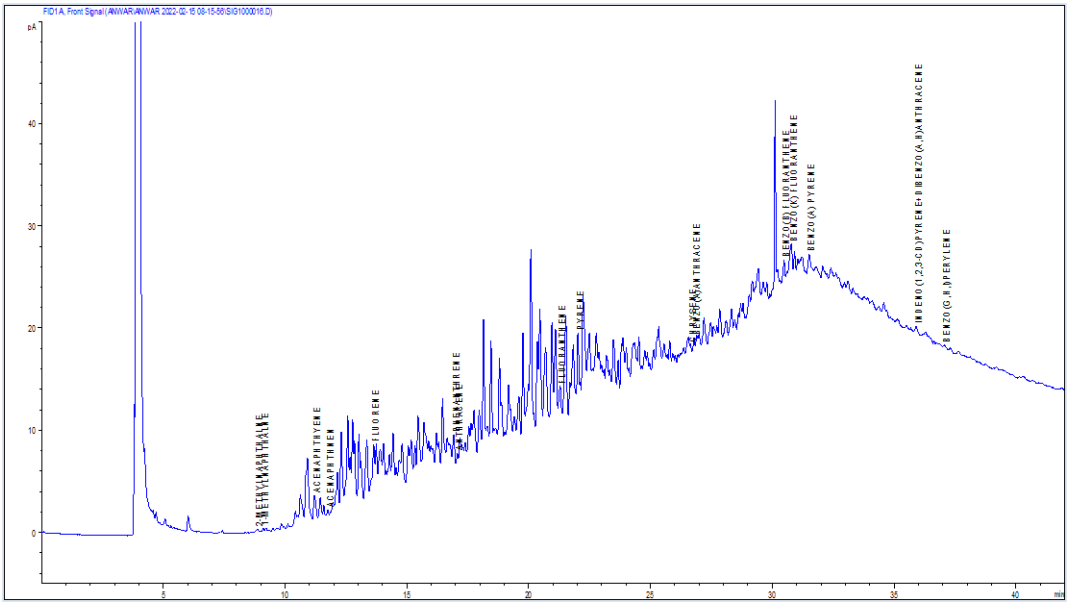

Figure 4-35: GC of residual control crude oil (aromatic portion) 0.5% after 7 days of incubation.

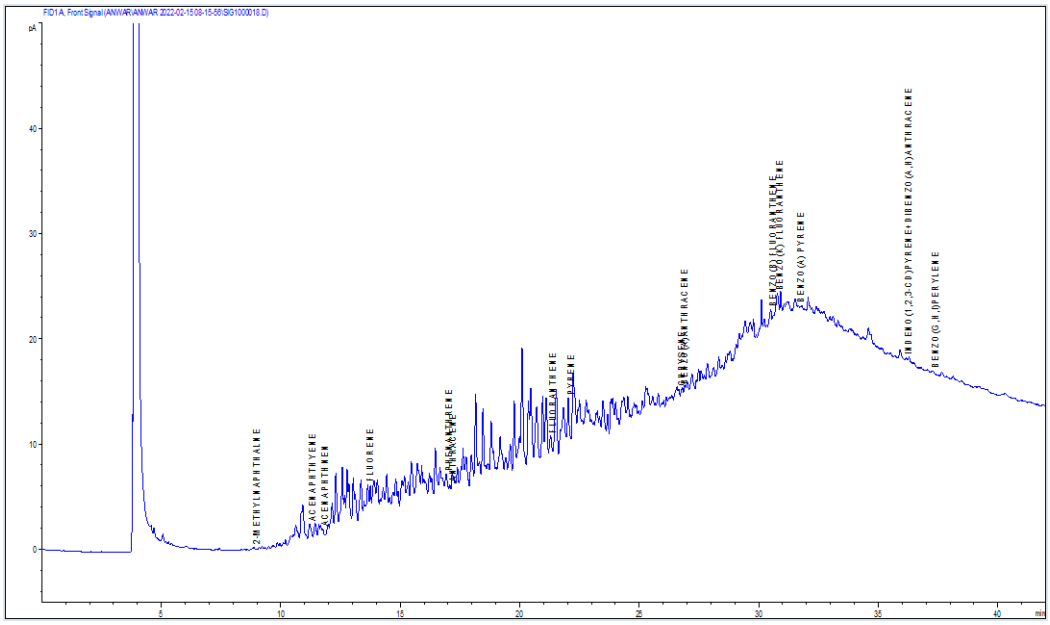

Figure 4-36: GC of residual crude oil (aromatic portion) of 0.5% after 7 days of incubation of uncured *M. extorquens.*

The percentage of degradation was computed based on the GC report of aromatic component residues after 7 days of incubation with 0.5% crude oil of cured *M. extorquens* with CRISPR- Cas9 system, and it was 78.23% (Fig. 4-37). The phenanthrene, fluorene, chrysene, and pyrene degradation percentages were 92.49%, 91.98%, 90.96%, and 87.60%, respectively. Conversely, fluorene, benzo(A)Anthrac, benzo(B), fluora, and acenaphthyene degraded at 74.03%, 73.34%, 72.65%, and 72.06%, respectively. While the 2-methylnaphth, Acenaphthnen, and indeno (1,2,3-CD) fractions were completely degraded.

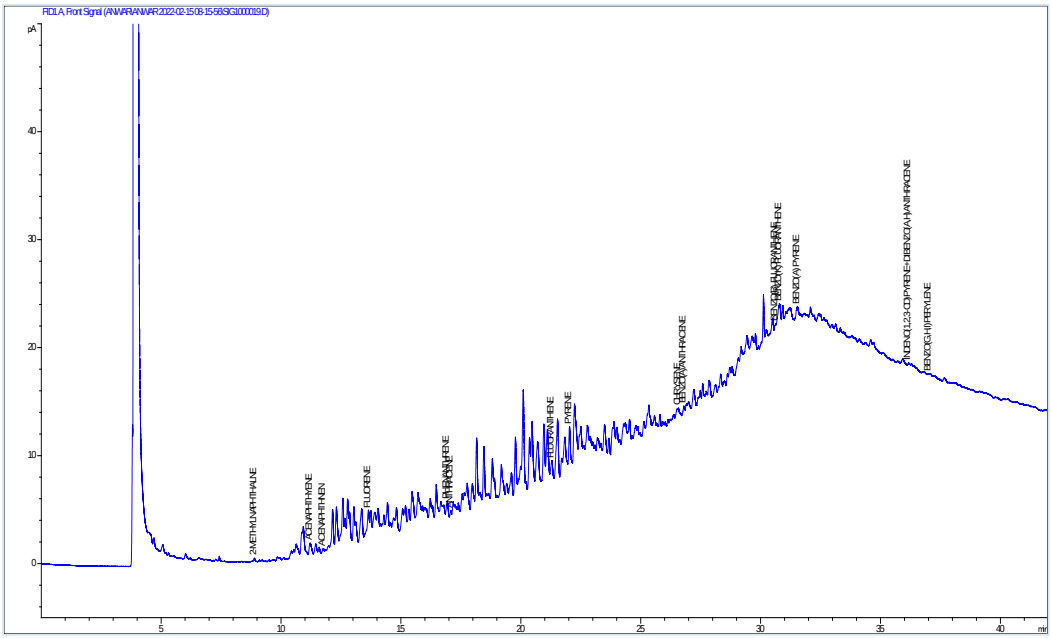

Figure 4-37: GC of residual crude oil (aromatic portion) of 0.5% after 7 days of incubation of cured *M. extorquens* with CRISPR-Cas9 system.

4.15.4. Estimation of Aromatic part biodegradation in *P. balearica*

The GC results showed that the aromatic component residues were degraded at a ratio of 83.16 % at 7 days' incubation of non-transformed *P. balearica* with 0.5% crude oil.

The degradation rates of Benzo(A)-Anthrac, Fluoranthene, Phenanthrene, and Chrysene were 90.82%, 90.74%, 90.44%, and 89.86%, respectively. On the other hand, Pyrene, acenaphthyene, benzo(B) fluora, benzo(A) pyrene, anthracene, and fluorene, degrade at 83.48%, 81.34%, 78.03%, 76.78%, 76.77%, and 72.44%, respectively. Meanwhile, the 2-methylnaphtha, acenaphthnen fractions, and indeno (1,2,3-CD) were completely degraded (Fig. 4-38).

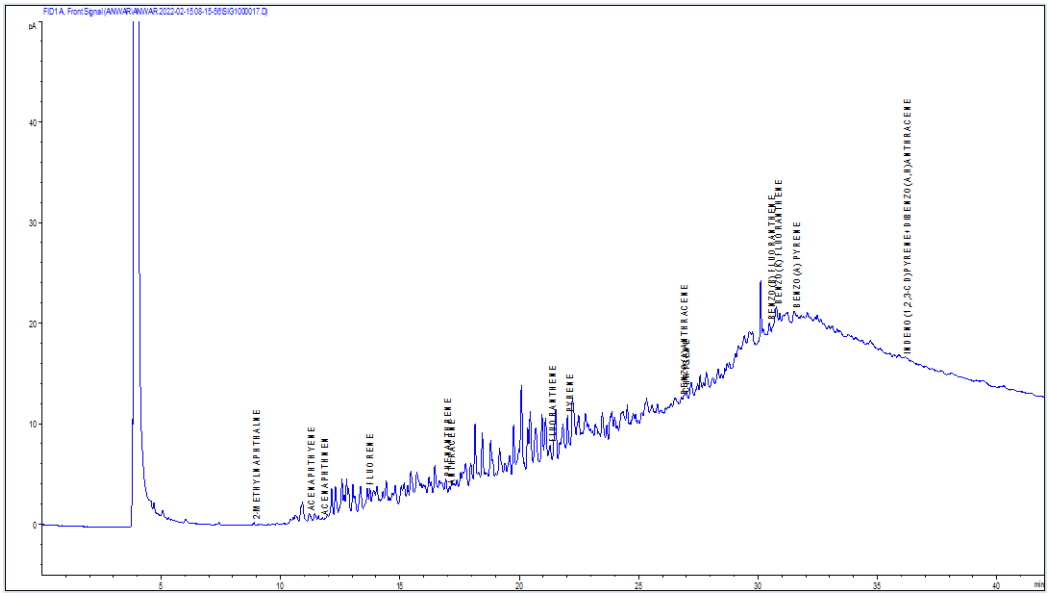

Figure 4-38: GC of residual crude oil (aromatic portion) of 0.5% after 7 days of incubation of non-transformed *P. balearica.*

Based on the GC report of aromatic component residues, it was determined that 94.07% of the crude oil of cured *P. balearica* was degraded after 7 days of incubation with 0.5% Cas9 (Fig. 4-39).

The phenanthrene, fluorene, benzo(A)Anthrac, benzo(B) and Benzo(A) Pyrene degradation percentages were 94.19%, 94.37%, 95.62%, 95.61% and 94.55%, respectively. Conversely, Fluoranthene, Pyrene and Benzo(K) degraded at 80.83%, 88.80%, and 89.99%, respectively. Anthracene and indeno (1,2,3-CD) degrade at 73.37 and 73.64%. While the 2-methylnaphtha, Acenaphthyene and Acenaphthnen, and fractions were completely degraded.

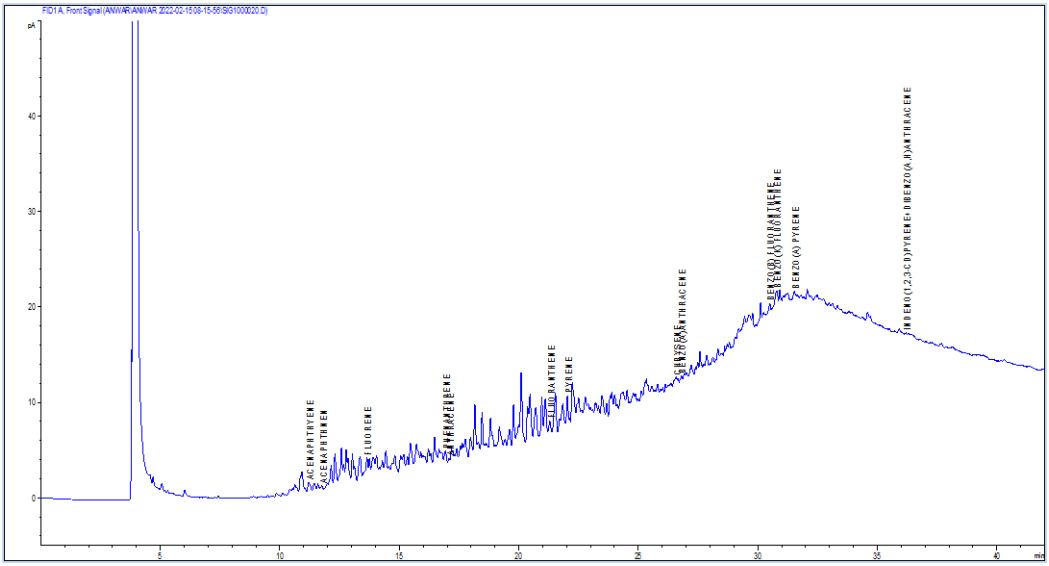

Figure 4-39: GC of residual crude oil (aromatic portion) of 0.5% after 7 days of incubation of cured *P. balearica* with Cas9.

Chapter Five

Discussion

5. Discussion

The majority of the world's energy needs are still met by petroleum hydrocarbons. Spills and leaks are commonplace across the petroleum and petroleum product supply chain, from exploration to refining to storage, making petroleum a significant worldwide environmental contaminant (Nafal and Abdulhay, 2020).

Toxic and dangerous aliphatic, cycloaliphatic, and aromatic hydrocarbons are the primary components that can be found in oil polluted soil. They disrupt the biological balance of the soil, lower the diversity of plants and microbes that live there, lessen the fertility of the soil, and even put people's health in danger (Ansari *et al.*, 2018; Suganthi *et al.*, 2018). Pollutants can also enter the body through skin contact, breathing, consuming petroleum-contaminated food, or eating, which can result in dermatitis, auditory and visual hallucinations, gastrointestinal issues, as well as significantly increasing the risk of childhood leukemia (Ozigis *et al.*, 2020; Kumari *et al.*, 2018). So, the aim of the study was to isolate, test the ability of methylotrophic bacteria and genetically modifying them to improve their performance.

The study samples were collected from locations that contained with oil residues and were situated in close proximity to oil installations such as Zubair and Nahran Omar, as well as the gas isolation station in Basra city, which is located in southern Iraq.

Many researchers have written articles on the bioremediation of crude oil, TPH, and other related petroleum products by employing bioaugmentation, biostimulation, or a combination of the two (Sayed *et al.*, 2021). As a consequence of this, a novel technique known as CRISPR-Cas9 will be implemented to create a long-term solution to the issue.

This will involve the introduction of genetic modifications within the bacteria that are the subject of the study. These modifications will improve the bacteria's ability to convert these hydrocarbon molecules into simpler compounds with lower risk to the environment.

5.1. Isolation, Identification and purification of bio-remediating bacteria

Methylotrophic bacterial strains were isolated from soil samples collected from sites polluted with a wide range of petroleum compounds. Bacterial species diversity is a direct result of the variety of soil contamination sources. In this study, we have used a novel modified medium that was designed to maximize the growth potential of the bacteria being studied. In fact, methylotrophs are slowly growing and many researchers developed special media to support a robust and consistent growth of them such as using PIPES buffer with citrate use as a chelator rather than EDTA (Delaney *et al.*, 2013) .

Our strategy to improve the methylotrophs growth is increases in biotin concentration promoted faster bacterial growth. Since biotin may increase the rate of nitrogen removal by bacteria by more than twofold, it is used to improve anaerobic ammonium oxidation (anammox). The anammox bacteria's growth was also greatly enhanced by biotin (Li *et al.*, 2021).

Thiamin, in its phosphorylated form, is a necessary cofactor for an enzyme involved in the transformation of carbon. Although many bacteria are capable of thiamine production, others, such as the methylotrophs, must acquire it or its precursors from the environment. Thiamin serial dilution in the growth medium enhanced the development of thiamin-requiring bacteria in the presence of thiaminase I. Thiaminase I releases the precursors that synthesize thiamin. This is an example of how bacteria manage nutrients in their environment (Sannino *et al.*, 2018; Tani, 1985).

Microorganisms that live in the phyllo sphere are able to use a variety of different sources of energy, including carbon and nitrogen, as well as trace cofactors like vitamins (Yoshida *et al.*, 2019). Most PPFMs, including *Methylobacterium sp.* OR01, that were isolated from living plant samples by Yurimoto *et al.* (2021) needed pantothenate (vitamin B5) to thrive on a minimal medium.

These organisms either failed to develop or grew extremely slowly on the methanol-salt medium when the inoculum was small. The medium used by Kouno *et al.* (1973) does not allow these organisms to grow. This may be due to the harsh environment that they are isolated from, which may have contributed to the problem. Because of this, the presence of an antifungal as well as an increase in biotin and thiamine led to the proliferation of these bacteria.

This mineral salt medium was essentially prepared from mineral salts. The medium was maintained with methanol as the only source of energy, which feeds the bacteria with organic carbon; thus, the predominately developing bacteria in this medium were methylotrophic, which is consistent with the findings of the study (Amaresan *et al.*, 2020; Belkhelfa *et al.*, 2019; Kim *et al.*, 2003).

5.2. Morphological and biochemical characteristics

The morphological properties of isolates are depicted in Figure 4-1. The selected isolate reflected biochemical features based on biochemical analyses as reported in Table 4-1 and known morphological characteristics.

The isolate was positive for catalase, which is used to determine which species is responsible for the production of catalase enzymes. Exhibiting the existence of the catalase enzyme, which has the ability to break down hydrogen peroxide into oxygen. As a consequence of this, it is presumed that

all of the isolated bacteria were aerobic in nature. The results were in line with those of a prior study by Waturangl *et al.* (2011) who identified the biochemical properties of methylotrophic bacteria included catalase-positive features.

Numerous isolates tested positive for the presence of oxidase. According to the results of this study, the vast majority of the isolates include cytochrome oxidase, an enzyme that catalysis the passage of electrons from electron donors to electron acceptors (Brown and Smith, 2014).

5.3. Molecular detection of the isolates: Amplification of the *16S rDNA* gene in bacterial isolates

16S rDNA gene was chosen and amplified in 15 different isolates using the universal primers F27 and R1492. This was done as a marker for all different kinds of bacteria (Figure 4-3). *Methylorubrum extorquens* was successfully recognized at the species level after processing the row data in which their sequences were compared to the sequences of the type strain in Gen Bank. Dourado *et al.* (2012) discovered *Methylobacterium* strains in mangrove samples collected near oil spill sites in Bertioga, Brazil. They applied *16S rDNA* detection system to identify them.

Pseudomonas balearica was also identified using *16S rDNA*, which is consistent with Zerrad *et al.* (2014) who identified *Pseudomonas balearica* strain U7 from Sea Lettuce (Ulva lactuca) using *16S rDNA* primers 27F and 1492R.

The results come in agreement with the Rojas-Gätjens *et al.* (2022) which investigated an old oil well and discovered a significant population of methylotrophic bacteria there. Some of the bacteria they discovered were *Paracoccus communis*, *Methylorubrum rhodesianum*, and *Pseudomonas* sp. The current results are also supported by the study of Godini *et al.* (2018)

who identified nine different bacterial species from oil-contaminated sites on the island of Kharg in Iran. Among them is *Methylobacterium persicinum* and *Pseudomonas* sp. In addition, it was not surprising that the methylotrophs were isolated specially in such environment, since they are widely distributed in soil, air, water, plant noodles and surfaces an implicated in degrading toxic compounds and detoxifying heavy metals (Salam *et al.*, 2015).

5.4. Detection of the *sMMO* and *MxaF* gene by cPCR

Through the use of PCR, bacterial isolates that belonged to the genus *Pseudomonas, sMMO* gene was successfully detected and amplified using their genomes as templates (Fig. 4-4). The *pMMO* is ubiquitous among all known obligate methanotrophs, whereas, *sMMO* appears only in some methanotrophs including facultative methanotrophs, and expresses itself only in the presence of less Cu^{2+} (Nielsen *et al.*, 1997). In fact, there is unusual form of monooxygenase was detected in *p. putida* three decades ago. This enzyme is implicated oxidizing 2-oxo-A3-4,5,5-trimethylcyclopentenylacetic acid (Ougham *et al.*, 1983).

MDH activity was detected for the first time in *M. extorquens*, and it is an essential part of methylotrophic growth. Because the highly conserved *MxaF* gene is present in all known methylotrophs, and based on that, it was used to identify methylotrophs found in their natural environments (Chistoserdova *et al.*, 2009) .

In order to identify methylotrophic bacteria and detect the presence of the *MxaF* gene, degenerate primers were employed. F1003 and R1561 were chosen from the conserved region of the *MxaF* gene (Fig. 4-5). These primers generated a 550-bp amplicon.

The effectiveness of degenerate primers relies on their capacity to identify degenerate base positions, which are sites where numerous nucleotide

codons define the same amino acid residue. The results were consistent with those observed by Lau *et al.* (2013) who used the *MxaF* gene as a biomarker for methanotrophic proteobacteria belonging to the families methylocystaceae and methylococcaceae.

5.5. Quantitative PCR detection

qPCR that is employed to detect/ quantify common functional genes expressed microorganisms with known physiological properties. At the moment, more than 100 functional genes with more than 14,000 DNA sequences were identified. Many of these functional gene products have potential applications in the environment such as methane oxidation genes (Zhang and Fang, 2006).

The use of pmoA, which encodes the α subunit of the particulate methane monooxygenase, was unable to show the existence of a class of methylotrophs that could use carbon-1 molecules other than methane as their exclusive source of carbon and energy, indicating that they participate in C1 metabolism in nature. Utilizing another functional gene marker, *MxaF*, which is found in all methylotrophs, could make up for pmoA's drawback in methylotrophs diversity study. Since formaldehyde is the intermediate of both methylotrophs' assimilative and dissimilative metabolism, all known gram-negative methylotrophs (Wang *et al.*, 2004)

In this study, qPCR was utilized to detect the *pMMO* gene (Fig. 4-6 and 4-7). This is responsible for encoding the methane monooxygenase enzyme, and this might explain the high sensitivity of the qPCR in detection. qPCR was successful in amplifying the *MxaF* gene. This gene is recognized as a functional marker for the identification of methylotrophic bacteria since it encodes for the methanol dehydrogenase enzyme, as shown in Figures 4–8 and 4–9.

5.6. Gene expression analysis of MDH in *M. extorquens*

Analysis of gene expression revealed that there was a 44-fold difference in the enzyme activity between *M. extorquens* and *P. balearica* when comparing the two organisms. It is possible that this can be attributed to the fact that the enzyme is clearly represented in the genome of *M. extorquens* and is able to be expressed at its highest level when the substrate is provided in an effective manner, whereas in *P. balearica*, the enzyme is a component of the cytochrome chain reactions that take place in that organism.

Both *M. extorquens* and *P. aeruginosa* pose a region that encodes for *XoxF*, and *P. aeruginosa* has shown that it can make methyl dehydrogenase. Due to the existence of respiratory Chan-linked dehydrogenase, the latter can oxidize the N-methyl l-glutamate. Using mutant insertion analyses, researchers noted that *P. aeruginosa* can catalyze the methyl-branched molecules (Förster-Fromme and Jendrossek, 2008; Hersh *et al.*, 1971).

5.7. Gene expression analysis of *pMMO* in *P. balearica*

Particulate methane monooxygenase (*pMMO*) is an enzyme that methane-oxidizing bacteria have, and the active site of this enzyme may include copper (Sluis *et al.*, 2002).

The comparison among *P. balearica* and *M. extorquens* for *pMMO* gene expression, we find 10-fold variation in the enzyme activity between them. This is may be due to that the enzyme is very well represented in the genome of *P. balearica*, allowing it to be expressed at highest level when the organism is grown on methane medium. Meanwhile, in *M. extorquens* it is expressed at a lower level. The reason for this may be due to the inability of bacteria to thrive on a substrate like methane. In bacteria, there is a growth rate dependent gene expression that regulates the gene expression of the target genes accordingly. Several factors affect such gaps in the level of gene

expression, for example the location of the gene whether it is in chromosome or plasmids or how close the gene to the origin of replication and the plasmid copy number (Klumpp and Hwa, 2014).

5.8. Growth in methane and Methane Monooxygenase enzyme assay

Extensive sampling across many biological niches has resulted in a continually evolving idea of methylotrophy as it relates to a special bacterial group, and other bacterial groups with the trait of methylotrophy have been uncovered (Macey *et al.*, 2020; Chistoserdova and Kalyuzhnaya, 2018; Boden *et al.*, 2008).

In this study, *P. balearica* were able to grow on NMS broth and to use methane as the only source of carbon and energy after 7 days of incubation. The qualitative measurement of soluble monooxygenase activity by naphthalene oxidation test revealed an encouraging result. This was accomplished by cultivating the organisms on solid NMS medium with CH_4 as the only source of carbon.

The producing of 1-naphthol is an indicator for the activity of the soluble monooxygenase on naphthaline and dye o-dianisidine. These findings are in agreement with Rani *et al.* (2021), who investigated a flooded paddy environment and found considerable methane use in 30 distinct bacterial taxa, including *Methylobacterium* and *Pseudomonas*, and obtained a positive naphthalene oxidation test.

In contrast to *P. balearica*, *M. extorquens* showed their inability to grow in NMS broth containing methane, tiny colonies were appeared either in the presence or absence of methane in NMS agar and this refer to that these colonies may be do not oxidize methane. These findings coincide with Dianou and Adachi (1999).

M. extorquens showed a negative result for the methane monooxygenase test, and this indicates the absence of the *sMMO* gene, which contradicts the study by Jhala *et al.* (2014), which showed a positive result for the use of the *sMMO* activity assay in *M. extorquens*. A negative result of this test suggests the presence of another form of the enzyme Methane Monooxygenase, which is *pMMO*.

Few reports suggested to use of methane in *Methylobacterium* (Jhala *et al.*, 2014; Van Aken *et al.*, 2004; Patt *et al.*, 1976). Another study by Ventorino *et al.* (2014) identified *Methylobacterium populi* VP2 from soil tainted with organic and inorganic contaminants. This strain was able to use methane, fructose, methylamine and ethanol as its only carbon source. It was proposed that the genetic capacity for CH_4 utilization may be plasmid-borne and may have been lost in the absence of CH_4 in inorganic environments, leading to an inconsistent outcome (Dedysh *et al.*, 2004).

This might explain why, despite *pMMO* amplification, the bacteria in the current investigation did not grow on NMS medium using methane as the source of carbon.

5.9. Growth at different concentrations of methanol (1-10) %

In this experiment, *M. extorquens* was tested for their ability to tolerate high methanol concentrations. When the concentration of methanol was increased, there was a corresponding drop in the growth rate.

Methanol is the only carbon and energy source for these bacteria, but at concentrations higher than 6% ($^{v}/_{v}$), the growth diminished. Methanol is oxidized to formaldehyde (which is harmful to organisms) by the methanol dehydrogenase enzyme found in methylotrophic bacteria.

Kim *et al.* (2003), discovered that the specific growth rate was substantially inhibited above a methanol concentration of 6%, and fell to

50% of the maximal specific growth rate as the methanol concentration was raised to 3%. Also they found that the maximum growth rate was at a concentration of 0.5% methanol. According to a study by Belkhelfa *et al.* (2019), *M. extorquens* AM1 grows at its fastest rate when exposed to 1% ($^{v}/_{v}$) methanol.

Pseudomonas balearica exhibited sluggish growth when grown on methanol, and the best growth occurred at concentrations 1% and 3%. Then it began to decrease by half until it reached a concentration of 6%. This result is consistent with the finding by Pacheco *et al.* (2003) that isolated *pseudomonas* could withstand methanol concentrations of up to 5% ($^{v}/_{v}$).

Methylotrophic bacteria use methanol as their only source of carbon and energy. It interacts with biological components as an alcohol solvent, changing their structure and chemical stability in a concentration-dependent manner. It also poses a chemical risk to the cells because of its metabolic conversion to the deadly substance formaldehyde (Belkhelfa, 2019). Methylotrophs are naturally resistant to the toxicity of formaldehyde due to the presence of many linear and cyclic formaldehyde dissimilation pathways (Khider *et al.*, 2021).

5.10. Gene expression analysis of MDH in *M. extorquens* at different concentrations of methanol

It was observed that, the bacterial growth affected when the methanol concentration is higher than4% and the MDH expression decline 5 folds at concentrations 5% or 6%. In fact, the correlation between substrate availability and enzyme expression or activity was revealed. A few decades ago, for example, when lactose is available, the lac operon is triggered, or when the medium content of amino acids is depleted, the biosynthesis of the concerned amino acid starts (Jacob and Monod, 1961; Zaslaver *et al.*, 2004).

Gene expression is strongly correlated to environmental changes since the enzymes involved in biosynthesis and substrate utilization are regulated by environmental-affected transcription factors (Wall *et al.*, 2004). However, down regulation of MDH gene expression with the increase of methanol to 6% agreed with Dyrda *et al.* (2019) who reported the high toxicity of methanol to *E. coli* where the low concentration of methanol in the increase and driven of MDH synthetics in methylotrophy and might be due to the reduction in toxicity of formaldehyde growth at high methanol concentrations (Bozdag *et al.*, 2015).

5.11. Growth in crude oil

M. extorquens and *P. balearica* displayed an ability to grow on crude oil by distributing the oil layers in the conical flasks at various levels and gradually increasing the turbidity of the mineral medium during the incubation time, which represents the growth of bacteria on crude oil as a carbon source. *M. extorquens*, a methylotrophic bacterium that produces a pink pigment, can obtain all of the carbon and energy it needs from either C1 compounds or Cn compounds (Hu and Lidstrom, 2014; Madhaiyan *et al.*, 2005).

The conclusion is consistent with research conducted by Godini *et al.* (2018) on Kharg Island in Iran. They found nine distinct types of bacteria in areas that had been polluted with oil. It has been demonstrated that *Methylobacterium persicinum* and *Pseudomonas* sp. are able to live with only a 2% concentration of crude oil as their only source of energy and carbon in their environment.

This findings matched those of a different study carried out by Salam *et al.* (2015). They used motor oil served as the only source of carbon and energy for the *Methylobacterium mesophilicum* strain RD1 isolate that grew in an incubator for a period of 21 days. As a direct result of this, the cell

densities in used engine oil bloomed up significantly, while many other parts of the oil slowed down.

5.12. Antibiotic resistance assay

Due to the emergence of bacterial resistance during work, the current study advised an antibiotic sensitivity test carried on plasmids for the purposes of genetically modifying the bacteria under study. Antibiotic resistance is a method used by microorganisms to survive in the natural world.

After a period of incubation lasting 72 h., it was found that *M. extorquens* was susceptible to kanamycin but resistant to ampicillin (Table 4-2). The findings of Belkhelfa (2019) are consistent with this observation.

At a concentration of 50 μgmL^{-1}, *Pseudomonas balearica* is susceptible to kanamycin. Pacheco *et al.* (2003) discovered that *Pseudomonas* sp. was susceptible kanamycin.

The morphology of bacterial colonies on culture medium, biochemical tests, serology, and antibiotic susceptibility are all methods that might be used to identify the different bacterial phenotypes (Li *et al.*, 2009).

5.13. Gene expression analysis before and after CRISPR-Cas9

One of the powerful techniques to study gene expression is reverse-transcription RT-qPCR using fluorescent DNA-binding dyes. However, RNA sample quality and reference gene selection must be carefully handled in the RT-qPCR process to provide trustworthy findings (Rocha *et al.*, 2020).

Development of genome-editing mechanism in *M. extorquens* based on the CRISPR-Cas9 system in order to develop a method of genetic modification that is both successful and easy to apply in *Methylorubrum* species', in order to modify the genomes of these bacteria we used the

CRISPR-Cas9 system. This system was implemented as a single-plasmid system called PHSPO2 and contained the *Streptococcus pyogenes* Cas9 (spCas9) protein as well as the appropriate sgRNA expression cassettes (Fig.3-3).

Comparing the control *M. extorquens* and CRISPR-modified *M. extorquens*, gene expression analysis indicated about 6-fold change in the MDH enzyme activity (Fig. 4-27). This confirms the success of the CRISPR-Cas9 system used in increasing the gene expression of MDH, and this result is consistent with what was concluded by Mo *et al.* (2020) which used CRISPRi to inhibit the transcriptional level of the squalenehopene cyclase gene, which is involved in hopnoid biosynthesis, by 64.9%. This led to an increase in carotenoid production that was 1.9-fold greater in *M. extorquens* than before.

Many trials of cloning *P. balearica* with *MMO* gRNA and a 1-kb DNA repair template led for no successful transformation (Fig. 3-5) Therefore, in order to achieve genetic modification, we added copper to the medium that contains the plasmid Cas9 to activate it. This allows the bacteria to self-repair. In fact, copper is used to optimize and rewiring the gene expression network through the copper responsive factors to undergo a conformational change in order to bind to DNA. The increase of the gene expression reaches hundreds times compared to the control in a dose dependent manner (Garcia-Perez *et al.*, 2022).

Analysis of gene expression showed a 25-fold difference in the activity of the *pMMO* enzyme between control *P. balearica* and modified *P. balearica* with the CRISPR system. While Tapscott *et al.* (2019) noted in their initial finding that evaluated Cas9 or Cas9D10A expression in *Methylococcus capsulatus* demonstrated that nuclease expression did not alter bacterial growth in the absence of gRNA expression.

The normally dormant Cas9 enzyme becomes active with the addition of Mn^{+2} to the media. This was concluded by Sundaresan *et al.* (2017) who found that Cas12a and Cas9 may cleave DNA in the presence of Mn^{2+} ions even in the absence of a guide RNA. Both Mn^{2+} and Co^{2+} ions have the extraordinary capacity to attach to the DNA strand between a guanine base and a neighbouring phosphate, which results in a localized unwinding impact on the DNA (Campbell and Jackson, 1980).

Without a guide RNA, Mn^{2+} and Co^{2+} may help DNA cleave in the active site. Mn^{2+} and Co^{2+} enhance a productive orientation of the protein's metal, phosphate, and active site residues in DNA ligase D 3́-phosphoesterase (PE) enzyme crystal structures, whereas Zn^{2+} does not. A large number of DNA repair enzymes require Mn^{2+} but are inactive in the presence of Mg^{2+} (Das *et al.*, 2012; Zhu and Shuman, 2005).

In this study, CRISPR-based gene editing with Cas9D10A nickase was employed successfully, this corresponds to Song *et al.* (2017) and Xu *et al.* (2015). This variant features a RuvC1 nuclease domain that has been deactivated so that it can cause single-stranded DNA (ssDNA) nicks in order to induce single-nick-assisted HDR (Jinek *et al.*, 2012).

Tapscott *et al.* (2019) found that the Cas9D10A-mediated nick provides higher chromosomal recombination efficiency than a Cas9 dsDNA break in *Methylococcus capsulatus*, resulting in more transformants and improved mmoX locus-editing efficiency. Previous research has shown that single-nick-assisted HDR is capable of undergoing repair through a separate mechanism with a better fidelity than dsDNA break-induced repair (Metzger *et al.*, 2011).

5.14. Biodegradation of crude oil

Oil pollution has a serious threat to the natural world, necessitating this field of investigation. This field needs the researchers to be better in comprehending the natural mechanisms that are used in oil bioremediation techniques to degrade oil components to improve the process.

Bacteria have the ability to change hydrocarbons from their inert condition as metabolically inactive molecules into more active forms that may then be used for further catalysis. This process is a relatively complex one (Ladygina *et al.*, 2006).

5.14.1 Estimation of aliphatic part biodegradation before and after CRISPR-Cas9

A GC analysis of the control (crude oil) without bacteria was carried out to corroborate the hydrocarbon-degrading activity in MSM, and the results showed that it was a mixture of different hydrocarbons (C_{12}-C_{37}). The outcomes of this analysis were then compared with the GC outcomes of oil extracts from the inoculated media.

The nearly complete removal of the corresponding peak of each chemical considered as proof of the conclusion. The ability of isolates to assimilate hydrocarbons in a liquid medium is thought to be the result of adaptation brought on by prior exposure to hydrocarbons. It might reveal the capacity for emulsifying hydrocarbons, which is crucial for absorbing and assimilating hydrocarbons (Abdulla *et al.*, 2019).

The n-alkanes, which are the components of aliphatic hydrocarbons that break down the quickest, are divided into four classes based on their molecular weight: gaseous alkanes, aliphatic hydrocarbons with molecular weights ranging from C_8 to C_{16} for the lowest, C_{17} to C_{28} for the middle, and

more than C_{28} for the highest. The n-alkanes that break down the quickest are the gaseous (Riazi, 2005).

In spite of the fact that the majority of bacteria that are capable of degrading hydrocarbons can only do so for the lowest category of hydrocarbons, Van Beilen and Funhoff (2007) found that aliphatic compounds exceeding C_{44} were also destroyed in their investigation. However, these long-chain alkanes can only be broken down if enzymes have first converted them into their active forms.

Petroleum hydrocarbons are the only carbon and energy that bacteria can use when living in contaminated environments since they can be digested. The ability of a bacterium to incorporate molecular oxygen into a hydrocarbon and produce intermediates that enter the cell's main metabolic pathway to produce energy is determined by genetics (Wemedo *et al.*, 2018).

The majority of alkane-degrading enzymes are capable of working with a wide variety of substrates. Not only do normal alkane monooxygenases have the capacity to degrade a wide variety of substrates, but other monooxygenases, such as those that include cytochromes as well as dioxygenases, also have this capability (Abbasian *et al.*, 2015).

Globally this is the first time found a new strain of *M. extorquens* has the ability to degrade aliphatic hydrocarbons compound at 61.14% within 7 days (Fig. 4-31). This degradation rate is higher than the 51.64% and 58.31% at 7 days reported for *Ochrobactrum anthropic* and *Sphingomonas paucimobilis*, respectively, However, it was lower than the 83.8% and 81.63% at 0.5% crude oil reported for *Vibrio vulnificus* and *Brevundimonas diminuta*, isolated from contaminated soils in the Khor Al-Zubair channel, southern Iraq (Al-Taee *et al.*, 2017).

This might be because these bacteria were taken from polluted environments where they had been exposed to a wide variety of chemicals and oils. In general, gram-negative bacteria are the most effective in breaking down petroleum hydrocarbons down (Dong *et al.*, 2020; Yang *et al.*, 2012).

During their exponential development phase, bacteria used the hydrocarbon substrate as a source of carbon and energy, as evidenced by a dramatic drop in peaks between days 0 and 7.

The levels of activity of the enzymes dehydrogenase and catalase can be used to figure out how much microbiological processes are going on in the soil. Catalase and dehydrogenase play important roles in the breakdown of hydrocarbons in environments where the soil has been contaminated with oil products. Catalase is an enzyme that speeds up the oxidation of hydrocarbons by eliminating the hydrogen peroxide that is produced when microbes do their necessary essential activities in order to obtain the oxygen that they require. Dehydrogenase is the enzyme that is responsible for catalyzing the dehydrogenation process, which is the removal of hydrogen from hydrocarbons and the products of their breakdown (Akhmetov *et al.*, 2022).

Additionally, *Methylobacterium mesophilicum* strain RD1 was discovered by Salam *et al.* (2015) to have destroyed between 61.2% and 89.5% of the initial concentration of used motor oil over the course of 12–21 days, they ascribe this to strain RD1's abundance of degradative genes.

After 7 days of incubation with 0.5% crude oil of CRISPR Cas9-cured *M. extorquens*, the percentage of breakdown of the aliphatic component residues was 74.35% (Fig. 4-32). The data showed the potentiality of the CRISPR system to improve biological therapy by increasing the rate of metabolism, which is consistent with the increased gene expression of bacteria modified with the CRISPR system.

CRISPRi has been shown to be incredibly useful. In a study by Mo *et al.* (2020) using the CRISPRi system with a small sequence of sgRNAs, screened rapidly out a new phytoene desaturase encoded by META1_3670 responsible for carotenoid biosynthesis. Lee *et al.* (2018) quickly discovered an unidentified carboxyl esterase for the breakdown of methyl acetate in *C. glutamicum* using CRISPRi technology. Traditional industrial strains like *Corynebacterium glutamicum* and *E. coli* have shown that CRISPRi is particularly effective at discovering targeted genes from a collection of candidate genes (Lee *et al.*, 2018; Wang *et al.*, 2018).

While *P. balearica* was able to consume crude oil and reduce the amount of aliphatic material by 90.32% (Fig. 4-33). These results confirm previous research that indicated that *P. balearica* was a component of bacterial group that degraded the total petroleum hydrocarbons (TPH) in crude oil (Yetti *et al.*, 2018). and another study by Pandey *et al.* (2018) isolated *P. balearica* from oil-contaminated soil near the Mathura Refinery, it was able to metabolize n-alkanes and polycyclic aromatic hydrocarbons in diesel fuel..

Hamad *et al.* (2021) showed that microorganisms isolated from locations that had oil pollution were more effective in degrading petroleum hydrocarbons than bacteria were isolated from locations did not have oil contamination. Almansoory *et al.* (2019) isolated *Pseudomonas* sp. from a variety of polluted locations in Iraq. Gas chromatography showed that the crude oil degradation rates were 65%. In another study, Liu *et al.* (2022) isolated a strain of *Pseudomonas aeruginosa* had the ability to break down n-alkanes (C_{13}-C_{35}) in crude oil, found the degradation rates were 87 to 100 %.

In addition, Fadhil and Al Baldawi (2020) noticed that *Pseudomonas stutzeri* could break down TPH at 68.6% while displaying a strong capacity to digest C_{10}-C_{12}, C_{17}, C_{19}-C_{20}, and C_{24}-C_{26} over a course of seven days.

In *p. balearica* which modified with CRISPR-Cas9, the percentage of aliphatic component residues that had been broken down after 7 days of incubation with 0.5% crude oil was 92.90% (Fig. 4-34). This study illustrates the power of the CRISPR system to improve biological treatment by boosting the rate of catalysis, which is compatible with the increased gene expression of bacteria modified with the CRISPR system.

5.14.2. Estimation of biodegradation of aromatic compounds before and after CRISPR-Cas9

For bioremediation to be effective, microorganisms must enzymatically attack the contaminants and convert them into harmless molecules. Its application often requires the management of natural barriers to enable microbial development to grow at a faster rate since it is potent when ecological conditions encourage microbial development and migration (Trimurtulu, 2021).

Historically, petroleum hydrocarbons have been rated in the following order of decreasing susceptibility to microbial attack: n-alkanes, branching alkanes, low molecular weight aromatics, and cycloalkanes (El-Sheshtawy *et al.*, 2022; Medić *et al.*, 2020; Paudyn *et al.*, 2008).

M. extorquens was successful in degrading 65.69% of the aromatic portion of crude oil after being incubated for a total of seven days (Fig. 4-36). Basically, this might be due to the fact they were originally isolated from oil-contaminated areas. The findings are consistent studies discovered that *Methylobacterium populi* VP2 strain had the ability to degrade polycyclic aromatic hydrocarbons (PAH) found in polluted areas. *Methylobacterium* strains that have been isolated from extremely contaminated areas obtain all of their necessary carbon requirements from a wide variety of organic compounds (Amaresan *et al.*, 2020; Ventorino *et al.*, 2014).

Because of the wide variety of substrates that may be broken down by the *MMO* enzyme, methylotrophic bacteria have the potential to degrade a significant quantity of aromatic pollutants (Giri *et al.*, 2021).

In comparison, this rate of degradation is higher than that of *Sphingomonas paucimobilis*, *Vibrio vulnificus*, and *Ochrobactrum anthropic* isolated from contaminated soils in the Khor Al-Zubair channel, southern Iraq, which were 27.26%, 22.91%, and 19.00% in 7 days, respectively. For *Brevundimonas diminuta*, it is less than the reported 62.08% at 0.5% of crude oil (Alkanany *et al.*, 2017).

According to Salam *et al.* (2015), *Methylobacterium mesophilicum* has the ability to break down PAH components such as anthracene and pyrene. After a 21-day incubation period, the fractions of pristane, pyrene, and anthracene totally disappeared, whereas phytane (96.8%) showed a noticeable drop.

CRISPR-Cas9 modified *M. extorquens* was incubated with 0.5% crude oil for 7 days, 78.23% of the aromatic component residues were degraded (Fig. 4-37). The enhanced breakdown of aromatic compounds was obvious and consistent with the gene expression of CRISPR-modified bacteria, as did the increased breakdown of aliphatic compounds. The broad host range applicability of these genetic tools may also make it possible to come up with innovative solutions to overcome the challenges of genetically engineering a number of non-model microorganisms that have significant economic potential.

P. balearica strain was effective in decomposing 83.16 % of the aromatic component of the crude oil after being cultured for a total of seven days (Fig. 4-38). According to a study that was conducted by Medić *et al.* (2020), *Pseudomonas aeruginosa* was able to decompose polycyclic aromatic hydrocarbons (fluorene, phenanthrene, and pyrene) with efficiencies of 96%,

50%, and 41%, respectively, at starting concentrations of 20 mgL^{-1} and in a span of seven days.

In addition, Smułek *et al.* (2020) discovered that *Pseudomonas mendocina* and *Brevundimonas olei* were capable of degrading more than 60% of the total concentration of PAHs during the course of a test that lasted for 28 days. According to the findings of our study, prolonged exposure to aromatic chemicals caused the bacteria to start using PAHs as a source of both carbon and energy.

Kumari *et al.* (2018) demonstrated that several PAHs, including naphthalene, phenanthrene, enzo(b)fluoranthene, and fluorene, can be biodegraded by *M. esteraromaticumat*, *S. maltophilia*, and *P. aeruginosa* rates greater than 50% in 45 days. Patowary *et al.* (2018) found that, in just three months, *P. aeruginosa* was able to breakdown more than 60% of the petroleum oil that they used in their experiment.

Adebusoye *et al.* (2007) showed that different hydrocarbon mixes had very different solubility, volatility, and susceptibility to degradation, proving the requirement for mixed cultures for effective biodegradation of crude petroleum oil.

P. balearica that had been modified using the CRISPR-Cas9 system had 94.07% of its aromatic component residues destroyed after being cultured with 0.5% crude oil for seven days (Fig. 4-39). Both the increased breakdown of aliphatic compounds and the accelerated breakdown of aromatic compounds were visible and consistent with the gene expression of the CRISPR-treated bacteria.

Conclusions

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Recommendations

Conclusions

The following were concluded findings from the current study:

1. Based on these findings, one might draw the conclusion that oil-degrading bacteria are present in high numbers in soils that have been polluted with oil. These sites are of significant interest for isolating novel hydrocarbon-degrading bacteria with high catabolic abilities, which are enhanced by living in a highly polluted site.
2. Elevated bioremediation ability is attributed to the high and continuous exposure to hydrocarbon chemicals over time.
3. The presence of *MMO* and *MxaF* in bacteria isolated from oil-polluted areas and the ability of these bacteria to utilize single-carbon molecules indicate the significant colonization of methylotrophic bacteria at polluted areas.
4. The most frequent genus was *Methylorubrum* sp. and *Pseudomonas* sp. which was isolated from soil contaminated with crude oil. These bacteria can be used to bioremediate the hydrocarbons in contaminated locations since they can use crude oil as their only source of carbon.
5. This study demonstrated the ability of the employed aerobic methylotrophic bacteria to break down petroleum hydrocarbons irrespective of presence of CRISPR-Cas9 gene.
6. The application of the CRISPR-Cas9 system in methylotrophic bacteria suggests a workable and suitable genetic alteration technique to boost its susceptibility to bioremediation of pollutants, particularly hydrocarbons.

Recommendations

1. Finding new species of bacteria that have the ability to grow in methane or polluted soils.
2. Applying more efficient techniques to easily transform these bacteria by CRISPR.
3. Establishing a university central laboratory capable of conducting bioremediating studies financed by oil companies present in the Basrah & other oil producing cities.

Publications

1. Maki AA, Al-Taee AMR, Atwan ZW. Molecular identification of *Methylorubrum extorquens* using PCR-Amplified MxaF Gene Fragments as a molecular marker. Accepted in Baghdad Science Journal. December 8,2022.
2. Maki AA, Al-Taee AMR, Atwan ZW. Measuring the Degradation of Aromatic Compounds using *Methylorubrum extorquens* Isolated from Oil-Contaminated Soils in Southern Iraq. Accepted in Marine Science Journal. February 7,2023.

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Appendices

Appendix 1: DNA purity and concentration for some samples

ID #	SampleType	A260	A280	260/280	ng/ul
8	dsDNA	3.788	3.087	1.23	189.4
7	dsDNA	0.757	0.652	1.16	37.8
6	dsDNA	6.811	5.941	1.15	340.6
5	dsDNA	4.732	3.501	1.35	236.6
4	dsDNA	8.264	7.108	1.16	413.2
3	dsDNA	0.584	0.419	1.39	29.2
2	dsDNA	4.348	3.627	1.20	217.4
1	dsDNA	10.595	8.933	1.19	529.7

Appendix 1: RNA purity and concentration for some samples

ID #	SampleType	A260	A280	260/280	ng/ul
7	RNA	3.073	1.393	2.21	122.9
6	RNA	2.854	1.414	2.02	114.2
5	RNA	2.462	1.141	2.16	98.5
4	RNA	3.994	1.888	2.12	159.8
3	RNA	4.400	2.065	2.13	176.0
2	RNA	0.993	0.459	2.17	39.7
1	RNA	1.043	0.481	2.17	41.7

Appendix 2: Sequencing results for *Methylorubrum extorquens* from Gen Bank

Methylorubrum extorquens strain IMB16-167 16S ribosomal RNA gene, partial sequence

GenBank: MG190762.1
FASTA Graphics
Go to:
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ACCESSION MG190762 REGION: 11...1363
VERSION MG190762.1
KEYWORDS.
SOURCE Methylorubrum extorquens
ORGANISM Methylorubrum extorquens
Bacteria; Proteobacteria; Alphaproteobacteria; Hyphomicrobiales; Methylobacteriaceae; Methylorubrum.
REFERENCE 1 (bases 1 to 1353)
AUTHORS Hu,Y.Y., Meng,J.Z. and Gan,M.L.
TITLE Direct Submission
JOURNAL Submitted (10-OCT-2017) National Laboratory for Screeening New Microbial Drug Institute of Medicinal Biotechnology, Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, No. 2 Nan Wei Poad, Beijing 100050, China
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Methylorubrum extorquens strain IMB16-167 16S ribosomal RNA gene, partial sequence

GenBank: MG190762.1
GenBank Graphics
>MG190762.1:11-1363 Methylorubrum extorquens strain IMB16-167 16S ribosomal RNA gene,
partial sequence

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Pseudomonas balearica DSM 6083, complete genome

GenBank: CP007511.1

FASTA Graphics

Go to:

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DEFINITION Pseudomonas balearica DSM 6083, complete genome.

ACCESSION CP007511 REGION: 1037389...1038607

VERSION CP007511.1

DBLINK BioProject: PRJNA240670

BioSample: SAMN02673660

KEYWORDS.

SOURCE Stutzerimonas balearica DSM 6083

ORGANISM Stutzerimonas balearica DSM 6083

Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Stutzerimonas.

REFERENCE 1 (bases 1 to 1219)

AUTHORS Bennasar-Figueras,A., Salva-Serra,F., Jaen-Luchoro,D., Segui,C., Aliaga,F., Busquets,A., Gomila,M., Moore,E.R. and Lalucat,J.

TITLE Complete Genome Sequence of Pseudomonas balearica DSM 6083T

JOURNAL Genome Announc 4 (2) (2016) PUBMED 27103708

REMARK Publication Status: Online-Only

FEATURES Location/Qualifiers source 1...1219

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Pseudomonas balearica DSM 6083, complete genome

GenBank Graphics

>CP007511.1:1037389-1038607 Pseudomonas balearica DSM 6083, complete genome

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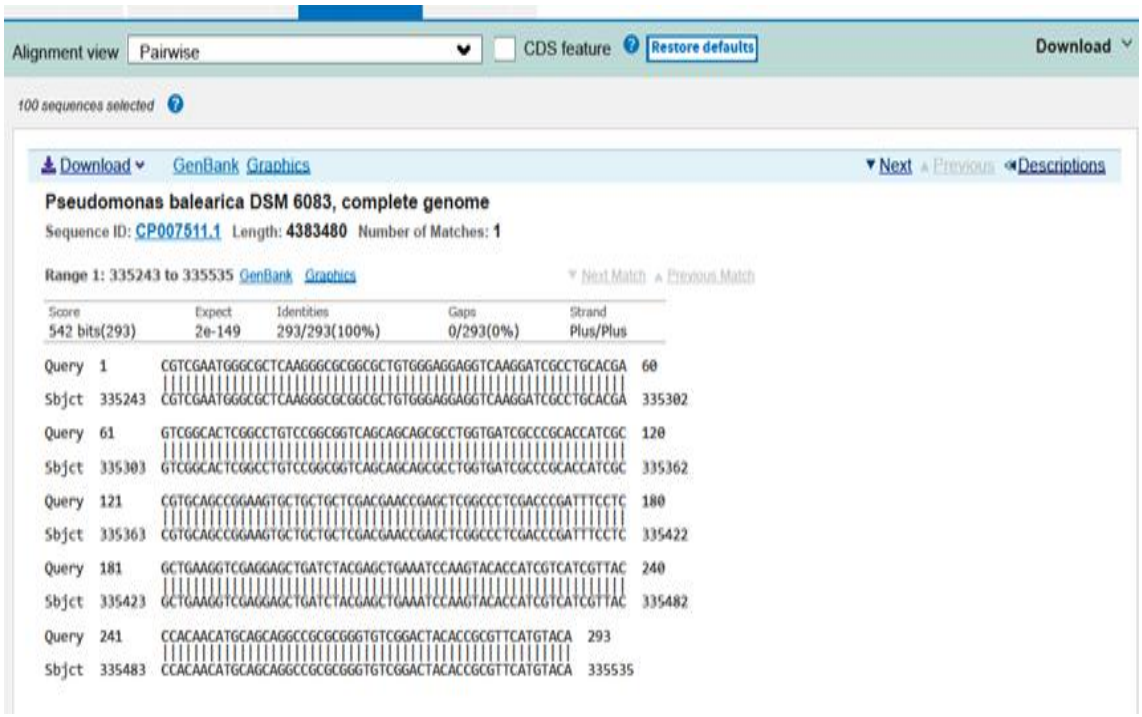
Appendix 3: Sequencing results for detect sMMO gene from Genbank



Pseudomonas balearica DSM 6083, complete genome

Gen Bank: CP007511.1

Gen Bank Graphics

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الخلاصة

يعد التلوث بالهيدروكربونات النفطية مشكلة خطيرة تهدد النظام البيئي في جميع أنحاء العالم. تتطلب إزالة مثل هذه النفايات العديد من الإجراءات المكلفة والمستهلكة للوقت، والتقنيات البيولوجية هي مثال فعال.

صُممت الدراسة لتطوير نهج تعديل وراثي جديد في بكتيريا Methylotrophic من خلال نظام تعبير فعال لتعزيز قدرة البكتيريا بيولوجيًا على استهلاك المركبات النفطية في البيئات الملوثة. حَقَّقَ هذا الهدف من خلال عزل بكتيريا الـ Methylotrophic من ثلاث محطات ملوثة بالمركبات النفطية مرتبطة بصناعات إنتاج البترول. عزلت بكتيريا الـ Methylotrophic باستخدام تقنية الاستزراع المخصب ووسط ميثانول قاعدي مدعوم بنسبة 2٪ من الميثانول كمصدر وحيد للكربون والطاقة. شُخصَت البكتيريا اعتماداً على الاختبارات الكيموحيوية والجينية باستخدام جين 16SrRNA كان لجميع التتابعات التي حللت قيمة متوقعة (E)تبلغ 0، وكان أكثر من 99 ٪ من الأجناس الصالحة المبلغ عنها مرتبطة ارتباطًا وثيقًا بنسبة هوية BLAST. وحددت على أنها *Methylorubrum extorquens* و*Pseudomonas balearica*.

فيما يتعلق بقدرة المعالجة الحيوية والنظام الأنزيمي المسؤول عن ذلك، استخدم بادئ متخصص لتضخيم جين *MMO*(المشفرلانزيم الميثان أحادي الأوكسجين) وجين *MxaF*(المشفر لانزيم ميثانول ديهيدروجينيز) لبكتيريا Methylotrophicالمحددة. اكتشفت حزمة بحجم 439 و550 زوج قاعدي تقريبًا من جين *MMO* الكروموسومي وجين *MxaF* المرتبط بالحمض النووي .أظهرت مقارنة التعبير الجيني MDH (انزيم ميثانول ديهايدروجينيز) عبر الأجناس المحددة زيادة بمقدار 44 ضعفًا في مزرعة *M. extorquens* مقارنةً بالسيطرة (*P. balearica*) .كان التعبير عن جين *pMMO* في *P. balearica* اعلى بعشر مرات من ذلك الذي حدد كميا في مجموعة السيطرة (*M.extorquens*).

أظهرت *P. balearica* نموًا على وسط مرق NMS في وجود الميثان كمصدر وحيد للكربون والطاقة، وكان اختبار النفثالين إيجابيًا حيث تحولت المستعمرات إلى اللون الأرجواني، بينما لم تنمُ *M. extorquens* على وسط مرق NMS وكان سلبي للنفتالين. استخدم وسط مرق MSM المضاف إليه الميثانول بتراكيز مختلفة كمصدر وحيد للكربون والطاقة، نمت *M. extorquens* بأعلى مستوى بتركيز 1٪ و2٪، حيث بلغت الكثافة الضوئية 1.9 و1.8 على التوالي، بينما زاد نمو *P. balearica* بنسبة 1٪ و3٪ وبلغت الكثافة الضوئية 0.1. أظهر كلاهما أبطأ نمو عند التركيز

6٪ من الميثانول. كان تعبير MDHفي *M. extorquens* 12 ضعف بتركيز 1-4٪، بينما قل إلى 1 عند تركيز 6٪ ميثانول.

أظهر كلا الجنسين القدرة على النمو في وسط معدني يحتوي على 1 ٪ من النفط الخام. أظهرت بكتريا *P. balearica* نمواً أعلى من *M. extorquens* بعد 7 أيام من الحضن في حاضنة هزازة عند 120 دورة في الدقيقة و30 س° وكانت الكثافة الضوئية 0.17 و0.09 على التوالي. أظهر اختبار الحساسية للمضادات الحيوية أن الحد الادنى للتثبيط في *M. extorquens* للكاناميسين والأمبيسيلين والسبيرامايسين كانت (20، 200.500) ميكروغرام / مل على التوالي. اما *P. balearica* كان الحد الأدنى للتثبيط (50، 200، 100) ميكروغرام / مل على التوالي.

ادخل نظام CRISPR-Cas9 المعالج بالبلازميد في DNA الكروموسومي البكتيري الذي أكده التعبير الجيني لنظام إدراج CRISPR-Cas9 باستخدام تقنيةqPCR . في بكتيريا *M. extorquens* المعالجة بـ CRISPR، زاد التعبير الجيني لـ MDH حوالي 6أضعاف اما في *P. balearica* المعالج بـ CRISPR، ارتفع التعبير الجيني لـ PMMO بمقدار 25 ضعفًا. اختيرت تقنية CRISPR Cas9 للدراسة والاستخدام في المختبر لتحسين كفاءة التحلل البيولوجي للهيدروكربونات (n-alkane وPAH).

في غضون 7 أيام من فترة الحضانة، زاد معدل التحلل الحيوي للمركبات الأليفاتية في *M. extorquens* المعدلة من 61.14٪ إلى 74.35٪ مقارنةً بالسيطرة، اما في بكتريا *P.* balearica المعدلة، زاد معدل التحلل البيولوجي من 90.32٪ إلى 92.90 ٪. اما بالنسبة للهيدروكربونات العطرية متعددة الحلقات في *M. extorquens* المعدلة، فقد ارتفعت النسبة من 65.69٪ إلى 78.23٪. أما بكتريا *P. balearica* المعالجة فقد ارتفع من 83.16٪ إلى 94.07٪. على حد علمنا في هذه الدراسة، تم عزل البكتيريا Methylotrophic لأول مرة في العراق، وبالإضافة إلى ذلك، فهذه هي المرة الأولى التي يتم فيها استخدام نظام CRISPR-Cas9 على مستوى العالم لزيادة فعالية هذه البكتيريا في التحلل البيولوجي لجزيئات الهيدروكربون.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة البصرة/كلية العلوم

قسم علوم الحياة

تحسين أداء بكتريا Methanotrophsباستخدام نظام تعديل الجينات CRISPR / Cas9

أطروحة مقدمة الى مجلس كلية العلوم-جامعة البصرة

كجزء من متطلبات نيل درجة الدكتوراه في علوم الحياة

أحياء مجهرية

من قبل

أنوار عبد الوهاب مكي

بكالوريوس علوم حياة/ جامعة البصرة (1999)

ماجستير علوم حياة/ جامعة البصرة (2015)

بأشراف

الأستاذ	الأستاذ
الدكتور أسعد محمد رضا الطائي	**الدكتورة زينة وحيد عطوان**

1444 هـ

2023م