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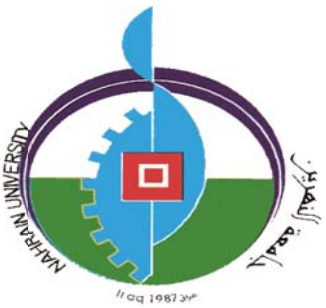

Desulfurization and Utilization of Dibenzothiophene by Some Local Bacterial Isolates

A Thesis

Submitted to the council Science College / Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology

By

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DEDICATION

To

My father

To my role model in this life, I never seen a man like him in the whole universe, he can be serious in serious things and warm affectionate all the time, he is so educated that we learn from him the important ethics and values that give us the push to keep going in life and stimulates us to give our best to the world, my father is the sunshine of our lives.

My mother

Your arms were always open when I needed a hug. Your heart understood when I needed a friend. Your gentle eyes were stern when I needed a lesson. Your strength and love has guided me and gave me wings to fly. All that I am or ever hope to be, I owe to my angel Mother.

My sisters (Nuha and Abrar)

For happy times shared through the years for the loyalty, love for the laughter and tears for the special things only they can do.

I dedicated my thesis work

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Albab

Summary

The main aim of this study was obtaining efficient bacteria capable of desulfurizing and/or utilizing organosulfur compounds that found in petroleum and its derivatives. For this purpose oil contaminated soil samples with a history of oil pollution were collected from forty different sites in Iraq.

Sixty three bacterial isolates were obtained and these isolates capable to utilize dibenzothiophene (DBT). Results showed that most of these isolates were capable to grow on DBT in the presence or without glycerol (carbon source). The growth density of bacterial isolates in presence of glycerol was more than growth density without glycerol. Results also showed that the growth density of bacterial isolates was a little more in medium containing ethanol plus magnesium sulfate compared to the growth in DBT plus ethanol medium. This means that these isolates were capable to utilize ethanol as C- source.

From all isolates, only three isolates (M9, M19 and S25) had the ability to desulfurize DBT (cleave C-S bond) and converted it to 2-hydroxybiphenel (2-HBP) or other phenolic end products and gave blue color in the presence of Gibb's reagent. Depending on this result it can be suggested the involvement of the 4S pathway in the utilization of DBT via a specific cleavage of only the C-S bond by these isolates.

In addition to the three isolates which gave positive results with Gibb's assay, twelve efficient isolates (M3, M7, M16, M20, P4, P13, P14, S21-1, S22, S26, S34 and S37) could be utilized C ands. However, P13 isolate was the best one that cleaved both C-C and C-S bonds of DBT were candidate for identification. These fifteen isolates were identified as: *Staphylococcus* spp. [6 isolates, (including P13 isolate)], three of them were *Staphylococcus aureus. Pseudomonas* spp. (4 isolates), three of them were *Pseudomonas aeruginosa*. *Micrococcus* spp. (2 isolates), and one isolate for each genus of *Neisseria* sp., *Corynebacterium* sp. and *Bacillus* sp. The DBT desulfurizing (*P. aeruginosa* M9, M19 and S25) and utilizing (P13) bacteria were grown in medium containing different carbon and sulfur sources. Highest growth of all isolates

was obtained in medium containing glycerol and magnesium sulfate as carbon and sulfur sources respectively, because these compounds are simple sources for carbon and sulfur.

To select one isolate from the three *P. aeruginosa* isolates (M9, M19 and S25), the concentration of the end product, resulted from culture grown with DBT was quantified by measuring the intensity of the corresponding blue color formed after addition of Gibb's reagent. The result showed that *P. aeruginosa* S25 was the efficient one for removing sulfur from DBT.

The optimum conditions for utilization of DBT by *P. aeruginosa* S25 and P13 isolate were investigated. It was found that these conditions are growing these bacteria in basal salt medium (pH 8) containing 0.6 mM DBT and incubated with shaking (150 rpm) at 35 °C for three days.

In order to determine the plasmid profile for *P. aeruginosa* (S25) and P13 isolate, a number of DNA extraction methods were used. Results showed that *P. aeruginosa* (S25) harbor small plasmid DNA bands, while there was no plasmid in P13 isolate.

The GC/MS analysis of bacterial cultures (*P. aeruginosa*–S25 and P13 isolate) on DBT-Basal salt medium, indicated that 12.89 % of DBT was consumed (consumption of sulfur) by the *P. aeruginosa*-S25, and the GC/MS analysis for the P13 isolate showed that this isolate utilize the DBT as sulfur and carbon source. The GC/MS analysis of bacterial cultures on diesel-BSM indicated that all compounds that have sulfur atoms were degraded to less complex compounds by *P. aeruginosa*–S25 and P13 isolate.

The sulfur content X-ray analysis of bacterial cultures indicated that these isolates utilize about 89 % and 34 % of total sulfur from DBT-BSM by P13 isolate and *P. aeruginosa*-S25 respectively. The percentage of sulfur consumption from diesel-BSM was about 5.9 % by P13 isolate and 5.4 % by *P. aeruginosa*-S25. Consequently both isolates showed a very good ability desulfurize and utilize DBT, they also showed good ability to consume sulfur from diesel.

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List of Abbreviations

Abbreviations	Term
API	American petroleum institute
BDS	Biocatalytic desulfurization
BSM	Basal salt media
C	Carbone
Cont.	Control
DBT	Dibenzothiophene
DBTO	Dibenzothiophene sulfoxide
$DBTO_2$	Dibenzothiophene sulfone
DNA	Deoxyribonucleic acid
D.W	Distilled water
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetic acid
EPA	Environmental Protection Agency
FCC	Fluid catalytic cracking
HBP	Hydroxybiphenyl
HBPS	Hydroxybiphenyl sulfunic acid
HDS	Hydrodesulfurization
Kb	Kilo Base
LB	Luria- Bertani
$MgSO_4$	magnesium sulfate
MR	Methyl red
NaCl	Sodium chloride
NO_2	Nitrogen dioxide
ODS	Oxidative desulfurization
ORFs	Open reading frames
PAH	Polyaromatic hydrocarbon
PASHs	Polyaromatic sulfur heterocyclic
PCR	Polymerase chain reaction
pH	Power of hydrogen (H^+)
PHC	Petroleum hydrocarbon
R. erythropolis	*Rhodococcus erythropolis*
S	sulfur
SDS	Sodium dodecyl sulfate
SO_x	Sulfur dioxide(So_2), So_3,So_4…. etc.

SRB	Sulfate reducing bacteria
TBE	Tris-Borate –EDTA
TH	Thiophene
TSI	Triple sugar iron
VP	Vogas-proskaure

Chapter one introduction and literatures review

1. Introduction and Literatures Review

1.1. Introduction.

Oil refineries are facing many challenges, including heavy crude oils, decreased fuel quality and a need to reduce air pollution emissions. Crude oil (Petroleum hydrocarbon) continues to be used as principle source of energy and hence a large global environmental pollutant, due to the possibility of air, water and soil contamination. Petroleum is a complex mixture of non-aqueous and hydrophobic components. Many of these components are toxic, mutagenic and carcinogenic (Mandal *et al.*, 2012).

Combustion of petroleum-derived fuels leads to the release of vast amount of sulfur dioxide (SO_2) into the atmosphere, which is the principle source of acid rain and air pollution. Thus, most countries have imposed strict regulations to control these releases mainly by enforcing stringent restrictions on the levels of sulfur in transportation fuels. However, a common problem, petroleum refineries are facing around the world is that crude oil reserves being used as feedstock for refining process are becoming heavier day after day with elevated sulfur contents (Bhatia and Sharma, 2012; Li and Jiang, 2013).

Clean fuel research, including improvement of the processes of desulfurization and dearomatization, has become an important focus on environmental catalysis studies worldwide. Sulfur contained in diesel fuel is an environmental concern because the sulfur is converted to So*x* during combustion, which not only contributes to acid rain, but also poisons the catalytic conversion widely installed for exhaust emission treatment. The problem of sulfur removal has become more apparent due to the high sulfur contents in crude oils and the low limit of sulfur content in finished fuel products specified by regulatory authorities. The Environmental Protection Agency of the United States (EPA) had set a target to reduce the sulfur content of diesel fuel from 500 ppm to 15 ppm at the year 2006 and 10 ppm will become the maximum content for sulfur by 2008 (Yang and Marison, 2005). Thiols,

sulphides and thiophenes are readily removed by hydrodesulfurization (HDS), but up to 70% of the sulfur in petroleum is found as dibebzothiophene (DBT) and substituted (methylated) DBTs, which are particularly recalcitrant to HDS treatment compared with mercaptans and sulfides (Le Borgne *et al*., 2003; Yang and Marison, 2005).

However, there was a direct correlation between the use of fossil fuel and environmental pollution. The impurities present in fossil fuel, primarily nitrogen and sulfur, after combustion lead to the emission of various gases such as sulfur dioxide (SO_2) and nitrogen dioxide (NO_2) (Gupta *et al.,* 2005). These gases contribute to acid rain, greenhouse effects, and particulate matter formation. Long-term exposure to particulate matter leads to various cardiopulmonary and lung diseases (Brook *et al*., 2004; Neuberger *et al*., 2004).

The increasing sulfur content of crude oil also results in an increase in sulfur content in automotive gasoline, diesel fuel, and jet fuel. To meet the needs for producing clean fuels, decreasing the sulfur content of crude oil becomes an urgent task. Studying new desulfurization technology and raising the efficiency of desulfurization processes are the keys to bringing more profits to the oil refining companies (Liu *et al*., 2007).

Microbial methods of removing sulfur from organosulfur compounds are of interest to the petroleum industry for reducing sulfur emissions and for reducing heavy oil viscosity. As conventional crude oils are consumed throughout the world, heavier oils are being exploited which, due to their high viscosity, cannot be transported from remote field sites to refineries without adding diluents. The vacuum residue fraction of crude oils (boiling point 524 °C [975 °F]) contributes to viscosity, and models indicate that alkyl sulfides compose important bridges in the network of high-molecular weight molecules in this fraction (Murgich *et al*., 1999).

Several microbial strains belonging to various genera such as *Pseudomonas, Rhodococcus, Gordonia, Paenibacillus,* and *Mycobacterium* have

been found to show desulfurization activity. These organisms use diverse biochemical mechanisms, such as Kodama and 4S pathways, to metabolize various polyaromatic sulfur heterocycles (PASHs). Of these, *R. erythropolis* IGTS8 was the first to be isolated for its ability to specifically cleave the C–S bond in PASHs without affecting the C–C bond (Kilbane and Jackowski, 1992). Since then, several *Rhodococcus* strains have been studied for specifically desulfurizing DBT and its derivatives via the 4S pathway (Honda *et al.,* 1998; Davoodi-Dehaghani *et al.,* 2010).

Global society is stepping on the road to zero-sulfur fuel, with only differences in the starting point of sulfur level and rate reduction of sulfur content between different countries. Among the new technologies one possible approach is biocatalytic desulfurization (BDS). The advantage of BDS is that it can be operated in conditions that require less energy and hydrogen. BDS operates at ambient temperature and pressure with high selectivity, resulting in decreased energy costs, low emission, and no generation of undesirable side products.

The aim of the current study was to obtain a potential biocatalytic (bacterial isolates) for desulfurization and / or bioremediation of dibenzothiophene (DBT) this was achieved by the following steps :

- Isolation and identification of bacteria capable of utilizing organosulfur compound (DBT).
- Screening the bacterial isolates and select the efficient isolates in utilizing DBT as a sole S-source and C and S source.
- Optimization of culture media to achieve high cell density of DBT-utilizing bacteria.
- Determine the plasmid(s) profile for the efficient isolate.
- Testing the utilization of DBT and diesel using GC/MS and sulfur content X-ray analysis.

1.2. Literatures Review.

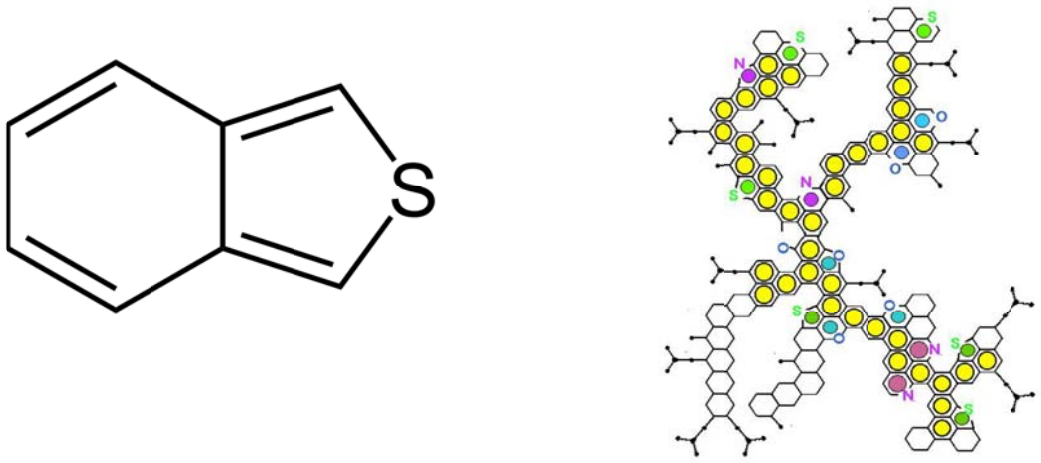
1.2.1. Crude Oil.

Petroleum is defined as “A complex combination of hydrocarbons". It consists predominantly of aliphatic, alicyclic and aromatic hydrocarbons. It may also contain small amounts of nitrogen, oxygen and sulfur compounds (Speight, 1999). Crude oil is a naturally occurring substance derived from the decomposition over millions of years of plant and animal organic matter under elevated temperature and pressure. In appearance, crude oils range from mobile, volatile, light colored liquids to dark, viscous tar-like materials with low vapor pressure. They are generally classified by their density, predominant type of hydrocarbon (paraffinic or naphthenic) present, and whether their sulfur content is high (sour) or low (sweet). As a general rule, crude oils with less than 1 % sulfur are “sweet” and crude oils with over 1 % are “sour". An “average” crude oil contains 84 % carbon, 14 % hydrogen, 1-3 % sulfur, and approximately 1.0 % nitrogen, 1.0 % oxygen and 0.1 % minerals and salts (Kaes, 2000).

Crude Oil or Petroleum is simply unprocessed oil found deep beneath the earth’s surface. It can range in color from clear to black and can be found as a liquid or solid. Overall properties of crude oils are dependent upon their chemical composition and structure. Generally all crude oils are made up of hydrocarbon compounds. The main hydrocarbons found in crude oil are Aliphatics, Alicyclics, and Polycyclic Aromatic Hydrocarbons (PAH). The aromatic carbon compounds (PAH’s) found in crude oils can range from simple structures like benzo-c-thiophene to very complex ones like asphaltene as shown in Figure (1-1). The Aromaticity of the compound plays a key role in determining its fluorescence intensity (Wilhelm and Spitz, 2003).

Crude oil continues to be used as the principal source of energy and play an important role in the global environmental pollutant consideration. On the other hand, oil will remain as a major source of energy in the next several decades,

because reliable alternative energy consumption has not yet been substituted. (Trindade *et al*., 2005; Al-saleh and Obuekwe, 2005).



(A) **(B)**

Figure (1-1): The aromatic carbon compounds found in crude oils, Benzo-c-thiophene simple structure (A); Asphaltene (B).

Liu *et al*.,(2010) reported that the poor quality of crude oil currently can obviously result in the high sulfur content of oil products, which can lead to corrosion, catalyst poisoning, environmental pollution and other negative consequences.

1.2.2. Sulfur level in oil fractions and legislative regulations.

Sulfur and sulfur containing compound can be termed the unwanted species found in the petroleum and petroleum products because their harmful effects on the refinery facilities, automotive and combustion engines which use the fuels and the environment in general (Speight, 1999). This has led to the stringent environmental regulations which have been placing considerable pressure on refinery operators worldwide to reduce sulfur contents of crude oil products, most especially the fuels. Sulfur is usually the third most abundant element in

crude oil (Pokethitiyook *et al*., 2008). All fossil fuels contain variety of organic and inorganic sulfur compound. Sulfur usually accounts for around 0.03-7.89 wt % of crude oil but depending on the sulfur content of any given crude oil supply (Mohebali *et al*., 2007). Fuel desulfurization represented an opportunity for a cost effective solution to acid rain and other health hazard caused by sulfurous emission (Rhee *et al*., 1998; Mohebali *et al*., 2007).

The American Petroleum Institute (API) mentioned that gravity of oil is decreasing and sulfur content is increasing resulting in an increase in the sulfur concentrations in finished petroleum products. Sulfur is preferentially associated with the higher molecular mass components of crude oils. When crude oil is refined the sulfur concentrates into the high molecular mass fractions (Swaty, 2005).

Sulfur in crude oil exists in two main forms based on their reactivity with metals as either "active" or "inactive" sulfur. The active sulfur can react directly with metal while the inactive dose not (Rasina *et al*., 1988). Active sulfur includes elemental sulfur, hydrogen sulfide and mercaptan while the inactive one includes sulfide, carbon disulfide, thiophene (TH), etc. It is found that in fluid catalytic cracking (FCC) gasoline, more than 90 % of the sulfur present exist in the form of thiophene and its derivatives, while in diesel, thiophenic sulfur accounted for 80 % of the total sulfur, and benzothiophene (BT) and dibenzothiophene (DBT) accounted for more than 70 % of this thiophenic category (Hagen *et al*., 2004; Anonymous, 2006; Gaofei *et al*.,2009).

A typical flue gas from the combustion of fossil fuels will contain quantities of NO_x, S and particulate matter. SO_2 gas at elevated levels can cause bronchial irritation and trigger asthma attacks in susceptible individuals. Potential health risks expand to a broader section of the public when the gas turns to particulate matter (see http://www.epa.gov/acidrain/). Long-term exposure to combustion related fine particulate air pollution is an important

environmental risk factor for cardiopulmonary and lung cancer mortality (Pope *et al.*, 2002).

1.2.3. Bioremediation technology.

Simply defined, bioremediation is the use of biological systems to destroy or reduce the concentrations of hazardous wastes from contaminated sites. Such systems have the potentially broad-spectrum site applications including ground water, soils, lagoons, sludge and process waste-streams, and it has been used in very large scale applications such as the shoreline cleanup efforts in Alaska, resulting from the oil tanker "Exxon Valdez" oil spill in 1989 (Caplan, 1993).

Bioremediation is also defined as any process that uses microorganisms or their enzymes to return the environment altered by contaminants to its original condition, is an attractive process due to its cost effectiveness and the benefit of pollutant mineralization to CO_2 and H_2O (Da Cunha, 1996). It also provides highly efficient and environmentally safe cleanup tools (Margesin, 2000).

At specific sites where the contaminants are petroleum products, the spectrum of necessary professional expertise is greatly expanded. However, four important aspects are necessary in bioremediation studies, and these include microbial composition, contaminant type, geology of polluted site and chemical conditions at the contaminated site (Aichberger *et al*., 2005).

However, the key component in bioremediation is the microorganisms, which produce the enzymes involved in the degradative reactions leading to the elimination or detoxification of the chemical pollutant. Due to the expected superiority and metabolic versatility of mixed cultures over pure cultures, they are being applied for the treatment of petroleum wastes in ferment or-based systems (Van Hamme *et al*., 2000).

Petroleum biotechnology is based on biotransformation processes. Petroleum microbiology research is advancing on many fronts, spurred on by knowledge of cellular structure and function gained through molecular and

protein engineering techniques, combined with more conventional microbial methods. Current applied research on petroleum microbiology encompasses oil spill remediation, fermenter- and wetland-based hydrocarbon treatment, biofiltration of volatile hydrocarbons, enhanced oil recovery, oil and fuel biorefining, fine-chemical production, and microbial community based site assessment (Van Hamme *et al*., 2003).

Among the major hydrocarbon products, benzene is of major concern as it is stable, water miscible, highly mobile, poisonous and cancer-causing aromatic compound. Successful degradation of benzene by microorganisms in an aerobic environment has been reported; however, under anaerobic conditions its rate of biodegradation is observed to be very slow and poor (Singh *et al.,* 2009; Vogt *et al*., 2011). The common bacterial genera exploited for benzene bioremediation are *Pseudomonas, Bacillus* (Mukherjee *et al.,* 2012), *Acinetobacter* (Kim and Jeon, 2009), *Gammaproteobacteria* (Sei and Fathepure, 2009), and *Marinobacter* (Berlendis *et al.,* 2010). The other bacterial species identified for diesel biodegradation were *Pseudomonas aeruginosa* (Mariano *et al.,* 2010), and *Staphylococcus aureus* (Shukor *et al.,* 2009).

Microorganisms in soil have a broad array of catabolic activities, and simple ways of degrading pollutants is to add the compounds or materials containing them to the soil and rely on the indigenous microflora. This procedure is called “land farming” or “land treatment”, and has been frequently used by the oil industry to decontaminate oily wastes. It has been utilized for many years. It is also employed if oily or hydrocarbon-rich materials are spilled on soil. The employment of microorganisms in the biodegradation of hydrocarbons over chemical or conventional treatment is preferred for many reasons. First of all, the potential and selected microbes can alter raw and crude oils in beneficial ways and the resulting end products are comparatively safer to the environment and all living beings. Microorganisms have been employed for bioremediation of hydrocarbon-rich waste material products, along with their

various recalcitrant noxious compounds, which are converted into environmentally friendly products. These microbes utilize waste material as carbon substrate, increase their population, and ultimately biodegrade hydrocarbon products to nontoxic products, such as H_2O and CO_2 (Toledo *et al.,* 2006).

1.2.4. Dibenzothiophene.

Dibenzothiophene (figure 1-2) is a sulfur heterocyclic compound and is quite persistent in the environment. Dibenzothiophene and its substituted analogus are found in several woody plants as stress chemicals (Gottstein and Gross, 1992). A variety of sulfur-containing heterocyclic organic compounds in fossil fuels have been characterized. Since dibenzothiophene (DBT) is a typical recalcitrant organic sulfur compound in fossil fuels, desulfurization of DBT has been a model reaction in the treatment of fossil fuels. Most of the sulfur in the crude oil is organically bound mainly in the form of condensed thiophenes. Up to 70 % of sulfur in petroleum is found as dibenzothiophene (DBT) and substituted DBTs (methylated DBTs and benzo DBTs) that are particularly recalcitrant to conventional hydrodesulfurization HDS (Li *et al*., 2005; Mohebali *et al*., 2007) especially when they are alkylated at position 4 and 6 (Grossman *et al*.,2001; Tao *et al*., 2006).

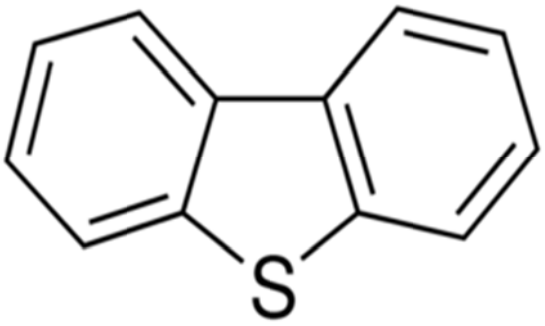


Figure (1-2): Chemical structure of Dibenzothiophene

The sulfur-containing compounds of crude oil have many different structures such as elemental sulfur, hydrogen sulfide, sulfides, mercaptans and heterocycles. Among these groups, heterocycles such as dibenzothiophene (DBT) or its derivatives are quite important, because they have higher boiling points (higher than 200 °C), which makes it is difficult to remove from the atmospheric tower outlet streams of oil refineries (Li *et al*., 2003).

Coal and crude oil contain many of the same organic sulphur structures, which include thiols, mercaptans, thiophenes and sulfides (Peinder *et al.,* 2010; Liu *et al.,* 2010; Lam *et al.,* 2012) which are required to be removed, prior to its use in automobiles and industry. The compound dibenzothiophene (DBT) has received most attention in biodesulphurization studies because it is an accepted model compound representative of thiophenic structures found in coal and petroleum. For this reason, biodesulfurization of crude oil and its fractions has been studied extensively (Kilbane 2006; Mohebali and Ball, 2008; Xu *et al.,* 2009). As thiophenic sulfur accounts for a substantial proportion of the sulfur which remains in the fuel after HDS, and dibenzothiophene (DBT) is a typical recalcitrant thiophenic sulfur component of fossil fuels, desulphurization of DBT has been a model reaction in the treatment of fossil fuels. Most of the organisms use the carbon-destructive pathway proposed by Kodama *et al.* (1973), in which degradation is initiated by the cleavage of carbon-carbon bonds. Thus reducing the caloric value of fuel and in this pathway water soluble sulfur compounds are produced which are then unavailable for burning and are therefore forfeited. Therefore kodama pathway is considered unsuitable (Hussein *et al.*, 2013).

Biodesulfurization (BDS) processes have been proposed as an alternative technology for removing the recalcitrant organic compounds found after the conventional hydrodesulfurization treatment, mainly polycyclic aromatic hydrocarbons (PAH) as dibenzothiophene (Mohebali *et al*., 2007; Caro *et al*.,

2007). DBT has therefore been widely used as a model compound for biodesulfurization studies (Mohebali *et al*., 2007).

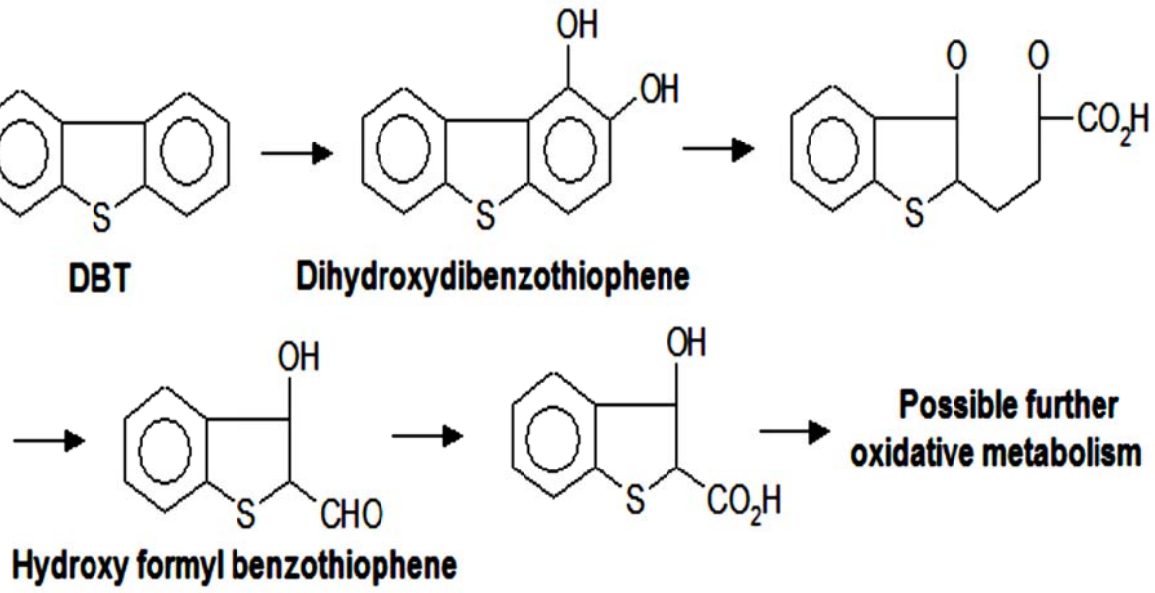


Figure (1-3): Kodama pathway for the degradation of DBT. The product, hydroxyl formyl benzothiophene, retains the sulfur moiety (Kodama *et al*., 1973).

Through 4S-pathway, dibenzothiophene is transformed to 2-hydroxybiphenyl and sulfite as end-products. Microbial systems have been reported to selectively take up the sulfur from the DBT molecule by consecutive enzymatic steps, leaving carbon skeleton intact of the DBT which is released intact as 2-hydroxybiphenyl (2-HBP); therefore, fuel value is not lost (Mohebali *et al*., 2007; Pokethitiyook *et al*., 2008).

Microbial desulphurization or biodesulfurization is expected to overcome the technical and economic problems associated with HDS as it has the potential benefits of lower capital and operating costs and will produce lesser greenhouse gases (Li *et al*., 2005).

1.2.5. Dibenzothiophene desulfurizing via C-S bonds cleavage pathway.

This process for removal of sulfur was first reported for *Rhodococcus rhodochrous* IGTS8 (Gallagher *et al.,* 1993). This Specific oxidative desulfurization involves a direct enzymatic attack on the sulfur-containing function. In the instance of DBT, the enzymatic attack occurs directly on the carbon-sulfur bond in the thiophenic ring of the molecule and not on the aromatic ring, as found in destructive desulfurization. The specific removal of sulfur via the biodesulfurization pathway involves formation of four S-containing compounds, namely sulfoxide, sulfone, sulfinate and sulfate. Hence this pathway is called the 4S pathway. The 4S pathway is a specific desulfurization pathway in which DBT is desulfurized and converted to 2-hydroxybiphenyl (2-HBP) (Soleimani *et al.*, 2007).

The desulfurization of DBT using the 4S pathway involves the consecutive oxidation of DBT to dibenzothiophene sulfoxide (DBTO), dibenzothiophene sulfone (DBTO2) and finally 2-hydroxybiphenyl 2-sulfinic acid (HBPS). HBPS is then converted to 2-hydroxybiphenyl (2-HBP) and sulfite. This requires the use of four enzymes (Gray *et al.,* 2003): (i) the oxidation of DBT into DBTO and then to $DBTO_2$ is catalyzed by the DBT monooxygenase (DszC); (ii) the conversion of $DBTO_2$ to HBPS is catalyzed by the DBT-sulfone monooxygenase (DszA); (iii) HBPS is catalyzed by the HBPS desulfinase (DszB) resulting in 2-HBP and sulfite; (iv) and the fourth enzyme is a flavin reductase enzyme (DszD) that is required to provide the reducing equivalents that are necessary to activate the molecular oxygen required by the flavin-dependent DszC and the DszA enzymes. The presence of DBTO is difficult to detect because it is readily converted to $DBTO_2$ through oxidation; in fact, the first oxidation step of DBT to DBTO is ten times slower than the second step, DBTO to $DBTO_2$ (Ma, 2010). DszA only oxidizes $DBTO_2$ and does not act on DBT or HBPS; however it has been shown to oxidize other sulfones and sulfines to form dihydroxybiphenyl (Ohshiro and Izumi, 2000). Isotopic labeling studies have shown that the oxygen

utilized by the monoxygenase enzymes comes from molecular oxygen, indicating that an aerobic environment is required for the desulfurization of DBT through the 4S pathway. DszB can specifically cleave the carbon–sulfur bond of HBPS resulting in 2-HBP and a sulfiteion. Since the activity of DszB is the lowest among the enzymes utilized in the 4S pathway, this step is considered rate-limiting (Ma, 2010).

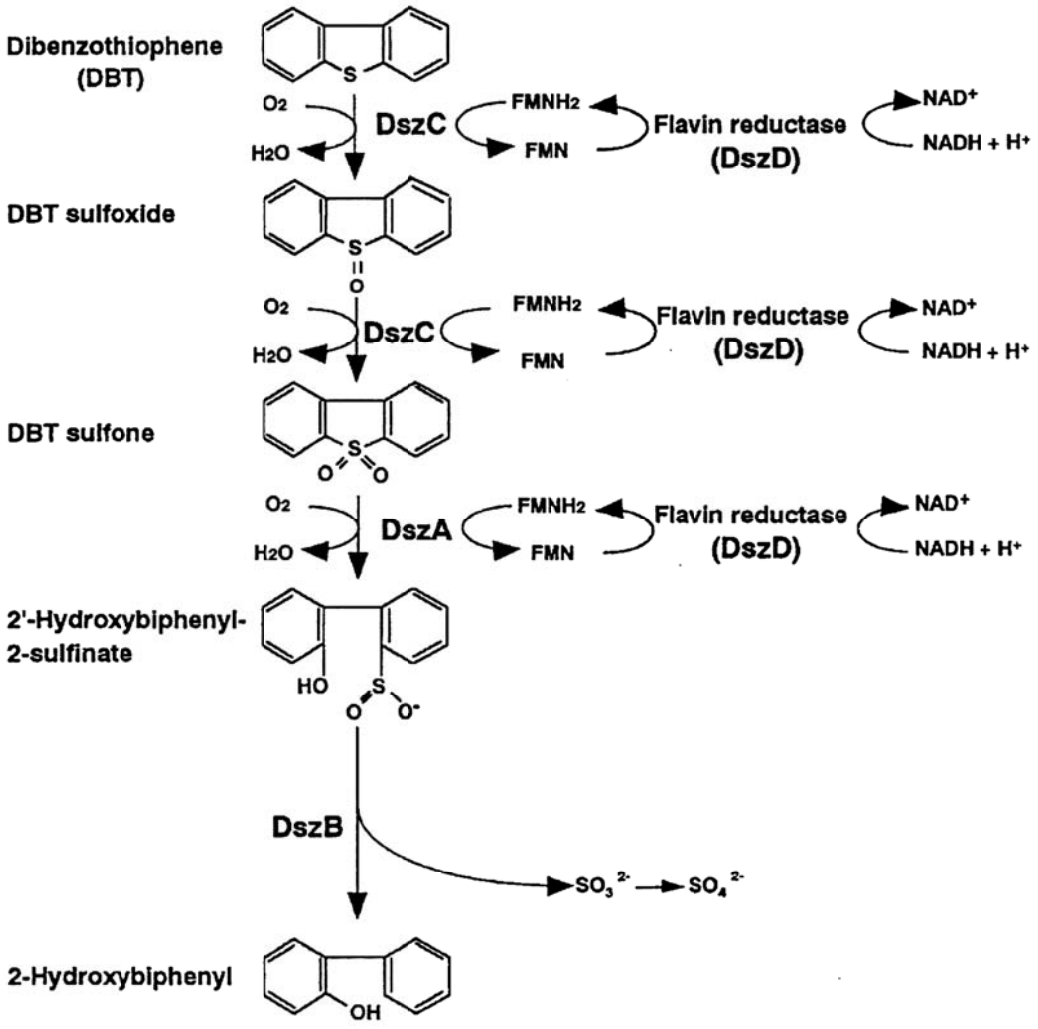


Figure (1-4): The 4S pathway for the biocatalytic desulfurization of DBT and its derivatives. DBT, dibenzothiophene; HPBS, hydroxyphenyl benzene sulfonate; 2-HBP, 2-hydroxybiphenyl; DszA, DBT sulfone monooxygenase; DszB, HPBS desulfinase; DszC, DBT monooxygenase (Kirimura *et al.*, 2001).

This pathway is energetically expensive because the carbon skeleton is not mineralized in order to get back the energy invested. The use of this pathway has been proposed for the desulfurization of petroleum in production fields and also refineries (McFarland, 1999). Through this pathway the carbon skeleton of DBT is released intact and thus the calorific value of the fuel is not lost (Ma, 2010).

1.2.6. General microbiology of biodesulfurization.

The natural environment, such as polluted soil or oil field, usually provides the best niches to source microorganisms with potential for biodesulfurizing (BDS) activities. As these microorganisms are cultivated and isolated in the laboratory for the purpose of BDS, they display different potentials arising from their different genetic make-ups and conditions that they were previously acclimatized to. Surprisingly, the BDS activity was discovered mainly in bacteria belonging to the gram-positive domain such as many strains of *Rhodococcus, Bacillus, Mycobacterium phlei, Paenibacillus, Gordona, Corynebacterium, Gordoniaalkanivorans, Xanthomonas, Nocardiaglobelula,* and *Agrobacterium*. A few gram-negative bacteria have been discovered, such as *Sphingomonas* (Nadalig *et al.,* 2002) and *Pseudomonas delafieldii* (Guobin *et al.,* 2006) which can desulfurize dibenzothiophene (DBT) has added another opportunity. A very distinct feature of these gram-positive bacteria is that the chemical nature of the molecular structure of its cell wall makes the *Rhodococci* cells hydrophobic. This hydrophobicity is taken advantage of by the *Rhodococci* in its attachment to the oil/water interface and its growth in an aqueous-hydrocarbon system (Borole *et al.*, 2002). Among eukaryotic organisms that initiate oxidation of DBT by attack of the S atom, although the 4S pathway is not utilized, by some fungi such as *Cunninghamell aelegan* (Crawford and Gupta, 1990), *Paecylomyces* sp. (Faison *et al.,* 1991) and the yeast *Rhodosporidium toruloides* (Baldi *et al.,* 2003). Although all the mentioned

organisms carry out the BDS under oxidative aerobic conditions, anaerobic bacterial strain *Desulfovibrio desulfuricans* has been shown to harbor the capability of reductive desulfurization of DBT to biphenyl and H_2S (Kim *et al.*, 1990).

In *Lysinibacillus sphaericus* DMT-7 from diesel contaminated soil in India, desulfurization of DBT started after a lag phase of 24 h and continued through 15 day, resulting in 60 % reduction in the sulfur content of DBT (Bahuguna *et al.*, 2010).

Other DBT desulfurizing microorganisms, mostly mesophilic and a few thermophilic, have been isolated; *Mycobacterium* sp. G3 (Nekodzuka *et al*., 1997); *Microbacterium* sp. ZD-M2 (Zhang *et al*., 2005); *Gordonia alkanivorans* RIPI90A (Mohebali*et al*., 2007); *Pantoea agglomerans* D23W3 (Bhatia and Sharma, 2010) and *Sphingomonas subarctica* T7b (Gunam *et al*., 2013).

1.2.7. Current technologies for desulfurization of crude oil.

1.2.7.1. Caustic washing method for oil desulfurization:

Caustic washing method is mainly aimed to removing the sulfides from the crude oil by using the caustic solution. This method is widely used in the oil industry. The advantage of this method is that the process is simple and its cost is low. But this method contains many defects such as the poor quality oil products and lower efficiency of desulfurization. However, this method can't remove all sulfur compounds from the crude oil, especially the organic sulfides. At the same time, it brings too much sulfide-containing waste water, which greatly pollutes the environment. So, it is urgent to improve this method or find a better way to reduce the sulfur contents in petroleum products (Liu *et al*., 2010) .

1.2.7.2. Dry gas desulfurization method.

According to the report of Tong *et al*. (2009), the first equipment in China for treating heavy crude oil through dry gas desulfurization method has been

used in the No.3 joint oil gathering station at Tahe Oil field of the northwest oil field branch company. The cost of this equipment was very high, and it is the first time in China to use this dry gas treatment method at the crude oil desulfurization system in reality. This dry gas desulfurization system works as follows:

Firstly, the sulfur compounds are removed from the crude oil; and secondly, after settling treatment and dehydration of crude oil, the sulfur compounds are removed again so that corrosion of the process equipment and the pipelines will be minimized to secure better working conditions and avoid accidents. Gas stripping process can reduce significantly the H_2S content in crude oil. But this method has its disadvantage because the efficiency of desulfurization will change the water content in the feedstock. Sulfate-reducing bacteria (SRB) can convert a part of organic sulfides into inorganic sulfides, causing the H_2S content to increase in the next phase of desulfurization (Xiang *et al*., 2004). And the rate for removal of other sulfur-containing compounds is very low.

1.2.7.3. Oxidative desulfurization (ODS).

Oxidative desulfurization (ODS) has the advantages of milder reaction conditions, higher efficiency and easier operation, lower investment, lower operating cost and energy efficient use compared with HDS. ODS methods include two steps: oxidation and extraction/adsorption. Firstly, sulfur compounds are oxidized by an oxidant, next the products are separated from the oil through extraction or adsorption. H_2O_2 as well as O_2, O_3, K_2FeO_4, and nitrogen oxides can be used as oxidants in ODS (Gao *et al*., 2009; Ma *et al*., 2013).

This method (ODS) is mainly aimed to removing most of mercaptans and few thioethers contained in the feed oil. But with increasing boiling points of petroleum fractions, their mercaptans and disulfides contents drop down immediately, and the thioethers content grows first, and then falls down, while

the content of thiophenes keeps rising. Most of sulfur in crude oil exists in the form of thioethers and thiophenes in higher boiling petroleum fractions, these inactive sulfur compounds are much more difficult to be removed than active sulfides (Liu *et al.*, 2004).

1.2.7.4. Hydrodesulfurization (HDS).

At present, numerous desulfurization methods have been developed. Among them hydrodesulfurization (HDS) has been widely applied to the field of petroleum fuel production around the world for a long time. HDS has short comings such as lower desulfurization efficiency, strict reaction conditions and so on, which leads to higher manufacturing costs and higher price of the resulting fuel, therefore, the search for new desulfurization technologies which offer higher efficiency and lower manufacturing costs has become a hot research topic in this field (Gao *et al.*, 2009; Ma *et al.*, 2013) .

Hydrodesulfurization (HDS), a reductive conventional physico-chemical technology, is the more effective and economical technology used worldwide for sulfur removal from crude oil and its fractions (Javadli *et al.*, 2012). It has been reported that more than 20 million barrels of oil are daily treated in the world by HDS (Ansari *et al.*, 2008). HDS involves the use of chemical catalysts containing metals at high pressures and temperatures to remove sulfur compounds. However, with the different classes of sulfur compounds found in the middle-distillate fraction, Cx-BTHs and Cx- DBTs with alkyl substitutions in positions four and six on the DBT ring are more resistant to HDS treatment than mercaptans and sulfides (Kabe *et al.*, 1992).

However, HDS has several disadvantages, in that it is energy intensive, costly to install and to operate, and does not work well on refractory organosulfur compounds. Research has therefore focused on improving HDS catalysts and processes and also on the development of alternative technologies. Compared with HDS process, the biodesulfurization (BDS) process using

microorganisms and/or enzymes could be carried out more safely, under mild conditions (Chen *et al*., 2009).

1.2.7.5. Biodesulfurization (BDS).

Biodesulfurization (BDS) is a process for the desulfurization of fossil fuel oils by means of enzyme-catalyzed reactions (Song and Ma, 2006). In this process, a biocatalyst in an aqueous solution is contacted with a fuel oil (organic phase) to be treated and the desulfurization reactions occur at the interface of the aqueous and organic phases. DBT and its derivatives were widely used for the desulfurization of simulated light fuel oils. The specific oxidative desulfurization of DBT is a pathway in which carbon-sulfur bond is targeted to produce 2-hydroxybiphenyl as the final product (Soleimani *et al.,* 2007). Based on the application of microorganisms that selectively remove sulfur atoms from organosulfur compounds, appears to be a viable technology to complement the traditional hydrodesulfurization of fuels (Monticello, 2000; Gray *et al.*, 2003; Kilbane, 2006; Soleimani *et al*., 2007).

Removal of inorganic sulfur from fossil fuels is possible by physical, chemical or biological means. Physical and chemical methods are expensive mean (Monticello, 2000), while biodesulfurization (BDS) is a process which is requires lower capital cost as it operates at mild conditions of atmospheric temperature and pressure and does not require molecular hydrogen (Alves and Paixão, 2011). It also produces far less carbon dioxide than HDS (Kilbane, 2006).

Biodesulfurization (BDS) has been considered as a novel biotechnology which is expected to be a complement or alternative to HDS. However, conversion rates are expected to be lower than the HDS technique. Therefore, BDS can be considered as a complementary process to achieve ultra-low-sulfur fuels, after the bulk sulfur is removed using the HDS process. The specific removal of S from fuels without attacking the caloric value *via* BDS, in addition to many other advantages such as mild operation conditions (ambient

temperature and pressure), decreased energy costs, low emission and no generation of undesirable side-products, designated the BDS as an environmentally benign biotechnological process. This prompted many research groups to improve the BDS process, making it feasible for commercialization. Accordingly, more emphasis will be given to commercializing the microbial BDS process (Zakariya, 2012).

1.2.8. The genetics of biodesulfurization.

In 1985, Isbister and Koblynski described a strain of *Pseudomonas sp.*, CB-1, that could accomplish sulfur specific metabolism of DBT. The intermediates were DBTO and $DBTO_2$ and the end product was dihydroxybiphenyl; unfortunately this strain was lost before the metabolic pathway could be fully characterized (Gallagher *et al*., 1993). Isolation of a suitable bacterium was achieved after 40 years of research effort when Kilbane (1990) at the Gas Technology Institute (formerly Institute of Gas Technology), USA, isolated *Rhodococcus erythropolis* IGTS8.

R. erythropolis IGTS8 and *Rhodococcus sp*. X309 were among the first strains to be characterized at the molecular level (Gallardo *et al.,* 1997; Oldfield *et al.,* 1997; Denis- Larose *et al.,* 1997). A gene cluster that could complement a desulfurization-negative mutant of IGTS8 has been cloned and sequenced (Denome *et al*., 1993; Piddington *et al*., 1995), and found to comprise three ORFs (open reading frames), designated dszA, B and C. Subclone analyses revealed that the product of dszC converts DBT directly to $DBTO_2$ and that the products of dszA and dszB act in concert to convert $DBTO_2$ to 2- HBP. The three genes are clustered on a 120 kb linear plasmid of strain IGTS8 (Denome *et al*., 1994). The desulfurization (Dsz) phenotype was conferred by a 4 kb gene locus on the large plasmid (Denis-Larose *et al*., 1997; Gray *et al*., 1998; Rambosek *et al*., 1999). The desulfurization ability of *Rhodococcus* sp. ECRD-1 appeared also to be an exclusive property of a 4 kb gene locus on a large

plasmid (Prince and Grossman, 2003). In *Rhodococcus* the dsz genes are located near insertion sequences (Denis-Larose *et al*., 1997; Kilbane and Le Borgne, 2004). Therefore, the desulfurization system is organized as one operon with three genes (dszA, dszB, dszC) transcribed in the same direction and under the control of a single promoter (Oldfield *et al*., 1997; Gray *et al*., 1998).

In biodesulfurization research, two monooxygenases, DszA and DszC, have been shown to be active in the desulfurization reaction with the aid of an oxidoreductase encoded by dszD. The dszD gene was cloned and sequenced from *R. erythropolis* IGTS8. The gene encoded a protein of ~20 kDa and was present on the chromosome IGTS8 rather than on the plasmid that contains dszABC (Gray *et al.*, 2003). Matsubara *et al.* (2001) purified and characterized the flavin reductase from *R. erythropolis* D-1. The N-terminal amino acid sequence of the purified flavin reductase was identical to that of DszD of *R. erythropolis* IGTS8. The gene (frm) encoding flavin reductase from *Mycobacterium phlei* WU-F1 has been cloned and the cloned enzyme has been purified and characterized. The deduced amino acid sequence of the from product shared only 33 % identity with that of the flavin reductase gene (dszD) from *R. erythropolis* IGTS8 (Furuya *et al*., 2005).

Ishii *et al*. (2000) isolated and cloned the gene *tdsD* encoding the flavin reductase which coupled with the monooxygenases of *Paenibacillus* sp. A11-2. There was no detectable sequence similarity between *TdsD* and the *Rhodococcus* DszD (Gray *et al*., 2003). Ohshiro *et al*. (2002) searched for non-DBT desulfurizing micro-organisms producing a flavin reductase that coupled more efficiently with DszC than that produced by the DBT-desulfurizing bacterium *R. erythropolis* D-1, and found *Paenibacillus polymyxa* A-1 to be a promising strain. The result of the coupling reaction may be beneficial in developing an efficient enzymic desulfurization system and in elucidating the mechanisms of interaction between the monooxygenases, reductases and reduced flavin species.

The conserved nature of the dsz genotypes among desulfurizing strains from different geographical locations has been documented (Denis-Larose *et al*., 1997); sulfate reducers showed no cross-reactivity, suggesting that the anaerobic sulfur-specific removal sometimes reported occurs by a different pathway. PCR amplification of dsz genes from soil samples revealed relatively few variations in dsz gene sequences, with the majority of variations found in dszA, and even then homology to the *R. erythropolis* IGTS8 dszA sequence was 95 % or more (Duarte *et al*., 2001). The distribution of dsz genes in aerobic bacterial cultures strongly supports the hypothesis that these genes are commonly subject to horizontal transfer in nature (Kilbane and Le Borgne, 2004).

Kilbane, (2006) finding is that bacterial cultures that possess identical dsz gene sequences can have very different Dsz phenotypes. This was clearly illustrated by examining the desulfurization activity of *Mycobacterium phlei* GTIS10 having dszABC gene sequences identical to *R. erythropolis* IGTS8; the temperature at which maximum desulfurization activity was detected in the cultures was about 50 ^{o}C and 30 ^{o}C, respectively. Characterization of four bacterial cultures capable of utilizing DBT as the sole source of sulfur revealed that these cultures had identical dsz genes, but the cultures differed significantly with regard to their substrate range, desulfurization activity and yield of metabolites (Abbad-Andaloussi *et al.*, 2003). A comparative study of *M. phlei* SM120-1 and *M. phlei* GTIS10 aimed at broadering the understanding of the Dsz trait at intra-species level revealed considerable differences in the phenotypic and genotypic characteristics of these two desulfurizing strains (Srinivasaraghavan *et al.*, 2006).

The range of Dsz phenotypes observed in different cultures may reflect the ability of each bacterial species or strain to provide cofactors and reaction substrates under the conditions tested. The transport of substrates and products might also contribute to desulfurization activity, as demonstrated by the fact that

cell-free lysates of desulfurization cultures can exhibit a broader substrate range than the intact cell culture (Kilbane, 2006).

1.2.9. Determination of the desulfurization activity by Gibb's assay.

Gibb's assay is used to determine the presence of DBT biodesulfurizing bacteria that exhibit the 4S pathway by the detection of 2-hydroxybiphenyl (2-HBP) which is the end product of the 4S pathway. The method is a standard method used for the detection of phenol (Mohler *et al.*, 1957).

The method is based on the condensation reaction between dichloroquinone-4-chloroimide with phenol compounds that do not have a successor group to form a compound of the 2,6-dichlorophenol. The reaction takes place in an alkaline medium at pH 9.4 of borate buffer. For the determination of phenol in the range of ppm, 2,6-dichlorophenol compounds give absorption at a wavelength of 595 to 630 nm. In addition, such factors as temperature, pH, and presence of other compounds such as sulphide, reducing agent, and thiocresol have been found to affect the reaction (Gibbs, 1926).

Desulfurization activity was monitored using the Gibb’s reagent (2,6-dichloroquinone-4-chloroimide) (figure 1-5) to detect the 2-hydroxybiphenyl (HBP) produced from DBT. Gibb’s reagent reacts with the aromatic hydroxyl groups at pH 8 to form a blue complex that can be monitored spectrophotometrically at 610 nm after 30 min incubation at room temperature (Kayser *et al.*, 1993).

Gibb’s reagent used in determination of phenols, for detection of antioxidants, primary and secondary aliphatic amines, secondary and tertiary aromatic amines, aromatic hydrocarbons, pharmaceuticals, phenoxy acetic acid herbicides, etc. (see http://www.drugfuture.com/chemicaldata/gibbs_reagent.ht.mail).

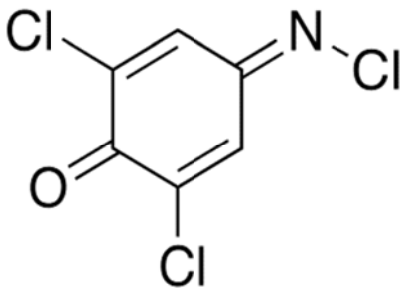


Figure (1-5): Chemical Structure of 2,6-dichloro-p-benzoquinone-4-chloroimide reagent.

Chapter Two Materials and Methods

2. Chapter two materials and methods

2.1. Materials.

2.1.1. Equipments and Apparatus.

The following equipments and apparatus were used in this study:

NO	Equipments	Manufacturing company	Origin
1	Autoclave	Hirayama	Japan
2	Compound light microscope	Olympus	Japan
3	Cooling centrifuge	Hettich	Germany
4	Distillator	GFL	Spain
5	Gas Chromatograph Mass Spectrometer (GC/Mass)	Shimadzu	Japan
6	Hot plate magnetic stirrer	Gallenkamp	England
7	Incubator	Sanyo	Germany
8	Laminar air flow hood	Sanyo	Germany
9	Micropipette	slamed	Germany
10	Millipore filter unit	Millipore corp	Spain
11	Minimal electrophoresis apparatus	Bio- Rad	Italy
12	Nanodrop system	Optizen	Korea
13	pH- meter	Martini	Germany
14	Refrigerator	Ishtar	Iraq
15	Sensitive balance	Denver	Germany
16	Shaker incubator	Gallenkamp	Germany
17	UV/VIS-Spectrophotometer	BUK	U.S.A
18	sulfur content X-ray	Chongqing Gold Mechanical & Electrical Equipment Co Ltd	Japan
19	Viteck2 / Viteck2 kit	Biomerieux	France
20	Vortex	Vortex-2-genie	U.S.A
21	Water bath	Grant	England

2.1.2. Chemicals.

The chemicals used in this study are the following classified according to the manufactured company:

Material	Manufacturing company	Origin
Chloroform, Urea, Peptone, Iodine, K_2HPO_4, NaOH, NaCl, $MgSO_4$, $MgCl_2.6H_2O$, $CaCl_2.2H_2O$, $FeCl_3.2H_2O$, Na_2HPO_4, NH_4Cl and Formic acid	BDH	U.K
Agar, Gelatin and Boric acid	Biolife	Italy
Sodium dodecyl sulfate (SDS), Tris (hydroxyl methyl), tris (hydroxymethyl) amino methane (Trise-base), Ethylene diamine tetra acetic acid (EDTA), Methyl red, Ethanol, Hydrogene peroxide, Crystal violet and $FeSO_4.7H_2O$	Fluka	Switzerland
Bromophenol blue and Cetrimide.	Riedel-Dehaeny	Germany
Yeast extract and Kovac's reagent.	Himedia	India
Dibenzothiophene (DBT) and Gibb's reagent(2, 6-Dichloroquinone-4-Chloroimide).	Sigma	U.S.A
Ethidium bromide, Agarose, Loading solution and Tris- Boric acid-EDTA (TBE buffer)	Promega	U.S.A

2.1.3. Bacteria strain.

Bacteria strain *(E. coli* HB101) harboring pBR322 (Ap^r, Tc^r) was obtained from Department of Biotechnology, Al-Nahrain University.

2.1.4. Soil samples.

The soil samples used in this study were taken from different places with a history of oil or its derivatives pollution.

2.1.5. Media.

2.1.5.1. Ready to use media.

The following media were ready-made media prepared according to the manufactured instructions:

Media	Manufacturing Company	Origin
Nutrient agar	Biolife	Italy
Nutrient broth	Difco	U.S.A
Triple sugar iron(TSI) agar	Biolife	Italy
MR-VP medium	Difco	U.S.A
Simmon citrate agar	Difco	U.S.A
Urea agar base	Biolife	Italy
Gelatine agar base	Biolife	Italy
Nitrate agar base	Difco	U.S.A

2.1.5.2. Laboratory prepared media.

- **King A (Starr *et al.*, 1981).**

This medium was prepared by dissolving peptone (20 g), $MgCl_2$ (1.4 g), and K_2SO_4 (10 g), agar (15 g) and glycerol (10 ml) in 950 ml D.W., pH was adjusted to 7, then the volume was completed to 1L and sterilized by autoclaving at 121^{o}C for 15 min.

- **King B (Starr *et al.*, 1981).**

This medium was prepared by dissolving peptone (20 g), $MgSO_4.7H_2O$ (10 g), and K_2SO_4 (1.4 g), agar (15 g) and glycerol (10 ml) in 950 ml D.W., pH was adjusted to 7, then the volume was completed to 1L and sterilized by autoclaving.

- **Cetrimide agar (Stolp and Gadkari, 1984).**

This medium was prepared by dissolving peptone (20 g), $MgCl_2$ (4.5 g), and K_2SO_4 (10 g), cetrimide (0.3 g), agar (15 g) and glycerol (10 ml) in 950 ml

D.W., pH was adjusted to 7, then the volume was completed to 1L and sterilized by autoclaving.

- **Luria-Bertani (LB) broth (Maeda *et al.*, 2004).**

This medium was prepared by dissolving peptone (10 g), yeast extract (5 g), NaCl (5 g) and agar (15 g) in 950 ml D.W., pH was adjusted to 7, then the volume was completed to 1L and sterilized by autoclaving.

- **Peptone water Medium (Atlas *et al.*, 1995).**

This medium was prepared by dissolving thoroughly 20 g peptone and 5 g of NaCl in a quantity of D.W., the volume was completed to 1L and pH was adjusted to 7 before distributing into test tubes (10 ml each) and autoclaving.

- **Urea Agar (Collee *et al.*, 1996).**

It was prepared by adjusting pH of the urea agar base to 7.0 and autoclaved, after cooling to 50 ^{o}C, 50 ml of 40 % urea (previously sterilized by filtration) was mixed with it and distributed into sterilized test tubes (10 ml each), then kept slant.

- **Gelatin medium (Stolp and Gadkari, 1984).**

This medium was prepared by dissolving 12 g of gelatin in nutrient broth and completed to 100 ml of nutrient broth medium, then distributed into tubes and sterilized by autoclaving.

- **Semi solid agar (Collee *et al.*, 1996).**

It was prepared by dissolving 0.4 % of agar in nutrient broth or peptone water, in which the final medium should be quite clear and transparent. Then 10 ml was dispensed in test tubes, sterilized by autoclaving and left to stand in a vertical position.

- **Basal (mineral) salt medium (BSM) (Kilbane and Bielaga, 1990).**

This medium was prepared by dissolving K_2HPO_4 (4 g), Na_2HPO_4 (4 g), NH_4Cl (2 g), $MgCl_2$. $6H_2O$ (0.2 g), $CaCl_2$. $2H_2O$ (0.001 g) and $FeCl_3.6H_2O$ (0.001 g) in 950 ml D.W., pH was adjusted to 7.2 then the volume was completed to 1L and sterilized by autoclaving.

2.1.6. Reagents, Solutions, Buffers and Dyes.

2.1.6.1. Catalase reagent (Atlas *et al*., 1995).

A solution of 3 % hydrogen peroxide was prepared.

2.1.6.2. Oxidase reagent (Atlas *et al*., 1995).

This reagent was prepared by dissolving 1 g of tetramethyl-*p*- phynylene diamine dihydrochloride in 100 ml D.W.

2.1.6.3. Methyl red (Colline and Lyne, 1987).

This indicator was prepared by mixing 0.1 g of methyl red, 300 ml of ethyl alcohol and 200 ml D.W.

2.1.6.4. Barritt's reagent (Collee *et al.*, 1996).

It consists of two solutions:

- **Solution A:** potassium hydroxide (40 %)
- **Solution B:** It was prepared by dissolving 5 g of α – naphtholamine into 100 ml of absolute ethanol.

Equal volumes of solution A and B were immediately mixed before use.

2.1.6.5. Nitrate test reagent (Atlas *et al.*, 1995).

This reagent consists of two solutions:

- **Solution A:** It was prepared by adding 0.8 g of sulfanilic acid to 5 N acetic acid up to 100 ml.
- **Solution B**: It was prepared by adding 0.5 g of dimethyle-α-naphthylamine to 5 N acetic acid up to 100 ml.

Equal volumes of solution A and solution B were immediately mixed before use.

2.1.6.6. Gibb's reagent (Kayser *et al*., 1993).

It was prepared by dissolving 10 mg of Gibb's reagent (2, 6-Dichloroquinone-4-Chloroimide) in 10 ml ethanol.

2.1.6.7. Gram stain (Atlas *et al.*, 1995).

It was composed of 4 reagents:

1- A primary stain- crystal violet.

2- Mordent- iodine solution.

3- A decolorizing agent- an organic solvent (alcohol).

4- A secondary stain- safranin.

2.1.6.8. Dibenzothiophene solution.

It was prepared as stock solution (100 mM) by dissolving 0.92 g of dibenzothiophene in 50 ml of ethanol (absolute ethanol).

2.1.7. Plasmid and Genomic DNA extraction buffers and solutions.

2.1.7.1. SET buffer (Green and Sambrook, 2012).

Tris-HCl 20 mM

EDTA 25 mM

NaCl 75 mM

pH was adjusted to 8.0 and sterilized by autoclaving.

2.1.7.2. NaCl solution (5M) (Pospiech and Neumann, 1995).

It was prepared in distilled water and sterilized by autoclaving.

2.1.7.3. SDS solution.

It was freshly prepared by dissolving 10 g in 100 ml of sterile distilled water.

2.1.7.4. Lysozyme solution (Pospiech and Neumann, 1995).

This solution was freshly prepared by dissolving 1mg lysozyme enzyme in 1ml of sterile D.W.

2.1.7.5. TE buffer (Green and Sambrook, 2012).

Tris-OH 10 mM

EDTA 1 mM

pH was adjusted to 8.0 and sterilized by autoclaving.

2.1.8. Electrophoresis buffers (Green and Sambrook, 2012).

2.1.8.1. Trise-Borate-EDTA 5X (TBE) (pH 8.0).

Trise-base 54 g

Boric acid 27.5 g

EDTA (0.5 M)	20 ml
Distilled water	1000 ml

2.1.8.2. Ethidium bromide.

Prepared in concentration of 10 mg/ml in D.W. and stored at 4 °C.

2.1.8.3. Gel loading buffer (6X).

Bromophenol blue	(0.25 % w/v)
Sucrose in H_2O	(40 % w/v)

2.1.9. Plasmid isolation by pure Yied™ plasmid minprep kit solutions (www.promega.com), USA.

- Cell Lysis Buffer (CLC) (Blue).
- Neutralization Solution (NSC).
- Endotoxin Removal Wash (ERB).
- Elution Buffer (EBB).

2.2. Methods.

2.2.1. Sterilization methods.

Three methods of sterilization were used:

❖ **Moist heat sterilization (autoclaving).**

Media and solutions were sterilized by autoclaving at 121 ^{o}C (15 Ib/ in^2) for 15 minutes.

❖ **Dry heat sterilization.**

Electric oven was used to sterilize Glassware's and other steel at 160 ^{o}C for 3 hrs and 180 ^{o}C for 2 hrs.

❖ **Membrane sterilization (filtration).**

Millipore filtering was used to sterilize heat sensitive solutions by using Millipore filter paper (0.22 µM in diameter).

2.2.2. Samples collection.

Oil- contaminated soil samples were collected from forty different sites with a history of oil pollution in Iraq. The soil samples (500 g) were collected randomly from the top soil layer (15 cm in depth) by auger. Soil samples were transferred to the laboratory using sterile plastic bags to isolate DBT utilizing and/or biodesulfurizing bacteria.

2.2.3. Isolation of Bacteria.

The microbial selection procedures were performed in basal salt media (BSM) (2.1.5.2.) constituted of minerals and DBT as sole source of sulfur. One hundred milliter of BSM were dispensed in the 250 ml Erlenmyer flasks supplemented with 0.1 mM of DBT and glycerol (10 mM) was used as the carbon source, autoclaved at 121 °C for 15 min. One percent (w/v) of soil samples were added to the flasks and incubated at 30 °C with shaking (150 rpm) for four days. Samples (0.1) ml of appropriate dilutions was spread onto plates of LB agar plates, incubated at 30 °C for 24 hrs. A single colony was picked with a sterile loop to prepare a pure subculture in a fresh LB agar plates by streaking. The purity of the isolated colonies was checked by microscopic examination. Pure isolates were growing in BSM medium containing DBT and glycerol (as mention above) to ensure their ability to utilize and/or desulfurize DBT.

2.2.4. Preparation of the bacterial inocula.

The bacterial isolates were grown in flasks containing DBT- glycerol-BSM medium at 37 ^{o}C with shacking (150 rpm) for 18 hrs. Cells were harvested by centrifugation (10,000 ×g), washed twice, and resuspended in BSM medium lacking DBT and glycerol. The resulting suspension was used as the bacterial inoculum. The size of the inoculums used in all experiments was 1% (v/v).

2.2.5. Maintenance of bacterial isolate.

Maintenance of bacterial isolates was performed according to Green and Sambrook (2012) as follows:

- **Short –term storage.**

Colonies of bacteria were maintained for periods of few weeks on the surface of LB agar medium; the plates were tightly wrapped in parafilm and stored at 4 °C.

- **Medium –term storage.**

Bacterial isolates were maintained in the stab culture for long periods of a few months. Such cultures were prepared in small screw capped bottles containing 2-3 ml of LB agar medium, the cultures were inoculated using a sterile straight platinum wire that is dipped into dense liquid culture of bacteria and then stabbed deep into agar medium. After proper incubation, the cap was wrapped tightly with the parafilm to prevent desiccation of the medium and kept at 4 °C.

- **Long-term storage.**

Bacteria can be stored for many years in the medium containing 15 % glycerol at low temperature without significant loss of viability. This was done by adding 1.5 ml of glycerol to an exponential growth of the bacteria in a small screw-capped bottle with final volume 10 ml and stored at-20°C.

- **Freeze drying (Lyophilization).**

Bacterial isolates can be stored for very long time in the form of powder by using a freeze-drying method. In this method, fresh culture of bacterial isolates were centrifuged (10000 ×g, 4 °C) and the bacterial pellet was frozen and lyophilized by lyophilizer apparatus after addition 5 ml of BSM .

2.2.6. Detection the ability of bacterial for utilization of DBT.

BSM medium (2.1.5.2) supplemented with (10 mM) Glycerol as the carbon source and 1mM of DBT as the only source of sulfur (medium A), that

sterilized by autoclaving. The same medium was prepared but without glycerol (medium B), to determined the ability of the isolates to utilizing the DBT as sole source of carbon and sulfur. One hundred milliliter of medium A or B were dispensed in conical flasks (250 ml) and inoculated with bacterial isolates. The inoculated flasks were incubated at 37 ^{o}C under shaking (150 rpm) for 4 days. Bacterial growth was determined by measuring the optical density at 580 nm.

2.2.7. Utilization of ethanol and $MgSO_4$ as alternative carbon and sulfur sources by DBT biodesulfurizing bacteria.

Since DBT was added to BSM from stock solution of DBT (100 mM) in ethanol, the utilization of ethanol as an alternative carbon source was examined in the presence of an inorganic sulfur source which was $MgSO_4$. The growth of DBT biodesulfurizing bacterial isolates at 37 ^{o}C in BSM containing 0.1 mM DBT plus 6 mM ethanol (control flask) was compared to the growth in medium containing 6 mM ethanol and 0.2 mM $MgSO_4$. Flasks (250 ml) containing 100 ml of the respective medium were inoculated and incubated under shaking (150 rpm) at 37 ^{o}C. At different time intervals, samples (2 ml) of bacterial cultures were withdrawn and the growth was measure at 580 nm.

2.2.8. Detection the biodesulfurization activity of bacterial isolates using Gibb's assay.

Gibb's assay was used to determine the presence of DBT biodesulfurizing bacteria that exhibit the 4S pathway by the detection of 2-hydroxybiphenyl (2-HBP) which is the end product of the 4S pathway.

The cell- free culture supernatant was obtained from 50 ml of bacterial cultures, in medium A and B, grown at different time intervals, by centrifugation (10,000 ×g, 10 min, at 4 ^{o}C). One ml of each cell-free supernatant was transferred to clean eppendorf and adjusted to pH 8 by adding 60 µl of 1 M $NaHCO_3$ (Konishi *et al.*, 1997) or by adding 3 µl of 4 M of NaOH.

Detection of the end products of the desulfurization of thiophene compound, was carried out in microtiter plates by adding 3 µl of Gibb's reagent (2.1.6.6) to 300 µl of cell- free supernatant (pH 8) (1:100) and the mixture was kept at room temperature for 30 min. Bacterial cultures capable of desulfurizing thiophene compound, accumulated phenolic compound in cultural supernatant and gave blue color in the presence of Gibb's reagent (Rambosek *et al.,* 1994).

2.2.9. Identification of bacteria.

Bacterial isolates were subjected to the following morphological, cultural and biochemical tests for their identification:

2.2.9.1. Morphological Characteristics (Atlas *et al.,* 1995).

A single colony was transferred by a loop onto clean slide. The smear was stained with crystal violet, treated with iodine, decolorized with absolute alcohol and counterstained with safranine, then examined under microscope to detect Gram reaction, shape, spore forming and capsule of isolated bacteria.

2.2.9.2. Cultural Characteristics.

Shape, color, edge, odor and viscosity of the colony were studied on the LB agar plate after incubation at 37°C for 24hrs.

2.2.9.3. Biochemical Tests.

The following biochemical tests were performed:

- **Growth on King A medium (Murray *et al.,* 2003).**

Inoculated the bacteria on plate by streaking and incubated at 37 °C for 24 hrs. This test was performed to study the production of the characteristic pigment, pyocyanin.

- **Growth on King B medium (Murray *et al.*, 2003).**

Inoculated the bacteria on plate by streaking and incubated at 37 °C for 24 hrs. Then the plates were exposed to U.V. This was performed to study the production of the characteristic pigment, fluorescein.

- **Growth on cetrimide agar (Greenwood *et al.*, 1997).**

This medium was used as a selective medium for *Pseudomonas* spp. The plate was inoculated with bacteria by streaking and incubated at 37 °C for 24 hrs.

- **Catalase production test (Maza *et al.*, 1997).**

This test was performed by adding drops of hydrogen peroxide (3 %) on a single bacterial colony grown on LB agar. The production of gaseous bubbles indicates the ability of bacteria to produce catalase enzyme.

- **Oxidase production test (Maza *et al.*, 1997).**

Filter paper was saturated with oxidase reagent (2.1.6.2), and then colony of bacteria to be tested was rubbed on the filter paper with a sterile wooden applicator stick. An immediate color change to a deep blue indicates the ability of bacteria to produce oxidase enzyme.

- **Nitrate reduction test (Atlas *et al.*, 1995).**

A single colony of each bacterial isolate was used to inoculate 5 ml of nitrate media, and then test tubs were incubated at 37^{o}C for 24 hrs. After incubation, 0.1 ml of the test reagent (2.1.6.5) was add to each tube. The immediate formation of red color indicates as positive result.

- **Gelatin liquefaction test (Stolp and Gadkari, 1984).**

Tubes of gelatin media were inoculated with a single colony of bacterial isolates and incubation overnight at 37 ^{o}C, and then transferred to refrigerator for an hour. Liquefaction of gelatin indicated positive result.

- **Citrate utilization (Atlas *et al.*, 1995).**

Slant of Simmons citrate was inoculated with single colony, incubated at 37 °C overnight. The appearance of blue color indicates that the pH is alkaline

end product and positive citrate test whereas the green color means a negative result. This test was used to examine the ability of bacteria to utilize citrate as sole source of carbon.

- **Triple Sugar Iron (TSI) Test (Atlas *et al*., 1995).**

The bacterial isolates were cultured on TSI agar slants by stabbing into the bottom and streaking on surface, and then incubated for (24-48) hrs at 37 ^{o}C. The results could be summarized as follows:

Slant / Butt	Color	Utilization
Alkaline / acid	Red /Yellow	Glucose fermented; peptones assimilate
Acid / acid	Yellow / Yellow	Glucose, lactose and sucrose fermented
Alkaline / alkaline	Red / Red	Glucose, lactose and sucrose not fermented/ peptones assimilate

The formation of black precipitate is an indication of H_2S production, whereas pushing the agar to the top indicates CO_2 formation.

- **Methyl- Red Test (Maza *et al*., 1997).**

MR-VP broth was inoculated and incubated at 37 ^{o}C for 48 hrs. Five drops of methyl red reagent was added and mixed. Positive results are bright red and negative ones are yellow.

- **Vogus Proskauer Test (Maza *et al*., 1997).**

MR-VP broth was inoculated and incubated at 37 ^{o}C for 24 hrs. Then 1ml of Barrett's reagent A (2.1.6.4) and 3 ml of Barrett's reagent B (2.1.6.4) were added to 5 ml of cultured broth and shacked for 30 seconds. The formation of pink to red color represents a positive test.

- **Urease production test (Atlas *et al*., 1995).**

Christensen urea agar slants were inoculated with bacteria, incubated at 37°C for 24-48 hrs, the appearance of pink color slant indicate a positive

reaction. This test was used to examine production of urease enzyme, which hydrolysis the urea to ammonia and CO_2.

- **Motility test (Collee *et al.*, 1996).**

Semisolid agar medium was inoculated with each bacterial isolate using a straight wire to make a single stab down the center of the tube to about half the depth of the medium. Motile bacteria typically give diffuse, hazy growth that spreads throughout the medium rending it slightly opaque.

2.2.10. VITEK2 system for identification of bacterial isolates.

VITEK2 system (figure 2-1) is used for diagnosis of bacterial isolates, it is consists of 64 biochemical tests and 20 antibiotic tests.

The VITEK2 system was used in this study to confirm the identification of DBT utilizing bacteria as follow:

- A single pure colony of bacterial isolate was suspended in 3 ml of physiological normal saline in a sterile manufacture's tube.
- Bacterial suspension was compared with standard turbid static solution (turbidity measurement system) to measure the turbidity supplier of the company, where the final concentration inside the tube must range between 0.5-0.63.
- The tubes were putt in its own rack after it was added to each tube examination VITEK 2 cassette depending on the diagnostic gram stain, where there is a special strip for Gram negative bacteria.
- The rack containing tubes and cassette was transferred to the system, putted in the first field of fillers automatically populates the cassettes with bacterial suspension and after the finishing of the process gave the end signal from the device.
- The second field reader was transferred to it, its first cut the tapes and gives order (burden) is a digital signal since kept. The rack containing tubes, moving from the device to give the data for each sample on a computer attached to the VITEK system.

- The taps were left for 24 hrs. at 37^{o} C then read the result for diagnosis of bacteria (appendix 1) .

Figure (2-1): VITEK2 apparatus

2.2.11. Monitoring growth of efficient isolates using different carbon and sulfur sources.

Basel salt medium (BSM) were prepared according to (2.5.1.2) and supplemented with DBT or $MgSO_4$ (0.1 mM) as the sole sulfur source and (10 mM) glycerol as the available carbon source (table 2-1). The media were dispensed in conical flasks (100 ml/ 250 ml flasks) and inoculated with bacterial isolates (M9, M19, S25 and P13).

The inoculated flasks were incubated at 37 ^{o}C under shaking (150 rpm) for 4 days and the bacterial growth was followed by measuring the optical density at 580 nm.

Table (2-1): Preparation of BSM containing different carbon and sulfur source

Contents	Media supplemented with differ carbon and sulfur source		
	Medium (C)	Medium (B)	Medium (A)
BSM	+	+	+
Glycerol (10 mM)	+	-	+
DBT (0.1 mM)	-	+	+
$MgSO_4$ (0.1 mM)	+	-	-

+: presence, -: absent

2.2.12. Selection the efficient isolates that capable of utilizing and/or desulfurizing DBT compound.

The efficient bacterial isolates capable of desulfurizing DBT (as S-source only) were chosen depending on Gibb's assay. The efficient isolates gave high readings at 610 nm after developing blue color (due to the reaction of phenolic end products with Gibb's reagent) of the doubling concentrations of DBT (0.1, 0.2, 0.4, 0.8 mM).

The efficient bacterial isolates capable of utilizing DBT as sulfur and carbon source were chosen depending on their growth (isolates that gave high $O.D_{580}$) in medium (B) at 37 ^{o}C for 4 days.

2.2.13. Determination of optimal conditions for utilizing and biodesulfurizing of DBT by selected isolates.

Optimization experiments were carried out through dispensed of 100 ml of BSM in 250 ml Erlenmyer flasks, inoculated with bacterial isolates. The flasks were incubated in shaker incubator (150 rpm). After incubation period, bacterial growth density was determined for each sample by measuring the $O.D_{580}$.

2.2.13.1. Effect of incubation time.

In order to determine the optimum incubation time, the basel salts medium (BSM) (pH 7.2) supplemented with 0.1 mM DBT was inoculated and incubated at 37 ^{o}C for different times (24, 48, 72, 96 and 168 hrs).

2.2.13.2. Effect of pH.

BSM supplemented with 0.1mM DBT was prepared at different pH values (6.5, 7, 7.5, 8, 8.5, 9 and 9.5) to determine the optimum pH. Then cultures were incubated at 37 ^{o}C for three days.

2.2.13.3. Effect of temperature.

BSM (pH 8) supplemented with 0.1 mM DBT was inoculated and incubated at different temperatures (25, 30, 35, 40, 45 and 50 ^{o}C) for three days. Optimum temperature was subsequently employed.

2.2.13.4. Growth at different DBT concentration.

Dibenzothiophene (DBT) was added to BSM (pH 8) at different concentrations (0.025, 0.050, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 mM), then inoculated and incubated at 35 °C for three days.

2.2.14. Plasmid extraction.

2.2.14.1. PureYield™ Plasmid Miniprep (Promega) kit.

1- Bacterial culture (600 µl) was added to a 1.5 ml microcentrifuge tube, and then 100 µl of cell lysis buffer was added and mixed by inverting the tube 6 times. Cold (4–8 °C) neutralization solution (350 µl) was added and mixed thoroughly by inverting, then centrifuged at maximum speed in a microcentrifuge for 3 minutes.

2- The supernatant (~900 µl) was transferred to a PureYield™ Minicolumn (without disturbing the cell debris pellet). Then the minicolumn was placed into a collection tube, and centrifuged at maximum speed in a microcentrifuge for 15 seconds.

3- The flow through minicolumn was discarded and placed into the same collection tube. Endotoxin removal wash (ERB) (200 µl) was added to the minicolumn, and then centrifuged at the maximum speed of microcentrifuge for 15 seconds.
4- Column wash solution (CWC) with 400 µl was added to the minicolumn, and then centrifuged at the maximum speed of microcentrifuge for 30 seconds.
5- The minicolumn was transferred to a clean 1.5 ml microcentrifuge tube, then 30 µl of elution buffer or nuclease-free water was added directly to the minicolumn matrix, it was kept for 1 minute at room temperature. After that, centrifuged for 15 seconds to elute the plasmid DNA and cap the microcentrifuge tube, and stored eluted plasmid DNA at –20 °C.

2.2.14.2. Salting out method (Pospiech and Neumann, 1995).

1- Culture of bacteria grown in 20 ml of LB broth was pelleted by centrifugation at 6000 rpm for 15 min.
2- The pellet was washed with 3 ml of STE buffer, and the cells were resuspend with 1.6 ml of STE buffer, and then freshly prepared lysozyme (final concentration 1mg/ml) was added and incubated at 37^{o}C for 30 min.
3- One ml of 10 % SDS was added and mixed by inversion, then incubated at room temperature for 30 min.
4- Two ml of 5 M NaCl was added and mixed by inversion at room temperature.
5- An equal volume of chloroform was added, mixed by inversion for 15 min, then centrifuged (6000 rpm at 4 ^{o}C) for 20 min.
6- The aqueous phase was transferred to another sterile tube, and 0.6 volume of isopropanol was added and mixed by inversion. It was kept at room temperature for 15 min. then, centrifuged at 13000 rpm for 15 min at 4^{o}C.
7- The isopropanol was discarded and the precipitated DNA dissolved in 100 µl TE buffer and stored at -20^{o}C.

2.2.15. Agarose gel electrophoresis (Green and Sambrook, 2012).

Agarose gels (0.7 %) were run horizontally in tris-borate-EDTA (TBE1X). Samples of DNA were mixed with 1/10 volume of the loading buffer and added to the wells on gel. Generally, gel was run for 2-3 hrs at 5 V/cm and the gel buffer added until it covered the gel for about 1-2 mm. Agarose gels were stained with the ethidium bromide by immersing them in the distilled water containing the dye at final concentration of 0.5 μg/ml for 15-20 min. DNA bands was visualized by U.V transillumination at 302 nm, gels were de-stained in distilled water for 30-60 min to get ride of background before photographs were taken.

2.2.16. Biodesulfurization of DBT and Diesel by resting bacterial cells.

Bacterial isolates were grown in 500 ml of BSM containing 0.6 mM DBT (as sole S-source) at 35 ^{o}C. The culture with $O.D_{580}$ of 1.421- 1.602 were centrifuged (10,000 rpm, 4^{o}C for 15 min) to harvest the cell pellets, which were washed twice with BSM and resuspended in 500 ml DBT-BSM and incubated at 35 ^{o}C for 48h to induce the enzymes of the biodesulfurization process.

The resulting cell pellets were washed once and then suspended in 50 ml of BSM lacking DBT to prepare bacterial cell suspension. The cell suspension of each culture was dispensed in two conical flasks (50 ml/ 250 ml) and 25 ml of diesel (diesel fraction of 4650 ppm total sulfur) was added to each flask. Other two conical flasks containing 70 ml of BSM supplemented with 0.6 mM DBT were inoculated with 5 ml of cell pellets suspension of each isolates to complete volume to 75 ml in each flask (250 ml).

These flasks in addition to non inoculated flasks, served as control for biotic losses, were incubated under shaking (150 rpm) at 35 ^{o}C for 3 days for DBT-BSM and 7 days for diesel-BSM. After incubation the cultures were centurifuged to separate bacterial cell, DBT and diesel layer were extracted from cell free supernatant. One ml of culture supernatant, from cultures growing with

DBT or diesel was extracted with 3 ml of ethylacetate, this solvent was evaporated by lyophilizer and the residue was dissolved in 100 µl ethanol. The total amount of sulfur contained in the DBT and diesel layer and the selective detection of the sulfur species were traced by GC/MS and X-ray analysis as follow:

2.2.16.1. Detection and verifying the chemical nature of biodesulfurizing products by GC/MS analysis (Smart *et al.*, 2010).

The identity and quantity of end products of extracted samples (as mentioned above) were determined by using gas chromatography mass-spectrometer (GC/MS) (figure 2-2).

Figure (2-2): Gas Chromatograph Mass Spectrometer (GC/Mass) apparatus

The GC/MS analysis was conducted with capillary ultra 2 columns (25 mm length, 0.32 mm inner diameter, 0.52 µm film thickness, and 3.2 cm flow). Helium gas (high purity) was used as a carrier gas. The temperature program for the analysis of 2-hydroxybiphenyl was 75^{o}C (1 min isothermal), 75^{o}C to 200 ^{o}C (20 ^{o}C/min), 200^{o} C to 280^{o}C (15^{o}C/min), and 280^{o}C (6.42 min isothermal).

The analytical procedure using GC/MS was carried out in the Research Department of Environment and Water in the Ministry of Science and Technology.

2.2.16.2. Detection of the DBT and diesel sulfur content by using sulfur content X-ray.

The analytical procedure using sulfur content X-ray was carried out in Chemical, Petrochemical Research Center. Department of quality control of The Ministry of Industry and Minerals.

Chapter Three Results and Discussion

3. Results and Discussion

3.1. Isolation of dibenzothiophene utilizing bacteria.

To isolate bacteria harboring the capability of desulfurization petroleum from nature, soil samples with a history of oil pollution were chosen because most of the indigenous isolates living in these soils have acquired adaptable mechanisms. A total of forty soil samples were collected from different sites in Iraq with a history of oil or its derivatives pollution.

To enrich the potential DBT desulfurizing bacterial isolates, BSM (2.1.5.2) constituted of minerals and 1 mM of DBT as sole source of sulfur with glycerol (10 mM) was used as the carbon source, inoculated with one percent (w/v) of soil samples. All the samples showed clear good growth after four days of incubation at 30 ^{o}C. After that, these cultures were plated on LB (2.1.5.2.) to get on pure isolates. Sixty three bacterial isolates were obtained and these isolates were examined for their capability to utilize DBT by culturing on two types of media (A and B)(2.2.6).

The growth patterns and utilization of DBT with and without glycerol (carbon source) in BSM were tested at 37 ^{o}C incubation temperature. The obtained results (table 3-1) showed that most bacterial isolates capable to grow on DBT in presence or without presence of glycerol (carbon source). However the growth density (O.D at 580 nm) of bacterial isolates in presence of glycerol (in medium A) was more than growth density without presence of glycerol (in medium B). And this may be attributed to the fact that glycerol is a simple source of carbon helps the bacteria to grow faster and stronger than in its absence (Kenny *et al.*, 2012).

Results (table 3-1) showed also that four isolate (S23, S27, S28 and S39) were unable to grow on DBT only (medium B). This means that these isolates need a simple C-source (e.g. glycerol) to support their growth.

Table (3-1): The growth density of bacterial isolates in basel salt medium (pH 7) supplemented with 0.1 mM of DBT along 4 days of incubation with shaking (150 rpm) at 37°C.

Bacterial growth (O.D 580 nm)					
Isolate NO.	Medium A with glycerol	Medium B without glycerol	Isolates NO.	Medium A With glycerol	Medium B Without glycerol
M1	1.124	0.702	**P9b**	1.100	0.629
M2	0.971	0.731	**P11**	1.130	0.545
M3	1.170	1.140	**P12**	0.424	0.382
M4	1.136	0.802	**P13**	1.468	1.253
M5	1.044	0.734	**P14**	0.850	0.820
M6	1.478	0.683	**P15**	1.224	0.810
M7	2.005	1.150	**P16**	1.324	0.806
M8	1.986	0.722	**P17**	1.030	0.790
M9	1.050	0.812	**P18**	1.402	0.642
M10	1.990	0.756	**P19**	1.214	0.680
M2b	1.002	0.803	**P20**	0.771	0.525
M9b	1.858	0.730	**S21-1**	1.790	1.200
M11	0.739	0.487	**S21-2**	0.939	0.625
M12	0.617	0.537	**S22**	1.244	1.054
M14	1.186	0.562	**S23**	0.118	0.054
M15	0.839	0.372	**S24**	0.754	0.361
M16	1.192	1.198	**S25**	0.785	0.663
M17	1.404	0.803	**S26**	0.895	0.878
M18	0.904	0.303	**S27**	0.607	0.056
M19	0.955	0.292	**S28**	0.697	0.066
M20	1.090	0.982	**S29**	1.240	0.420
P1	0.586	0.487	**S30**	0.517	0.450

P2	0.763	0.131	**S31**	0.336	0.231
P3	0.994	0.158	**S32**	0.927	0.481
P4	1.080	0.964	**S33**	0.586	0.217
P5	0.828	0.498	**S34**	0.983	0.833
P6	0.584	0.213	**S35**	0.670	0.401
P7	0.711	0.207	**S36**	1.272	0.580
P8	0.812	0.728	**S37**	1.302	0.840
P9	0.860	0.527	**S39**	1.394	0.055
P10	0.581	0.543	**S40**	0.645	0.198
P2b	0.520	0.390			

The growth of bacterial isolates in medium (B), which not contained C-source, might be attributed to ability of these isolates to utilize DBT as C-and S-source or utilize ethanol as C- source, because DBT in this medium was dissolved in ethanol.

3.2. Utilization of ethanol and $MgSO_4$ as alternative carbon and sulfur sources.

To confirm that the isolates are able to utilize ethanol as C-source, all bacterial isolates were grown in BSM containing DBT plus ethanol and in BSM containing ethanol plus $MgSO_4$ (see item 2.2.7). Results indicated that the growth density ($O.D_{580}$) of bacterial isolates was a little more in medium containing ethanol plus $MgSO_4$ compared to the second medium. This means that these isolates are capable to utilize ethanol as C- source. The utilization of ethanol as a carbon source was reported for *Rhodococcus erythropolis* KA2-5-2 (Tanaka *et al.,* 2002) and *Gordonia* sp. CYKSI (Choi *et al.,* 2003), which are DBT desulfurizing bacteria. The cultures containing 0.1 or 1.0 % ethanol exhibited a shorter lag time and more rapid exponential growth than cultures grown with glucose alone. However, the presence of ethanol in a medium at

concentrations higher than 1.0 % produced progressively decreased exponential growth rates and slightly reduced extents of growth (Yoshikawa *et al.,* 2002). The little difference in the capability of these isolates in utilizing both DBT and $MgSO_4$ as S-source, could be related to the fact that $MgSO_4$ is a simple source of sulfur compared to DBT. However, the presence of ethanol increases the solubility (bioavailability) of DBT. It was suggested that NADH, which is produce by the biochemical reaction of NAD with ethanol catalyzed alcohol dehydrogenase, might contributed to the conversion of FMN to $FMNH_2$, which is coenzyme for the activities of desulfurization enzymes (Yan *et al.,* 2000). It was reported that the addition of DBT dissolved in ethanol provided more rapid growth and desulfurization than DBT powder only (Setti *et al.*, 1999).

3.3. Screening of DBT desulfurizing bacteria.

Gibb's reagent (2,6-dichloro-*p*-benzoguinone-4-chloroimine), which detects the formation of corresponding phenolic end-products resulting after desulfurization of DBT, was used to screen this activity in our DBT utilizing bacterial isolates.

All the sixty three isolates were subjected to Gibb's assay after four days of incubation. The results showed that three isolates (M9, M19 and S25) have the ability to desulfurizing DBT and converted it to 2-HBP or other phenolic end products and gave blue color in the presence of Gibb's reagent. It was noticed that these isolates gave positive results only when grown in medium (B). All the isolates (including M9, M19 and S25) gave negative results when grown in medium (A). These isolates were repeatedly cultured in BSM supplemented with DBT, in order to ensure their desulfurization ability. Results (figure 3-1) confirmed their ability and referred that these isolates have metabolic capability of C-S bond cleavage and lacks the metabolic capability of attacking the carbon skeleton of DBT via the C-C bond cleavage. This preliminary result suggests the involvement of the 4S pathway in the utilization of DBT via a specific cleavage

of only the C-S bond by bacterial cultures exhibiting positive results with Gibb's reagent (Gupta *et al.,* 2005; Mohebali and Ball, 2008).

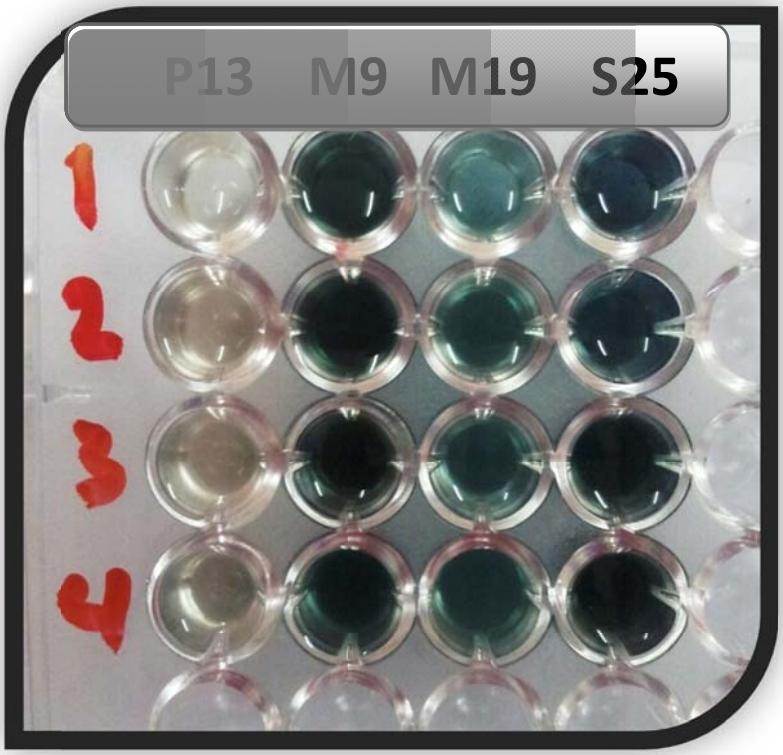


Figure (3-1): Gibb's assay guided desulfurization. The formation of blue color is an indication of desulfurization and formation of phenolic end products.

The inability of the other isolates for desulfurizing DBT might be due to: firstly, a bacteria attacked the aromatic skeleton of the compound and utilizing carbon and sulfur in DBT, so no 2-HBP will formed, it would be concluded that almost all bacterial isolates growing on DBT attacked this compound via cleavage of both C-C and C-S bonds in a manner similar to degradation by the common Kodama pathway (Gupta *et al.,* 2005; Mohebali and Ball, 2008). Secondly, the isolates (that gave negative Gibb's results) may have other specific enzymes that attack the 2-HBP and degraded it to less complex compounds and utilizing 2-HBP as carbon source (Al-Sayegh, 2005).

However, Gibb's is not enough to distinguish whether these phenolic compounds are intermediates or the final end product, the accumulation of the blue color may give prediction of the presence of the end product but still further experiments are needed (Al-Hassar, 2010).

In addition to the three isolates which gave positive results with Gibb's assay, twelve efficient isolates (cleaved both C-C and C-S bonds of DBT) were candidate for subsequent study. The twelve isolates (M3, M7, M16, M20, P4, P13, P14, S21-1, S22, S26, S34 and S37; however, P13 isolate was the best one) showed respectable growth density in medium (B) (high $O.D_{580}$) compared with other isolates throughout the incubation period (table 3-1), and this might be attributed to physiological and genetic properties of these isolates. However, it is difficult to predict which molecular change can be expected by a specific microbe, since each group of microorganisms, even various strains of one genus, can alter a selected molecule differently (Zipper *et al.,* 1998).

3.4. Identification of isolates.

Fifteen isolates were selected for identification according to their ability to utilize DBT, as S- source only or S- and C- source, as mentioned above. These isolates were identified depending on morphological, cultural and biochemical characteristics. Results indicated in table (3-2) show that these isolates were identified as *Staphylococcus* spp. (6 isolates, including P13 isolate, figure 3-2), three of them were *Staphylococcus aureus. Pseudomonas* spp. (4 isolates), three of them were *Pseudomonas aeruginosa* (figure 3-3). *Micrococcu* spp. (2 isolates), and one isolate for each genus of *Neisseria* sp., *Corynebacterium* sp. and *Bacillus* sp.

The morphological, cultural and biochemical characteristics for these isolates (table 3-2) were as prescribed by Bergey's Manual of Systematic Bacteriology (2000) and in agreement with Holt *et al.* (1994).

Table (3-2): Morphological, physiological and biochemical characteristics of Bacterial isolates.

Characters		Isolates				
		M3	**M7**	**M16**	**M20**	**P4**
Colony color		Cream to yellow	Cream	Cream to Yellow	Yellow	Cream
Cell shape		Cocci	Cocci	Cocci	Cocci	Cocci
Gram stain		+	-	+	+	+
Catalase		+	+	+	+	+
Oxidase		N	+	+	N	N
Motility		-	-	+	-	-
Urease		W	+	-	W	-
Gelatinase		-	-	-	-	N
Nitrate reduction		+	-	-	+	-
Citrate utilization		-	+	-	-	+
MR		+	-	+	W	-
VP		-	+	-	-	-
Indol		-	-	-	-	-
Mannitol		+	-	-	+	-
Bile salt		N	N	N	N	N
King A		N	N	N	N	N
King B		N	N	N	N	N
Growth on Cetrimide		N	N	N	N	N
TSI	H_2S	-	-	-	-	-
	CO_2	-	+	-	-	-
	Slant	Acid	Acid	Alkaline	Acid	Alkaline
	Butt	Acid	Acid	Acid	Acid	Alkaline

Characters		Isolates				
		P13	**P14**	**S22**	**S21-1**	**S26**
Colony color		Cream	Cream	Cream to Yellow	Yellow	White
Cell shape		Cocci	Cocci	Cocci	Rod	Rod
Gram stain		+	+	+	-	+
Catalase		+	+	+	+	+
Oxidase		N	N	N	+	N
Motility		-	-	-	+	-
Urease		W	+	-	-	N
Gelatinase		N	N	-	-	-
Nitrate reduction		-	-	-	+	+
Citrate utilization		+	+	-	+	+
MR		-	W	+	-	-
VP		W	+	+	W	+
Indol		-	-	-	-	-
Mannitol		+	+	-	-	+
Bile salt		+	N	N	N	N
King A		N	N	N	+	N
King B		N	N	N	+	N
Growth on Cetrimide		N	N	N	+	N
TSI	H_2S	-	-	-	-	-
	CO_2	-	+	-	-	-
	Slant	Acid	Acid	Alkaline	Acid	Acid
	Butt	Acid	Acid	Acid	Acid	Acid
Characters		**Isolates**				
		S34	**S37**	**M9**	**M19**	**S25**
Colony color		White	Yellow	Green	Green	Green

Cell shape		Spore bearing bacilli	Cocci	Rod	Rod	Rod
Gram stain		+	+	-	-	-
Catalase		+	+	+	+	+
Oxidase		+	N	+	+	+
Motility		-	-	+	+	+
Urease		-	W	-	-	-
Gelatinase		+	-	+	+	-
Nitrate reduction		+	+	-	+	+
Citrate utilization		+	-	+	+	+
MR		-	W	W	+	+
VP		+	W	-	W	-
Indol		-	-	-	-	-
Mannitol		-	+	-	-	-
Bile salt		N	N	N	N	N
King A		N	N	+	+	+
King B		N	N	+	+	+
Growth on Cetrimide		N	N	+	+	+
TSI	H_2S	-	-	-	+	+
	CO_2	-	-	-	+	-
	Slant	Alkaline	Acid	Alkaline	Alkaline	Alkaline
	Butt	Alkaline	Acid	Alkaline	Acid	Acid

+: Positive result (-): Negative result N: Not tested W: Weak test

TSI: Triple sugar iron agar MR: Methyl red VP :Vogas-Proskauer

Staphylococcus spp.= P4, P13, P14; *Staphylococcus aureus*=M3, M20, S37; *Pseudomonas aeruginosa*=M9, M19, S25; *Neisseria* sp.= M7; *Bacillus* sp.=S34; *Pseudomonas* sp.=S21-1; *Corynebacterium* sp.= S26; *Micrococcus* spp.= M16, S22

Figure (3-2): Growth of P13 on LB agar plate.

Figure (3-3): Growth of *Pseudomonas aeruginosa* on LB agar plates.

Many studies, showed that the maximum hydrocarbon degrading potential followed by *Staphylococcus* spp. (Debajit and Yaday, 2012). Degradation activity of the hydrocarbon from environmental samples revealed that *Staphylococcus aureus*, was the potent degraders of hydrocarbons (petrol and diesel). The ability of these isolates (*Staphylococcus* spp.) to degrade hydrocarbons was clear evidence that their genome harbors the relevant degrading gene (Shukor *et al.,* 2009; Jyothi *et al.,* 2012). Also, *Micrococcus* showed potential degrading of petroleum hydrocarbon (PHC), benzene and diesel when isolated from oil-contaminated soil (Ajeet *et al.,* 2014). The usage of petroleum hydrocarbon products increases soil contamination with diesel and engine oil is becoming one of the major environmental problems. Bioremediation provides an effective and efficient strategy to speed up the clean up processes. For this purpose, isolation of the indigenous hydrocarbon degrading microorganisms i.e. *Staphylococcus* spp., and *Corynebacterium* sp. was important step. (Debajit, 2011).

It was reported that *Neisseria elongate* showed potential degradation of crude oil by more than 50 %. Maximum growth of the isolates in the mineral salt medium (pH 7) occurs at 37 ^{o}C, and shaking (130 rpm) (Makred *et al.,* 2008).

The three isolates (M9, M19 and S25), that showed desulfurizing activity of DBT, were belonged to the species *Pseudomonas aeruginosa* (table 3-2) and constituted 4.7 % from the total sixty three isolates.

Boboye *et al.* (2010) recorded that biodegradation, which was the destruction of organic compounds by microorganisms, carried out largely by diverse bacterial populations, mostly by *Pseudomonas* species. The common bacterial genera exploited for benzene bioremediation are *Pseudomonas* and *Bacillus* (Mukherjee and Bordoloi, 2012). Nasier *et al*. (2002) referred that *Pseudomonas* spp. were the most abundant and efficient bacterial species in utilizing crude oil and different hydrocarbon compounds.

3.5. VITEK2 system for identification of DBT desulfurizing isolates.

The three isolates that desulfurized DBT and suspected as *Pseudomonas aeruginosa,* as well as the *Staphylococcus* isolate (P13), which was the efficient isolate in utilizing DBT as C- and S- source, were subjected to further identification using VITEK2 system, in order to confirm their identification.

Results illustrated in appendix (2) confirmed the results obtained by morphological, cultural and biochemical tests. For the identification of the three isolates which is previously identified as *Pseudomonas aeruginosa.*

The *Staphylococcus* sp. (P13), was unidentified by this system (appendix 3). Although the isolate was pure, and the test was repeated several time in two different centers used VITEK 2 system.

The VITEK 2 system provides biochemical as well as antibiotic susceptibility tests. Appendix (4), (5) showed positive results of the *Pseudomonas aeruginsa* and results of the unidentified (P13) respectively.

To shed more light on the identification of P13 isolate, it is recommended to subjecte this isolate to molecular identification.

3.6. Bacterial growth with different carbon and sulfur sources.

The basel salt medium (BSM) supplemented with different carbon and sulfur sources, prepared according to table (2-1), were used to select the best medium for growing the DBT utilizing bacteria. The growth of the three desulfurizing isolates (*P. aeruginosa* M9, M19 and S25), in addition to P13 isolate (utilizing DBT as carbon and sulfur source), was followed at 580 nm in three culture media. The obtained result clearly demonstrated highest growth of all tested isolates in medium (C), with glycerol and $MgSO_4$ as carbon and sulfur sources respectively (Table 3-4), when compared with their growth in the two other media. The isolates with less cell density in medium (B) could be attributed to the absence of glycerol. The bacterial growth in medium (A) was more than in medium (B) and lower than in medium (C) and this might be due to

the glycerol which is simple and rich source for carbon (as mentioned before). The highest growth in medium (C) could be related to glycerol and $MgSO_4$ (inorganic), which is simple S-source compared to DBT (organic) (Akbar, 2008).

Table (3-3): Bacterial growth (optical density at 580 nm) in Basel salt media with different carbon and sulfur sources at 37 ^{o}C for 4 days.

Isolates	Bacterial growth ($O.D_{580}$ nm) after 4 days of incubation		
	Medium (C)	Medium (B)	Medium (A)
P13 isolate	1.706	1.2 77	1.538
***P. aeruginosa* (M9)**	2.540	0.733	0.952
***P. aeruginosa* (M19)**	2.690	0.620	1.080
***P. aeruginosa* (S25)**	1.406	0.688	0.942

3.7. Selection the efficient isolate that capable of desulfurizing DBT.

To select one isolate from the three efficient *P. aeruginosa* isolates (M9, M19 and S25) on removing sulfur atom from DBT, Gibb's assay was performed. The concentration of the end product (2-HBP or other phenolic end products), resulted from 4S pathway in culture grown with DBT (medium B) was quantified by measuring the intensity of the corresponding blue color formed after addition of Gibb's reagent (Akbar, 2008).The obtained results (table 3-4) showed increase the concentration (intensity) of the 2-HBP with increase the concentration of DBT. *P. aeruginosa* S25 gave obvious blue color upon addition of Gibb's reagent compared to other isolates. This indicated that *P. aeruginosa* S25 isolate is the efficient one for utilizing sulfur in DBT, that was clear from the results that showed a higher concentration (intensity) of the phenolic compounds accumulation.

Table (3-4): Detected the 2-HBP using Gibb's reagent in culture supernatants of bacteria growing with doubling concentrations of DBT in medium (B) at 37 ^{o}C for 4 days.

Isolates	Concentration of DBT				
	0.1 mM	0.2 mM	0.4 mM	0.8 mM	
***P. aeruginosa*-M9**	0.013	0.079	0.145	0.175	**$O.D_{610}$**
***P. aeruginosa*-M19**	0.020	0.039	0.109	0.145	
***P. aeruginosa*-S25**	0.082	0.115	0.185	0.294	

3.8. The optimal conditions for desulfurizing and utilizing of DBT by *P. aeruginosa* S25 and P13 isolate.

3.8.1. Effect of incubation time:

P. aeruginosa S25 and P13 isolates were grown and incubated at different duration time (24, 48, 72, 96 and 168 hrs) to determine the optimum time for bacterial growth. The obtained results (figure 3-4) show that the highest optical density of bacterial growth was after 72 hrs. (three days) of incubation in BSM supplemented with 0.1 mM of DBT at shaking (150 rpm) at 37 ^{o}C and the bacterial growth was decreased gradually after this time for both isolates. However, it has been reported that hydroxybiphenel (HBP) is toxic to bacterial cells; hence biodesulfurization were inhibited by accumulation of HBP (Zhang *et al.,* 2005). There was a complicating factor in batch experiments, i.e. the end product HBP was some what inhibitory to cell growth and possibly therefore to desulfurization activity. Although some of the characterized biodesulfurizing bacteria showed high rates of catalytic activity to desulfurize thiophene compounds, the overall biodesulfurization process has been shown to be inhibited by the final end products 2-HBP and sulfate (Chen *et al.,* 2008).

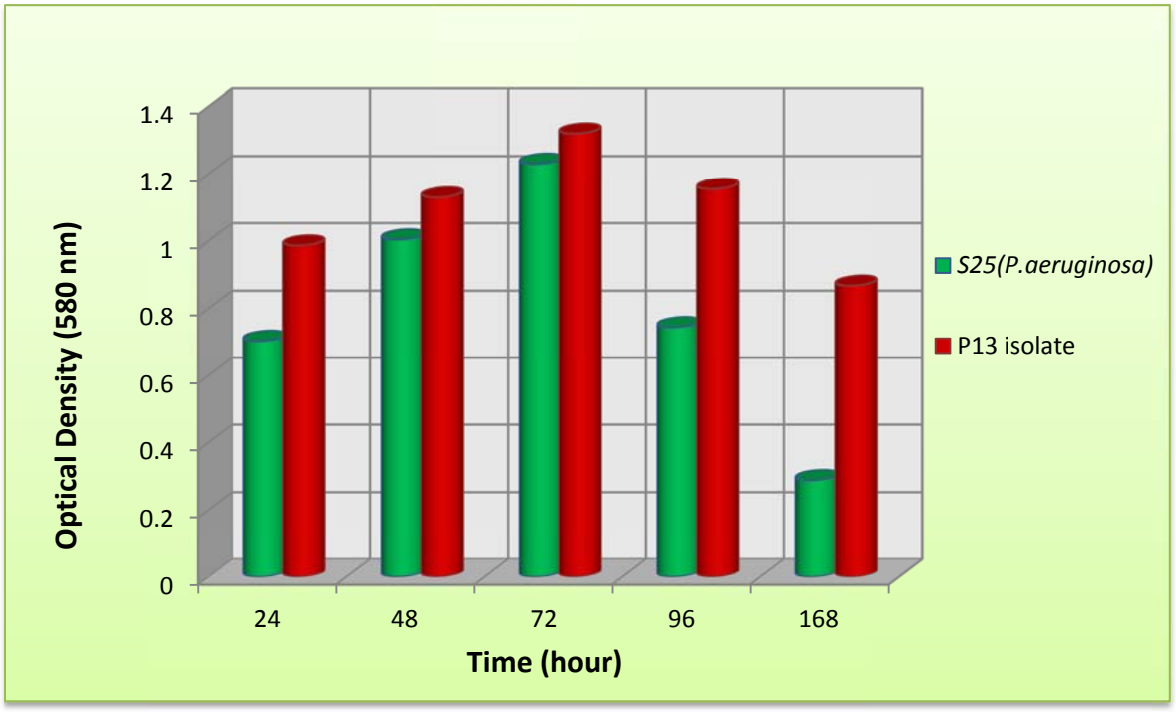


Figure (3-4): Effect of incubation time on *P. aeruginosa* S25 and P13 isolate grown in basel salt medium (pH 7.2) in shaker incubator (150rpm, 37 °C).

3.8.2. Effect of pH:

The basel salt medium (BSM) supplemented with 0.1mM DBT was prepared at different pH values (6.5, 7, 7.5, 8, 8.5, 9 and 9.5) in an attempt to determine the optimum pH required for growth of *P. aeruginosa* S25 and P13 isolate. Results presented in Figure (3-5) elucidate that an optimum growth occurred at pH 8, the optical density for bacterial growth was reached 1.292 and 1.442 for *P. aeruginosa* S25 and P13 isolate respectively. While bacterial growth was decreased at other pH values compared with growth at pH 7.5 and 8. This mean that both bacteria preferred slightly alkaline media.

Results showed that the pH values of the cultures slightly decreased companies with initial pH. This decrease in pH value could be explained by the production of intermediates or end products which might cause the decline in the pH of the medium (Hussein *et al.*, 2013).

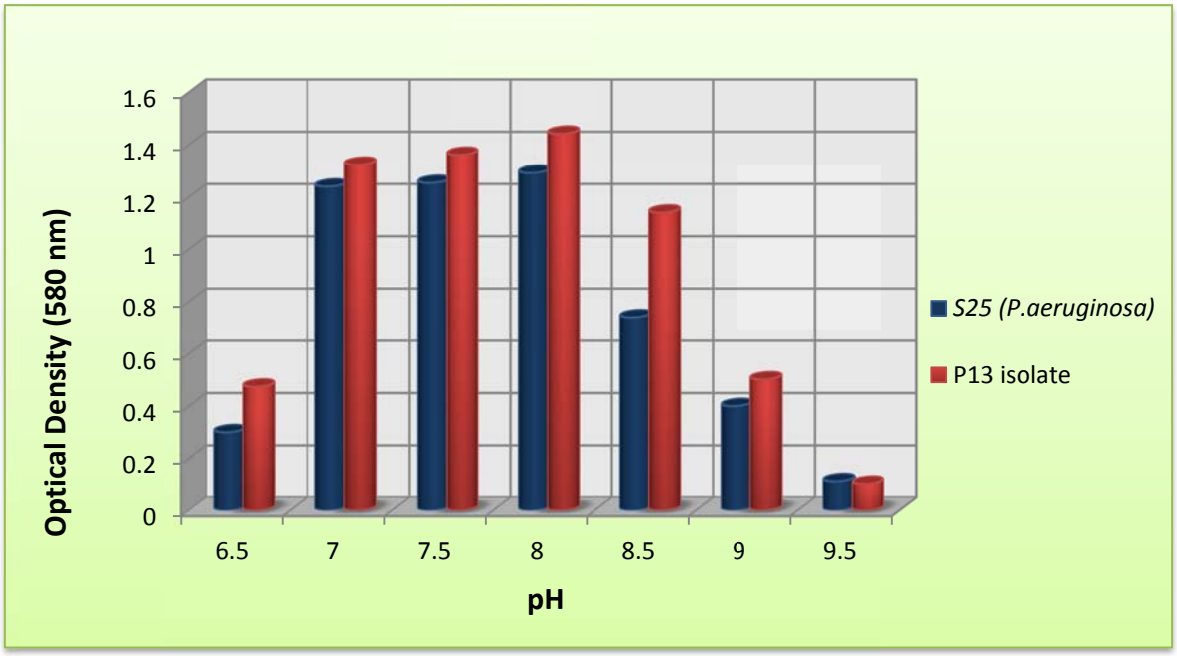


Figure (3-5): Effect of pH on DBT utilization by *P. aeruginosa* S25 and P13 isolate grown in Basel salt medium containing 0.1 mM of DBT in shaker incubator (150 rpm, 37 °C) for 3 days.

3.8.3. Effect of temperature:

P. aeruginosa S25 and P13 isolate were grown at different temperatures (25, 30, 35, 40 45 and 50 °C). Results shown in Figure (3-6) declare that the optical density ($O.D_{580}$) of bacterial growth at 35 °C was 1.302 and 1.485 for *P. aeruginosa* S25 and P13 isolate respectively. These bacteria exhibited highest cell densities only around 35^{o}C and decreased above and below this temperature. Therefore, this temperature was suggested as the optimum temperature for bacterial growth. Some of the important DBT desulfurizing bacteria such as *Rhodococcus erythropolis* IGTS8, *R. erythropolis* H-2 and *R. erythropolis* KA2-5-1 have been isolated and investigated to belong to mesophilic bacteria. All of them desulfurize DBT through a sulfur specific pathway without reducing the energy content of the molecule (Kirimura *et al.*, 2001).

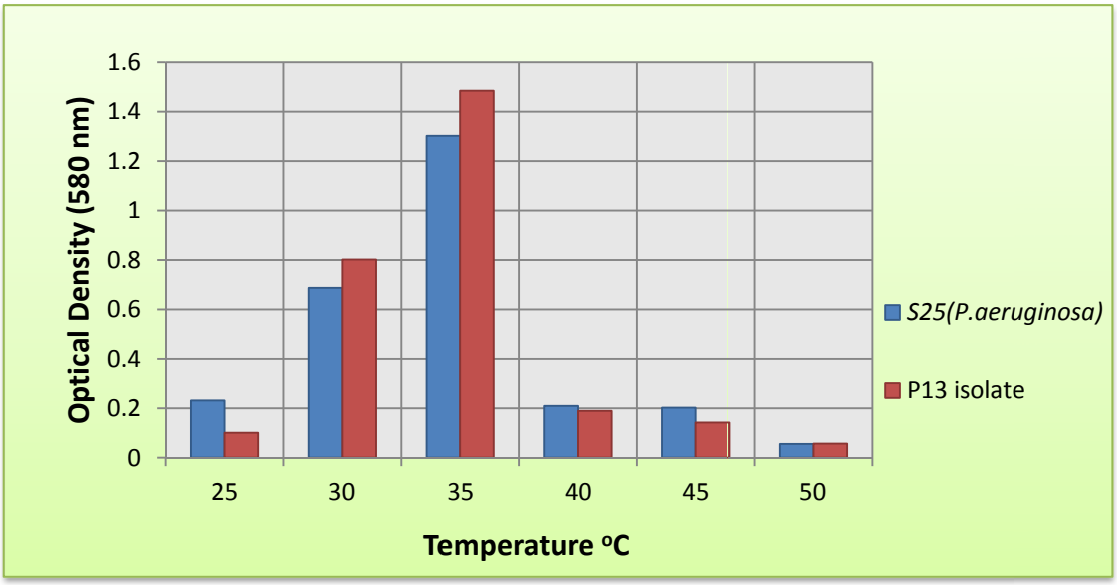


Figure (3-6): Effect of temperature on DBT utilization by *P. aeruginosa* S25 and P13 isolate grown in basal salts medium (pH 8) containing 0.1mM of DBT in shaker incubator (150rpm) for 3 days.

Only a few thermophilic DBT desulfurizing bacterial strains such as *Paenibacillus* strain (Konishi *et al.*, 1997), *Bacillus subtilis* WU-S2B (Kirimura *et al.*, 2001), *Mycobacterium phlei* (Furuya *et al.*, 2001) and *Mycobacterium phlei* WU-0103 (Ishii *et al.*, 2005) have been reported to carry out the biodesulfurization process in the temperature rang 40-60^{o}C (Kirimura *et al.*, 2004).

3.8.4. Growth at different DBT concentrations:

Different concentrations (between 0.025 mM and 1 mM) of DBT were used as S-source to grow *P. aeruginosa* S25 and P13 isolate in order to determine the optimum concentration. Results in Figure (3-7) indicated that the optimum concentration for bacterial growth was 0.6 mM, the optical density ($O.D_{580}$) of bacterial growth, at this concentration, were 1.410 and 1.615 for *P. aeruginosa* S25 and P13 isolate respectively. Figure (3-7**)** showed also that gradual increasing of DBT concentration accompanied with increasing of bacterial growth, until reached to its optimum at a concentration of 0.6 mM,

while DBT concentrations higher than 0.6 mM caused gradual decrease of bacterial growth.

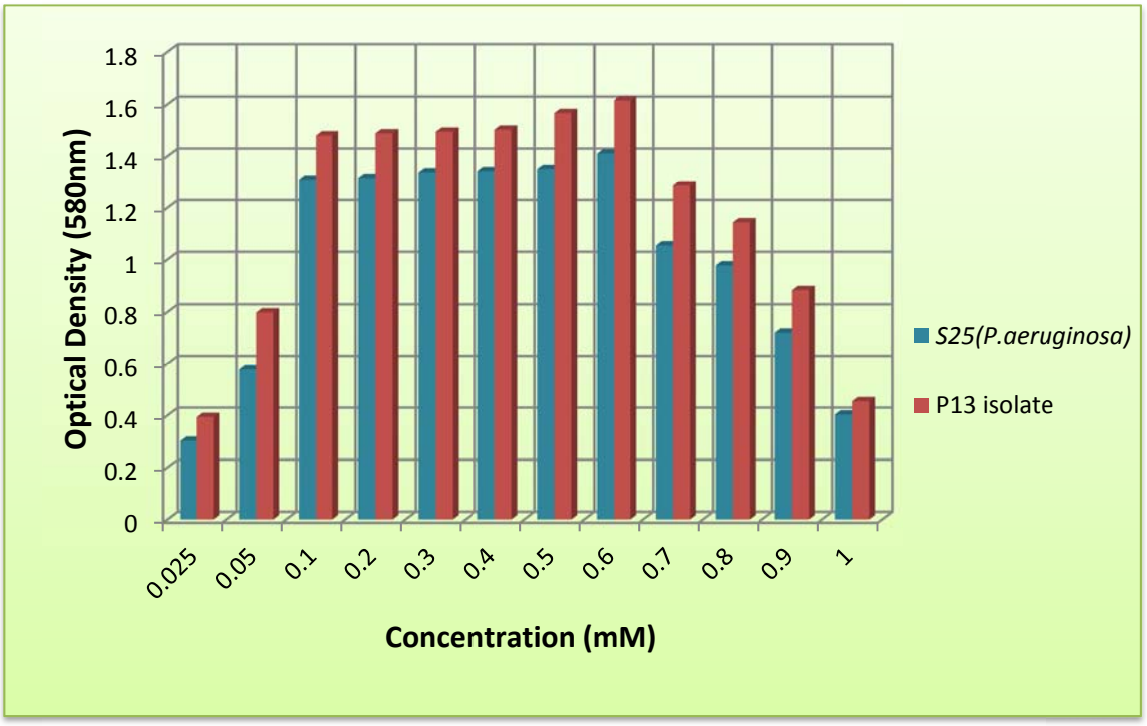


Figure (3-7): Effect DBT concentration on *P. aeruginosa* S25 and P13 35°C for 3 days.

3.9. Plasmid profile.

In order to determine the plasmid profile of *P. aeruginosa* S25 and P13 isolate, Pure Yield™ Plasmid Miniprep and salting out method were used (Pospiech and Neumann, 1995).

Results indicated that no plasmid DNA bands were shown for *P. aeruginosa* S25 and P13 isolate when plasmid miniprep kits were used.

DNA extraction by salting out method gave the best results. Figure (3-8) indicated that *P. aeruginosa* harboring a small plasmid DNA bands and these bands are approximately in the same size compared with the pBR322 plasmid (4.363 Kb), while P13 isolate has no plasmid DNA.

Several studies referred that the small plasmid DNA bands are prevalenced in *Pseudomonas* spp. and responsible for degradation of different hydrocarbon and synthetic compounds (Abd Al-Hussan, 2006; John and Okpokwasili, 2012). Several studies also indicated that a different *Pseudomonas* isolates containing plasmids with same size but have different set of genes (Bayly and Barbour, 1984), or the plasmids are the same plasmids, there were transferred between the soil population of bacteria by conjugation or transformation (Devereux and Sizemore, 1982; Trevors, 1998).

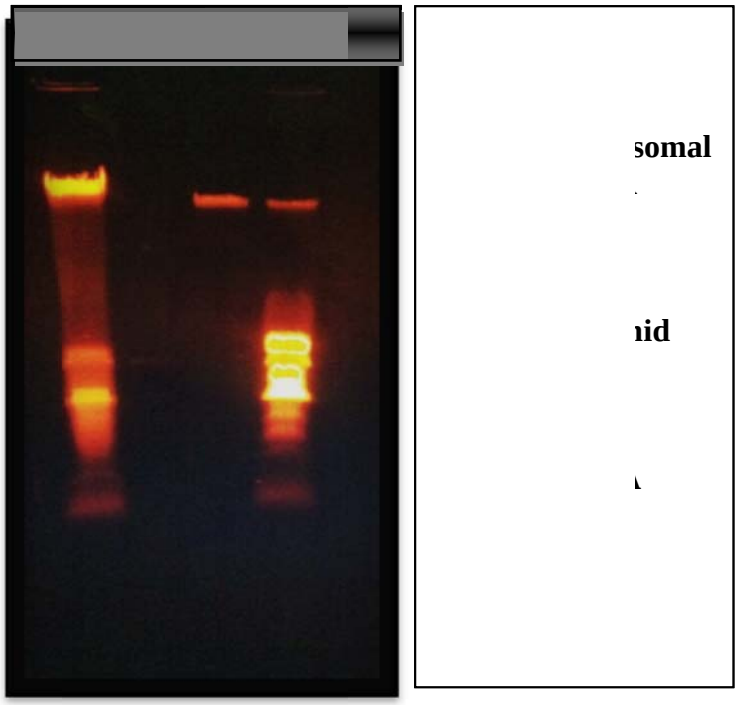


Figure (3-8): Gel electrophoresis for plasmid isolation by salting out method. Electrophoresis was performed on (0.7 %) agarose gel with 5 V/cm for one hour. 1:P13 isolate, 2: *P. aeruginosa* S25, M: *E. coli* HB101.

Mirdamadia *et al.*(2010) elucidated that certain plasmids play an important role in adaptation of natural microbial populations to oil and other hydrocarbons. Some of the microbial catabolic pathways responsible for the degradation, including the *alk* (C5 toC12 n-alkanes), *nah* (naphthalene) and *xyl* (toluene)

pathway have been extensively characterized and are generally located on large catabolic plasmids.

The *dsz* genes are arranged in an operon-regulated system in a 4-kb conserved region of a mega-plasmid (Oldfield *et al.,* 1998). It is a cluster of three genes (*dszA, dszB, dszC*) transcribed in the same direction, coding for three proteins DszA, DszB, DszC, respectively (Piddington *et al.,* 1995). These genes, when cloned on a Dsz phenotype, confer the ability to desulfurize DBT to 2-HBP. The *dsz* operon was found on a large plasmid of 150 kb in *R. erythropolis* IGTS8 and on a 100 kb plasmid in other strains. In biodesulfurization research the gene encoded a protein of ~20 kDa was present on the chromosome of IGTS8 rather than on the plasmid that contains dszABC. This indicate that genes responsible for desulfurization are located on the chromosome and/or large plasmid rather than to be on small plasmids, therefore small plasmid DNA bands of *P. aeruginosa* S25 may not have a role in desulfurization process in this bacterium.

3.10. Biodesulfurization of DBT in DBT-Basel salt media and in Diesel by resting bacterial cells.

The capability of the two bacterial isolates for desulfurizing DBT-BSM and hydrotreated diesel (with a total sulfur content ≈4650 ppm) in phase consisting 1/3 diesel and 2/3 BSM was determined .

DBT adapted cell suspension was inoculated in diesel for 7 days under shaking. As well as cell suspension was inoculated in DBT-BSM and incubated for 3 days, for the quantification and qualification the end products. Similar treatments lacking bacterial cells, served as control for non-biological loss of sulfur.

To verify the identity of the end products formed after the biodesulfurization of DBT by the bacterial isolates, cell free culture supernatants

(for both types of media with diesel and with DBT-BSM) were extracted with ethylacetate and subjected to GC/MS and X-ray analysis.

3.10.1. Detection and verifying the chemical nature of biodesulfurizing end products by GC/MS analysis.

The GC/MS analysis for the control (DBT-BSM) showed only one peak (figure 3-9), which represented DBT. Analysis of bacterial cultures on DBT-BSM indicated that, DBT showed as much as 12.89 % consumption (which represent consumption of sulfur) by the *P. aeruginosa*-S25 (which gave 32 peaks (figure 3-10), depending on the area of peaks (this is a direct proportion between area of peak and the concentration). *P. aeruginosa*-S25 showed good ability to consume sulfur when compared with other bacterial isolates.

Al-Hassar (2010) isolated two bacterial isolates assigned as 233 and 343, that have ability to desulfurization 8 % and 4 % of sulfur respectively.

The peak area appeared at a retention time of 9.867 for the control sample (DBT-BSM) was 8,942,827 (figure 3-9), this area was reduced to 1,153,056 and appeared at retention time of 9.817 (figure 3-10 and appendix 6). The analysis of DBT control sample gave only one peak, and this mean that DBT was pure and probably its purity was 91 % (SI: 91) with molecular weight 184 (appendix 7).

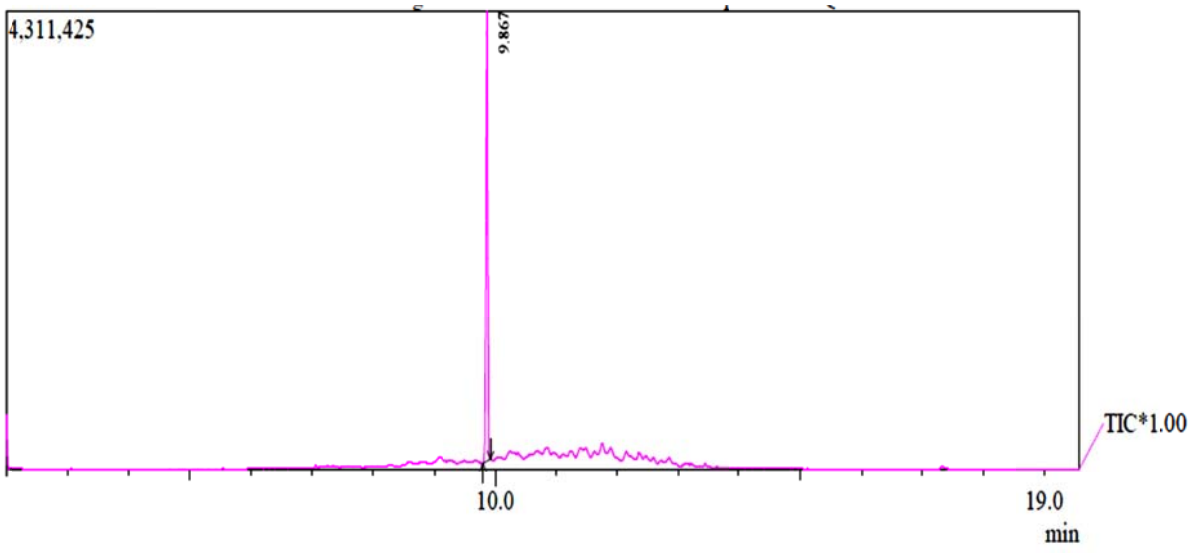
Results (appendix 8) showed also that there was further converted of 2-HBP to 1, 2-Naphthalenediol, 2-ethyl-1, 2, 3, 4-tetrahydro-, cis and to 4-Methoxybenzhydrol at retention time 9.150 and 12.367 respectively. This is like the conversion of 2HBP to 4-MBP by methylation at the hydroxyl group (Rhee *et al.,* 1998).Therefore, it could be a novel pathway of DBT degradation by *P. aeruginosa*-S25, similar to the 4S pathway except that 2-HBP was further converted to 2-MBP (by addition methyl group).

In biodesulfurization, DBT is converted to 2-HBP, which will increase the possibility of environmental pollution (Ichinose *et al.,* 1999). In contrast, methylation of the hydroxyl group of 2-HBP by bacteria may partially eliminate

the inhibitory effect of product and the pollution from diesel oil combustion (Monticello *et al.*, 2000). Therefore, it was suggested that the pathway followed by this bacteria (*P. aeruginosa*-S25) is the sulfur-selective pathway for utilization of DBT as reported earlier by Oldfield *et al.* (1997).

The GC/MS analyses for the P13 isolate which act on degradation of DBT to give 20 peaks (figure 3-11). The result indicated the ability of this isolate to utilize the DBT as sulfur and carbon source, where the analysis showed the absence of any atom of sulfur in the resulting compounds which were mostly as carbon chains, like Hexadecane (appendix 9).

Soleimani *et al*. (2007) referred that DBT might also serve as a carbon source.



Peak Report TIC

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	9.867	9.800	9.925	8942827	100.00	4223480	100.00	2.12	MI	
				8942827	100.00	4223480	100.00			

Figure (3-9): GC/MS chromatogram of control sample (DBT-BSM lacking bacterial cell). one peak appeared at retention time 9.86 which represent DBT peak.

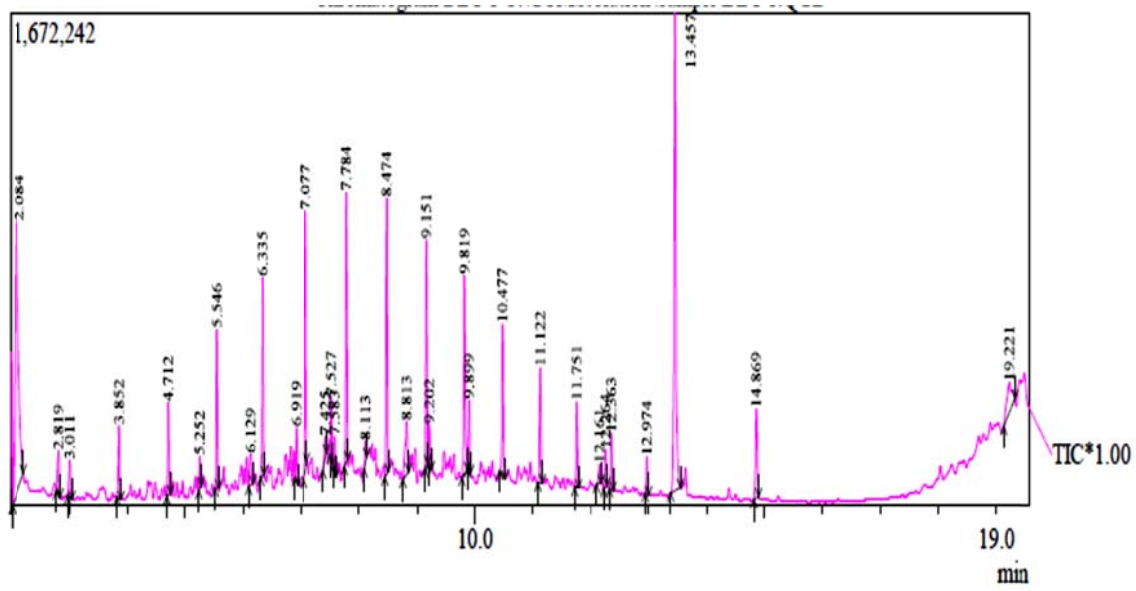


Figure (3-10): GC/MS chromatogram of *P. aeruginosa*-S25 DBT-BSM supernatant extracted culture. 32 peaks appeared with DBT peak (21) at retention time 9.81.

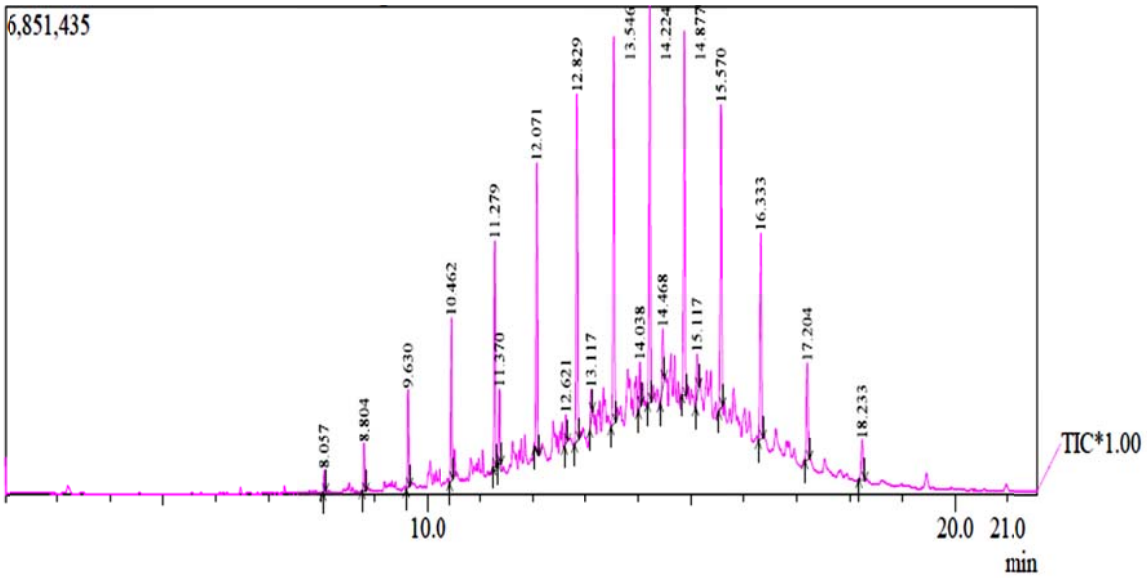


Figure (3-11): GC/MS chromatogram of P13 isolate DBT-BSM supernatant extracted culture. 20 peaks appeared.

Its recommended to studying the biochemical pathways of DBT desulfurization by these isolates.

The GC/MS analysis for the second group which included the extract of diesel (control) and samples of culture supernatant extracted after growth of *P. aeruginosa*–S25 (figure 3-13), gave 17 peaks and after growth of P13 isolate (figure 3-14), which gave 25 peaks. Analysis of diesel (control) gave 32 peaks (figure 3-12), no one of these peaks contain a DBT but instead contain other sulfur containing compounds like Thiazole, 2, 5-diethyl-4-methyl; 3-(Benzoylthio)-2-methylpropanoic acid and 2-Isopropyl-4, 5-dimethylthiazole, at a retention time 8.025, 8.083 and 9.617 respectively (see appendix 10). All these compounds that have sulfur atom were degraded by *P. aeruginosa*–S25 and P13 isolate to give less complex compounds in the form of carbon chain like Heneicosane (appeared at the retention time 9.825) which is resulted from diesel degradation by *P. aeruginosa*- S25 (appendix 11) and n-Eicosane (appeared at the retention time 8.642) which is result from diesel degradation by P13 isolate (appendix 12).

Consequently, the both isolates showed a very good ability to consume sulfur from different organosulfur compounds, and detailed studies about the biochemical pathway are required.

On the other hand, *P. aeruginosa*- S25 isolate can be considered the first isolate belong to species *P. aeruginosa* which capable of removing sulfur from DBT without any manipulation (recombinant DNA technology). Recombinant technology was used to enhance the biodesulfurization efficiency using *Pseudomonas aeruginosa* through the duplication of the *dsz* cluster in this organism. The recombinant *Pseudomonas* sp. was able to desulfurize dibenzothiophene (Raheb *et al.,* 2005). The *dsz* desulfurization gene cluster from *Rhodococcus erythropolis* KA2-5-1 was transferred into the chromosome of *Pseudomonas aeruginosa* strain NCIMB9571 using a transposon vector (Noda *et al.,* 2003)

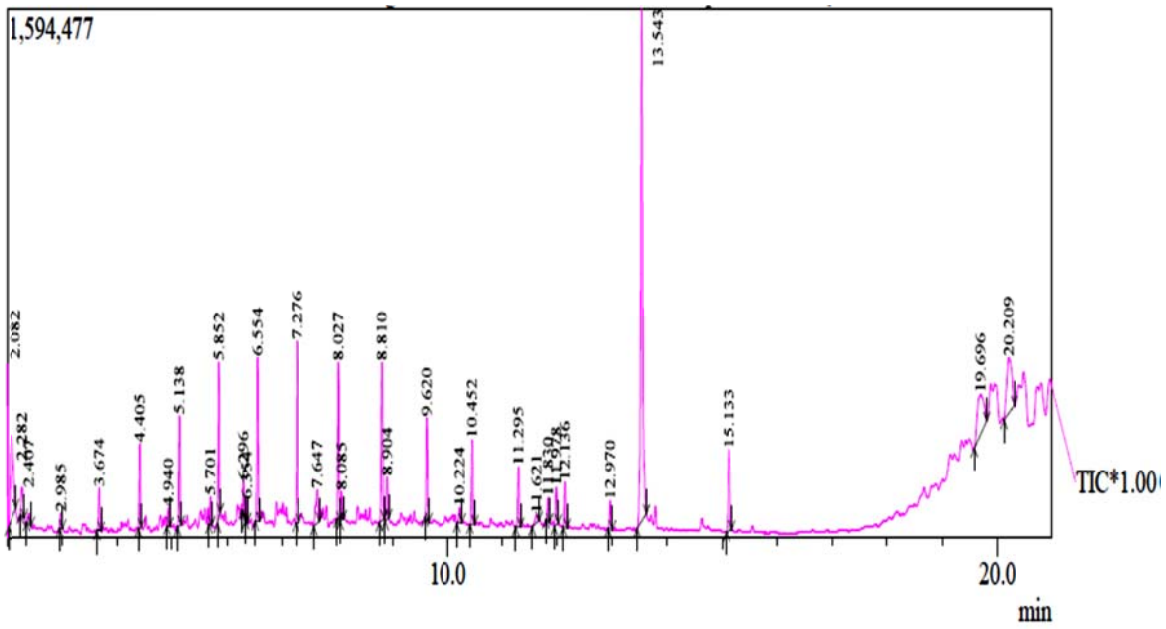


Figure (3-12): GC/MS chromatogram of diesel-BSM (control) extract. That gives 32 peaks.

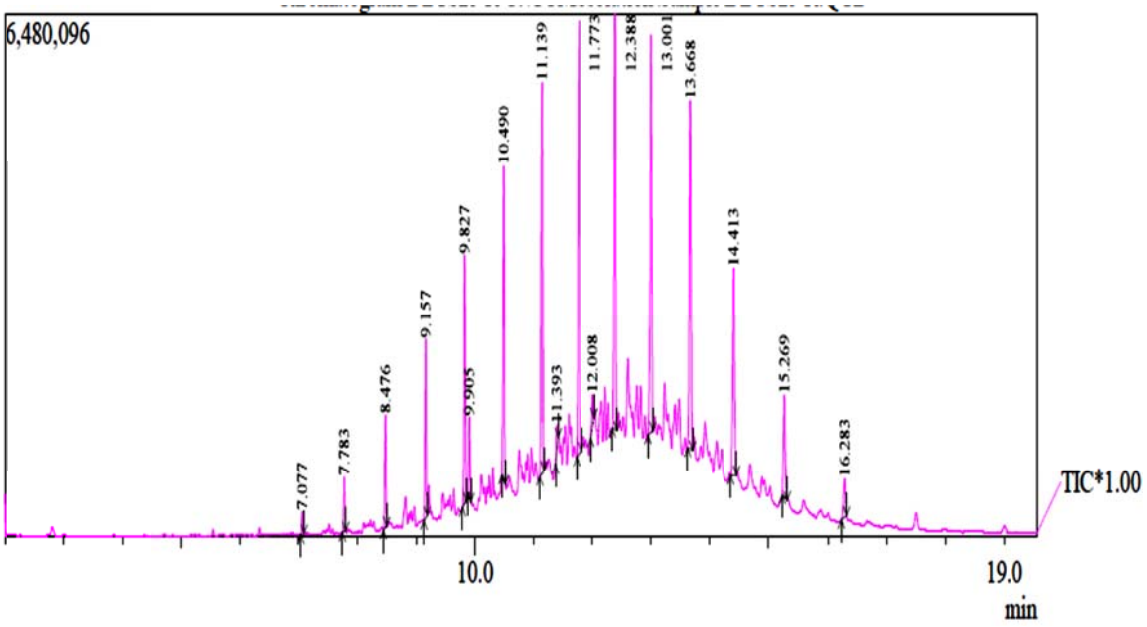


Figure (3-13): GC/MS chromatogram of *P. aeruginosa*-S25 diesel-BSM supernatant extracted culture. That gives 17 peaks.

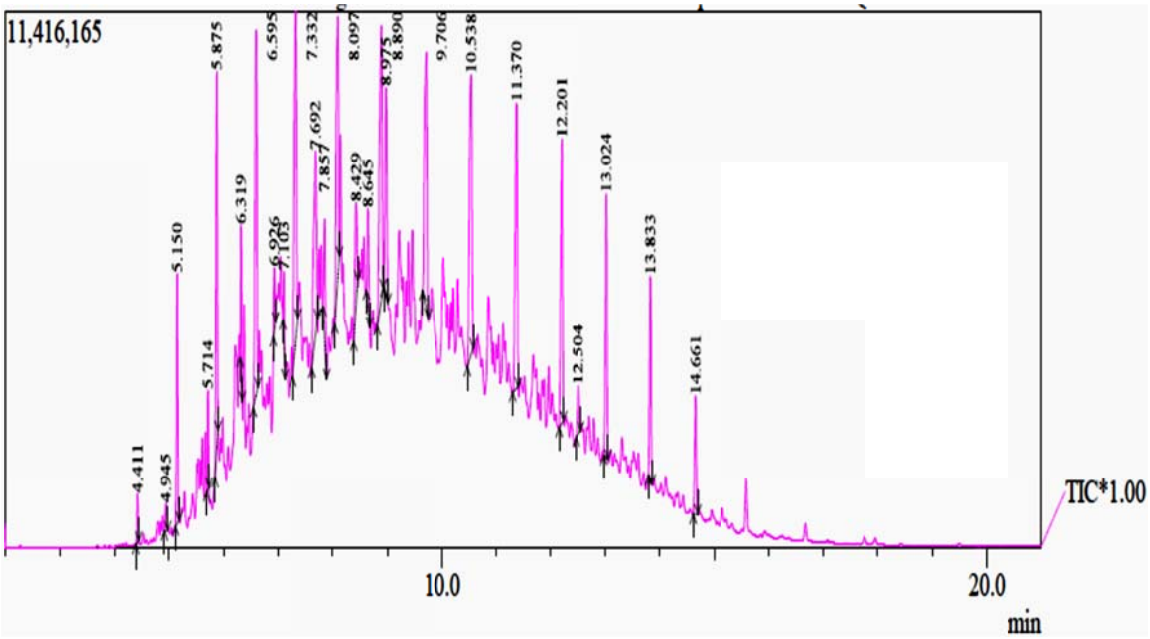


Figure (3-14): GC/MS chromatograms of P13 isolate diesel-BSM supernatant extracted culture. That gives 25 peaks.

The difference in the conversion of DBT and other sulfur containing compounds by different isolates might be due to different enzyme systems which act to desulfurize these compounds and/or might be due to other factors other than kinetics, physical and chemical parameters required by each isolate to utilize these compounds. Several factors could also be responsible for this behavior, such as substrate mass transfer through the cell wall, the pathway through which they could utilize these compounds and/or inhibition of activity by the intermediates and products produced from organosulfur compound metabolism (Monticello, 2000; Caro *et al*., 2007; Irani *et al*., 2011).

3.10.2. Detection of the DBT and diesel sulfur content by using sulfur content X-ray.

The basis for the work of this device is to take three readings per sample during 100 seconds and calculate the average of the readings, which is the final result.

The results (table 3-5), indicated that the initial concentration of sulfur in DBT-BSM was about 0.0036 wt % and reduced to be 0.0004 and 0.0024 wt % by P13 isolate and *P. aeruginosa*-S25 isolates respectively. That means these isolates utilized about 89 % and 34 % of total sulfur by P13 isolate and *P. aeruginosa*-S25 respectively (figure 3-15).

Also, the result declared that the initial concentration of sulfur in diesel sample was about 0.465 wt % (4650 ppm) and reduced to be 0.438 wt % (4380 ppm) and 0.440 wt % (4400 ppm) by P13 isolate and *P. aeruginosa*-S25 respectively, which means that the percentage of sulfur consumption in diesel-BSM was about 5.9 % by P13 isolate and 5.4 % by *P. aeruginosa*-S25 (figure 3-16).

Consequently both isolates showed a very good ability to desulfurize and utilize DBT, they also showed good ability to consume sulfur from diesel.

Table (3-5): Determination of the total sulfur content in DBT-BSM and Diesel-BSM for two biocatalysts (P13 isolate and *P. aeruginosa*), compared to the no-cell controls.

Items	The three reading for sulfur content			Average of sulfur content
DBT cont.	0.0042 wt%	0.0033 wt%	0.0033 wt%	0.0036 wt%
P13- DBT	0.0004 wt%	0.0003 wt%	0.0004 wt%	0.0004 wt%
S25-DBT	0.0017 wt%	0.0025 wt%	0.0030 wt%	0.0024 wt%
Diesel cont.	0.0464 wt%	0.466 wt%	0.467 wt%	0.465 wt%
P13- diesel	0.436 wt%	0.438 wt%	0.439 wt %	0.438 wt%
S25-diesel	0.440 wt%	0.440 wt%	0.439 wt%	0.440 wt%

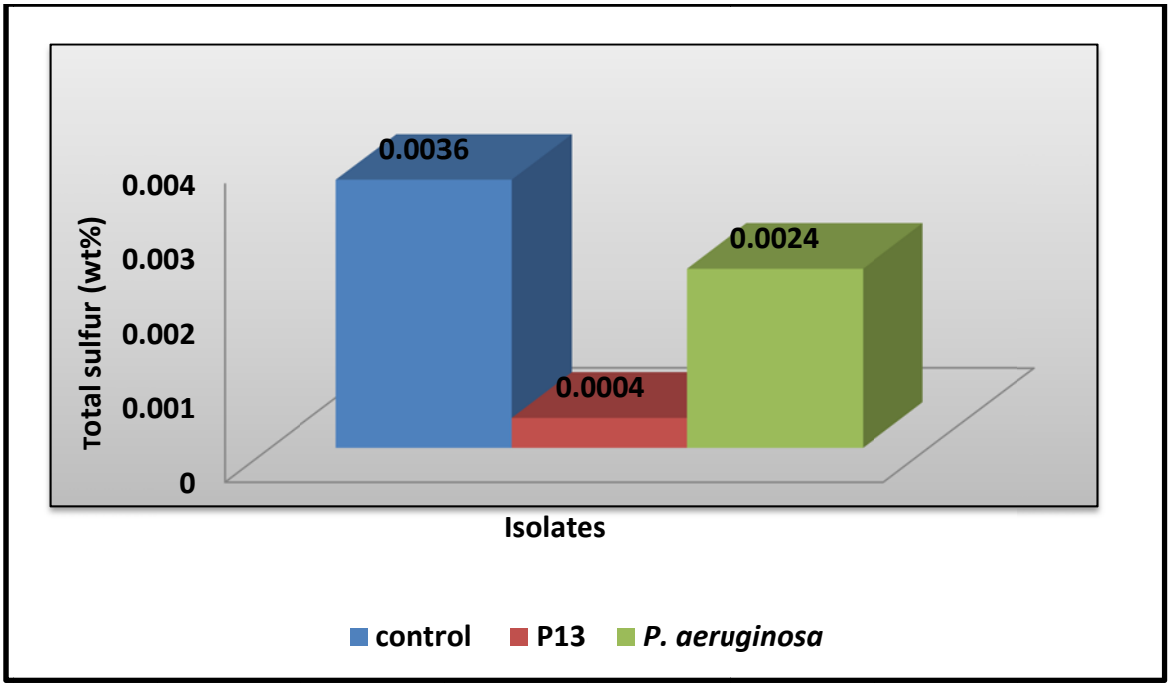


Figure (3-15): Total sulfur amount in DBT-BSM for two biocatalysts (P13 isolate and *P. aeruginosa*).

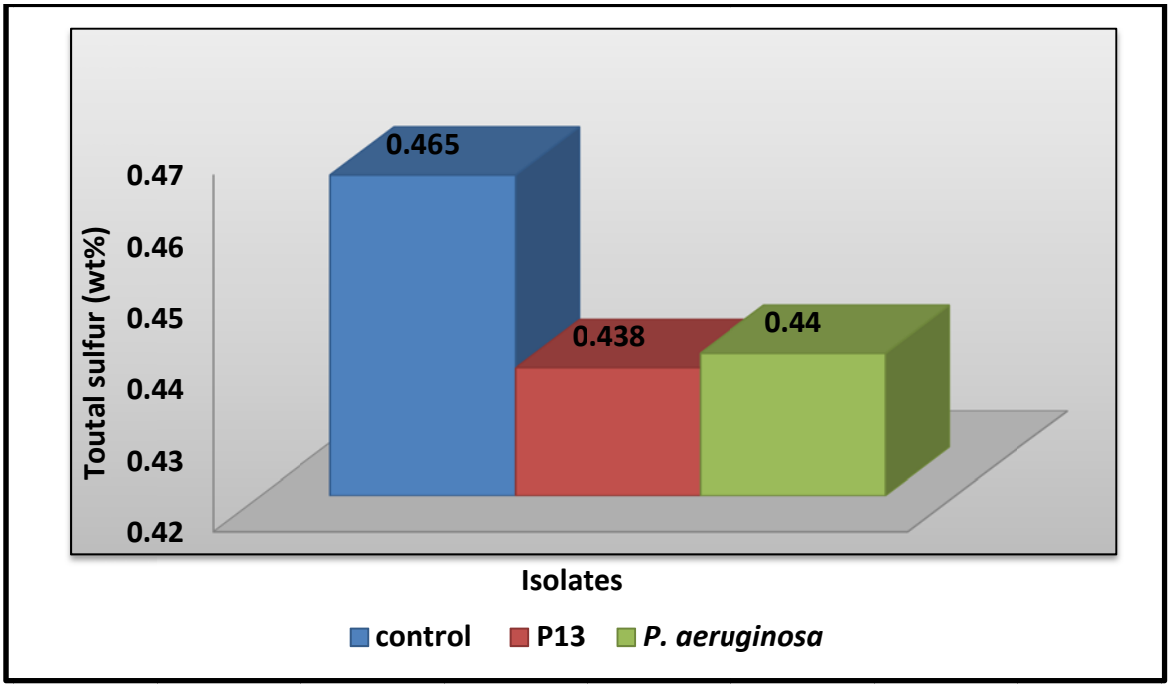


Figure (3-16): Total sulfur amount in Diesel-BSM for two biocatalysts (P13 isolate and *P. aeruginosa*).

Diesel fuels are difficult to desulfurize in general, because of their complex composition. As the bacteria showed promising results in their ability to desulfurize DBT, the possibility of their application for biodesulfurization of diesel oils was investigated. Diesel oil, was extensively hydrodesulfurized and it showed substantial decrease in sulfur content. It is well known that the extent of biodesulfurazation (BDS) depends on the chemical composition of oil especially sulfur containing species (Folsom *et al.,* 1999).

The production of fuels has been adapted to a worldwide trend towards a decrease of harmful substances in combustion products. The diminishing of the sulfur content in fuels is one of the significant steps to the environmental protection. It's a reliable and robust method for the detection of low-level sulfur in fuel (Mehrnia *et al.*, 2004).

Chapter Four

Conclusions and Recommendations

4. Conclusions and recommendations

4.1. Conclusions:

- Soil contaminated with hydrocarbons and oil derivatives were rich with dibenzothiophene (DBT) utilizing bacteria.
- Only three isolates (M9, M19, and S25) showed ability to attack DBT (desulfurizing DBT) via a specific cleavage of only the C-S bonds and converted it to 2-HBP or other phenolic end products. These isolates were belonged to *Pseudomonas aeruginosa* and *P. aeruginosa* S25 was the most efficient one.
- Twelve DBT utilizing isolates were also candidated for identification. They were belonged to *Staphylococcus aureus* (3 isolates), *Staphylococcus* spp. (3 isolates), *Micrococcus* spp. (2 isolates) and one isolate for each of *Neisseria* sp., *Pseudomonas* sp., *Corynebacterium* sp. and *Bacillus* sp. The efficient one was unidentified (P13 isolate).
- Ability of *P. aeruginosa* S25 and P13 isolate to utilize DBT was enhanced under optimum conditions growing these bacteria in Basel salt medium (pH 8) containing 0.6 mM DBT and incubated with shaking (150 rpm) at 35 °C for three days.
- DNA extraction by salting out method, indicated that *P. aeruginosa* S25 harboring a small plasmid DNA bands, while P13 isolate has no plasmid DNA.
- The GC/MS analysis for bacterial cultures on DBT-BSM indicated that the *P. aeruginosa* S25 consumed as much as 12.89 % (which represent consumption of sulfur) and P13 isolate have the ability to utilize the DBT as sulfur and carbon source. Also, both isolates showed a very good ability to consume sulfur from diesel-BSM.
- The sulfur content X-ray analysis indicated that these isolates utilize about 89 % and 34 % of total sulfur in DBT by P13 isolate and *P. aeruginosa*-S25 respectively, and the percentage of sulfur consumption in diesel-BSM was

about 5.9 % by P13 isolate and 5.4 % by *P. aeruginosa*-S25. Consequently, both isolates showed a very good ability to desulfurize and utilize DBT, they also showed good ability to consume sulfur from diesel.

- *P. aeruginosa* S25 considered as biodesulfurizing bacteria, when its attacked DBT via the specific cleavage of only the C-S bond. This bacterium could have a novel pathway similar to the 4S pathway excepted that 2-HBP was further converted to other phenolic compond.Thus, no carbon loss is observed.

4.2. Recommendations:

- Screening the ability of the efficient isolates to degrade other commonly used thiophenic compound(s) that found in crude oil.
- Further studies are required to isolate other bacteria (e.g. thermophilic bacteria) capable to degrade different types of organosulfur compounds and search for other biodesulfurizing novel organism(s).
- Studying the molecular genetics of biodesulfurizing bacteria, the dsz genes (involved in 4S pathway) which responsible for biodesulfurization process in these bacteria.
- Studying the biochemical pathways of desulfurizing DBT by these isolates and characterizes the intermediate compounds.
- Another analytical techniques are required like sulfur chemiluminescence detector (SCD) to determine the quantity of sulfur after desulfurization process.
- The biodesulfurizing isolate (*P. aeruginosa* S25) possess some promising features that make it potential candidate for developing a biocatalyst for desulfurization process of fuels or crude oil in refineries.
- The efficiency of P13 isolate in utilizing DBT as C-and S- source make it potential candidates for developing a biocatalyst for bioremediation process in environment.

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Appendix

Appendix (1): Biochemical tests in the VITEK2 system.

A-Test substrates on GN (gram negative) card.

Well	Test	Mnemonic	Compound conc. (mg)
2	Ala-Phe-Pro- ARYLAMIDASE	APPA	0.0384
3	ADONTOL	ADO	0.1875
4	L-Pyrrolydonyl-ARYLAMIDASE	PyrA	0.018
5	L-ARABITOL	IARL	0.3
7	D-CELLOBIOSE	dCEL	0.3
9	BETA-GALACTOSIDASE	BGAL	0.036
10	H_2S PRODUCTION	H2S	0.0024
11	BETA-N-ACETYL-GLUCOSAMINDASE	BNAG	0.0408
12	Glutamyl Arylamidase pNA	AGLTp	0.0324
13	D-GLUCOSE	dGLU	0.3
14	GAMMA-GLUTAMYL-TRANSFERASE	GGT	0.0223
15	FERMENTATION /GLUCOSE	OFF	0.45
17	BETA-GLUCOSIDAES	BGLU	0.036
18	D-MALTOSE	dMAL	0.3
19	D-MANNITOL	dMAN	0.1875
20	D-MANNOSE	dMAE	0.3
21	BETA-XYLOSIDASE	BXYL	0.0324
22	BETA-Alanine aryamidase Pna	BAlap	0.0174
23	L-Proline ARYLAMIDASE	ProA	0.0234
26	LIPASE	LIP	0.0192
27	PALATINOSE	PLE	0.3
29	Tyrosine ARYLAMIDASE	TyrA	0.0276
31	UREASE	URE	0.15
32	D-SORBITOL	dSOR	0.1875
33	SACCHAROSE /SUCROSE	SAC	0.3
34	D-TAGATOSE	dTAG	0.3
35	D-TREHALOSE	dTRE	0.3
36	CITRATE (SODIUM)	GIT	0.054
37	MALONATE	MNT	0.15
39	5-KETO-D-GLUCONATE	5KG	0.3
40	L-LACTATE alkalipisation	ILATK	0.15
41	ALPHA-GLUCOSIDASE	AGLU	0.036
42	SUCCINATE alkalipisation	SUCT	0.15

43	Beta-N-ACETYL-GALACTOSAMINIDASE	NAGA	0.0306
44	ALPHA-GALACTOSIDASE	AGAL	0.036
45	PHOSPHATASE	PHOS	0.0504
46	Glycine ARYLAMIDASE	Gly A	0.012
47	ORNITHINE DECARBOXYLASE	ODC	0.3
48	LYSINE DECARBOXYLASE	LDC	0.15
52	DECARBOXYLASE BASE	ODEC	NA
53	L-HISTIDINE assimilstion	IHISa	0.087
56	COURMARATE	GMT	0.126
57	BETA-GLUCORONIDASE	BGUR	0.0378
58	O/129 RESISTANCE (comp. vibrio)	O129 R	0.0105
59	GLU-GLY-Arg ARYLAMIDASE	GGAA	0.0576
61	L-MALATE assimilation	IMLTa	0.042
62	ELLMAN	ELLM	0.03
64	L-LACTATE assimilation	ILATa	0.186

B-Test substrate on GP (gram positive) card.

well	Test	Mnemonic	Compound conc. (mg)
2	D-MAYGDALIN	AMY	0.1875
4	PHOSPHATIDYLINOSITOL PHOSPHOLIPASE C	PIPLC	0.015
5	D-XYLOSE	dXYL	0.3
8	ARGININE DIHYDROLASE 1	ADH1	0.111
9	BETA-GALACTOSIDASE	BGAL	0.036
11	ALPHA-GALACTOSEDASE	AGLU	0.036
13	Ala-Phe-Pro- ARYLAMIDASE	APPA	0.384
14	CYCLODEXTRIN	CDEX	0.3
15	L-Aspartate ARYLAMIDASE	AspA	0.024
16	BETA GALACTOPYRANOSIDASE	BGAR	0.00204
17	ALPHA-MANNOSIDASE	AMAN	0.036
19	PHOSPHATASE	PHOS	0.0504
20	Leucine ARYLAMIDASE	LeuA	0.0234

23	L-Prolin ARYLAMIDASE	ProA	0.0234
24	BETA-GLUCURONIDASE	BGURr	0.0018
25	ALPHA-GLUCURONIDASE	AGAL	0.036
26	L-Pyrrolidonyl-ARYLAMIDASE	PyrA	0.018
27	BETA-GLUCURONIDASE	BGUR	0.0378
28	Alanine ARYLAMIDASE	AlaA	0.0216
29	Tyrosine ARYLAMIDASE	TyrA	0.0276
30	D-SORBITOL	dSOR	0.1875
31	UREASE	URE	0.15
32	POLYMIXIN B RESISTANCE	POLYB	0.00093
37	D-GALACTOSE	dGAL	0.3
38	D-RIBOSE	dRIB	0.3
39	L-LACTATE alkalinization	ILATk	0.15
42	LACTOSE	LAC	0.96
44	N-ACETYL-D-GLUCOSAMINE	NAG	0.3
45	D-MALTOSE	dMAL	0.3
46	BACITRACIN RESISTANCE	BACI	0.0006
47	NOVOBIOCIN RESISTANCE	NOVO	0.000075
50	GROWTH IN 6.5% NaCl	NC6.5	1.68
52	D-MANNITOL	dMAN	0.1875
53	D-MANNOSE	dMAE	0.3
54	METHYL-B-D-GLUCOPYRANOSIDE	MBdC	0.3
56	PULLULAN	PUL	0.3
57	D-RAFFINOSE	dRAF	0.3
58	O/129 RESISTANCE (comp. vibrio)	O129R	0.0084
59	SALICIN	SAL	0.3
60	SACCHAROSE/SUCROSE	SAC	0.3
62	D-TREHALOSE	dTRE	0.3
63	ARGININE DIHYDROLASE 2	ADH2s	0.27
64	OPTOCHIN RESISTANCE	OPTO	0.000399

Appendix (2): Result of *Pseudomonas aeruginosa*-S25 diagnosed by VITEK2 system.

No.	Test	Result
1	APPA	-
2	ADO	-
3	PyrA	-
4	IARL	-
5	dCEL	-
6	BGAL	-
7	H2S	-
8	BNAG	-
9	AGLTp	+
10	dGLU	+
11	GGT	+
12	OFF	-
13	BGLU	-
14	dMAL	-
15	dMAN	-
16	dMAE	+
17	BXYL	-
18	BAlap	+
19	ProA	+
20	LIP	+
21	PLE	-
22	TyrA	-
23	URE	-
24	dSOR	-
25	SAC	-
26	dTAG	-
27	dTRE	-
28	CIT	+
29	MNT	+
30	5KG	-
31	ILATK	+
32	AGLU	-
33	SUCT	+
34	NAGA	-
35	AGAL	-
36	PHOS	-

37	Gly A	-
38	ODC	-
39	LDC	-
40	IHISa	-
41	GMT	+
42	BGUR	-
43	O129 R	-
44	GGAA	-
45	IMLTa	+
46	ELLM	-
47	ILATa	-

Appendix (3): Result of P13 (unidentified organism) diagnosed by VITEK2 system.

No.	Test	Result
1	AMY	-
2	PIPLC	-
3	dXYL	+
4	ADH1	+
5	BGAL	+
6	AGLU	-
7	APPA	-
8	CDEX	-
9	AspA	-
10	BGAR	+
11	AMAN	-
12	PHOS	+
13	LeuA	+
14	ProA	-
15	BGURr	-
16	AGAL	+
17	PyrA	+
18	BGUR	-
19	AlaA	+
20	TyrA	-
21	dSOR	+
22	URE	+
23	POLYB	-
24	dGAL	+
25	dRIB	+
26	ILATk	+
27	LAC	+
28	NAG	+
29	dMAL	+
30	BACI	+
31	NOVO	+
32	NC6.5	-
33	dMAN	+
34	dMAE	+
35	MBdG	+
36	PUL	+

37	dRAF	+
38	O129R	-
39	SAL	+
40	SAC	+
41	dTRE	+
42	ADH2s	-
43	OPTO	+

Appendix (4): Examination report of S25 isolate with VITEK2 system.

bioMerieux Customer:
System #:

Laboratory Report

Printed Apr 8, 2014 08:08 CDT
Autoprint

Isolate Group: 9-1

Bionumber: 0043053003500240
Selected Organism: Pseudomonas aeruginosa

Comments:	

Identification Information	Card:	GN	Lot Number:	241268740	Expires:	Apr 23, 2014 13:00 CDT
	Completed:	Apr 7, 2014 16:08 CDT	Status:	Final	Analysis Time:	5.00 hours
Selected Organism	99% Probability		Pseudomonas aeruginosa			
	Bionumber:	0043053003500240			Confidence:	Excellent identification
SRF Organism						
Analysis Organisms and Tests to Separate:						
Analysis Messages:						
Contraindicating Typical Biopattern(s)						

Biochemical Details

2	APPA	-	3	ADO	-	4	PyrA	-	5	IARL	-	7	dCEL	-	9	BGAL	-
10	H2S	-	11	BNAG	-	12	AGLTp	+	13	dGLU	+	14	GGT	+	15	OFF	-
17	BGLU	-	18	dMAL	-	19	dMAN	-	20	dMNE	+	21	BXYL	-	22	BAlap	+
23	ProA	+	26	LIP	+	27	PLE	-	29	TyrA	-	31	URE	-	32	dSOR	-
33	SAC	-	34	dTAG	-	35	dTRE	-	36	CIT	+	37	MNT	+	39	5KG	-
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	-	53	IHISa	-	56	CMT	+	57	BGUR	-
58	O129R	-	59	GGAA	-	61	IMLTa	+	62	ELLM	-	64	ILATa	-			

Installed VITEK 2 Systems Version: 06.01
MIC Interpretation Guideline:
AES Parameter Set Name:

Therapeutic Interpretation Guideline:
AES Parameter Last Modified:

Page 1 of 1

Appendix (5): Examination report of P13 isolate with VITEK2 system.

bioMerieux Customer:
System #:

Laboratory Report

Printed Apr 7, 2014 13:43 CDT
Autoprint

Isolate Group: 1-1

Bionumber: 430513557757531
Selected Organism: Unidentified Organism

Comments:	

Identification Information	Card:	GP	Lot Number:	242298240	Expires:	Feb 12, 2015 12:00 CST
	Completed:	Apr 7, 2014 13:54 CDT	Status:	Final	Analysis Time:	2.75 hours
Selected Organism	Bionumber:	430513557757531	Unidentified Organism			
SRF Organism						
Analysis Organisms and Tests to Separate:						
Analysis Messages:						
Contraindicating Typical Biopattern(s)						

Biochemical Details

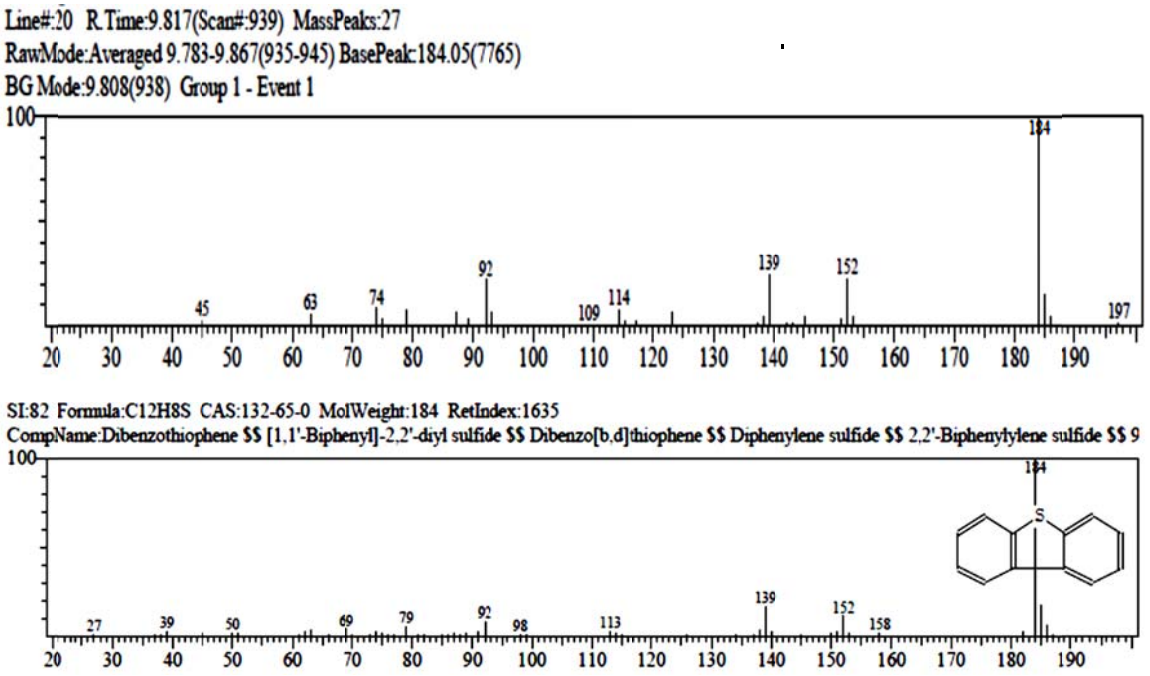
2	AMY	-	4	PIPLC	-	5	dXYL	+	8	ADH1	+	9	BGAL	+	11	AGLU	-
13	APPA	-	14	CDEX	-	15	AspA	-	16	BGAR	+	17	AMAN	-	19	PHOS	+
20	LeuA	+	23	ProA	-	24	BGURr	-	25	AGAL	+	26	PyrA	+	27	BGUR	-
28	AlaA	+	29	TyrA	-	30	dSOR	+	31	URE	+	32	POLYB	-	37	dGAL	+
38	dRIB	+	39	ILATk	+	42	LAC	+	44	NAG	+	45	dMAL	+	46	BACI	+
47	NOVO	+	50	NC6.5	-	52	dMAN	+	53	dMNE	+	54	MBdG	+	56	PUL	+
57	dRAF	+	58	O129R	-	59	SAL	+	60	SAC	+	62	dTRE	+	63	ADH2s	-
64	OPTO	+															

Installed VITEK 2 Systems Version: 06.01
MIC Interpretation Guideline:
AES Parameter Set Name:

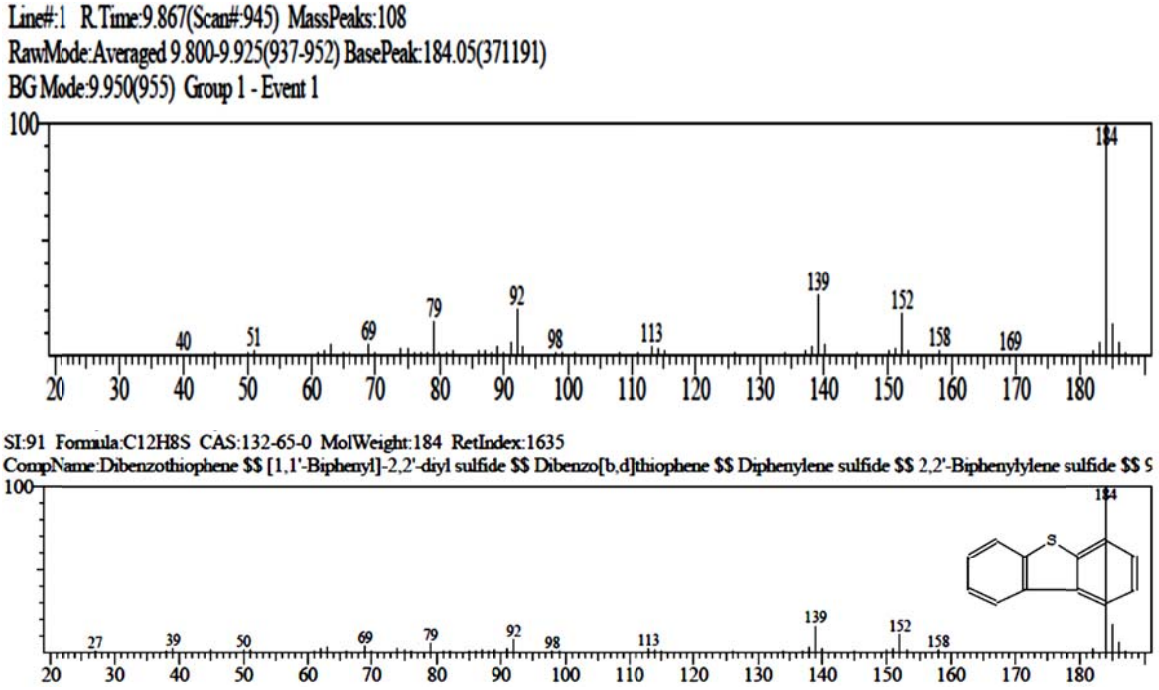
Therapeutic Interpretation Guideline:
AES Parameter Last Modified:

Page 1 of 1

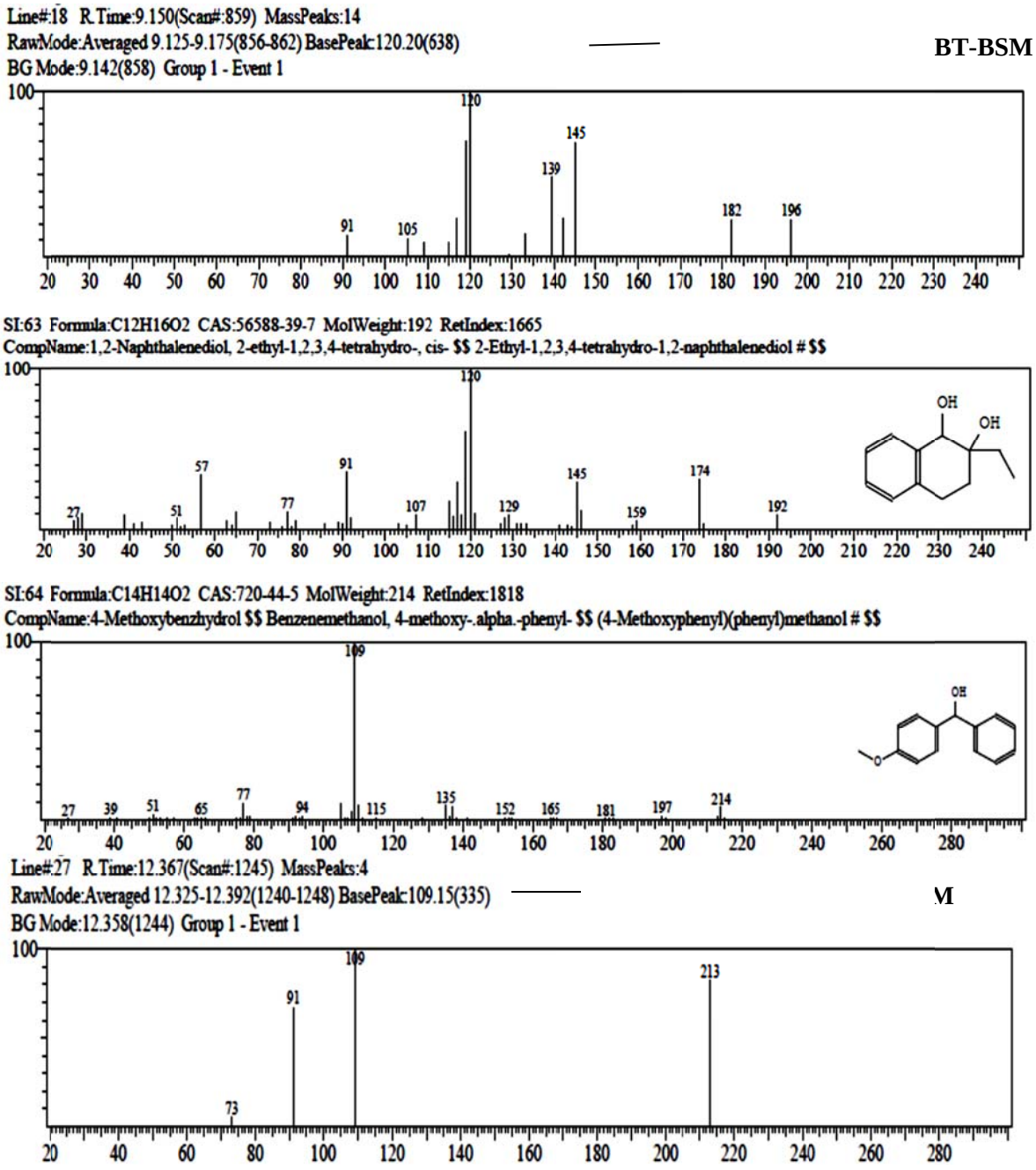
Appendix (6): GC/MS analysis showing the mass spectrum of DBT (molecular mass, 184) with specific identity 82.



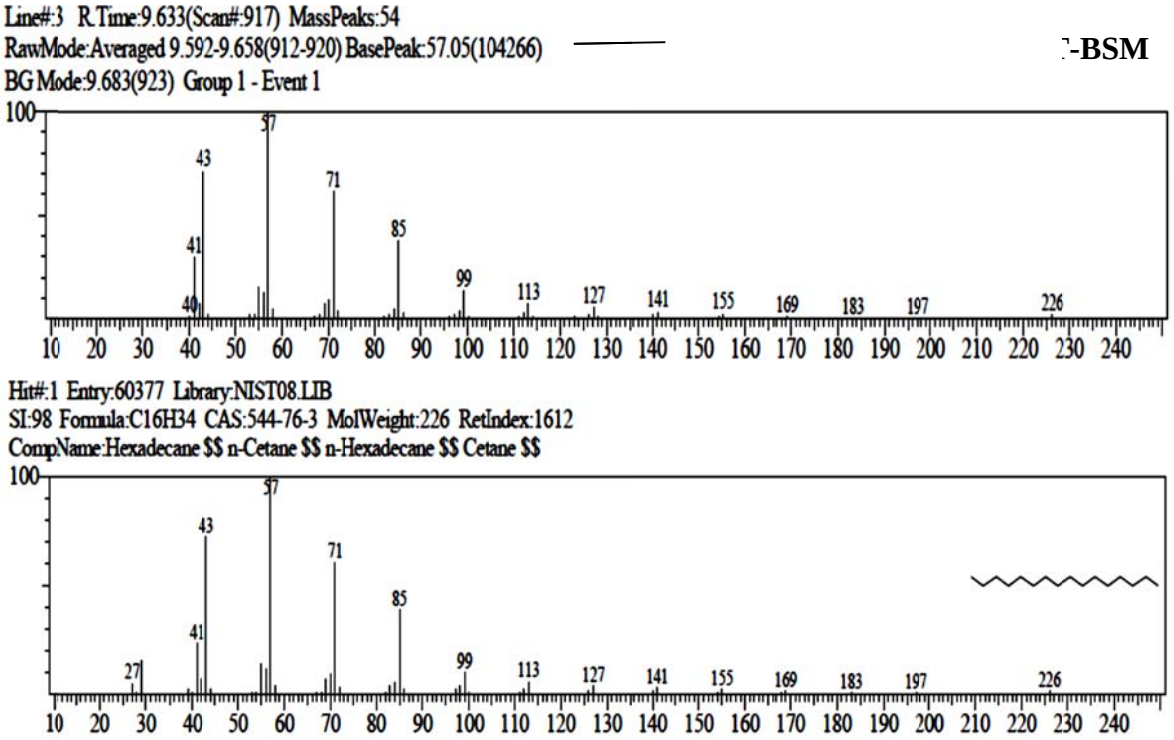
Appendix (7): GC/MS analysis showing the mass spectrum of DBT (molecular mass, 184) with specific identity 91.



Appendix (8): GC/MS analysis showing the mass spectrum of 1, 2 Naphthalenediol, 2- ethyl-1, 2, 3, 4-tetrahydro-, cis (molecular mass, 192) with retention time 9.15 and the mass spectrum of 4-Methoxybenzhydrol (molecular mass, 214) with retention time 12.36.

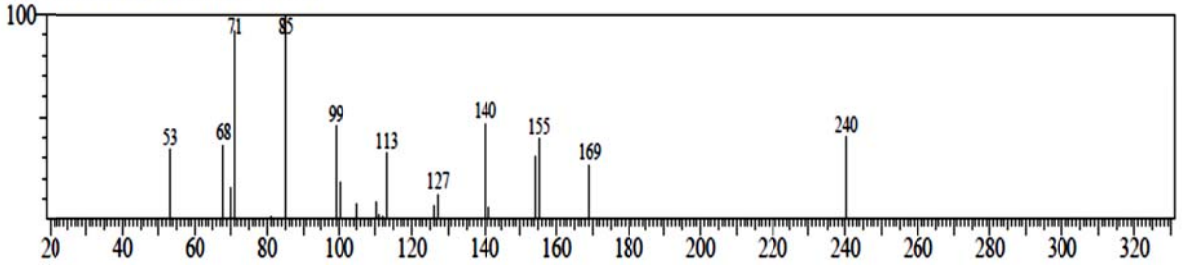


Appendix (9): GC/MS analysis showing the mass spectrum of Hexadecane (molecular mass, 226) with retention time 9.63.

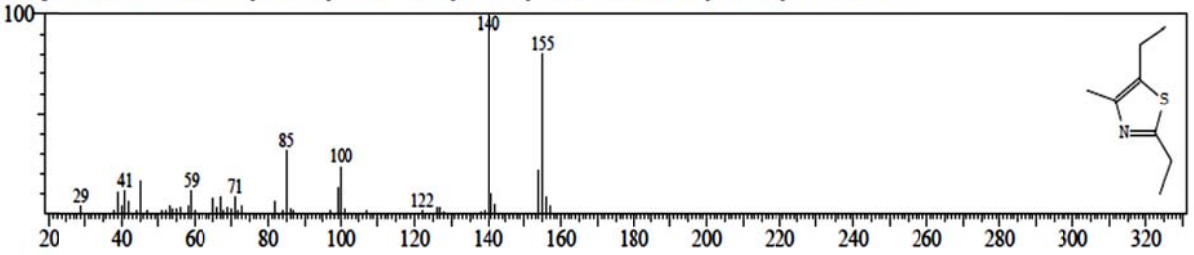


Appendix (10): GC/MS analysis showing the mass spectrum of Thiazole,2,5-diethyl-4-methyl (molecular mass, 155) with retention time 8.025, the mass spectrum of 3-(Benzoylthio)-2-methylpropanoic acid (molecular mass, 224) with retention time 8.083 and the mass spectrum of 2-Isopropyl-4,5-dimethylthiazole (molecular mass, 155) with retention time 9.617.

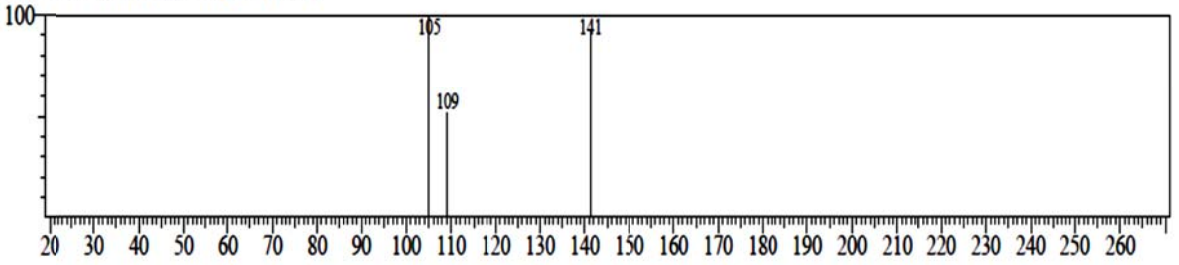
Line#:15 R.Time:8.025(Scan#:724) MassPeaks:24
RawMode:Averaged 8.000-8.058(721-728) BasePeak:85.15(1658)
BG Mode:8.042(726) Group 1 - Event 1



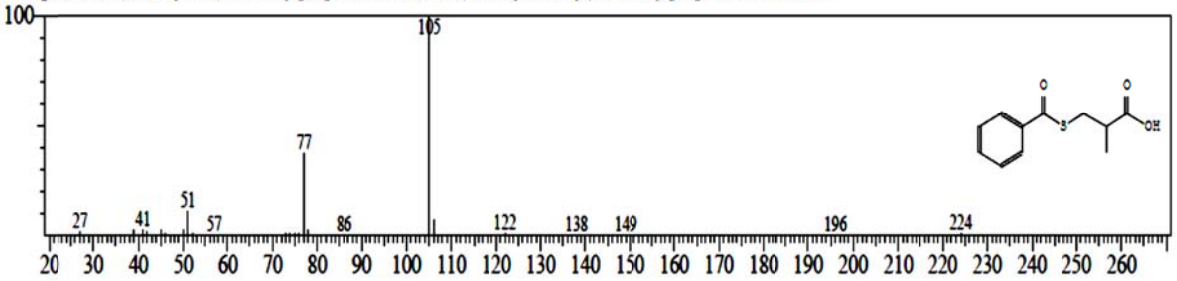
SI:60 Formula:C8H13NS CAS:41981-71-9 MolWeight:155 RetIndex:1210
CompName:Thiazole, 2,5-diethyl-4-methyl- $$ 2,5-Diethyl-4-methylthiazole $$ 2,5-Diethyl-4-methyl-1,3-thiazole # $$



Line#:16 R.Time:8.083(Scan#:731) MassPeaks:3
RawMode:Averaged 8.058-8.117(728-735) BasePeak:105.10(136)
BG Mode:8.092(732) Group 1 - Event 1

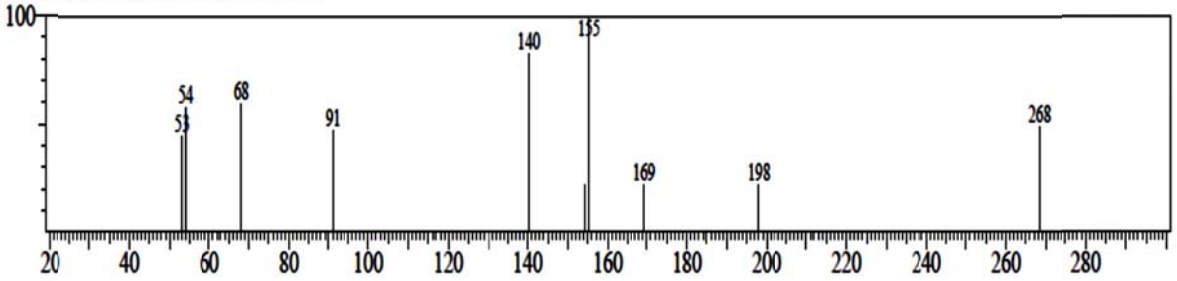


SI:77 Formula:C11H12O3S CAS:67714-34-5 MolWeight:224 RetIndex:1870
CompName:3-(Benzoylthio)-2-methylpropanoic acid $$ 3-(Benzoylsulfanyl)-2-methylpropanoic acid # $$

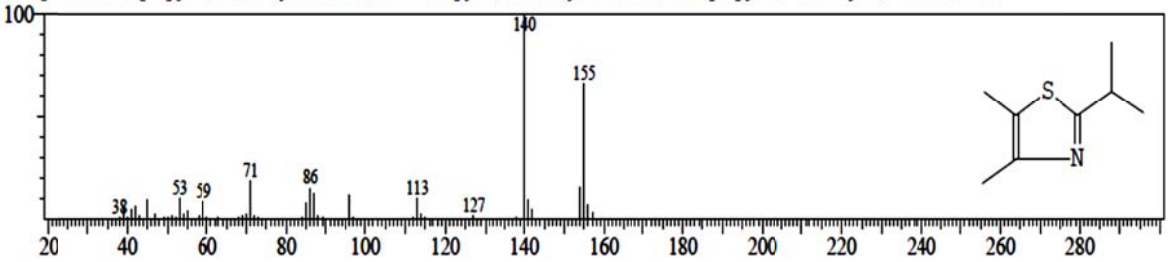


Line#:19 R.Time:9.617(Scan#:915) MassPeaks:10
RawMode:Averaged 9.583-9.667(911-921) BasePeak:155.25(439)
BG Mode:9.633(917) Group 1 - Event 1

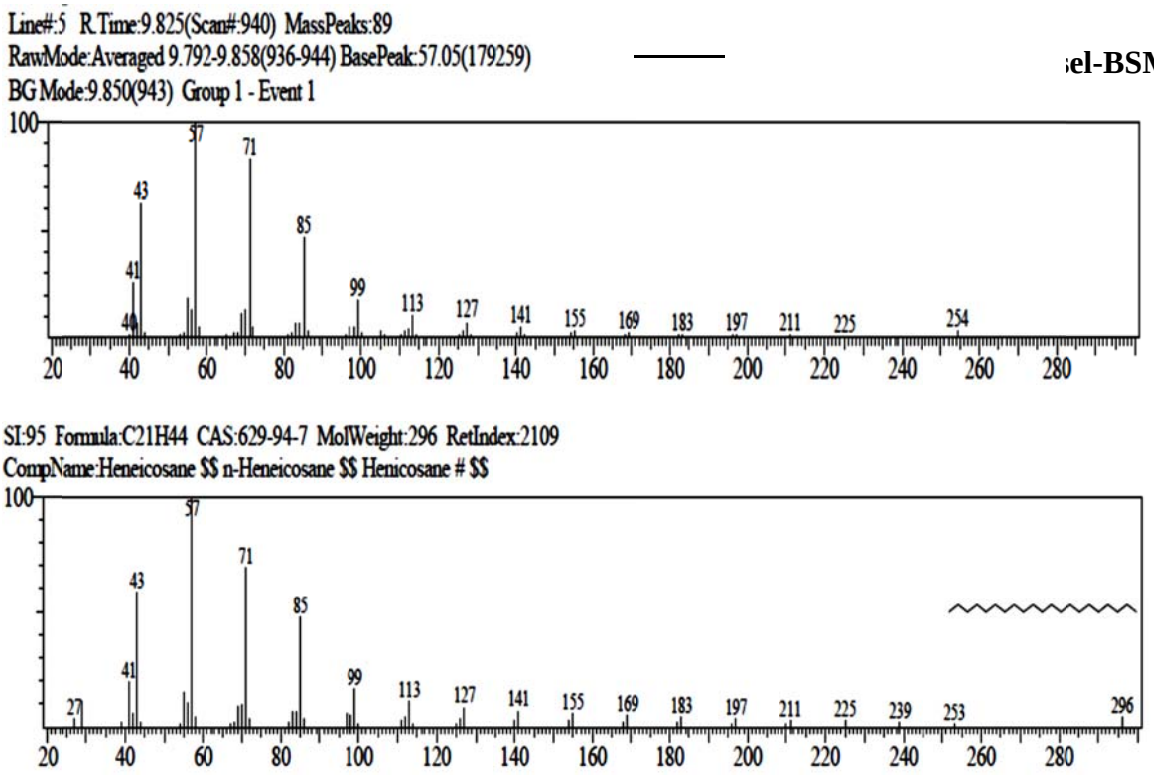
ıtrol



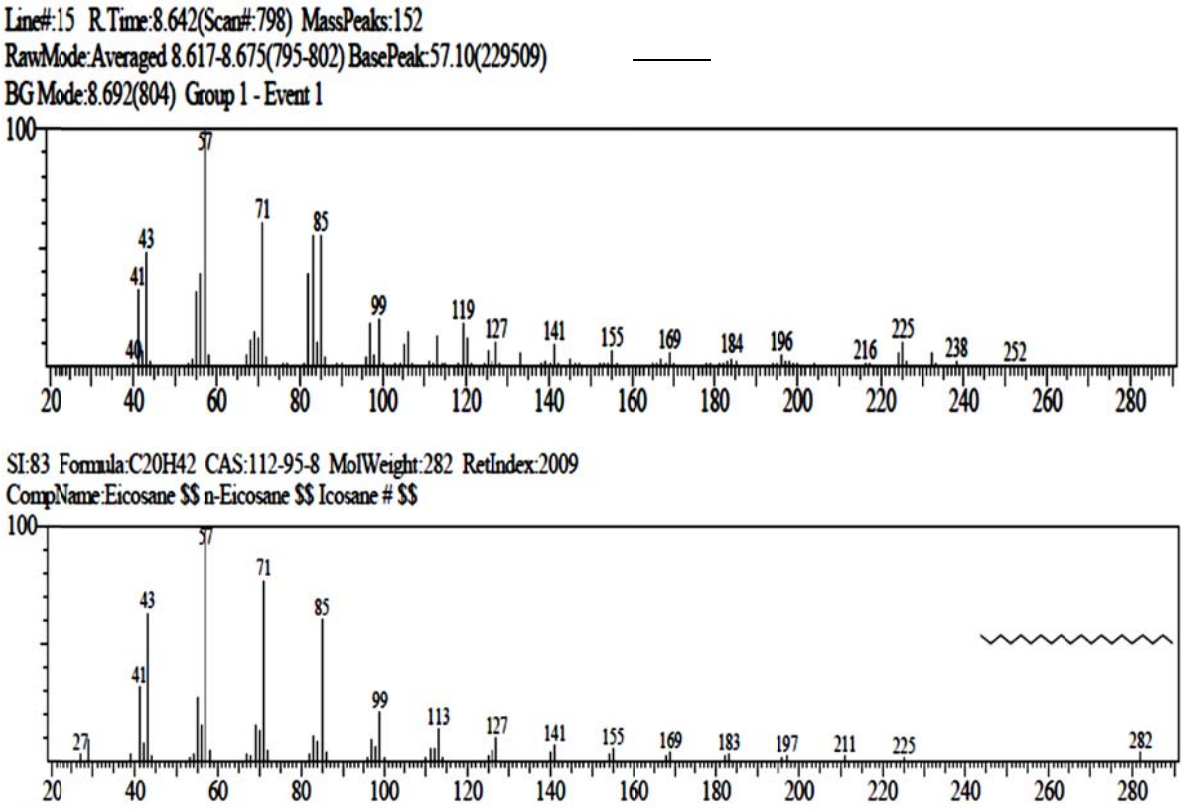
SI:55 Formula:C8H13NS CAS:53498-30-9 MolWeight:155 RetIndex:1146
CompName:2-Isopropyl-4,5-dimethylthiazole $$ 2-iso-Propyl-4,5-dimethylthiazole $$ 2-Isopropyl-4,5-dimethyl-1,3-thiazole # $$



Appendix (11): GC/MS analysis showing the mass spectrum of Heneicosane (molecular mass, 296) with retention time 9.825.



Appendix (12): GC/MS analysis showing the mass spectrum of n-Eicosane (molecular mass, 282) with retention time 8.642.



الملخص

اختبرت الظروف المثلى لاستغلال DBT بفعل *P. aeruginosa* S25 و عزلة P13. وقد تبين أن الظروف المثلى هيه بتنمية هذه البكتيريا في الوسط الملحي (BSM (pH 8)، الحاوي على 0.6 ملي مولار من DBT والحضن في حاضنة هزازة (150 دورة / الدقيقة) بدرجه حرارة 35 مئوية لمدة ثلاث أيام.

ولتحديد النمط البلازميدي لبكتريا *P. aeruginosa*-S25 وعزله P13، تم استخدام عدة طرق لاستخلاص الدنا. أظهرت النتائج ان *P. aeruginosa*-S25 تمتلك حزم بلازميدية صغيره، بينما لم يكن هناك أي بلازميد في العزلة P13.

تحليل كروماتوغرافيا الغاز / الكتلة (GC / MS) لمزارع البكتريا على وسط DBT-BSM، أوضح أن 12.89٪ من DBT استغل (استغلال الكبريت) من قبل *P. aeruginosa*-S25 ، وأظهر هذا التحليل أن عزلة P13 قد استغلت مركب DBT كمصدر للكبريت والكاربون. واظهر تحليل GC/MS لمزارع البكتريا على وسط diesel-BSM، إن جميع المركبات الحاوية على ذرات كبريت تم تحليلها الى مركبات اقل تعقيداً بفعل *P. aeruginosa*-S25 وعزلة P13.

أوضحت نتائج فحص sulfur content X-ray لمزارع البكتريا على وسط DBT-BSM، ان هذه البكتريا استغلت حوالي 89٪ و 34٪ من الكبريت بفعل كل من عزلة P13 و*P.* -S25 *aeruginosa* على التوالي. وبلغت نسبة استغلال الكبريت من وسط diesel-BSM حوالي 5.9٪ بفعل عزلة P13 و 5.4٪ بواسطة *P. aeruginosa*-S25. وعلية فان كلا العزلتين أظهرتا مقدره جيدة جدا على أزاله الكبريت و استغلاله من مركب DBT، كما أظهرتا قدرة جيدة على استغلال الكبريت من الديزل.

الملخص

كان الهدف الرئيسي من هذه الدراسة هو الحصول على بكتريا كفؤة قادرة على إزالة الكبريت و / أو استغلال مركبات الكبريت العضوي الموجودة في البترول ومشتقاته. لهذا الغرض تم جمع عينات من الترب الملوثة بالنفط منذ مدة طويلة، من أربعين موقع مختلف في العراق.

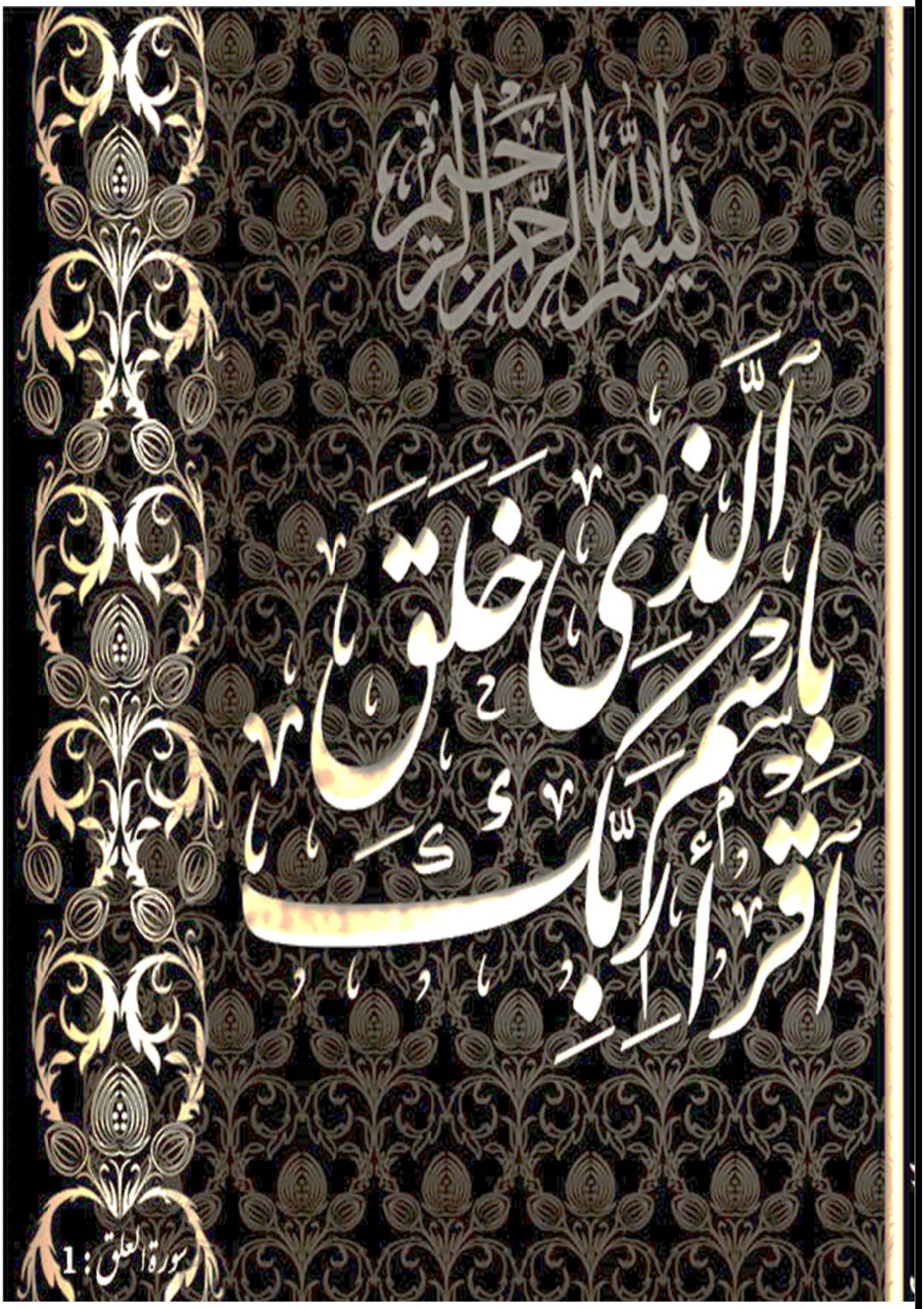
تم الحصول على ثلاثة وستون عزله بكتيرية قادرة على استغلال مركب dibenzothiophene (DBT). أظهرت النتائج أن معظم هذه العزلات قادرة على النمو على DBT بوجود أو بدون وجود الجلسرين (مصدر الكربون). وكانت كثافة نمو العزلات البكتيرية في وجود الجلسرين أكثر من كثافة النمو بدون وجودهِ. وأظهرت النتائج أيضا أن كثافة نمو العزلات البكتيرية في الوسط الحاوي على الإيثانول و كبريتات المغنيسيوم أكثر بقليل مقارنة مع النمو في الوسط الحاوي على DBT والإيثانول. هذا يعني أن هذه العزلات كانت قادرة على استغلال الإيثانول كمصدر كاربون.

ومن بين جميع العزلات، كان لثلاثه منها فقط (M9، M19 وS25) القدرة على ازالة الكبريت من DBT (كسر اصرة C-S) وتحويلهُ إلى 2-HBP أو إلى نواتج نهائيه فينوليه أخرى وإعطائها اللون الأزرق بوجود كاشف Gibb's. إعتماداً على هذه النتائج يمكننا القول ان المسار الذي تتبعه هذه العزلات في عمليه ازالة الكبريت من DBT هو مسار 4S، عن طريق كسر الآصرة الموجودة بين C-S.

إضافة إلى العزلات الثلاثة التي أعطت نتائج إيجابية مع فحص Gibb's، فإن اثنتي عشر عزله من العزلات الكفوءة (M3,M7,M16,M20, P4, S21-1,S22,S26,S34,S37, P14, P13,): ومع ذلك، كانت العزلة P13 افضلها)، (تكسر كلا الآصرتين C-C و C-S الموجودة في DBT) رشحت للتشخيص. شخصت هذه العزلات (15) بكونها:.*Staphylococcus* spp . [6 عزلات، (بما في ذلك عزلة P13)]، وكانت ثلاثة منها *Staphylococcus aureus*. *Pseudomonas* spp (4عزلات) ، كان ثلاثة منها *Pseudomonas aeruginosa*. *Micrococcus* spp (عزلتان)، وعزلة واحدة لكل \ من الأجناس التالية .*Corynebacterium* sp., *Neisseria* sp و .*Bacillus* sp.

نميت العزلات التي تقوم بإزالة الكبريت (*P. aeruginosa* M9, M19, S25) من DBT أو تستغل هذا المركب (العزلة P13 على أوساط تحوي على مصادر مختلفة من الكبريت والكاربون. وكان أعلى معدل لنمو جميع العزلات في الوسط الحاوي على جليسرين كمصدر كربون وكبريتات المغنيسيوم كمصدر كبريت، لكون هذه المركبات مصادر بسيطة للكبريت والكاربون.

لاختيار عزلة واحده من عزلات *P. aeruginosa* الثلاث (M9، M19 و S25)، فقد تم تحديد تركيز الناتج النهائي، بعد نمو البكتريا مع DBT, كميا وذلك بقياس كثافة اللون الأزرق الذي يتكون بعد إضافة كاشف Gibb's. وأظهرت النتائج أن*P. aeruginosa*-S25 هي العزلة الأكثر كفاءة في أزاله الكبريت من DBT.


بسم الله الرحمن الرحيم
اقرأ باسم ربك الذي خلق
سورة العلق : 1

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العوم
قسم التقانة الإحيائية

إزالة الكبريت واستغلال الداي بنزوثايوفين (Dibenzothiophene) بفعل بعض العزلات البكتيرية المحلية

رسالة

مقدمة إلى مجلس كلية العلوم / جامعة النهرين

كجزء من متطلبات نيل درجه الماجستير علوم في التقانة الإحيائية

من قبل

الباب فواز إبراهيم الفراس

بكالوريوس تقانة إحيائية / كلية العلوم / جامعة النهرين

(2012)

بإشراف

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(أستاذ)

د. عبد الغني إبراهيم يحيى

(أستاذ)

كانون الثاني (2015)

ربيع الأول (1436)