Biocatalyst Development for Biodesulfurization

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Abstract

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Engineering and Physical Sciences

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All fossil fuels contain varying levels of sulfur compounds which are undesirable because they cause environmental pollution, corrosion, acid rain and lead to health problems. There is strict international legislation for the permissible levels of sulfur compounds in fossil fuels. The aim of this research therefore was the biocatalyst development for biodesulfurisation using two approaches. In the first approach, *Rhodococcus erythropolis* IGTS8-5 and IGTS8-5G were immobilised in porous coke particles and tested in repeated cycles successfully. Both bacterial strains grew well in the chemically defined medium with glucose as the main carbon and energy source and the model sulfur compound dibenzothiophene (DBT) as the sole sulfur source. 0.8 g of cells was immobilized on 250 g of coke particles without refreshing the medium over 72 h while 1.8 g of cells were immobilised on 250 g of coke when the media was refreshed every 24 hours for 120 h after the initial immobilisation batch of 72h. The latter, were used repeatedly in twelve consecutive batch desulfurisation cycles during which the biodesulfurisation activity progressively decreased from over 95% removal of 100 ppm DBT to around 45% removal. DBT removal is often expressed in terms of 2-hydroxybiphenyl which is the end product of biodesulfurisation. The biodesulfurisation activity of immobilised bacteria was equivalent to 310 umol 2-HBP h⁻¹ g⁻¹ dry cell weight during the first hour. Freely suspended cells on the other hand exhibited biodesulfurisation activity equivalent to 91 umol 2-HBP h⁻¹ g⁻¹ dry cell weight. Unfortunately, after the first 24 h, the activity of the immobilised cells decreased to 12 umol 2-HBP h⁻¹ g⁻¹ dry cell weight. Use of plant cell cultures for biodesulfurisation is the other novel aspect of this work. *Armoracia rusticana* (horse radish) cell culture was chosen as the novel biocatalyst since this plant is a well known source of peroxidase enzyme which is involved in the biodesulfurisation metabolism according to the literature on bacterial biodesulfurisation. *Arabidospsis thaliana* (thale cress) was also used since its genome is completely sequenced and it is a model organism in genomics studies. Our results indicate that cell suspensions of both plants did show biodesulfurisation activity by reducing the level of sulfur compounds, mainly DBT and other three derivatives from both aqueous and oil phase. When compared to the bacteria, in terms of DBT consumption, the activity of *A. rusticana* was calculated as 55 umol DBT h⁻¹ g⁻¹ DCW and 65 umol DBT h⁻¹ g⁻¹ DCW for *A. thaliana* while in bacteria it was 91 umol DBT h⁻¹ g⁻¹ DCW for IGTS8-5 and 73 umol DBT h⁻¹ g⁻¹ DCW for IGTS8-5G. Transcriptomics analysis of the plant cell cultures after exposure to the DBT when compared to control cultures showed alterations in gene expression levels several of which were related to sulfur metabolism and transmembrane transporters of sulfate.
Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.
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# LIST O F ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ppi</td>
<td>Pores per inch</td>
</tr>
<tr>
<td>6, BAP</td>
<td>6, Benzyl Amino Purine</td>
</tr>
<tr>
<td>2,4 D</td>
<td>2,4 Dichloro Phenoxy Acetic acid</td>
</tr>
<tr>
<td>AECC</td>
<td>Association for Emissions Control by Catalyst</td>
</tr>
<tr>
<td>BT</td>
<td>Benzothiophene</td>
</tr>
<tr>
<td>BDS</td>
<td>Biodesulfurization</td>
</tr>
<tr>
<td>CEC</td>
<td>Central Environment Council</td>
</tr>
<tr>
<td>CFC</td>
<td>Chlorofluorocarbon</td>
</tr>
<tr>
<td>CDM</td>
<td>Chemical Defined Media</td>
</tr>
<tr>
<td>D.C.W</td>
<td>Dry cell weight</td>
</tr>
<tr>
<td>DBT</td>
<td>Dibenzothiophene</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>4MDBT</td>
<td>4 Methyl dibenzothiophen</td>
</tr>
<tr>
<td>4, 6-DMDBT</td>
<td>4,6-Dimethyl dibenzylthiophene</td>
</tr>
<tr>
<td>4,6-DEDBT</td>
<td>4,6-Diethyl dibenzothiophen</td>
</tr>
<tr>
<td>EPA</td>
<td>Environmental Protection Agency</td>
</tr>
<tr>
<td>EPRI</td>
<td>Electric Power Research Institute</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin Adenine Dinucleotide</td>
</tr>
<tr>
<td>FMN</td>
<td>Flavin Mononucleotide</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography mass spectra</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>H₂</td>
<td>Hydrogen Gas</td>
</tr>
<tr>
<td>HDS</td>
<td>Hydrodesulfurization</td>
</tr>
<tr>
<td>H₂S</td>
<td>Hydrogen Sulfide</td>
</tr>
<tr>
<td>2-HBP</td>
<td>2-Hydroxybiphenyl</td>
</tr>
<tr>
<td>LSF</td>
<td>Low-sulfur Fuels</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>Magnesium sulfate</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide Adenine Dinucleotide</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate</td>
</tr>
<tr>
<td>NRDC</td>
<td>Natural Resource Defense Council</td>
</tr>
<tr>
<td>NOₓ</td>
<td>Nitrogen Oxide</td>
</tr>
<tr>
<td>O.D</td>
<td>Optical Density</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>SOₓ</td>
<td>Sulfur oxides</td>
</tr>
<tr>
<td>Tg</td>
<td>teragram (10^{12}) g</td>
</tr>
<tr>
<td>ULSD</td>
<td>Ultra Low Sulfur Diesel</td>
</tr>
<tr>
<td>ULSF</td>
<td>Ultra Low Sulfur Fuel</td>
</tr>
<tr>
<td>S-Zorb</td>
<td>Sulfur Adsorption</td>
</tr>
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</table>
CHAPTER ONE

1. Introduction

1.1 Background

Main source of energy comes from fossil fuel (85%), while (8%) comes from nuclear power and (7%) from all other sources, mostly hydroelectric power oil and natural gas (United States Energy Information Administration U.S.E.I.A. ). Almost all fossil fuels are used by burning, which causes pollution.

Sulfur is the third most abundant element in crude oil. Nearly 70% of the sulfur present in crude oil is in the form of heterocyclic organic compounds, such as benzothiophene (BT), dibenzothiophene (DBT), highly condensed thiophenes and their alkylated derivatives. Crude oil sulfur content can vary from 0.03 to 7.89% w/w (kilbane and Borgne, 2004). Combustion of sulfur-containing fuels results in emission of sulfur-oxides(SO\(_x\)) to the atmosphere causing serious environmental pollution problems and hazards to human health.

Recognizing these problems, several governments across the world are introducing stringent legislation on transportation fuel quality, in order to limit and restrict the levels of sulfur, nitrogen, metals as well as other harmful aromatics found in fuel, with the aim of reducing the harmful emissions and the risks associated with it. In relation to these regulations, in 2006 the US targeted 30ppm for sulfur contents in gasoline and 15 ppm in diesel ( Song, 2003) and was expected to reach 10 ppm S content in diesel ( Zhou et al. 2006). Similar legislated levels were adopted in Europe and Japan (Fujikawa et al. 2006). The tightening legislation on the transportation fuel quality and emissions on one hand and the declining availability of low sulfur fuels (LSF), have created incentives for the search of efficient techniques for desulfurization. This is becoming an urgent and pressing necessity. To reduce the sulfur content in petroleum, the hydrodesulfurization (HDS) process has been used since a long time (Gupta et al. 2005). However, most of the thiophene heterocyclic
compounds present in petroleum are recalcitrant to HDS and escape the treatment by this process. In addition, HDS is a high cost process which operates under severe conditions such as extremely high temperature and pressure and with catalysts which are vulnerable to catalyst poisoning. These situations prompted many researchers to search for new alternative techniques for desulfurization of petroleum using BT and DBT as model compounds.

In this introduction we will review briefly sulfur compounds present in oil and its emissions, after which an attempt is made to review the existing as well as the promised technologies that share the same objective and that is the reduction of sulfur content in oil, in order to meet the target emissions set by many governments worldwide. Needless to say, this constitutes a major challenge to the industry as well as to the academia.

Governments all over the world have recognized these problems and enacted legislations to reduce these emissions. As environmental regulations become even more stringent and availability of low-sulfur fuels (LSF) decreases, efficient techniques for desulfurization of petroleum products are required. To reduce the sulfur content in petroleum, the hydrodesulfurization (HDS) process has been commonly used. However, most of the thiophene heterocyclic compounds present in petroleum are recalcitrant to HDS and escape the treatment by this process. In addition, HDS is a high cost process which operates under severe conditions such as extremely high temperature and pressure and with catalysts which are vulnerable to catalyst poisoning. These situations promoted researchers to search for new alternative techniques for desulfurization of petroleum using BT and DBT as model compounds. During the last decade, an innovative biotechnology has been discovered for the specific removal of sulfur from these model compounds using the metabolic machinery of microorganisms and the process was designated as biodesulfurization (BDS).

Hydrodesulfurized diesel oil can be subjected to further biodesulfurization to give very low sulfur fuel that meets recent environmental strict regulations (Kilbane 2006). Dibenzothiophene (DBT), benzothiophene (BT) and their derivatives represent a broad range of heterocyclic sulfur compounds found in
petroleum that are resistant to desulfurization via the traditional hydrodesulfurization method, but can easily be desulfurized using the biodesulfurization process (Gupta et al. 2005).

Besides being a specific bioprocess for the removal of S from compounds escaping the HDS, the BDS is also a promising technology in terms of safety and low cost treatment due to its mild operating conditions. BT- and DBT-utilizing microbes with C-S bond cleavage activity are expected to be useful as biocatalysts for biodesulfurization of petroleum feedstock’s in refineries. However, there are still several challenges limiting the implementation and commercialization of the process. These include biocatalyst stability in organic phases, desulfurization rate, the need for a carbon source to regenerate cofactors, oil-water separation, product recovery and the need for thermostable microbial strains to desulfurize hot distillate oil fractions during the refinery process. Recently, researchers followed two approaches to overcome these limitations. The first approach involves the enrichment and isolation of new strains fulfilling the criteria needed for implementation of BDS in refineries, whereas the second approach focused on using molecular biology tools and directed evolution towards design recombinant strains with enhanced and improved BDS activity. Recently, researchers’ are looking for techniques enabling the refineries to re-use of the biocatalysts several times before it gets depleted.

Biodesulfurisation has been attempted using only bacterial cultures so far but despite a few decades of research there is no industrial application yet. This is because of the several problems inherent in the use of bacteria for biodesulfurisation. These are further elaborated in the literature review section. The main problem is the handling of the bacteria; if they are freely suspended then they are lost in the treatment process necessitating continuous growth and supply of bacteria. They may also attack the hydrocarbon components of the crude oil. Immobilization of bacteria has its own problems creating a mass transfer barrier to the crude oil if they are encased in a gel-like support or behind membranes. Although they can grow fast, they can also die fast if the nutritional requirements are not met.
1.2 Aims and Objectives

The problems with the current chemical hydrodesulfurisation and biodesulfurisation formed the justification of the research presented in this thesis: biocatalyst development for biodesulfurisation. The main aim of the thesis was therefore biocatalyst development for biodesulfurisation.

Two approaches were used that can be considered as the objectives:

   i. immobilization of biodesulfurising bacteria.
   ii. Search for other novel organism(s) other than bacteria for biodesulfurisation based on ability to consume sulfur and easily maintained.

In objective i, the immobilization method and material were chosen from several available options in order to reduce mass transfer problems naturally present in any immobilized cell system and also in order to allow the repeated use of the bacteria after refreshments in between biodesulfurisation cycles. For this purpose biodesulfurising bacteria were immobilized in porous coke particles and used in repeated cycles in a glass column.

In objective ii, cell cultures of two plants were tested for their biodesulfurisation activity which yielded positive results. As an extension of objective ii, a transcriptomics analysis was performed on the plant cell cultures that were exposed to model sulfur compounds typically present in crude oil. When compared to the plant cell cultures without exposure the results showed alterations in gene expression levels related mostly to sulfur metabolism. Although the work performed in both objectives are not exhaustive and several aspects need further experimentation, nevertheless they are novel and form the basis for exciting new research.

1.3 Scope

This work is only experimental and involves shake flask level of experiments for both bacterial and plant cell cultures. Since the initiation and growth of plant cell
cultures take much longer times compared to microbial cultures, there was only very limited amount of plant cell cultures to perform the experiments. The work did not involve any theoretical aspects, nor did it involve any molecular biology such as metabolic pathway elucidation or genetic manipulation. Only some transcriptomics analysis were performed on plant cell cultures.

1.4 Thesis organization

After this introduction that covers the brief background, justification for this research and aims and objectives, the relevant literature is reviewed in chapter 2. The routine procedures and materials are described in the materials and methods chapter next. Chapter three is presented in four main sections involving analyses, bacterial cultures, plant cell cultures and transcriptomics. Immobilisation of bacterial and plant cell cultures are also described according to the organism involved. Result and their discussion are presented in chapter four which also includes some details of experimental methods that were not routine but performed in a specific experiment. The thesis finishes with conclusions and recommendations for future and the appendices.
CHAPTER TWO

2. Literature Review

2.1 Fundamental sources of sulfur emissions

The emission of sulfur oxides (SO$_x$) by combustion of S-containing fuels is a global problem in terms of the hazardous effects exerted on the environment and human health. Other sources for sulfur emissions include volcanic eruptions, bacterial processes, evaporation from water and decaying of organisms. The emission of sulfur gases from vegetation has also been documented. The emission of H$_2$S from plant leaves increases with the intensity of light flux and drops to very low values in the absence of light (Rennenberg, 1984). The large quantities of reduced sulfur gases, especially H$_2$S, are not able to escape into the atmosphere, because they are precipitated by metal ions forming sulfide minerals in the sediment. Most active volcanoes are expected to release sulfur mainly as SO$_2$, then SO$_2$ to sulfuric acid and sulfate. Although biogenic sulfur emissions from the oceans contribute to air pollution, it has been reported that about 90% of gaseous sulfur emissions in the USA is a result of human-caused sources.

It has been established that the main global source of anthropogenic sulfur is burning of high-sulfur coal, which contributes 70-80 Tg of sulfur per year, mostly in the form of SO$_2$.

Biomass burning contributes 0.8-2.5 Tg to the global total. Table 2.1 clearly shows that anthropogenic emissions now dominate natural sources of sulfur in the atmosphere.

In the atmosphere, SO$_2$ gases are oxidized in the presence of oxygen and react with rain forming acid rain which falls back onto earth. Acid rain causes a serious environmental pollution problem that is hazardous to human health and life on earth.
Table 2.1 Estimated sources of short-lived sulfur gases (Tg S per year) (Houghton et al., 1992).

<table>
<thead>
<tr>
<th>Source</th>
<th>Estimated Sulfur Emissions (Tg S per year)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthropogenic emissions (mainly SO₂)</td>
<td>70-80</td>
</tr>
<tr>
<td>Biomass burning (SO₂)</td>
<td>0.8-2.5</td>
</tr>
<tr>
<td>Oceans (DMS)</td>
<td>10-50</td>
</tr>
<tr>
<td>Soils and plants (DMS and H₂S)</td>
<td>0.2-4</td>
</tr>
<tr>
<td>Volcanic emissions (mainly SO₂)</td>
<td>7-10</td>
</tr>
</tbody>
</table>

2.2 Sulfur compounds present in crude oil and the consequence of its emissions

2.2.1 Sulfur in crude oil

Petroleum is a complex mixture of hundreds of different compounds whose chemical composition and physical properties vary significantly from a crude oil to another depending on the location, origin and nature of the crude. After carbon and hydrogen elements, sulfur is the third most abundant element in crude oils and can account for 0.05% to 14% (w/w) (Van Hamme et al., 2003), depending on the source and type of the crude (Monticello and Finnerty, 1985; Kropp & Fedorak, 1998; Marcelis, 2002). Organic sulfur compounds are the major S-constituents present in crude oils, but inorganic sulfur i.e. elemental sulfur, hydrogen sulfide and pyrites can also be present (Tissot and Welte, 1984). The ranges of organic sulfur contents present in crude oils found in different countries according to Marcelis are given in Table 2.2.

More than 200 sulfur-containing organic compounds have been identified from crude oils. These include sulfides, thiols, thiophenes, substituted benzothiophenes, dibenzothiophene, and more condensed thiophenes (Monticello and Finnerty, 1985).
The molecular structure of some of these compounds is presented in Figure 2.1. The thiophene organic sulfur compounds can account for about 70% of the sulfur content present in higher boiling point oil fractions (Kropp and Fedorak, 1998). Thiophenes condensed with one or more benzene rings form benzo- and dibenzothiophenes (BTs, DBTs), and their alkylated derivatives. This is the reason behind choosing BT and DBT as model compounds in bio-desulfurization research.

Small amounts of organic compounds containing oxygen, nitrogen as well as compounds containing metallic constituents particularly vanadium nickel, iron and copper are found in crude oils (Speight, 2006).

Table 2.2 Organic sulfur contents in crude oils. Modified from (Marcelis, 2002).

<table>
<thead>
<tr>
<th>source</th>
<th>wt. % sulfur</th>
<th>source</th>
<th>wt. % sulfur</th>
<th>source</th>
<th>wt. % sulfur</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argentina</td>
<td>0.06 - 0.42</td>
<td>Iran</td>
<td>0.25 - 3.23</td>
<td>Norway</td>
<td>0.03 - 0.67</td>
</tr>
<tr>
<td>Australia</td>
<td>0-0.1</td>
<td>Iraq</td>
<td>2.26 - 3.3</td>
<td>Russia</td>
<td>0.08 - 1.93</td>
</tr>
<tr>
<td>Canada</td>
<td>0.12 - 4.29</td>
<td>Italy</td>
<td>1.9 - 6.36</td>
<td>Saudi Arabia</td>
<td>0.04 - 2.92</td>
</tr>
<tr>
<td>Cuba</td>
<td>7.03</td>
<td>Kuwait</td>
<td>0.01 - 3.48</td>
<td>United Kingdom</td>
<td>0.05 - 1.24</td>
</tr>
<tr>
<td>Denmark</td>
<td>0.2 - 0.25</td>
<td>Libya</td>
<td>0.01 - 1.79</td>
<td>USA</td>
<td>0.29 - 1.95</td>
</tr>
<tr>
<td>Egypt</td>
<td>0.04 - 4.19</td>
<td>Mexico</td>
<td>0.9 - 3.48</td>
<td>Venezuela</td>
<td>0.44 - 4.99</td>
</tr>
<tr>
<td>Indonesia</td>
<td>0.01 - 0.66</td>
<td>Nigeria</td>
<td>0.04 - 0.26</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.1 Molecular structure of some S-containing organic compounds encountered in crude oils (Oil Spill Environmental Forensics: Fingerprinting and Source Identification)
2.2.2 Impacts of sulfur emissions

Sulfur emissions if left unchecked can lead to profound damage to the environment and life on earth in general. It is no wonder that there is a big drive by the government as well industrialists towards drastic decrease in the sulfur emissions.

2.2.2.1 Impact of sulfur emissions on environment

Acid rain has a lasting deleterious effect on aquatic and forest ecosystems, as well as on agricultural areas located downwind of combustion facilities (Kropp et al. 1998). The increased acidity in bodies of water caused by acid rain can cause the death of fish and other aquatic creatures. It also releases toxic metals which contaminate bodies of water (Environmental Protection Agency, 2003). Moreover, acid rain harms vegetation. It is believed that acid rain damages the protective wax that coats leaves, allowing acids to diffuse into the leaves, which interrupts water evaporation and gas exchange. Furthermore, acid rain adds hydrogen ions to the soil which reacts with soil minerals, displacing calcium, magnesium, and potassium from soil particles and causing nutrient leaching and plant starvation. In addition, toxic metals such as lead, copper, zinc, inhibit the growth of trees (Environmental Protection Agency, 1999). Bad air quality is a direct effect of sulfur emissions in the atmosphere. However, sulfur deposition on S-deficient agricultural land may induce crop growth. Furthermore, sulfate aerosol formed in the stratosphere from $\text{SO}_2$ could increase the reflection of sun radiation back into space, consequently causing cooling of the earth. This is in contrast to the global warming effect caused by the emitted $\text{CO}_2$ from the burning of fossil fuels. The sulfate aerosols also accelerate chemical reactions that, together with increased stratospheric chlorine levels, form man-made chlorofluorocarbon (CFC) pollution and destroy the ozone layer.
2.2.2.2 Impacts of sulfur emissions on health

Sulfur-oxides (SO$_x$) resulting from fuel combustion and inhaled lead to many health problems not only to humans but also to animals. Inhalation of that gas leads to brain and nervous system damage. Also kidney and lungs are seriously affected by (SO$_x$), eyes irritation, coughing and chronic bronchitis and other respiratory tract infection developed due to exposure of (SO$_x$) gas (Lazarus et al. 1997). Sulfur-containing heterocyclic compounds, besides being pollutants, have a high potential for bioaccumulation; their accumulation in tissues of organisms is known to be mutagenic and carcinogenic (Kropp and Fedorak, 1998).

2.2.2.3 Impacts of sulfur-containing fuels on economy

Some of the economic impacts of (SO$_x$), emitted due to the incomplete combustion of high-sulfur fuels, have to do with acid rain as well. Acid rain affects monuments and buildings that are made of certain materials such as limestone and marble. The acid dissolves the calcium carbonate in the stone creating cracks, leading the structure to collapse (Electric Power Research Institute, 2005).

Sulfuric acid can cause enormous economic losses due to metal corrosion that occurs in vehicles and machineries in oil plants. It has been estimated that the transition to low sulfur fuels would be economically beneficial due to the avoidance of thousands of hospital admissions and emergencies, lost working days, reducing agricultural crop, and commercial forest damage (Natural Resource Defense Council, 2002).
2.3 Metabolic pathway of microorganisms for thiophene compounds

Recognizing the adverse impacts of sulfur emitted from burning S-containing fuels, many research groups invested great efforts for more than four decades to isolate microorganisms capable of degrading these pollutants using DBT and BT as the model compounds. However, during the last two decades, more emphasis was given to the isolation of desulfurizing microorganisms rather than degrading ones.

There are a vast number of reports on the microbial utilization of condensed thiophenes (reviewed by Kropp & Fedorak, 1998; Ohshiro & Izumi, 1999; Bresseler & Fedorak, 2000; Gupta et al., 2005). These studies denoted three distinctive metabolic pathways for DBT and related compounds.

In the first degradative pathway, DBT serves as the sole source of carbon and involves C-C bond cleavage of the aromatic ring mediated by ring hydroxylating and ring cleavage dioxygenases (Kodama et al., 1973). In the second degradative pathway, DBT serves as the sole source of carbon and sulfur and involves C-C and C-S bond cleavage mediated by angular dioxygenation (Nojiri et al., 2001). The third pathway is not a degradative one, and involves the utilization of DBT as the sole source for S (biodesulfurization) via C-S bonds cleavage leaving the aromatic rings intact and hence, keeping the caloric value of the fuels (Kilbane, 1989).

Recently, Gupta et al., (2005) outlined three categories for microbial metabolism of DBT and related compounds according to the mode of action. The first category involves the oxidative C-C bonds cleavage (Kodama pathway). The second category is concerned with the oxidative C-S bond cleavage (biodesulfurization) and the last category is concerned with the reductive C-S bond cleavage (anaerobic biodesulfuration). Accordingly, the different pathways involved in the microbial attack of DBT and related compounds, namely the partial oxidation pathway via C-C bonds cleavage and the mineralization pathway via angular dioxygenation that involves C-C and C-S bond cleavage, oxidative C-S bonds cleavage (biodesulfuration), and the
reductive C-S bonds cleavage (anaerobic desulfurization) will be treated in more details in the next section.

2.4 Major Pathways of microbial utilization of thiophene compounds

2.4.1 Partial oxidation pathway via oxidative C-C bond cleavage (Kodama pathway)

In this pathway, DBT serves as the sole source of carbon and energy. The microbial attack of DBT via the Kodama pathway Figure 2.2 involves the degradation of one of the homocyclic rings forming 3-hydroxy-2-formyl benzo-thiophene (HFBT). This pathway starts by oxidation of the aromatic ring mediated by DBT 1,2-dioxygenase in analogy to naphthalene-1,2-dioxygenase forming cis-1,2-dihydroxy-1,2-dihydro DBT, and 1,2-dihydroxy DBT. The dihydroxylated ring in 1,2-dihydroxy DBT is subjected to further attack by the ring cleavage 1,2-dihydroxy DBT dioxygenase and subsequent reactions leading to the formation of HFBT which accumulates in supernatant of pure cultures (Kodama et al., 1970; Kodama et al., 1973). Mormile and Atlas (1988, 1989) suggested that HFBT can be biodegraded further, but did not reveal anything about the fate of the carbon and sulfur atoms. Bressler and Fedorak (2001) reported some chemical properties of the purified HFBT and described the abiotic condensation of HFBT to form cis- and trans- thioindigo. They also showed that HFBT was mineralized by a mixed bacterial culture and identified benzo thiophene-2,3-dione in extracts of these acidified cultures. Biodegradation of BT via kodama pathway has also been reported Figure 2.2 and Figure 2.3 (Kropp et al., 1998). However, it was also reported that the oxidation of BT and DBT via the Kodama pathway is a co-metabolic process, because other substrates were required for growth and DBT, and BT oxidation to occur (Kropp et al., 1998; Frassinetti et al., 1998; Nadalig et al., 2002; soleimani et al.2007). It seems likely that many microbial type Table 2.3
Harbor the metabolic machinery involved in the degradation of DBT and BT via the kodama pathway. Depending on high sequence identity between the genes coding for naphthalene dioxygenase $ndo\ A-C$ and three of the ORFs ($dox\ A, B, and\ D$) encoding DBT oxidation by the Kodama pathway in $P.\ putida$, Denome et al., (1993) showed that a single genetic pathway controls the metabolism of DBT to HFBT and the metabolism of naphthalene to salicylaldehyde. Thus, it appears that there may not be a separate set of genes or enzymes for degradation of DBT via Kodama pathway. The similarity of $dox$ genes with $ndo$ genes and the ability of naphthalene dioxygenase from $Pseudomonas$ strain NCIB to oxidize DBT suggest that DBT may likely serve as an alternate substrate for naphthalene degrading enzymes (Resnick & Gibson 1996). This finding runs in accordance with the accumulation of HFBT in supernatant of cultures growing with DBT because DBT is merely a substrate analogue to naphthalene and is acted upon by naphthalene dioxygenase.
Figure 2.2 The proposed Kodama pathway of the biodegradation of dibenzothiophene by dibenzothiophene dioxygenase (adapted from Kropp et al., 1998).
Figure 2.3 The proposed pathway of the biodegradation of benzothiophene via dioxygenation (adapted from Kropp et al., 1998).
2.4.2 Angular dioxygenation through oxidative C-C bond cleavage and DBT mineralization

In this pathway DBT serves as the sole source of C, S, and energy Figure 2.4. The microbial attack of DBT via angular dioxygenation starts on the angular position adjacent to the sulfur atom (Nojiri et al., 2001). DBT-sulfoxide, DBT-sulfone, and benzoic acid were isolated and identified as metabolic intermediates of DBT degradation by the angular dioxygenation pathway (Van Afferden et al., 1990).

During the degradation of DBT by this pathway the C-S bonds are also cleaved and sulfite was released in stoichiometrical amounts and was further oxidized to sulfate. In this pathway, DBT was first oxidized via monooxygenases, leading to the formation of DBT sulfone which is then subjected to the attack by angular dioxygenase, leading to the formation of 2,3-dihydroxy biphenyl-2-sulfinate. This compound undergoes degradation by meta cleavage and the ring fission product is degraded to sulfite and benzoate, which is further metabolized to TCA cycle metabolites. Accordingly, this pathway results in complete mineralization of DBT with the release of S as sulfite which is oxidized afterwards to sulfate. Among the bacterial species that attack DBT via angular deoxygenation are Brevibacterium sp. (Van Afferden et al., 1988) and Arthrobacter sp. (Dahlberg et al., 1993).
Figure 2.4 Overview of the bacterial degradative pathway for DBT via angular dioxygenation. The structures shown in brackets have not been characterized. The arrows, with solid and broken lines indicate the enzymatic and spontaneous conversion, respectively, (Nojiri et al., 2001).
2.4.3 Biodesulfurization via C-S bonds cleavage pathway

This process for removal of sulfur was first reported for *Rhodococcus rhodochrous* IGTS8 (Gallagher et al., 1993). 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 Figure 2.5. In this oxidative pathway, DBT and methyl DBT serve as the sole source of sulfur. The C-S bonds are specifically cleaved and the carbon skeleton remains intact as 2-hydroxy biphenyl (2-HBP), which accumulates, thus preserving the fuel value. It has been shown that this pathway is inhibited in the presence of sulfate. The microbiology, biochemistry, and genetics of the biodesulfurization pathway will be emphasized in details hereafter.
Figure 2.5 Proposed (4S) pathway for desulfurization of DBT by *R. erythropolis* IGTS8. DBTO, DBT sulfoxide; DBTO$_2$, DBT sulfone; HPBS, 2-(2''-hydroxyphenyl)-benzene sulfinate; HBP, 2-hydroxybiphenyl; DBTMO, DBT mono oxygenase; DBTO$_2$-MO, DBTO$_2$ monooxygenase; FMN, flavin mononucleotide adapted from (Kirimura et al., 2001; Matsubara et al., 2001).
2.4.4 Anaerobic biodesulfurization via Reductive C-S bond cleavage

In contrast to aerobic BDS, the anaerobic BDS operates under sulfate reducing conditions. Accordingly, the possibility of non-specific oxic attack of hydrocarbons in diesel will be eliminated under sulfate-reducing conditions. Moreover, the anaerobic BDS does not liberate sulfate as a bio-product that must be disposed of by some appropriate treatment.

However, the cost needed to establish the reducing equivalent (generation of H\(_2\)) and to maintain anaerobic conditions limits the commercialization of the anaerobic BDS (Gupta et al., 2005). In addition, anaerobic microorganism effective enough for practical petroleum desulfurization have not been found yet. It has been demonstrated that the bacterium *Desulfovibrio desulfuricans M6* is capable under sulfate-reducing conditions of desulfurizing some organic sulfur compounds present in diesel, such as DBT (Kim et al., 1990; Kim et al., 1995). In this pathway, DBT is used as the sole electron acceptor which is transformed and accumulated as 2-HBP, whereas the sulfur is eliminated as H\(_2\)S Figure 2.6.
Figure 2.6 Principle of reductive DBT desulfurization pathway by *Desulfovibrio desulfuricans* M6 (adapted from Kim et al., 1995).
2.5 Biodesulfurization process of microbial biodesulfurization of thiophene compounds

After distillation of crude oil, DBT and alkylated DBTs are concentrated in the middle distillate fractions and they may represent up to 70% of the S present in diesel oil. Conventional chemical hydrodesulfurization (HDS) processes used in refinery industry cannot completely remove these heterocyclic organosulfur compounds.

Recognizing this and other shortcomings of HDS, many investigators invested more effort looking for an alternative technology for ultra low sulfur fuel production. Biodesulfurization (BDS) has been considered as a novel biotechnology which is expected to be a complement or alternative to ultra deep 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.
2.5.1 General microbiology of biodesulfurization

Since the discovery of *Rhodococcus* sp. strain IGTS8 (Kilbane et al., 1989) and the exploration of its ability to desulfurize DBT (Kayser et al., 1993), there has been considerable interest in isolating new potential microbial strains (biocatalysts) with novel capabilities which are crucial for industrial implementation of BDS. Among the desired capabilities are mass production of biocatalyst with high desulfurization activity, stable catalytic activity in aqueous as well as organic phases (solvent tolerance), a broad substrate desulfurization spectrum (relaxed specificity), working at high temperature, production of biosurfactant to increase substrate mass transfer (bioavailability) and hence speed up BDS rate, and end products-inhibition tolerance which is exerted by 2-HBP and SO$_4$.

Isolation of microorganisms harboring this fascinating microbial machinery will strengthen the feasibility of BDS as a new challenge for a biotechnological approach to obtain sulfur-free fuels. Over the past two decades, a number of microbial strains which possess BDS activity via the 4S pathway has been isolated Table 2.3. 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 Gordonia alkanivorans, Xanthomonas, Nocardia globelula, and Agrobacterium*. A few gram-negative bacteria have been recently discovered, such as *Sphingomonas* (Nadalig et al., 2002) and *Pseudomonas delafieldii* (Guobin et al., 2006). A very distinct feature of this 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 (Neu, 1996; 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, are some fungi such as *Cunninghamamella elegan* (Crawford & 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, a new anaerobic bacterial strain *Desulfovibrio desulfuricans* has been shown to harbor the capability of reductive desulfurization of DBT to biphenyl and H$_2$S (Kim et al., 1990). Only few strains of these microorganisms can desulfurize thiophene compounds at elevated temperature (40-60º C) and therefore are designated as moderate thermophilic desulfurizers such as *Paenibacillus*, *Mycobacterium*, and *Bacillus Sulfolobus acidocaldarius* (Kargi, 1987; Kankipati & Ju, 1994). A facultative autotroph which can grow in the temperature range (60-90º C) has been shown to desulfurize DBT. However, 4-hydroxybenzoic acid was tentatively identified as a metabolite, suggesting that the carbon backbone was further degraded after the sulfur atom was removed as sulfate.

Unfortunately, so far most of the isolated and identified bacteria having biodesulfurizing activity exhibited narrow range of substrate specificity, limited to either DBT or BT with few possessing desulfurization activity to both and few of their derivatives present in oil. The other major problem related to these thermophilic biodesulfurizers is the inhibition of its desulfurization activity by the final end products 2-HBD or other biphenyl derivatives as well as the sulfate produced ((Ohshiro et al., 1995; Ohshiro et al., 1997; Setti et al., 1999). Kim et al. (2004), reported the inhibition of the desulfurization activity and cell growth of of *Gordonia* sp. CYKSY at a 2-HBP concentration of 0.15 mM or higher. This seems to be in contrast to Guobin et al (2006), who reported that the DBT-desulfurization activity of the gram negative *Pseudomonas delafeldii* R-8 in HDS-treated diesel oil was not affected when the concentration of 2-HBP reached 1.0 mM. However, a drastic decline (80% inhibition) in the in the DBT-desulfurization activity was observed at 1.0mM 2HBP when tested in aqueous phase. The same authors reported that the desulfurization activity for DBT was not affected by sodium sulfate, even at 15.0 mM concentration.

These findings brings to attention the importance of the media in which such investigations are conducted and the caution required for any extrapolation of the results obtained from one medium to another.
There is still a need to isolate new strains since the majority of the identified biodesulfurizing bacteria exhibit narrow substrate specificity where most of them desulfurize either DBT or BT and few of them can desulfurize both compounds and some of their derivatives. Although some of the characterized biodesulfurizing bacteria showed high rates of catalytic activity to desulfurize thiophene compounds, the overall BDS process has been shown to be inhibited by the final end products 2-HBP and sulfate.

Table 2.3 Desulfurization of DBT and thiophene compounds in diesel by various bacterial species.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Substrat</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rhodococcus erythropolis</em></td>
<td>DBT</td>
<td>Guerinik and Al-Mutawah, 2003</td>
</tr>
<tr>
<td>and <em>Rhodococcus globerulus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Rhodococcus erythropolis</em> XP</td>
<td>Crude Oils</td>
<td>Bo Yu et al., 2006</td>
</tr>
<tr>
<td><em>Rhodococcus erythropolis</em> XP</td>
<td>Diesel, DBT</td>
<td>Bo Yu et al., 2006</td>
</tr>
<tr>
<td><em>Rhodococcus erythropolis</em> XP</td>
<td>DBT</td>
<td>Bo Yu et al., 2006</td>
</tr>
<tr>
<td><em>Rhodococcus sp.</em></td>
<td>Diesel</td>
<td>Labana. et al., 2005</td>
</tr>
<tr>
<td><em>Arthrobacter sulfurous</em></td>
<td>Diesel</td>
<td>Labana. et al., 2005</td>
</tr>
<tr>
<td><em>Rhodococcus globerulus</em></td>
<td>Diesel</td>
<td>Gladys. et al., 2002</td>
</tr>
<tr>
<td><em>Rhodococcus, Gordonia sp.</em></td>
<td>Diesel</td>
<td>Abbad-Andaloussie et al., 2003</td>
</tr>
<tr>
<td><em>Rhodococcus strain WU-BT</em></td>
<td>BT</td>
<td>Kirimura et al., 2002</td>
</tr>
<tr>
<td><em>Mycobacterium goodie X7B</em></td>
<td>DBT</td>
<td>Li et al., 2005</td>
</tr>
<tr>
<td><em>Paenibacillus sp. A11-2</em></td>
<td>DBT</td>
<td>Konishi et al., 1997; Onoka et al., 2001</td>
</tr>
<tr>
<td><em>Gordona sp.</em></td>
<td>BT</td>
<td>Gilbert et al., 1998</td>
</tr>
<tr>
<td><em>Corynebacterium</em></td>
<td>DBT</td>
<td>Omori et al., 1992</td>
</tr>
</tbody>
</table>
2.5.2 Biochemistry of biodesulfurization

The 4S microbial biodesulfurization pathway represents the oxidative attack of C-S bonds in the heterocyclic five-membered thiophene ring moiety in BT, DBT or their derivative compounds. During this pathway, the sulfur atom oxidized and is selectively released as sulfite, which spontaneously oxidized further to sulfate, whereas the hydrocarbon backbone (C-C bonds) of the desulfurized compounds remains unattacked, thereby preserving the caloric value of the fuel.

The thermodynamic of C-S and C-C bonds cleavage in thiophene compounds and the effect of the introduction of oxygen atoms on the bond strength have been treated in an excellent review by Bressler et al. (1998). Cited data in this review Table 2.4 from Bressler et al. (1998) showed the calculated strength of the C-S bonds in thiophene, BT and DBT to be near 340 kJ/ mol. The calculated average strength of the C-C bond in benzene is around 505 kJ/ mol, and in ethane is 376 kJ/ mol, whereas the strength of the C=C bond in ethene is 733 kJ/ mol. Accordingly, comparing the C-C bond strengths to the C-S bond strength in thiophenones suggests that the heteroatomic bonds are the weakest in these molecules. Thus, one would predict that the C-S bond would be the most susceptible to cleavage rather than the C-C or C=C bonds.

This feature is common in all aerobic biodesulfurizing bacteria that follow the 4S pathway. Another common feature in the aerobic microbial attack of sulfur heterocycles is the addition of one or more oxygen atoms to the molecule. Oxygen is typically added to the sulfur atom and/or to the carbon atom adjacent to the sulfur atom. As the oxygen element possesses the second highest

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Compound</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gordonia strain CYKS1</td>
<td>DBT</td>
<td>Oldfield et al., 1997</td>
</tr>
<tr>
<td>Xanthomonas sp.</td>
<td>DBT</td>
<td>Constanti et al., 1994</td>
</tr>
<tr>
<td>Nocardia strain</td>
<td>DBT</td>
<td>Chang et al., 1998</td>
</tr>
<tr>
<td>Agrobacter MC501</td>
<td>DBT</td>
<td>Constanti et al., 1996</td>
</tr>
</tbody>
</table>
electronegativity of all the elements, it is expected that addition of oxygen atom to C-C or C-S bonds will weaken the bond strength (Marcelis, 2002). Given that the C-S bond in sulfur heterocycle is likely the weakest bond in the molecule, the addition of oxygen to the sulfur atom or to a carbon atom adjacent to the sulfur atom would further weaken the C-S bond, thereby increasing the possibility of ring cleavage between these two atoms.

Table 2.4 Bond strengths of various C-S, and C-C bonds, adapted from Marcelis, (2002).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Bond energy kJ/mol</th>
<th>Compound</th>
<th>Bond energy kJ/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-S bond</td>
<td></td>
<td>C-C bond</td>
<td></td>
</tr>
<tr>
<td>Thiophene</td>
<td>341</td>
<td>H₂C-CH₃</td>
<td>376</td>
</tr>
<tr>
<td>Benzo thiophene</td>
<td>339</td>
<td>H₂C=CH₂</td>
<td>733</td>
</tr>
<tr>
<td>Dibenzothiophene</td>
<td>338</td>
<td>Benzene</td>
<td>505</td>
</tr>
</tbody>
</table>

The sulfur specific metabolic pathway for DBT desulfurization involves four enzymatic steps Figure 2.5. To date, all of the reported DBT-desulfurizing microorganisms share this pathway. The four enzymes operating in this pathway are two monooxygenases (DsxC and DszA), a desulfinase (DsxB), and a flavin redutase (DsxD). The first three enzymes (DsxC, DszA, and DszB) are encoded on a plasmid forming one operon, whereas the last enzyme (DsxD) is encoded chromosomally. The monooxygenases and the desulfinases are colorless, indicating that they contain no tightly associated chromophores and none of them was inhibited by EDTA, indicating that they did not require metal ions as cofactors (Gray et al., 1996). It has been shown that the activity of DszD is essential for the catalytic activity of the monooxygenases, as it delivers the reducing equivalent (FMNH₂) required for the monooxygenation reactions.
DsxC is the first enzyme operating in the 4S pathway. It is a monooxygenase that oxidizes DBT to sulfoxide and then to sulfone in a two-step monooxygenation process Figure 2.5. The first oxidation step is about one-tenth the rate of the second step (Gray et al., 1996). This enzyme appears to be specific for sulfoxidation because no other oxidized products of DBT were detected. Using $^{18}O_2$, Lei and Tu (1996) confirmed that both oxygen atoms in the sulfone were derived from molecular oxygen. They also showed that the over expressed DszC from *Rhodococcus* is a homodimer of 90.2 kDa. However, a homotetramer DszC with a subunit of 50 kDa (Gray et al., 1996) and a homohexamer DszC version with a subunit of 45 kDa (Ohshiro et al., 1994) have been reported. This enzyme can act on derivatives of DBT such as 4,6-dimethyl DBT, 2,8-dimethyl DBT, 3,4-benzo-DBT but does not show any activity on carbazole, dibenzofuran and fluorine, i.e., DBT substituted for sulfur atom. The enzyme requires FMNH$_2$, for its catalytic activity, which is delivered by the flavin reductase (DszD). Xi et al., (1997) suggested that flavin is not a cofactor of DszC, but the reduced form of flavin serves as a co-substrate. However, NAD(P)H is not utilized by DszC.

Dsza is the second monooxygenase operating in the 4S pathway Figure 2.7. This enzyme catalyzes the cleavage of one of the two C-S bonds and the conversion (hydroxylation) of DBT sulfone (DBT-5,5 dioxide) to 2-hydroxybiphenyl-2-Sulfinate (HBPS). DszA also requires the flavine reductase in equimolar quantities for the oxidative catalytic activity (Xi et al., 1997) where FMNH$_2$ serves as a cosubstrate for DszA. Figure 2.7 Similar to DszC, the NAD(P)H is not utilized by DszA. The native molecular mass of the purified DszA from *R. erythropolis* IGTS8 was 100 kDa for a dimmer protein. A thermophilic DszA was also purified from *Paenibacillus* sp. (Konishi et al., 2000) and was found to be a homodimer with a subunit molecular mass of 50kDa. The reaction rate of DszA was about 5-6 fold higher than DszC. This would explain why there was no accumulation of DBT sulfone in cell-free desulfurization assay. Gray et al., (1996) reported that the enzymes catalyzing the 4S pathway do not require metal ions as a cofactor. However, Ohshiro et al.
purified DszA and suggested that a metal might be involved in the activity of DszA from R. erythropolis D-1.

The desulfinase enzyme DszB catalyses the cleavage of the second C-S bond and hence the desulfination of HBPS to form 2-hydroxybiphenyl (2-HBP) and sulfite. This reaction is unusual because no enzyme has previously been known to catalyse such a reaction (Ohshiro & Izumi 1999). The DszB reaction seems to be a rate-limiting step of the whole pathway since it is the slowest of the three enzymatic reactions the produced 2-HBP accumulates as dead end product. This enzyme is produced in very small amounts, and therefore is the least studied enzyme (Gupta et al., 2005). DszB is a monomer with a subunit molecular mass of 40 kDa and shows enzyme activity over a wide temperature range (25-50º C), the optimum being 35º C (Watkins et al., 2003). The working pH range for DszB is 6.0-7.5. A cysteine residue is shown to be conserved in the sequence of this enzyme and it is found to be critical for enzyme activity.

The complete removal of sulfur from the thiophene ring by desulfurizing strains has been shown to involve, in addition, a fourth protein (flavin reductase,
DsD), which provides the reducing equivalent required for the monoxygenation reactions. The flavin reductase has been shown to be chromosomally coded (Gray et al., 1996; Matsubara et al., 2001).

2.5.2.1 Effect of concentration of 2-HBP and sulfate in the media

Inhibitions of cell growth and desulfurization activity by the end products from DBT desulfurization, 2-HBP and sulfate, are known to be severe (Kayser et al., 1993). Most of the strains previously reported which desulfurized DBT via sulfur-specific pathway were observed to be inhibited by sulfate, L-methionine and L-cysteine and not by DBT and dibenzothiophene sulfone DBTO2 (Li et al., 1996).

It has been reported that a concentration of 200 µM is inhibitory and 400 µM of 2-HBP completely prevents growth and desulfurization activity of Corynebacterium, Rhodococcus and Gordonia cultures. M. Phlei GTIS10 are able to grow in medium containing concentrations of 2-HBP as high as 750 µM and have difficulty in growing in 1mM 2-HBP (Kilbane, 2002). The cell growth of Gordonia sp. CYKS1 was also inhibited by the 2-HBP even in the presence of sulfates implying that 2-HBP not only inhibited the enzymes involved in desulfurization but also certain enzymes for cell growth (Kim et al., 2004). Sulfur-specific DBT degradation pathway, which is capable of transforming DBT to sulfite and 2-HBP, was identified in the soil bacterium R. erythropolis (Denome et al., 1994) and found to be catalyzed by the following three enzymes DszA, DszB, and DszC. is reported to be inhibited in the enzyme level by biphenyl-derivatives such as biphenyl (BP), 2-HBP and 2,2'-DHBP(Oshiro et al., 1995). The reaction catalyzed by DszB is the slowest in the pathway and thus was proposed as the rate-limiting step in desulfurization (Gray et al., 1996). DszB is inhibited by the reaction product of 2-HBP but not by biphenyl, which indicates that the hydroxyl group is required for the inhibition. DszA and DszC are flavin-dependent monooxygenase and responsible for oxidation of DBT to 2'-hydroxybiphenyl-2-sulfinic acid (HBPS). DszB, 39-kDa protein participates in the last desulfurization step and hydrolyzes the sulfinate group of HBPS. However, DszB can accept biphenyl-2-sulfinic acid (BPS) as a
substrate, and the hydroxyl group of HBPS does not seem to be essential for the activity (Nakayama et al., 2002).

The inhibition of desulfurization activity by sulfate is considered to be a gene-level regulation. The expression of dsz genes that are involved in desulfurization of DBT is strongly repressed by sulfates (Li et al., 1996). The desulfurization activity of Mycobacterium G3 is repressed by sulphate ions of concentration about 0.15 mM in the growth medium. Bacteria strain G3-2, grown with 0.1 mM MgSO$_4$ as the sulfur source, had desulfurization activity. However, expression of desulfurization genes was completely repressed when G3-2 was grown with 0.5 mM MgSO$_4$ (Masaki et al., 2005). The biodesulfurization can be enhanced and effective by the application of a sulfate non-repressor promoter. The construction of promoterless by a promoter- probe and red-shifted green fluorescence protein gene (rsgfp). The isolation of 340bp putative promoter, designated kapl from bacterial strain R. erythropolis KA2-5-1 recombinant that had shown high fluorescence intensity. 1 mM Sulfate did not effect the activity of kapl (Noda et al., 2002).

2.5.2.2 Effect of ethanol and glucose on desulfurization Activity of DBTutilizing bacteria

The effect of ethanol as sole of carbon source was reported by Rhodococcus erythropolis KA2-5-1. The desulfurization activity increased when ethanol containing DBT feed up to 135 mmol 2-HBP kg$^{-1}$ h$^{-1}$. 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 media at concentrations higher than 1.0% produced progressively decreased exponential growth rates and slightly reduced extents of growth (Yoshikawa et al., 2002). It was reported that the addition of DBT dissolved in ethanol provided more rapid growth and desulfurization than DBT powder only (Setti et al., 1995). Gordonia sp. CYKS1 has ability to utilize ethanol as well as glucose as carbon source. The growth rate on ethanol was observed to be
higher than that on glucose. Ethanol is used as de-emulsifier in one of the diesel desulfurization processes under development, to promote phase separation for the recovery of desulfurized diesel oil and microbial cells from the very stable emulsion from the reactor (Choi et al., 2003). The major portion of the added ethanol remains in the aqueous phase containing microbial cells after the phase separation. It can serve as a good carbon source for the growth of the recovered cells to be recycled to the reactor, replacing, at least partially, the carbon source requirements.

The bacterial strains harboring the DNA sequence which encodes DszD (flavin reductase) can be grown under conditions which maximize the expression of the gene. *Rhodococcus* species which contain the gene can be grown in the presence of an alcohol (such as ethanol, ethanolamine, glycerol or propanol). The expression of the gene in microorganism may induce or increase by presence of these compounds (Kevin et al., 1996).

### 2.5.2.3 Increasing the bioavailability of thiophene compounds and biosurfactant production

Many bacteria, yeasts, and fungi produce extracellular or membrane-associated surface active compounds called biosurfactants (Desai and Banat, 1997). Biosurfactants are a diverse group of surface-active chemical compounds produced by wide variety of microorganisms, members of genera *Arthrobacter, Bacillus, Candida, Pseudomonas* and *Rhodococcus* as shown in Table 2.5 (Banat, 1995).

#### Table 2.5 Microbial Biosurfactants (Banat, 1995).

<table>
<thead>
<tr>
<th>Type of biosurfactant</th>
<th>Bacterial strain</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhamnolipids</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Lang and Wagner (1993); Haba et al. (2000)</td>
</tr>
<tr>
<td>Biosurfactant</td>
<td>Source</td>
<td>Reference</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>Sophorolipids</td>
<td>Candida bombicola</td>
<td>Schippers et al. (2000)</td>
</tr>
<tr>
<td>Neutral lipid</td>
<td>Nocardia erythropolis</td>
<td>MacDonald et al. (1981)</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>Acinetobacter sp., Corynebacterium insidiosum</td>
<td>Kappelli and Finnerty (1979); Akit et al. (1981)</td>
</tr>
<tr>
<td>Lipopeptide</td>
<td>Arthrobacter MIS38, Bacillus subtilis, Pseudomonas fluorescens</td>
<td>Akpa et al. (2001); Braun et al. (2001); Morikawa et al., (1993).</td>
</tr>
<tr>
<td>Fatty acid</td>
<td>Corynebacterium lepus</td>
<td>Desai and Banat (1997)</td>
</tr>
</tbody>
</table>

Sorosurfactants are amphiphilic molecules with both hydrophobic and hydrophilic domains, which are able to lower surface and/or interfacial tension (Banat, 1995) by partitioning at the water/air and water/oil interfaces (Tarek et al., 2006). Based on their chemical structure the hydrophilic moiety consists of amino acids, peptides- anions, mono-di- cations, or polysaccharides; and the hydrophobic moiety consists of, saturated or unsaturated fatty acids. Accordingly, the major classes of biosurfactants include glycolipids, fatty acids, phospholipids, lipopeptides, and polymeric biosurfactants (Georgiou et al., 1992).

Glycolipids are the most known biosurfactant. They are carbohydrates in combination with long chain aliphatic or hydroxyaliphatic acids. Among the glycolipids, the best known are rhamnolipids as shown in Figure 2.8, trehalolipids and sophorolipids. They are less sensitive to extreme conditions of temperature, salt concentration, and pressure (Sullivan, 1998).
Biosurfactant-producing microorganisms can be screened using different assays. The production of rhamnolipids by colorimetric measuring of the amount of methylpentoses in culture supernatants and by the ability to form clear halos in methylene blue/cetyltrimethylammonium bromide (CTAB) plate or N-cetylpyridinium chloride-methylene blue agar plate (Maneerat, 2005).

Recent advances in the field of microbial surfactants are largely attributed to development of quick, reliable methods for screening biosurfactant-producing microbes and assessing their potential. Siegmund and Wanger (1991) developed an agar plate method that helps in the detection of extracellular rhamnolipids (a group of anionic biosurfactants). These biosurfactants form an insoluble ion pair with the cationic tenside cetyltrimethylammonium bromide and the basic dye methylene blue which are included in mineral agar plates. This technique facilitates the detection of rhamnolipids producing colonies by colour reaction. On coloured plates, colonies producing extracellular anionic biosurfactants are surrounded by halos. Another method for detection of the rhamnolipids depending on their hemolytic activity was reported for *B. subtilis* by Bernheimer and Avigad 1970. Hemolysis on blood agar has been widely used to screen biosurfactant producing bacteria and for preliminary identification of many types of clinically important bacteria (Rodrigues et al., 2006). Blood agar is purposely used as an enriched medium for growing fastidious bacteria and as a differential medium. It has been used previously to
quantify surfactin (Rodrigues et al., 2006) and rhamnolipids (Johnson and Boese-Marrazzo, 1980). Many researchers have used this technique to screen for biosurfactant production by new isolates (Yonebayashi et al., 2000).

At present, biosurfactants plays an important application in petroleum-related industries which are used in enhanced oil recovery, cleaning oil spills, oil-contaminated tanker cleanup, viscosity control, oil emulsification and removal of crude oil from sludge (Kosaric, 1992).

For designing a biocatalyst capable of desulfurizing DBT, BT and their alkylated derivative, the bioavailability of these compounds was increased using genetic engineering tools. A recombinant Pseudomonas was constructed harboring the genes responsible for production of biosurfactant as well as the dsz genes coding for the specific cleavage of C-S bond via the common desulfurization 4S pathway (Gallardo et al., 1997). The same technique was used in construction of the recombinant Bacillus subtilis carrying dszABC and dszD genes (Ma et al., 2006)

2.5.3 Genetics of biodesulfurization

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).

Although expressed as an operon, DszB is present at concentrations several-fold less in the cytoplasm, as compared with DszA and DszC (Li et al., 1996). 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. An insertion sequence (IS 1166) was found to be associated with the dsz gene. Promoter and regulatory regions of the dsz operon were also studied and it was found that enzymes are strongly repressed in the presence of readily bioavailable sulfur (Li et al., 1996), i.e., sulfate, sulfide, methionine and
cysteine, a phenomenon that is similar to responses to other sulfur compounds under sulfur starvation. Accumulation of 2-HBP also inhibits growth and desulfurization. Monticello (1998) postulated that repression of the enzyme synthesis occurs at the transcription level, but 2-HBP does not act as an inhibitor of the enzyme. Analogous to the dsz operon in mesophiles, the tds thermal desulfurization operon is located on an 8.7 kb DNA fragment in the thermophile *Paenibacillus* sp. All-2 (Ishii et al., 2000; Konishi et al., 2000). The tdsA, tdsB, and tdsC nucleotide sequences and the deduced amino acid sequence showed significant homology to the dszA, dszB and dszC genes of *R. erythropolis* IGTS8. However, several local differences were observed between them. Several gram-positive and gram-negative organisms are known to have desulfurization genes and they show 70% homology (McFarland, 1999).

The genes specifically involved in desulfurizing DBT by *R. erythropolis* IGTS8 were identified and cloned in a non-desulfurizing mutant derived from the strain IGTS8 and another strain *R. fascians* that is normally unable to desulfurize DBT. Both of these strains gained the desulfurization function after cloning the isolated genes. This indicates that the genes involved in the desulfurization pathway were entirely isolated. The DNA sequence of the 4.0-kb fragment coding for the desulfurization activity was determined. A frame shift and deletion mutations showed that three open reading frames were required for DBT desulfurization, and the genes were designated as soxABC (Denome et al., 1993). The desulfurization genes of *R. erythropolis* IGTS8 were characterized and sequenced in two reports in 1994. The first, report (Denome et al., 1994) showed that SoxC is a protein that oxidizes DBT to DBT-5,5'-dioxide and that SoxA metabolizes DBT-5,5'-dioxide to an undefined intermediate and that SoxB together with SoxA complete the desulfurization of DBT-5,5'-dioxide to 2-HBP. In the other report by Piddington et al. (1995) the three genes were designated dszABC. It was stated in the report that the gene product of dszC converts DBT directly into DBT-sulfone and that dszA and dszB work together on converting DBT-sulfone to 2-HBP. At that point it was
still not clear whether the DBT-sulfone is directly converted to DBT-dioxide or converted stepwise to DBT-dioxide with DBT-sulfoxide as an intermediate.

Further investigation gave a more clear understanding of the desulfurization genes in *R. erythropolis* IGTS8. Three of the genes involved in desulfurizing DBT in this strain are clustered on a 120-kb linear plasmid. The three genes *dszA*, *dszB* and *dszC* encode the enzymes DszA, DszB and DszC respectively. DszC is a 45-kDa protein responsible for the conversion of DBT to DBTO$_2$ through two consecutive monooxygenation reactions. DszA is a 50-kDa protein responsible for the second monooxygenation reaction that converts DBTO$_2$ to HBPS. DszB is a 40-kDa protein. This enzyme is a desulfinase that catalyzes the last step in the pathway where 2-HBP and sulfinate are formed as final products. The *dszABC* genes are transcribed in the same direction. The termination codon for the gene *dszA* and the initiation codon for the gene *dszB* overlap. A 13-bp gap separates the genes *dszB* and *dszC* (Ohshiro & Izumi 1999). The fourth desulfurizing gene, *dszD*, is located on the chromosomal DNA and not on the plasmid. This gene code for the protein DszD, a flavin reductase involved in the monooxygenation reactions (Gray et al., 1996).

### 2.5.4 Desulfurization of S-containing compounds by thermophilic microorganisms

There has been growing interest during the last few years in isolating potential thermophilic biodesulfurizers to be used as a biocatalyst in biorefining processes at higher temperatures. Performing BDS at high temperature is not only compatible with the hot HDS-treated oil fractions, but may also result in higher catalytic rates, and the reduced viscosity of petroleum at higher temperature will allow lower processing costs (Ishii et al., 2005).

The HDS processes operate under high temperature. It is expected that a successful biocatalytic BDS process would be implemented in refiners to desulfurize HDS-treated hot diesel fractions. Accordingly, the thermophilic
bacterial strains harboring BDS activity will be preferable to tolerate the high temperature of the feedstock that is going to be desulfurized. The use of thermophilic bacteria is most beneficial in terms of saving energy necessary to cool the feedstock after HDS processes. *Paenibacillus* sp. Strain A11-2 has been shown to biodesulfurize BT and DBT (Konishi, 2000; Onaka, 2000; Konishi et al., 1997). Another moderately thermophilic strain, *Bacillus subtilis* WU-S2B, has been reported with high desulfurization activity for DBT at 50°C through the selective cleavage of C-S bonds (Kirimura et al., 2001). *Mycobacterium pheli* GTIS10 has been proved to have the ability to desulfurize DBT at 52°C through a pathway identical to that of *R. erythropolis* IGTS8 (Kayser et al., 2002). Also *Mycobacterium phlei* WU-F1 has been shown to reduce sulfur of HDS light gas oil at 50°C (Furuya et al., 2003). The three enzymes involved in the microbial DBT desulfurization were purified and characterized from the thermophilic *M. phlei* WU-F1. The first two enzymes, DBT monooxygenase (BdsC) and DBT sulfone monooxygenase (BdsA), were purified from the wild-type strain, whereas the third enzyme, 2'-hydroxybiphenyl 2-sulfinic acid desulfinase (BdsB), was purified from a recombinant *Escherichia coli* after expressing the gene, *bdsB*, with chaperoning genes. The genes of BdsC and BdsA were also over expressed. The three enzymes were characterized and compared with the corresponding enzymes (DszC, DszA, and DszB) of mesophilic desulfurization bacteria. The specific activities of BdsC, BdsA, and BdsB were 84.2, 855, and 280 units/mg, respectively, and the latter two activities were higher than those of DszA and DszB. The heat stability and optimum temperature of BdsC, BdsA, and BdsB were higher than those of DszC, DszA, and DszB. (Ohshiro et al., 2005). Accordingly, the thermophilic bacterium *Mycobacterium phlei* WU-F1 could be used as a biocatalyst for practical biodesulfurization of diesel oil (Furuya et al., 2003).
2.6 Universal tendencies toward low sulfur fuels

2.6.1 Trials towards improving biodesulfurization activity

Although the BDS process has shown to work successfully with model compounds at the laboratory scale, some bottlenecks that limit the rate of this process and its implementation at the industrial scale should be overcome. Among the factors that limit the integration of a biocatalytic BDS process in refineries are:

1. feed back inhibition of the process by the phenolic end products such as 2-HBP, 2. limited supply of the reduced cofactor FMNH$_2$ which is necessary for the two monooxygenases catalyzing the early steps in the 4S pathway, 3. limited bioavailability of the desulfurization substrates in organic phase, 4. narrow substrate spectrum for desulfurization of various S-containing organic compounds, 5. obtaining high cell density of the biocatalyst and recovery of the biocatalyst for long run operations, 6. the need for thermostable enzymes to treat hot diesel fractions after HDS process, and 7. the need of a biocatalyst that does not attack C-C bonds in various chemical compounds present in diesel.

Recently, some of these obstacles have been tackleed by many research groups to overcome the previously mentioned limitations. An overview of the efforts made to improve the BDS process reveals the employment of three main approaches. The first approach deals with genetic manipulations of the genes coding for the BDS process aiming to 1. improve substrate bioavailability, 2. reduce product inhibition, 3. increase substrate spectrum, 4. improve thermostability of the biocatalyst, and 5. increase the enzymatic activity. The second approach tacklest the limited bioavailability of substrate and product inhibition via physical and chemical tools. The third approach focuses on isolation of new microbial strains with elevated BDS activity and novel characteristics that are crucial for implementation of BDS in refineries.

Among the genetic manipulations involved in improving the BDS activity are: 1. over expression of the genes encoding the BDS activity in other hosts to obtain
recombinant biocatalyst with improved and higher BDS activity than wild-type strain (Alves et al., 2006; Kilbane, 2006; Ma et al., 2006; Kirimura et al., 2004; Ohshiro et al., 2004; Gallardo et al., 1997), 2. manipulations of the dsz operon to remove gene overlap (Li et al., 2007), 3. activity improvement via directed evolution (Arensdorf et al., 2002), and 4. achievement of higher BDS rates via multiple copies of the dsz genes and place them under the control of alternative promoters (Hirasawa et al., 2001; Franchi et al., 2003).

Two stages are adapted during biocatalyst mass production of oil biodesulfurization process, cell growth phase were separated from active desulfurization phase (Chang et al. 2001). A progress has been made to increase the rate of biodesulfurization by improving the transfer rate of DBT via emplying γ-Al₂O₃ nanosorbents. The nanosorbents have the ability to adsorb DBT from the oil phase at higher rate than that of the biodesulfurization. Thus, DBT could be quickly transferred to the biocatalyst surface where nanosorbent particles were located (Guobin et al., 2005). For preparation of stable biocatalyst which could be repeatedly used in BDS processes, biodesulfurizing Pseudomonas delafieldii cells were immobilized on magnetic poly vinyl alcohol (PVA). Cell immobilization on magnetic PVA is one of the most promising techniques for increasing the efficiency of biodesulfurizing cells and decreasing the cost of operations (Guobin et al., 2004). Chang et al. (2000) reported the immobilization of Gordona sp. CYKS1 and Nocardia sp. CYKS2 in celite for desulfurization of light oil. Guerinik and AI-Mutawah (2003) presented data showing enhanced BDS by promoting the oxidoreductase activity via the preferential utilization of carbon sources. The same authors discussed the effect of DBT concentration and its bioavailability from different solvents on the BDS process.

### 2.7 Further maneuvers toward applying technologies for sulfur removal

A survey for the most common desulfurization technologies has been recently reviewed by Babich & Moulijn (2003) and Eri Ito & Rob (2006). Refiners can
reduce the sulfur content of their diesel fuel to a limited extent by switching to crude oil containing less sulfur. However, most of the wells producing low-sulfur crudes have been depleted. The availability of technologies for producing ultra-low-sulfur diesel fuel (ULSD) is one of the issues that gains priority concern in many refineries. Gasoline, diesel and non-transportation fuels account for 75-80% of the total refinery products. Most of the desulfurization processes are therefore dealing with the streams forming these end products. Accordingly, the following paragraphs will highlight the most common techniques employed for desulfurization of crude oil and refinery streams such as gasoline and diesel.

### 2.7.1 Hydrodesulfurization (HDS)

Hydrodesulfurization (HDS) is a catalytic chemical process carried out with the aim of removing sulfur compounds from the refined petroleum products. Hydrosulfurization technology has been used since 1933 by which the organosulfur compounds are removed to lower levels by the use of metal catalyst (Speight, 2006). The objective of removing the sulfur as has been mentioned earlier, is to reduce the SO₂ emissions associated with the use of these fuels in all means of transportation, power stations, domestic as well as industrial furnaces. However, another main reason for the removal of sulfur in particular from the naptha fraction within a petroleum refinery, is the fact that the platinum/rhenium bimetallic catalyst used in the reforming of the naptha fraction, with the purpose of raising its octane number is very sensitive and vulnerable to poisoning even at extremely low concentration of sulfur in the feed. The reduction in the sulfur content also reduces the extent of corrosion in the refining process and prolongs the life of the catalytic converter used in cars and some other means of transport.

In the hydrosulfurization process the sulfur is converted to hydrogen sulphide which is usually separated and converted to elemental sulfur that can be used in the synthesis of sulfuric acid.

The catalysts used for hydrosulfurization process is molybdenum sulfide catalysts containing cobalt supported on the high surface alumina support. The
support allows a high dispersion of the active material, hence optimize the use and activity of the expensive catalyst. The catalyst bed is kept at a temperature of 300 ± 44°C and the pressure used depends on the fraction used in the feed. For low and middle boiling point fractions hydrogen pressure in the range of 0.7 to 5 MPa is used, while for the higher boiling point fraction, with higher sulfur content more severe operating conditions are used. Hydrodesulfurization involves the catalytic reaction of hydrogen to convert the various sulfur compounds to hydrogen sulfide, at high pressure ranging from 5 up to 10 MPa and temperatures between 300 and 350°C, depending on the oil fraction and the required level of desulfurization (Gary and Handwerk, 1994). Current designs for desulfurization plants carry out the desulfurization in two successive hydrogenation stages where increased purity and concentration of hydrogen are used in the catalysis. The HDS of DBT proceeds via the path of minimal H2 consumption, where the hydrogenation of biphenyl and cyclohexylbenzene (CHB) proceeds slowly. The rate of DBT hydrogenation increases at higher H2S concentrations at the expense of hydrogenolysis. Furthermore, the CHB concentrations depend on the catalyst type applied as shown in Figure 2.9 (Marcelis, 2002).

![Figure 2.9 Proposed reaction mechanism for DBT hydrodesulfurization (Catalyst, Houalla et al., 1980).](image)
There is a wide spectrum of sulfur containing compounds in the fractions that are subjected to hydroprocessing treatment whose reactivity is different in such processes. Reactivity of organosulfur compound varies widely depending on their structure and local sulfur atom environment. Low boiling point oil fractions containing mainly aliphatic organosulfur compounds, such as mercaptans and sulfides, are very reactive and react to form hydrogen sulphide ($\text{H}_2\text{S}$) and hydrocarbons. The sulfur content can be completely removed in such reactions. On the other hand the higher boiling point crude oil fractions such as heavy straight run naptha and straight run diesel, the organosulfur compounds contain thiols and thiophenes, benzothiophenes and their alkylated derivatives.

Thiophene containing compounds are less reactive than mercaptans and sulfides and hence more difficult to convert via hydroprocessing treatment. Thiophene, benzothiophene, dibenzothiophene, other condensed-ring thiophenes, and substituted forms of these compounds are particularly difficult to remove by hydrodesulfurization (Kabe et al., 1992). Heavy part of crude oil mainly consist of aliphatic and aromatic of Sulfur compounds. The high cost, production of hydrogen sulfides, ineffective removing of organosulfur compounds in many crude classes and energy intensive processes requirement (high temperature and pressure) (Monticello & Finnerty, 1985), stimulated researchers to look for a new alternatives or complementary technology to produce ultra-low sulfur fuels.

The reactivity of sulfur compounds in HDS follows this order (from most to least reactive): thiophene > alkylated thiophene > BT > alkylated BT > DBT and alkylated DBT without substituents at the 4 and 6 positions > alkylated DBT with one substituent at either the 4 or 6 position > alkylated DBT with alkyl substituents at the 4 and 6 positions (Ma et al., 1994; Gates & Topsoe, 1997).
2.7.2 Oxidative desulfurization

In contrast to the hydrodesulfurization method where sulfur compounds are reduced to form hydrogen sulphide, this method oxidize the sulfur species to their corresponding sulfoxides (1-oxides) and sulfones (1,1-dioxides).

Figure 2.10 illustrates the desulfurization of dimethyl dibenzyl thiophene to its sulfone form (Frank & Han, 2003).

![4,6-DMDBT, Sulfoxide, Sulfone]

When compared to the conventional hydroprocessing, oxidative desulfurization provides many advantages, as it can be carried out using mild conditions of temperature and pressure and it does not require the use the expensive hydrogen. On the hand however, the process requires large amounts of oxidizing agent and involves separation procedure to recover the catalysts. The reaction also suffers from low selectivity and activity, hence long reaction times are needed.

### 2.7.3 Adsorption process

This technique uses adsorbing agents (S-Zorb as they are called) having an affinity to adsorb sulfur containing compounds. The adsorption could be of destructive type or what also could be termed as reactive adsorption (Babich & Moulijn, 2003), in which the adsorbed sulfur containing compound is converted to a hydrocarbon after the stripping of its sulfur which is left adsorbed on the adsorbing agent. On the other hand adsorption could be of non-destructive type (physical adsorption) i.e one in which the chemical identity of the adsorbed sulfur containing species is preserved. In both cases the adsorbing agent could be discarded or regenerated as convenient.

### 2.7.4 Reactive adsorption

’S Zorb’ was reported by Phillips Petroleum (ConocoPhillips, 2004). The adsorption process was carried out on the fluid fuel in the presence of hydrogen. An example on the use of this S-Zorb was given in which the benzothiophene is converted to ethyl benzene with the sulfur species left adsorbed on the sorbent as shown below.
Transition metals supported on basic oxides can form an ideal adsorption system for this purpose (Babich & Moulijn, 2003). Ni on ZnO is the prototypical formulation most often found in literature and patents. In this formulation Ni functions as the hydrodesulfurization site, while ZnO has the crucial role of scavenging the H₂S produced and converting it into zinc sulfide ZnS in the process Tawara et al. (2001). When ZnO is depleted through that conversion, it can be discarded or regenerated.

The potential of using the S-Zorb technique in the removal of sulfur from diesel feeds has been demonstrated by Slater et al. (2002). According to Kim et al. (2006), the efficient removal of the sulfur in this method has no effect on the octane number which is usually preserved in the process.

### 2.7.5 Physical adsorption

In this method the chemical identity of the sulfur containing species in diesel and gasoline are kept since the molecules are adsorbed intact (Tucker et al., 2003). Ni-Al₂O₃ and active carbon have been reported as sorbents for removing mercaptans, sulphides and thiophene under mild conditions (Salem, 1994). Using this system, the adsorption strength for various thiophene becomes like 4,6-DMDBT > DBT > BT > 2-methyl thiophene" > thiophene, which is interestingly the reverse of their HDS propensities.

Although at a first glance this method looks interesting and easy, however, in practice this is the case. This is mainly due to the low sulfur uptake capacity of the sorbent material, when considering the percentage of sulfur in the oil fractions of interest. Another important drawback of this adsorption method is
the presence of competitive adsorption of aromatics and olefins, which leads to a reduction in the sulfur uptake by the adsorbing material (Hernandez-Maldonado & Yang, 2004; Hernandez-Maldonado & Yang, 2003). The use of pre-adsorbent for removing aromatics and some of the larger sulfur containing molecules, has been suggested as one way of improving the sulfur uptake capacity of the adsorbing material used.

2.8 Latest applied biodesulfurizer

New strain named *Rhodococcus erythropolis* SHT87 has been isolated from oil-contaminated soils in Iran. Specific desulfurization activity of strain SHT87 resting cells in aqueous and biphasic organic–aqueous systems at 30 °C was determined to be 0.36 and 0.47 mmol 2-HBP min⁻¹ (g dry cell)⁻¹, respectively, (Davoodi-Dehaghani, Vosoughi et al. 2010).

Genetically modified microorganism Pseudomonas putida CECT 5279 can act as a desulfurizing biocatalyst. It has the ability of 4S pathway from *Rhodococcus erythropolis* IGTS8, it has been proved that a combined cells of age (23h) were (DszA and DszC) monooxygenase enzyme are active and (5h) were (DszB) desulfinase enzyme is active yields much more desulfurizing ability (Calzada J et al. 2009).

Sumedha Bhatia and Sharma D.K. have reported that mesophilic bacterium, characterized as *Pantoea agglomerans* D23W3 could degrade 93% of the 100ppm DBT within 24 h of culture. It could also desulfurize 4,6-dimethyl DBT and BT (Bhatia and Sharma, 2010).

Yu-Guang Li et al., (2009) was able to immobilize *Rhodococcus erythropolis* LSSE8-1 cells by adding the magnetic fluids to the culture broth, were immobilized by adsorption, 530 g DCW/g particles of cell mass was adsorbed.
2.9 Cell immobilization

Several trials had been made in the last decade to immobilize the bacterial cells in order to develop a durable and instant biocatalyst. Immobilization was tried on several materials, usually in gel entrapment such as Alginate, calcium alginate gels, Polyacrylamide gel, and gelatin, or by using porous materials such as reticulate foam matrices and PolyHIPE. Various types of cells have been immobilized: bacteria, fungi, yeasts, plant tissues, and mammalian tissues. Although, not many trials have success.

There are major problems facing this technique that can limit its applications. Mass transfer resistance is one of the problems that emerged during process of substrate diffusion to the active site of the immobilised cells and elimination of toxic or inhibitory metabolic end product from the medium or designated bioreactor. Another imposed problem to the immobilized cells is the oxygen transfer, it is a rate limiting factor in multilayers or mass immobilized cells were oxygen needs to reach a deep layer of the immobilized cells. Pore diameter and cross-linking method are important factors in the immobilization efficiency (Gao et al., 2010).

Bacteria and lower microorganisms like yeasts and fungi can be easily immobilized by various methods: porous ceramics, entrapment, ion exchange adsorption (Alejandro et al., 2010).

Plant cells can be immobilized by physical adsorption, and entrapment in synthetic and natural polymer matrices.

A well-established process for preparing microporous polyHIPE polymers is described in WO-A-00/34454. (Akay, Galip).

Various methods are used for immobilization including covalent coupling, adsorption, entrapment in a three-dimensional scaffold, confinement in a liquid-liquid emulsion and entrapment within a semi permeable membrane. Bioreactors with immobilized cells and enzymes have several advantages over bioreactors with free cells and free enzymes.
2.10 Advantages and disadvantages of desulfurisation processes

The major and most known techniques in desulfurizing the oil and oil products listed in Table 2.6 below,

<table>
<thead>
<tr>
<th>Process/Reference</th>
<th>Condition used</th>
<th>End product</th>
<th>Advantage</th>
<th>Disadvantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrodesulfurisation (Shafi and Hutching, 2000)</td>
<td>Use of molecular hydrogen and high temperature (200-450 C) and pressure (150-200 psig)</td>
<td>Biphenyl and H₂S</td>
<td>Most Efficient</td>
<td>Needs high temperature and pressure with hydrogen</td>
</tr>
<tr>
<td>Photochemical (Bobinger et al, 1999) (Shiraishi et al, 1999)</td>
<td>Methanol, water (1:2), sunlight simulation (300W), Acteonitrile.</td>
<td>DBT Sulfoxide, 2-sulfobenzoic acid,</td>
<td>Mild operation temperature</td>
<td>Small laboratory scale</td>
</tr>
</tbody>
</table>
2.11 Transcriptomics

The study of the RNA transcripts of a cell, tissue, or organism (i.e. the transcriptome). Transcriptomics is concerned with determining how the transcriptome, and hence pattern of gene expression, changes with respect to various factors, such as type of tissue, stage of development, hormones, drugs, or disease. It complements and overlaps with proteomics. The complete set of RNA transcripts produced by the genome at any one time is the transcriptome.

Whole genome arrays produce splendid information about genetic and expression variation occurred in the cell during a particular tested time under designed circumstances intended to be tested for their effect. The most common integrative approach for measuring gene expression is microarray analysis (Schuler et al., 2011). The responsive genes can be grouped according to functional categories or biosynthetic pathways (Nikiforova et al., 2003).

Various study for detecting transcript level variation, splicing variation specific expression were performed in Arabidopsis thaliana cell suspension. (Zhang et al., 2008)
Schuler et al., (2011) generated transcriptome data of root and leaf responses to Fe deficiency and the Arabidopsis metal homeostasis mutant nas4x-1, they proved that GeneTrail offers a flexible and user-adapted way to identify functional categories in large-scale plant transcriptome data sets. The distinguished feature that allowed analysis of individually assembled functional categories facilitated the study of the *Arabidopsis thaliana* transcriptome.

The full strength of microarray interpretation lies in the possibility of extracting information beyond the single gene level to address questions on the co-regulation of genes, on the identification of gene networks and entire extensive pathways of genes acting in the same physiological process. Specialized software tools like Genevestigator (Zimmermann et al., 2004), the Botany Array Resource (BAR) (Toufighi et al., 2005), MapMan(Thimm et al., 2004), ATTED-II (Obayashi et al., 2007; Obayashi et al., 2009) or VirtualPlant (Katari et al., 2009), for example have been developed to answer such complex questions in plants. The analysis software tool GeneTrail (Backes et al., 2007) can be used for comparative analysis of transcriptome data to identify functional clusters or pathways rather than single genes that are affected in one experimental condition compared to another. This user-friendly and freely available tool covers analysis of a wide spectrum of available biological categories assembled from information of the Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Ontology (GO), TRANSPATH pathways and transcription factors from TRANSFAC.

The effect of single feature polymorphisms (SFPs) (Borevitz et al., 2003) can be significant when the analyzed unit is interrogated by only a small number of probes, or when the locus has a high level of genetic variation (Borevitz et al., 2007)

Microarrays provide a comprehensive platform for the study of natural transcriptome variation between closely related genomes. Gene expression arrays and exon arrays, on which each annotated gene or exon is interrogated
by approximately the same number of probes, have been widely used in gene expression studies (Zhang et al., 2008)

Nikiforova et al., (2003) examined the sulfur depletion in *Arabidopsis thaliana* using the transcriptome analysis, he indicated that 632 genes responded specifically to sulfur deficiency by significant over-expression.
CHAPTER THREE

3. Materials and Methods

The routine procedures used in this work are presented in this chapter in four main sections:

- Analytical procedures including equipment, calibration and standardization used frequently in this work.
- Materials and methods used for the maintenance and cultivation of bacterial cell cultures and the relevant experimental procedures used in order to monitor and their biodesulfurization activity.
- Materials and methods used for the initiation and cultivation of plant cell cultures and the relevant procedures used in order to monitor their biodesulfurization activity.
- Transcriptomics analysis of plant cell cultures

The detailed procedures for specific experiments that were not routinely performed will be given in the relevant sections of the results and discussion chapter in order to clarify the interpretation and discussion of the results.

3.1 Analyses

This part includes the analytical instruments and procedures as well as the preparation of chemicals that used as standards, reactants, or an end products of the biodesulfurization pathway which were needed for the calibration.

3.1.1 GC-FID analysis

Gas chromatograph equipped with a flame ionization detector (FID) (HP-6890 GC / FID) was used for the analysis of DBT and 2-HBP in the samples. The filtrate was J&W Scientific DB-5MS and the column was fused silica capillary column (30 meter long) (250μm x 0.10μm nominal). The equipment was supplemented with Helium carrier gas at 1.5 ml / min of flow, hydrogen at 50 ml / min, and air at 450 ml / min with helium make up flow at 30 ml / min. The GC / FID oven was programmed from 80° C to 250° C with initial hold time of 4 min
and ramp rate at 8°C / min and final hold at 250°C for 1.0 min. The sample volume injected was 1.0 μl. The samples were analysed twice and from the analysis the amount of DBT in the sample could be estimated.

3.1.2 High Performance Liquid Chromatography (HPLC)
High Performance Liquid Chromatography (HPLC) equipment was Varian (USA). PL Hi-Plex Ca column (300 x 7.7 mm) (Polymer labs) with a disposable guard cartridge (Polymer labs) was used for sugar analyses. Mobile phase was HPLC grade water and it was passed through the column at a flow rate of 0.4 ml / min. The analyser was equipped with Evaporative Light Scattering Detector (ELSD, Polymer Laboratories, UK) and computerised integrator (Prostar / Dynamax System).

3.1.3 Spectrophotometer
SHIMADZU UV-VIS spectrophotometer UV mini 1240 was used for optical density measurement for the growth of bacterial cells and for the phenol reaction in Gibb’s assay (section 3/1/6) in order to determine the 2-HBP concentration.

3.1.4 FEI Quanta 200 ESEM
The Quanta 200 ESEM is a special type of high performance scanning electron microscope (SEM). The instrument is called ESEM (Environmental SEM) because it can be used in high vacuum mode (HV) and low low-vacuum mode (LV). It was used to in the visualisation of the bacterial cells immobilised in the coke particles (section 4.4).

Instrument data are:

Spatial Resolution 30 kV: 2.0 nm, 15 kV: 2.1 nm, 8 kV: 2.5 nm, 3 kV: 3.5 nm, 1 kV: 5 nm, 500 V: 7 nm (at 7 mm).
Operating voltage: 0.5-30kV, Gun: Field emission gun (FEG).

Equipped with: Oxford Inca 300 EDX system, Peltier stage (-20°C - +100°C).

3.1.5 Filtration Method
25 mm GD/X sterile disposable filter device used to filter the plant cell suspension solutions in the determination of wet and dry weights of the cells in the samples. The filter device had a Whitman filter paper with polypropylene housing and the pore size of 0.2 µm.

3.1.6 Detection of 2-HBP by Gibb’s assay
Gibb’s assay was used to detect 2-HBP (section 3.1.8) produced from DBT-biodesulfurizing bacteria that exhibit the 4S pathway (Rambosek et al., 1995). 1.0 ml of the bacterial culture was withdrawn and centrifuged at 10,000 rpm for 5 min. The supernatant was transferred to a clean Eppendorf tube and the pH was adjusted to pH 8.0 using 1M NaHCO3 (pH 8.0) (Konishi et al., 1997) or by adding 3 µl NaOH (4M). After adjusting the pH, 300 µl of the supernatant were transferred to a microtiter plate and 3 µl of Gibb’s reagent was added to the culture supernatant (1:100 v/v). The development of blue color was observed within 30 min of incubation under room temperature (Rambosek et al., 1995).

3.1.7 Calibration curves for model sulfur compounds and metabolic end products
In order to calculate the amount of sulfur consumed by designated biocatalyst, calibration curves were obtained for sulfur compounds using GC/ FID, or Spectrophotometer after preparing known concentrations of the relevant standard solutions.
3.1.8 Calibration curve for 2HBP using spectrophotometer in Gibb’s assay

Gibb's reagent (2, 6-dichloro-p-benzoquinone 4- chloroimine), is used for the detection of phenol derivatives. The reagent adds to the para position of the phenol ring to give an indophenol. Indophenols are brightly colored and undergo a dramatic colour change with pH due to the ionization of the phenolic proton. Remarkably, many phenol derivatives bearing substituents at the para position react readily with Gibb's reagent. The intensity of the colour produced due to the reaction is measured using optical density and is taken as a measure of the concentration of phenol derivative.

In this assay, (2,6-dichloroquinone-4-choroimide) reacts with 2-hydroxybiphenyl, forming 2,6-dichlorobenzenoneindophenol the salt of which gives an intense blue color to the solution which can be measured at 610nm (Gibbs, 1926).

The quantity of the accumulated 2-HBP or 2-(2-hydroxyphenyl)ethan-1-al (the end products of the biodesulfurization of DBT and BT, respectively) in cell-free supernatants was determined from the standard curves of these compounds with Gibb's reagent.

10 mg of Gibb’s reagent (2,6-dichloroquinone-4-chloroimide) was dissolved in 10 ml ethanol to prepare Gibb’s solution.

10 µl of Gibb’s reagent was added to 1 ml of 2-HBP solution and the pH adjusted to 8.0 by adding 3 µl of 4M NaOH, the mixture was kept at room temperature for 30 min. The intensity of the blue colour (due to the reaction of phenolic end products with Gibb's reagent) was measured at 610 nm.

Spectrophotometer was used as a quick method to determine the quantity of 2-HBP produced by biodesulfurizing bacteria. Every mol of DBT will give one mol of 2-HBP as a result of 4S pathway of biodesulfurization. Therefore, knowing the concentration of 2-HBP will give the consumption of DBT from the medium.

Standard concentrations (mM) of DBT dissolved in ethanol were prepared as in Table 3.1 knowing that the molecular weight of 2-HBP is 170.21 g/mol, and 1.0
mM of DBT is equal to 170 ppm 2-HBP, so that 0.588 Mm of 2-HBP is equal to 100 ppm 2-HBP.

The concentration of 2-HBP in (mM) was plotted against the optical density reading obtained by the spectrophotometer in Figure 3.1 to create a calibration curve. This was used in the to calculate the unknown concentration of 2-HBP in the samples from the bacterial cultures.

Table 3.1 Concentration of DBT standards dissolved in absolute ethanol used in the calibration curve of 2-HBP.

<table>
<thead>
<tr>
<th>Concentration of 2-HBP (mM)</th>
<th>Optical density (O.D.)_{610nm}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>0.15</td>
</tr>
<tr>
<td>0.05</td>
<td>0.2</td>
</tr>
<tr>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>0.2</td>
<td>0.6</td>
</tr>
<tr>
<td>0.4</td>
<td>0.9</td>
</tr>
<tr>
<td>0.6</td>
<td>1.2</td>
</tr>
<tr>
<td>0.8</td>
<td>1.65</td>
</tr>
<tr>
<td>1</td>
<td>2.1</td>
</tr>
<tr>
<td>1.2</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Figure 3.1 Calibration for 2-HBP.
3.1.9 Calibration for DBT in ethanol

The DBT was purchased from Sigma. Due to the insolubility of DBT in water, an organic solvent was chosen according to its solubility. The organic solvent chosen was absolute ethanol because DBT was soluble in ethanol at a ratio of 1:100. In this, the DBT was prepared at a concentration of 10000ppm in absolute ethanol. Then the solution was sterilised using syringe filtration and then it was used as the stock solution for the preparation of standards. The stock solution was diluted to 5000ppm, 2000ppm, 1000ppm, 500ppm, 100ppm and 10ppm in absolute ethanol. The serial dilution was used as a standard concentration to obtain a calibration plot using GC/ FID as shown in Figure 3.2 and Figure 3.3. The relevant chromatogram is given in the appendix.

![Calibration plot of DBT at retention time of 17.5 min](image)

Figure 3.2 Calibration plot of DBT shows plot of concentration verses area for DBT dissolved in ethanol. Retention time is 17.5 minutes in GC/ FID analyser.

3.1.10 Calibration for DBT in crude oil

Two calibration plots are obtained, first one by spiking the crude oil directly with the specific amount of solid DBT and the second calibration by spiking the crude oil with specific amount of DBT dissolved in ethanol.
Crude oil sample used in the calibration process was obtained from Stanlow Oil Refinery and was known to contain total sulfur of (2200 ppm).

100 ml of crude oil was spiked with 10.0 ml of the standard solution of DBT dissolved in ethanol in a concentration of 10,000 ppm, this gives the concentration of 1000 ppm DBT in the crude oil sample. Serial concentrations of crude oil spiked with DBT dissolved in ethanol were prepared in the same manner to have a concentration range from 5000 to 50 ppm in order to be tested in GC/FID. The calibration plot is given in Figure 3.2. The relevant chromatogram is given in the appendix.

![Calibration of DBT](image)

Figure 3.3 Calibration plot of DBT as concentration versus area for DBT in crude oil. Retention time is 17.2 minutes in GC/FID analyser.

### 3.1.11 Calibration for 4,6 DEDBT, 4MDBT, and 4,6DMDBT

4,6 DEDBT, 4MDBT and 4,6DMDBT are other main sulfur compounds found in crude oil. In three labelled and cleaned glass containers, standard solutions of 4,6 DEDBT, 4MDBT, and 4,6DMDBT were prepared separately by dissolving 5.0 mg in 10.0 ml absolute ethanol of each compound to attain a concentration of 500 ppm. Furthermore, serial dilutions from each container were made using
absolute ethanol to have concentrations of 400, 300, 200, and 100 ppm of each compound. These samples were then analysed in GC/ FID under the same conditions mentioned earlier. The calibration plots are given in Figure 3.4

The GC/ FID analyser with the standards gives the retention times of 17.2, and 17.5 minutes for the DBT Figure 3.2 and Figure 3.3 in aqueous and oil phase correspondingly, and 21.9 min. for 4,6 DEDBT, 18.8 min. for 4 MDBT, and 19.8 min. for 4,6 DMDBT. Plotting the data in Excel application provides a straight line equation and the retention time coefficient that used to calculate the concentration of the DBT in the samples analysed after.

The GC/ FID chromatograph of the three sulfur compounds 4,6 DEDBT, 4 MDBT, and 4,6 DMDBT are shown in Figure 3.5, Figure 3.6, and Figure 3.7, respectively.
4,6DEDBT Calibration

\[ y = 0.6188x - 4.52 \]
\[ R^2 = 0.998 \]

4MDBT Calibration

\[ y = 0.227x - 4.3 \]
\[ R^2 = 0.9989 \]

4,6DMDBT Calibration

\[ y = 0.2006x + 0.16 \]
\[ R^2 = 0.9955 \]
Figure 3.4 Three calibration plots for 4,6 DEDBT, 4 MDBT, and 4,6 DMDBT. Retention times are 21.9 min. for 4,6 DEDBT, 18.8 min. for 4 MDBT, and 19.8 min. for 4,6 DMDBT in the GC/ FID analyser.

Figure 3.5 GC/ FID chromatogram shows the retention time for 4,6 DEDBT is 21.9 min.
3.1.12 Calibration of glucose, sucrose, and fructose concentrations using HPLC analyser

Standard solutions of glucose, sucrose, and fructose were prepared separately in three different labelled clean containers at concentrations of 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10, and 20 g L\(^{-1}\) to be used with the HPLC analyser. Calibration plots for glucose, fructose and sucrose were obtained using the standard solutions for each running from 1.0 – 10 g/L with increments of 1.0 g / L and also at 20 g / L. The concentration of the standards was plotted against the area obtained from the analyses and the trend line was drawn.

These calibrations shown in Figure 3.8 were then used calculate the sugar concentrations in the samples.
Figure 3.8 Calibration for glucose, fructose, and sucrose concentrations using HPLC.
3.2 Bacterial cultures

3.2.1 Chemicals and Biochemicals
All chemical and biochemical compounds were of analytical grade. All chemicals, biochemicals, and culture media were purchased from Sigma Aldrich (UK).

3.2.2 Bacterial strains
The bacterial strains used in this study were reference bacterial strains harbouring the biodesulfurization activity Rhodococcus erythropolis ITGS8 (ATTC 53968), currently renamed as R. erythropolis IGTS8-5 and R. erythropolis IGTS8-5G. These strains were kindly provided by Professor J. Kilbane of Illinois Institute of Technology, USA.

3.2.3 Culture media

3.2.3.1 Complete Media
Luria-Bertani (LB) agar and broth media (Miller, 1972) was prepared and sterilized according to the instruction of suppliers.

3.2.3.2 Chemically defined media (CDM)
Sulfur-free CDM was prepared as shown in Table 3.2, Gilbert et al. (1998):

<table>
<thead>
<tr>
<th>Table 3.2 composition of CDM</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-phosphate buffer (1M, 20x fold, PH 7.2)</td>
</tr>
<tr>
<td>K₂HPO₄</td>
</tr>
<tr>
<td>K₂H₂PO₄</td>
</tr>
<tr>
<td>Chemical Formula</td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>NH₄Cl (1M, 1000 x fold)</td>
</tr>
<tr>
<td>MgCl₂·6H₂O (1M, 1000 x fold)</td>
</tr>
<tr>
<td>CaCl₂·2H₂O (0.3 M, 1000 x fold)</td>
</tr>
<tr>
<td>S-free trace element (1000 x fold) (Van Hamme et al, 2000)</td>
</tr>
<tr>
<td>FeCl₂·4H₂O</td>
</tr>
<tr>
<td>MgCl₂</td>
</tr>
<tr>
<td>MnCl₂·4H₂O</td>
</tr>
<tr>
<td>CuCl₂</td>
</tr>
<tr>
<td>CoCl₂·6H₂O</td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
</tr>
<tr>
<td>NiCl₂·6H₂O</td>
</tr>
<tr>
<td>H₃BO₃</td>
</tr>
<tr>
<td>Vitamins (1000x fold) (Pfennig 1978):</td>
</tr>
<tr>
<td>Cyanocobalamin (B₁₂)</td>
</tr>
<tr>
<td>Pyridoxamin –HCl (B₆)</td>
</tr>
<tr>
<td>Thiamin –HCl (B₁)</td>
</tr>
<tr>
<td>Nicotinic acid</td>
</tr>
<tr>
<td>P- Aminobenzoic acid</td>
</tr>
<tr>
<td>Biotin</td>
</tr>
<tr>
<td>Ca-Panthothenate</td>
</tr>
<tr>
<td>Glucose solution (2 M)</td>
</tr>
</tbody>
</table>
3.2.3.3 Preparation of chemically defined medium (CDM)

The used CDM (50 mM phosphate buffer) contained per 1 L the following: 50 ml phosphate buffer (1M), 10 ml NH₄Cl (1M), 1.0 ml CaCl₂ .2H₂O (0.3 M), 1.0 ml MgCl₂ 6.H₂O (1M), 1.0 ml trace elements, 1.0 ml vitamins solution, 10.0 ml glucose, 10.0 ml DBT (to give final concentration of 100 ppm) and completed to 1L using sterile distilled H₂O. In some experiments, the glucose was replaced by 10.0 ml of 30% glycerol. The pH of the medium was adjusted to 7.2.

The stock solutions of phosphate buffer, NH₄Cl, MgCl₂, and CaCl₂ were separately prepared and sterilized by autoclaving. Trace elements, vitamins, and glucose solutions were sterilized by filtration using 0.24 μm (Nalgene) and kept protected from light. All the stock solutions mentioned above were prepared from sulfur-free pure chemicals.

3.2.4 Model sulfur compounds

Dibenzothiophene (DBT) stock solution was prepared by dissolving 1.0 g of DBT in 100 ml ethanol; the concentration acquired is 10,000 parts per million (ppm).

4,6 diethyl dibenzothiophene (4,6 DEDBT) stock solution was prepared by dissolving 1.0 g in 100 ml ethanol giving the concentration of 10,000 ppm.

4,6 dimethyl dibenzothiophene (4,6 DMDBT) stock solution was prepared by dissolving 1.0 g in 100 ml ethanol giving the concentration of 10,000 ppm.

4 Methyl dibenzothiophene (4MDBT) stock solution was prepared by dissolving 1.0 g in 100 ml ethanol giving the concentration of 10,000 ppm.
3.3 Cultivation of bacterial cultures

3.3.1 Inoculum preparation

Inocula of the tested bacterial strains *R. erythropolis* IGTS8-5 and *R. erythropolis* IGTS8-5G were prepared by taking a loop-full of stock cultures previously maintained on a slant of LB medium and placed in to a flask containing CDM (30 ml/ 100 ml flask) with glucose (10.0 g/L) as the sole C source and (100 ppm) DBT as the sole S source and incubating the flasks at 30° C for 24 h. This inoculum was used to inoculate the flasks used for the next set of experiments with these bacteria.

3.3.2 Growth and maintenance of bacterial cells

For the cultivation of strain IGTS8-5 and IGTS8-5G, which utilizes glucose as the carbon source, 2.0 L flask containing 500 ml of CDM supplemented with 0.6 mM DBT(100 ppm) and 10 g / L glucose were incubated at 30° C with shaking (200 rpm) for 120 hours. During the course of cultivation, aliquots of the culture were collected every 10 hours for measurements of cell growth by turbidimetric assay (O.D.) at 600 nm.

The growth of the bacterial strains was followed at 600 nm and by measuring the mass of dry cells present in 20 ml of the withdrawn culture sample.

3.4 Bacterial biodesulfurization

3.4.1 Biodesulfurisation using freely suspended cells

In order to keep the plasmid that confers biodesulfurisation ability, *R. erythropolis* strains were maintained on sulfur-free chemically defined medium supplemented with model sulfur compounds such as DBT.

Flasks containing CDM (30 ml/100 ml flask) with two different concentrations of DBT (100 ppm and 200 ppm) as the sole S-source and 10 g/ L glucose as the sole carbon source were prepared. These flasks were inoculated (1%v/v
inoculum) from inocula cultures growing with glucose and DBT and incubated at 30° C on an orbital shaker 200 rpm. At different time intervals, samples were withdrawn aseptically in order to measure growth at 600 nm and formation of the 2-HBP by by Gibb’s assay as an indicator of DBT concentration.

3.4.2 Immobilization of bacterial cells on coke particles

Immobilization of biocatalysts benefits the bio desulfurization process in several aspects. It reduces the cost of mass production both in terms of lower capital and operating costs, mainly due to the mild condition of operation (Hartdegen F. J. J. M 1984). Immobilization of biodesulfurizing bacteria has been an active area of research for many years.

In this study porous coke particles were used to naturally immobilize *Rhodococcus erthropolis*. Coke particles were a kind gift from Dr. M J Dempsey, department of biological sciences, Manchester Metropolitant University. Coke particles with a size of 1.0 ± 0.2 mm and pores size vary from 5 um to 0.2 mm were washed with distilled water and rinsed with 70% ethanol and washed again with distilled water before sterilizing in an autoclave at 121° C for 20 minutes. 250 g of it was then transferred aseptically to a jacketed sterilised glass column. The column was connected to another column containing the CDM, bacterial inoculum, 100 ppm DBT and 10 g/ L glucose. The whole set up was sterilised in the autoclave. Filter sterilised air was continuously bubbled in the column containing the medium inoculated by bacterial strain *R. erythropolis* IGTS8-5 to be immobilized on the coke. Immobilization was achieved by circulating the medium containing bacteria using peristaltic pump. The whole set up is shown in Figure 3.9 below. Silicone tubing was used to join the two columns in order to affect the circulation. The medium was introduced from the bottom of the column containing the coke particles in order to insure the presence of void between the coke particles (slight fluidisation), hence maximizing the area of contact between the coke particles and the circulated medium.
Temperature was maintained at 30 °C and the pump was operated at 15 rpm for the first 48 hours after which it was reduced to 4 rpm to increase the residence time of the media in the column until the end of the experiment in order to facilitate and enhance the immobilization.

![Immobilization system design](image)

Figure 3.9 Immobilization system design: feed inlet (1), air inlet (2), aeration column (3), coke column (4), ceramic air diffuser (5), peristaltic pump (6), outlet for air attached to filter (7), outlet for excess liquid attached to filter (8), flow control valve (9), fluid drainage (10).

### 3.5 Plant cell cultures

As a novel approach to biodesulfurisation, it was thought that plant cell cultures could be used. This section describes the routine procedures for the two plant cell cultures used; *Arabidopsis thaliana* (a model plant used in the genomic research) and *Armoracia rusticana* (horse radish).
3.5.1 Plant species

Callus cultures of *Armoracia rusticana* (horse radish), and *Arabidopsis thaliana* were initiated to be used in this study. Horse radish (*A. rusticana*) is a perennial plant and it belongs to Brassicaceae family. This plant cannot be propagated from seeds because of its sterility. The plant was propagated vegetative by explants form a potted plant. Suspension cultures were acquired from Professor Mavituna’s group. The *Arabidopsis thaliana* cell suspension culture was given by Noor Illi Mohamad Puad, a PhD student of Professor Mavituna.

3.5.2 Medium for *Arabidopsis thaliana*

Murashige and Skoog medium with Gamborg’s vitamins (Axelos et al. 1992) was prepared by adding 4.41 g of the ready-made medium (Sigma) to a liter of distilled water and supplementing it with 30 g/L sucrose which was the carbon and energy source. The pH was adjusted to 5.7 using (KOH). After autoclaving, 0.5 ml/L of hormones (growth regulators) stock solution was added to the medium. The hormone stock solution contained 1.0 mg/ml naphthyl acetic acid (NAA) and 0.1 mg/ml 6-benzyl aminopurine (BAP) in EtOH that was prepared by dissolving 50mg NAA and 5mg BAP in 50ml EtOH.

3.5.2.1 Growth regulators for plant cells

Growth regulators (hormones), 2, 4-Dichloro phenoxy acetic acid (2, 4 D) and 6-Benzyl Amino Purine(6, BAP) were prepared as shown in Table 3.3 and explained below in detail.

Preparation of 2, 4-Dichloro phenoxy acetic acid (2, 4 D):

0.1 mM stock solution of (2, 4 D) was prepared by adding 0.2 mg to 100 ml distilled water. To dissolve the salt crystals of 2, 4 D, a few drops of sodium hydroxide were added before adding the water. It was then sterilized by filtration and stored at 4°C. Reagents were bought from Sigma Aldrich (UK).

Preparation of 6-Benzyl Amino Purine (6, BAP):
1.0 mM stock solution was prepared by adding 2.25 mg of 6-BAP to 100 ml distilled water and the solution was stored at 4°C. Filtration method used for sterilization. Reagent was bought from Sigma Aldrich (UK).

Table 3.3 Growth regulators used for plant cell cultures.

<table>
<thead>
<tr>
<th>GROWTH REGULATORS</th>
<th>DESIRED CONCENTRATION</th>
<th>STOCK SOLUTION CONCENTRATION</th>
<th>VOLUME ADDED TO THE MEDIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kinetine</td>
<td>0.05 mg / L</td>
<td>5 mg / 10 mL</td>
<td>0.1 ml / L</td>
</tr>
<tr>
<td>2,4-D</td>
<td>0.5 mg / L</td>
<td>50 mg / 10 mL</td>
<td>0.1 ml / L</td>
</tr>
<tr>
<td>BAP</td>
<td>0.005 mg / L</td>
<td>1 mg / 10 ml</td>
<td>0.5 ml / L</td>
</tr>
<tr>
<td>NAA</td>
<td>0.05 mg / L</td>
<td>10 mg / 10 ml</td>
<td>0.5 ml / L</td>
</tr>
</tbody>
</table>

3.5.2.2 Vitamins for plant cells

Vitamins used for plant cell cultures are shown in Table 3.4 below.

Table 3.4 Vitamins for plant cells cultures.

<table>
<thead>
<tr>
<th>VITAMINS</th>
<th>DESIRED CONCENTRATION</th>
<th>STOCK SOLUTION CONCENTRATION</th>
<th>VOLUME ADDED TO THE MEDIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inositol</td>
<td>100 mg / L</td>
<td>5 g / 50 mL</td>
<td>1 mL</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>10 mg / L</td>
<td>0.5 g / 50 mL</td>
<td>1 mL</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>1 mg / L</td>
<td>0.05 g / 50 mL</td>
<td>1 mL</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>1 mg / L</td>
<td>0.05 g / 50 mL</td>
<td>1 mL</td>
</tr>
</tbody>
</table>
3.5.2.3 Preparation of Murashige-Skoog (MS) Medium

Murashige-Skoog (MS) medium were used for plant cell cultivation as shown in Table 3.5. 20 g / L sucrose was added to the medium as a carbon and energy source. 1.0 ml / L of 2, 4 D stock solution and 1.0 ml / L of 6, BAP stock solution were added to the medium for *Armoracia rusticana* cultivation as specific hormones for growth, whereas, 1.0 ml / L of kinetin stock solution and 1.0 ml / L final conc 0.5 mg/ L NAA of naphthalene acetic acid (NAA) stock solution were added to the medium as specific hormones for growth of *Arabidopsis thaliana*. pH was adjusted to 5.8 by sodium hydroxide as base and hydrochloric acid as acid as needed. Medium components shown in Table 3.5 Murashige & Skoog (1962).

To prepare petri dishes for sub culturing of callus cultures, 2.0 g / L of gellan gum were added to the medium for solidification. Medium with gellangum was heated with stirrer until homogeneous, then autoclaved at 121° C for 20 minutes. After cooling, the solution was poured into petri dishes to solidify giving about 3 mm thick solid medium.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (mg / L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium nitrate (NH₄NO₃)</td>
<td>1,650</td>
</tr>
<tr>
<td>Boric acid (H₃BO₃)</td>
<td>6.2</td>
</tr>
<tr>
<td>Calcium chloride (CaCl₂ · 2H₂O)</td>
<td>440</td>
</tr>
<tr>
<td>Cobalt chloride (CoCl₂ · 6H₂O)</td>
<td>0.025</td>
</tr>
<tr>
<td>Magnesium sulfate (MgSO₄ · 7H₂O)</td>
<td>370</td>
</tr>
<tr>
<td>Cupric sulfate (CuSO₄ · 5H₂O)</td>
<td>0.025</td>
</tr>
<tr>
<td>Potassium phosphate (KH₂PO₄)</td>
<td>170</td>
</tr>
</tbody>
</table>
### 3.5.2.4 Composition of Gamborg’s B5 medium

GB5 medium was prepared according to the compositions as shown in Table 3.6.

**Table 3.6 Gamborg’s B5 medium**

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration (mg / L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium sulfate (NH₄)₂SO₄</td>
<td>134</td>
</tr>
<tr>
<td>calcium chloride (CaCl₂·H₂O)</td>
<td>150</td>
</tr>
<tr>
<td>Magnesium sulfate (MgSO₄·7H₂O)</td>
<td>246</td>
</tr>
<tr>
<td>Potassium nitrate (KNO₃)</td>
<td>2,528</td>
</tr>
<tr>
<td>Boric Acid (H₃BO₃)</td>
<td>3.0</td>
</tr>
<tr>
<td>Colbalt chloride (CoCl₂·6H₂O)</td>
<td>0.025</td>
</tr>
<tr>
<td>Cupric Sulfate (CuSO₄·5H₂O)</td>
<td>0.025</td>
</tr>
<tr>
<td>Ferrous sulfate (FeSO₄·7H₂O)</td>
<td>27.8</td>
</tr>
</tbody>
</table>

Source: Murashige and Skoog (1962)
Manganese sulfate, monohydrate (MnSO$_4$.H$_2$O) | 10
---|---
Potassium iodine (KI) | 0.75
Sodium molybdate (Na$_2$MoO$_4$.2H$_2$O) | 0.25
Sodium phosphate (NaH$_2$PO$_4$.H$_2$O) | 150
Zinc Sulfate (ZnSO$_4$.7H$_2$O) | 2.0
Na$_2$EDTA.2H$_2$O | 37.2

Source: Gamborg, O.L et al (1976)

### 3.5.2.5 Composition of sulfur-free medium (SFM)

Sulfur-free medium was prepared using the concentrations in Table 3.7 for testing the growth of plant cells after adding DBT as a sole source of sulfur.

#### Table 3.7 Composition of sulphur free Medium (SFM)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (mg / L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium Chloride</td>
<td>108</td>
</tr>
<tr>
<td>Boric acid (H$_3$BO$_3$)</td>
<td>3</td>
</tr>
<tr>
<td>Calcium chloride (CaCl$_2$. H$_2$O)</td>
<td>150</td>
</tr>
<tr>
<td>Cobalt chloride (CoCl$_2$. 6H$_2$O)</td>
<td>0.025</td>
</tr>
<tr>
<td>MgCl$_2$. 7H$_2$O</td>
<td>219</td>
</tr>
<tr>
<td>Cupric chloride (CuCl$_2$. 5H$_2$O)</td>
<td>0.023</td>
</tr>
<tr>
<td>Potassium phosphate (KH$_2$PO$_4$)</td>
<td>170</td>
</tr>
</tbody>
</table>
3.6 Preparation and initiation of callus culture of *Armoracia rusticana* (Horse Radish)

Fresh roots of *Armoracia rusticana* was bought from a supplier from the internet and grown in a pot in order to obtain a full growing plant. After plant was established, samples of roots, leaves, and leaf stems were collected to be used as primary explants to initiate callus cultures.

In a laminar flow cabinet, *Armoracia rusticana* explants were sterilized by washing with water, then with 70% ethanol for 10 seconds, followed by another washing with 1.0% sodium hypochlorite solution for 10 minutes, finally the explants were rinsed three times with sterile distilled water for 2-3 minutes.

Under aseptic conditions sterilized leaves were cut into small pieces (1.0 x 1.0 cm) with maintaining the structural veins intact. The small pieces of leaves were cultivated on solid MS medium. The MS medium supplemented with 2g / L of gellan gum for solidification and 20 g / L sucrose as a carbon source for growth, 1.0 ml of 6, benzyl amino purine stock solution and 1.0 ml of 2,4 D-
chloro phenoxy acetic acid stock solution were added as required hormones for growth. The small cut leaves were cultivated on MS solid medium plates in a way making leaf edges come in contact with the medium surface to allow nutrient access to the plant cells. Incubation of the plates was carried out at 25°C under constantly lit fluorescent lamps (400 Lux) for 15-20 days.

### 3.6.1 Liquid suspension of *Armoracia rusticana* (horse radish) cells

#### 3.6.1.1 Liquid suspension using Gamborg’s B5 medium

GB5 medium prepared as shown in Table 3.6. It was supplemented with 20g/l of sucrose and 1ml/l (2, 4 D) hormone stock solution. Four 250 ml flasks each containing 100 ml liquid GB5 medium was used for liquid suspension of plant callus after sterilization in an autoclave for 20 minutes at 121°C. Small pieces of callus were obtained by using a sterilised glass mortar to push the callus aggregates through a sterilised stainless steel sieve before transferring them into the sterilised liquid medium in the flasks. Flasks were kept on a shaker at a rotation speed of 110 rotations per minute (rpm) and 25°C incubation temperature with continuous illumination of 400 Lux. The incubation period was 10-15 days.

Subculturing was repeated in order to obtain adequate amount of fine cell suspension.

#### 3.6.1.2 Liquid suspension using MS medium

Another method used for the preparation of liquid suspension cultures involved using MS medium to subculture *Armoracia rusticana*. The composition of basal salts is listed in MS medium composition in Table 3.5
3.6.2 Cultivation of *A. rusticana* (horse radish)

Twenty 250 ml flasks containing 50 ml of Gamborg’s B5 medium (50 ml / 250 ml flasks) supplemented with 20 g / L of sucrose and 1.0 ml / L of hormone 2, 4 Dichloro phenoxy acetic acid stock solution and 1.0 ml/ L Kinetin stock solution (May and Leaver, 1993) were inoculated each with 5.0 ml, 10 % v/v of *Armoracia rusticana* (Horse Radish) cell suspension and incubated at 25° C, at 110 rpm, under continuous illumination. Every day, one flask was sacrificed for weight measurements. The relevant results are presented in Section 4.8

3.6.3 Cultivation of *A. thaliana*

Cell suspensions acquired from a PhD student of Professor Mavituna were used in this culture and the same procedure was followed as with *Armoracia rusticana* suspensions. *A. thaliana* grows well in the MS medium supplemented with GB5 vitamins shown in Table 3.4 and supplemented by adding BAP and NAA as growth regulators as shown in Table 3.3. The relevant results are presented in Section 4.8

3.6.4 Sugar analysis for batch growth of *Armoracia rusticana* (Horse Radish) and *Arabidopsis thaliana* cells

Plant cell suspensions of *Armoracia rusticana* (Horse Radish) and *Arabidopsis thaliana* cells were sub-cultured into two separate flasks using GB5 medium. A set of two 2.0 L flasks each containing 500 ml of GB5 medium with the relevant amount of growth regulators and vitamins were inoculated with 50 ml of *Armoracia rusticana* (Horse Radish) cell suspension in the first flask and 50 ml of *Arabidopsis thaliana* cell suspension in the second flask. The carbon source used in both flasks was 20 g/ L sucrose. Flasks were incubated at 25° C with shaking 110 rpm and under continuous illumination.
At the starting time of incubation and at day 2, then every third day, 5.0 ml samples were withdrawn from each flask to analyse growth, and sucrose, fructose, and glucose levels in HPLC analyser.

### 3.6.5 Immobilization of plant cells

Reticulated polyurethane foam particles containing open pore network were used for the natural (passive) immobilisation of the plant cells.

#### 3.6.5.1 Immobilisation of *Armoracia rusticana* (Horse Radish)

*Armoracia rusticana* cell suspension was immobilized in 1x1x1 cubes of polyurethane foam matrix. The reticulated polyurethane foam cubes had 10 pores per linear inch (ppi). Cubes were immersed in 70% v/v ethanol for overnight in order to be decontaminated and dissolving out any toxic chemicals from its surface. Cubes were then washed with distilled water and then submerged in a flask containing distilled water and autoclaved at 121 °C for 20 minutes. After complete dryness of the cubes they were weighed. Cubes were strung on to a piece of L-shaped stainless steel wire and plunged in a flask containing Gamborg’s B5 medium and growth regulators and vitamins as required. The cubes lay horizontally at the bottom of the flask attached to the L-shape wire. After autoclaving, the flask containing the sterilized cubes in GB5 medium was inoculated with fine cell suspension. Immobilization took place while the flask was on a shaker with 110 rpm for 7 days. Then, every eight days the depleted medium was removed from the culture by decanting the old medium and adding the fresh medium under aseptic conditions. The medium refreshment step was repeated several times until cell immobilization was clearly noticable.
3.6.5.2 Immobilisation of *Arabidopsis thaliana*

The same procedures applied for *A. rusticana* (H.R) were also used for the immobilisation of *Arabidopsis thaliana*.

3.7 Plant biodesulfurization

In the following section, some routine details are given for the series of experiments designed to examine the effect of DBT on the plant cell suspensions and the ability of cells to tolerate the presence of crude oil in the culture media. Furthermore, the amount of DBT reduced (consumed) during the growth of plant cells was measured by GC/ FID analyser to assess the biodesulfurisation activity of the plant cells.

3.7.1 Growth of plant cells in Gamborg’s B5 medium in the presence of 100 ppm (0.6 mM) DBT

*A. rusticana* and *Arabidopsis thaliana* were subcultured in GB5 Medium in the presence of 100 ppm (0.6 mM) DBT as an extra source of sulfur essential for the plant growth. This experiment was to determine whether there is any adverse effect of DBT on the growth and proliferation of plant cell suspension.

3.7.2 Growth of plant cells in Gamborg’s B5 medium in presence of crude oil spiked with 200 ppm (1.2 mM) DBT

The previous experiment was repeated in presence of crude oil spiked with 200 ppm (1.2 mM) DBT as an extra source of sulfur essential for the plant growth. This experiment was carried out with the aim of establishing the capability of plant cells to tolerate the crude oil and whether they use the sulphur in crude oil as source of sulfur needed for growth. The oil was 20 % v/v. Every three days 1.0 ml sample was withdrawn from supernatant oil layer and centrifuged to remove any aqueous fraction and the supernatant oil layer was used in the GC-
FID for analysis of DBT, also mass variation of cells was determined by weight difference.

3.7.3 Growth of *Armoracia rusticana* in sulfur-free medium (S.F.M) with 100 ppm DBT as a sole sulfur source

Sulfur-free Gamborg’s B5 medium was prepared as described before and supplemented with 20 g/ L sucrose as carbon source and 100 ppm dibenzothiophen DBT as sole sulfur source. 150 ml of this medium was dispensed into two 500 ml Erlenmeyer flasks before sterilization by autoclaving at 121 °C for 20 minute. At 25 °C, the flask inoculated with 5.0 ml of free cell suspension of *Armoracia rusticana* and 1.0 ml / L of 2, 4, D stock solution and 1.0 ml / L of 6, BAP stock solution were added to the medium as specific hormones for culture growth. The flask was placed on a shaker 110 rpm in 25°C incubation temperature. Every third day of the incubation, 5.0 ml aliquots were withdrawn to determine the mass increase during the growth.

3.7.4 Growth of *Arabidopsis thaliana* in sulfur free medium (SFM) with 100 ppm DBT as a sole sulfur source

Sulfur free Gamborg’s B5 medium was prepared as described before and supplemented with 20 g / L sucrose as carbon source and 100 ppm dibenzothiophen DBT as sole sulfur source. 150 ml of this medium was dispensed into 500 ml Erlenmeyer flasks before it was sterilized by autoclaving at 121 °C for 20 minute and At 25 °C, the flask was inoculated with 5.0 ml of free cell suspension of *Arabidopsis thaliana* and 1.0 ml / L of kinetin stock solution and 1.0 ml / L of NAA (naphthalene acetic acid) stock solution were added to the medium as specific hormones for growth. The flask was placed on a shaker at 110 rpm at 25°C incubation temperature. Every third day of the incubation; 5.0 ml aliquots were withdrawn to determine the mass increase during the growth.
3.7.5 Plant cell suspensions consuming DBT in aqueous medium

Two cultures of the plant cells that grew in 250 ml of GB5 medium were separately harvested at exponential phase of growth (day 14) by leaving to settle for 30 minutes at room temperature, then the supernatant (depleted medium) were discarded and the cells were washed with buffer solution twice and re-suspended in 250 ml fresh sulfur-free Gamborg’s B5 medium.

Sulfur-free Gamborg’s B5 medium was prepared as described before and was supplemented with 20 g / L sucrose as carbon and energy source and 100 ppm dibenzothiophene (DBT) as the sole sulfur source. 250 ml of SFGB5 medium was dispensed into two 1.0 L Erlenmeyer flasks before sterilization by autoclaving at 121 °C for 20 minutes. The first flask was inoculated with free cell suspension of Armoracia rusticana and the second flask was inoculated with Arabidopsis thaliana cells. The specific hormones for growth and vitamins were added to the medium as required for each plant cells under aseptic conditions in a laminar flow cabinet. Flasks were then placed on the shaker at 110 rpm at 25° C incubation temperature. Samples were collected at the beginning of the experiment and every 8 hours of the start of the experiment to test the DBT level in GC/ FID analyser. The relevant results are presented in Section 4.10

3.7.6 Plant cell suspensions consuming DBT from oil

The same procedure was followed as the above experiment except for the medium used did not containing any DBT. The crude oil was spiked with DBT in two ways: in one, DBT was dissolved in ethanol first and then added to the crude oil; in the second way DBT was directly added into the crude oil without any extra solvent.

In both cases 20% v/v spiked crude oil were added to batch growth of the plant cells growing in sulfur-free Gamborg’s B5 medium.
10 g of fresh weight plant cell suspension was used and the oil volume was 10 ml added to 50 ml sulfur free medium and oil was spiked with 200 ppm DBT.

Frequent Samples withdrawn every 8 hours and centrifuged at speed 6000 rpm for 20 minutes, then the upper oily layer were tested for DBT level. Also the watery layer was tested for the DBT in GC/ FID analyser. The results are presented in Sections 4.16 and 4.17.

3.7.7 Growth of each of Horse Radish Armoracia rusticana and Arabidopsis thaliana in sulfur free (S.F) GB5 medium supplemented with 100 ppm 4,6 DEDBT or 100 ppm 4,6 DMDBT or 100 ppm 4MDBT as sole source of sulfur

The previously mentioned experiment for the growth of two plant cell suspensions supplemented with 100 ppm DBT as the sole sulfur source were repeated separately for each plant cell suspension but with three different sole sources of sulfur. In these experiments, sulfur-free GB5 medium was supplemented with 100 ppm 4,6 DEDBT or 100 ppm 4,6 DMDBT or 100 ppm 4MDBT as the sole source of sulfur. Two negative control flasks run in parallel with these flasks but did not contain any sulfur source required for the growth; the two control negative flasks had the same medium and contained all other compounds and hormones like other flasks in this experiment. The results are given in Section 4.15.

3.7.8 Growth of plant cells in Gamborg’s B5 medium and the production of DBT compound during the growth

Two 1.0 L flasks each containing 300 ml of GB5 medium were supplemented with 20 g / L of sucrose and 1.0 ml / L of hormone 2, 4 Dichloro phenoxy acetic acid. One flask was inoculated with 30 ml, 10% v/v of Armoracia rusticana (Horse Radish) cell suspension, while the other was inoculated with 30 ml, 10%
v/v *Arabidopsis thaliana*, after which both were incubated at 25 °C, at 160 rpm, under continuous illumination. Every 8 hours, 1.0 ml sample was withdrawn from each flask, and centrifuged and the supernatant was analysed by GC-FID for DBT content. The results are given in Section 4.14

### 3.7.9 Control negative experiment with dead plant cell suspension

In order to check the possibility of DBT being removed from the aqueous medium by biosorption (filtered or entrapped inside the plant cells without any metabolic activity) an experiment was designed. In this case, the plant cells of both species were killed by heating the cells in a flask containing sterile distilled water for 30 minutes at 70°C before adding the DBT solution. After the same period of contact as with the previous experiments with the living cultures, samples were taken from the medium for DBT analysis. Furthermore, plant cells were extracted first freezing them in liquid nitrogen and then by crashing the cells and re-suspending them in ethanol in order to test the presence of any DBT residue. The results are discussed in Section 4.18

### 3.8 Cell viability test

Fluorescein di-acetate (FDA) reagent was bought from (Sigma); stock solution of (FDA) was prepared by dissolving 20 mg of FDA in 10 ml acetone (BDH Chemicals) and stored in a refrigerator 2-5 °C. Widholm (1972) uses FDA to estimate viable cells in cell culture. Esterase enzyme in living cell cleaves the stain added to the cells on the examination slide; consequently gives a fluorescent yellow / green colour when using Ultra Violet UV microscope (Dixon and Gonzales, 1994). 3-7 drops of FDA stock solution stain were added to 5.0 ml distilled water as a further diluent before using this solution for determination cells’ viability. On a microscope slide few drops of diluted FDA solution were mixed gently with cells and left for 2 minutes period of time to allow proper immersion of stain into cells. Microscope slice was then covered with a glass cover slip in order to be viewed under UV fluorescent microscope (NIKON
Eclipse E600); using blue/violet filter. Living cells which can cleave the stain appears fluorescent and can easily count.

3.9 Transcriptomic analysis for plant cell suspension

The genome of *Arabidopsis* is only about 115 million base pairs long with approximately 26,000 genes on 5 chromosomes (An Sun, 2000).

In order to understand the metabolic activity of the recently studied plant cell suspensions of *Arabidopsis thaliana* from a gene expression point of view, the following experiment was designed in order to monitor the alteration and triggering of the genes during contact with DBT. The normal cell cultures without exposure to DBT served as the control.

3.9.1 Genetic effects after exposure to DBT in *Arabidopsis thaliana* cell suspension

*Arabidopsis thaliana* cell suspension inoculum was sub-cultured in MS medium supplemented with GB5 vitamins as indicated in Section 3.5.2. The inoculation volume was 10% v/v and the culture was kept under a photo-regime of 16 hours illumination 8 hours darkness at 25°C. The medium was also enhanced with growth regulators BAP and NAA as indicated.

Two 100 ml cultures of *Arabidopsis thaliana* suspensions were pooled 5 days after subculturing. The cells were allowed to settle and the turbid medium was decanted. The cells were washed by adding the same volume of medium and allowing them to settle again. Finally, cells were resuspended at four times the original density in fresh medium, 12 ml suspension aliquoted into 25ml Erlenmeyer flasks and put onto a rotary shaker for 2h until the beginning of treatment Table 3.8.

Plant cell suspensions of *Arabidopsis thaliana* were exposed to 30 ppm DBT dissolved in ethanol, the exposure times were 2 hours and 6 hours, separately. Samples were taken in triplicate for each control and time set exposure.
Triplicate control samples were also incubated with ethanol only to inspect the possible effect of ethanol on the cell suspensions and consequently distinguish it from the effect of DBT on the cells.

The concentration of the ethanol in the medium was 0.3% v/v.

Cells were harvested separately from each flask by filtration and frozen by liquid nitrogen and stored in a -70°C freezer in order to perform the RNA extraction for microarray transcriptomics analysis.

Table 3.8 Samples of *Arabidopsis thaliana* used in the RNA extraction experiment. Mock samples have ethanol only added without DBT.

<table>
<thead>
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<th>Flask number</th>
<th>Description</th>
<th>Time of incubation/ hours</th>
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<td>2h</td>
</tr>
<tr>
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<tr>
<td>12</td>
<td>30 ppm DBT</td>
<td>6h</td>
</tr>
</tbody>
</table>
3.9.2 RNA isolation

QIAGEN kit was used for the isolation of total RNA and the protocol attached in the kit was followed for this experiment.

Important points were taken into consideration before starting the Protocol 12 of RNA isolation:

Not to use too much plant tissue for each ml of Trizol.

Not to use too much RNA in the DNase treatment step.

Not to use too much RNA for the reverse transcriptase reaction.

To Use sterile MilliQ water.

Using a mortar and pestle allows tissue to be ground thoroughly.

A brief description of the procedure followed after filtration of the Arabidopsis thaliana cell suspension is given below:

Cells were frozen in liquid nitrogen and grounded to a fine powder and before the tissue thawed and 500 µl of Trizol was added. Grinding resumed for a further 30 sec and another 500 µl of Trizol was added. 1.0 ml of the grounded cells in Trizol was transferred to eppendorf tube and placed in the shaker or rotator for 15 minutes. After 15 minutes of shaking, 200 µl chloroform was added and shaken for 2 minutes followed by centrifugation at 11000 rpm for 15 minutes in 4°C centrifuge. From the aqueous phase 450-460 µl was collected and equal volume of isopropanol was added and the mixture stood for 10 minutes. After centrifugation of speed 11000 rpm for 15 minutes and temperature of 4°C, supernatant was then decanted and 700 µl 70% ethanol was added. Tube was inverted several times to wash the RNA pellet. Another centrifugation at 11000 rpm for 5 minutes in temperature of 4°C, then supernatant was decanted. Pellet was dried and resuspended in 15 µl H₂O or TE Buffer pH 8.0 (RNase free).
3.9.3 Electrophoresis Assay

Quantities of the isolated RNA were checked on gel using Affymetrix analyser adjusted as per the manufacturer’s instructions as follows:

**Electrophoresis File Run Summary**

Instrument Name: DE13804716

Firmware: C.01.069

Serial#: DE13804716

Type: G2939A

Assay Information:

Assay Origin Path: C:\Program Files (x86)\Agilent\2100 bioanalyzer\2100 expert\assays\RNA\Eukaryote Total RNA Nano Series II.xsy

Assay Class: Eukaryote Total RNA Nano

Version: 2.6

Assay Comments: Total RNA Analysis ng sensitivity (Eukaryote)

3.9.4 Electrophoresis Assay Details

**General Analysis Settings**

Number of Available Sample and Ladder Wells (Max.): 13

Minimum Visible Range [s] : 17

Maximum Visible Range [s] : 70

Start Analysis Time Range [s] : 19

End Analysis Time Range [s] : 69

Ladder Concentration [ng/μl] : 150
Lower Marker Concentration [ng/μl] : 0
Upper Marker Concentration [ng/μl] : 0
Used Lower Marker for Quantitation
Standard Curve Fit is Logarithmic
Show Data Aligned to Lower Marker

**Integrator Settings**
Integration Start Time [s] : 19
Integration End Time [s] : 69
Slope Threshold : 0.6
Height Threshold [FU] : 0.5
Area Threshold : 0.2
Width Threshold [s] : 0.5
Baseline Plateau [s] : 6

**Filter Settings**
Filter Width [s] : 0.5
Polynomial Order : 4

**Ladder**

<table>
<thead>
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<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
</tr>
</tbody>
</table>
3.9.5 GeneChip Arabidopsis ATH1 Genome Array

The genechip contained more than twenty thousand probe sets that represented approximately twenty four thousand genes. The sequences used by the manufacturer in this design were selected and also clustered in collaboration with TIGR. They were derived from TIGR's ATH1-121501 database.

Oligonucleotide probes were synthesized in situ to each corresponding sequence. Eleven pairs of oligonucleotide probes were used to measure the level of transcription of each sequence represented on the GeneChip Arabidopsis ATH1 Genome Array.

3.9.6 Hybridization

For each target, a hybridization cocktail was made using the standard array recipe as described in the GeneChip Expression Analysis Technical Manual. The cocktail was hybridized to Arabidopsis thaliana (Arab) chips by incubating the GeneChips in a rotisserie box in a 45°C oven rotating at 60rpm. Following hybridization for 16 h, the chips were loaded onto a Fluidics station for washing and staining with R-phycoerythrin conjugated to streptavidin (Molecular Probes, Inc.) using the EukGe W S2v5 program controlled using Affymetrix GCOS (V1.4) software.
3.9.7 Scanning
The chips were loaded onto the Agilent GeneChip scanner 3000 and quality control checks for control hybridizations were performed, again using Affymetrix GCOS (V1.4) software.

RNA quality was checked using the RNA 6000 Nano Assay, and analyzed on an Agilent 2100 Bioanalyser (Agilent Technologies). RNA was quantified using a Nanodrop ultra-low-volume spectrophotometer (Nanodrop Technologies).

3.9.8 Total RNA Extraction
Total RNA was extracted by using QIAGEN kit. Procedure was explained in details in Material and Methods section.

3.9.9 Quality Check for the RNA
RNA quality was checked using the RNA 6000 Nano Assay, and analyzed on an Agilent 2100 Bioanalyser (Agilent Technologies). RNA was quantified using a Nanodrop ultra-low-volume spectrophotometer (Nanodrop Technologies). Samples 1-12.

3.9.10 Biotinylation and fragmentation of cRNA
Biotin labeling of cRNA was carried out using Genechip IVT labeling kit (Affymetrix). 12 µl of cDNA was used and the resultant cRNA was purified using the GeneChip Sample Clean Up Module with a final elution volume of 19 µl in RNase free water. cRNA was quantified using the Nanodrop spectrophotometer. 15 µg cRNA was used for fragmentation. The reaction was carried out in 5X fragmentation buffer at 94°C for 35 min.
3.9.11 cDNA synthesis

Approximately 100 ng total RNA was used to synthesize cDNA. Synthesis was carried out using an Two-Cycled cDNA Synthesis Kit (Affymetrix). A GeneChip Sample Clean Up Module kit (Affymetrix/QIAGEN) was used for cDNA cleanup. The final elution step resulted in approximately 12 µl cDNA.
CHAPTER FOUR

4. Results and discussion

4.1 Introduction

Oil refining industries have to cope with more stringent specifications on the sulfur content that are driven by environmental concerns (Anabtawi et al., 1996; Konishi et al., 2007). During the last decade, clean air considerations have led to drastic reduction in the allowable sulfur content for gas and oil. These stricter regulations on sulfur content stimulated many research groups to perform more research on deep desulfurization to obtain ultra-low-sulfur fuels (ULSF).

The adverse effects accompanying burning S-containing fuels have been documented (Literature Review). The sulfur content of crude oil from different sources ranges from 0.05 to 5% w/w. In some rare cases higher contents reaching around 14% were documented. Most of the sulfur present in crude oil is organically bound sulfur, with minor portions of inorganic and elemental sulfur (Kropp & Fedorak, 1998). Approximately 80% of the organic sulfur in crude oil is estimated to be thiophenic in nature Czogalla and Boberg (1983).

The removal of the bulk of sulfur present in gas and oil (S-containing oil fractions with boiling points ranging 260-350°C) is conventionally performed by the hydrodesulfurization (HDS) process that operates between 200 and 350°C and pressure from 5-10 Mpa, depending on the desulfurization severity required. Unfortunately, HDS is not suitable to meet future specified deep desulfurization levels because many thiophene compounds escape this treatment (Marcelis, 2002).

The use of microorganisms might offer an alternative and complementary way to remove sulfur specifically from hydrocarbon fractions without altering the caloric value of fuels. In recent years the isolation of microbial isolates harboring the BDS capability for establishment of a biocatalytic process, for specific removal of S from fuel fractions was tackled by many research groups. To achieve a successful biocatalytic BDS process, the following criteria should be fulfilled: 1. working in organic phase, 2. the biocatalyst should tolerate high
concentrations of substrate, 3. the biocatalyst should have broad substrate spectrum, 4. the biocatalyst should tolerate the inhibitory effect of the end-products, 5. the biocatalyst should have high and stable turnover rates, 6. reasonable cost for mass production and recovery of biocatalyst, and 7. biocatalyst working at high temperature is preferable. To date, no one microorganism has met all these criteria. Therefore, the present study was dedicated to tackling some of these objectives using plant cell suspension to establish a biocatalytic BDS process for the petroleum and oil industry.

The experimental approach was set to test the following: 1. establishment of analytical techniques to follow up and evaluate the biodesulfurization process, 2. evaluating the biodesulfurization activity compared to a reference biodesulfurizing bacterium. 3. working in diesel and crude oil to mimic the biocatalytic process at the industrial scale, 4. testing the substrate spectrum and the preferential utilization of organic sulfur compounds encountered in diesel and crude oil, and 5. evaluating the biodesulfurization activity compared to a reference biodesulfurizing bacterium.

### 4.2 Bacterial cells growth and its consumption of glucose and DBT during the growth

Two CDM flasks (1.0 L medium / 3.0 L flask) containing glucose (10.0 g/L) as the sole C source and (100 ppm) DBT as the sole S source were inoculated with 5.0 ml of R. erthropolis IGTS8-5 and R. erythropolis IGTS8-5G respectively from the previously prepared inoculant and incubating the flasks at 30° C for 80 hours. 1.0 ml culture was withdrawn every 8 hours and the optical density was measured at 600 nm. Gibb’s assay guided biodesulfurization activity in the different collected samples was tested as recommended in Gibb’s test, DBT was tested in the GC/FID and glucose was tested in HPLC.

Both strains of IGTS8-5 and IGTS8-5G proliferated in CDM containing 10 g/ L glucose as a sole carbon source and 100 ppm DBT as sole sulfur source. Results are presented as a plot of optical density and consumption of DBT in ppm, both as a function of time Figure 4.5.
IGTS8-5 yielded a maximum of 18.1 g/ L fresh cell weight giving 3.25 g/ L dry cell weight corresponding to an optical density of 4.0 measured at 600 nm. IGTS8-5G on the other hand yielded a maximum of 16.6 g/ L fresh cell weight giving 2.98 g/ L dry cell weight, corresponding to an optical density of 3.5 measured at 600 nm.

These results show that a value of 1.0 O.D is equal to 0.8 g dry cell weight per litre (D.C.W/ L).

The two strains show very similar growth pattern with an exponential growth starting at approximately 24 hours after inoculation.

The lag phase, where the rate of growth is equal to the rate of death of the bacterial cells, commenced after 48 hours from inoculation, extending to nearly 72 hours, as shown in Figure 4.1

![Growth and DBT consumption](image)

**Figure 4.1** Plots of optical density and consumption of DBT during the growth of bacterial strains as a function of time.
Both strains show similar behaviour towards glucose and DBT consumptions. A sharp decline in the concentration of DBT see Figure 4.1 and glucose see Figure 4.2 is observed in the exponential growth regime. In this regime ~ 60% and ~ 40% of DBT and glucose were consumed respectively. The obtained result showed a rapid consumption of DBT (within 10-24 h) by both strains of IGTS8-5 and IGTS8-5G cells. However, growth and 2-HBP production were at a slow rate. Remarkably, low DBT was detected (30 ppm or less) in culture supernatants during the period of 50-80 h incubation. However, during that period the growth of both strains reached the maximum values and remained high for a long stationary phase. Although, nearly equimolar amounts of 2-HBP were produced from the consumption of DBT, the time course of substrate consumption did not match the time course of end product (2-HBP) formation. When nearly 80% of DBT was consumed after 32 h, around 50% of 2-HBP was detected see Figure 4.3. This phenomenon was reported for a growing culture of *Rhodococcus* sp. strain IGTS8, where the substrate was entirely consumed in the early stages of growth but the 2-HBP concentration did not reach the theoretic maximum until the end of the exponential growth phase (Oldfield et al., 1997). Therefore, it was concluded that the rapid disappearance of the
substrate (DBT) from the medium is most probably due to the transient adsorption of DBT to the cell envelope prior to conversion to 2-HBP. However, the presence of a regulatory mechanism that enables the cells to collect the substrate from the medium before the production of a possible inhibitory end product is a reasonable explanation.

Figure 4.3 plots of DBT consumption and 2-HBP production during the growth of bacterial strains as a function of time.

A gradual decline in both DBT and glucose was observed after the exponential growth period, reaching nearly to 10 ppm and 5 mM for DBT and glucose respectively.

In comparison, the consumption of 100 ppm DBT in 80 hours resulted in the production of 3.25 g/ L dry cell weight of IGTS8-5 strain and 2.98 g/ L dry cell weight of IGTS8-5G.

The GC/ FID chromatogram shows the consumption of DBT and clearly converted to 2-HBP as in Figure 4.4
Figure 4.4  GC/ FID chromatogram shows the reduction of DBT at 17.5 minutes showed by black solid arrow and the production of 2-HBP at 14.1 showed by red scattered arrow.
4.3 Biodesulfurization activity assessment in dense culture

Concentrated cells of strain IGTS8-5 and the IGTS8-5G were compared for their biodesulfurization of DBT as a model compound as follows. Two sets of 3 L flasks containing 1L CDM with 10 g/L glucose were supplemented with 100 ppm DBT. One set was inoculated with strain IGTS8-5 and the other with strain IGTS8-5G. The flasks were incubated at 30° C under shaking 200 rpm. The cells of both strains were harvested in exponential growth phase by centrifugation at 9000 rpm and 4° C. Each cell pellet was washed twice with phosphate Table 3.2 and concentrated to prepare dense cell suspension by resuspending in 50 ml of CDM in a 250 ml flask containing 10 g/ L glucose and 100 ppm DBT giving the cell concentration of 3.25 – 3.0 g/L as shown in Table 4.1. The dense cells were incubated at 30° C under shaking and samples were taken at Tzero, T1, T3, and T7 to analyzed by GC/ FID for concentration of 2-HBP after testing the samples with Gibb’s reagent.

The BDS activity of dense cell suspensions of strains *R. erythropolis* IGTS8-5 and IGTS8-5G using DBT as a model compound (100 ppm) was assessed by following the formation of the end-product 2HBP. The result of this experiment and the calculated BDS activity were given in Table 4.1.
Table 4.1 Amount of 2-HBP produced by dense cell suspensions of strains IGTS8 and 12a and the calculated BDS activity units (µmol 2-HBP. h⁻¹. g⁻¹ dry cell weight).

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Cell density g/L</th>
<th>(ppm) 2-HBP detected after</th>
<th>BDS activity, µmol 2-HBP. h⁻¹. g⁻¹ dry cell weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 h</td>
<td>3 h</td>
</tr>
<tr>
<td>IGTS8-5</td>
<td>3.25</td>
<td>50</td>
<td>93</td>
</tr>
<tr>
<td>IGTS8-5G</td>
<td>3.0</td>
<td>37</td>
<td>75</td>
</tr>
</tbody>
</table>

The obtained result revealed that nearly half of the added DBT was recovered as 2-HBP after 1h and the calculated BDS activity was nearly 20% higher for IGTS8-5 than for IGTS8-5G see Table 4.1. However, after 3 h and 7 h the added DBT (100 ppm) was mostly recovered 95 % as equimolar amount of 2-HBP (100 ppm) by cells of strain IGTS8-5 and only 86% of DBT was recovered as 2-HBP by cells of strain IGTS8-5G. Accordingly, the desulfurization of DBT ran at higher BDS activity during that period and accounted for 91 µmol. h⁻¹. g⁻¹ dry cell weight (DCW) for both IGTS8-5 and 73 µmol. h⁻¹. g⁻¹ DCW for IGTS8-5G cells.

The BDS activity measured for IGTS8-5 and IGTS8-5G against DBT is comparable to those values reported for other biodesulfurizing bacteria. The reported BDS activity for *R. erythropolis* IGTS8 varied considerably depending on the experimental design. Oldfield et al. (1997) reported BDS activity for DBT by *Rhodococcus* sp. IGTS8 strain between 35-42 µmol. h⁻¹. g⁻¹ dry cell weight depending on the method of quantifying the accumulated 2-HBP, whereas, Kilbane and Le Borgne (2004) reported BDS activity of 72 µmol. h⁻¹. g⁻¹ DCW for the same strain. The maximum BDS activity we measured (91 µmol. h⁻¹. g⁻¹ DCW) for the same strain is very close to those reported values.
Since many *Rhodococci* and *Mucobacteria* strains have been reported to desulfurize DBT, it seems likely that the BDS activity is conserved in genus *Rhodococci* and *Mucobacteria*. However, considerably different BDS activities were measured for different strains of the same species. BDS activities between 4 and 74 µmol. h⁻¹. g⁻¹ DCW and between 4 and 211 µmol. h⁻¹. g⁻¹ DCW were reported for different wild strains of *R. erythropolis* and *Mycobacterium* sp., respectively (Kilbane, 2006). BDS activities against DBT by other bacterial strains have been reported. Activities of 1, 5-10, and 11-40 µmol. h⁻¹. g⁻¹ DCW were reported for *Gordonia alkanivorans* strain 1B (Alves et al., 2005), recombinant *E.coli* (Kirimura et al., 2004) and *Pseudomonas dellafieldi* R-8 (Luo et al., 2003; Guobin et al., 2005), respectively.

The BDS activity measured for strain IGTS8-5 against DBT (91 µmol. h⁻¹. g⁻¹ DCW) is higher than the values reported for some other bacterial species. This result highlights the capability of strain IGTS8-5 as a potential candidate for developing a biocatalyst for BDS process. However, further studies for optimizing this activity and elucidating the genetic makeup encoding the BDS activity are needed to achieve higher BDS activity.

### 4.4 Effect of DBT concentration on the growth and biodesulfurization activity

The effect of DBT concentration as the sole S source on growth and BDS activity was tested at 100 ppm, and 200 ppm for reference biodesulfurizer *R. erythropolis* strain IGTS8-5 which exhibited the highest BDS activity and for *R. erythropolis* strain IGTS8-5G. The observed result showed that cultures with 200 ppm DBT stimulated the growth of strain IGTS8-5 (approximately 20%) over culture containing 100 ppm DBT. However, nearly all the added DBT was recovered as 2-HBP in both cultures of strains IGTS8-5. The culture of the reference strain *R. erythropolis* IGTS8-5G showed nearly the same pattern of growth and BDS activity as IGTS8-5 cells.
Results showed that strain IGTS8-5 tolerate 200 ppm DBT (1.1 mM) in culture media and produce stoichiometric amounts of 2-HBP which accumulates and is not further degraded. This also points out that accumulation of 200 ppm 2-HBP (≈ 1 mM 2-HBP) or sulfate seems to have no inhibitory effect on the growth of strain IGTS8-5. This is in contrast to what is reported for the same strain in a previous study (30% growth inhibition when the cells were grown with 1 mM DBT plus 1 mM 2-HBP (Al-Ajmi, 2006). The expression of dsz genes under a dsz promoter has been shown to be completely repressed by 0.3 mM 2-HBP or 1.4 mM sulfate (Ma et al., 2002).

Guobin et al. (2006) reported that the DBT-desulfurization activity of the gram-negative *Pseudomonas delafieldii* R-8 in HDS-treated diesel oil was not affected when 2-HBP concentration reached 1.0 mM. However, when the BDS activity was tested in the aqueous phase, dramatic inhibition (80%) was observed at 1.0 mM 2-HBP. No inhibition was observed when the sulfate (as sodium sulfate) concentration reached 15 mM in cultures of *Pseudomonas delafieldii*. The growth and the BDS process of the desulfurizing *Gordonia* CYKS1 was shown to be completely inhibited when the concentration of 2-HBP was 0.15 mM or higher (Kim et al., 2004). Also, Omori et al. (1992) reported that 0.4 mM of 2-HBP completely inhibited the growth and BDS activity of *Corynebacterium* sp. Strain SY1. It should be emphasized that the highest concentration tested for DBT was 200 ppm (approximately 1.2 mM). The effect of higher (more than 200 ppm) DBT concentration was not tested due to the very low solubility of DBT in aqueous media that limits the bioavailability of DBT due to its precipitation above 1 mM.

### 4.5 Investigation of progressive immobilization of bacterial cells on the coke particles

Eleven set ups of the previously described system were used with the aim of investigating the extent of immobilization of the bacteria on the coke, as well as
studying the biodesulfurization activity of the immobilised bacteria. The bacteria used for this experiment was IGTS8-5.

Two sets of experiments were carried out for the investigation of the extent of immobilization. For each set of these experiments, five coke columns were used.

For both sets the increase in weight of coke after drying was taken as a measure of immobilization of the bacteria i.e. how much of the bacteria cells clung to the coke. For this measurement, the coke was removed from the column and dried overnight in an aired oven kept at 60° C, after which it was weighed to determine the dry cell weight of the attached bacteria.

For the first set, the five separate columns were left running for 24 hours before the commencement of the first measurement. One system was sacrificed to carry on dry cell weight measurement of the bacterial cells attached to the coke particles.

As for the other remaining four systems, one system at a time was sacrificed every twelve hours for measuring the dry cell weight.

For the second set, measurements were carried out to investigate the effect of refreshing the media on the extent of immobilization. For this experiment, circulation were kept running for 48 hours before the start of the first measurement where the first coke column was sacrificed. After this measurement, the media for each system was refreshed every 24 hours, and a series of measurements were carried out every 24 hours, at which one column at a time was sacrificed.

The results obtained in the investigation of a progressive immobilization of bacterial cells on the coke particles (250 g) are summarized in Figure 4.6 and Figure 4.7. Dry cell weight of the immobilized bacterial cells was obtained after removing the coke particles from the column, followed by drying it in an air circulated oven kept at 60° C overnight.

The results in Figure 4.6 are those obtained from the first set of experiments where five of the systems were left under circulation for 24 hours after which
measurements commenced after every 12 hours interval between successive measurements. For each measurement, one system was sacrificed at a time to obtain the dry cell weight of immobilized bacteria.

Figure 4.6 Plots of optical density and dry cell weight as a function of time in hours for the first set of experiments including the first five systems.

Figure 4.6 shows a progressive increase in the dry cell weight of the bacterial cells immobilized on the coke particles used. Maximum immobilization of bacterial cell is reached (0.8 g/ 250 g of coke particles) after 48 hours of circulation. This finding prompted another set of experiments where the effect of refreshing the media on the extent of immobilization was investigated.

For the second set of experiment the systems were left under circulation for 48 hours, before the commencement of the first measurement, when the first coke column was sacrificed for obtaining the dry cell weight. This corresponds to the first point at zero hours (0h) in Figure 4.7. At this time the media in the four other systems were refreshed by completely replacing the whole medium with a fresh one with the same composition. The systems were left under circulation for another 24 hours when another system was sacrificed for measuring the dry cell weight of the immobilized bacteria on that column. The process of
refreshment, time of circulation and measurement was repeated for the other remaining systems.

![Refreshing the media every 24h](image)

*Figure 4.7: Plot of dry cell weight of bacteria immobilized on coke particles for the second set of experiments including the second five systems.*

As can be seen from Figure 4.7, refreshment has proved to be beneficial for the immobilization of the bacterial cells on the coke particles. This is manifested by the increase observed in the dry cell weight from 0.8 g/250 g of coke to a maximum of 1.8 g/250 g of coke particles used in this immobilization process after 72 hours. After 72 hours, no appreciable change in the dry cell weight of bacterial cells is observed.

The pictures below depict the different stages of bacterial cell growth in the media as shown by the increase in turbidity (Figure 4.8 a and b), and immobilization of the bacterial cells on the coke particles (Figure 4.9 a, b and c).
Figure 4.8 a and b Bacterial growth in the media as shown by the increase in turbidity

Figure 4.9 a, b, & c Immobilization of the bacterial cells on the coke particles.

Environmental Scanning Electron Microscopy (ESEM) was used to investigate the porosity of the coke particles as well as the growth and immobilization of the bacteria cells on these particles. The porous structure of the coke used is clearly shown in ESEM image in Figure 4.10, where various sizes of pores can be observed. Pores size vary from 5 um to 0.2 mm and not connected.
Figure 4.10  ESEM image for the coke particles used in bacterial immobilization showing multi porous structure.

ESEM images of some samples showing the bacteria growth with varying magnification are shown in the Figure 4.11 (a-d). The figures reveal three dimensional growths of the bacteria cells on the surface of the coke particles as well as its growth in the pores.
Figure 4.11 ESEM images depicting the growth and immobilization of bacterial cell on the porous coke particles used; surface three dimensional (3D) growth a & d and growth in pores b & c.

4.5.1 Biodesulfurization activity of the immobilized bacterial cells on the coke particles

Biodesulfurization activity of the immobilized bacterial cells on the coke particles in system eleven was investigated as described in detail in the material and method section 3.2.10.

The last column (number 11) that was subjected to continuous medium refreshment for enhancing the growth and immobilization of the bacterial cell onto the coke particles was used to investigate the biodesulfurization activity of IGTS8-5. Based on the last coke column of the previous set (five systems) the dry cell weight of bacteria is estimated to be 1.8 g/250 g coke particles on this coke column (number 11).

Before the commencement of the biodesulfurization experiment, the circulation was stopped and the coke column was detached from the system to drain the whole media present in both columns. The columns were connected again and the drained media was replaced by 500 ml of Phosphate buffer pH (7.2) containing 10 g/L glucose and 100 ppm DBT. The circulation was resumed for 24 hours after which it was stopped and the system was drained completely and a sample of the solution was taken to determine the amount of DBT.
remaining in the solution. The difference between the initial and the remaining amount of DBT in the buffer solution is assumed to have been consumed by the immobilized bacteria after 24 hours of circulation. The drained buffer solution containing the $10 \text{ g/ L}$ glucose and $100 \text{ ppm DBT}$ was replaced by a fresh identical solution and the circulation was resumed for another 24 hours after which it was stopped, the solution was drained to measure the amount of DBT remaining. The process of replacement of buffer solution, circulation and testing the amount of DBT remaining in solution every 24 hours was repeated for a period of 288 hours at 24 hours intervals from the start of the experiment.

For each of the drained solutions at the different times, its optical density was measured and recorded.

This part includes all experiments and analytical methods used to examine the visibility of using plant cell as future bio catalyst for sulfur removal from crude oil and its product. The behaviour of plant cell suspension will be examined in parallel with well-known biodesulfurizing bacteria in order to find the mechanism of sulfur removal using plant cell suspension.

Results obtained in this experiment are depicted in Figure 4.12, where the residual DBT measured in ppm and the optical density of solutions under investigation are plotted as a function of time.
As can be seen from Figure 4.12, almost 90% of the DBT present in the solution was consumed by the immobilized bacterial cell on the coke particles. This percentage of DBT consumption declined gradually with time reaching to 60-70% between the third and the seventh run (72-168 hours), followed by a consumption of nearly 50% DBT in time interval (192-288 hours).

These values show a decline in the activity of the immobilized bacterial cells for biodesulfurization. This decline in the activity can be seen mainly as a result of two factors. The first of which is the leaching of some of the immobilized bacteria cells on the coke particles, indicated by the rise in the optical density at 600 nm which is taken as a measure of the turbidity of the media solution. The second factor to be considered here which is regarded of great importance is the accumulation of the end product namely 2-hydroxy biphenyl (2-HBP). This compound has been reported to be inhibiting the desulfurization activity (Ohshiro et al., 1996; Ohshiro et al., 1997; Setti et al., 1999). Inhibition of the
biodesulfurization activity as well as cell growth of bacteria by the 2-HBP was also reported by Kim et al. (2004). The presence of 2-HBP in circulated media was confirmed by using Gibbs’ reagent and the appearance of the blue colour, as shown in Figure 4.13 and also the GC/ FID shows accumulation of 2-HBP as an end product of the 4S bio desulfurization pathway, see Figure 4.14 below.

![Figure 4.13 Blue colour with Gibbs’ reagent indicating the presence of 2-HBP in the circulating medium.](image)

No appreciable change in the biodesulfurization activity is observed between 192 and 288 hours. One reason which could be envisaged for this behaviour is that no significant leaching is taking place here and that the main activity is mainly due to the residual surface bacterial cells together with that imbedded in the pores. It is reasonable to assume that surface bacterial leaching is much easier than that bacteria located in the network of pores present in the porous coke used.
4.6 Biodesulfurization of bacteria using crude oil spiked with 200 ppm DBT

So far, the biodesulfurization activity of the bacteria strains namely \textit{R. erythropolis} IGTS8-5 and \textit{R. erythropolis} IGTS8-5G has been studied in aqueous media. In the following section results obtained for the biodesulfurization activity of these strains using crude oil spiked with 200 ppm DBT is going to be presented.

Two 3.0 L sterilized Erlenmeyer flasks each containing 1.0 L CDM, glucose 10.0 g/L as the sole C source and 100 ppm DBT as the sole S source were inoculated with \textit{R. erythropolis} IGTS8-5 and \textit{R. erythropolis} IGTS8-5G strains respectively and cultures were incubated at 30° C under shaking at 200 rpm for 36 hours. The cells of both strains were harvested separately while in exponential growth phase by centrifugation at 9000 rpm and 4° C. The cell pellet of each strain was washed twice with phosphate buffer solution and re-suspending in 100 ml of CDM containing 10 g/L glucose in order to obtain a concentrated cell suspension. Each re-suspended strain cells was divided equally into two 250 ml flasks (each containing 50 ml culture). For each of the flasks, 10 ml of crude oil spiked with 200 ppm DBT was added. Both flasks were incubated at 30° C under 200 rpm shaking and samples were taken at the
beginning of the experiment T0 and after five hours T5. Samples were centrifuged and the upper oily layer was extracted to register the amount of remaining DBT in the organic layer using GC equipped with flame ionization detector (FID).

Figure 4.15 shows the consumption of DBT for a crude oil sample spiked with 200 ppm DBT by the bacteria strains used after 24 hours and 3 days of incubation at 30° C. as can be seen from the Figure 4.15, the amount of the initial DBT present is reduced, indicating that both strains of bacteria has consumed part of the DBT. More DBT is consumption by IGTS8-5 than that of IGTS8-5G.

![Reduction of Crude Oil DBT by Bacteria Cells](image)

Figure 4.15 GC/ FID result showing the consumption of DBT from crude oil spiked with 200 ppm DBT by the bacteria strains used after 24 hours and 3 days incubation at 30° C (IGTS8-5 blue, IGTS8-5G red).

4.7 Preparation and initiation of callus culture of *Armoracia rusticana* (Horse Radish)

After 20 days of incubation on plates, growth of the callus started to be visible at the edges of leaves. Calluses were taken for sub culturing in new plates for another 10-15 days as in Figure 4.16
Sub culturing process was repeated until sufficient amount of callus obtained. Sub culturing solid MS medium were used under aseptic condition and continuous illumination.

Culture then transferred to aqueous medium for further investigation of their growth and activity toward consumption of DBT Figure 4.17.

Figure 4.16 Growth of callus at the edge of leaves and sub culturing it for another 10-15 days.
4.8 Batch cultures of *A. rusticana* (horse radish) and *A. thaliana* in Gamborg’s B5 medium

In three different medium (Gamborg’s B5, GB5 with 100 ppm DBT, and SFGB5 with 100 ppm DBT) a batch growth curve plotted using wet and dry weight and convert it to gram per litre scale. Every third day during the batch growth, two flasks were sacrificed for weight measurements. Plant cells in each flask were filtered using Whatman filter papers. Net weight of plant cells in flask was calculated after subtracting the original weight of filter paper. Plant cells and the filter paper were placed in hot air oven for overnight at 80°C before reading the dry weight of the cells.
Plant cell suspension grows and proliferates reaching a maximum 30 g/L and 25 g/L fresh weight in a period of 14 days for Armoracia rusticana and Arabidopsis thaliana respectively when growing in GB5 medium Figure 4.18 and Figure 4.19 (a) and (b). Fresh cell weight was 40 g/L and 35 g/L fresh weight in a period of 14 days for Armoracia rusticana and Arabidopsis thaliana respectively when growing in GB5 medium supplemented with an extra 100 ppm DBT as an additional sulfur source Figure 4.22 and Figure 4.23 (a) and (b). Whereas the maximum fresh cell weight was 20 g/L and 18 g/L in a period of 14 days for Armoracia rusticana and Arabidopsis thaliana respectively when growing in SF medium supplemented with 100 ppm DBT as a sole source of sulfur Figure 4.28 and Figure 4.29 (a) and (b)
Figure 4.18 plots of the fresh cell weight and dry cell weight of *Armoracia rusticana* (Horse Radish) as a function of time (days) in GB5 medium.

Figure 4.19 plots of the fresh cell weight and dry cell weight of *Arabidopsis thaliana* (Arab.) as a function of time (days) in GB5 medium.

Cell suspensions of *Armoracia rusticana* (Horse Radish) and *Arabidopsis thaliana* (Arab) growing in MS medium supplemented with GB5 Medium vitamins.
and containing the growth hormones and the necessary vitamins for the growth of each cell suspension as mentioned in Materials and Methods.

The medium of growth containing 20 g/L sucrose as a carbon source. One sample of 1.0 ml in volume were taken from each batch growth ment to be tested at the beginning of incubation (day zero) shows that sucrose level is 20 g/L at the beginning of two batches of growth for the two tested plant cell suspension.

The second samples taken at day 2 of incubation then followed by one sample from each flask of plant cell suspension culture every third day gives the following results in Figure 4.24 and Figure 4.25.

For Armoracia rusticana cell suspension, sucrose has been converted completely to fructose and glucose during the first 11 days of incubation Figure 4.24. Glucose reaches 5 g/L and Fructose reaches 4 g/L in the second day of incubation when sucrose level was dropped to 13 g/L.

Glucose reaches its maximum level 8 g/L in day 5 while fructose reaches its maximum level 10 g/L in day 8. Glucose level continues drop reaching 4 g/L in day 17th. Fructose level was almost stable at level of 8.5 g/L from day 8 to day 23 except for day 24 when its level was 7 g/L then it drop to 6 g/L in day 26 when the whole experiment terminated.

Arabidopsis thaliana (Arab) cell suspension also converts sucrose to fructose and glucose during its growth Figure 4.25. The sucrose level reduced from 20 g/L in day zero (0) to only 2 g/L in day 11 of the start of incubation. The plot of sugar level concentration in a function of time (days) in Figure 4.25 shows that glucose level was 2 g/L on day 5 and reaching 10 g/L in day 8 followed by another reduction to 2 g/L in day 11 and continuously consumed at the determination time of the experiment in day 26th. Fructose level shows the same behaviour also during this experiment, its level was 2 g/L in day 5 and increased to 8 g/L in day 8 to be decline steadily reaching 2 g/L in day 14, then it continuously reduced to reach less than 1 g/L in day 26 were the experiment determinated.
Result shows that during the growth; sucrose has been converted to glucose and fructose in order for plant cells to utilize it as simple carbon source. Plant cells also show higher preferences toward glucose than fructose as a simple sugar for growth and proliferation.

Figure 4.20 Arabidopsis thaliana & Armoracia rusticana grows in GB5 medium

4.9 Batch culture of plant cells in Gamborg’s B5 medium in the presence of 100 ppm DBT

In the presence of 100 ppm DBT as an extra source of sulfur, cell mass of two plant cell suspension were reaching a maximum of 40.0 g / L in 14 days for Armoracia rusticana and 35.0 g / L for Arabidopsis thaliana.

The dry weight of Armoracia rusticana was 3.0 g / L growing in GB5 medium increased to 4.0 g / L when growing in GB5 medium supplemented with 100 ppm DBT.

Improvement in growth was noticed also in Arabidopsis thaliana., the dry cell weight was 2.0 g / L when growing in GB5 and increased to 3.0 g / L when growing in GB5 medium supplemented with 100 ppm DBT.
The whole 100 ppm DBT added to the media at the time of inoculation have been adsorbed by plant cells and disappeared from the media, also no BDT produced observed during this experiment when the media supplemented with 100 ppm DBT. This indicates that Armoracia rusticana and Arabidopsis thaliana needs DBT during the growth and proliferation stage and it can use the existing DBT which has been added to the medium and stop producing its own DBT.

Figure 4.21 Plant cells grows in GB5 medium in presence of 100 ppm DBT, A. thaliana (a) and Armoracia rusticana (b).
Figure 4.22 plots the fresh cell weight and dry cell weight of *Armoracia rusticana* (Horse Radish) as a function of time (days) in GB5 medium with an extra 100 ppm DBT dissolved in ethanol.

Figure 4.23 plots the fresh cell weight and dry cell weight of *Arabidopsis thaliana* (Arab) as a function of time (days) in GB5 medium with extra 100 ppm DBT dissolved in ethanol.
Figure 4.24 sugar analysis for Horse Radish (*Armoracia rusticana*).

Figure 4.25 sugar analysis for *Arabidopsis thaliana* (Arab).
4.10 **Batch culture of plant cells in sulfur-free Gamborg’s B5 medium in the presence of 100 ppm DBT**

Sulfur is essential for plants in various aspects, it is a macronutrient required for cell growth and development (Leustek et al., 2000; Grossman, and Takahashi, 2001) and to test the capability of nominated plant cells to utilize sulfur from DBT; this experiment was designed for that purpose. Sulfur free medium (SFM) was prepared and 100 ppm of DBT was added as a sole sulfur source as mentioned in materials and methods. Results of the fresh and dry weight obtained from the aliquots withdrawn from batch culture growth reveals noticeable mass increase of both plant cells suspension during two weeks of incubation. This growth considered to be an indicator for the capability of the cells to use the sulfur in DBT for their metabolic activity for their growth and proliferation as in Figure 4.27. 2-HBP is measured in any experiment of plant cells because plant cells usually produce phenolic compounds during the metabolic activity.
Figure 4.27 Growth of *Arabidopsis thaliana* in GB5 medium (a) and in sulfur free medium (SFM) supplemented with 100 ppm DBT (b), and Horse Radish *Armoracia Rusticana* in GB5 medium (c) and (SFM) with 100 ppm DBT as a sole sulfur source (d)
Figure 4.28 plots of the fresh cell weight and dry cell weight of *Armoracia rusticana* (Horse Radish) as a function of time (days) in SF medium containing 100 PPM DBT as sole sulfur source.

Figure 4.29 plots of the fresh cell weight and dry cell weight of *Arabidopsis thaliana* (Arab) as a function of time (days) in SF medium containing 100 PPM DBT as sole sulfur source.
Immobilisation occurred naturally since the plant cells aggregate get impeded in between reticulated pores and grows in size those giving more physical support for these trapped aggregate to stay in between reticulated polyurethane foam.

Immobilization occurred naturally when fine cells callus diffused through the reticulated foam and get trapped inside the small pores and grow around reticulated matrix. Cells immobilised by attaching to each other's naturally as shown in Figure 4.31.
Armoracia rusticana (Horse radish) cultures were immobilised in the open pore network of reticulated polyurethane foam cubes 10 ppi size

Arabidopsis thaliana cell cultures were immobilised in the open pore network of reticulated polyurethane foam cubes 10 ppi size

Figure 4.31 Natural immobilization of the plant cells when entrapped inside and on the surface of polyurethane porous foam.

4.12 DBT consumption by plant cell suspension

Plant cell suspension grows and proliferates reaching a maximum fresh cell weight of 20 g/ L and 18 g/ L in a period of 14 days for Armoracia rusticana and Arabidopsis thaliana respectively when growing in SF medium supplemented with 100 ppm DBT as a sole source of sulphur, Figure 4.22 and Figure 4.23 (a) and (b). in this experiment, 4.0 g D.C. of Armoracia rusticana and 3.2 g D.C. of Arabidopsis thaliana were used to evaluate the activity of the particular plant cell and furthermore compare it with the activity of biodesulfurizing bacteria obtained by using 3.25 g D.C. of Rhodococcus erythropolis IGTS8-5 and 3.0 g D.C. of Rhodococcus erythropolis IGTS8-5G earlear in the result of bacteria activity assessment.

The activity of the plant cells were calculated depending on the measurement of DBT reduction from the medium. The amount of DBT added in the beginning of the experiment was 100 ppm and the consumption of DBT illustrated below in Table 4.2.
Figure 4.32 Plot of reduced DBT concentration as a function of time in a comparison between reference bacterial desulphurizing strains *Rhodococcus erythropolis* IGTS8-5 (5) and *Rhodococcus erythropolis* IGTS8-5G (5G) and the plant cell suspension *Armoracia rusticana* (HR) and *Arabidopsis thaliana* (Arab).

Table 4.2 Activity of the two plant cells suspension calculated in respect of DBT consumpnsion. The calculated BDS activity units (µmol DBT. h⁻¹. g⁻¹ dry cell weight)

<table>
<thead>
<tr>
<th>Plant cells</th>
<th>Cell density (g/L)</th>
<th>(ppm) DBT consumed after</th>
<th>BDS activity units</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 h</td>
<td>3 h</td>
</tr>
<tr>
<td><em>Armoracia rusticana</em> (HR)</td>
<td>4.0</td>
<td>55</td>
<td>65</td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em> (Arab)</td>
<td>3.2</td>
<td>65</td>
<td>70</td>
</tr>
</tbody>
</table>
4.13 Production of phenolic compounds by plant cell suspension as end-products of biodesulfurisation

Sulfur compound can be stored in vacuoles and act a key water structure of the cytoplasm or can enter the metabolic stream (Leustek et al., 2000).

Sulfur involved in the catalytic and electrochemical functions in plants biological functions and molecules structure as well (Thomas Leustek et al., 2000).

Sulfur is one of the essential macronutrients for plants. It is found in the aminoacids cysteine and methionine and in various plants metabolites (Thomas Leustek and Kazuki Saito 1999).

The GC/ FID results of DBT level in culture medium revealed that the DBT level reduced over time indicating the possible consumption of DBT by plant cells. The GC/ FID chromatogram was showing also accumulation of phenolic compound in the medium and detected in the same area of 2-HBP retention time, see Figure 4.33. That behaviour indicates that plant cells tested in this research has a similar mechanism toward utilizing the sulfur from DBT as that presents in biodesulfurizing bacteria. The exact mechanism is not yet confirmed, therefore further investigation is needed in this particular area.
Figure 4.33 GC/ FID chromatogram shows several phenolic compounds accumulated in the area of 2-HBP retention time 14.1 minutes during the growth of bacteria (a), and Arabidopsis thaliana. and Armoracia rusticana (b) in GB5 medium sublimated with DBT.
4.14 Growth of plant cells in GB5 medium and the production of DBT compound during the growth

Plant cell suspension grows and proliferates reaching 40.0 g / L in 14 days for Armoracia rusticana and 35.0 g / L for Arabidopsis thaliana. Monitoring the outcome during the period of time revealed obvious production and accumulations of DBT in the media.

This behaviour of producing DBT during the growth and proliferation is ceased if DBT has been added to the media in the first day of inoculation. This seems to suggest that somehow the addition of DBT to the medium is inhibiting the production of DBT by the plant. The question as to the mechanism of inhibition is unclear and further investigation into thus is needed. The other question that poses itself here is to why the plant cells produce DBT is it part of defence mechanism. Furthermore, consumption of the added DBT was pragmatically observed to commence on the third week of incubation.

Figure 4.34 Plant cells growth in sulfur free medium and accumulation of DBT during the exponential phase.
4.15 Growth of each of Horse Radish Armoracia rusticana and Arabidopsis thaliana in sulfur free medium (SFM) supplemented with 100 ppm 4,6 DEDBT or 100 ppm 4,6 DMDBT or 100 ppm 4MDBT as sole source of sulfur

The experiment was run each time using different sole source of sulfur in the medium to check the ability of plant cell suspension to consume different common sulfur compounds present in crude oil.
Plant cell suspensions have been subjected separately to three different sulfur sources commonly present in crude oil. After proving the ability of plant cell suspensions to grow in presence of DBT as a sole source of sulfur, similar experiments repeated separately with 4,6 DEDBT, 4,6 DMDBT, and 4 MDBT each as sole sulfur source. The positive growth revealed the broad spectrum of plant cell suspension toward sulfur compounds.

Growth and proliferation was very clear as a circular ring in interior wall of all incubated flasks that contains 100 ppm 4,6 DEDBT or 100 ppm 4,6 DMDBT or 100 ppm 4MDBT as sole source of sulfur needed for the growth, also increase in size and amount of the originally inoculated cells were noticed by naked eyes. No growth were noticed in two control negative flasks that contain no sulfur and the two plant cell suspensions turned to yellowish brown in colour after 10 days of incubation. The positive growth revealed the broad spectrum of plant cell suspension toward sulfur compounds.

GC/ FID analyzer results of the designated sulfur compound consumption can be seen in Figure 4.36.

Figure 4.36 Histogram shows the reduction of three different sulfur compounds from 100 ppm in the beginning of incubation when incubated with plant cell suspensions for 96 hours of incubation.
4.16 Growth of plant cells in GB5 medium in presence of crude oil spiked with 200 ppm (1.2 mM) DBT

Growth was normal in all flasks and there was a visible increase in size of fine cell culture during the entire experiment period. In day 9, double cell weight is achieved by cells growing with crude oil spiked with 200 ppm DBT and in day 11th another 50% increase in weight is also noticed. Fluctuation in cell masses was observed beyond day 14th. In the sets of flasks growing in the presence of 100 ppm DBT dissolved by ethanol, the cells were much greenish but there were a slight increase in cell weight comparing to the obvious mass increase occurring in the presence of crude oil spiked with 200 ppm DBT.

The initial weight of plant cells in each flask was 5.0 g and the final weight at the determination time of the experiment was reaching 6.5 g for each plant cell suspension and the incubation period was seven days.

Figure 4.37 Plant cell suspensions grow in four different sole sulfur sources

In the GC / FID chromatogram; the crude oil spiked with DBT that previously dissolved in ethanol shows DBT peak at 17.5 minutes elution time. GC / FID show reduction in DBT concentration after incubation with plant cells, suggesting that plant cells has used up some of the DBT added for its growth.
Figure 4.38 *Arabidopsis thaliana* incubated with crude oil, starting day (a) and after two weeks (b). *Armoracia rusticana* incubated with crude oil, starting day (c) and after two weeks (d).

### 4.17 Consumption of DBT from oil by two of the plant cell suspension

5.0 g of plant cell suspension of *Armoracia rusticana* (H.R) and *Arabidopsis thaliana* (Arab) in there exponential phase were separately re-suspended in several 75 ml flasks containing 25 ml of Sulfur free Gamborg’s B5 medium and
3.0 ml of crude oil spiked with 200 ppm DBT were able to grow and increase in mass Figure 4.39. The DBT concentration was measured by GC/ FID revealing that the concentration of DBT in the crude oil was tremendously reduced from the aqueous layer and samples from the oily phase analysed by GC/ FID also shows reduction in the DBT level as shown in Figure 4.40 and Figure 4.41. This behaviour was noticed in biodesulfurizing bacteria.

Figure 4.39 growth of plant cell suspension in presence of crude oil containing 200 ppm DBT
Figure 4.40 GC/ FID chromatogram shows DBT in water phase (blue colour) getting consumed by plant cell suspension.

DBT transferred from oil to water before it consumed by plant cells

Figure 4.41 Plot of DBT concentration as a function of time for two reference bacterial desulfurizing strains (5) and (5G) and two plant cell suspensions (Armoracia rusticana) and (Arabidopsis thaliana) showing the reduction of DBT in the crude oil.
4.18 Control negative experiment with dead plant cell suspension

Sulfur compound can be stored in vacuoles and act a key water structure of the cytoplasm or can enter the metabolic stream (Leustek et al., 2000). Therefore an experiment designed to expose heat killed plant cells to a known quantity (100 ppm) of DBT in order to monitor any possible diffusion of DBT into ded cells or any form of internal vacuoles storage of DBT. Heat killed plant cells dose not reduce the DBT concentration in the medium more than 5% which can be considered as an error due to possibility of precipitation of DBT. Simultaneously, the cells extract of killed cells shows a slight amount of no more than 2% DBT in their contents after grinding the cells and extract the contents to be tested for the presence of any DBT. The cell extracts of living cells incubated in medium contain DBT shows 12-18% DBT in their content. It is believed that this small amount was in their way to be utilised in cells metabolic pathway or stored in vacuoles inside the cell for possible future use.

![Figure 4.42](image)

Figure 4.42 Monitoring DBT at 17.5 from day 1 to day 21 incubated with dead plant cells shows stability in the quantity of that compound in presence of 200 ppm DBT at the start of incubation, reduction in DBT is negligible.
4.19 Cell viability test

Fluorescein di-acetate (FDA) reagent was used to estimate viable cells in cell culture. Esterase enzyme in living cell cleaves the stain added to the cells on examination slide; consequently gives a fluorescent yellow / green colour when using Ultra Violet UV microscope (Dixon and Gonzales, 1994). UV fluorescent Microscope shows living cells which can cleave the stain appears fluorescent and can easily count. Percentages of fluorescent and non-fluorescent cells were calculated using the following equation

\[
\% \text{ Viability} = \frac{\text{Number of fluorescent cells}}{\text{Total number of cells}} \times 100 \%
\]

Cells were remained alive after exposure to DBT in the medium and no adverse effect were noticed during the longest experiment exposure period assessment of 21 days. Figure 4.43 shows cluster of plant cells varies in there activity. Some sells looks fresh and very active and appears in dense green colour.

![Ultra violet fluorescent microscope image showing living cells.](image)

Figure 4.43 Ultra violet fluorescent microscope image showing living cells.
4.20 Transcriptomics analysis of plant cells’ biodesulfurisation activity

4.20.1 Introduction

Genechip probe arrays has been developed by Affymetrix for *Arabidopsis thaliana* by synthesized more than 22500 oligonucleotides. Therefore it is possible to detect the possible gene trigaring as a response to thiyophene compound (DBT).

4.20.2 Cell culture and sampling

*Arabidopsis thaliana* cell suspension inoculum 10% v/v has been sub cultured in MS medium supplemented with GB5 vitamins, and incubated in 25°C incubation temperature under illumination 16 hours/ 24 hours. The medium was also enhanced with growth regulators BAP and NAA as indicated in Table 3.3 that makes very good growth for the culture.

Two 100 ml cultures of *Arabidopsis thaliana* suspension cultured cells were pooled 5 days after subculturing. The cells were allowed to settle and the turbid medium was decanted. The cells were washed by adding the same volume of medium and allowing them to settle again. Finally, cells were resuspended at four times the original density in fresh medium, 12 ml suspension aliquoted into 25ml Erlenmeyer flasks and put onto a rotary shaker for 2h until the beginning of treatment summarized in Table 3.8.

Plant cell suspension of Arabidopsis thaliana were exposed to 30 ppm DBT dissolved in ethanol, the exposure time were 2 hours and 6 hours separately. Samples were done in triplicate for each control and time set exposure.
Triplicate control samples also incubated with ethanol only to inspect the possible effect of ethanol only on the cell suspensions and consequently distinguish it from the effect of DBT on the cells.

The concentration of the ethanol in the medium was 0.3% v/v.

Cells were harvested separately from each flask by filtration and frezed by liquid nitrogen and stored in -70°C freezer in order to perform the RNA extraction for microarray transcriptomics analysis.

### 4.20.3 Electrophoresis File Run Summary

Gel electrophoresis results confirm the quantity and the quality of the samples showing distinctive line in gel separation, sample number 6 was repeated due to mechanical jamming in the analyser and it gives positive result.

![Gel electrophoresis result](image)

*Figure 4.44 Presence of distinctive line in the gel chromatography revealed sufficient amount of RNA in the sample.*
Electropherogram Summary

Sample 1

Overall Results for sample 1: Sample 1

RNA Area: 1,819.9
RNA Concentration: 1,800 ng/μl
rRNA Ratio [28s / 18s]: 1.6
RNA Integrity Number (RIN): N/A (B.02.08)
Result Flagging Label: RIN N/A

Fragment table for sample 1: Sample 1

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<th>End Time [s]</th>
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<td>44.71</td>
<td>47.82</td>
<td>382.5</td>
<td>21.0</td>
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</tbody>
</table>
Overall Results for sample 2 : Sample 2

RNA Area: 1,996.0

RNA Concentration: 1,974 ng/μl

rRNA Ratio [28s / 18s]: 1.6

RNA Integrity Number (RIN): N/A (B.02.08)

Result Flagging Label: RIN N/A

Fragment table for sample 2 : Sample 2

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<td>28S</td>
<td>44.62</td>
<td>47.75</td>
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</table>
Overall Results for sample 3 : Sample 3

RNA Area: 1,771.9

RNA Concentration: 1,752 ng/μl

rRNA Ratio [28s / 18s]: 1.6

RNA Integrity Number (RIN): N/A (B.02.08)

Result Flagging Label: RIN N/A

Fragment table for sample 3 : Sample 3

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<td>44.57</td>
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</table>
Sample 4

Overall Results for sample 4 : Sample 4

RNA Area: 1,565.3

RNA Concentration: 1,548 ng/μl

rRNA Ratio [28s / 18s]: 1.5

RNA Integrity Number (RIN): N/A (B.02.08)

Result Flagging Label: RIN N/A

Fragment table for sample 4 : Sample 4

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<td>44.72</td>
<td>47.98</td>
<td>261.4</td>
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</table>
Sample 5

Overall Results for sample 5 : Sample 5

RNA Area: 1,910.6

RNA Concentration: 1,889 ng/μl

rRNA Ratio [28s / 18s]: 1.6

RNA Integrity Number (RIN): N/A (B.02.08)

Result Flagging Label: RIN N/A

Fragment table for sample 5 : Sample 5

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<td>44.43</td>
<td>47.54</td>
<td>405.7</td>
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</table>
Sample 6

Overall Results for sample 6 : Sample 6

RNA Area: 1,408.3
RNA Concentration: 1,393 ng/μl
rRNA Ratio [28s / 18s]: 0.1
RNA Integrity Number (RIN): N/A (B.02.08)
Result Flagging Label: RIN N/A

Fragment table for sample 6 : Sample 6

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<td>28S</td>
<td>44.50</td>
<td>47.48</td>
<td>3.8</td>
<td>0.3</td>
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</tbody>
</table>
**Overall Results for sample 7 : Sample 7**

RNA Area: 1,139.7

RNA Concentration: 1,127 ng/μl

rRNA Ratio [28s / 18s]: 1.5

RNA Integrity Number (RIN): N/A (B.02.08)

Result Flagging Label: RIN N/A

**Fragment table for sample 7 : Sample 7**

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<td>28S</td>
<td>44.42</td>
<td>47.33</td>
<td>236.7</td>
<td>20.8</td>
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</table>
Sample 8

Overall Results for sample 8 : Sample 8

RNA Area: 1,104.1
RNA Concentration: 1,092 ng/μl
rRNA Ratio [28s / 18s]: 1.6
RNA Integrity Number (RIN): N/A (B.02.08)
Result Flagging Label: RIN N/A

Fragment table for sample 8 : Sample 8

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<td>42.26</td>
<td>129.8</td>
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<tr>
<td>28S</td>
<td>44.46</td>
<td>47.54</td>
<td>205.5</td>
<td>18.6</td>
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</tbody>
</table>
Sample 9

Overall Results for sample 9 : Sample 9

RNA Area: 996.7
RNA Concentration: 986 ng/μl
rRNA Ratio [28s / 18s]: 1.5
RNA Integrity Number (RIN): N/A (B.02.08)
Result Flagging Label: RIN N/A

Fragment table for sample 9 : Sample 9

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<tr>
<td>18S</td>
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<td>42.44</td>
<td>115.0</td>
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</tr>
<tr>
<td>28S</td>
<td>44.41</td>
<td>47.56</td>
<td>170.5</td>
<td>17.1</td>
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</table>
Overall Results for sample 10 : Sample 10

RNA Area: 1,232.3

RNA Concentration: 1,219 ng/μl

rRNA Ratio [28s / 18s]: 1.5

RNA Integrity Number (RIN): N/A (B.02.08)

Result Flagging Label: RIN N/A

Fragment table for sample 10 : Sample 10

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<td>138.7</td>
<td>11.3</td>
</tr>
<tr>
<td>28S</td>
<td>44.45</td>
<td>47.82</td>
<td>205.5</td>
<td>16.7</td>
</tr>
</tbody>
</table>
Sample 11

Overall Results for sample 11 : Sample 11

RNA Area: 1,399.7

RNA Concentration: 1,384 ng/μl

rRNA Ratio [28s / 18s]: 1.4

RNA Integrity Number (RIN): N/A (B.02.08)

Result Flagging Label: RIN N/A

Fragment table for sample 11 : Sample 11

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</tr>
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<tbody>
<tr>
<td>18S</td>
<td>40.23</td>
<td>42.27</td>
<td>164.1</td>
<td>11.7</td>
</tr>
<tr>
<td>28S</td>
<td>44.30</td>
<td>47.83</td>
<td>231.2</td>
<td>16.5</td>
</tr>
</tbody>
</table>
Overall Results for sample 12: Sample 12

RNA Area: 1,646.2

RNA Concentration: 1,628 ng/μl

rRNA Ratio [28s / 18s]: 1.5

RNA Integrity Number (RIN): N/A (B.02.08)

Result Flagging Label: RIN N/A

Fragment table for sample 12: Sample 12

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<th>End Time [s]</th>
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</tr>
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<td>42.23</td>
<td>232.2</td>
<td>14.1</td>
</tr>
<tr>
<td>28S</td>
<td>44.16</td>
<td>47.52</td>
<td>341.2</td>
<td>20.7</td>
</tr>
</tbody>
</table>
4.20.4 Bioinformatics Analysis

Technical quality control and outlier analysis were performed with dChip (V2005) ([www.dchip.org](http://www.dchip.org)), Cheng Li and Wing Hung Wong (2001) using the default settings.

Background correction, quantile normalization, and gene expression analysis were performed using RMA in Bioconductor (Bolstad et al., 2003) to establish relationships and compare variability between samples.

4.20.5 Principal component analysis

Principal component analysis (PCA) was used since this method is able to reduce the effective dimensionality of complex gene-expression space without significant loss of information, Quackenbush, J (2001). Principal component analysis (PCA) was performed with Partek Genomics Solution (Parket 2010). Principal component analysis for the Arabidopsis thaliana cell suspension shows close similarity in the biological triplicates samples, see Figure 4.45.
Figure 4.45 Principal component analysis shows similarity of the biological triplicate samples.

Differential expression analysis was performed using Limma using the functions lmFit and eBayes (Smyth, 2004). Gene lists of differentially expressed genes were controlled for false discovery rate (fdr) errors using the method of QVALUE (Storey, and Tibshirani, 2003).

Functional annotation of the genes was performed using DAVID version 2.
Figure 4.46 GeneChip Arabidopsis ATH1 Genome Array image

4.20.6 Microarray analysis to examin the effect of adding DBT to the medium of growth of *Arabidopsis thaliana*.

Clear molecular mechanisms regulating the inorganic sulphate uptake in plants are still mostly unknown (Rouached et al., 2008). Maruyama-Nakashita et al., (2003) mentioned that Arabidopsis has two high-affinity sulfate transporters (SULTR1;1 and SULTR1;2) that represent the sulfate uptake activities at the root surface. The transcript level of SULTR1.1 was altered generally in response to sulfur-related treatments. In split-root experiments performed by
Rouached et al., (2008) show that the expression of SULTR1.1 is locally regulated in response to sulfate starvation. In contrast, accumulation of SULTR1.2 transcripts seemed to be mostly related to metabolic demand and is directly controlled by photoperiod (Rouached et al., 2008). High-affinity sulfate transporters SULTR1;1 and SULTR1;2 are expressed at epidermis and cortex of Arabidopsis thaliana roots during sulfur restriction (Yoshimoto et al., 2007). In Arabidopsis thaliana, SULTR1;1 and SULTR1;2 are two genes anticipated to be involved in high-affinity sulphate uptake from the soil (Barberon et al., 2008), also Kataoka et al. (2004) describe SULTR3;5 as an essential factor of the sulfate transport system that enables the root-to-shoot transport of sulfate in the vasculature (Kataoka et al., 2004).

In the following part of data analysis for the result obtained from microwarray analysis I am focusing on the effect of DBT on the genes of plant cells after 2 hours (2h) exposures and 6 hours (6h) exposures and compare it verses the mock samples that contain no DBT as a function of fold increase of gene expression that related to sulfur uptake. Furthermore, comparisons of folds increase among 2h and 6h of exposures to DBT.

4.20.6.1 Grope of genes increased several folds after 2 hours exposure followed by another folds increased after 6 hours exposures to DBT

General Screening for more than 22,000 gene involved in this microarray for the effect of DBT on three biological repetition samples after 2 hours and 6 hours exposures shows 9 genes increase in there folds after 2 hours and continue their increase in 6 hours exposures. Those genes are listed in Table 4.3 in probe set ID with correlated representative public ID in parallel with their target description. Gene At2g02930; Affimix probe ID266746_s_at is one of those genes that shows that behaviour toward DBT and its function is for glutathione S-transferase.
Two genes showing the maximum consecutive folds increased after 2 and 6 hours are plotted in Figure 4.48. Their proposed functions are listed in Table 4.3, as putative transcription factor by gene At2g22330 and protein involved in transmembrane function by gene At4g15620.

Figure 4.47 Plot of the nine genes increased several folds after 2 and 6 hours exposure to DBT.
Figure 4.48 Plot of only two genes that mostly have the highest folds increase after two and six hours exposure to DBT.

Table 4.3 list of genes increased several folds after 2 hours and 6 hours exposure to DBT

<table>
<thead>
<tr>
<th>Probe Set ID</th>
<th>Representative Public ID</th>
<th>Target Description</th>
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</thead>
<tbody>
<tr>
<td>262047_at</td>
<td>At1g80160</td>
<td>hypothetical protein</td>
</tr>
<tr>
<td>246781_at</td>
<td>At5g27350</td>
<td>putative sugar transporter protein SUGTL2</td>
</tr>
<tr>
<td>265067_at</td>
<td>At1g03850</td>
<td>hypothetical protein</td>
</tr>
<tr>
<td>260435_at</td>
<td>At1g68320</td>
<td>putative transcription factor</td>
</tr>
<tr>
<td>258791_at</td>
<td>At3g04720</td>
<td>(PR-4) hevein-like protein precursor similar to wound-induced protein (WIN2)</td>
</tr>
<tr>
<td>264052_at</td>
<td>At2g22330</td>
<td>putative cytochrome P450</td>
</tr>
<tr>
<td>266746_s_at</td>
<td>At2g02930</td>
<td>putative glutathione S-transferase</td>
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<td>254889_at</td>
<td>At4g11650</td>
<td>Osmotin precursor</td>
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<tr>
<td>245501_at</td>
<td>At4g15620</td>
<td>Related to a membrane protein involved in function in membrane or transmembrane</td>
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### 4.20.6.2 Genes show several fold increase (2h * DBT vs. 2h * mock)

Table 4.4 list of genes show folds increase after 2 hour exposure to DBT versus mock

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<thead>
<tr>
<th>Probe Set ID</th>
<th>Gene Title</th>
<th>Uni. Gene ID</th>
<th>Gene Ontology Biological Process/ Cellular Component/ Molecular Function</th>
<th>Fold Change (2h * DBT vs. 2h * mock)</th>
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</thead>
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<tr>
<td>261763_at</td>
<td>PDR12 (Pleiotropic Drug Resistance 12)</td>
<td>At.49303</td>
<td>drug transmembrane transport, movement of substances, integral to membrane, ATPase activity, coupled to transmembrane movement of substances</td>
<td>37.9</td>
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<tr>
<td>266486_at</td>
<td></td>
<td>At.44883</td>
<td></td>
<td>19.5</td>
</tr>
<tr>
<td>246310_at</td>
<td>SULTR3;1 (Sulfate Transporter 3;1)</td>
<td>At.11928</td>
<td>sulfate transport, transmembrane transport, integral to membrane</td>
<td>3.9</td>
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<td>248676_at</td>
<td>ATSDI1 (Sulfur Deficiency-Induced 1)</td>
<td>At.29820</td>
<td>regulation of sulfur utilization, cellular response to sulfur starvation</td>
<td>2.8</td>
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<td>249721_at</td>
<td>LSU4 (Response To Low Sulfur 4)</td>
<td>At.30947</td>
<td></td>
<td>2.2</td>
</tr>
<tr>
<td>256244_at</td>
<td>SULTR4;2; sulfate transmembrane</td>
<td>At.43106</td>
<td>Transport, sulfate, transmembrane transport, integral to membrane</td>
<td>1.9</td>
</tr>
<tr>
<td>Accession</td>
<td>Description</td>
<td>Gene ID</td>
<td>Description</td>
<td>Log2 Fold Change</td>
</tr>
<tr>
<td>-----------</td>
<td>-------------</td>
<td>---------</td>
<td>-------------</td>
<td>------------------</td>
</tr>
<tr>
<td>245855_2</td>
<td>SULTR4;1; sulfate transmembrane transporter</td>
<td>At.21951</td>
<td>response to stress, sulfate transport, transmembrane transport, chloroplast membrane, symporter activity</td>
<td>1.7</td>
</tr>
<tr>
<td>263703_2</td>
<td>SRX (Sulfiredoxin)</td>
<td>At.40405</td>
<td>response to oxidative stress, oxidation reduction, chloroplast, DNA binding, acting on a sulfur group</td>
<td>1.3</td>
</tr>
<tr>
<td>252119_2</td>
<td>ATTRX1</td>
<td>At.193</td>
<td>Transport, positive regulation of catalytic activity, Cytoplasm, enzyme activator activity, protein disulfide oxidoreductase, acting on a sulfur group of donors, disulfide as acceptor</td>
<td>1.2</td>
</tr>
<tr>
<td>255443_2</td>
<td>SULTR3;2 (sulfate transporter 3;2)</td>
<td>At.393</td>
<td>sulfate transport, transmembrane transport, integral to membrane</td>
<td>1.2</td>
</tr>
<tr>
<td>255105_2</td>
<td>SULTR1;1 (Sulfate Transporter 1;1)</td>
<td>At.4168</td>
<td>sulfate transport, transmembrane transport, integral to membrane, symporter activity</td>
<td>1.1</td>
</tr>
<tr>
<td>262133_2</td>
<td>SULTR1;2 (Sulfate Transporter 1;2)</td>
<td>At.10549</td>
<td>sulfate transport, cellular response to sulfate starvation, plasma membrane, integral to membrane, symporter activity</td>
<td>1.1</td>
</tr>
<tr>
<td>255958_2</td>
<td>SULTR1;3; Sulfate Transmembrane Transporter</td>
<td>At.5346</td>
<td>sulfate transport, transmembrane transport, integral to membrane, symporter activity</td>
<td>1.08</td>
</tr>
</tbody>
</table>
4.20.6.3 Genes show several fold increase (6h * DBT vs. 6h * mock)

More than 10 gene were distinctively altered and shows many fols increase after 6 hours exposure to DBT in the media. In Table 4.5 the Probe Set ID and Universal gene ID for those gene are listed with number of folds increased showing also the ontology biological process and cellular components involved and genes molecular function correlated.

ATGSTF3 (GLUTATHIONE S-TRANSFERASE F3); glutathione transferase, 266746_s_at has increased by 15 folds. LSU1 (RESPONSE TO LOW SULFUR 1), 252269_at has increased 13.4 folds. SULTR1;2 (SULFATE TRANSPORTER 1;2); sulfate transmembrane transporter, 262133_at, has increased 7 folds. ATGSTU27 (GLUTATHIONE S-TRANSFERASE TAU 27); glutathione transferase, 252712_at, has increased by 5 folds. Also 255284_at APR1 (APS REDUCTASE 1) and 262133_at SULTR1;2 (Sulfate Transporter 1;2) resposible for sulfate assimilation and transportation of sulfur were increased more than 7 folds after the 6 h exposure to DBT in the medium.

Table 4.5 list of genes showing many folds increase after exposure to BDT in the medium.

<table>
<thead>
<tr>
<th>Probe Set ID</th>
<th>Gene Title</th>
<th>Uni. Gene ID</th>
<th>Gene Ontology Biological Process/ Cellular Component/ Molecular Function</th>
<th>Fold-Change (6h * DBT vs. 6h * mock)</th>
</tr>
</thead>
<tbody>
<tr>
<td>266746_s_at</td>
<td>ATGSTF3 (Glutathione S-Transferase F3)</td>
<td>At:22 195</td>
<td>toxin catabolic process, response to salt stress defense response to bacterium, Cytoplasm, chloroplast stroma, membrane apoplast, glutathione transferase</td>
<td>14.8</td>
</tr>
<tr>
<td>Gene ID</td>
<td>Description</td>
<td>Activity</td>
<td>Activity Score</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>-----------------------------------------------</td>
<td>----------------</td>
<td></td>
</tr>
<tr>
<td>252269</td>
<td>LSU1 (Response to Low Sulfur 1)</td>
<td>Sulfur transporter</td>
<td>13.4</td>
<td></td>
</tr>
<tr>
<td>255284</td>
<td>APR1 (APS REDUCTASE E 1); adenyllyl-sulfate reductase</td>
<td>Sulfate assimilation response to stress</td>
<td>7.3</td>
<td></td>
</tr>
<tr>
<td>262133</td>
<td>SULTR1;2 (Sulfate Transporter 1;2)</td>
<td>Sulfate transmembrane transporter</td>
<td>7.1</td>
<td></td>
</tr>
<tr>
<td>245855</td>
<td>SULTR4;1</td>
<td>Sulfate transmembrane transporter</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>250475</td>
<td>SULTR2;1; AST68;</td>
<td>Sulfate transmembrane transporter</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>249752</td>
<td>LSU2 (Response To Low Sulfur 2)</td>
<td>Sulfur transporter</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>256835</td>
<td>APS1 (ATP SULFURYL)</td>
<td>Sulfate assimilation plasma membrane</td>
<td>1.8</td>
<td></td>
</tr>
</tbody>
</table>
Table 4.6 list genes that show folds increased and related to sulfur transportation and metabolism.

<table>
<thead>
<tr>
<th>Probe Set ID/ Affymetrix ID</th>
<th>Transcript ID (Array Design)</th>
<th>Gene Name/ Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>261763_at</td>
<td>At1g15520</td>
<td>Drug transmembrane</td>
</tr>
<tr>
<td>266486_at</td>
<td>At2g47950</td>
<td>Membrane system</td>
</tr>
<tr>
<td>246310_at</td>
<td>At3g51895</td>
<td>Sulfate transporter 3.1</td>
</tr>
<tr>
<td>248676_at</td>
<td>At5g48850</td>
<td>Regulation of sulfur utilization</td>
</tr>
<tr>
<td>249721_at</td>
<td>At5g24655</td>
<td>LSU4 (Response to low sulfur 4)</td>
</tr>
<tr>
<td>256244_at</td>
<td>At3g12520</td>
<td>Probable sulfate transporter 4.2</td>
</tr>
<tr>
<td>263703_at</td>
<td>At1g31170</td>
<td>SRX oxidoreductase</td>
</tr>
<tr>
<td>252119_at</td>
<td>At3g51030</td>
<td>Redox reactions</td>
</tr>
<tr>
<td>255443_at</td>
<td>At4g02700</td>
<td>Sulfate transporter 3.2</td>
</tr>
<tr>
<td>255105_at</td>
<td>At4g08620</td>
<td>Sulfate transporter 1.1</td>
</tr>
<tr>
<td>262133_at</td>
<td>At1g78000</td>
<td>Sulfate transporter 1.2</td>
</tr>
<tr>
<td>255958_at</td>
<td>At1g22150</td>
<td>Sulfate transporter 1.3</td>
</tr>
<tr>
<td>252269_at</td>
<td>At3g49580</td>
<td>LSU1 (Response to low sulfur 1)</td>
</tr>
<tr>
<td>255284_at</td>
<td>At4g04610</td>
<td>APR1 Sulfate assimilation</td>
</tr>
<tr>
<td>245855_at</td>
<td>At5g13550</td>
<td>Sulfate transporter 4.1,</td>
</tr>
<tr>
<td>250475_at</td>
<td>At5g10180</td>
<td>Sulfate transporter 2.1</td>
</tr>
<tr>
<td>249752_at</td>
<td>At5g24660</td>
<td>LSU2 (Response to low sulfur 2)</td>
</tr>
<tr>
<td>256835_at</td>
<td>At3g22890</td>
<td>APS1 Sulfate assimilation</td>
</tr>
<tr>
<td>245912_at</td>
<td>At5g19600</td>
<td>SULTR3,5 sulfate transmembrane transporter</td>
</tr>
<tr>
<td>258219_at</td>
<td>At3g17880</td>
<td>Probable sulfate transporter 3.5</td>
</tr>
</tbody>
</table>

at ASE 1) sulfate adenyltransferase (ATP) 053 assay
256244_at SULTR4;2; At.43 106 Sulphate transmembrane transporter Transport, sulfate transport, transmembrane transport integral to membrane 3.3
4.20.6.4 Genes show Fold-Changes (6h * DBT vs. 2h * DBT)

Genes related to sulfur expressed after 6 hours of exposure to DBT verses the gens that also related to sulfur expressed after 2 hours of exposure to DBT are listed in this part Table 4.7 to try to determine the optimum time frame of gene functioning in response to extra source of sulfur.

Table 4.7 Comparison of folds after 2h DBT and 6h DBT exposure

<table>
<thead>
<tr>
<th>Probe Set ID</th>
<th>Gene Title</th>
<th>Fold-Change(2h * DBT vs. 2h * mock)</th>
<th>Fold-Change(6h * DBT vs. 6h * mock)</th>
<th>Fold-Change(6h * DBT vs. 2h * DBT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>252269_at</td>
<td>LSU1 (Response To Low Sulfur 1)</td>
<td>4.8</td>
<td>13.4</td>
<td></td>
</tr>
<tr>
<td>255284_at</td>
<td>APR1 (APS Reductase 1); adenyllyl-sulfate reductase</td>
<td>3.2</td>
<td>7.2</td>
<td></td>
</tr>
<tr>
<td>262133_at</td>
<td>SULTR1;2 (Sulfate Transporter 1;2); Sulfate Transmembrane Transporter</td>
<td>1.1</td>
<td>7.1</td>
<td></td>
</tr>
<tr>
<td>250475_at</td>
<td>SULTR2;1 AST68; Sulfate Transmembrane Transporter</td>
<td>-1.6</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>256244_at</td>
<td>SULTR4;2; Sulfate Transmembrane Transporter</td>
<td>1.9</td>
<td>3.3</td>
<td>1.1</td>
</tr>
<tr>
<td>249752_at</td>
<td>LSU2 (Response To Low Sulfur 2)</td>
<td>1.3</td>
<td>1.9</td>
<td>-1.15</td>
</tr>
<tr>
<td>256835_at</td>
<td>APS1 (ATP Sulfonylase 1); Sulfate Adenylyltransferase (ATP)</td>
<td>1.4</td>
<td>1.8</td>
<td>-1.5</td>
</tr>
<tr>
<td>245912_at</td>
<td>SULTR3;5 (Sulfate Transporter 3;5); sulfate transmembrane</td>
<td>-1.3</td>
<td>-1.5</td>
<td>2.1</td>
</tr>
<tr>
<td>gene symbol</td>
<td>gene name</td>
<td>chromosome</td>
<td>catalytic activity</td>
<td>function</td>
</tr>
<tr>
<td>-------------</td>
<td>-----------</td>
<td>-------------</td>
<td>-------------------</td>
<td>----------</td>
</tr>
<tr>
<td>258219_at</td>
<td>ATTDX</td>
<td>4</td>
<td>AMP + sulfite + glutathione disulfide = adenylyl sulfate + 2 glutathione.</td>
<td>Reduces sulfate for Cys biosynthesis. Uses glutathione or DTT as source of protons.</td>
</tr>
<tr>
<td>248676_at</td>
<td>ATSDI1</td>
<td>4</td>
<td>binding</td>
<td></td>
</tr>
</tbody>
</table>

4.20.6.5 Further details on titled genes

Some details were not mentioned previously about the designated genes involved in the effect of DBT on *Arabidopsis thaliana*, below are more details that indicates the gene location on chromosome and their specific functions.

- **255284_at**

  Chromosome 4
  Catalytic activity: AMP + sulfite + glutathione disulfide = adenylyl sulfate + 2 glutathione.
  Function: Reduces sulfate for Cys biosynthesis. Uses glutathione or DTT as source of protons.
  Induction: By sulfate starvation.
• **245912_at**, Chromosome 5, Probable sulfate transporter Colocalization with SULTR2;1, and co-expression in sulfate transport. Function: sulfate co-transporter that may play a role in the regulation of sulfate assimilation.

• **256244_at**, Chromosome 3, Probable sulfate transporter Function: sulfate co-transporter that may play a role in the regulation of sulfate assimilation.

• **255105_at**, Chromosome 4, Sulfate transporter The identification of a sulfur-responsive element in the promoter region of SULTR1;1, sulphate membrane transport, Function: regulation of sulfate assimilation and sulfate co-transporter that mediates the uptake of the environmental sulfate by plant roots under low-sulfur conditions.
• **262133_at**

Chromosome 1,
Sulfate transporter
Involved in protein-protein interactions that could control sulfate transport, the domain is critical for both the activity and biosynthesis/stability of the transporter.
Function: sulfate co-transporter that mediates the uptake of the environmental sulfate by plant roots. Plays a central role in the regulation of sulfate assimilation.

• **255958_at**

Sulfate transporter
Chromosome 1,
Function: sulfate cotransporter that mediates the loading of sulfate into the sieve tube. Plays a central role in the regulation of sulfate assimilation.
Induction: In roots and leaves by sulfate starvation.
Tissue specificity: Expressed in the phloem of cotyledons, hypocotyls and roots.

• **250475_at**

Sulfate transporter 2.1
Chromosome 5,
Colocalization with SULTR3,5 and coexpression in sulfate transport.
Function: sulfate cotransporter that may be involved in root-to-shoot translocation of sulfate. Plays a central role in the regulation of sulphate assimilation.
Induction: In roots by sulfate starvation or after selenate treatment.
Tissue specificity: Expressed in root cap, central cylinder of roots and in vascular tissues of leaves.
- **246310_at**
  Sulfate transporter 3.1
  Chromosome 3,
  Function: sulfate cotransporter that may play a role in the regulation of sulfate assimilation.
  Induction: By nitrogen starvation, but not by sulfate starvation.
  Tissue specificity: Expressed only in leaves.

- **255443_at**
  Sulfate transporter 3.2
  Chromosome 4,
  Function: sulfate cotransporter that may play a role in the regulation of sulfate assimilation.
  Tissue specificity: Expressed only in leaves.

- **245855_at**
  Sulfate transporter 4.1,
  Chromosome 5,
  Function: sulfate cotransporter that may play a role in the regulation of sulfate assimilation.
  Induction: By sulfate starvation in leaves.
  Tissue specificity: Expressed both in roots and leaves.
252119_at

Thioredoxin H-type 1
Chromosome 3,
Structural model of thioredoxin h1 from Arabidopsis thaliana in the oxidized state displays the conserved thioredoxin fold.
Function: Participates in various redox reactions through the reversible oxidation of the active center dithiol to a disulfide. It is known to activate a number of cytosolic enzymes.
Thioredoxins catalyze disulfide/dithiol exchange with various substrate proteins. While the human genome contains a single thioredoxin gene, plant thioredoxins are a complex protein family. A total of 19 different thioredoxin genes in six subfamilies have emerged from analysis of the Arabidopsis thaliana genome (Peterson et al., 2005)
Chapter Five

5. Conclusions and Recommendations

5.1 Conclusions based on biodesulfurisation by bacteria and plant cell cultures

*Rhodococcus erythropolis* IGTS8-5 and *Rhodococcus erythropolis* IGTS8-5G were chosen because of their well known biodesulfurisation activity so that novel immobilisation methods could be tested against the freely suspended cells that would constitute the control for comparisons. They were also used as comparison basis for the biodesulfurisation activity of the novel organisms used in this research, i.e., plant cell cultures.

The model sulfur compounds used were dibenzothiophene (DBT), 4,6-dimethyldibenzothiophene (4,6DMDBT), 4,6-diethyldibenzothiophene (4,6DEDBT) and 4-methyl-dibenzothiophene (4MDBT).

Both bacterial strains grew well in the chemically defined medium with glucose as the main carbon and energy source and the model sulfur compound DBT as the sole sulfur source. The immobilisation of bacterial cells in and on the porous coke particles was novel since there was no mention of it in the literature. Immobilisation was physically mediated when bacterial cells got entrapped inside the pores of the coke particles 0.8 g of cells were immobilized on 250 g of coke particles without refreshing the medium over 72 h while 1.8 g of cells were immobilised on 250 g of coke when the media was refreshed every 24 hours for 120 h after the initial immobilisation batch of 72h (over 192 h in total). The latter, were used repeatedly in twelve consequetive batch desulfurisation cycles during which the biodesulfurisation activity progressively decreased from over 95% removal of 100 ppm DBT to around 45% removal. This biodesulfurisation activity is equivalent to $310 \mu\text{mol } 2\text{-HBP (h}^{-1})(g \text{ dry cell weight})^{-1}$ during the first hour. Freely suspended cells on the other hand exhibited biodesulfurisation activity equivalent to $91 \mu\text{mol } 2\text{-HBP (h}^{-1})(g \text{ dry cell weight})^{-1}$. Unfortunately, after the first 24 h, the activity of the immobilised cells
decreased to 12 μmol 2-HBP (h⁻¹)(g dry cell weight)⁻¹. Efficiency gains in terms of time gained should be considered in the repeated use of the same immobilised biocatalyst twelve times.

Use of plant cell cultures for biodesulfurisation is the other novel aspect of this work. Plant cells are much larger than bacteria and tend to grow and exist as aggregates, fine (as in the case of *Arabidopsis thaliana*) or coarse (as in the case of *Armoracia rusticana*) which allows them to settle easily from suspending fluids. They can be easily handled and packed in to the columns, and crude oil and/or diesel can be made to flow through the packed bed of biocatalysts. Although they can grow slowly relative to bacteria, they survive much much longer under adverse conditions and they can be revived easily. Furthermore, plant cells have plasmodesmata (intercell pore channels) that allow the transport of various compounds from one cell to another that can be several hundred cells away. This property renders them more suitable for natural immobilisation systems such as the one used in this work; i.e. reticulated porous polyurethane matrix or PolyHipe materials. All these advantages of plant cell cultures were observed during this research.

*Armoracia rusticana* cell culture was chosen as the novel biocatalyst since this plant is a well known source of peroxidase enzyme which is involved in the biodesulphurisation metabolism according to the literature on bacterial biodesulphurisation. *Arabidospsis thaliana* on the other hand was chosen since its genome is completely sequenced, it is a model organism in genomics studies, and various bioinformatics databases, procedures and test kits such as the Affymetrics chip are available for research.

Our results indicate that cell suspensions of both plants do show biodesulphurisation activity by reducing the level of sulfur compounds, mainly DBT and other three derivatives mentioned above, from both aqueous and oil phase. The biodesulphurisation activity of both plant cell cultures is summarised in Table 5.1 which indicates that they were similar.
When compared to the bacteria, in terms of DBT consumption, the activity of *A. rusticana* was calculated as 55 μmol DBT h\(^{-1}\) g\(^{-1}\) DCW and 65 μmol DBT h\(^{-1}\) g\(^{-1}\) DCW for *A. thaliana* while in bacteria it was 50 μmol DBT h\(^{-1}\) g\(^{-1}\) DCW for IGTS8-5 and 37 μmol DBT h\(^{-1}\) g\(^{-1}\) DCW for IGTS8-5G.

Table 5.1 Summary of the biodesulfurisation activity of the plant cell cultures

<table>
<thead>
<tr>
<th>Experiment</th>
<th>DBT produced</th>
<th>DBT consumed</th>
<th>Armoracia rusticana growth, g FW/L</th>
<th>Arabidopsis thaliana growth, g FW/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Armoracia rusticana and Arabidopsis thaliana growth in GB5 medium</td>
<td>Yes</td>
<td>NA</td>
<td>30</td>
<td>25</td>
</tr>
<tr>
<td>Armoracia rusticana and Arabidopsis thaliana growth in GB5 medium containing 100 ppm DBT</td>
<td>No</td>
<td>Yes</td>
<td>40</td>
<td>35</td>
</tr>
<tr>
<td>Armoracia rusticana and Arabidopsis thaliana growth in sulfur-free medium containing 100 ppm DBT</td>
<td>No</td>
<td>Yes</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>Armoracia rusticana and Arabidopsis thaliana growth in GB5SF medium with 20% crude oil containing 200 ppm DBT</td>
<td>No</td>
<td>Yes</td>
<td>35</td>
<td>30</td>
</tr>
</tbody>
</table>
The GC-FID chromatogram of the sample from plant cell cultures growing in sulfur-free GB5 medium spiked with 100 ppm DBT as the sole sulfur source shows a noticeable accumulation of compounds with elution times similar to 2-HBP elusion time of 14.1-14.2 minutes. Since 2-HBP is the end product of the 4S pathway of bacterial biodesulfurisation, plant cells may be following either a similar pathway or share similar metabolic reactions in their biodesulfurisation.

Transcriptomics analysis of the plant cell cultures after exposure to the DBT when compared to control cultures showed alterations in gene expression levels several of which were related to sulfur metabolism and transmembrane transporters of sulfate.

In conclusions, although immobilisation of bacteria for biodesulfurisation in suitable materials using procedures that do not reduce activity should be a step forward in biocatalyst development for biodesulfurisation, plant cell cultures seem to be a more convenient and improved biocatalyst for biodesulfurisation.

### 5.2 Recommendations for future work

This work involved novel approaches to biocatalyst development for biodesulfurisation using immobilisation of bacteria on porous coke particles and using the plant cell cultures for their biocatalytic activity that seemed to reduce the levels of model sulfur compounds from both aqueous and oil phase. Because of the novelty of both approaches, most of the experiments although groundbreaking were preliminary. There was also a shortage of plant cell material that limited the amount of experiments that could be performed. It is therefore recommended that further experiments should be conducted in order to reproduce some of the results and to clarify some aspects as summarised below.

Bacterial biodesulfurisation with immobilised cells should be tested further using oil phase and quantified as much as possible. Effect of long term use, repeated batch cycles compared to continuous flow of aqueous and oil phase
through immobilised cell columns should be tested and compared quantitatively. Other methods of immobilisation, such as the use of polyHIPE should be tested.

As for the plant cell biodesulfurisation, first of all, other plants should be also screened for their biodesulfurisation activity since there may be plants with better activities. Then, for the chosen plant species, callus and cell suspension cultures should be bulked up so that experiments can be performed with due replication to check reproducibility. Also, it would be useful to store the plant cell culture long-term using suitable cryogenic techniques (although they are not as efficient as those for microbial and animal cell cultures).

The metabolic pathway for biodesulfurisation in plants is not known although obviously they are capable of reducing the levels of sulfur compounds. Therefore, labelled compounds and if possible gene deletion should be used for such research.

As shown in this research, transcriptomics analysis can reveal changes in gene expression levels. More experiments should be performed in this area in order to identify properly which genes or family of genes are affected alongside the metabolic pathway elucidation experiments under different conditions.

Another research approach can involve the transfer of bacterial biodesulfurisation genes to plant cells.

Plant cell biodesulfurisation is a novel area and therefore it can lead to a very large research activity involving several interdisciplinary areas.
References


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Appendix

6. A

Figure 6.1 correlation between od and dry cell weight concentration.
Figure 6.2 GC/ FID chromatogram shows DBT consumption by plant cell suspension
Figure 6.3 Oil spiked with DBT dissolved in ethanol retention time at 17.5 minutes

Figure 6.4 DBT dissolved in ethanol retention time at 17.5 minutes
Figure 6.5 GC/ FID chromatogram shows reduction of DBT in diesel
From 55.6 mV (100 ppm) to 41.3 mV (74 ppm) 4,6DEDBT at 21.88 minutes

From 19.0 mV (100 ppm) to 14.4 mV (83 ppm) 4MDBT at 18.8 minutes

From 21.7 mV (100 ppm) to 13.2 mV (66 ppm) 4,6 DMDBT at 19.8
Figure 6.6 GC/ FID chromatogram shows the reduction of (4,6DEDBT, 4MDBT, and 4,6 DMDBT) after three days incubation with plant cell suspension.

Figure 6.7 Oil spiked with DBT dissolved in ethanol and incubated with Armoracia rusticana & Arabidopsis thaliana

Figure 6.8 Oil directly spiked with DBT without ethanol retention time at 17.2 minutes
Figure 6.9 GC/ FID chromatogram shows (a) Crude oil contain 200 ppm DBT incubated with Plant cells, (b) Crude oil contain 40 ppm DBT, (c) Crude oil contain 200 ppm DBT, (d) Crude oil contain 200 ppm DBT incubated with plant cells for 15 Days.
Figure 6.10 GC/ FID chromatogram shows control (high peak) and consumed (low peak) of DBT in aqueous phase incubated with plant cells elution time at 17.2 minute.
Figure 6.11 GC/ FID chromatograms (a, b, c, and d) shows various stages of DBT consumption during the growth of plant cells in GB5 medium supplemented with 100 ppm DBT.
Figure 6.12 GC/FID chromatograms shows consumption of DBT at time 17.2 minutes after incubation with plant cells.

Figure 6.13 GC/FID chromatogram shows reduction of the DBT from the oil after incubation with plant cell suspension (a) 120 hours incubation, (b) 24 hours incubation, (c) control sample contain 200 ppm DBT.
Figure 6.14 several GC/FID chromatogram shows different concentration of DBT reduced after incubation with plant cell

Table 6.1 Array quality check

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Figure 6.15 GC/FID chromatogram shows reduction of DBT content elution time 17.2 minutes

Using Gibb's reagent

Molecular weight of 2-HBP = 170.21 g/mol

1.0 mM = 170 ppm 2-HBP

0.588 Mm 2-HBP = 100 ppm 2-HBP

DBT = 184.26 g/mol

Glucose = 180.06 g/mol

Glucerol = 92.09 g/mol