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Chapter 3

Diverse Groups of Isolated Bacteria for Biodesulfurization of Petroleum Products in Petroleum Refinery Industry

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Abstract

Various energy sources are required for different needs of daily life including use of electrical appliances and moving the automobiles and other transport machinery etc. A major source (~82%) for energy production is fossil fuels, and nearly half of it comes from petroleum. Incidentally, the naturally occurring crude oils contain numerous sulfur compounds, which upon combustion convert into sulfur-dioxide that lead to various environmental and health problems. For this reason, several countries have prescribed the guidelines to reduce the sulfur content in transportation fuels 0 to 10 ppm by the year 2020. In India also, Euro IV guidelines (maximum sulfur level at 50 ppm) has been implemented in 13 major metropolitan cities. While in other parts of the India, Euro III (50-350 ppm) stage is being followed.

Conventionally, organic sulfur from crude oil-derived fuels is removed by a 'hydrodesulfurization' (HDS) process, wherein the crude oil fractions are reacted with hydrogen at high pressure (150-3000 psi) and temperature (290-455°C) in the presence of metal catalysts i.e. CoMo/Al2O3 or NiMo/Al2O3. In this process, the organic sulfur gets converted into hydrogen sulfide and total sulfur concentration in oil-fractions can be reduced from 1-5% to 0.1%. Many sulfur compounds e.g. 4, 6-dimethyl dibenzothiophene (4, 6-DMDBT) are refractory to desulfurization and are often removed by 'deep HDS', where higher temperatures and pressures are used that makes the process very expensive.

Bio-desulfurization (BDS) has been suggested as an alternative method for achieving low sulfur levels in the crude oil fractions. It is remarkable for its mild operating conditions, greater reaction specificity afforded by the nature of biocatalysts and for not requiring molecular hydrogen. For this reason, biodesulfurization of thiophenic compounds e.g. dibenzothiophene and benzothiophene has been studied extensively. Several diverse groups of microorganisms have been isolated and reported for these groups of organosulfur compounds (Thiophenic and non-thiophenic) found in crude oil. They have shown Biodesulfurization mostly by reductive 4S pathway and some by related to degardative pathway like Kodama pathway. Studies on non-thiophenic compounds, however, are rare. Microbes for broad substrate range organo sulfur compounds desulfurization are being isolated, but little progress has been made. Isolated strain like Rhodococcus sp., Mycobacterium sp., Bacillus sp., Pseudomonas sp. have shown limited substrate range for organosulfur compounds found in crude oil/Diesel/Petrol. Recently isolated our lab isolate Gordonia sp. IITR100 has shown broad substrate range and biodesulfurized model compounds Dibenzyl sulfide, thianthrene, Dibenzothiophene, Benzonaphthothiopphene.

Hence we could say that we can try from even more new sources for microbe's isolation which could full fill the demand of industrial activity of 3.2-100 µmol/dry cell weight. Diverse group of microbes even from different sources may be explored to obtain the even enhanced features bacterium from industrial points of view. In this book chapter, I have shown various microbes isolated for Biodesulfurization along with nature of problem of desulfurization/Biodesulfurization.

Keywords: desulfurization; hydrodesulfurization; organosulfur compounds; biodesulfurization; diverse group of microbes

1. Sulfur Compounds in Crude Oil

Sulfur is nearly ubiquitous in fossil fuels, where it arises both as inorganic (e.g., elemental sulfur, hydrogen sulfide and pyrites) and as organic sulfur (e.g., sulfur atom or moiety present in a wide variety of hydrocarbons for example, disulfides, mercaptans, sulfones, thiols, thiophenes, thioethers and other more complex forms) that can account for approximately to 100% of the total sulfur content of petroleum liquids, crude oil and many petroleum distillate fractions. Organic sulfur in crude oils can range from 5 wt % down to about 0.1 wt % [1]. Al-

though the organic forms are predominant with over 200 sulfur containing organic compounds [2]. These compounds can be divided in three groups: (I) aliphatic and aromatic thiols and its oxidation products (bisulfides); (II) aliphatic and aromatic thioethers; (III) heterocycles based in thiophenic ring: thiophene, benzothiophene (BT), dibenzothiophene (DBT) and its alkyl derivatives [3].

Petroleum recovered from different reservoirs varies widely in compositional and physical properties [4] having different concentration of sulfur (0.4 to 5.0 wt %). In general, a crude oil from a region of a higher density contains a higher percent of sulfur [5].

The organosulfur compounds in petroleum include thiols, thioethers, and thiophenes, but the sulfur compounds that predominate in the so-called heavy fractions, where sulfur content is the highest, are primarily the condensed thiophenes [2]. In some cases the sulfur content is very high, e.g., the crude oil in Utah and California at the USA and in Germany contain 13.9, 5.5, and 9.6% of sulfur, respectively [3]. Sulfur in gasoline is mainly found in thiophenic and non-thiophenic compounds, and in diesel oil is found in benzo and dibenzothiophenes [6]. Total sulfur content varies on average, organic and inorganic sulfur comprise equal amounts [7], although there are some exceptions [8]. Organic sulfur is present in several forms; the principal moieties are sulfides, disulfides, thiols and thiophenes, while inorganic moieties are found in the form of pyrites [7].

While refiners gain some advantage in using higher quality crude, the price premium more than cancels the cost advantage for low-sulfur fuel refining [9]. Since there are many different sulfur-containing compounds in petroleum-derived fuels, the sulfur content is usually expressed as the weight percent (wt %) of sulfur in the fuel as demonstrated on 78 different crude oil types [10]. As mentioned before, the most important constituents are organic sulfur compounds, but inorganic sulfur can also be present. An overview of the range of organic sulfur contents in crude oils found in different countries over the world is given here in Figure 1.1 below.

Sulfur compounds in crude oil include thiols, sulfides, polysulfides, thiophenic and alkyl substituted isomers of thiophenic compounds containing a variety of aromatic rings (i.e.polycyclic aromatic sulfur heterocycles such as thiophene, benzothiophene, dibenzothiophene and benzonaphthothiophene) which are carcinogenic. The molecular structures of some of these sulfur components are presented in **Scheme 1.1**.

Distribution and amount of organic sulfur compounds reflect the reservoir and maturity of the oil. Chemically immature oils are rich in sulfur and often have a high content of non-thiophenic sulfur compounds [12]. Mercaptans in crude oils are generally of low molecular weight (less than eight carbon atoms). They are readily removed from crude oil during refinery processing and are negligible in petroleum products.

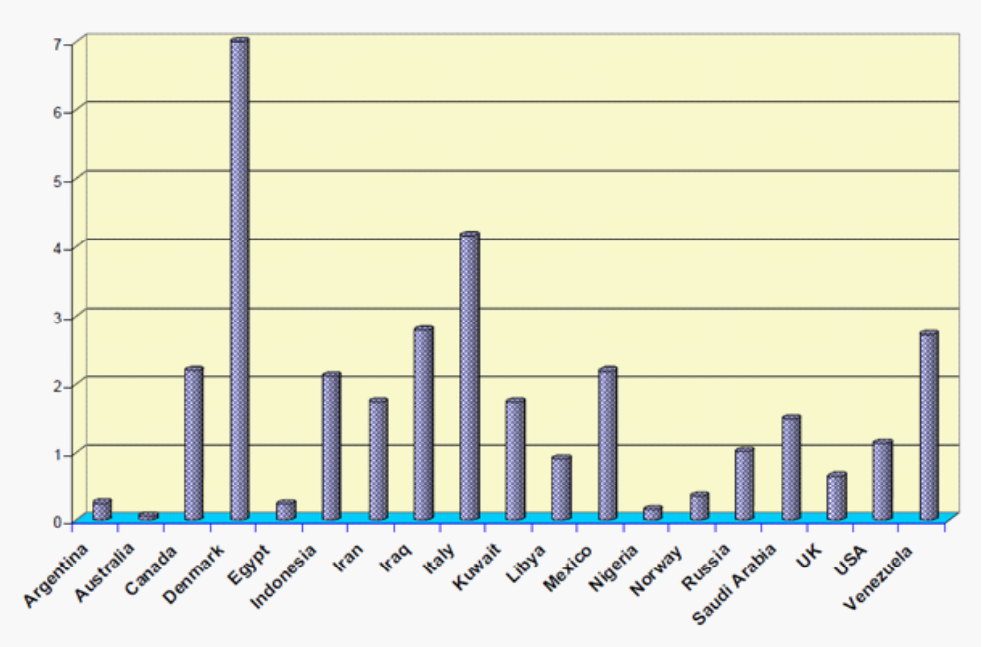
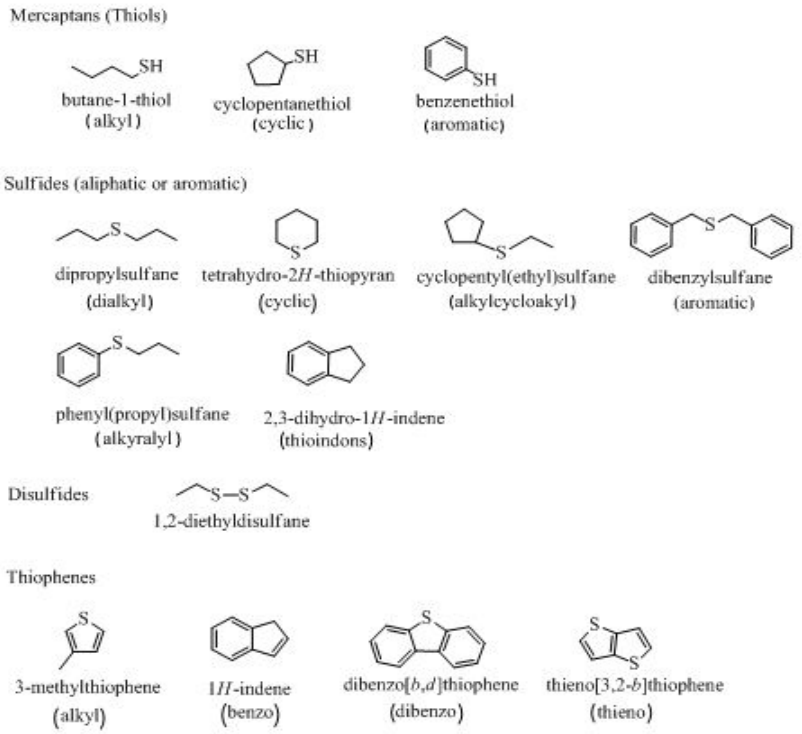


Figure 1.1: Average organic sulfur contents in crude oils [11].



Scheme 1.1: Different types of sulfur-containing organic compound identified in crude oils

Aliphatic sulfides (cyclic or acyclic) are major components of the sulfur-containing fraction of petroleum products, e.g. diesel fuels and heating oils. Aromatic sulfides are of lower concentration in the heavier cuts. Thiophenic sulfur is normally the most plentiful form but as mentioned before, depending on the reservoir history of the oil, other sulfur compounds are often present in appreciable quantities. The unsaturated five member heterocyclic ring, thiophenes, is an important constituent of high-sulfur oils and its derivatives are the most abundant sulfur compound in distillates and residues, including heavy fuel oils and bitumens.

2. Problems due to Sulfur Compounds in Crude Oil

Most of the hydrocarbons mined from the Earth are burned for energy and since most

liquid and solid (i.e., oil and coal) reserves are contaminated with sulfur, direct combustion of this fuel will lead to the release of vast amounts of sulfur oxides into the atmosphere [13,14]. These oxides (together with acidic nitrogen oxides) are responsible for poor air-quality, acid rain [15] and for ozone layer depletion [16].

SO_2 is also responsible for various health hazards, such as respiratory tract cancer and cardio-respiratory diseases [16]. SO_2 concentrations >100ppm in the atmosphere are harmful to the respiratory system of humans and a short-period exposure to 400-500 ppm is lethal [17] The mixting of SO_2 with dust in the atmosphere or with fog increases the noxious effect. The plant kingdom is also very sensitive to the SO_2 concentration; exposure to 1-2 ppm SO_2 provokes damages in few hours [17].

The presence of sulfur in oil has been related to the corrosion of pipeline, pumping, refining equipment, and with prior breakdown of combustion engines. Sulfur also contaminates many catalysts which are used in the refining and combustion of fossil fuels. Burning of gasoline and diesel emits sulfur dioxide (SO_2) or sulfate particulate matter leading to acid rain, which has harmful effects on aquatic and forest ecosystems, as well as on agricultural areas [18].

In oil spill accidents, some sulfur heterocyclic compounds are introduced in the environment. Some of these compounds (e.g. benzothiophene and its derivatives) present carcinogenic, mutagenic activities and acute toxicity to the organisms living in that ecosystem and environment. Condensed thiophenes are bioacumulated in organism tissues, which associated with their mutagenic, carcinogenic and toxic potential; considerably contribute to the negative effect [7].

3. Biodesulfurization

Since 1935, when an early account of microbial desulfurization of crude oil was published [19,20] there have been expended lots of interest in applying biodesulfurization (BDS) processes in the oil industry to demonstrate this ability of microorganisms. In the early 1950s, a series of U.S. patents were issued covering the use of bacteria to reduce the sulfur content of petroleum. However, early attempts were not found to work because of inability to control the bacteria [18]. From 1970s to 1980s, the U.S. Department of Energy (DOE) and other organisations have sponsored work to explore this technology. The isolated bacteria were not appropriate for commercial BDS technologies at that time, because they attack the hydrocarbon portion [21]. In the late 1980s, bacteria that could liberate sulfur from DBT (as the model sulfur compound) without attacking the hydrocarbon were identified [22,23]. During these decades significant contributions have been made to construct and operate a large scale oil biodesulfurization process that involve many challenges and in order to develop biological desulfurization processes numerous attempts have been made up until the present time.

Biological processes require relatively mild conditions (low pressures and low temperatures), which could be a major advantage of BDS. It can be noticed that biocatalytic desulfurization offers the petroleum industry several benefits over hydrodesulfurization (HDS) processes: capital cost savings, operating cost saving, flexibility to handle a wide range of petroleum streams, more rapid engineering and construction time, safer and milder conditions. Testimonies of Naser [24] have indicated that by BDS technology, the small refinery at Woodlands (Texas) achieved capital cost savings of approximately 50 percent and operating cost saving of 10-20 percent. In addition to cost savings, BDS will result in up to 80 percent less greenhouse gas emissions over HDS, because bioprocess operates at essentially room temperature and pressure. The scheme of the biocatalytic desulfurization process is presented in **Figure 1.3.** The biocatalyst is mixed with water/media and transferred to the bioreactor. The biocatalyst slurry and petroleum containing sulfur compounds are mixed with oxygen and stirred continuously in a tank reactor. The petroleum is desulfurized and separated from the aqueous/biocatalyst output stream. The biocatalyst and water are separated and the sufur by-product is removed from the process in the aqueous phase as sulfate, which can be disposed of sodium sulfate or ammonium sulfate. After the spent biocatalyst is removed the biocatalyst/water mixture is recycled to the bioreactor.

Basic microbiological researches have been done on the BDS mechanisms which have made a lot of progress in the recent years. Whereas significant removal of organic sulfur compounds from oil has not been demonstrated to date. To enable technological applications, to improve in knowledge on this field, the flux through this pathway must be enlarged considerably on activity, selectivity and stability of microorganisms applied in the process. For commercial applications, mixtures of microorganisms may be needed [10]. Therefore, currently genetic and metabolic engineering efforts are applied by researchers. They tried to identify and clone the genes involved in the sulfur removal pathway, therefore efficiency of biological process could be enhanced by increasing the number of copies of the genes, altering the gene to produce a more active or efficient product and increasing the amount of expression from each gene [25,26,27,28,29]. For example recombinants of *Rhodococcus erythropolis* containing multiple copies of the dsz-genes were used to study the conversion of sulfur compound [30,31].

4. Biodesulfurization Application

An industrial-scale process for petroleum biodesulfurization using aerobic microorganisms has not yet been demonstrated. However, through an improved understanding of the biochemistry and genetics of the desulfurization pathway, it is anticipated that improved biocatalysts with activities suitable for an industrial process will be developed [1]. Until the present date, studies on sulfur oxidative pathways have mainly been focused in model compounds, which limit the ability to demonstrate the commercial potential of BDS [32]. However, some

reported works involved several fractions of crude oil refining, including gasoline and diesel [33-38]. Efforts to increase the rate of sulfur removal from aromatic sulfur heterocycles have been possible due to the use of genetic engineering techniques or the use of immobilization matrices [39].

The selection of the petroleum feedstock in biodesulfurization will play a large role in the overall economic viability of the process. BDS may be utilized as a pre-treatment to crude oil before entering pipelines. It may also be applied as an alternative to hydro treating the crude at the refinery or it may be applied in the polishing of refinery products such as diesel or gasoline. As pre-treatment, the BDS unit may be used to treat marginally sour crudes (0.6-0.7% S), converting them to sweet crudes (<0.5% S). For this application, the extent of desired desulfurization is quite low, and this may serve as an attractive initial niche for BDS [40]. Inherent to all of the current bio processing of fossil feedstock's schemes is the need to contact a biocatalyst-containing aqueous phase with an immiscible or partially miscible organic substrate [41]. Factors such as liquid-liquid and gas-liquid mass transport, amenability for continuous operation and high throughput, capital and operating costs, as well as ability for biocatalyst recovery and emulsion breaking, are significant issues in the selection of a reactor for aqueous-organic contacting [40]. Biodesulfurization studies of fossil fuels usually involved intact cells as biocatalyst, which avoids the Dsz enzymes purification and facilitate the BDS industrialization. The immobilization of cells can be used to desulfurize DBT efficiently [42] being the life-time of immobilized cell biocatalysts more than 600 h [43].

Traditionally, impeller-based stirred reactors are used for such mixing, because of their ease of operation and wide acceptance in the chemical and biological processing industries. This kind of reactors promotes the contact between the aqueous and organic phases by imparting energy to the entire bulk solution, achieving water or oil droplet sizes of 100-300 μm in diameter when surfactants are not present. To obtain droplets of about 5 μm, the energy consumed by the reactor will be 5-fold higher [44]. In BDS processes, oil is mixed with an aqueous medium that contains biocatalytically active bacterial cells. Recovery of oil from the oil-water-bacteria mixture follows the biodesulfurization step as a separated batch process [45].

To date, there are some microbial desulfurization studies at laboratory scale involving petroleum fractions and coal. Energy BioSystems Corporation (ECB) was the only commercial venture dedicated to the development of biodesulfurization technology [1]. EBC's concept for a biodesulfurization process was not only to treat diesel, but also to produce a value-added surfactant byproduct to achieve a more economical process [13]. There was a plan to construct a demonstration-scale biodesulfurization process at the Petro Star refinery in Valdez, Alaska. The date for the construction of a demonstration plant was progressively postponed [1]. For the industrial application, DBT desulfurization was optimized by using response surface meth-

odology [46].

5. Oil Biodesulfurization

Early biodesulfurization research used model compounds like DBT, sometimes in aqueous systems bearing little resemblance to the conditions the biocatalyst would encounter in commercial applications [47]. In fact, the desulfurization rates of diesel oil were much smaller than those obtained for pure DBT [33].

Biodesulfurization of petroleum results in total sulfur removals between 30 and 70% for mid-distillates [48] 24 to 78% for hydro treated diesel [33,49,50], 20-60% for light gas oils [51,52] and 25-60% for crude oils [53]. Taking into account that BDS can be a complementary method to HDS, the study of fractions of pre-desulfurized oil is important. Grossman *et al.* (2001) reported a treatment by *Rhodococcus* sp. strain ECRD-1 of middle distillate oil whose sulfur content was virtually all substituted DBTs containing 669 ppm of total sulfur. Analysis of the sulfur content of the treated oil revealed that 92% of the sulfur had been removed, reducing the sulfur content from 669 ppm to 56 ppm.

In addition, studies of desulfurization with *Rhodococcus erythropolis* I-19, involving hydrodesulfurized middle distillate oil, showed that after 0, 1, 3 and 6 h, the sulfur concentrations were 1850, 1620, 1314 and 949 ppm, respectively. The first 230-ppm drop in total sulfur, observed after 1 h, corresponded primarily to a biotransformation of DBT and midboiling-range sulfur compounds. Between 1 and 3 h, another 300-ppm sulfur reduction occurred, with some evidence for more highly alkylated DBTs being affected. At 3 h, most of the DBT and much of the C1-DBTs were consumed. Between 3 and 6 h, desulfurization shifted to the higher-boiling-range sulfur compounds, resulting in an additional 365-ppm drop in total sulfur. Analysis of this middle distillate oil biodesulfurized from 1850 to 615 ppm sulfur showed the majority of the remaining sulfur to be thiophenes (75%), with 11% sulfides, 2% sulfoxides and 12% sulfones [31]. More recently, Zhang *et al.* (2007) reported a total reduction of 97% (to 6 μg ml-1) of the sulfur content of previous hydrodesulfurized diesel oil.

There are also some studies on desulfurization of oil fractions involving thermophilic bacteria such as *Paenibacillus* sp. [54]. When *Paenibacillus* sp. strains A11-1 and A11-2 were cultured in the presence of light gas oil containing 800 ppm of total sulfur at a high temperature, the bacteria grew well. Light gas oil is known to contain small amounts of sulfur and limited species of heterocyclic organosulfur compounds composed mainly of alkylated DBT derivatives. In conformity with the stimulated bacterial growth, the content of sulfur in the oil phase was significantly decreased, indicating that both *Paenibacillus* strains can desulfurize at high temperatures from the processed light gas oil.

However, these strains presented a very low desulfurization rate [55] lower than the

desulfurization rate obtained with *R. rhodochrous* [54]. The use of thermophilic bacteria has some advantages since it is not necessary to cool-down the oil fractions after the HDS, which makes this process less expensive [56].

Another advantage is the fact of reducing the possibility of contamination by undesirable bacteria that can negatively affect the BDS process [57]. Although the obtained removals are significant, this level of desulfurization is insufficient to meet the required sulfur levels for all oil derivatives [35].

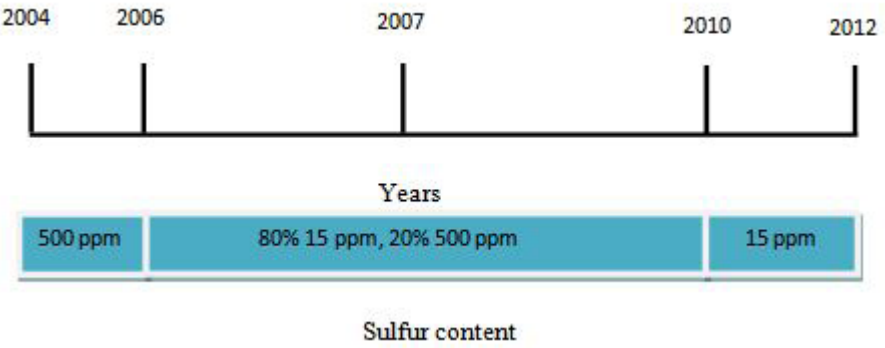
8. The need for Enhancement of Biodesulfurization

Clean fuels research including desulfurization has become an important subject of environmental catalysis studies worldwide. Sulfur content in diesel fuel is an environmental concern because sulfur is converted to (sulfur oxides) SO_x during combustion, which not only contributes to acid rain, but also poisons the catalytic converter for exhaust emission treatment. The problem of sulfur removal has become more apparent due to the increasing high sulfur contents in remaining reservoirs of crude oils and the low limit of sulfur content required in finished fuel products by regulations specified by the authorities. It is impossible to have clean atmospheric air, or in particular to reduce air pollution from the transport sector, without removing sulfur from fuels. No significant air pollution reduction strategy can work successfully without reducing sulfur to near-zero level. The Environmental Protection Agency of the United States (EPA) has set a target to reduce the sulfur content of diesel from 500 ppm (2010 regulation) to 15 ppm for the year 2012 [58].

Hydrodesulfurization (HDS) involves the catalytic reaction of hydrogen and the organic matter in the feed, at pressures ranging from 5 up to 10 MPa and temperatures between 300 and 350^{o}C, depending on the oil fraction and the required level of desulfurization [59,60]. Due to the high costs and inherent chemical limitations associated with HDS, alternatives for this technology are of great interest to the petroleum industry. The benefits of sulfur reduction would be more important than the costs, even though required refinery investments continue to be significant. The U.S. EPA found human health benefits and environmental profits due to sulfur reduction ten times higher than the costs [61]. Furthermore, a European study showed that ultralow sulfur fuels significantly reduce total fuel costs by increasing fuel economy from which the considerable potential for greenhouse gas emission reductions adds further payback to the health, environmental, and social benefits of sulfur reduction.

The global refining industry has spent about $37 billion on new desulfurization equipment and an additional $10 billion on annual operating expenditures for sulfur removal through the last 10 years to meet the new sulfur regulations [62]. More than 70 million barrels of crude are worldwide produced each day, of which half is considered to be "high sulfur" (>1%) Energy [63]. This concern will become more serious owing to the decreasing availability of low-

sulfur fuels. So in many areas of the world industry needs new technologies to reduce sulfur to the ultralow levels now acceptable in use. Current costs might be reasonable; the refining industry continues to develop more active catalysts and novel processes for the removal of sulfur in order to reduce costs even more.



Evolution of permissible sulfur content of diesel fuel [64].

9. Attempts Made for Improved Biodesulfurization

9.1. Search for new strains

In order to develop biologically enhanced desulfurization technology, a range of bacteria have been previously isolated which have been shown to be able to remove sulfur from organic compounds that commonly exist in petroleum [65]. However, this activity is unlikely to be sufficient for commercial applications where the requirement is for microorganisms with high activity and selectivity for different sulfur compounds [66]. To achieve this requirement, there needs to be about a 500-fold increase in the rate of biodesulfurization of currently used bacteria [1]. Therefore due to a range of problems (one of which is the low desulfurization capability of the bacteria employed, which makes the process uneconomical) this technology area needs to be enhanced by new developments. Bacteria, which exist in the oil fields, have a great potential to degrade sulfur compounds in fuel [67]. Research over the last few years has shown that oil fields contain microbial communities that influence the petroleum quantity and yield of petroleum produced and its quality. Professionals in the petroleum industry today clearly understand the critical role that regulating microbial activity has in enhancing the beneficial effects. Accordingly newly isolated strains of bacteria continue to attract attention for their potential application to desulfurization. Since DBT is a typical recalcitrant organic sulfur compound in petroleum, it has been a model reactant in the treatment of oil [68, 69]. In 1985 a strain of *Pseudomonas* that could desulfurize DBT was described by Isbister and Koblynski, but unfortunately before the metabolic pathway could be fully characterized, this strain was lost [70]. After 40 years of research effort, Kilbane isolated a suitable bacterium named *Rhodococcus erythropolis* IGTS8 (Kilbane, 1990). Since then many researchers have isolated bacteria capable of degrading DBT via the 4S pathway and a variety of DBT- desulfurizing bacteria have been reported. A list of which has been given by Mohebali and Ball, **table 1.1.**

Table-1.1: List of isolated bacteria those are capable of selectively degrading DBT and its derivatives via the 4S pathway [71].

Bacterium	Reference
Agrobacterium sp. strain MC501	Constanti *et al.* (1994)
Arthrobacter sp. strain ECRD-1	Lee *et al.* (1995)
Arthrobacter sulfureus	Labana *et al.* (2005)
Bacillus subtilis strain WU-S2B	Kirimura *et al.* (2001)
Bacillus brevis strain R-6	Jiang *et al.* (2002)
Bacillus sphaericus strain R-16	Jiang *et al.* (2002)
Bacillus subtilis strain Fds-1	Ma *et al.* (2006c)
Corynebacterium sp. strain SY1	Omori *et al.* (1992)
Corynebacterium sp. strain P32C1	Maghsoudi *et al.* (2000)
Corynebacterium sp. strain ZD-1	Wang *et al.* (2006)
Desulfovibrio desulfuricans	Yamada *et al.* (1968)
Gordona sp. strain CYKS1	Rhee *et al.* (1998)
Gordona sp. strain WQ-01	Jia *et al.* (2006)
Gordonia alkanivorans strain 1B	Alves *et al.* (2005)
Gordonia sp. strain F.5.25.8	Duarte *et al*. (2001)
Gordonia sp. strain ZD-7	Li *et al.* (2006)
Gordonia alkanivorans RIPI90A	Mohebali *et al.* (2007)
Klebsiella sp.	Dudley & Frost (1994)
Mycobacterium sp. strain G3	Nekodzuka *et al.* (1997)
Mycobacterium sp. strain X7B	Nekodzuka *et al.* (1997)
Mycobacterium sp. strain ZD-19	Li *et al*. (2003)
Mycobacterium goodii strain X7B	Chen *et al.* (2008)
Mycobacterium phlei strain SM120-1	Srinivasaraghavan *et al.* (2006)
Mycobacterium phlei strain GTIS10	Kayser *et al.* (2002)
Nocardia globelula	Wang & Krawiec (1994)
Nocardia globerula strain R-9	Jiang *et al*. (2002)
Nocardia asteroids	Olson (2000)
Paenibacillus sp. strain A11-2	Konishi *et al.* (1997)
Pseudomonas abikonensis strain DDA109	Yamada *et al.* (1968)
Pseudomonas jianii strain DDC279	Yamada *et al.* (1968)
Pseudomonas jianii strain DDE 27	Yamada *et al.* (1968)
Pseudomonas delafieldii strain R-8	Jiang *et al.* (2002)
Pseudomonas sp. strain ARK	Honda *et al.* (1998)
Pseudomonas putida strain CECT5279	Alcon *et al*. (2005)
Rhodococcus erythropolis strain IGTS8	Kilbane (1992)
Rhodococcus sp. strain UM3	Purdy *et al.* (1993)

Rhodococcus sp. strain UM9	Purdy *et al.* (1993)
Rhodococcus erythropolis strain D-1	Izumi *et al.* (1994)
Rhodococcus sp. strain ECRD-1	Lee *et al.* (1995)
Rhodococcus erythropolis strain H-2	Ohshiro *et al*. (1995)
Rhodococcus sp. strain X309	Omori *et al.* (1995)
Rhodococcus sp. strain B1	Denis-Larose *et al*. (1997)
Rhodococcus erythropolis strain I-19	Denis-Larose *et al.* (1997)
Rhodococcus erythropolis strain KA2-5-1	Folsom *et al.* (1999)
Rhodococcus sp. strain P32C1	Kobayashi *et al.* (2000)
Rhodococcus sp. strain T09	Maghsoudi *et al.* (2000)
Rhodococcus sp. strain IMP-S02	Matsui *et al.* (2001)
Rhodococcus sp. strain DS-3	Akbarzadeh *et al.* (2003)
Rhodococcus sp.	**Ma** *et al.* **(2006d)**
Rhodococcus erythropolis strain XP	Labana *et al.* (2005)
Rhodococcus sp. strain 1awq	Yu *et al.* (2006)
Rhodococcus erythropolis strain XP	Ma *et al.* (2006a)
Rhodococcus erythropolis strain DS-3	Yu *et al.* (2006)
Rhodococcus erythropolis strain DR-1	Ma *et al.* (2006b)
Rhodococcus erythropolis strain NCC-1	Li *et al.* (2007a)
Rhodococcus erythropolis strain LSSE8-1	Li *et al*. (2007b)
Sphingomonas sp. strain AD109	Zhang *et al.* (2007)
Sphingomonas subarctica strain T7b	Darzins & Mrachko (1998)
Xanthomonas sp.	*Gunam et al. (2006)*
Gordonia sp. IITR100	Ahmad *et al*. (2014)

9.2. Bacteria, characterized for the desulfurization of model thiophenic compound i.e. dibenzothiophene and benzothiophene

Heterocyclic compounds such as dibenzothiophene, benzothiophene and their alkylated derivatives have been found to be very recalcitrant against the attacks by the chemical catalysts. Henceforth, development of the catalysts capable of desulfurizing these HDS-resistant thiophenes is now needed for deeper desulfurization of petroleum fractions. Biodesulfurization (BDS) in which microbial catalysts are used to remove sulfur from the petroleum fractions has come to public notice as an environmentally benign process (Monticello et al., 1995) as mentioned earlier. It may be the best choice to employ a microbial catalyst capable of desulfurizing dibenzothiophene, benzothiophene, benzonaphthothiophene and their alkyl derivatives in the BDS process.

Many microorganisms such as *Rhodococcus sp*. IGTS8 [72], *Rhodococcus erythropolis*

D-1 [73] and N1-36 [74] have been reported to mediate desulfurization of dibenzothiophene. The reaction is accompanied by the production of sulfite ions and 2-hydroxybiphenyl. In nature, many bacterial strains capable of desulfurizing dibenzothiophene have also been reported to desulfurize benzothiophene. On the other side, it has been shown that benzothiophene is also susceptible to microbial transformation. Fedorak et al. have reported that a *Pseudomonas* strain BT1 converted benzothiophene to benzothiophene-2, 2-dione and 3-methyl benzothiophene to its sulfoxide and sulfone [75]. Boyd *et al.* (1993) have demonstrated that a toluene-degrading mutant strain *Pseudomonas putida* UV4 transforms benzothiophene to three dihydrodiols; cis-4, 5-dihydro benzothiophene, cis- and trans-2, 3-dihydro benzothiophene. However, none of these bacteria produced desulfurized metabolites from benzothiophene and its derivatives. *Gordonia* sp. strain 213E (NCIMB 40816) desulfurizing benzothiophene had been isolated and it had been shown that this strain cannot grow in a mineral salt medium containing dibenzothiophene as the sole sulfur source [76]. Thermophilic *Paenibacillus* sp. strain A11-2 that desulfurize dibenzothiophene [77], was found to degrade benzothiophene. *Klebsiella* sp. 13T was found to remove 22-53 % of sulfur from different petroleum oils [78].

G. alkanivorans strain 1B can remove selectively the sulfur from DBT producing 2-hydroxybiphenyl the only detected metabolic product. In equimolar mixture of DBT/BT as a source of sulfur in the growth medium, *G. alkanivorans* strain 1B utilized both compounds in a sequential way; BT was the preferred source of sulfur. When BT concentration was decreased to a very low level, then only DBT was utilized as the source of sulfur for bacterial growth [79]. *Rhodococcus* sp. KT462, which can grow on either benzothiophene (BT) or dibenzothiophene (DBT) as the sole source of sulfur, was newly isolated and revealed that strain KT462 has the same BT desulfurization pathway as that reported for *Paenibacillus* sp. A11-2 and *Sinorhizobium* sp. KT55. The desulfurized product of DBT produced by this strain, as well as other DBT-desulfurizing bacteria such as *R. erythropolis* KA2-5-1 and *R. Erythropolis* IGTS8 was 2-hydroxybiphenyl. A resting cells of this strain can degrade various alkyl derivatives of BT and DBT [80]. Sinorhizobium sp. KT55 was the first Gram-negative isolate to be capable of utilizing benzothiophene as the sole source of sulfur. Benzothiophene desulfurization pathway of this strain is 3-benzothiophene sulfoxide, 3-benzothiophene sulfone, 3-benzo[e][1, 2] oxathiin S-oxide, 3-o-hydroxystyrene [81,82].

A benzothiophene-desulfurizing bacterium with novel desulfurization pathway was isolated and identified as *Gordonia rubropertinctus* strain T08. Metabolites formed were detected to be benzothiophene sulfoxide, benzothiophene sulfone, benzo[e]- [1,2]oxathiin S-oxide (BT-sultine), benzo[1,2]oxathiin S,S-dioxide (BT-sultone), o-hydroxystyrene, & 2-coumaranone, but not 2-(2'-hydroxyphenyl)ethan-1-al, that has been reported to be a desulfurized product of mesophilic Nocardio forms [83]. Naphtho [2, 1-b] thiophene (NTH) is an asymmetric structural isomer of dibenzothiophene (DBT), NTH derivatives can also be detected in diesel oil

following hydrodesulfurization treatment. *Rhodococcus* sp. strain WU-K2R could grow in a medium with NTH as the sole source of sulfur. WU-K2R could also grow on NTH sulfone, benzothiophene (BTH), 3-methyl-BTH, or 5-methyl-BTH as the sole source of sulfur but could not utilize DBT, DBT sulfone, or 4,6-dimethyl-DBT. On the other hand, WU-K2R did not utilize NTH or BTH as the sole source of carbon. Desulfurized NTH metabolites were identified as NTH sulfone, 2′-hydroxynaphthylethene, and naphtha [2, 1-b] furan. Moreover, since desulfurized BTH metabolites were identified as BTH sulfone, benzo[c] [1,2] oxathiin S-oxide, benzo[c] [1,2] oxathiin S, S-dioxide, o-hydroxystyrene, 2-(2′-hydroxyphenyl) ethan-1-al, and benzofuran,thus WU-K2R desulfurized NTH and BTH through the sulfur-specific degradation pathways with the sulfur selective cleavage of carbon-sulfur bond [84]. The biodesulfurization of DBT by resting cells of *Pseudomonas putida* CECT5279 was enhanced by 140 % in a batch process and 122 % in a fed-batch process, in the presence of a co-substrate 1.5 % acetic acid [85].

9.3. Bacteria desulfurizing sulfidic and thiophenic organosulfur compounds

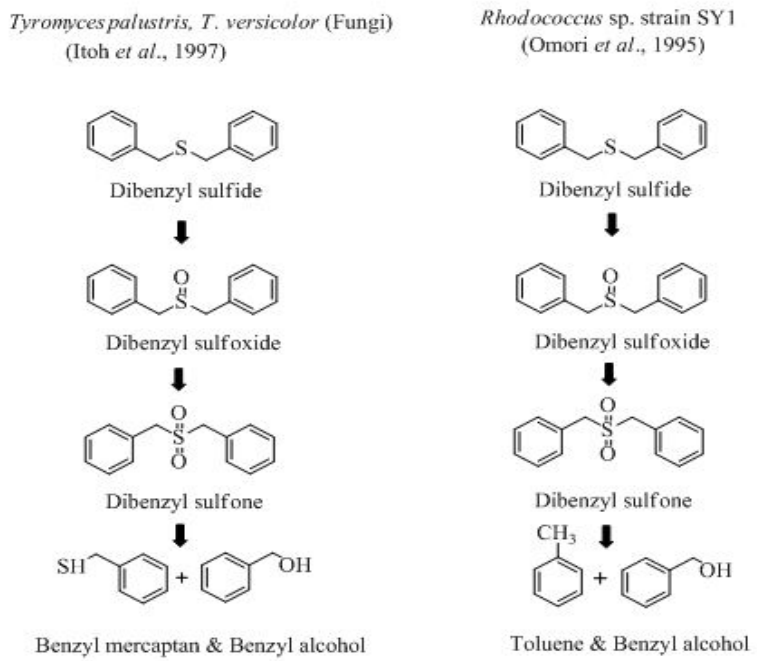
Large reserves of different heavy crude oils which have high viscosity, correlated to the average molecular weight of the material and to the asphaltene content have been reported [86, 87]. Transportation of such heavy and viscous oils by pipeline requires the addition of low molecular weight hydrocarbon diluents, which are increasingly expensive and hard to manage. Decreasing average molecular weight of the crude oil on-site before it is sent by pipeline to the refinery would decrease reliance on diluents and make transportation more economic and easiear. In particular, reducing the molecular weight of the asphaltene fraction could contribute to a decrease in the viscosity of the oil. According to the molecular model proposed by [88], the asphaltenes found in different heavy crude oils are polycyclic aromatic structures linked by aliphatic chains of various lengths. Ethers sulfides and esters have been identified as linking structures in these aliphatic bridges [89]. Selective chemical cleavage of aliphatic sulfide bonds results in as much as a fourfold reduction in the molecular weight of heavy asphaltene fractions [89]. Biocatalytic cleavage of these carbon-sulfur bonds should achieve the same effect.

The use of a biocatalyst avoids the problems of available technologies for viscosity reduction such as avoiding liberation of hydrogen sulfide, thermal visbreaking and the production of unstable oil. The focus of petroleum biodesulfurization research has been on the aromatic compound dibenzothiophene and on alkyl-substituted dibenzothiophenes. Several bacteria can remove the sulfur from dibenzothiophene by the 4S pathway, leaving the carbon structure intact as 2-hydroxybiphenyl [90,91,92]. Infact, this kind of attack would not reduce the molecular size of asphaltenes and therefore is inappropriate for the viscosity reduction approach. Instead, the ideal biocatalyst would affect selective carbon-sulfur bond cleavage in a broad range of aliphatic sulfides, but would not attack aromatic sulfur compounds such as

dibenzothiophene. Thus, the goal is not to achieve general biodesulfurization of the crude oil, but rather to target cleavage of key carbon-sulfur bonds that would depolymerise asphaltenic molecules and decrease the crude oil viscosity.

There are comparatively few reports of the degradation of aliphatic sulfides, particularly of carbon-sulfur bond cleavage in high molecular weight representatives of this type of compounds. Smaller compounds, including methyl, ethyl, propyl and butyl sulfides [93,94], 2-chloroethyl ethyl sulfide [95,96], are cleaved at the sulfur atom. Jenisch-Anton *et al.* (2000) showed bacterial degradation of the n-alkyl chain of phytanyl octadecyl sulfide and Van Hamme *et al.* (2003) showed fungal oxidation of the sulfur atom in dibenzyl sulfide, but carbon-sulfur bond cleavage was not observed in these larger compounds. A mutant of the dibenzothiophene-desulfurizing bacterium *R. erythropolis* strain IGTS8 is able to use di-n-octyl sulfide as a sulfur source, but this compound is not a substrate for the wild-type organism [97]. Recently, Van Hamme *et al.* (2004) used the fluorinated compound bis-(3-pentafluorophenylpropyl) sulfide to isolate *Rhodococcus* sp. strain JVH1, which uses this compound as a sulfur source by a mechanism analogous to the 4S pathway, although JVH1 does not use dibenzothiophene as a sulfur source.

Compared to sulfur-containing ring structures such as thiophenes and DBTs, relatively little information is available on the microbial metabolism of compounds with sulfur moieties present within alkyl chains. These structures are important as bridges in the high-molecular-weight asphaltene components of petroleum [98]. Therefore, biological attack on sulfides is of considerable interest for biological heavy-oil viscosity reduction. Bioremediation efforts directed towards Yperite [bis (2-chloroethyl) sulfide; mustard gas] contamination have prompted some work in this area. For example, IGTS8 is able to use 1-chloroethyl sulfide as the sole sulfur source [99]. Rhee *et al.* (1998) reported that the DBT-desulfurizing *Gordona* strain CYKS1 can also use dibenzyl sulfide (DBS) as the sole sulfur source, but the intermediate metabolites were not identified. In a study on fungi, Itoh *et al.* (1997) reported that *T. versicolor* IFO 30340 and *Tyromyces palustris* IFO 30339 metabolize DBS to benzyl alcohol and benzyl mercaptans. Similarly, *Rhodococcus* sp. strain SY1 reportedly converts dibenzyl sulfoxide to benzyl alcohol and toluene [100], **scheme 1.6.**



Scheme 1.6: Pathways reported for the desulfurization of DBS by [101,102].

9.4. Bacteria desulfurizing non-thiophenic organosulfur compounds

Studies on the desulfurization of non-thiophenic sulfur compounds like 1, 4-dithiane or thianthrene, however, are scarce [103,104]. Thus, characterization of three bacterial strains *Rhodococcus erythropolis* EPWF, *Pseudomonas* sp. K1oA and *Rhodococcus* sp. KIbD has been reported, which were able to grow on 1, 4-Dithiane under sulfur limiting conditions [103]. No metabolites, however, were detected in either of the culture extracts. Further, while the desulfurization of 1, 4-dithiane by *Rhodococcus sp*. K1bD was inhibited by >90% in presence of DBT [103], results with other bacteria are not available. Similarly, growth of an isolated strain *Rhodococcus* sp IGTS8 by using TA as sulfur source [105], and its oxidation by a thermophilic Sulfolobus acidocaldarius strain, has been shown [106]. But, again no information is available about the formed metabolites. In another study, oxidation of TA to TA-monosulfoxide by the ligninase from *Phanerochaete chrysosporium*, in the presence of hydrogen peroxide, has also been reported [107]. No reports, however, are available where in influence of other sulfur compounds on the desulfurization of TA has been evaluated.

9.5. Genetic engineering approaches to improve biodesulfurization

Even with this number of isolated bacteria, the desulfurization activity of naturally occurring bacterial cultures is not high enough for the requirements of industry and a successful commercial process is still awaited. To achieve this, therefore we need to isolate new species and identify the genes responsible for desulfurization and manipulate the system involved by genetic engineering techniques and it is highly likely that future biodesulfurization research will focus on development of this promising research area along these lines. One of the popular strategies in metabolic engineering of the bacteria is to change host strains in order to take

advantage of another strain's properties to get a higher metabolic rate. For example, several research studies have focused on over expression of the enzymes involved in microbial desulfurization.

The DNA encoding the Dsz enzymes can be transferred into a host cell [108] or the gene amplified with designed primers [109]. Matsui *et al.* (2001) used a recombinant strain capable of desulfurizing both DBT and BT as the sole source of sulfur [110, 111]. To improve the uptake of sulfur compounds in oil fractions, Watanabe *et al.* (2003) transferred the *dsz* genes from *Rhodococcus erythropolis* KA2-5-1 into the *Rhodococcus erythropolis* MC1109. The desulfurization activity of the new strain was about twice that of the previous strain. In order to enhance the expression of the genes involved, the *dsz* genes from *Rhodococcus erythropolis* DS-3 were integrated into the *Bacillus subtilis* and yielded recombinant strains with higher desulfurization efficiency [112]. Li et al. (2007) enhanced the desulfurization ability of *Rhodococcus erythropolis* DR-1 by removing the gene overlap in the operon.

Other efforts were made include (1) supplying the oxidoreductase gene from *Vibrio harveyi*, which supplements the *Dsz*D activity in Trans, and enhanced the activity by around 7 fold [113]. (2) By supplementing the existing *dsz* genes in *R.erythropolis* KA2-5-1by a plasmid that contained two additional copies of *dsz*ABC genes and one *dsz*D gene which led to an increase by 4 fold [114]. (3) By making a transposon mutant in the membrane leading to enhancement in the uptake of organosulfur compounds and increase in the desulfurization activity by 2 fold [115]. (4) By supplying the *dsz*B gene in a plasmid where 16 nucleotides in the 5'-untranslated region were changed led to an increase in the activity by 9 fold [116]. [5] By expressing the desulfurization genes from *Mycobacterium* sp.G3 under phsp60 promoter leading to an increase in activity by 1.2 fold [117,6] changing the order of desulfurization genes from *dsz*ABC to *dsz*BCA which led to an increase in activity by 12 fold [118]. Separately, in a chemostat approach a gain of function phenotype i.e. capability to additionally desulfurize 5-methyl benzothiophene and octyl sulfide appeared due to a mutation V261F in dszC gene [119].

10. Bottlenecks for Biodesulfurization Application

A significant stumbling block to the commercialization of BDS is the rate at which whole bacterial cells can remove sulfur [120]. Biocatalyst activity, the oil/water volume ratio and biocatalyst stability constitute the most important technical bottlenecks in the development of biodesulfurization processes. The highest bioconversion values were obtained by unspecific aerobic microorganisms such as *Rhizobium meliloti* [1200 mg DBT removed $(g^{-1}(DCW)\ h)^{-1}$]. However, these destroy the hydrocarbon structure of the sulfur compound [121]. The involvement of three enzymes and two coenzymes in biocatalytic desulfurization makes the use of isolated free or immobilized enzymes difficult. Consequently, schemes using whole cells ap-

pear more feasible because they will allow cofactor regeneration in situ. Another problem to the implementation of a BDS process is the fact that the sulfur requirement of bacteria is low when compared to the level of sulfur found in fuels. In *Rhodococcus* sp., the cells were found to require 0.1 mM of sulfur for normal growth [122]. Only 1% of bacterial dry weight is sulfur [123], which implies a very low need in relation to this element. The sulfur content in fossil fuels is about 100 mM and thus, the bacteria cease desulfurization before its total removal.

The utilization of organic solvents and emulsifiers supports protein solubilisation and enzymatic reactions in hydrophobic environments [124]. These compounds allow the organo-sulfur compounds, which have very low water solubility, to be more available to enzymes and microbial cells [125].

As mentioned above, changing the genes of the host cell for those involved in desulfurization is a popular strategy in metabolic engineering to take advantages of desirable properties another strain such as its physical properties, growth properties or higher intrinsic metabolic rate. Since these are not yet understood, a better understanding of the factors that contribute to the biodesulfurization pathway is needed so as to achieve high level expression of the gene [1] and future research to isolate new strains and identify the biocatalyst would be helpful to develop this promising research area.

Despite significance progress in biodesulfurization, the desired rates and broad substrate range are yet to be realized and more efforts are needed in this direction. Recently, we described characterization of a bacterium *Gordonia* sp. IITR100, isolated from a petroleum-contaminated soil which affects the desulfurization of several organosulfur compounds thiophenic (DBT), aliphatic (DBS) and non-thiophenic (thianthrene) [126,127,128,129]. Pathway and genes of desulfurization of DBS, Thianthrene, Benzonaphathothiophene have been characterized by *Gordonia* sp. IITR100 which is in accordance of 4S pathway [126,12,128,129,130]. Final metabolites of Dibenzyl sulfide was well characterized by the using the analytical technique of GC/GC-MS, and LC/LC-MS, was found to be Benzoic acid. Optimization of this strain has also been reported for desulfurization of crude oil, petrol and diesel. 76% sulfur reductions in crude oil, 98% sulfur reduction in diesel were reported by *Gordonia* sp. IITR100 [131]. Finding such a industrially applicable bacterium, this IITR100's whole genome sequence have been elucidated recently, [132].

Here it can be concluded that if efforts were being made, microbes may be employed for industrial Biodesulfurization which would be cheap in comparison to current process of costly Hydrodesulfurization and deep desulfurization. So more emphasis should be given to microbes which have shown potential for wide substrate range, high temperature tolerance and better activity for sulfur removal from crude oil, diesel and petrol.

11. References

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