DEVELOPMENT OF A MODIFIED METHOD FOR DESULFURIZATION OF DBT USING A RECOMBINANT BACTERIAL STRAIN

A DISSERTATION

Submitted in partial fulfillment of the requirements for the award of the degree of

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By

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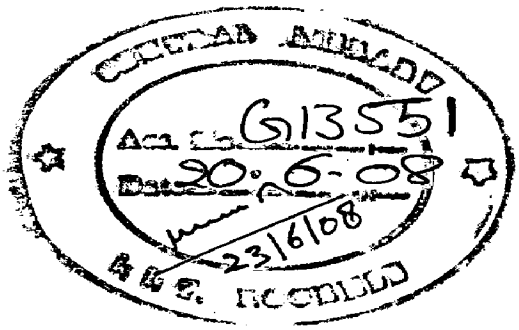

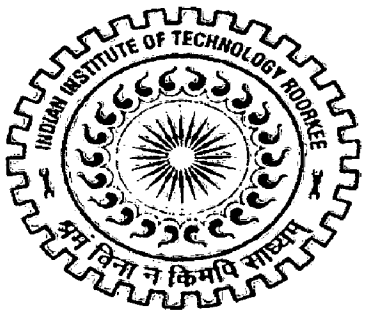

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ABSTRACT

Sulfur content in crude oil available from various sources ranges from 0.03 to values as high as 8.0 wt%. These significantly high quantities of sulfur must be removed before the crude oil processing otherwise the combustion of this oil results in severe environmental pollution like acid rain. Due to high utility and operating costs, the conventional Hydrodesulfurization process (HDS) is considered to be uneconomic. The biotechnological option, Biodesulfurization (BDS) seems to be the attractive low cost, environmentally benign technology.

Here we reported the development of recombinant bacterial strain which was designed by introducing the plasmid pSAD 225-32 containing desulfurization *dsz* genes, which was isolated from *Rhodococcus erythropolis* IGTS8 into a gram negative solvent tolerant bacterium, *Pseudomonas putida* (MTCC 1194). The recalcitrant heterocyclic organic sulfur compound Dibenzothiophene (DBT) was used as model compound for the biodesulfurization studies. This recombinant bacterium can desulfurize DBT in the sulfur specific 4S-pathway with out breaking the carbon-carbon bond.

It has been observed that for the same concentration of DBT, the recombinant strain's growth rate and the consumption of DBT are greater than that of parent strain. The growth parameters μ_{max} and K_s were determined to be 0.0466 h^{-1} and 8.6875 mg/l respectively. This genetically modified bacteria desulfurized 73.1% of 1.2 mM DBT in 67 h during cultivation.

The actual biodesulfurization reaction industrially occurs in two-phase mixture containing oil to be desulfurized and the bacterial cell suspension, in aqueous phase. The desulfurization studies were performed using model oil. DBT dissolved in n-hexadecane is known as model oil, hexadecane represents hydrocarbons present in the 40% of the diesel oils. The optimum oil to water (O/W) operating ratio was determined to be 1:2. At the optimum O/W ratio the recombinant bacteria desulfurized 96.95% of 2 mM DBT with in

24 h. The effect of surfactant, Tween-80 in enhancing the end product, 2-Hydroxybiphenyl (2-HBP) formation was also studied. After 36 h of desulfurization reaction, the 2-HBP produced was 1.5 times higher with the reaction mixture containing 1 g/l than that formed without surfactant. At the optimum O/W ratio containing optimum surfactant concentration of 1 g/l, the resting cells of recombinant bacterium desulfurized 97.88% of 25.2 mM of DBT dissolved in n-hexadecane (model oil) with in 56 h with the specific desulfurization rate of 0.4397 mg sulfur/g biomass/l and it also desulfurized the diesel oil containing 22.025 g/l to 2.934 g/l in 90 h with desulfurization rate 6.67 mg sulfur/g biomass/l.

We conclude that the developed recombinant bacteria can efficiently desulfurize DBT in both aqueous as well as two phase media and also the real fraction of petroleum, diesel. Further research on this area of biodesulfurization using genetically modified microorganism may remove the bottlenecks present in the way of commercialization of BDS process.

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NOMENCLATURE

BDS	Biodesulfurization
CHNS analyzer	Carbon, hydrogen, nitrogen and sulfur elemental analyzer
DBT	Dibenzothiophene
FID	Flame ionization detector
GC	Gas chromatography
2-HBP	2-hydroxybiphenyl
HDS	Hydrodesulfurization
K_s	Half saturation constant (mg/l)
O.D	Optical density
O/W	Oil to water ratio
S	Substrate concentration (mg/l)
t	Time (h)
μ	Specific growth rate of cells (h^{-1})
μ_{max}	Maximum specific growth rate of cells (h^{-1})

CHAPTER-1

INTRODUCTION

BACKGROUND

In recent years, an increasing awareness of environmental pollution has become manifest, particularly with respect to air pollution. This awareness has, in turn, led to recognition of pollution sources. Chief sources of air pollution are the exhaust vapor emanating from internal combustion engines, i.e., automobiles and emissions that arise in the combustion of fossil fuels used in the furnaces. Fossil fuels contribute over 85% of the worldwide energy production. For energy production these fuels are to be combusted, which results in severe environmental pollution. Combustion processes emits greenhouse gases like CO_2, which are responsible for global warming. Sulfur oxides, nitrogen oxides emitted from these processes combines with water vapor present in the atmosphere to form sulfuric acid, nitric acid respectively and results into "acid rain" by condensation process, which dissolves buildings, kills forests and poisons lakes. In addition to this, the sulfur compounds corrode the oil pipelines.

The sulfur content in crude oil available from various sources ranges from 0.03 to values as high as 8.0 wt%. These objectionable quantities of sulfur must be removed before the crude oil processing otherwise the combustion of this oil results in severe environmental pollution. Conventionally to reduce sulfur content in crude oil, refineries adopted hydrodesulfurization process (HDS) using conventional catalysts, which is an effective treatment technique in reducing high sulfur contents. Conventional HDS process involves temperatures between 200°C and 450°C and pressures up to 10 Mpa depending on the desulfurization level to be achieved. It uses hydrogen gas to reduce the sulfur in petroleum to hydrogen sulfide, which is then readily separated from the fuel.

Due to high utility and operating costs, the conventional Hydrodesulfurization process (HDS) is considered to be uneconomic. The biotechnological option, Biodesulfurization (BDS) is a process which uses harmless microorganisms as catalysts to desulfurize petroleum fractions at mild conditions (low temperature, low pressure)

by removing organically bound sulfur from fuels without degrading the fuel value is seems to be the attractive low cost, environmentally benign technology.

1.1 IMPORTANCE OF SULFUR

Sulfur is a multivalent non-metal, abundant, tasteless and odorless element. In its native form sulfur is a yellow crystalline solid. In nature it occurs as the pure element or as sulfide and sulfate minerals. Although sulfur is infamous for its smell, frequently compare to rotten eggs, that odor is actually characteristic of hydrogen sulfide (H_2S). The crystallography of sulfur is complex. Depending on the specific conditions, sulfur allotropes form several distinct crystal structures. (http://www.lenntech.com/Periodic-chart-elements/S)

Table 1.1: Properties of sulfur

Atomic number	16
Atomic mass	32.06 g/mol
Electro negativity according to Pauling	2.5
Density	2.07 g/cm^3 at 20 °C
Melting point	113°C
Boiling point	445°C
Vanderwaals radius	0.127 nm
Ionic radius	0.184 (-2) nm ; 0.029 (+6)
Isotopes	5
Electronic shell	[Ne] $3s^2 3p^4$
Energy of first ionisation	999.3 kJ.mol^{-1}
Energy of second ionisation	2252 kJ.mol^{-1}
Energy of third ionisation	3357 kJ.mol^{-1}
Standard potential	- 0.51 V
Identified by	Ancients

1.1.1 Health Effects of Sulfur

All living things need sulfur. It is especially important for humans because it is part of the amino acid methionine, which is an absolute dietary requirement for us. The amino acid cysteine also contains sulfur. The average person takes in around 900 mg of sulfur per day, mainly in the form of protein. Elemental sulfur is not toxic, but many simple sulfur derivates are, such as sulfur dioxide (SO_2) and hydrogen sulfide.

Sulfur can be found commonly in nature as sulfides. During several processes sulfur bonds are added to the environment that are damaging to animals, as well as humans. These damaging sulfur bonds are also shaped in nature during various reactions, mostly when substances that are not naturally present have already been added. They are unwanted because of their unpleasant smells and are often highly toxic.

Globally sulfuric substances can have the following effects on human health:

- Neurological effects and behavioral changes
- Disturbance of blood circulation
- Heart damage
- Effects on eyes and eyesight
- Reproductive failure
- Damage to immune systems
- Stomach and gastrointestinal disorder
- Damage to liver and kidney functions
- Hearing defects
- Disturbance of the hormonal metabolism
- Dermatological effects
- Suffocation and lung embolism

1.1.2 Effects of Sulfur on the Environment

Sulfur can be found in the air in many different forms. It can cause irritations of the eyes and the throat with animals, when the uptake takes place through inhalation of sulfur in the gaseous phase. Sulfur is applied in industries widely and emitted to air,

due to the limited possibilities of destruction of the sulfur bonds that are applied. The damaging effects of sulfur with animals are mostly brain damage, through malfunctioning of the hypothalamus, and damage to the nervous system. Laboratory tests with test animals have indicated that sulfur can cause serious vascular damage in veins of the brains, the heart and the kidneys. These tests have also indicated that certain forms of sulfur can cause foetal damage and congenital effects. Mothers can even carry sulfur poisoning over to their children through mother milk (http://www.lenntech.com/Periodic-chart-elements/S)

Acidic deposition or "acid rain" occurs when emissions of sulfur dioxide (SO_2) and oxides of nitrogen (NOx) in the atmosphere react with water, oxygen, and oxidants to form acidic compounds. These compounds fall to the Earth in either dry form (gas and particles) or wet form (rain, snow, and fog). Some are carried by the wind, sometimes hundreds of miles, across state and national borders. In the U.S., about 70 percent of annual SO_2 emissions and 30 percent of NOx emissions are produced by electric utility plants that burn fossil fuels.

Among sulfur containing compounds in fuels, the main pollutants are alkyl dibenothiophene and benzothiophenes with ethyl, propyl and butyl groups. Sulfur containing heterocyclic compounds, besides being pollutants, have a high potential for bioaccumulation. Their accumulation in tissues of the organisms is known to be mutagenic and carcinogenic. (S. Krishnan, 2001).

1.1.3 Sulfur Emission Standards

Many countries have taken steps to reduce the sulfur emission levels by setting stringent standards. Early in 2000, the U.S, E.P.A issued the final rule for reduced sulfur content of gasoline with an average of 30 ppm. In December 2000, new sulfur standards for diesel fuel reduced the allowable sulfur content to 15 ppm from 50 ppm ('no sulfur' specification). This was 97 % decrease. In India, in order to reduce sulfur content in High Speed Diesel (HSD) from the present 1 % weight to 0.25 %, refineries were directed by the Hon'ble Supreme Court of India to put up required facilities by 1.4.1999. (S. Krishnan, 2001).

Table 1.2: Proposed sulfur emission standards for petroleum oil refineries in India (MINAS) **(http://www.cpcb.nic.in)**

Table 1.2.1 Proposed standards for emissions from furnace and boilers

S. No.	Parameter		Limiting concentration in mg/Nm^3, unless stated	
			Existing refineries	New refineries, furnaces, boilers commissioned after January 01, 2006
1	Sulfur Dioxide (SO_2)	Gas firing	50	50
		Liquid firing	1700	850
2	Oxides of Nitrogen (NO_x)	Gas firing	350	250
		Liquid firing	450	350
3	Particulate Matter (PM)	Gas firing	10	5
		Liquid firing	100	50
4	Carbon Monoxide (CO)	Gas firing	150	100
		Liquid firing	200	150
5	Nickel + Vanadium (Ni + V)	Liquid firing	5	5
6	Hydrogen Sulfide (H_2S) in fuel gas	-	150	150
7	Sulfur content in liquid fuel, weight %	-	1.0	0.5

Table 1.2.2 Proposed standards for emissions from Sulfur Recovery Units

S. No.	Plant capacity (tonnes/d)	Parameter	Existing refineries	New refineries or SRU commissioned after January 01, 2006
1	Above 20	Sulfur recovery, %	98.7	99.5
		H_2S, mg/Nm^3	15	10
2	5 – 20	Sulfur recovery, %	96	98
3	1 – 5	Sulfur recovery, %	94	96
4	-	Oxides of Nitrogen (NO_x)	350	250
5	-	Carbon Monoxide (CO)	150	100

Table 1.3: International emissions regulations for sulfur

Country & Product	Sulfur (Current) (ppm)
European Union, Heating Oil	1,000
European Union, Bunker Oil	10,000
South Korea, Diesel fuel	500
Thailand, Diesel Fuel	500
United States Clean air act Amendments, Gasoline	400
European Union, Diesel Fuel	500
European Union, Diesel Fuel	350

1.2 SULFUR COMPOUNDS IN OIL

The sulfur content in crude oil available from various sources ranges from 0.03 wt% to values as high as 8 wt% which was demonstrated on 78 various crude oil types (Rall et al., 1972). Organic sulfur compounds are the most important constituents, but inorganic sulfur i.e. elemental sulfur, hydrogen sulfide and pyrites can also be present (Tissot et al., 1984). An overview of sulfur levels in the global supplies of crude oil is given in Table 4 (S. Krishnan., 2001).

Table 1.4: Sulfur Levels in the Global Supplies of Crude oil

Region	Crude Oil gravity (API)	1990 Sulfur weight (%)	Production (tpd)	Crude oil gravity (API)	2010 Sulfur weight (%)	Production (tpd)
Alaska	26.970	1.11	1,954	28.340	0.99	1,645
Canada	32.000	1.52	2,000	31.400	1.62	2,500
California	18.430	1.59	970	17.730	2.60	951
Rest of USA	36.110	0.86	4,510	35.930	0.85	2,470
Africa	31.280	0.17	7,000	32.640	0.16	6,100
Europe	33.800	1.09	16,330	33.300	1.10	15,530
Latin America	27.060	1.62	7,770	25.100	1.82	9,850
Middle East (Indla)	33.730	1.69	29,100	34.350	1.61	35,760
Far East	39.800	1.09	16,330	33.300	1.10	15,530
World Average	32.300	1.13	70,800	31.810	1.27	83,450

Table 1.5: Typical sulfur compounds and corresponding refinery streams for fuels (Beverly L. McFarland et al., 1998)

Sulfur compounds	Refinery streams	Corresponding fuels
Mercaptanes, RSH; sulfides, R_2S; disulfides, RSSR; thiophene (T) and its alkylated derivatives, benzothiophene	SR-naphtha; FCC naphtha; coker naphtha	Gasoline (BP range: 25°C–225°C)
Mercaptanes, RSH; benzothiophene (BT), alkylated benzothiophenes	Kerosene; heavy naphtha; middle distillate	Jet fuel (BP range: 130°C–300°C)
Alkylated benzothiophenes; dibenzothiophene (DBT); alkylated dibenzothiophenes.	Middle distillate; FCC LCO; coker gas oil	Diesel fuel (BP range: 160°C–380°C)
Greater than or equal to three-ring polycyclic sulfur compounds, including DBT, Benzonaphthothiophene (BNT), phenanthro[4,5-b,c,d]thiophene (PT) and their alkylated derivatives and naphthothiophenes (NT)	Heavy gas oils; vacuum gas oil; distillation resides	Fuel oils (non-road fuel and heavy oils)

In addition to the data depicted in Table 1.4 it should be mentioned that the Middle East and Venezuela have the most oil reserves with high organic sulfur contents. More than 200 sulfur-containing organic compounds have been identified from crude oils; these compounds include sulfides, mercaptanes and thiophenes. The distribution and amount of organic sulfur compounds reflect the source and maturity of the crude oil. Chemically immature oils are rich in sulfur and often have a high content

in non-thiophenic sulfur compounds. During maturation non-thiophenic compounds are degraded and the sulfur content decreases. Mature oils contain mainly high molecular weight alkylated benzo and dibenzothiophene derivates, the benzothiophene: dibenzothiophene ratio decreases with maturity (Tissot et al., 1984). Because of the ubiquity of alkylated benzo and dibenzothiophenes in practically all crude oils, these compounds represent the bulk of sulfur. The majority of the alkylated benzothiophenes can be found in the boiling point range of 220°C up to 300°C, and then alkylated dibenzothiophenes are found with boiling points up to approximately 350°C. In Table 1.5, the different sulfur compounds present in the various refinery streams with their corresponding fuels are summarized.

1.3 METHODS FOR DESULFURIZATION OF PETROLEUM FRACTIONS

1.3.1 Physico-Chemical Methods

Various physico-chemical methods have been developed for desulfurization of petroleum fractions are here discussed in this section. The biotechnological option, biodesulfurization which is of interest was discussed in the later sections.

1.3.1.1 Hydrocatalytic desulfurization (HDS)

This process is industrially adopted in refineries. In all HDS processes the main reaction is the elimination of sulfur in the form of hydrogen sulfide. The units in the process consist essentially of heater to bring the mixture of feed and hydrogen containing gases up to the reaction temperature, and the heated stream, vapor, liquids, or mixed phase, is passed over the catalyst in the reactor section (Fig 1.1). After heat exchange and cooling the reactants pass into a high-pressure separator where the hydrogen rich gases are removed from the liquid product and recycled to the feed heater together with any make-up gas found necessary. Depending upon the process conditions and type of unit, the hydrogen sulfide gases in the recycle gases may be removed in a treating tower using amine or other hydrogen sulfide absorbent (Ranney et al., 1975)

A large variety of catalysts can be employed for catalytic desulfurization using hydrogen but the most commonly used is a mixture of cobalt, molybdenum and alumina. Catalysts of this type can be used in granular, powdered, pelletted or extruded

form and they usually consist of 1 to 5% cobalt oxide and 5 to 20 % molybdenum trioxide on an alumina base. The basic methods for catalyst preparation are impregnation, co-precipitation, and wet mixing. The following are the normal range of most commercial units:

Catalyst	Cobalt-molybdenum-alumina type
Temperature	315°C to 455°C
Pressure	200 to 1000 psig
Space velocity	Up to 20 v/ v/ h

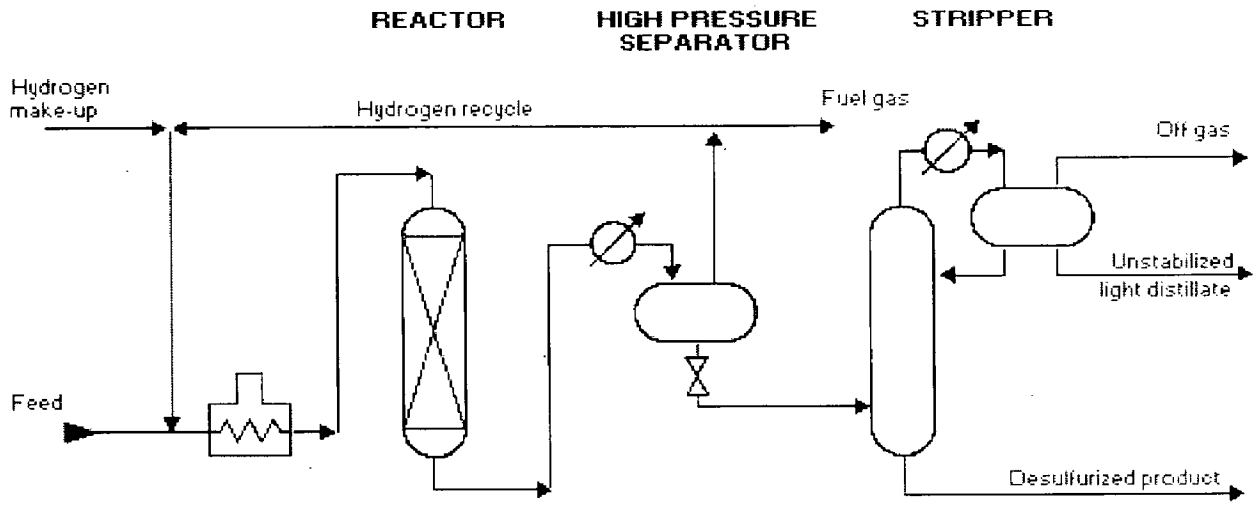


Fig 1.1: Hydrodesulfurization schematic flow diagram (http://www.osha.gov/dts/)

Fig 1.2 presents a qualitative relationship between the type and size of sulfur molecules in various distillate fuel fractions and their relative reactivities. Various refinery streams are used to produce three major types of transportation fuels, gasoline, jet fuels and diesel fuels that differ in composition and properties. The common types of sulfur compounds in liquid fuels are outlined in Table 1.5, which corresponds to Fig 1.2 for transportation fuels. The reactivity ranking in Fig. 1.2 is based on well-known experimental observations and a large amount of literature information. For the sulfur compounds without a conjugation structure between the lone pairs on S atom and the Π-electrons on aromatic ring, including disulfides, sulfides, thiols, and tetrahydrothiophene, HDS occurs directly through hydrogenolysis pathway. These sulfur compounds exhibit higher HDS reactivity than that of thiophene by an order of

magnitude, because they have higher the electron density on the S atom and weaker C–S bond. The reactivities of the 1- to 3-ring sulfur compounds decrease in the order thiophenes > benzothiophenes > dibenzothiophenes. In naphtha, thiophene is so much less reactive than the thiols, sulfides, and disulfides that the latter can be considered to be virtually infinitely reactive in practical high-conversion processes. Similarly, in gas oils, the reactivities of (alkyl-substituted) 4-methyldibenzothiophene and 4, 6 - dimethyldibenzothiophene (4, 6-DMDBT) are much lower than those of other sulfur-containing compounds. Consequently, in deep HDS, the conversion of these key substituted dibenzothiophenes largely determines the required conditions. Gates and Topsoe pointed out in 1997 that 4-methyldibenzothiophene and 4, 6-DMDBT are the most appropriate compounds for investigations of candidate catalysts and reaction mechanisms (Chunshan Song et al., 2003)

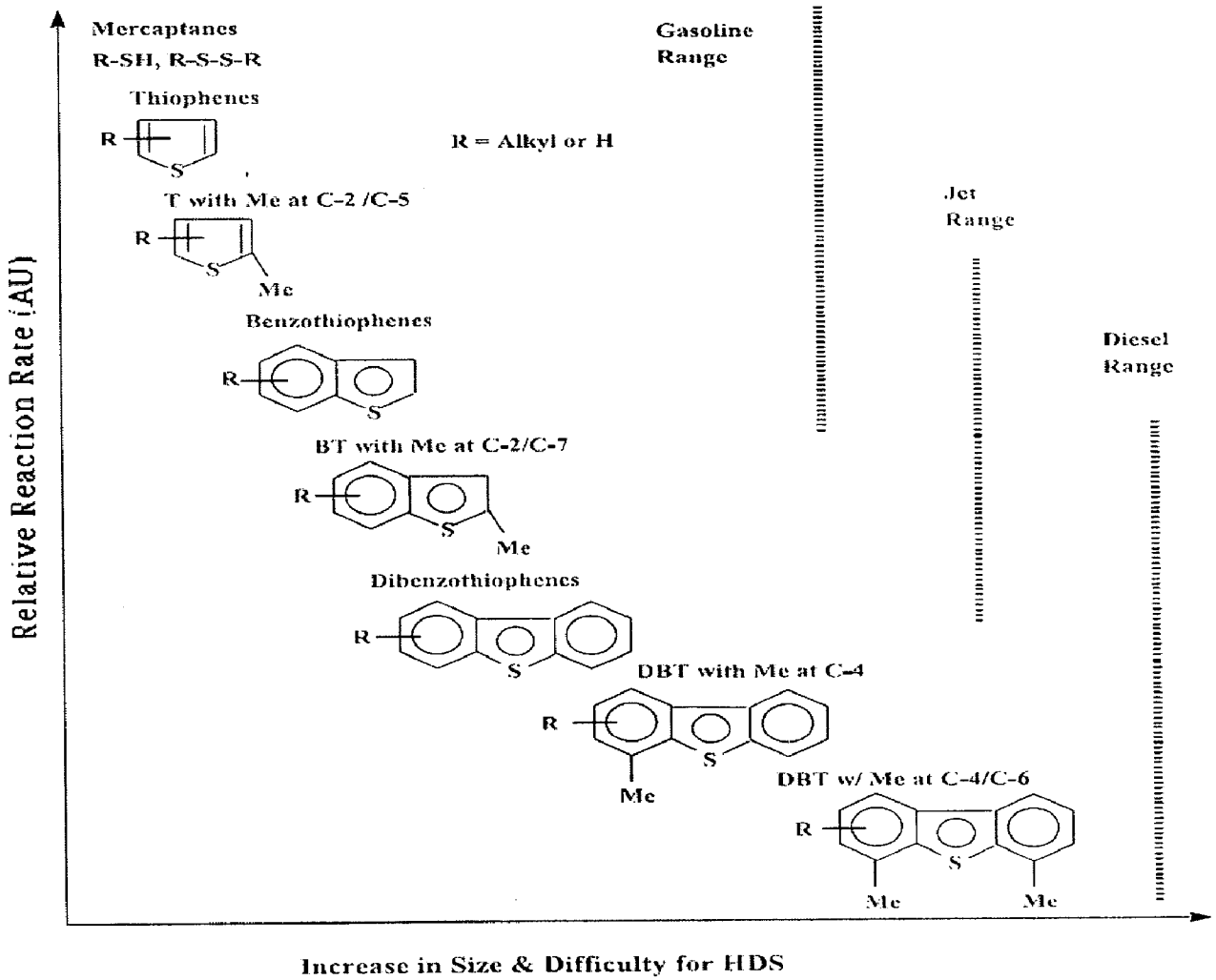


Fig 1.2: Reactivity of various organic sulfur compounds in HDS versus their ring sizes and positions of alkyl substitutions on the ring (Chunshan Song et al, 2003)

To reduce the sulfur content in crude oil, refineries adopted hydrodesulfurization process (HDS) using conventional catalysts, which is effective treatment technique in reducing high sulfur contents. Conventional HDS process involves temperatures between 200°C and 450°C and pressures up to 10 Mpa depending on the desulfurization level to be achieved. It uses hydrogen gas to reduce the sulfur in petroleum to hydrogen sulfide, which is then readily separated from the fuel. But to achieve stringent standards deep desulfurization is required which needs still high pressure, temperature and expensive catalysts. In addition to these, heavy metals present in the crude oil are catalyst poisons which during hydroprocessing causes irreversible deactivation. To protect the catalysts hydrometallation catalysts have been developed, but the use of these catalysts further increases the cost of the desulfurization process (Hung et al., 1986). These are the limitations of HDS, which lead researchers to search for alternative technique. The other treatment options for desulfurization include reactive adsorption, non-destructive adsorption, biodesulfurization, extractive distillation, alkylation etc.

1.3.1.2 Reactive adsorption

One idea has been to remove sulfur species by reactive adsorption. The chemistry involved is adsorbents are usually comprised of transition metals supported on base oxides. Ni on ZnO is the prototypical formulation most often found in literature and patents. Ni functions as hydrodesulfurization sites, while ZnO has the crucial role of taking up the resulting H_2S, converting into ZnS in the process. After completion of the ZnO to ZnS conversion, the adsorbent can either be discarded, or regenerated. Conoco Phillips's S-Zorb process is the first real commercial process of importance based on reactive adsorption. The process is schematically illustrated in Fig 1.3.

The process consists of a desulfurization section (reactor on the left) and the sorbent regeneration section (regenerator on the right). It looks quite similar to fluid catalytic cracking in its process scheme, equipped with a reactor and a regenerator section in which fluidized sorbent particles are transported. However, the reactor circulation speed is slow, viz. a residence time of a few days, comparable to that in a continuous regeneration reformer. The feed with hydrogen is brought into the reactor to make contact with the sorbent, which is then pushed upwards slowly until it is finally

separated into product and sorbent at the separator. The sulfided spent sorbent is then regenerated in the regenerator under a proper oxidative atmosphere. The process is claimed to be able to preserve octane number well, while removing sulfur species effectively (G. Germana et al., 2004). The clue seems to lie in the use of a tuned reaction temperature, probably on the high side, which balances the hydrogenation and dehydrogenation equilibrium, therefore minimizing hydrogen consumption and effectively minimizing olefin hydrogenation. The usual problem of coking on the catalyst is not an issue, since the sorbent is anyway regenerated.

The S-Zorb has some disadvantages like the relatively high complexity of the process there are relatively many pieces of equipment, and the proximity of hydrogen (in the reactor) and oxygen (in the regenerator) in the process could mean some operational complexity, as known in FCC.

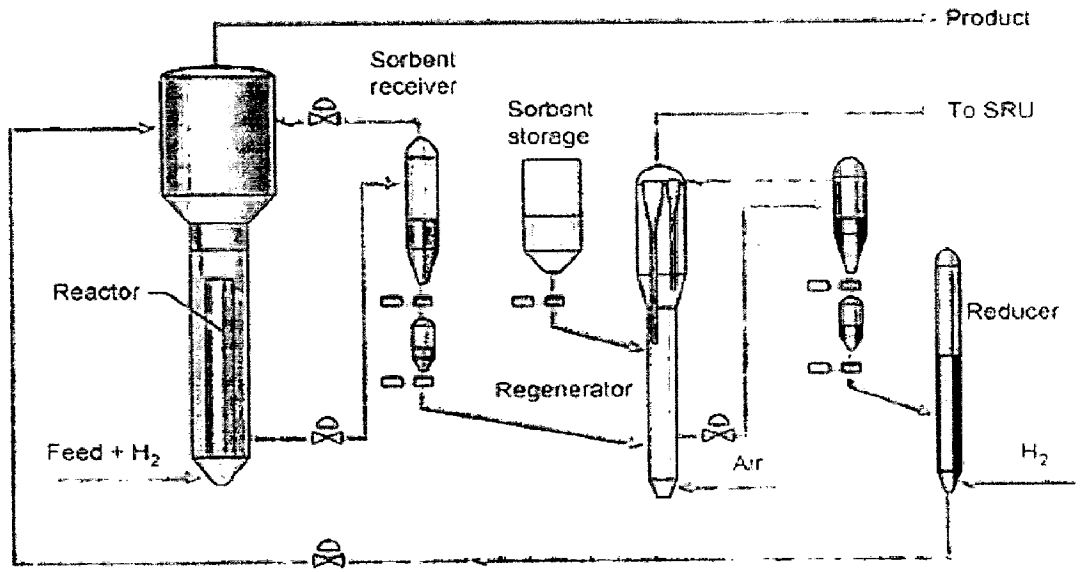


Fig 1.3: S-Zorb process scheme.

1.3.1.3 Non-destructive adsorption:

An alternative to 'reactive' adsorption could be 'nondestructive' adsorption (or molecular adsortion, i.e., physisorption and some forms of chemisorption) where sulfur species are adsorbed as molecules as such. Of course, this generates an adsorbent loaded with S-bearing molecules that has somehow to be disposed off. In this field,

zeolites like 5X and 13A and active carbon have been reported as sorbents for removing mercaptans, sulfides and thiophene under mild conditions already some time ago. Furthermore, some commercial materials like Ni–Al_2O_3 are available for adsorbing thiophene type sulfur species for the purpose of applying it as a guard bed of naphtha reformer platinum catalyst. However, the Irvad process, named after its developer Mr. Irvin and sulfur adsorption, could probably be mentioned as one of the first commercially oriented desulfurization processes reported for gasoline sulfur reduction. They use an in situ regenerative process with fluidizable sorbent particles like S-Zorb, but then without applying H_2. They have disappeared from the field upon the rise of the S-Zorb process. The adsorbent for the Irvad process was to be supplied by Alcoa and mentioned to be alumina with an inorganic promoter (Irvin et al., 1999). The fate of the sulfur species is not well described in the publications on Irvad, but they seem to adsorb physically, given the typical reaction conditions of 30°C, 13 bar without applying hydrogen. The sulfur-loaded adsorbent is then regenerated by 'reactive gases. After Irvad has disappeared from the commercial field, Alcoa still continued its adsorbent development, as seen in its report on Ag–Al_2O_3.

1.3.1.4 Alkylation:

Alkylation chemistry of thiophenic sulfur over acidic catalysts can also give an opportunity of reduction of sulfur in gasoline as the case in OATS process. Alkylation of thiophenic sulfur leads to a boiling point shift of the resultant sulfur compounds toward a diesel range, thus reducing the sulfur content in the gasoline range product. A recent patent proposed also the possibility of alkylation of sulfur and nitrogen containing compounds in the presence of ionic liquid containing alkylation. There are, however, several issues. The most important issue is the fact that it is only applicable for a narrow boiling range fraction, thus a large fraction of the gasoline will still require treatment. Furthermore, the high boiling product should be also further treated.

1.3.2 Other physical separation methods

Apart from adsorption and alkylation routes, other physical separation methods have been looked into in some detail as well. For example, a membrane-based process, called S-Brane, is offered by Grace Davison and CB& I (Fig 1.4). Though it is apparently commercially available, a full commercial unit has not been announced yet,

to our knowledge. They reported on their demonstration project of 2003 in the NPRA conference of 2004, announcing that they successfully produced 30 ppmw S stream with their per-vaporation membrane technique (X.Zhao et al., 2004). The biggest issue of this process would, however, appear to be the 'stage-cut' (related to the ratio of S-rich to S-poor product) to be applied. The typical stage cut of the present S-Brane process is high at around 30%, which means that only 70% of the stream can be desulfurized to, for instance, 30 ppmw S, while the remaining 30% is the sulfur-rich stream, to be routed to HDS or other processes. Such a high stage cut is possibly also connected with the necessity of retaining the olefins in the low-S stream, selectivity always being a problem. May be the process is only suitable to treat certain boiling-range fractions, in which case integration with hydrotreating is still required when full-range FCC gasoline is treated.

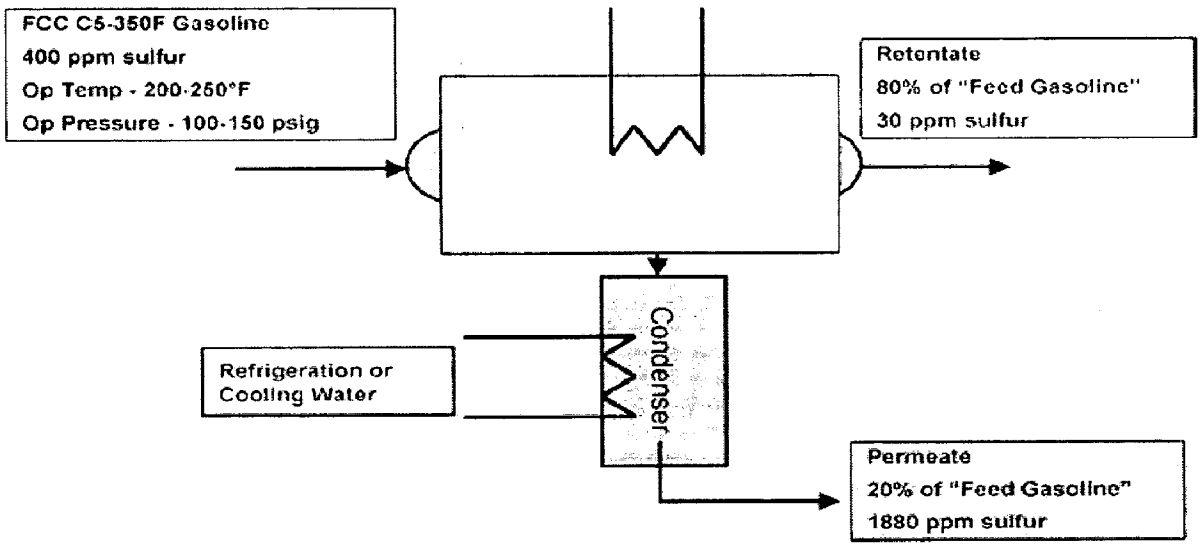


Fig 1.4: S-Brane schematic process flow.

An extractive distillation process

GT- DeSulf™ has been reported by GTC in 2002. The process scheme is shown in Fig. 6. The GT-DeSulf™ utilizes a proprietary aromatics selective solvent, which is effective in extracting thiophenic sulfur species and aromatics, and to a limited extent, mercaptans and sulfides as well. Since olefin is not extracted, it is indeed a 'selective' desulfurization system. The application is, however, limited to a light and a middle FCC cut, with specified boiling point range up to180°C (Gentry et al 2004). This is

probably due to a substantial amount of aromatics present in the heavier FCC fraction, which would make an effective sulfur species separation difficult. This process has been announced in 2002–2003, but nothing much has been heard about it since then. Several drawbacks are apparent. The first is a high volume of the sulfur-rich stream, like 10–30% of the total volume. This should be separately treated in HDS later. Another point of concern is the complexity of the extraction process, i.e., many pieces of equipments, due to the separation and recovery of extractant. That often seems to make such an extraction process a potentially high CAPEX process.

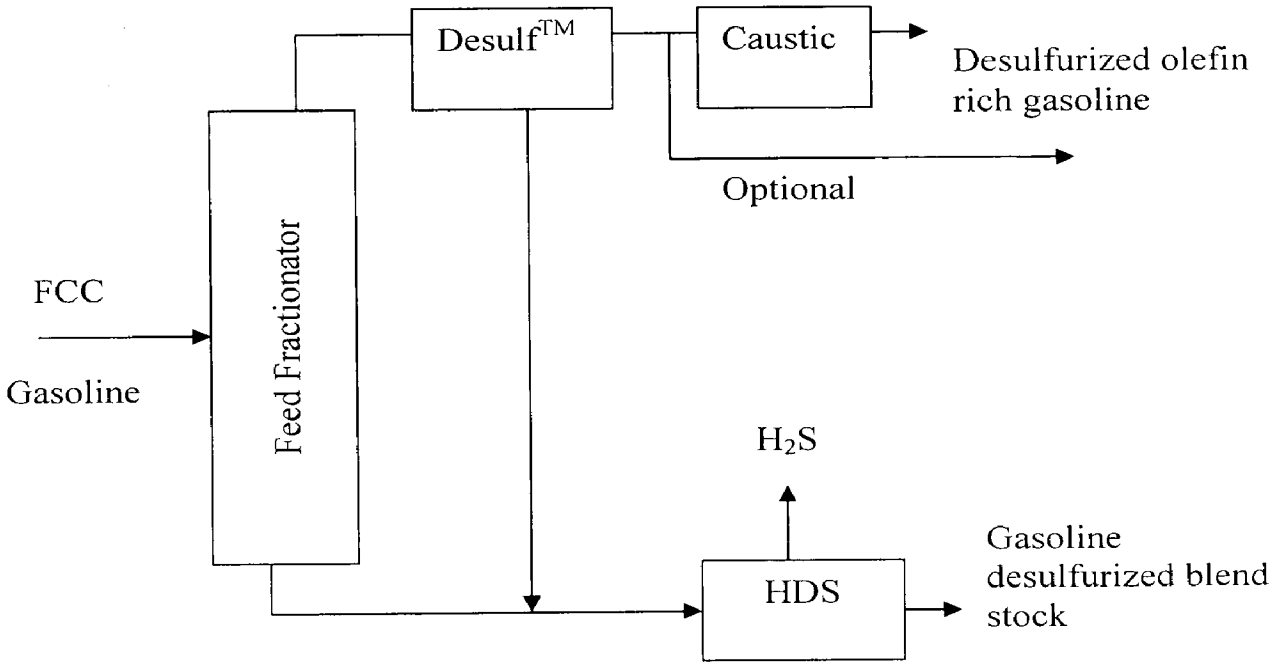


Fig 1.5: GT-DeSulf™ schematic process flow

1.3.3 Miscellaneous

Among other intriguing options to reduce sulfur content in gasoline, precipitation and polymerization can be named. Precipitation of sulfides and thiophenes is realized by alkylating the sulfur atom in such molecules to form S alkylsulfonium salts, $(R–S^{+}– CH_3)\ BF_4^{-}$, that precipitate from the matrix. The desulfurization of FCC gasoline from 100 to less than 30 ppmw has been reported. The problems with this route, however, can be envisaged to be some olefin loss, consumption of expensive

chemicals and the waste treatment of alkylsulfonium salts. Another interesting approach is electrochemical polymerization of sulfur compounds (R.C. Schucker et al., 2001). The idea is to electrochemically oxidize thiophene and aniline type species to form dimer/ oligomer of them, which can be separated easily from the matrix by gravity or boiling point. This, however, seems to be limited in the type of species that are polymerizable. Furthermore, the cost of electrochemical processing and of ionic liquid should be also considered (E. Ito et al., 2006)

1.3.4 Biodesulfurization

The microbial desulfurization of fossil fuels has been under active investigation for over 60 years. It is one of several methodological approaches that can be used for producing cleaner fuels. Biodesulfurization is intended to augment the existing process of hydrodesulfurization, which tends to be expensive, energy-intensive, and relatively inadequate for the deep desulfurization of fuel that will increasingly be required, as low sulfur crude becomes scarcer and regulations for low levels of sulfur in fuels become more stringent; although it has significant limitations, hydrodesulfurization (HDS) also removes nitrogen and metals. Finding strategies for making low-sulfur fuels to meet environmental regulations in a cost effective manner is critical for the petroleum and automotive industries, it is the major driving force for the evaluation of biocatalytic removal of sulfur from fuels, including diesel and gasoline.

Biodesulfurization (BDS) can be defined as a process which uses harmless microorganisms as catalysts to desulfurize petroleum fractions at mild conditions (low temperature, low pressure) by removing organically bound sulfur from fuels without degrading the fuel value.

While there are alternative ways of reducing the sulfur content of fuels and any use of biocatalysis will have to be integrated into the processing stream and be cost effective when compared with alternate methods, removing sulfur from fuels, including diesel and gasoline, by biocatalysis using enzymes is receiving significant attention. The next chapter will examine the underlying biochemical basis for considering biocatalytic sulfur removal from fuels and the bioreactors and bioprocess designs that may be used in commercial applications of such biotechnological applications.

Biological desulfurization of petroleum feedstocks and products may offer an attractive alternative to conventional high temperature, high-pressure thermochemical treatment (hydrotreatment) because of the high substrate specificity and the relatively mild conditions for biological reactions that tend to make them safer and less energy intensive. Efficiency, safety, and cost effectiveness are essential factors when considering the advantages of a biological process versus a conventional chemical catalytic process. This must include consideration of both capital investment and operating costs.

Most of the research done on the biodesufurization targetted compound was dibenzothiophene (DBT, Fig 1.6) since it is the most abundant organic compound found in the petroleum and DBT concentrations can be high as 70% of the total organic sulfur found in petroleum, it is an appropriate compound to study the biodesulfurization of petroleum and its fractions.

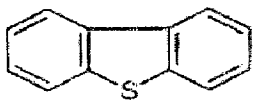


Fig 1.6: Chemical structure of dibenzothiophene

Table 1.6: Physical-chemical properties of dibenzothiophene

Chemical name	Dibenzothiophene
Molecular formula	$C_{12}H_8S$
Molecular weight	184.257
Melting point	98.2°C
Boiling point	332.5°C
Molar volume	191.3 cm^3/mol
Vapor pressure	0.267 Pa at 25°C
Volatilization	Half life 140 h in river, Half life 720 h in eutrophic pond
Biodegradation	5.3 X 10^{-7} ml cells/h

The aim of biodesulfurization research is to develop a commercially viable biodesulfurization process for petroleum, and it has been estimated that a successful commercial process would require a biocatalyst with desulfurization activity of 1.2 mM DBT/ g DCW/h to 3 mM DBT/ g DCW/h (Monticello D.J, 2000). From the literature cited we can say that this can be possibly achieved by genetically manipulating the wild type cultures to improve copy number of the desulfurization gene cluster which enhances the desulfurization rate, substrate specificity and mass transfer rate of oil into the cell from two phase mixture.

The objectives of the present study are

1. Isolate the desulfurization genes (*dsz*) containing plasmid, pSAD 225-32 from *Escherichia coli* XL1 Blue.
2. Development of recombinant bacterial strain by transforming pSAD225-32 into a solvent resistant gram-negative host *Pseudomonas putida* MTCC 1124.
3. Optimization of desulfurization assays to confirm the sulfur specific 4S-pathway in the recombinant bacterial cells.
4. Determination of kinetic parameters μ_{max} and K_s and perform desulfurization studies using DBT in ethanol as a model compound by recombinant bacterial cells in aqueous phase.
5. Desulfurization studies on model oil (DBT dissolved in n-hexadecane) using resting cells of recombinant strain.
    - Determination of optimum oil to water ratio for desulfurization
    - Determination of effect of surfactant, Tween- 80 in enhancing the efficiency of biodesulfurization.
6. Desulfurization studies on model oil at optimum oil to water ratio using resting cells of recombinant strain.
7. Desulfurization studies on diesel oil at optimum oil to water ratio using resting cells of recombinant strain

CHAPTER-2

LITERATURE REVIEW

Biological transformations of sulfur-containing molecules in fuels have been the subject of intensive investigations for several decades. Research has been conducted on various aspects of sulfur transformations, ranging from specific biocatalytic sulfur removal from fuels (biodesulfurization) to biodegradation of sulfur-containing compounds in petroleum pollutants (bioremediation) (Table 2.1). Studies have tended to focus on specific fuels, for example, coal, crude oil, diesel fuel, gasoline, and on specific compounds in these fuels (e.g., thiophenes, benzothiophenes, dibenzothiophenes). The initial attention was on ring cleavage and the biodegradation of heterocyclic compounds. Most recently the attention has been primarily on biodesulfurization of diesel fuel and gasoline, with biocatalytic sulfur cleavage from dibenzothiophenes the principal target of investigation. In most studies, the emphasis has been placed on determining which heterocyclic compounds are subject to biotransformation and identifying metabolic products and elucidating pathways.

2.1 Metabolic Pathways

Microoraganisms reported in literature can be classified into three different groups depending upon the pathways followed by them to desulfurize DBT. These pathways are discussed below.

Kodama et al. in 1970 first reported on the aerobic conversion of DBT. The transformation of DBT with the 'Kodama' pathway results in the oxidative cleavage of one of the aromatic rings of DBT rings. Various intermediates formed during the oxidation of DBT are identified by Kodama et al. It involves three major steps: hydroxylation, ring cleavage and hydrolysis (Fig 2.1). It was reported that the end product of DBT degradation via the Kodama pathway is 3-hydroxy-2-formyl benzothiophene (HFBT). This pathway is followed by many microorganisms like *Pseudomonas jijani*, *P.abikonesis* (Kodama et al. 1970), *Rhizobium meliloti* (Frassinetti et al.1998), and *Beijerincika* sp. (Laborde et al. 1977). Because of the degradation of C-C bond cleavage the calorific value of oil is altered, consequently this route is not desirable in BDS.

The first sulfur selective pathway was explained by Kilbane et al. using *Rhodococcus erythropolis* in 1989. This species is able to carry out a stepwise selective oxidation of the hetero sulfur atom, while the carbon skeleton is not metabolized thus preserving the fuel value. The sulfur specific metabolic pathway for DBT desulfurization involves four enzymatic steps and is designated as 4S-pathway (Fig 2.2). The whole reaction is summarized as follows:

Step 1: Transfer of the molecules from the oil fraction into the bacterium.

Step 2: Action of DBT-Monooxygenase, the bacterial enzyme, which spe specifically acts on DBT, which yields sulfoxide and finally sulfone by oxidation of DBT-Sulfoxide. It has been found that gene *dsz*C codes for this important enzyme. It transfers an electron from Flavin Mono Nucleotide ($FMNH_2$) to DBT, to produce oxidized FMN, DBTO, and $DBTO_2$.

Step 3: Cleavage of first C-S linkage by DBT sulfone-monooxygenase coded by gene *dsz*A. It appears to operate in the cell as a dimer.

Step 4: Production of sulfite and an intact hydrocarbon molecule is the last reaction in the pathway. This is characterized by a "Desulfinase" coded by *dsz*B gene, leads to the release of sulfur as sulfite, and the production of the oil-soluble product Hydroxy biphenyl (HBP).

In parts similar to the 4S-pathway another reaction pathway proposed by van Afferden et al. A *Brevibacterium* sp. which was isolated by its ability to use DBT as sole source of sulfur also starts with two sulfoxidation steps. In this special case however an angular dioxygenation follows as it was identified by Engesser et al. for the degradation of dibenzofuran. During rearomatization the C-S bond is cleaved. The degradation of the resulting dihydroxydibenzylsulfinic acid follows the biphenyl pathway. Sulfite is released by a not known mechanism.

In contrast to aerobic biodesulfurization, evidence for the anaerobic conversion of organic sulfur compounds is equivocal. Kim et al. (1995) reported conversion of various sulfur compounds under anaerobic conditions, accompanied with formation of

H_2S. The conversion measured using an enzyme assay with a concentrated cell suspension of *Desulfovibrio desulfuricans* M6 in the presence of the artificial electron donor methyl viologen. Biphenyl was found as the major end product when DBT is reductively converted, as depicted in Fig 2.3.

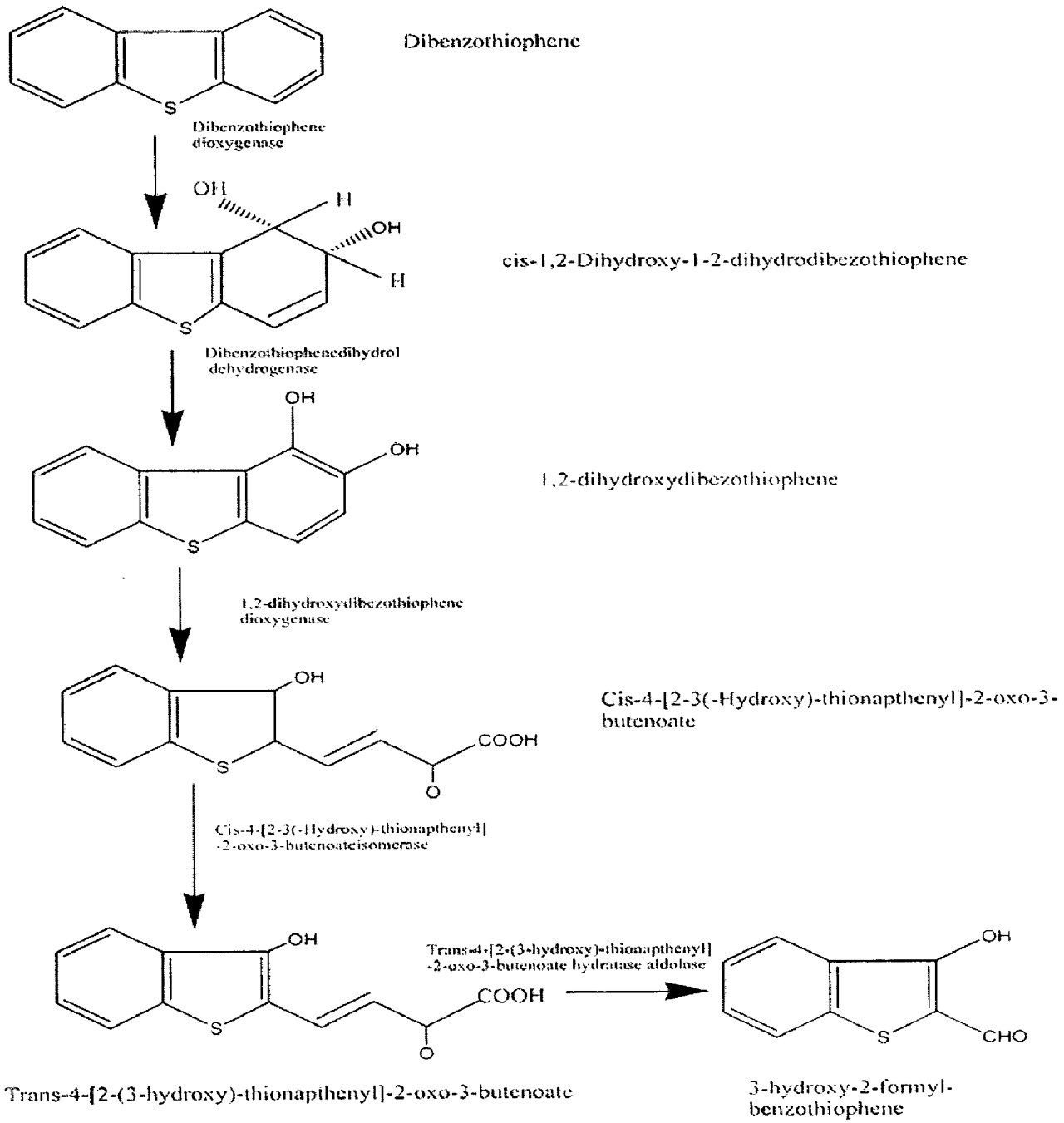


Fig 2.1: Kodama pathway of DBT oxidative degradation

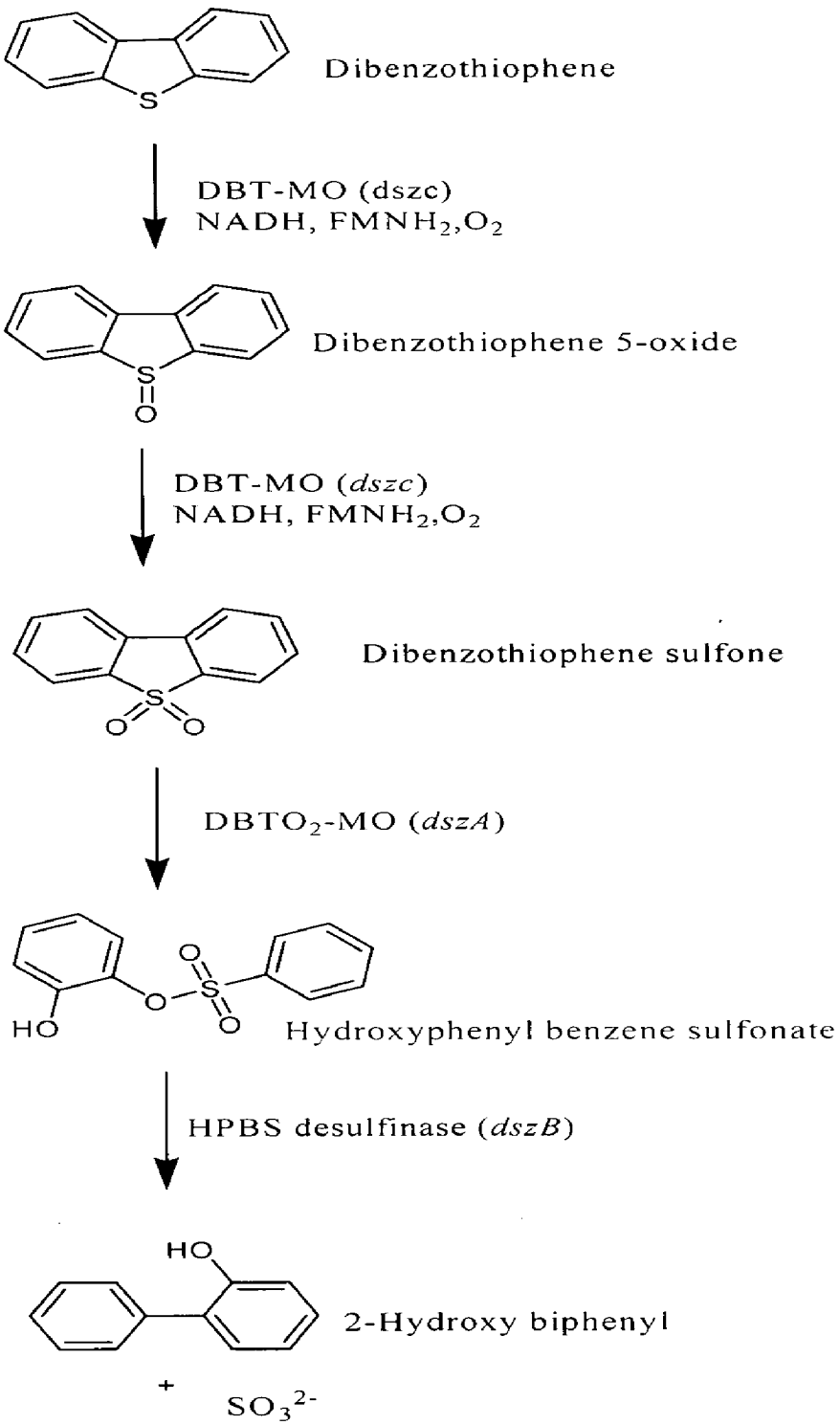


Fig 2.2: Systematic representation of 4S pathway

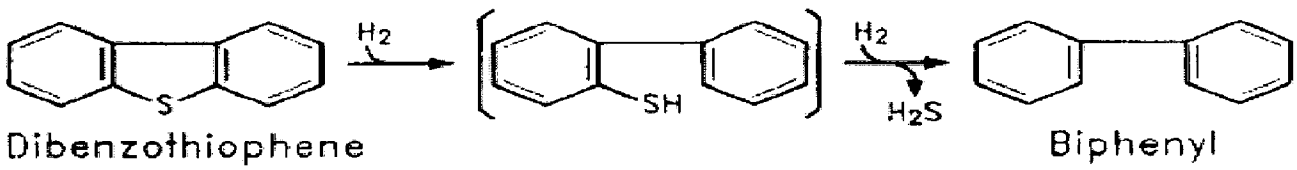


Fig 2.3: Principle of reductive biodesulfurization

2.2 Comparative Studies on Microorganisms

The next phase of research in biodesulfurization was concerned with biotransformation rates; various microorganisms reported in the literature by researchers, which can desulfurize the organic sulfur compounds, are classified into two categories. One is mesophilic organisms and another one is thermophilic organisms which can desulfurize organic sulfur compounds in oil.

The systematic study of microbial desulfurization was initiated by Yamada et al. (1968) DBT was used as a model substrate for its predominance in many crudes. Desulfurization studies were conducted in batch experiments at 30°C. Six bacterial cultures (*Pseudomonas* species) were found to be capable to degrade DBT to water-soluble products. Two of the isolates were new species that were subsequently named as *P. abikonensis* and *P. jianii.* A DBT conversion efficiency of 40% was achieved in 3 days; conversion efficiencies were less when the organisms were used singly. But these organisms follow the Kodama pathway (Kodama et al.,1973) forming 3-hydroxyl-2 formyl benzothiophene as end product by C-C ring cleavage which reduces the calorific value of oil.

Here in this part we concentrated on the mesophiles which can degrade the organic sulfur compounds in sulfur specific 4S- pathway are more emphasized due to as mentioned above through the sulfur specific pathway microorganisms degrade the sulfur compounds without reducing the caloric value of oil.

The first sulfur selective *Rhodococcus erythropolis* strains were isolated by Kilbane et al. in 1989. This species able to carry out a stepwise selective oxidation of the hetero sulfur atom, while the carbon skeleton is not metabolized. Different species of *R. erythropolis* were reported in the literature. *R. erythropolis* D-1 can completely degrade 2.2 mmol DBT with in 150 min at 37°C and pH of 7.0.(Izumi et al.,1994). Afterwards Kobayashi et al. (2000) reported that *R. erythropolis* KA2-5-1 can utilise DBT as well as other alkylated DBTs as substrates at 37°C with 74.2 mmol/ kg DCW/ h desulfurization activity for DBT. Honda et al reported, using a high cell density culture of *R. rhodochorous* IGTS8 by pH stat feeding the specific rate of

desulfurization of DBT was 6.1mmol/ kg DCW/ h. They also studied the effect of different concentrations of ammonia and acetic acids (nitrogen and carbon sources respectively) on the cell growth. Two phase cultivation, cell growth phase and induction phase is proposed in order to obtain a high cell density and full induction of DBT degrading enzymes.

Apart from DBT as a model compound, there are some reports using fractions of crude oil for experimentation. M.J.Grossman et al evaluated *Rhodococcus* sp.strain ECRD-1's ability to desulfurize a 232°C to 343°C middle-distillate (diesel range) fraction Oregon basin (OB) crude oil. This strain removed 30 % of total sulfur present in OB oil. The desulfurization activity of *Gordonia* sp. CYKS1 with model oil is 0.12 mg sulfur/ g cell/ h and it is also reported that the resting cells of these species has higher desulfurization rates as 0.34 mg sulfur/ g cell/ h with diesel oil, light oil and middle distillate unit feed (Chang et al). Recently, another *Gordonia* sp. ZD-7 isolated which has desulfurization rate of 5 mmol DBT/ kg DCW/ h at 30°C (Wei Li et al., 2006).

Various mesophiles with their temperature, and the enzymes/ genes responsible for desulfurization that can desulfurize DBT, its derivatives and fractions of crude oil are summarized in Table 2.2.

To date the mesophiles, which have been reported to degrade DBT in a C-S bond, targeted fashion can do it usually near 30°C. On the other hand, distillation and hydrodesulfurization are performed at much higher temperatures in the course of the conventional refining process. If the biodesulfurization reaction which could be applied at mild but still higher temperatures without cooling the treated petroleum fractions to normal temperature were integrated into the refining process, it would be far more desirable. Moreover, the rate of chemical reaction is in general increases with the increasing reaction temperature (Konishi et al., 1997).

Thermophilic degradation of DBT by *Sulfolobus acidocaldarius*, a reduced-sulfur, iron-oxidizing bacterium with sulfur removal rate 2.56 mg S/ l. day at 70°C has been reported (Kargi, 1987). Two thermophilic bacterial strains capable of degrading

DBT and several methylated DBTs even in the vicinity of 60°C have been isolated which are subsequently named as *Paenibacillus* sp. Strain A11-1, *Paenibacillus* sp. Strain A11-2 degradation of DBT and its related compounds by these bacteria is optimal at around 55°C and takes place via specific cleavages of their two C-S bonds. These thermophilic bacteria were also found to further desulfurize light gas oil, which had been treated by the hydrodesulfurization process. *Bacillus Subtilis* WU-S2B is another thermophile reported which can degrade 0.54 mmol DBT in 120 h using growing cells and the resting cells of this bacteria can degrade 0.81 mmol DBT in 12h using resting cells at 50°C (Kirimura et al., 2001). Anaerobic microbial degradation of DBT was studied using thermophilic bacteria obtained from crude oil. A mixed culture was obtained that degraded 98% of DBT at 0.5 mg/ml at 65°C over 15 days both in the presence and in the absence of methyl viologen (A. Bahrami et al., 2001). Several *Mycobacterium* species have been reported in the literature using various substrates ranging from model heterocyclic compound DBT to various grades of light oils.

Various thermophiles with their temperature and the enzymes/ genes responsible for desulfurization that can desulfurize DBT, its derivatives and fractions of crude oil are summarized in Table 2.3.

Here in this report, graphically represented the desulfurization activity of various microorganisms including both mesophiles and thermophiles by plotting graphs between the cultivation time and the concentration of DBT in Fig 2.4, though the reaction conditions are not similar. In the graph data for the thermophiles, *Bacillus Subtilis* WU-S2B and *Mycobacterium phlei* WU-F1 has taken at 50°C from the literature. There are some mesophiles, which have more desulfurization activity much better than that of thermophiles, but at low temperatures. Here from Fig 2.5 though we cannot compare the performance of microorganism but from Fig 2.6 where 2-hydroxybiphenyl-production rate with cultivation time is plotted we can say that *Rhodococcus* sp. P32C1 has high 2-HBP-production rate (among the microorganisms compared though reaction conditions are not similar).

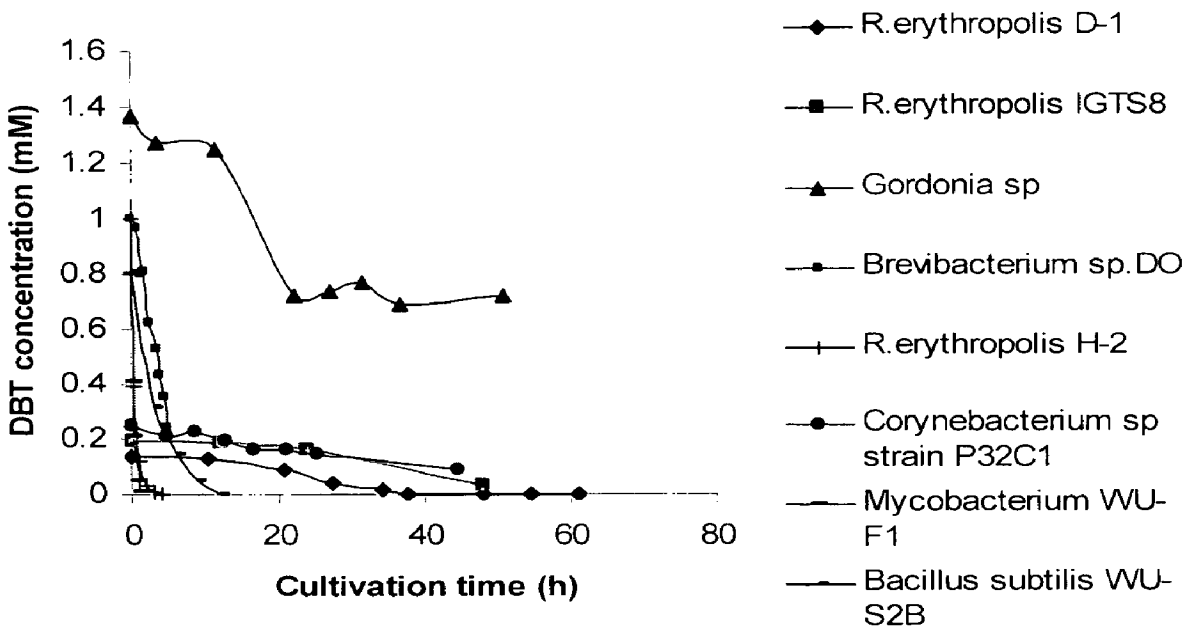


Fig 2.4: Graphical comparison of various microorganism's DBT desulfurization rate.

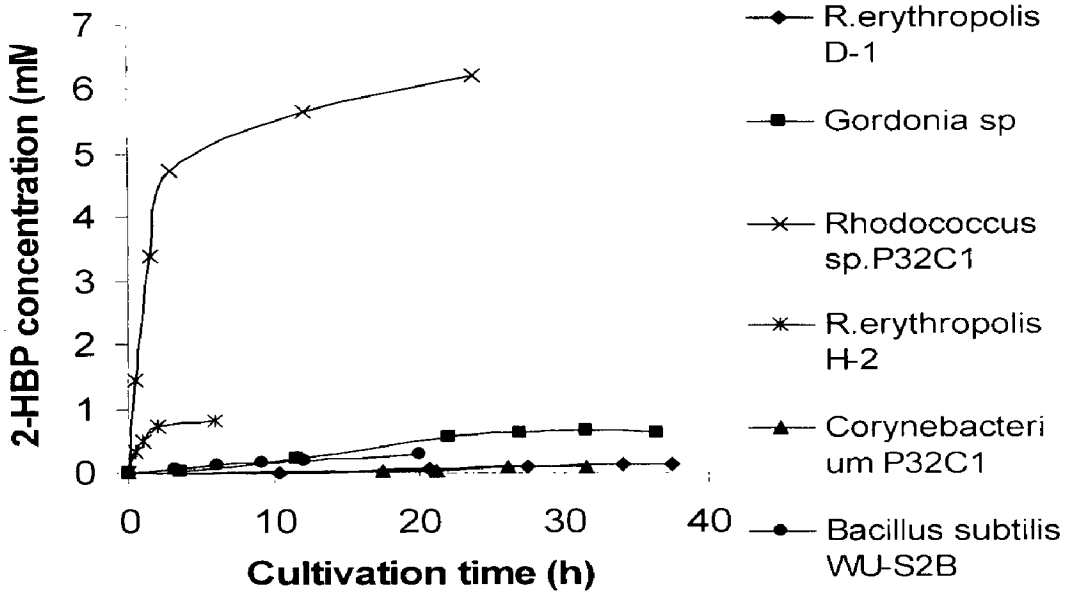


Fig 2.5: Comparison of various microorganism's 2-HBP production rate

Table 2.1: Recent Biodesulfurization Research Efforts

Organization	Researchers	Focus of effort	Collaborative alliances
Energy Biosystems Corporation (EBC)	Monticello, Gray, Squires and others	Diesel, gasoline, crude oil desulfurization public corporation, DOE	Total, Texaco, Petrolite, Kellogg, Petrostar, Notre Dame University
Petroleum Energy Center (Japan)	Yasui	Diesel biorefining	Consortium of Japanse Companies and Universities
Tonori University	Izumi	Diesel biorefining	Consortium of Japanse Companies
University of Tokyo (Japan)	Omori, Kodoma	Diesel biorefining	Consortium of Japanse Companies
University of Bologna (Spain)	Semi	Diesel	AGIP
Exxon	Grossman	Diesel, heating oil, other fuels biodesulfurizaton	Canadian National Research Council
Institute of Gas Technology (IGS)	Kilbane	Coal Biodesulfurization, bioremediation	Energy Biosystems Corporation
Korean Institute of Science and Technology (Korea)	Kim	Residual crude	EBC
Oak Ridge National Laboratory	Kaufman	Crude oil	EBC, Exxon, Chevron, Texaco, Unocal, Baker Chemicals
Brookhaven National Laboratory	Premuzic	Crude oil	Several companies
University of Alberta (Canada)	Fedorak	Crude oil, refined fuels	EBC
University of Riovira (Spain)	Comtani	Crude oil, diesel	
TATA Research Institute (TERI)	Srinivasa Raghava Krishnan	Gasoline biodesulfurization	

Table 2.2: Mesophiles reported as desulfurising microorganisms:

Microorganism	Genes / Enzymes responsible	Substrate	Temperature (°C)	pH	Result	Reference
Gordonia sp..ZD-7		DBT in n-hexadecane	30	7.0	Desulfurization rate: 5 mmol DBT/ kg DCW/ h	Wei Li et al.,(2006)
Gordonia sp.strain F.5.25.8	*dsz* ABC located on chromosome	DBT	30	7.0	70% desulfurization[1] specific desulfurization rate: 30 mmol 2-HBP/ (kg DCW.h)[2]	Santos et al ., (2005)[1] Kaufman et al.,(1998)[2]
Rhodococcus erythropolis IGTS8	*dsz* operon	DBT	30	6.5	70% decrease in S content	Delolmo et al., (2005)
R. erythropolis AC-1514D		DBT (0.43 mM)	27		78-80% desulfurization	Zakharyants et al.,(2004)
Pseudomonas sp. HKT54		1,2 – naphthothiophene DBT	30		34.1% degradation 41.3% degradation	Matsui et al .,(2003)
Immobilized *Pseudomonas delafieldii* R-8		DBT	30	7.0	Activity is 8.7 to 9 mmol S/ kg DCW /h.	Shan et al.,(2003).

Microorganism	Genes / Enzymes responsible	Substrate	Temperature (°C)	pH	Result	Reference
Staphylococcus sp stain S3/C		DBT/n-hexadecane	30		57% reduction in S	Goindi et al.,(2002)
R. erythropolis KA2-5-1	*DSZA*,*DSZB*, *DSZC*	2-amino methane sulfonic acid	30	7.0	Desulfurizing activity is 111.1 mmol of 2-HBP/kg DCW/ h.	Yoshikawa et al., (2002)
		DBT	30	7.0	109.7 mmol of 2-HBP/ kg DCW/ h	
Rhodococcus strain WU-K2R		Naphthothiophene	30		0.27 mM NTH degraded in 7 days	Kirimura et al., (2002)
Rhodococcus sp. strain P32C1		Benzothiophene	30		Desulfurizing activity is 30 mmol/ kg DCW/ h	Maghsoudi et al., (2001)
		Diesel oil with 303 ppm S content	30		48.5% of decrease in S content	
		Diesel oil with 1000 ppm S content.	30		23.7% of decrease in S content.	
Rhodococcus strain ECRD-1		Catalytic cracker middle distillate light cycle oil	25	7.0	669±40 ppm S is reduced to 56±4 ppm.	Grossman et al., (2001)

Microorganism	Genes / Enzymes responsible	Substrate	Temperature (°C)	pH	Result	Reference
R. erythropolis KA2-5-1	*dsz* ABC	DBT 4-methyl DBT 4,6- dimethyl DBT 2,8- dimethyl DBT	37		Desulfurizing activity (mmol /kg DCW/ h) 74.2 44.4 29.2 27.4	Kobayashi et al., (2000)
Corynebacterium sp strain P32C1		DBT	30		37 mmol/ kg DCW/ h of 2-HBP was produced.	Maghsoudhi et al.,(2000)
Rhodococcus rhodochrous - IGTS8 by pH - stat feeding	*dsz*ABC	DBT	30	7.0	6.1 mmol HBP produced /kgDCW/h	Honda et al., (1998)
R.erythropolis H-2		3,4 di methyl DBT	30	7.5	1 mM DBT was completely degraded within 12 h.	Oshiro et al., (1996)
R.erythropolis D-1		DBT	37	7.0	2.2 mM DBT was completely degraded in 150 min.	Izumi et al.,(1994)

Table 2.3: Thermophiles reported as desulfurising microorganisms:

Microorganism	Substrate	Temp (°C)	Result	Reference
Sulfolobus acidocaldarius	Thianthrene	70	Maximum sulfur removal rate: 2.56 mg S / l. day.	Kargi et al., (1987)
Paenibacillus sp. Strain A11-2	4-methyl DBT 4,6-dimethyl-DBT 2,8-dimethyl DBT 3,4,6-trimethyl DBT	50	Decrease in total S content: 26.0% 25.5% 23.5% 29.5%	Konishi et al..(1997)
Bacillus Subtilis WU-S2B	DBT	50	0.54 mmol DBT was degraded in 120 h using growing cells. 0.81 mmol DBT was degraded in 12h using resting cells	Kirimura et al., (2001)
Anaerobic thermophile	DBT	65	98% of DBT degraded in 15 days.	Bahrami et al.,(2001)
Mycobacterium phlei GTIS10	DBT	50	1.1±0.07 μ mol 2-HBP/min/g DCW. Was produced.	Kayser et al.,(2002).

Microorganism	Substrate	Temp (°C)	Result	Reference
Mycobacterium sp. X7B	Hydrotreated diesel oil	45	86% of total S content is removed	Fu Li Li et al., (2003)
Mycobacterium phlei WU-F1	Hydrodesulfur -ised light gas oil DBT Naphthothioph -ene (0.81mM) 2-ethyl naphthothioph -ene	45 50 50 50	60-70% of S is removed. 0.81 mM DBT was degraded in 90 min. 67% of S content was removed. 83% of S content was removed.	Furuya et al.,(2003) Furuya et al.,(2001)
Mycobacterium phlei WU-0103	Light gas oil (with 1000ppm S content)	45	52% reduction	Ishii et al. ,(2005)
Mycobacterium goodie X7B	Gasoline	40	S content is decreased from 275 ppm to 54 ppm.	Fu Li Li et al., (2005)
Mycobacterium sp.G3-1	DBT in presence of 0.1 mM of $MgSO_4$	45	DBT degradation activity: 211 μ mol/g DCW/ h	Takada et al., (2005)
Mycobacterium phlei SM120-1	DBT Light gas oil	45	0.17± 0.02umol 2-HBP/ min/ g DCW. 70% of total S is removed.	Srinivasaragh-avan et al.(2006)

2.3 Application of genetic engineering

As reported by Monticello D.J. (1998), manipulating the basic genetic map of bacteria is crucial forwarding step in enhancing the biodesulfurization activity. Recently number of reports has been published concerning the genetic manipulation of wild type of strains. The well characterized gram positive organism *Rhodococcus erythropolis* IGTS8 has been widely used for genetic manipulation to enhance the desulfurization activity. The *dsz* ABC gene cluster cloned into alternative hosts like *Escherichia coli* and *pseudomonas sp.* to take advantage of their growth properties, physical properties for mixing and separations, or a higher intrinsic metabolic rate (Monticello 1998). It has been reported that, several properties of biotechnological interest for design of biocatalysts for biodesulfurization are present in pseudomonas species such as solvent tolerance of *Rhodococcus* is the lowest reported (log P values from 6.0 to 7.0), that of the genus pseudomonas (log P values from 3.1 to 3.4) is the highest known (Inoue et al 1991), and several *pseudomonas* strains highly resistant to heavy metals present in fossil fuels (Atlas 1994).

Gallardo et al constructed a recombinant *Pseudomonas* strain by engineering *dsz* gene cluster from *R. erythropolis* into *P. aeruginosa* PG201, that combines two traits of industrial interest that is a desulfurization phenotype and the ability to produce a biosurfacant that results in increased mass transfer rates in two-liquid-phase bioreactors (Gallardo et al 1997). Increasing the copy number of the dsz cluster in *P. aeruginosa* ATCC9027 would increase the desulfurization rate, this is not the case seen for strains that contained either a plasmid located copy of *dsz* cluster or chromosomally–located copy of the cluster (J. Raheb et al., 2005). By harboring *hcu* ABC genes into the recombinant *Pseudomonas putida* IFO13696 increases the uptake of DBT in the oil phase into the cell, 82% of 1mM DBT in n-tetra decane was degraded in 24 h by resting cells of this strain (Noda et al 2003).

Rhodococcus erythropolis strain KA2-5-1 is another wild type strain, which has been widely used for genetic manipulation. Genetically improved *R. erythropolis* KA2-5-1 has higher production rate of 2- hydroxybiphenyl than the wild type strain about 196 mmol

2-HBP/ kg DCW/ h (Kobayashi et al 2001). *R. erythropolis* KA2-5-1 is unable to desulfurize 4, 6- dipropyl dibenzothiophene, a more recalcitrant organo sulfur compound than DBT. By transferring *dsz* gene cluster from *R. erythropolis* KA2-5-1 to *Mycobacterium* sp. using transposon-transposase complex results in a recombinant MR65 strain which can desulfurize 68 mg/ l of sulfur in light gas oil (LGO) containing 126 mg sulfur/l. strain MR65 had about 1.5 times the desulfurization activity of *R. erythropolis* KA2-5-1 and is able to utilize 4, 6-dipropyl dibenzothiophene in the oil phase (Ken-Ichi Noda et al 2002). Another transposon-mutant strain of *R. erythropolis* KA2-5-1 named *R. erythropolis* MC0203 resulted from the *R. erythropolis* strain MC1109 has the LGO desulfurization capacity of about twice that of wild type strain (K.Watanabe et al 2003). Using *Rhodococcus- E. coli* shuttle vector the desulfurizing operon, *dsz* was introduced into various benzothiophene (BT)-desulfurizing bacteria. Of the tested *Rhodococcus* sp. strain T09 expressed the *dsz* operon and about 30 mg/ l of 2-HBP was detected at 45 h (T. Matsui et al 2001). Afterwards by introduction of the NAD(P)H/FMN oxidoreductase gene(*dsz*D), the DBT degradation activity of the recombinant *Rhodococcus* sp. strain T09 was increased by about 3.5 fold (T. Matsui et al 2001). Various genetically modified organisms reported in the literature are listed in the Table 2.4.

The aim of biodesulfurization research is to develop a commercially viable biodesulfurization process for petroleum, and it has been estimated that a successful commercial process would require a biocatalyst with desulfurization activity of 1.2 mM DBT/g DCW/h to 3 mM DBT/ g DCW/h (Monticello DJ 2000). From the literature cited we can say that this can be possibly achieved by genetically manipulating the wild type cultures to improve copy number of the desulfurization gene cluster which enhances the desulfurization rate, substrate specificity and mass transfer rate of oil into the cell from two phase mixture. From Fig 2.6 we can conclude that the genetically modified microorganisms have higher desulfurization activity than that of the parent strain. By genetic modification of the wild type strains we can improve the substrate specificity of the microorganisms, for example genetic manipulation work done by researchers on the *Rhodococcus erythropolis* KA2-5-1 attained (Fig 2.7) recombinant strains which have

greater desulfurization activity for sulfur present in light gas oil (LGO) which proves by genetic manipulation more efficient strains can be designed.

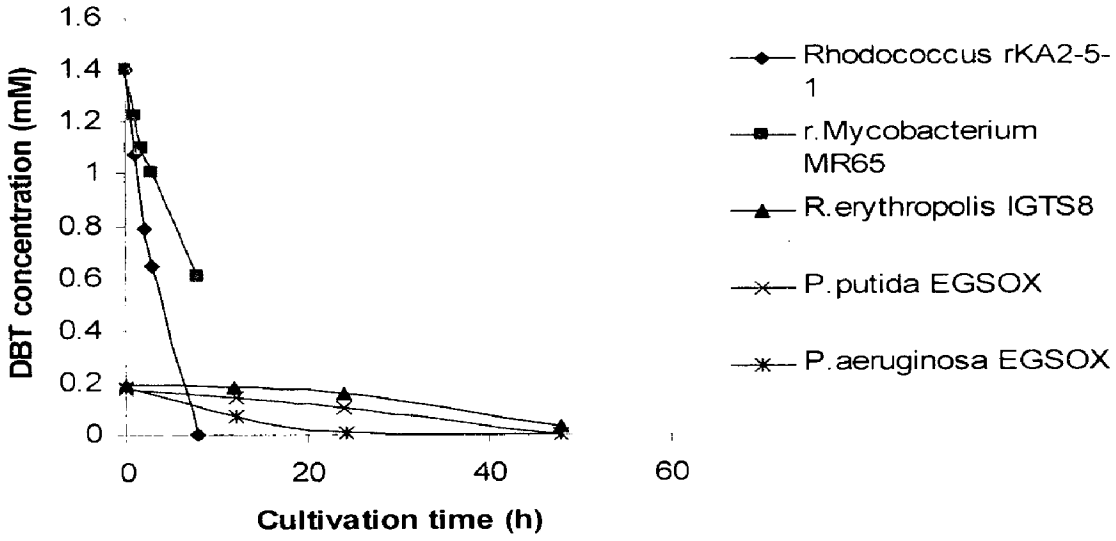


Fig 2.6: DBT degradation pattern of genetically modified microorganisms

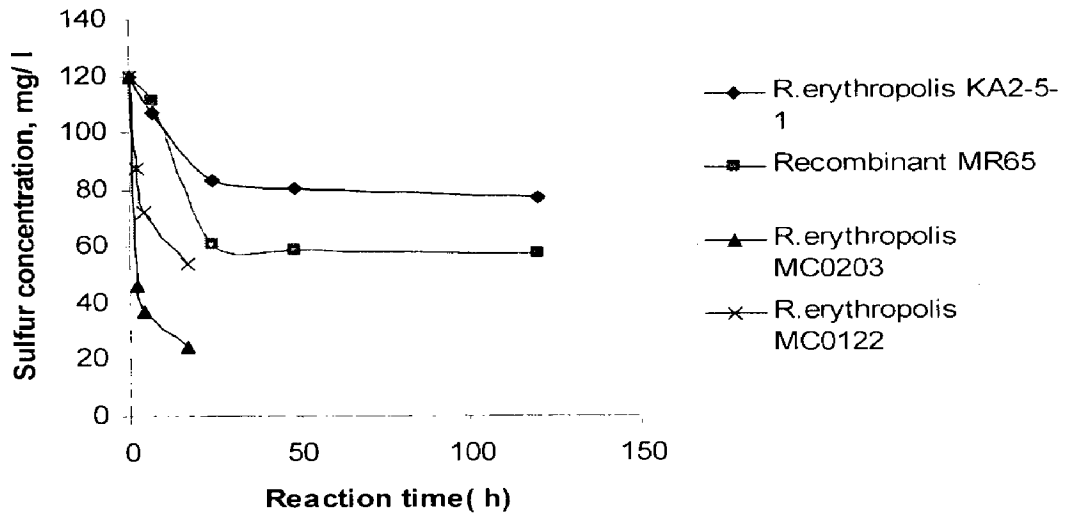


Fig 2.7: Desulfurization of Light gas oil (LGO) by genetically modified organisms

Table 2.4: Biodesulfurization activities of genetically modified organisms

Microorganism	Genes responsible	Result	Reference
Pseudomonas aeruginosa EGSOX	*dsz* gene cluster from *R.erythropolis* IGTS8 was inserted into *pseudomonas* strain	It transforms 95% of the DBT at 24h of incubation.	Gallardo et al (1997)
Pseudomonas putida EGSOX	*dsz* gene cluster from *R.erythropolis* IGTS8 was inserted into pseudomonas strain	40% of the DBT was consumed at 24h of incubation.	Gallardo et al (1997)
R.erythropolis I-19	*dsz* ABC operon cloned from *R.erythropolis* IGTS8	Desulfurization rate: 5.0-μ mol/ g DCW/ min.	Folsom et al (1999)
Rhodococcus sp. T09/pRKPP	With an additional dsz operon induction	DBT desulfurising activity is 31 μmol/g dry cell/h	Toru Matsui et al (2001)
Rhodococcus erythropolis rKA2-5-1	Genetically improved strain of *Rhodococcus erythropolis* KA2-5-1	Specific rate of desulfurization 196 mmol 2-HBP/ kg DCW.h	Kobayashi et al (2001)
Recombinant *Mycobacterium* MR65	Dsz gene cluster from *Rhodococcus erythropolis* KA2-5-1 was transefered to *Mycobacterium* sp.	It could desulfurize sulfur content in LGO from 126 mg/ l to 68 mg/ l	Ken-ichi Noda et al (2002)
Rhodococcus erythropolis strain MC0203	Transposon mutant strain of *Rhodococcus erythropolis* KA2-5-1	Decreases sulfur concentration of LGO from 120 mg/ l to 70 mg/ l.	Kimiko Watanabe et al (2003)
Pseudomonas putida IFO13696	*dsz* ABCD gene cluster from *R. erythropolis* KA2-5-1 and and hcu genes from *P.aeruginosa* NCIMB9571	82% of 1mM DBT in tetra decane was degraded in 24h by resting cells	Ken-ichi Noda et al (2003)

2.4 BIOREACTORS AND BIOPROCESSES FOR BIODESULFURIZATION

Various bioreactors may be used and different bioprocesses may be designed for the biodesulfurization of fuels. The nature of the biocatalyst, the type of fuel, volume, etc. will all dictate the applicability of specific bioreactor designs and the operational parameters of a biodesulfurization process. In the end, performance and economics will be the determining factor.

A biocatalytic process may be designed as a batch process in which the reactants and biocatalyst are maintained in a reaction vessel (bioreactor) for a period of time. Alternatively, the biocatalytic process can be designed as a continuous flow process in which the reactants are only brought into contact with the biocatalyst for a limited period of time. The choice of the process design depends on the economics of production and recovery of the desired product. It is relatively easy to maintain constant reaction conditions in a batch process and to prevent the entry of contaminating microorganisms since the bioreactor can be sealed. Batch reactors, however, require significant start-up times to initiate the fermentation process as well as incubation times to allow products to accumulate. Batch processes also require additional time during which the product is separated from the spent medium and microbial cells. The downtime involved in filling, emptying, and cleaning reduces the volumetric efficiency of these reactors.

Compared with batch processes, flow through processes is more prone to contamination with undesired microorganisms, making quality control difficult to maintain. The flow-through design, however, has the advantage of producing a continuous supply of product that can be recovered at a constant rate for commercial distribution. Continuous processes have higher volumetric productivity, that is, more efficient use of fermenter capacity because they are always full.

In contrast, the use of immobilized enzymes is an alternative method for the biocatalytic production of a product. In such processes, microbial enzymes or microbial cells are adsorbed or bonded to a solid surface support, such as cellulose, or encased in a

gel, such as alginate. The bonded and thus immobilized enzymes act as a solid-surface catalyst. A solution containing the chemicals to be transformed by the enzymes is then passed across the solid surface. Temperature, pH, and oxygen concentration are set at optimal levels to achieve maximal rates of conversion. This process is very useful when the desired transformation involves a single metabolic step, but it is more complex when many different enzymatic activities are required to convert an initial substrate into a desired end product (Beverly L. McFarland et al., 1998). Immobilized enzymes or immobilized cells make an industrial process far more economical than batch or continuous processes because the expense of continuously growing microorganisms and discarding the unwanted biomass is avoided. In immobilized enzyme systems, it is essential to maintain enzymatic activity so that the enzymes are not washed off the surface or inactivated during the process. When whole cells, rather than cell-free enzymes, are employed in such immobilized systems, it is necessary to maintain viability of the microorganisms during the process. This generally involves adding necessary growth substrates, but far lower amounts of nutrients are needed to maintain the viability of immobilized cells than would be required to support actively growing cells.

There are no published reports that describe the engineering design or operation of a process to biodesulfurize gasoline in terms that can be used to estimate the economics of the process. There are several reported research efforts, however, that address the biodesulfurization of crude oil, middle distillates, or diesel, which from an engineering perspective may have general application to gasoline biodesulfurization. Although developers of biodesulfurization processes have not published details of their process design approximate costs can be estimated by analogy to other processes. With this in mind, the following observations can be made: the estimated cost of biodesulfurization processing of 1400 ppm sulfur FCC gasoline to 150 ppm sulfur (which will meet the 50 ppm average sulfur national gasoline pool) is in the range of 2 to 3 cent/ gallon. On a pool basis, this yields a cost of about 0.6 to 1.0 cent/gallon of total refinery gasoline; capital costs in the context of the model refinery would be about $18 million; consideration is given to alternative reactor designs that present potential reductions for processing costs; it is apparent that there is a positive driving force for biodesulfurization even within the

context of these approximate data as installation of a new hydrotreating reactor would require closer to $30 million in capital costs. Except for the efforts of Energy Biosystems Corporation and to some extent Brookhaven National Laboratory, research in this area has generally centered on bioreactor designs and operations, leaving the biocatalyst preparation and product recovery/ separation steps as laboratory/ batch operations. In all cases, the research has generally addressed crude oil, middle distillates, or diesel as the feedstock material, not gasoline.

2.4.1 Continuous Stirred Tank Reactors (CSTR)

Energy Biosystems Corporation (EBC) has invested significant time and effort in development of a biocatalytic process to desulfurize petroleum distillate streams. Based on a microbial desulfurization approach using a *Rhodococcus* sp. strain isolated at the Institute of Gas Technology through funding from the U.S. Department of Energy, (Kilbane, 1989) EBC has proceeded to isolate the enzymes responsible for the desulfurization trait; enhance the efficiency of the microorganism for desulfurization; and design, engineer, and construct a pilot plant to desulfurize diesel fuel. A schematic of the EBC pilot operations is shown in Fig 2.8. The pilot plant system that has been used by EBC in its developmental process is a modular 0.5 to 5 barrels of oil/day plant operating at water-to-oil ratios of 0.1:1 to 5:1 and with residence times of 0.2 to 40 hours. Early design concepts involved nearly a straight translation from shaker flasks to a biotechnology process based on batch reactors. Later versions incorporated a continuous stirred tank reactor (CSTR) (Johnson, 1995). The EBC process mixes the biocatalyst in water with oil in a CSTR (OECD 1998). EBC reports that the parameters that appear important to biodesulfurization include pH, agitation rate, aeration rate, temperature, and cell loading. The pH should be between 6 and 8, and the temperature about 30°C. The target reaction time is one hour; the target water-to-oil ratio is 3:1. The material leaving the CSTR is an emulsion. This mixture is separated by centrifugation and a deoiling hydrocyclone. The sulfur byproduct from the reaction is sulfate, collected as gypsum. Other reports from EBC suggest that the oil/water emulsion may be difficult to separate. This work suggests that a phase-inversion process coupled with centrifugation can be used to separate the oil-water bacteria mixture. Biocatalyst enhancement has been a key focus of the EBC development work. According to EBC, the

biocatalyst can be whole, viable bacteria cells that express their desulfurization trait in the resting state. The specific activity of the biocatalyst is from about 8 to 30 mg product/h/g dry cell weight. EBC suggests that the activity must be increased to 600 in order to be cost competitive. Similarly, the half-life of the biocatalyst is currently in excess of 20 h, which according to EBC is sufficiently long to be cost competitive. Through the research conducted to date, EBC reports sulfur reductions using diesel feedstocks of 1900 to 570 ppm, 649 to 314 ppm, and 262 to 79 ppm (Monticello DJ, 1996). EBC plans call for this technology to be scaled up from a 5-barrels/day pilot-scale unit so as to operate on a commercial level. EBS has predicted commercialization of the technology in the next few years for diesel and in 3 to 5 years for crude oil. It has the potential to rival ethanol production as the largest process application of biotechnology to the energy sector and to have a significant impact on the economics of fuel processing. Like ethanol production, however, it must compete with existing solutions and faces daunting economic challenges before becoming a widespread technology. EBC has not published specific cost data for gasoline processing. EBC has, however, stated in various publications that the capital cost of a biodesulfurization facility will be about 50% of that for a hydrodesulfurization unit in the same service. EBC further claims that the operating costs will be about 10 to 15% less. In reference to data from the 1991 to 1992 National Petroleum Council Study of the Refining Industry, a 30,000-barrel/day hydrotreater is estimated to have an onsite capital cost of $25 million and operating costs (without capital charges) of $0.70/barrel. Allowing for off sites and escalation leads to an estimate of $40 million for the hydrodesulfurization. Applying this information with that obtained from EBC provides a biodesulfurization capital cost estimate of about $20 million. If capital charges are estimated at 20% /year and operating costs are 85% of the hydrodesulfurization case, a processing cost for biodesulfurization of $0.98/ barrel (2.3 cent/ gallon) is derived.

A second system employing stirred reactors is under development at Brookhaven National Laboratory (BNL) through funding from the U.S. Department of Energy, Office of Fossil Fuels. BNL is pursuing development of a biochemical technology for upgrading heavy crude oils and residuum, which includes desulfurization. The system can apparently employ either thermophilic (temperatures in excess of 55°C) or extremophilic organisms

(temperatures in excess of 100°C). BNL reports that the biological process can simultaneously reduce sulfur concentrations by 25 to 35%, trace metals by 20 to 50%, and nitrogen compounds by 20 to 50%. Product recoveries appear to exceed 90%, with 100% recoveries reported for some experimental runs (McFarland et al 1998).

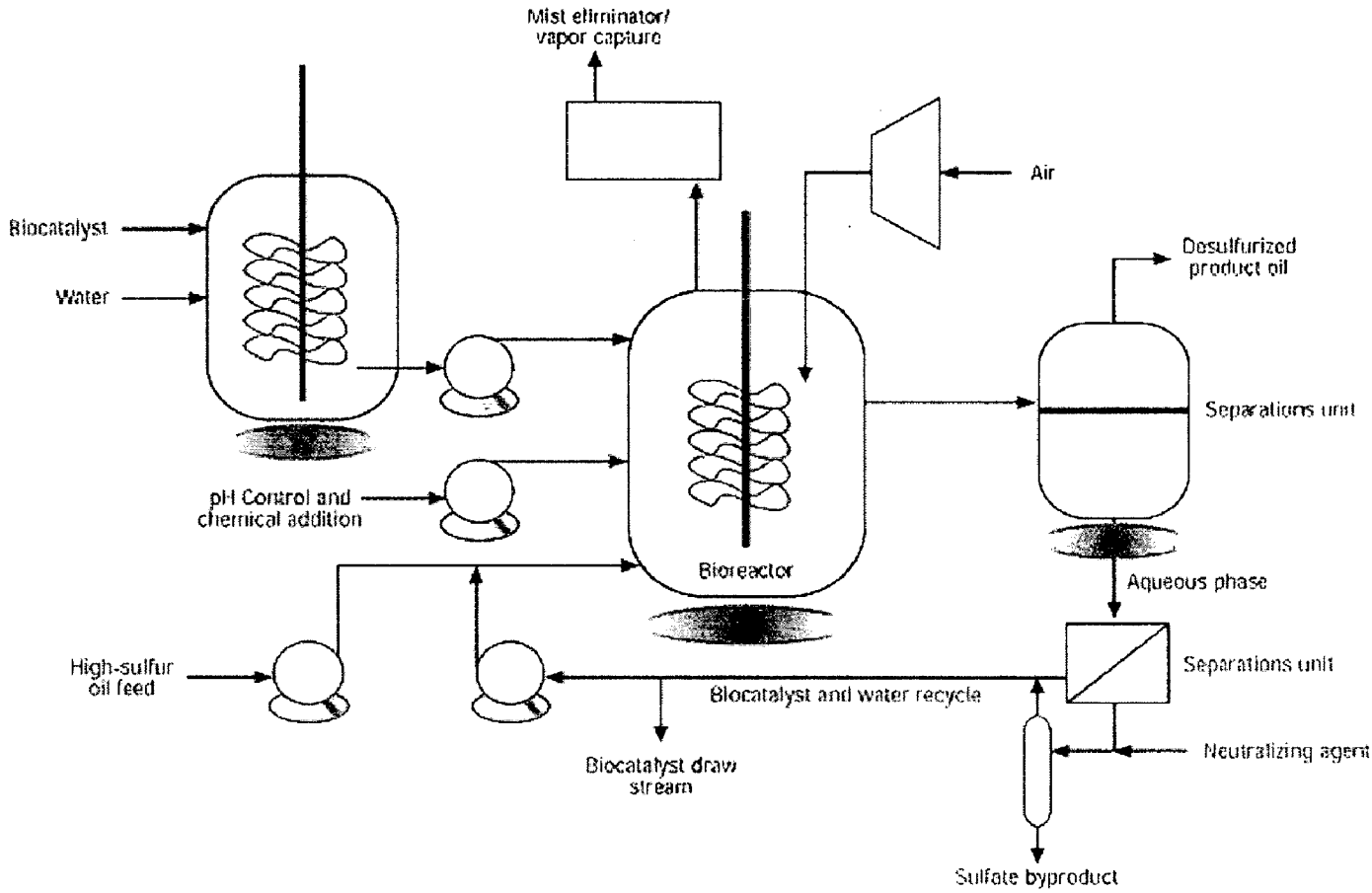


Fig 2.8: Bioprocess design for desulfurization of oil feed of 5bbl/ day (Developed by EBC)

2.4.2 Emulsion Phase Contactor:

A novel bioreactor system for treating crude oil is under development at Oak Ridge National Laboratory (ORNL) through funding from the U.S. Department of Energy, Office of Oil and Gas Processing. The research is novel in that it addresses the development of a bioreactor system that minimizes the introduction of water into hydrocarbon process

streams. This approach may also have application for the biological removal of nitrogen and trace metals from crude oil. The key operation unit in the ORNL system is the emulsion phase contactor (EPC). In this system, biocatalyst water slurry is sprayed through the EPC, which is immersed in a tank of the reaction media (e.g., hexane containing solubilized dibenzothiophene for model solutions, or crude oil). The spray action creates an emulsion of biocatalyst/water/ oil droplets. This mixture is then subjected to an electric field that serves to maintain the micron-sized particles formed by the action of the EPC as well as to help create new micron- sized droplets.

ORNL reports that the EPC minimizes the energy requirements of the system by selectively imparting energy at the liquid/liquid interface rather than imparting energy to the bulk solution. This action produces high surface area water/oil/bacteria droplets, which should serve to increase the desulfurization capability of the system if mass transfer were limiting desulfurization rates. The system uses water volumes of less than 5% and produces 3 to 5 μm aqueous droplets containing the biocatalyst with power consumption of about 3 W/L (Kaufmann et al 1997). The electric field applied to the system does not appear to adversely affect the activity of the biocatalyst, and the desulfurization capability of the system does not appear to be mass transfer limited. Using the IGTS8 *Rhodococcus* sp., test work to date has focused on model solutions of dibenzothiophene and several crude oil samples. When comparing the EPC reactor with batch stirred reactors based on model compound solutions, the EPC reactor uses nearly half as much biomass, but about 2.5 times the reaction volume (Kaufmann et al 1997). Although the reaction rates are comparable with batch stirred reactors at equivalent or excess cell densities, it appears that the extent of desulfurization is not appreciable, due perhaps to the excess reaction volumes. A study conducted by Cooney et al, (1998) employed a process simulator (Bio Pro Design) to develop process design and cost estimates for the biodesulfurization processing of high sulfur residual fuel oil using the IGTS8 *Rhodococcus* species. (This is the original strain employed by EBC in their process.) The study assumed residual sulfur reduction from 3.0% to 0.7%, the sulfur removed was dibenzothiophene, the oil to water ratio was 1 to 4, and the biodesulfurization plant employed a 10,000 barrel/day bioreactor system with 1 h residence time. Where lime was used to remove the sulfate, a processing cost of $2.50/

barrel (6 cent/gallon) was estimated. Of this, $0.40/barrel (1 cent/gallon) was attributed to lime addition and calcium sulfate disposition.

2.4.3 Fluidized Bed Reactors:

A proprietary bioreactor system is under development at the Argonne National Laboratory (ANL) in collaboration with EFX Systems, Inc. A presentation by ANL at the DOE Biodesulfurization Coordination Meeting17 indicates that this is a two-phase, fluidized bed reactor (FBR) adapted from technology developed by EFX for bioremediation. The system employs (1) oxygen/air dissolution in an external loop that uses no mechanical agitators to minimize explosion hazards and emissions, (2) very facile separation between the hydrocarbon phase and the biocatalyst bead, (3) immobilized biocatalysts on a suitable support system, and (4) a continuous bioprocessing operation. The FBR system is a high-rate bioreactor in which process liquid is passed up through a bed of a hydrophilic matrix containing a film of biocatalyst. The process liquid passes through the bed at a velocity sufficient to fluidize the particles on the bed. The reaction takes place in the biocatalyst film with simultaneous mass transfer between the film and the continuous phase process liquid (Datta et al., 1997).

The ANL's FBR is based on developments of Ecolotrol, Michigan Biotechnology Institute, and Envirex, Inc. EFX Systems was created in 1992 to further develop and commercialize this technology. To date, more than 30 commercial systems are in operation for treatment/remediation of contaminated industrial groundwater. These commercial systems range from 10 gallon/min to 3500 gallon/min. In a typical example for treating wastewater, containing toxics with 99% destruction, costs have ranged from 0.05 to 0.1 cent/gallon for systems ranging from 3000 to 100 gallon/min (McFarland et al 1998). While the ANL biodesulfurization system and biocatalyst requires further development, ANL has estimated the cost for FBR processing of gasoline for sulfur removal to 50 ppm at 5 to 10 times the cost for wastewater treatment. This implies a processing cost of 0.5 to 1.0 cent/gallon. To this one would need to add the cost of wastewater and biomass disposal.

CHAPTER-3

EXPERIMENTAL PROGRAMME

3.0 GENERAL

A recombinant *Pseudomonas putida* was developed to desulfurize dibenzothiophene (DBT) and it was tested for sulfur specific 4S pathway. Batch studies were conducted to desulfurize DBT in both aqueous and biphasic media for different concentrations of DBT. The effect of surfactant on biodesulfurization was also observed. The recombinant cells were immobilized on agar using entrapment technique and those cells were used for the biodesulfurization of model oil (DBT in n-hexadecane) and diesel.

Detailed procedures for developing recombinant strain and the desulfurization studies were explained in this section.

3.1 MATERIALS

3.1.1 Chemicals

Dibenzothiophene 98% purity obtained from MERCK-Schuchardt, n-hexadecane from Hi-Media chemicals and all other all the chemicals used were analytical reagent grade with more than 99% purity.

3.1.2 Microorganisms

Rhodococcus erythropolis IGTS8 was kindly provided by Dr. John Kilbane, Institute of Gas Technology, U.S.A., *Pseudomonas putida* (MTCC 1194) was procured from Microbial collection and Gene Bank, Institute of Microbial Technology (IMTECH) Chandigarh, India. *Escherichia coli* XL-1•Blue containing 4.0 kb plasmid pSAD 225-32 has been kindly provided by Dr. Kevin.D.Young, North Dakota University, U.S.A.

3.1.3 Plasmid

Plasmid pSAD 225-32 is of 4kb length contains functional desulfurization genes *dsz* genes, formerly known as SOX genes (Fig 3.1). The plasmid is of ampicillin resistant.

The plasmid was cloned from parent strain *Rhodococcus erythropolis* IGTS8 under lac control into *E. coli* XL1 Blue

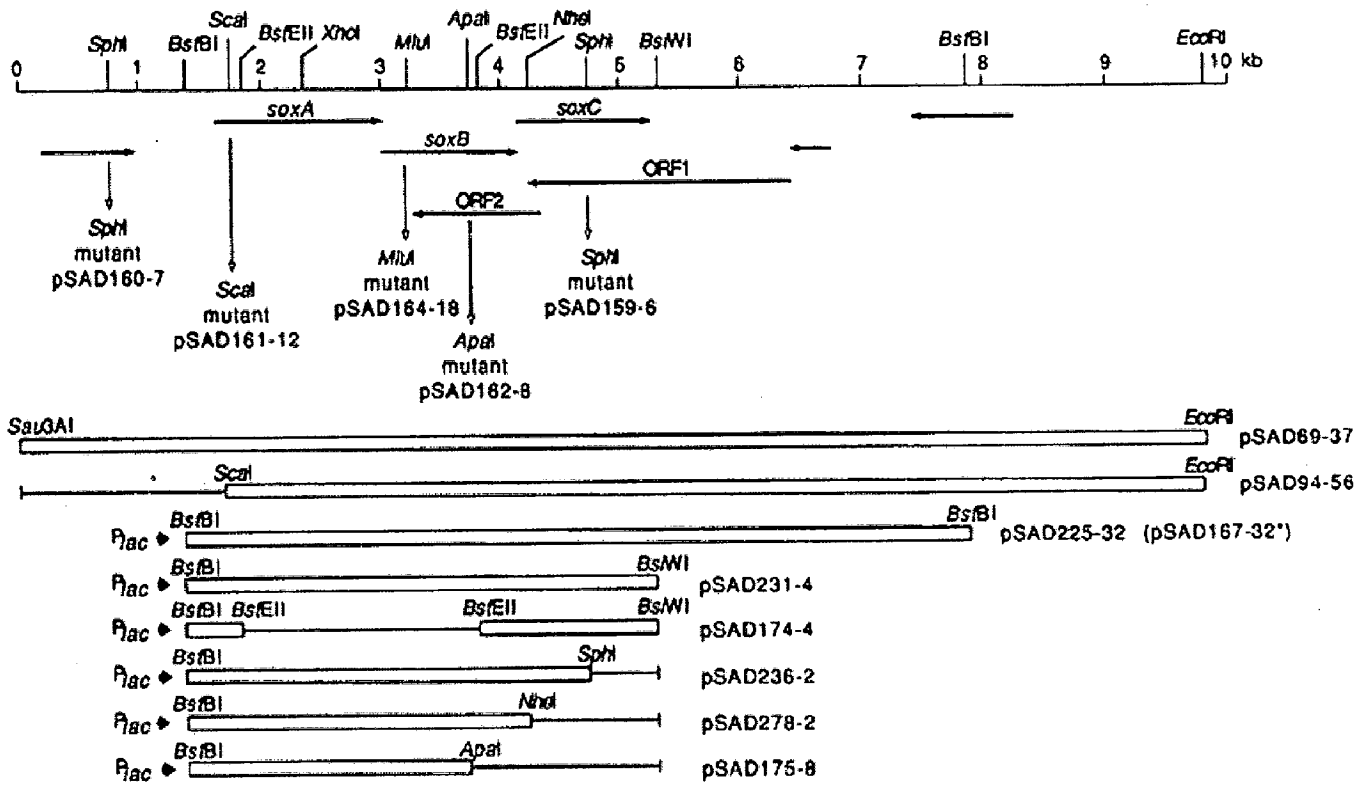


Fig 3.1: Partial restriction map of the 9.7-kb Sau3AI-EcoRI fragment that encodes the desulfurization genes from *Rhodococcus* sp. Strain IGTS8. (Denome et al., 1993)

3.1.4 Media for Cultivation

For plasmid isolation and transformation of cells Luria Bertani (LB) broth of Hi-Media Chemicals and for the selection of transformant cells selection LB agar plates were used of with ampicillin. The cultivation of transformant cells was done in two types of minimal salts media, one is basal salts media (BSM) of the composition given in Table 3.1 and another minimal salts media is NK of the composition given in Table 3.2. For both media DBT was used as sole source of sulfur. Composition of LB broth and agar were given in Table 3.3

Agar is a complex polysaccharide (carbohydrate) consists of 3, 6- anhydro-L-galactose and D-galacto pyranose, free of sulfur.

Table 3.1 Composition of Basal salts medium

Nutrient	Concentration (g/l)
$NaH_2PO_4.2H_2O$	4
K_2HPO_4	4
$MgCl_2.6H_2O$	0.0245
$CaCl_2.2H_2O$	0.001
Glycerol	2%
$FeCl_3.6H_2O$	0.001
NH_4Cl	2
Glucose	20

Table 3.2 Composition of NK medium

Nutrient	Concentration (g/ l)
KH_2PO_4	1.41
K_2HPO_4	6.13
NH_4Cl	2
Sodium citrate. $2H_2O$	1
$MgCl_2$	0.1
$FeCl_3.6H_2O$,	0.008
$CaCl_2$. $2H_2O$	0.02
Glycerol	20
Glucose	6

Table 3.3 Composition of Luria Bertani broth

Nutrient	Concentration (g/l)
Casein enzymic hydrolysate	10
Yeast extract	5
Sodium chloride	10
Agar (for solid medium)	15

3.1.5 Stock Solution

For aqueous phase studies, DBT was dissolved in the ethanol at concentration of 50 mM. For biphasic studies DBT was dissolved in the n-hexadecane, this mixture is known as model oil. Hexadecane is a representative aliphatic hydrocarbon in diesel oil, and it contains up about 40% of certain diesel oils. Diesel oil for the desulfurization was procured from Indian Institute of Petroleum, Dehradun, India.

3.2 METHODS

3.2.1 Sterility

All the glasswares used in the bacteriological studies were properly sterilized. Conical flasks, petridishes and test tubes were properly washed with lab wash and rinsed with distilled water for several times, dried in an oven at a temperature of 200°C and cotton plugged. These glasswares along with the solid and liquid medium were sterilized in an autoclave at 15 psi for 20 min. transfer of bacteria and media all were done in laminar hood over the flame of spirit lamp.

3.2.2 Isolation of Pure Culture

3.2.2.1 Preparation of stab culture of bacteria:

After getting the culture from the source, we revived the culture in the following procedure.

1. The medium of 10 ml of LB broth was prepared by mixing 0.25 g of LB broth in 10 ml of distilled water. The medium was sterilized in an autoclave for 30 min at 121°C.

2. In a laminar hood the bacteria from the eppendrof was carefully transformed into broth with sterilized wire loop.
3. The culture was incubated in an incubator at 37°C, which is an optimum temperature for *E. coli* growth, after overnight incubation considerable growth was observed in the medium. This culture was stored at 4°C.

3.2.2.2 Plating of bacteria:

From the fresh culture obtained by overnight growth of bacteria plating of the bacteria was done in the following manner.

1. A 25 ml of LB agar solution was prepared by mixing 1g of LB agar in 25 ml of distilled water. Autoclave the solution at 121 ° C for 30 min.
2. Wait for the solution to cool (should be cool enough to handle but not cold) and pour carefully into the petri dishes avoiding bubbles as much as possible.
3. Allow to gel or solidify at room temperature overnight (O/N). LB-Plates can be stored in a refrigerator for up to 2 months or at room temperature for a couple of days. .
4. After the wire loop has been flamed, without touching the end to anything, waiting several seconds for the loop to cool (otherwise bacteria will be killed). The wire loop was cooled; a very small amount of bacteria was scooped from the stock culture plate with the loop.
5. The bacteria was spreaded onto the media in the Bacteria Culture Plate. Plates were incubated for 48 hr. at 37 ° C.
6. After incubation of 48 hr, considerable number of bacterial colonies is observed on the plates. This plate was stored in the refrigerator for further batch studies.

3.2.3 Revival of Cultures

Rhodococcus erythropolis IGTS8 and *Escherichia coli* XL-1 Blue was obtained in the form of slant culture. *Escherichia Coli* XL-1 Blue was revived by inoculating the culture in Luria Bertani (LB) broth of Hi-Media Chemicals and incubating the broth at 37°C for overnight. *Rhodococcus erythropolis* IGTS8 was revived in BSM medium and incubated at 30°C for 24 h following the above mentioned procedure. *Pseudomonas putida*

(MTCC 1194) was obtained in the freeze-dried culture, which cannot be directly used for the batch studies; therefore the culture was revived according to the procedure prescribed by the Curator Chandigarh, India. The culture was grown in the LB medium at 30°C for 24 h.

3.2.4 ISOLATION OF PLASMID

The recombinant plasmid pSAD 225-32 (ampicillin resistant) from *E. coli* XL1 Blue was isolated by alkaline lysis with sodium dodecyl sulphate (SDS) following the protocols given by Sambrook et al., 1989. The stock solutions, buffers and reagents needed for the isolation are listed below:

3.2.4.1 Buffers and solutions

Alkaline lysis solution I (after autoclaving stored at 4°C)

Composition: 50mM glucose
25 mM Tris-Cl (pH 8.0)
10mM EDTA (pH 8.0)

Alkaline lysis solution II (solution II should be freshly prepared and used at room temperature)

Composition: 0.2N NaOH
1% (w/v) SDS

Alkaline lysis solution III

Composition: 5 M Potassium acetate-60 ml
Glacial acetic acid - 11.5 ml
H_2O- 28.5 ml

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate stored at 4°C.

Ethanol

Phenol: chloroform (1:1, v/v)

STE buffer

Composition: 10 mM Tris-Cl

0.1 M NaCl

1 mM EDTA (pH 8.0)

5% (v/v) Triton X-100

TE (pH 8.0) containing 20µg/ml Rnase A

Stock solution of ampicillin (100mg/ l) for plasmid selection

Media: LB

3.2.4.2 Procedure

Preparation of cells

1. 5 ml of LB medium was inoculated with a single colony of *E. coli* XL1 Blue. The culture was incubated overnight at 37°C with vigorous shaking.
2. 1.5 ml of the above culture was centrifuged at 10,000 rpm for 30 seconds at 4°C in a microfuge.
3. After centrifugation the supernatant medium was removed by aspiration, leaving the bacterial pellet as dry as possible. This step was accomplished using disposable pipette tip.

Lysis of cells

4. The bacterial pellet was resuspended in 100 µl of ice-cold alkaline lysis solution I by vigorous shaking.
5. 200 µl of freshly prepared alkaline solution II was added to the bacterial suspension and the contents were mixed by inverting the tube rapidly several times. Tube was stored on ice.
6. After that 150 µl of alkaline solution III was added and dispersed through the viscous bacterial lysate by inverting the tube several times tube was stored on ice for 5 min.
7. Bacterial lysate was centrifuged at 12000 rpm for 5 min at 4°C in an eppendrof and the supernatant was transformed to a fresh tube.
8. An equal volume of phenol:chloroform was added and by vortexing the organic and aqueous phases were mixed and then the emulsion was centrifuged at 10000 rpm for 2 min.

Recovery of plasmid:

9. By adding 2 volumes of absolute ethanol to the supernatant nucleic acids were precipitated. After vortexing the mixture was allowed to stand at room temperature for 2 min.
10. Precipitated nucleic acids were collected by centrifugation at maximum speed for 5 min at 4°C at room temperature.
11. Supernatant was removed by gentle aspiration. Tube was kept in an inverted position on a paper towel to allow all of the fluid drains away. Disposable pipette was used to remove the drops of fluid adhering to the walls of the tube.
12. 1 ml of 70% ethanol was added to the pellet and the tube was inverted several times. The DNA was recovered by centrifugation.
13. Again remove all the supernatant by gentle aspiration
14. Open tube was stored at room temperature until the ethanol has evaporated and no fluid is visible in the tube (approximately for 10 min).
15. Nucleic acids were dissolved in 50 µl of TE (pH 8.0) containing 20µg/ ml Dnase-free Rnase A. Solution was vortexed for few seconds and the plasmid DNA was stored at –20°C.

3.2.5 AGAROSE GEL ELECTROPHORESIS

The resulting plasmid DNA was screened by agarose gel electrophoresis. The protocol was described below.

3.2.5.1 1 x TBE Running Buffer

1. The volume of 1 x TBE buffer stock solution required to prepare 350 ml of running buffer for gel electrophoresis was determined.
2. Using 50 ml graduated cylinder, the calculated volume of buffer was transformed into 500 ml graduated cylinder.
3. The buffer was diluted to the final volume of 350ml using deionized water.

3.2.5.2 Preparing the Agarose gel

1. The amount of agarose needed to prepare 50 ml of 0.8% concentration was calculated and was added to the 50 ml of 1x TBE in a 100 ml flask.

2. The agarose solution was microwaved at the highest power for about 1 min.
3. When agarose solution was cool enough that can be easily handled by hand, ethidium bromide was added and gently swirled to mix. The agarose was ready to pour.

3.2.5.3 Casting the Agarose Gel and Preparing the Gel Chamber

Gel casting

1. The end of casting tray was sealed and the comb was inserted in the notch closed to the end of the tray. The casting tray was set in a place on lab bench where it will not be disturbed.
2. The warm 0.8% agarose solution was poured, containing ethidium bromide, into the casting tray to a depth of about 5 mm. The gel appears cloudy when it was solidified. The casting tray was not removed during solidification.

The gel and gel chamber preparation

1. When the agarose gel has solidified, the end of the casting tray was unsealed and placed in its correct alignment in the gel chamber. The comb was at the negative pole.
2. The chamber was filled with just enough 1xTBE buffer to cover the entire surface of the gel. The comb was gently removed, without damaging the gel. The gel was to be loaded with the DNA.

3.2.5.4 Loading the gel and performing gel electrophoresis

1. 1 µl of loading dye was added to each tube. Fresh tips used for each DNA sample. The loading dye was mixed with the DNA by pulsing the tube in a centrifuge.
2. An aliquot of DNA marker containing loading dye was also run.
3. 5 µl from each tube was taken using pipette, including the DNA marker were loaded into separate wells of the gel chamber. Fresh tips were used for each tube. Sample identification with well number was correlated and recorded.
4. The cover of the chamber was slided into place and the electrical leads were connected to the power supply.

5. The power supply was turned on, and electrophoresed at 100V until the blue dye front in near the end of the gel chamber.
6. When the electrophoresis was completed after 60 min, the power supply was turned off. The leads were disconnected from the power supply and the top of the gel chamber.
7. The casting tray was removed from the gel chamber and the buffer was discarded.

3.2.5.5 Gel visualization

DNA bands were visualized by placing the gel over by placing the gel on a transilluminator (Bio Rad, U.S.A). DNA bands appear orange in color as shown in the next section and the size of the plasmid was determined by comparing with DNA markers.

3.2.6 DEVELOPMENT OF RECOMBINANT *Pseudomonas putida*

The isolated plasmid DNA pSAD 225-32 was transformed to *Pseudomonas putida* by preparing competent cells of *Pseudomonas* sp following the protocol developed by Liu et al., with slight modifications. Briefly the procedure adopted is as follows.

3.2.6.1 Buffers and solutions

1. $CaCl_2.2H_2O$ of 1M in the preparation of competent cells, 10 ml aliquot of the stock solution was thawed and diluted to 100 ml with 90 ml of Millipore water. The solution was sterilized by filtration through a 0.4 μm filter and then chilled to 0°C.
2. Piperazine-1, 4-bis [2-ethanesulfonic acid] (PIPES, pH 7.2)
3. LB media for initial growth of culture

Nucleic acids Plasmid DNA (recombinant plasmid)

3.2.6.2 Apparatus

1. Refrigerated centrifuge
2. Temperature controlled water bath (preset at 42°C)
3. Incubator cum shaker
4. Polypropylene tubes chilled in ice

3.2.6.3 Procedure

Preparation of competent cells

1. 1 ml of an overnight Luria Bertani broth (LB) culture of *Pseudomonas putida* was transferred into 50 ml fresh LB and the incubation was continued at 30°C until the OD reached approximately 0.6.
2. Bacterial cells were transformed to sterile, disposable, ice-cold 50 ml polypropylene tubes. The culture was cooled to 0°C by storing the tubes on ice for 10 min.
3. The cells were collected by centrifugation at 4000 rpm for 10 min at 4°C.
4. The medium was decanted from the cell pellet and the tubes were made to stand on paper towel for 1 min to allow the last traces of media to drain away.
5. Cell pellet was resuspended in 10 ml of 100 mM $CaCl_2$, 10 mM PIPES (Piperazine-1, 4-bis [2-ethanesulfonic acid]), pH 7.2.
6. The cells were then incubated on ice for 30 min. The cells were collected by centrifugation, and gently resuspended in 0.5 ml of 100 mM $CaCl_2$, 10 mM PIPES, pH 7.2, 15% (v/v) glycerol, followed by a 30 min incubation on ice. At this point the competent cells were ready for transformation.

Transformation

7. To transform the $CaCl_2$ treated cells directly, to 100 µl of the competent cells from above, 10 µl (approximately 100 ng) of purified plasmid pSAD225-32 was added, mixed well by gentle swirling and the mixture was incubated on ice for 30 min.
8. The mixture was heat shocked at 42°C for 2 min and was immediately chilled on ice for 1 hour.
9. One ml of prewarmed (30°C) LB was then added to the mixture and 60 min of incubation at 30°C was allowed (with shaking) for the expression of the antibiotic resistance marker encoded by the plasmid.
10. In order to select transformants 50 µl of transformation mixture was plated on LB-agar plates containing amounts of antibiotics that prohibited growth of the untransformed strain, i.e., ampicillin of 0.1 g/ml was added.

11. The plates were incubated at 30°C and the transformed colonies were appeared in16h.

The transformant cells (Fig 3.2) are tested for the plasmid existence by preparing the cell cultures from the transformant colonies and isolated the plasmid following the same protocol as mentioned above. The resulting plasmid DNA preparation was screened by agarose gel electrophoresis.

3.2.7 DESULFURIZATION ASSAYS

The spray plate assay was performed to identify the pathway of the recombinant pseudomonas sp, as described by Denome et al., 1993. Briefly, large patches of genetically modified bacteria from the ampicillin resistant plates were incubated on BSM-2% agarose plates, which were previously sprayed with 0.1% DBT solution in ethyl ether. DBT was immediately precipitated as a white film on the plate surface. After 24 h of incubation at 30°C plates (Fig 3.3) were observed under short wave UV illumination, the bright blue white colonies were observed around the patches, which confirmed the 2-HBP production from DBT. This spray plate assay demonstrated that recombinant cells follow the sulfur specific 4S pathway (Fig 2.2). The desulfurization assay was performed in the liquid culture also. The details were discussed in the next chapter.

3.2.8 BATCH STUDIES

3.2.8.1 Preparation of Inoculum and Media Selection

Transformant bacterial colonies from nutrient agar plate were inoculated into the 50 ml of LB broth containing 50 µM of DBT in ethanol and incubated it for 24 hr at 30°C and 210 rpm. This culture was used for the further inoculation. To select the suitable media for the desulfurization studies using this strain, the growth rate of bacteria was monitored in two types of minimal salt media namely BSM and NK media at 30°C and 210 rpm. Growth was monitored by measuring the optical density (O.D) using Spectrophotometer at 600nm.

3.2.8.2 Growth Characteristics of Microorganism

Recombinant *Pseudomonas* cells were inoculated into NK medium of 100ml in 250 ml flask with final concentration of 0.2 mM of DBT as ethanolic solution and 0.1 g/l of ampicillin and incubated at 30°C and 210 rpm in incubator shaker. As a control the parent strain *R. erythropolis* IGTS8 also cultivated in BSM medium of optimized composition else where in the literature (Carolina et al., 2005) with the same DBT concentration as above. Further the recombinant cells were grown with increasing concentrations of DBT such as 0.4 mM, 0.8 mM, 1.2 mM and the growth patterns were observed and kinetic parameters μ_{max} and K_s were determined

3.2.8.3 Biodesulfurization Studies

Desulfurization of DBT was carried out in aqueous and in biphasic media (model oil) using growing cells and resting cells respectively.

3.2.8.3.1 Desulfurization of DBT in aqueous phase

A stock solution of 50 mM DBT was prepared by dissolving DBT in ethanol. This stock solution was added to the 100 ml NK medium to the final concentration of 0.2 mM and to the other broth with final concentration of DBT up to 1.2 mM and to each broth 0.1 g/l of ampicillin was added. These media were inoculated with 3% inoculum and incubated in incubator shaker at 30°C and 210 rpm. The desulfurization ability of recombinant *Pseudomonas putida* was studied by collecting and analyzing the periodically taken samples.

3.2.8.3.2 Preparation of resting cells

To prepare resting cells of recombinant *Pseudomonas* strain, 250 ml of NK medium was prepared with 0.2 mM of DBT as sole source of sulfur in 500 ml Erlenmeyer flask and 1 g/l ampiciliin was added. This medium was inoculated with 5% inoculum and incubated in incubator cum shaker at 30°C and 210 rpm. After 36 h, when the culture was in mid exponential growth phase the cells were harvested by centrifugation at 8000 rpm for 15 min. The supernatant culture was decanted from the cell pellet and the cells were

washed twice with saline of 0.9% and finally suspended in the 0.1 M potassium phosphate buffer. Now the resting cells are ready for the desulfurization assay.

3.2.8.3.3 Desulfurization of model oil by resting cells

Optimum oil to water ratio

The volume ratio of oil to water (O/W) affects the bioavailability of DBT when biodesulfurization occurs in the interface between the organic and the aqueous phases. The O/W factor is also an important factor in determining the reactor productivity and thus the reactor volume.

The optimum O/W ratio was determined as follows: model oil was prepared by dissolving 2 mM DBT in n-hexadecane. The model oil was mixed to the resting cell suspension in 1:4, 1:3, 1:2, 1:1, and 2:1 ratios for total volume of 10 ml in 50 ml conical flasks. The BDS reaction was performed by incubating the flasks in incubator shaker at 30°C at 210 rpm. 500 µl of samples were taken into aliquots and analyzed for DBT degradation and 2-HBP production.

Effect of surfactant on biodesulfurization

The effect of surfactant, Tween-80 on desulfurization was studied at different concentrations of Tween-80. In this study model oil containing 2 mM DBT was used. At the optimum O/W ratio 1:2 for the total volume of 10 ml reaction mixture containing resting cells and model oil, different concentrations of Tween-80 was added ranging from 0 to 3000 mg/l. After 36 h of BDS reaction samples were analyzed for residual DBT.

3.2.8.3.4 Desulfurization of diesel oil by resting cells

The model oil containing 25.2 mM (4666.66 ppm) DBT dissolved in n-hexadecane was desulfurized using resting cell suspension containing 31.8 g biomass/ l. The model oil was mixed with resting cell suspension adding 1 g/l Tween-80 as surfactant and the desulfurization reaction was carried on for 56 h. The model oil was mixed with the resting cells at the 1:2 ratio of total volume of 10 ml in 50 ml conical flask. The periodic samples

were analyzed for residual sulfur content. Similar experiment was conducted with diesel oil and the samples were analyzed by CHNS analyzer

3.3 ANALYTICAL METHODS

3.3.1 Quantification of DBT and 2-HBP

During desulfurization studies at each sample point samples were collected in aliquots. These samples are stored at 4°C until the analysis. These samples were acidified to less than pH 2 with 85% phosphoric acid, and then samples were extracted with an equal volume of ethyl acetate. The aliquots were centrifuged at 16000 rpm for 10 min at the room temperature and then the supernatant was analyzed using Gas chromatograph (GC) (NETEL (India) Limited model- MICHRO 9100) equipped with Flame ionization detector (FID). The HP-5 capillary column (30m×0.249mm×0.25μm film thickness, temperature limits: 60°C to 325°C) was used in the GC-FID with oven temperature held at 180°C for 1 min afterwards ramped up to 250°C at the rate of 10°C/ min. Injector and detector temperatures were maintained at 270°C and 280°C respectively. Nitrogen was used as carrier gas at the flow rate 20 ml/min and hydrogen gas as fuel. The peaks for DBT and 2-HBP were observed at 5.44 min and 3.99 min respectively. The intermediate products formed like $DBTO_2$, HPBS were not measured. The calibration curves, peak area versus concentration were plotted for the known concentrations of DBT as well as for 2-HBP dissolved in ethyl acetate.

3.3.2 Cell Growth

Bacterial growth was measured by measuring optical density at 600nm using UV-Vis spectrophotometer (Perkin Elmer model Lambda 35).

3.3.3 Biomass Concentration

The dry weight of cells was determined as follows the samples were centrifuged at 16000 rpm for 15 min and the supernatant was decanted and the cell pellet was dried by inverting it on paper towel until the medium was drained out. After that the pellet was washed with alcohol for several times to dehydrate the pellet and dried in the oven at 50°C

for 5-10 min. the dry cell weight was measured and the calibration curve was plotted between optical density and biomass concentration per liter.

3.3.4 Microscopic view of the microorganism

The microscopic view of the bacteria was obtained by using Gram Stains –Kit supplied by Himedia chemicals. Recombinant bacterial culture was diluted in 0.9% saline and a thin smear was prepared by taking 2 to 3 drops of culture on microscopic slide. This was dried in air and fixed by gentle heat. After that following the manufacturer's instructions the slide was observed under the microscope with 10000X resolution and microscopic view of the recombinant *Pseudomonas putida* was obtained.

3.3.5 Scanning electron microscopic image of bacteria

Bacteria was made to grow on cover plates in a test tube and incubated for 24 h. Cover plates removed from the media and dipped in the beaker containing 2% glutaraldehyde solution for 2 h. after discarding the solution, plates were consequently dipped in 30%, 50%, 70%and 90% alcohol for 30 min in each solution. Finally it was dehydrated in pure alcohol for 2 h and dried in air for 15 min. These plates were analyzed by scanning electron microscope (SEM, U.K).

3.3.6 CHNS Analysis

The sulfur content present in the diesel after biodesulfurization reaction was determined by analyzing the samples with CHNS analyzer (Elementar, Vario EL III, Germany).

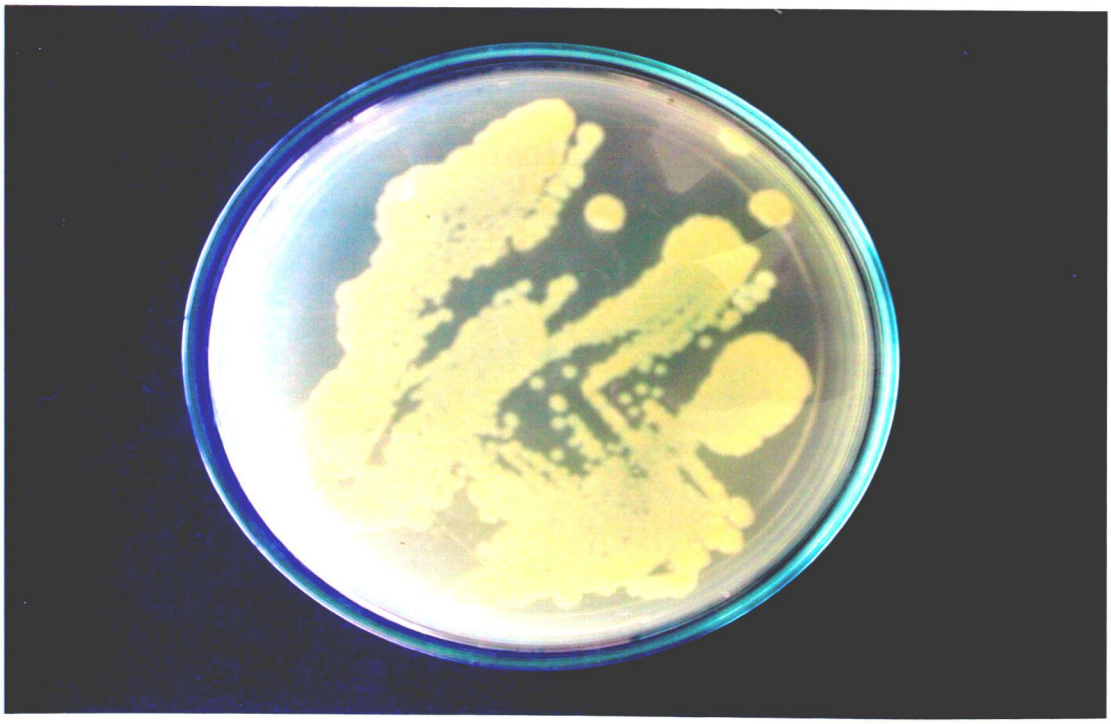


Fig 3.2 Photograph showing growth of transformant colonies of *Pseudomonas putida* on petri dish.

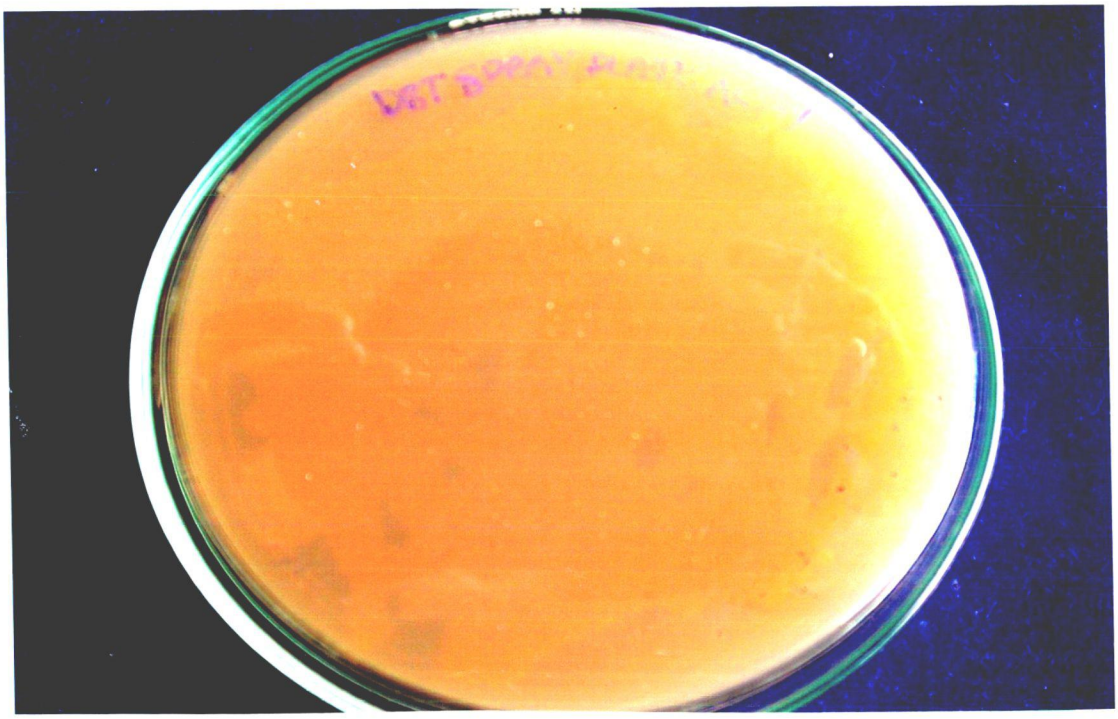


Fig 3.3 Photograph showing DBT spray plate assay test on BSM-agarose plate

CHAPTER-4

RESULTS AND DISCUSSIONS

4.0 GENERAL

The main aim of the present study is to develop a recombinant *Pseudomonas putida* that can desulfurize DBT in both aqueous and biphasic media. Experiments conducted in the present study include the isolation of the plasmid pSAD 225-32 which contains the *dsz* desulfurization genes (parent strain is *R. erythropolis* IGTS8) from *E. coli* XL1 Blue and transform the plasmid into *P. Putida* (MTCC 1194) to develop a recombinant strain. The protocols used for the plasmid isolation and transformation were discussed in the previous section.

This recombinant strain was used in the batch studies to determine its desulfurization ability in aqueous phase using DBT dissolved in ethanol for different concentrations of DBT. The growth characteristics of recombinant strain were determined by cultivating the bacteria with various concentrations of DBT. Desulfurization experiments were conducted on DBT dissolved in n-hexadecane (model oil), which represents diesel oil using resting cells of recombinant strain. The optimum oil to water ratio (O/W) was determined and the effect of surfactant on desulfurization at the optimum O/W was also determined. Similar experiment was conducted to desulfurize hydrodesulfurized diesel oil. The strain was immobilized on agar gel and the entrapped cells were used to desulfurize model oil and diesel oil.

4.1 DEVELOPMENT OF A RECOMBINANT STRAIN

By following the protocols discussed in the previous section a recombinant *Pseudomonas putida* strain was developed. The transformant cells are tested for the plasmid existence by preparing the cell cultures from the transformant colonies and isolated the plasmid following the same protocol as mentioned earlier. The resulting plasmid DNA preparation was screened by agarose gel electrophoresis, the gel band of 4

kb plasmid was obtained as shown in the Fig 4.1 confirmed the plasmid presence in the transformant cells.

The recombinant bacteria was tested for the sulfur specific 4-S pathway by performing desulfurization assays as described by Denome et al, 1993.. The plate assay for the identification of recombinant bacteria capable of modifying DBT was performed as described in the previous section. Two distinct metabolic phenotypes are distinguishable when plates are viewed under short wave UV illumination. First, when 2- HBP was produced from DBT, a bright blue-white UV- fluorescent product is produced. This fluorescence can spread across the entire Petri dish with prolonged incubations. The substrate sprayed on the plate completely disappeared beginning as a cleared zone around the colony and eventually spreading across the entire plate. A second fluorescent phenotype was observed when DBT was metabolized to $DBTO_2$. This fluorescence was less bright than the fluorescence seen with 2-HBP and the $DBTO_2$ fluorescence is closely associated with the bacterial colony. Also, the production of $DBTO_2$ from DBT was not associated with complete clearing of DBT from the assay plate as seen with the production of 2 HBP.

Desulfurization assays were also performed in liquid culture. For these assays, cells were incubated overnight at 30°C in LB medium, pelletted by centrifugation, and then washed three times in BSM medium. Washed cells were used to inoculate fresh BSM containing the test substrate DBT at a 0.1% concentration. The assay cultures were shaken at 30°C for 48 h and the culture supernatants were analyzed using GC-FID as described in the previous section for detection of DBT degradation and HBP production. These two desulfurization assays demonstrated that the recombinant bacterial cells follow the sulfur specific 4S desulfurization pathway.

The microscopic and scanning electron microscopic (SEM) images of host strain *Pseudomonas putida* MTCC 1124 and the recombinant *Pseudomonas putida* had been shown in the Fig 4.2 to Fig 4.7. These images show that the rod shaped gram-negative

pseudomonas host strain morphology was not changed due to the recombination or by the toxic substrate DBT.

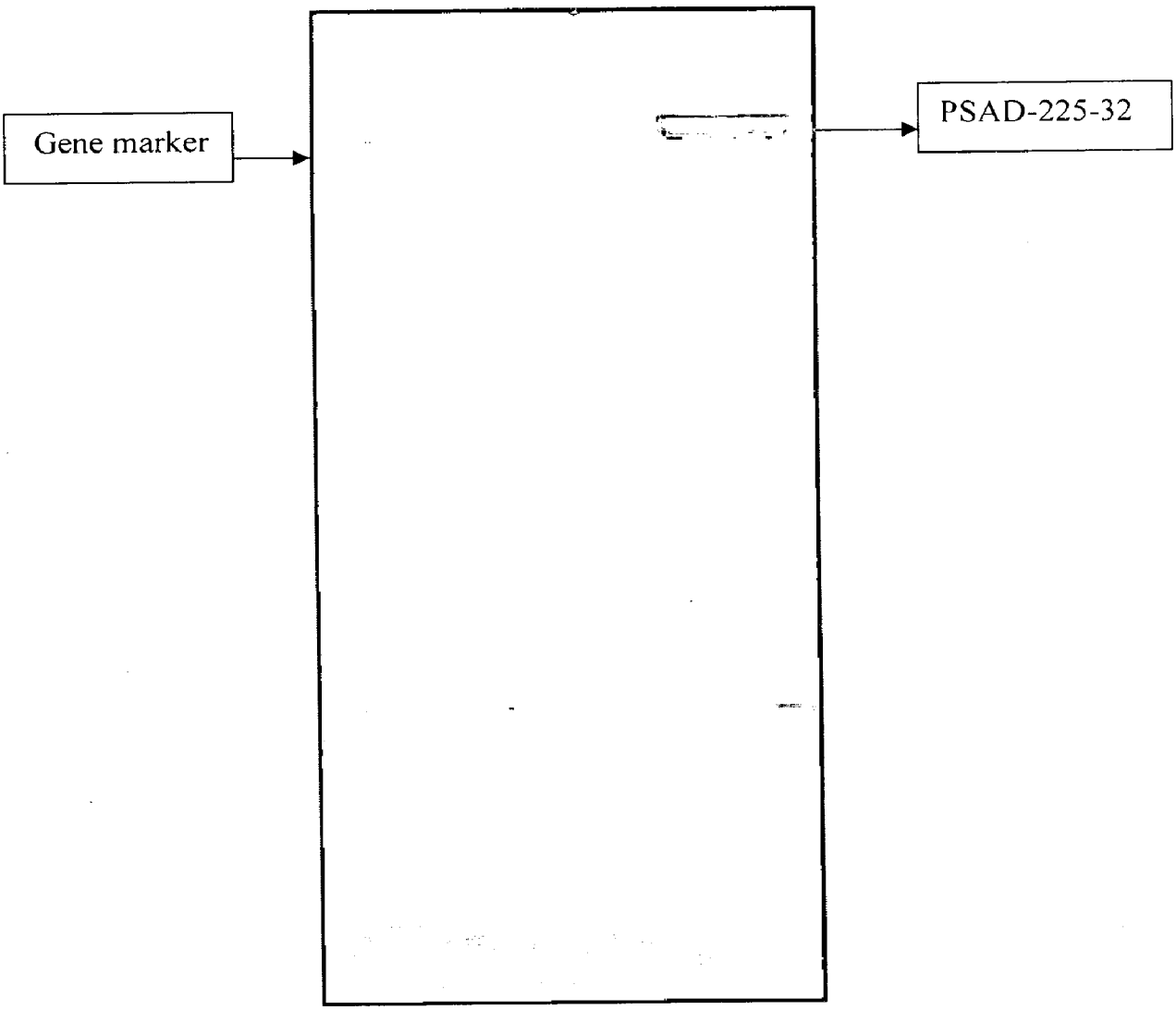


Fig 4.1: The gel band of 4-kb plasmid pSAD 225-32 obtained during plasmid screening by agarose gel electrophoresis.

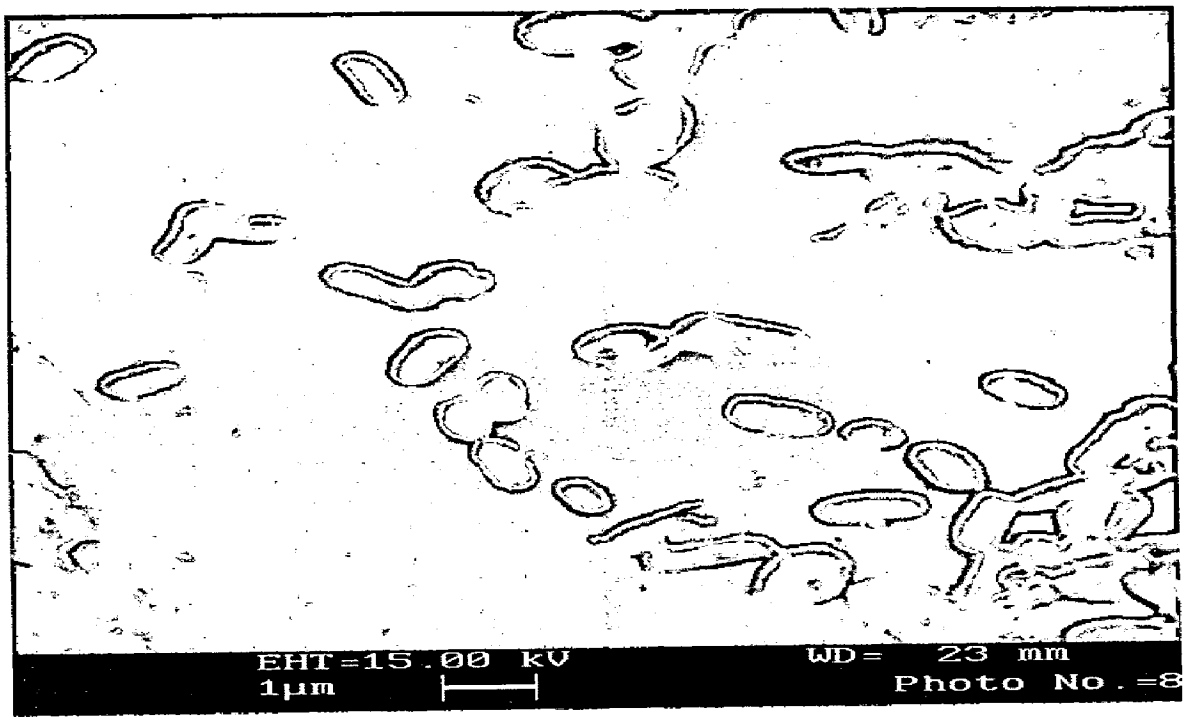


Fig 4.2: The scanning electron microscopic image of *Pseudomonas putida* MTCC 1194

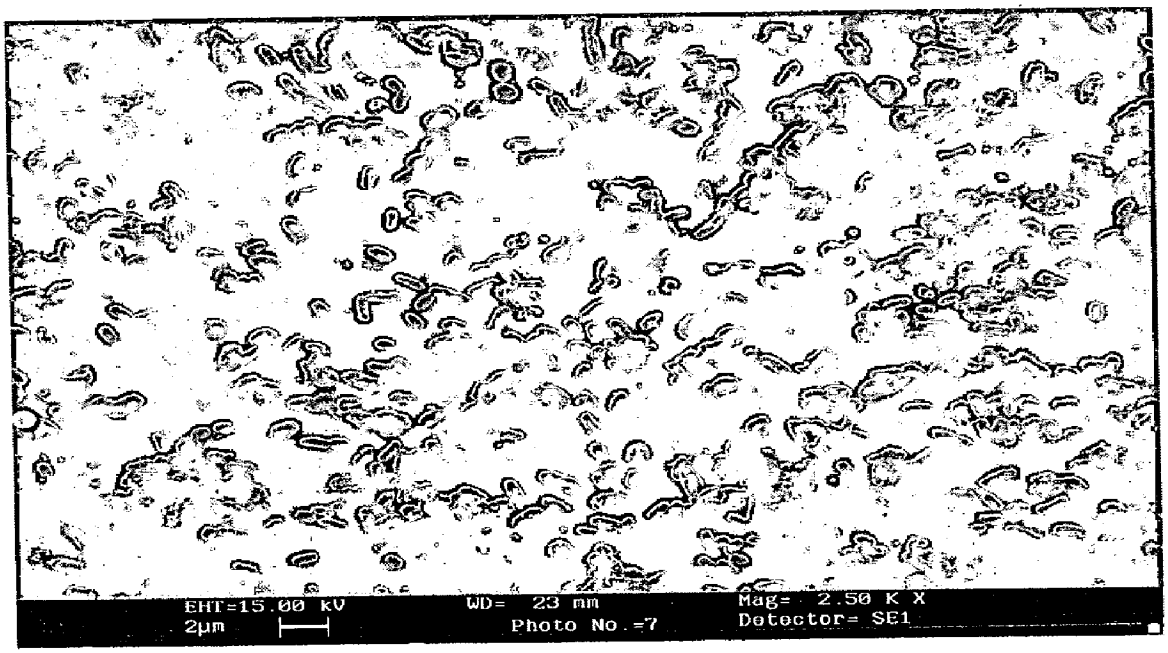


Fig 4.3: The scanning electron microscopic image of recombinant *Pseudomonas putida*

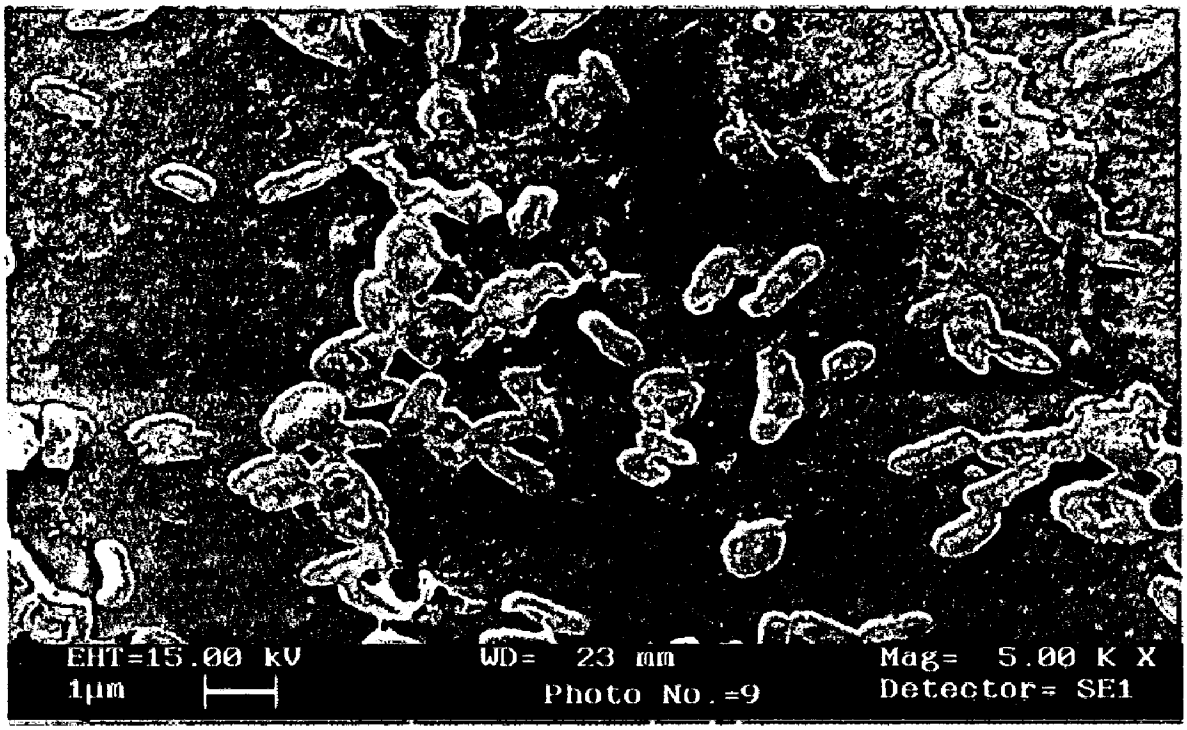


Fig 4.4: The scanning electron microscopic image of recombinant *Pseudomonas putida* cultivated with DBT

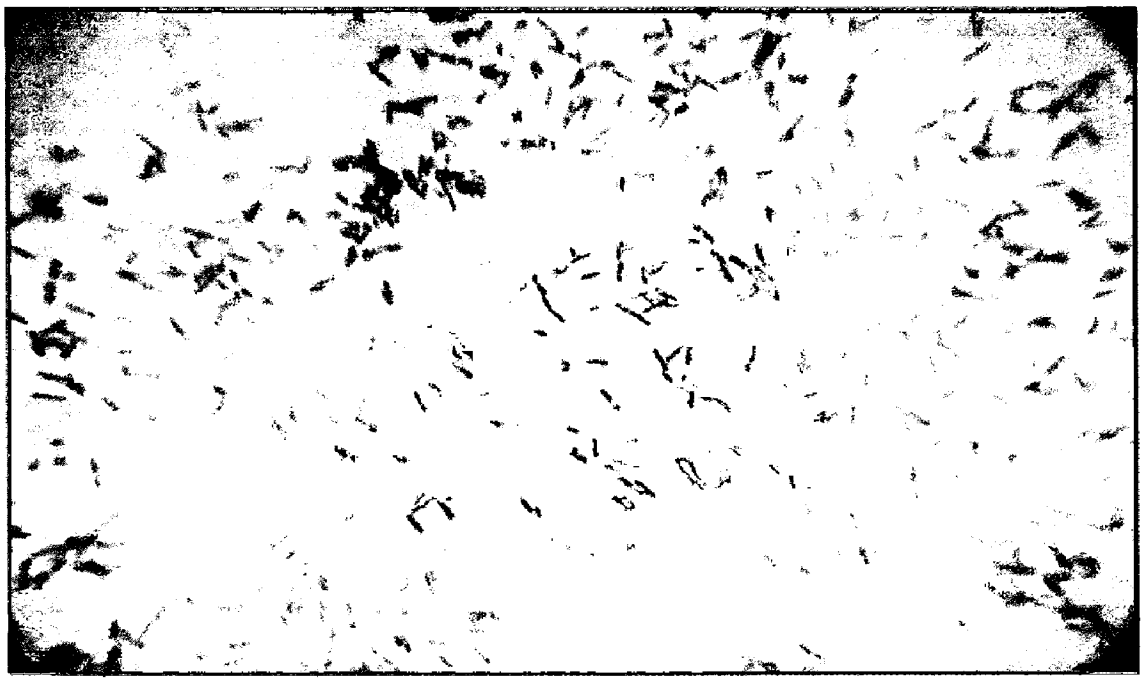


Fig 4.5: The microscopic image of host strain *Pseudomonas putida* MTCC 1194

Fig 4.6: The microscopic image of recombinant *Pseudomonas putida* cultivated with DBT

4.2 BATCH STUDIES

4.2.1 Selection of media for desulfurization studies

To select the suitable media for the desulfurization studies using this recombinant *Pseudomonas putida* strain, the growth rate of bacteria was monitored in two types of minimal salt media namely BSM and NK media at 30^0C and 210 rpm. Growth was monitored by measuring the optical density (O.D) using Spectrophotometer at 600nm. Recombinant cells shown higher growth rate in N.K medium than in BSM (Fig 4.7). Further experiments were carried out using the NK media.

4.2.2 Comparative growth study of recombinant bacteria with parent strain

Recombinant *Pseudomonas* cells were cultivated in NK medium of 100 ml in 250 ml flask with final concentration of 0.2 mM of DBT as ethanolic solution at 30°C and 210 rpm in incubator shaker. As a control the parent strain *R. erythropolis* IGTS8 also cultivated in BSM medium of optimized composition else where in the literature (Carolina et al, 2005) with the same DBT concentration as above. The parent strain had taken 94 h of cultivation time to reach stationary phase where as recombinant bacterium came to stationary phase in 67 h with higher growth rate of showing O.D 3.1164. Recombinant cells demonstrated growth rate than that of parent strain for the same cultivation conditions at the same concentration of DBT (Fig 4.8).

4.2.3 Growth characteristics of recombinant *P. putida*

Batch studies were conducted to study the growth of recombinant cells for increasing concentrations of DBT such as 0.4 mM, 0.8 mM, and 1.2 mM as sole source of sulfur. The growth patterns were determined by determining the optical density at 600 nm. Fig 4.9 shows the curves for cell growth Vs time. The calibration curve for optical density Vs biomass concentration was plotted Fig 4.10. Bacterial growth measurement with increasing DBT concentration shown that the lag phase is increasing with the increasing concentration of DBT and also the growth rate was decreased.

Specific was also found out for the different concentrations of DBT by using the below stated formula.

$$\mu = \frac{1}{t_2 - t_1} \ln\left(\frac{OD_2}{OD_1}\right)$$

Where,

OD_1 = optical density at time t_1

OD_2 = optical density at time t_2

t = time

The maximum specific growth rate μ_{max} and half saturation constant K_s were determined by following simple Monod kinetics. A double reciprocal plot was plotted between inverse of growth rate, 1/ μ and the inverse of substrate concentration, 1/S (fig 4.11). From that graph μ_{max} and K_s were determined to be 0.0466 h^{-1} and 8.6875 mg/l respectively.

Monod kinetics

Monod kinetics can be explained by the following equation:

$$\mu = \frac{\mu_m S}{(K_S + S)}$$

Where,

μ_m = Maximum specific growth rate (h^{-1})

K_s = Half saturation constant (mg/l)

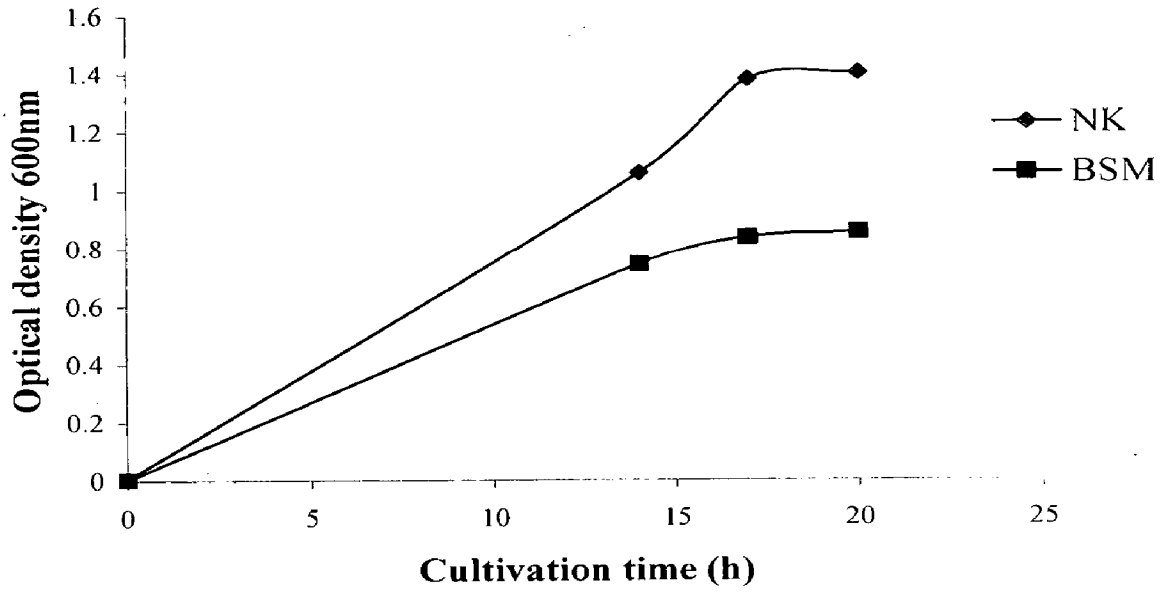


Fig 4.7: Growth characteristics of recombinant *Pseudomonas putida* in different minimal media

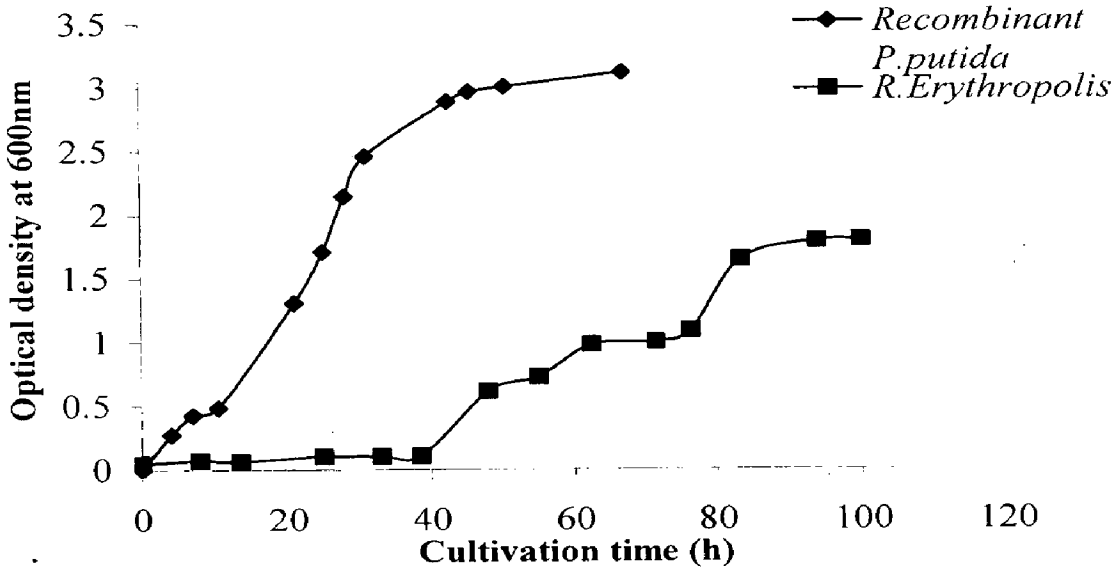


Fig 4.8: Comparison of growth rate of *R. erythropolis* IGTS8 and recombinant *P. putida* in the media supplemented with 0.2 mM DBT as sole source of sulfur.

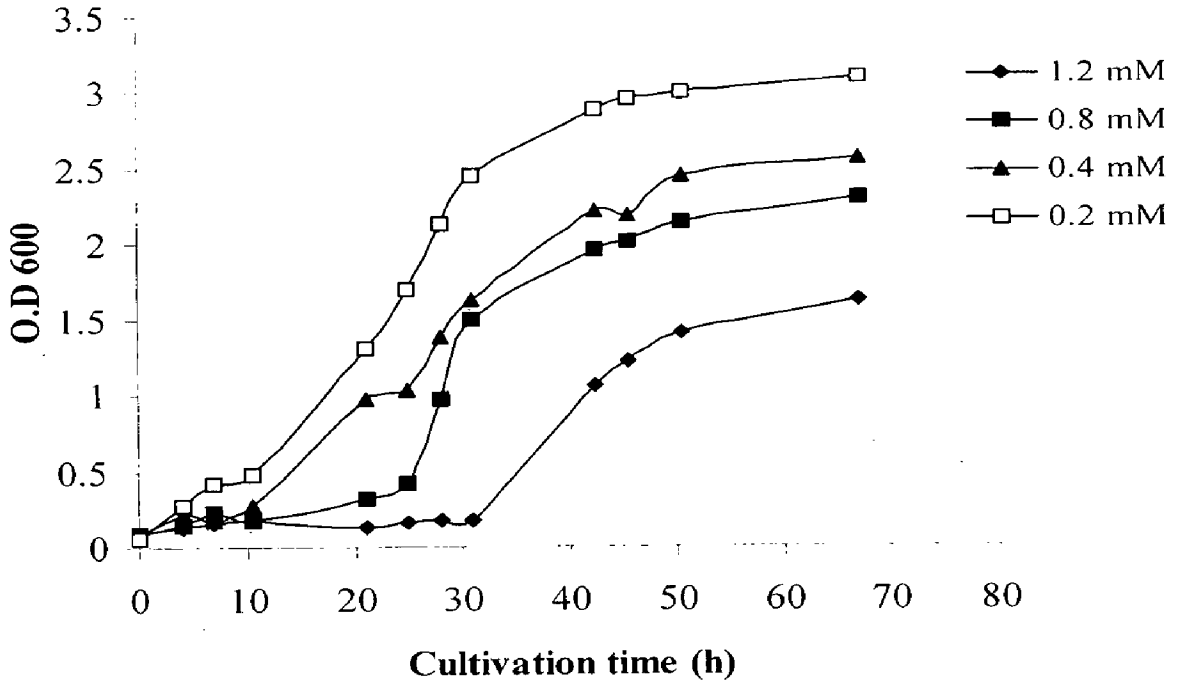
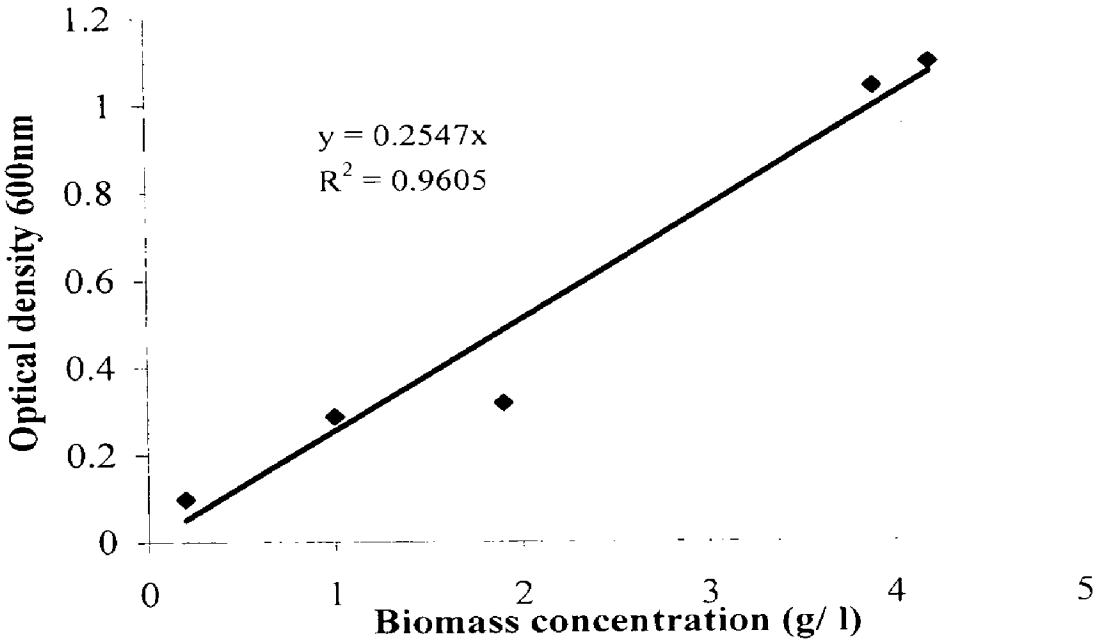


Fig 4.9: Effect of increasing DBT concentration on the growth of recombinant *Pseudomonas putida*



. Fig 4.10: Calibration curve for biomass Vs optical density

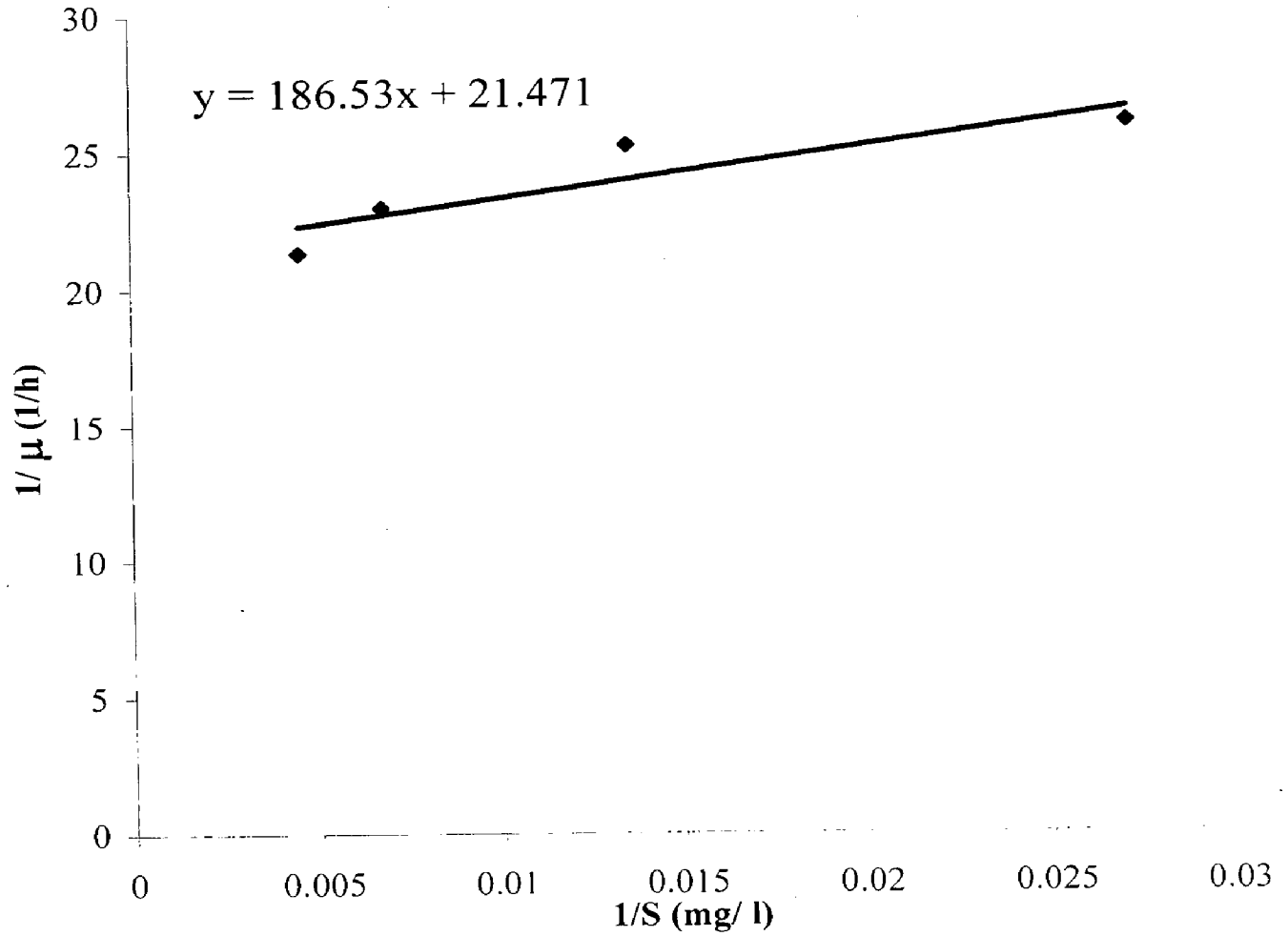


Fig 4.11: A double reciprocal plot- determination of growth kinetic parameters μ_{max} and K_s

4.2.4 Dibenzothiophene desulfurization study in aqueous phase

The desulfurization ability of recombinant *Pseudomonas putida* was studied in aqueous phase for the initial concentrations of DBT 0.2 mM and 1.2 mM dissolved in ethanol. Besides this desulfurization studies were conducted using 0.1 mM DBT. The periodically taken samples were analyzed by using GC- FID. The calibration curves for known concentrations of DBT and HBP Vs the peak areas were plotted (Fig 4.12, Fig 4.13). This genetically modified bacterium desulfurized the 1.2 mM DBT upto 73.1% in 67 h of cultivation time using growing cells where as parent strain *R. erythropolis* has taken 100 h to desulfurize 69.8 % of 0.1 mM DBT. The DBT and 2-HBP production patterns of both parent strain and recombinant strain were shown in Fig 4.14 and Fig 4.15 respectively and the same profiles were determined for the desulfurization of low concentration of DBT of 0.2 mM with recombinant bacteria (Fig 4.16). It was observed that the 2-HBP production rate was not much increased when DBT concentration was increased from 0.2 mM to 1.2 mM. The values are 0.1126 mM and 0.1046 mM respectively. The probable reason may be for this is that the HBP formed in the cell is not efficiently discharged out through the cell walls.

4.2.5 Desulfurization of DBT in Two Phase Mixture

The actual biodesulfurization reaction industrially occurs in two-phase mixture containing oil to be desulfurized and the bacterial cell suspension in aqueous phase. So, it is required to determine the optimum oil to water ratio for the operation.

4.2.5.1 Optimum oil to water ratio

The volume ratio of oil to water (O/W) affects the bioavailability of DBT when biodesulfurization occurs in the interface between the organic and the aqueous phases. The O/W ratio is also an important factor in determining the reactor productivity and thus the reactor volume. As shown in Fig 4.17 when the O/W ratio was 1:2, the production of 2-HBP was almost 50% higher than the others. While under higher oil held-up, with O/W ratio of over 2:1, far less DBT degradation activity was detected. The DBT desulfurization pattern and HBP production profile with reaction time for different O/W ratios had been

demonstrated (Fig 4.18, Fig 4.19). This was similar to the study with *Pseudomonas delafieldii* R-8 in dodecane (Luo et al., 2003) with an optimum of O/W ratio at 1:4 and to the study with *Corrynebacterium sp.* strain SY1 in hexadecane with an optimum ratio at 1:2 (Wang et al, 2006). Under low concentration, an oil-in-water emulsion was formed with the oil/water interface increasing with the proportion of oil. Turbidity of oil after desulfurization reaction was determined by measuring turbidity at 310 nm Fig 4.20. At O/W ratio 1:2 has shown greater O.D than the others. Meanwhile hexadecane decreased the feed back inhibition of the biocatalyst due to the by-products accumulation in water phase. Because water was necessary for enzyme activity, too high concentration of hexadecane resulted in low desulfurization rate.

4.2.5.2 Effect of surfactant on BDS

Surfactants are amphiphilic compounds (containing hydrophobic and hydrophilic portions) that reduce the free energy of the system by replacing the bulk molecules of higher energy at the interface. They have been used industrially for their desirable properties including solubility enhancement, surface tension reduction, wettability and foaming capacity. Only few were reported for surfactant's application in biodesulfurization. Marzona et al., (1997) found that the binding of benzothiophene (BT) and DBT with cyclodextrins (CD) could strongly enhance their solubility in water. Jiang et al. (2002) found that surfactants could improve the desulfurization rate. The *pseudomonas delafieldii* strain R-8 could remove 72% of the organic sulfur from low sulfur diesel oil (S<300 mg/L) in 72 h at 250 r/min with Tween-80 present.

Adding various concentrations of surfactant Tween-80 ranging from 0 to 3000 mg/l to the mode oil containing 2 mM DBT and resting cell suspension in 1:2, optimum O/W ratio, studied the effect of surfactants on BDS. After 36 h of desulfurization reaction, the 2-HBP produced was 1.5 times higher with the reaction mixture containing 1 g/l than that formed without surfactant (Fig 4.21). From the results it had been demonstrated that surfactant can increase the desulfurization ability of bacteria, the reason may be that the Tween- 80 enhances the solubility of DBT in aqueous phase and improve the mass transfer of DBT between organic and aqueous phases.

4.2.5.3 Desulfurization of model oil and diesel oil with increased resting cell concentration

The model oil containing 25.2 mM (4666.66 ppm) DBT dissolved in n-hexadecane was desulfurized using resting cell suspension containing 31.8 g biomass/l. the concentration of DBT in model oil was equivalent to 0.8064 g sulfur/l. the model oil was mixed with resting cell suspension adding 1 g/l Tween-80 as surfactant and the desulfurization reaction was carried on for 56 h. the resting cells of recombinant bacteria desulfurized 97.88% of DBT in 56 h of reaction time. Desulfurization of DBT with reaction time was shown in Fig 4.22. The specific desulfurization rate was 0.4397 mg sulfur/g biomass/l. In the first 12 h of reaction time it was observed that the desulfurization activity was 1.4844 mg sulfur/g biomass/l. Similar experiment was conducted with diesel oil and the samples were analyzed by CHNS analyzer. The sulfur content in diesel oil was desulfurized from 22.025 g/l to 2.934 g/l in 90 h (Fig 4.23) with 6.67 mg sulfur/g biomass/l. The increase in the desulfurization activity was may be due to the presence of other sulfur sources that can be degraded easily than DBT.

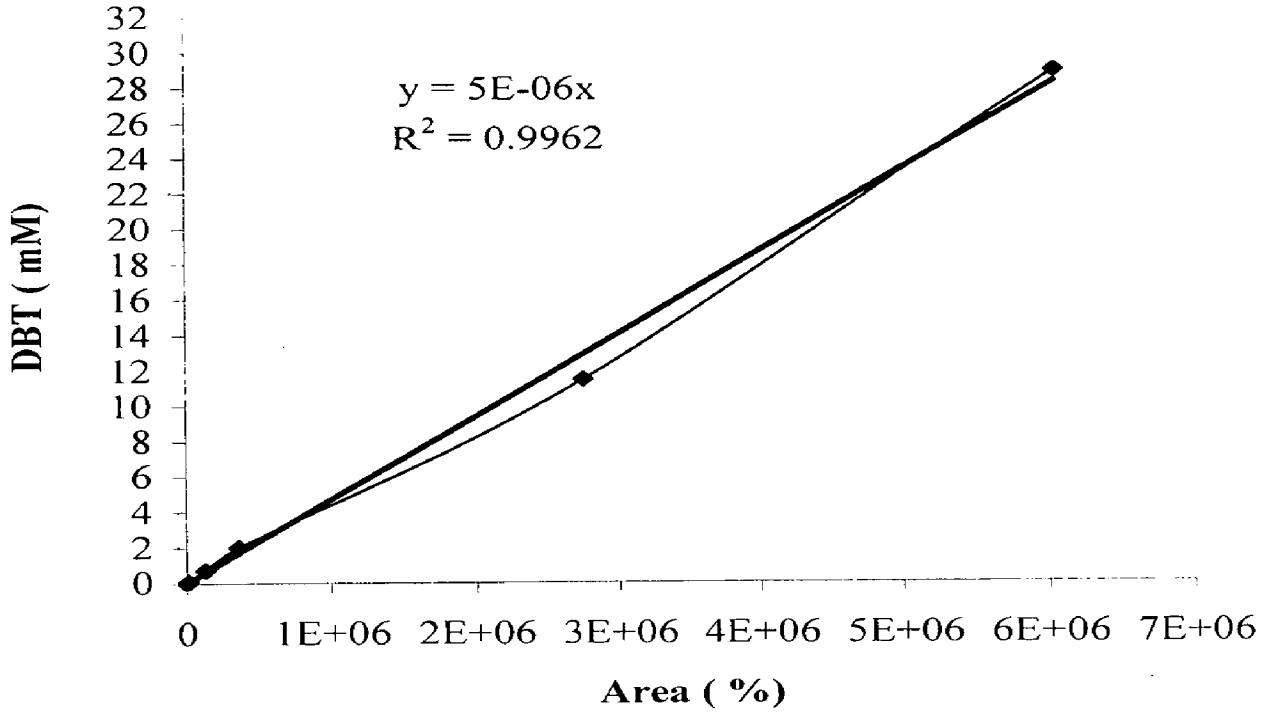


Fig 4.12: Calibration curve for Dibenzothiophene

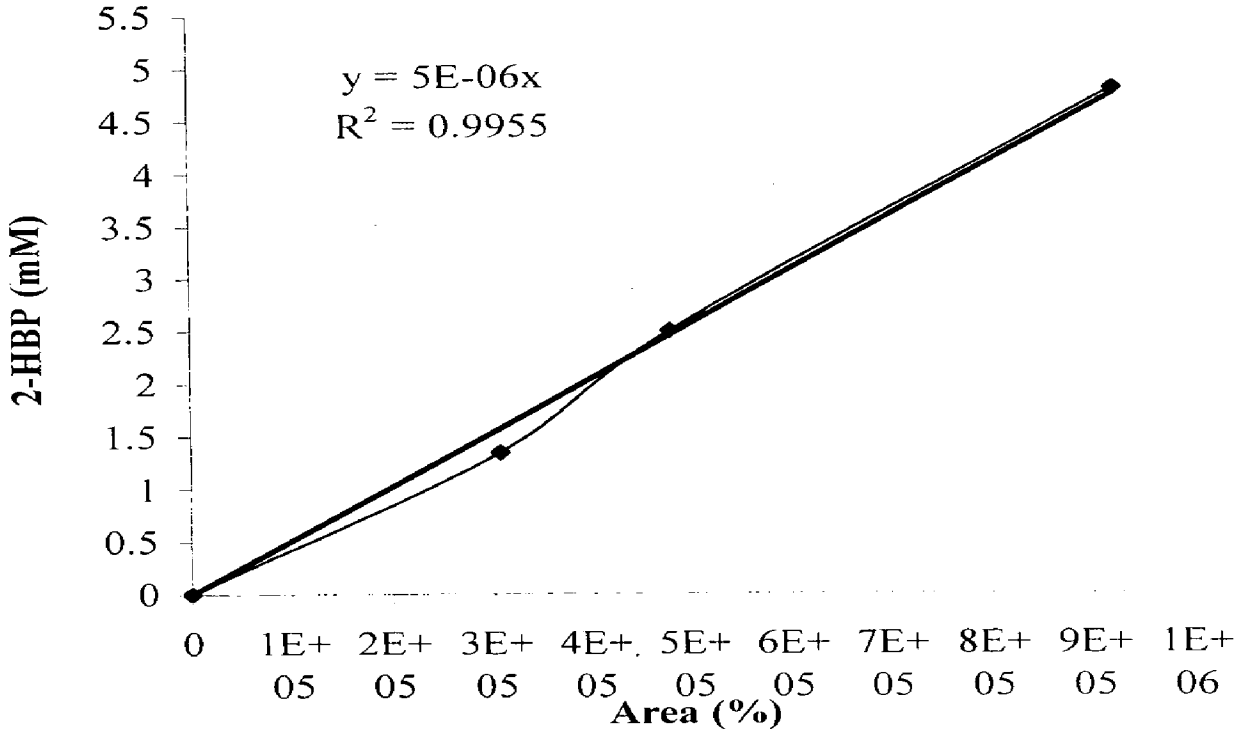


Fig 4.13: Calibration curve for 2-Hydroxy biphenyl

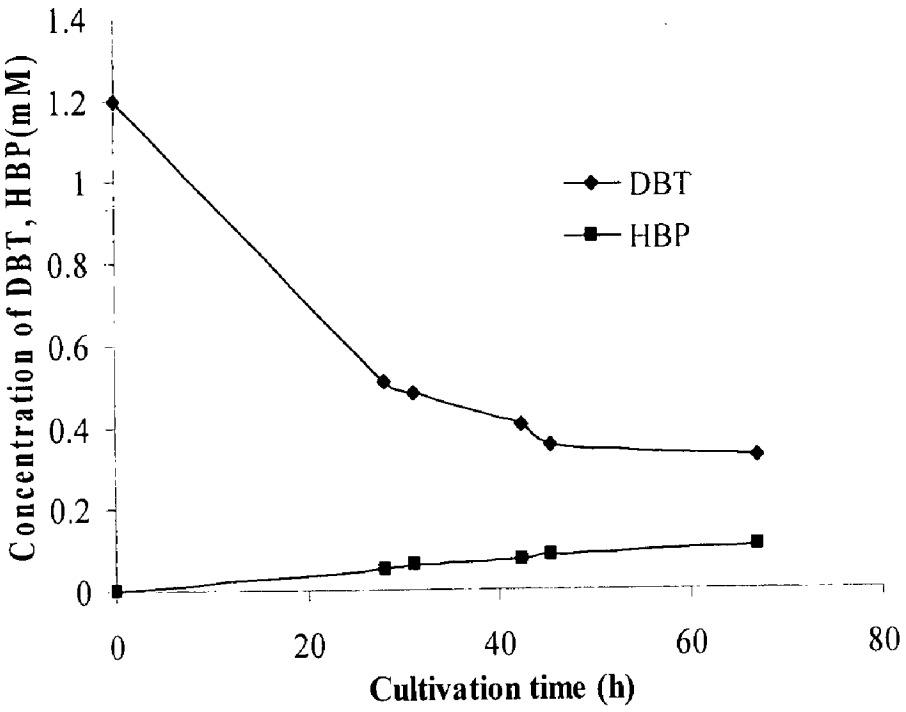


Fig 4.14: Desulfurization studies using recombinant *Pseudomonas putida* with 1.2 mM DBT in ethanol.

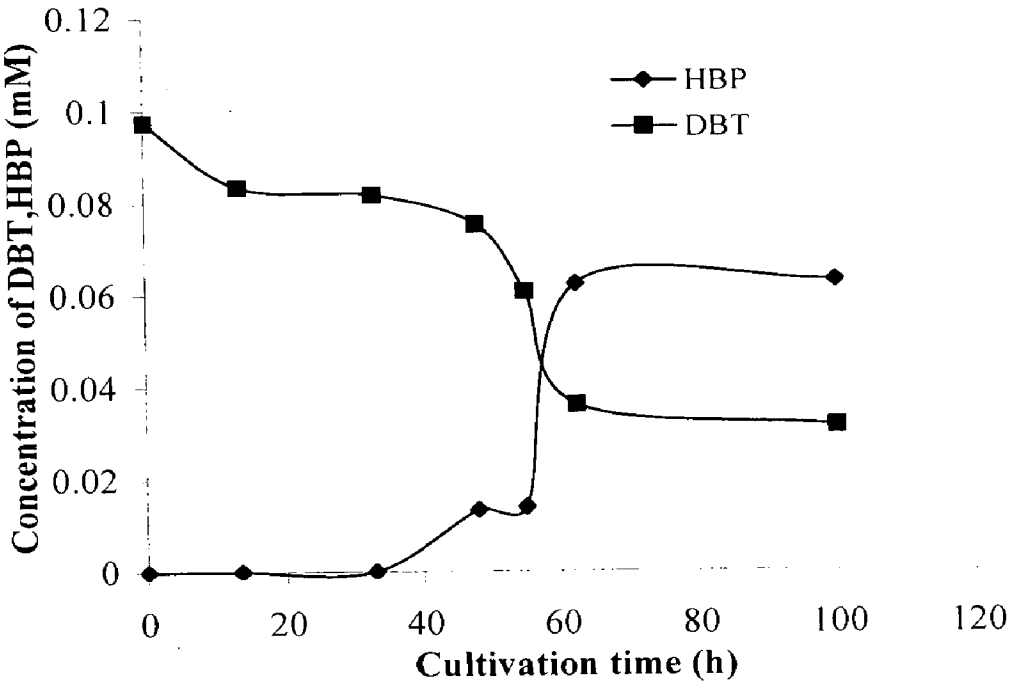


Fig 4.15: Desulfurization studies using parent strain *R. erythropolis* IGTS8 with 0.1 mM DBT in ethanol.

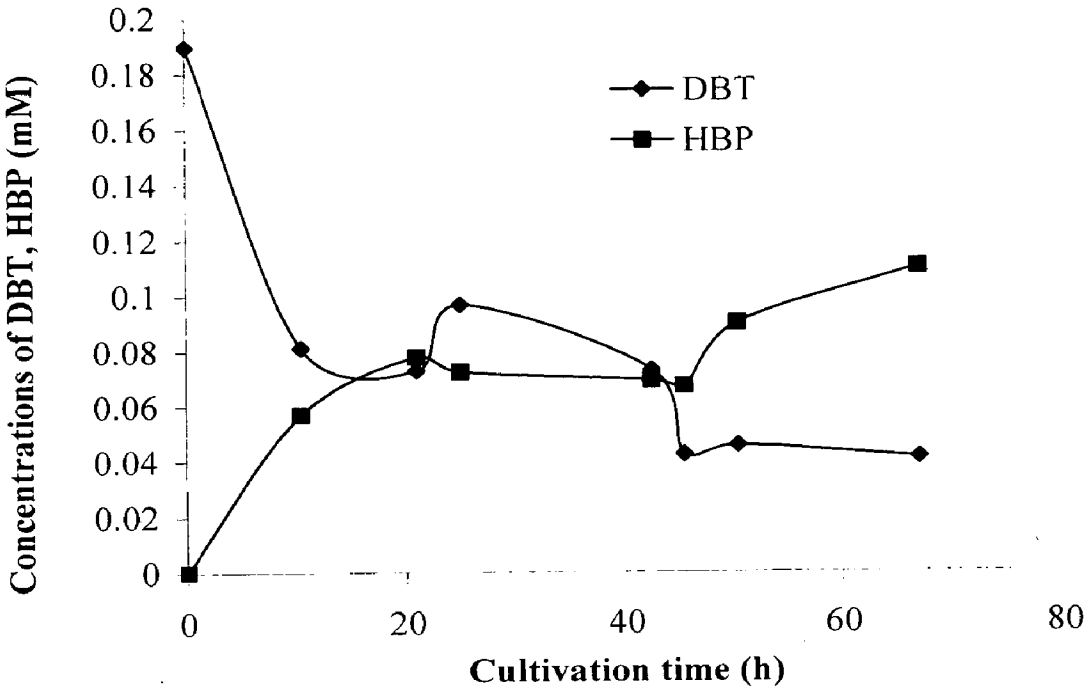


Fig 4.16: Desulfurization studies using recombinant *Pseudomonas putida* with 0.2 mM DBT in ethanol.

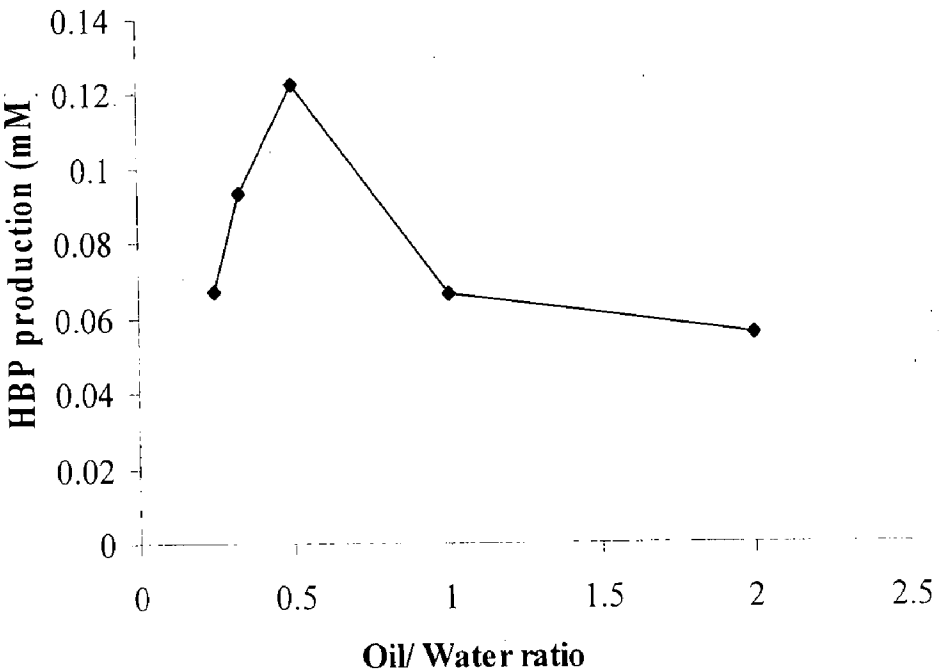


Fig 4.17: Determination of optimum oil to water (O/W) phase ratio- production of 2-HBP for various O/W ratios.

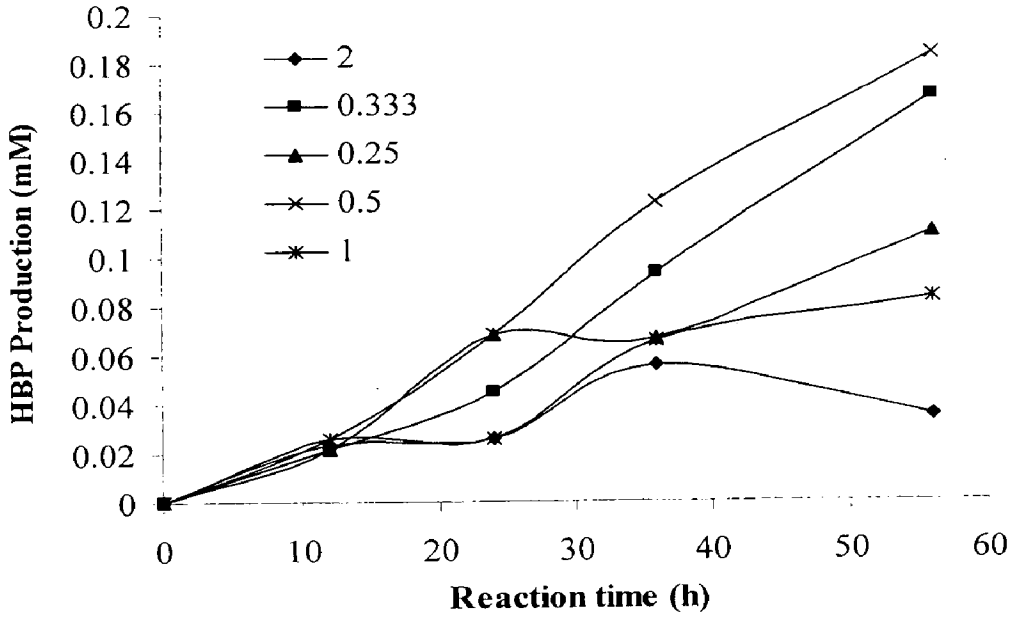


Fig 4.18: Production of 2-HBP during the desulfurization of model oil containing 2 mM DBT using resting cells for various O/W ratios

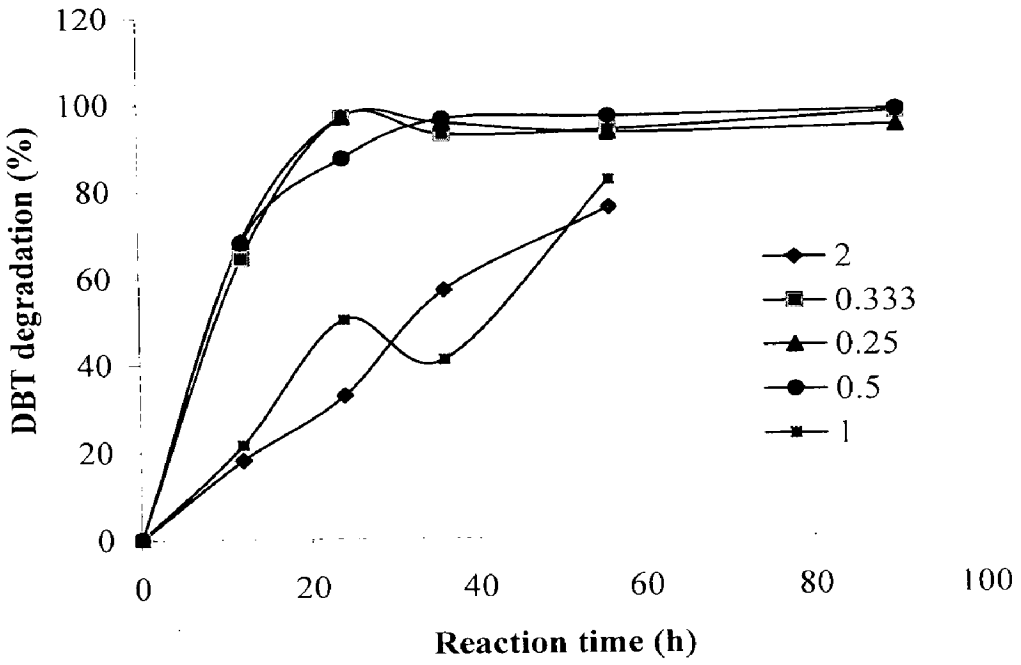


Fig 4.19: Percentage degradation of DBT during the desulfurization of model oil containing 2 mM DBT using resting cells for various O/W ratios

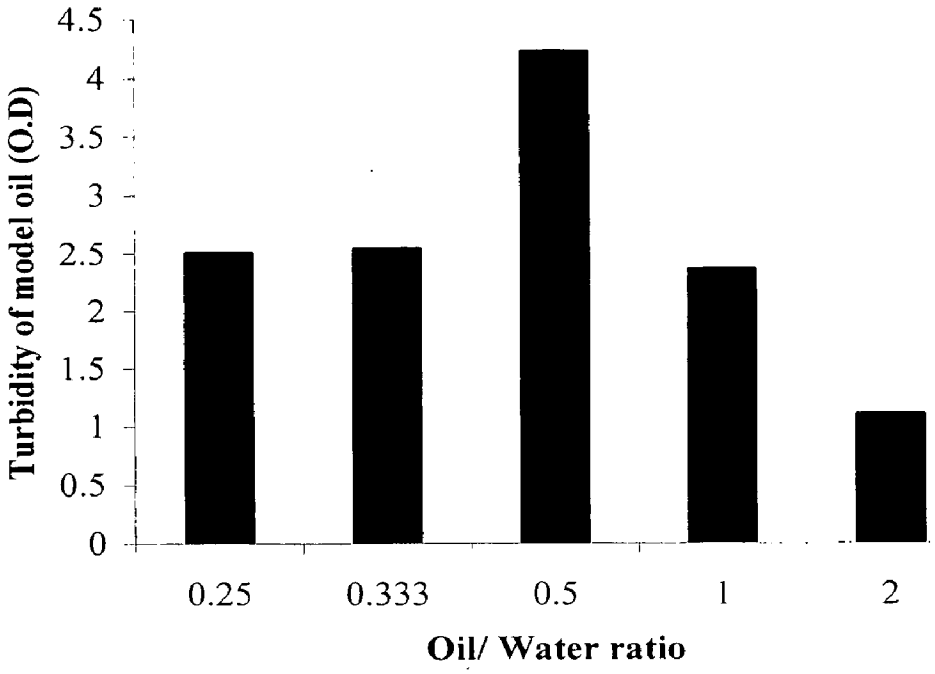


Fig 4.20: Turbidity of model oil after 36 h of BDS reaction with different oil to water ratios

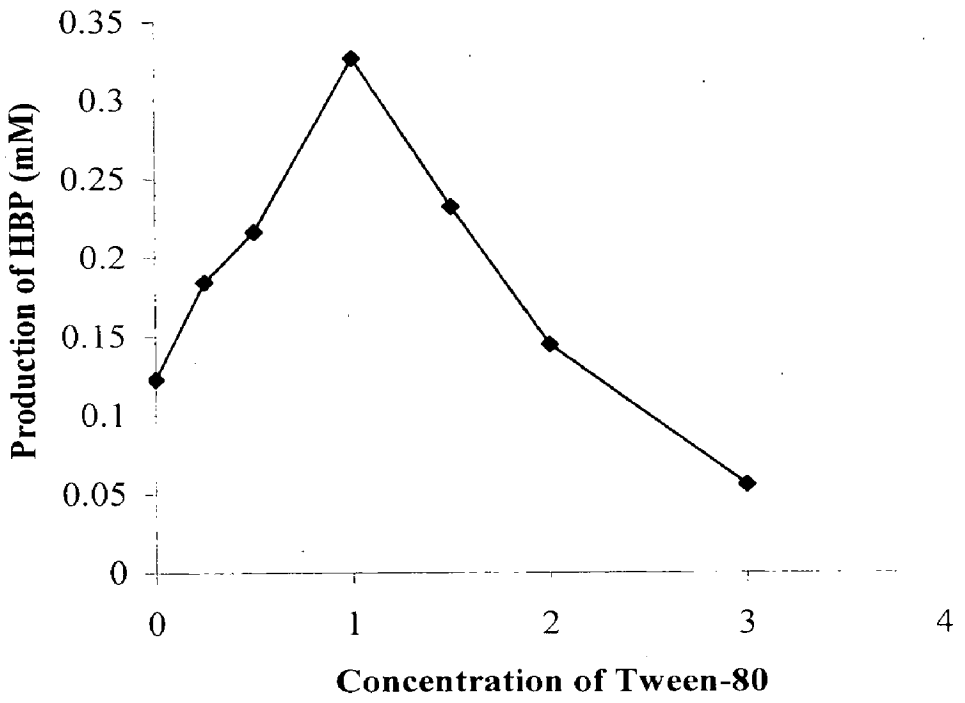


Fig 4.21: Effect of surfactant concentration on production of 2-HBP from DBT during desulfurization.

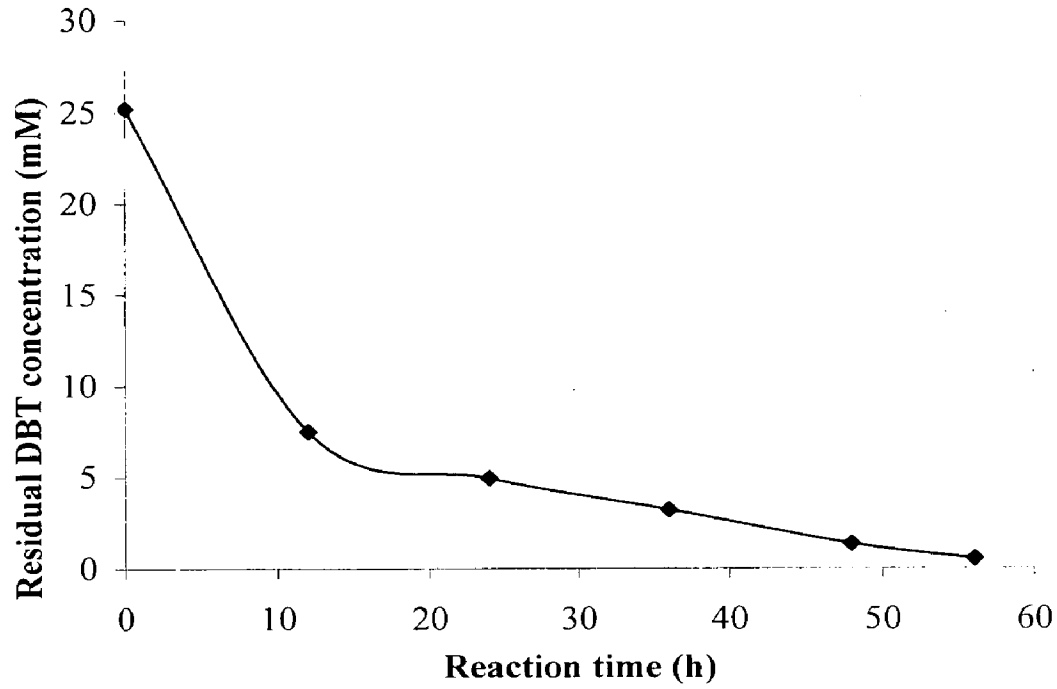


Fig 4.22: Desulfurization studies of model oil containing 25 mM DBT dissolved in n-hexadecane

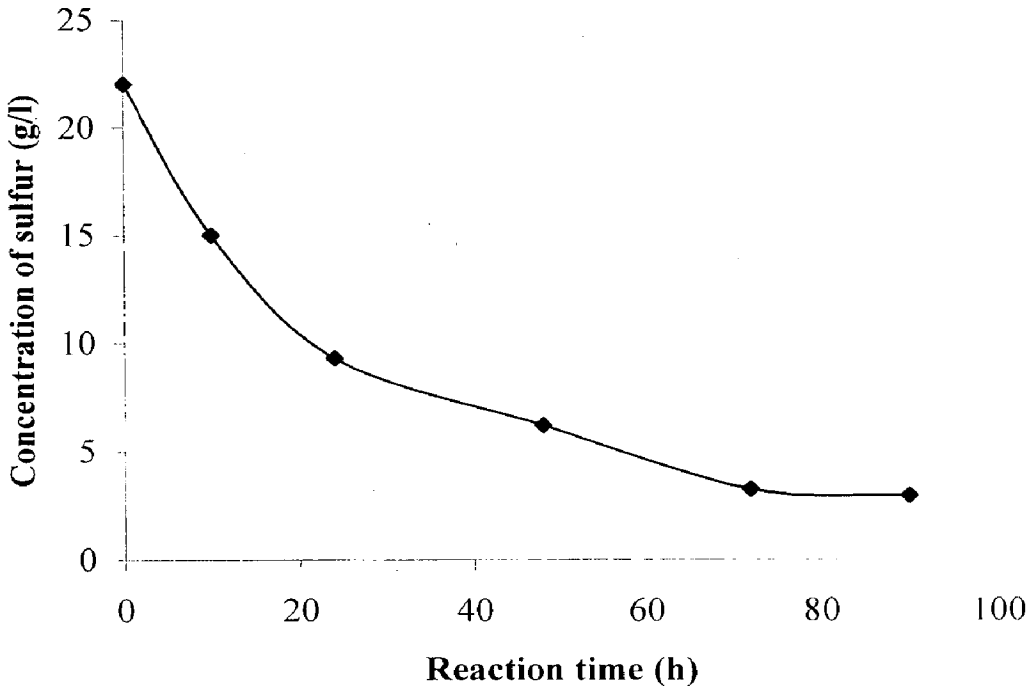


Fig 4.23 Desulfurization studies of diesel oil using resting cells of recombinant bacterial strain.

CHAPTER-5

CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

In summary we have developed a recombinant bacterial strain by transforming the plasmid pSAD 225-32 containing *dsz* genes from the parent strain *R. erythropolis* IGTS8 into a gram negative host strain *Pseudomonas putida* MTCC 1194 using *E. coli* XL1 Blue as a donor by competent cell- calcium chloride method. The desulfurization assays were demonstrated that the recombinant bacterial cells follow the sulfur specific 4S pathway in which there is no reduction of calorific value of the fuel oil. Using the dibenzothiophene as a model compound the growth characteristics of recombinant bacteria were determined. The recombinant cells demonstrated higher growth rate than that of parent strain for the same cultivation conditions at the same concentration of DBT. From the growth and desulfurization studies in the aqueous phase we arrived to the conclusion that the recombinant cells can utilize and desulfurize the DBT at higher rate during the cultivation than that of the gram-positive parent strain.

To represent the practical Biodesulfurization conditions the desulfurization studies were carried on using the DBT dissolved in n-hexadecane, the hydrocarbon, which represents the 40% of the diesel oils. This solution is mostly known as model oil. In this studies it was observed that the oil to water phase ratio (O/W) greatly influence the desulfurization ability of bacteria. At 1:2 optimum O/W ratio 96.95% of the model oil containing 2 mM DBT was desulfurized in 24 h of reaction time using resting cell suspension of recombinant bacteria. From these results we can conclude that the desulfurization activity of recombinant bacteria was greater in the resting cell state than that of during the cultivation period. The use of surfactant Tween-80 enhanced the product 2- HBP formation. At the optimum concentration of Tween-80, 1g/l, it was observed that the 2-HBP produced was 1.5 times higher with the reaction mixture containing 1 g/l than that formed without surfactant. Hence the use of surfactant increases the desulfurization activity.

Finally the desulfurization reactions carried out at optimum O/W and surfactant concentration using model oil and diesel oils. This time model oil used which contains high concentration of DBT of 25.2 mM, which is almost equivalent to the sulfur present in some of the crude oils. With the increased cell concentration 97.88% of the model oil was desulfurized with in 56 h. The sulfur content in diesel oil was desulfurized from 22.025 g/l to 2.934 g/l in 90 h with 6.67 mg sulfur/ g biomass/l. From this we can conclude that the recombinant strain can efficiently desulfurize the real fraction of crude petroleum.

We conclude that the use of gram negative hosts which have the high solvent resistance and can perform well under the two phase conditions than that of parent strains can remove the bottleneck mass transfer resistance between oil and aqueous phase that stood in the way of commercialization of BDS process.

5.2 RECOMMENDATIONS

Increasingly severe sulfur emission standards have force refiners to make lower sulfur petroleum. Although more hydrotreating capacity will be certainly added to the conventional chemical desulfurization system, that will be very expensive. Subsequently the Biodesulfurization will be recognized to be better because of the simplicity of the biotechnology based approach results in significantly lower capital and operating expenses. Microorganisms with sulfur specific pathway selectively remove sulfur from the heterocyclic organic sulfur compounds that are recalcitrant to the chemical desulfurization. Generally, the drawback with biological systems is that reaction rate is slower than the chemical reaction. Detailed information on the genes involved in the Biodesulfurization allows designing powerful recombinant strain with high specific activity and solvent tolerance. The use of alternative host system that is transforming genes from gram positive wild type strain to gram negative hosts gives provision to utilize some of the useful properties like solvent resistant and heavy metal resistance generally present in the fossil fuels. The over expression of such type of strains and the stabilization of a biocatalyst will lower the cost of the process. The recombinant strain developed in the present study can be tested for its desulfurization ability in the continuous process immobilizing it on the suitable bio support material.

The metabolic pathway of the microbial desulfurization consists of two oxygenases supported by flavin reductase and a sulfinase catalyzing the uncommon enzyme reaction, which are very unusual enzyme reactions. Therefore in the future work, to investigate the details of the enzyme structure and mechanism will be not only interesting but also useful for developing more active biocatalyst for the desulfurization. These developments may make the biological desulfurization process more attractive and cost effective than the chemical process.

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