

Biodesulfurization: Biochemical and Genetic Engineering Aspects

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ABSTRACT

Biodesulfurization (BDS) offers the potential for an effective method for lowering the sulfur content of petroleum products because insufficiently desulfurized distillates of petroleum products is a significant source of environmental pollution. This review describes the development of BDS; and compares destructive and non-destructive pathways as well as aerobic and anaerobic BDS. The process variables affecting growth and activity of microorganisms of BDS are described. Also genetic modifications and bioreactor designs, which lead to an increased BDS efficiency and commercial aspects, are discussed. Finally, the critical factor for industrial application of BDS as an efficient process is an adequate bioreactor design. The application of mixtures of biocatalysts is necessary for an efficient desulfurization of crude oil containing a wide range of sulfur compounds.

Keywords: aerobic, biocatalytic desulfurization, destructive pathway, genetic engineering, 4S pathway, dibenzothiophene (DBT), sulfur specific pathway

Abbreviations: BDS, biodesulfurization or biocatalytic desulfurization; BT, benzothiophene; Cx-DBT, dibenzothiophene and its derivatives; DBT, dibenzothiophene; *dsz*, desulfurization; **DszA**, DBT-sulfone monooxygenase; **DBTO**, dibenzothiophene sulfoxide; **DBTO₂**, dibenzothiophene-5, 5-dioxide or dibenzothiophene sulfone; **DszB**, 2-hydroxybiphenyl sulfinatase; **DszC**, DBT-monooxygenase; **DszD**, (NADH-FMN) flavin-oxidoreductase; **FMN**, flavin mononucleotide; **HBP**, hydroxy biphenyl; **HDS**, hydrodesulfurization; **HFBT**, 3-hydroxyl-2-formyl-benzothiophene; **HPBS**, 2-hydroxybiphenyl-2-sulfinate or hydrophenyl benzene sulfinate; **sox**, sulfur oxidation

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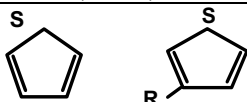
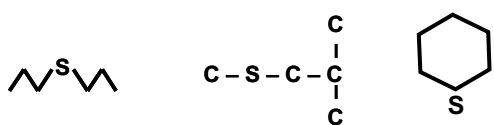
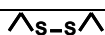
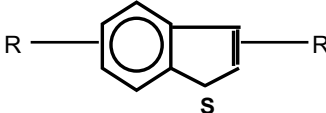
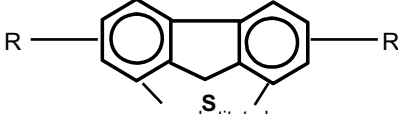
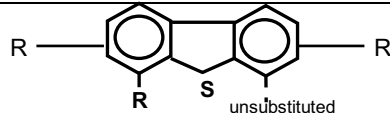
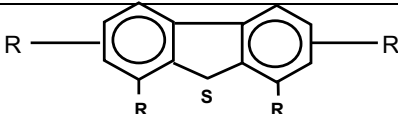
INTRODUCTION

Crude oil contains up to 5% (w/w) of organic and inorganic sulfur compounds (Grossman *et al.* 1999; Kertesz 1999; Marcelis 2002), and its combustion causes the distribution of corrosive sulfur oxides into the atmosphere, which return to earth as acid rain. The sulfur oxide is not only harmful to humans but is also a cause of environmental contamination and deactivation of catalysis. For this reason, strict regula-

tions against emissions of sulfur oxides have been implemented worldwide (Onaka *et al.* 2001a, 2001b; Marcelis 2002). Conventional hydrodesulfurization (HDS) is a physicochemical process, which can very easily remove different types of inorganic sulfur compounds, such as mercaptans, sulfides and disulfides as well as simple-structured thiophenes from crude oils. The molecular structure of the same inorganic sulfur compounds is shown in **Table 1**.

HDS needs high pressure (10-17 atm) and high tempera-

Table 1 Some molecular structures of inorganic sulfur compound present in crude oil.

Sulfuric compounds	Molecular structure	Difficulty of HDS
Non-thiophenic	R-S, R-S-R, R-S-S-R	Moderate
Thiophenic		Easy
Sulfides		Easy
Disulfides	 C-C-S-S-C-C	Moderate
Benzothiophenes		Very Easy
Non-substituted dibenzothiophenes (DBTs)		Easy
Beta substituted dibenzothiophenes (DBTs)		Moderate
Two beta substituted dibenzothiophenes (DBTs)		Difficult
Circular molecules (undefined S situations; 3, 4)	Variable	Moderate
Circular molecules (undefined S situations; 1, 2)	Variable	Moderate

tures (200–400°C) in which sulfur compounds are converted to hydrogen sulfide gas (H₂S) by reacting crude oil fractions with hydrogen gas (Marcelis 2002; Breyse *et al.* 2003). This technique is costly and produces hazardous products in the environment; also, it is not effective for removing heterocyclic sulfur compounds, such as benzothiophene (BT), dibenzothiophene (DBT) and its derivatives (C_x-DBT) from crude oil. Biocatalytic desulfurization or biodesulfurization (BDS) is a biological method in which microbes or their enzymes are used as the catalyst for removing organic sulfur compounds (especially recalcitrant sulfur compounds such as DBT and its derivatives) from fuels (Marcelis 2002; Breyse *et al.* 2003; McFarland 1999).

BDS is performed under mild conditions (low pressure and temperature, approximately ambient temperature and pressure) with no harmful reaction products. The conversion rates are expected to be lower than HDS because the rates of biochemical reactions are generally slower than chemical reactions. Therefore, BDS can be considered as a complementary process to HDS and can reduce capital and operating costs of desulfurization. This process will produce substantially less greenhouse gases. Therefore it is considered as an environmentally benign process (McFarland 1999; Monticello 2000; Kobayashi *et al.* 2001; Marcelis 2002). After the first study on BDS was published in 1950, many researchers' interests focused on this field and significant financial support was assigned to conduct research on BDS (Table 2). The most important aims are industrial-scale application of a microorganism with selective desulfurization of a carbon-sulfur bond in a sulfur-selective pathway (4S pathway) with intact carbon skeleton in final products.

This review describes the development of BDS; and compares destructive/non-destructive pathways and aro-

bic/anaerobic BDS as well as the process variables and commercial aspects of BDS. Also genetic modifications and bioreactor designs that lead to an increased BDS efficiency and commercial aspects, are also discussed.

Table 2 Research budgets in BDS

Company	Budget (10 ⁶ \$)	Year
Energy Biosystems	50	1992-1998
Center of Oil Energy	50	1994
Petroleum Companies	10-50	-
USA Government	10	1990

DESTRUCTIVE AND NON-DESTRUCTIVE BDS PATHWAYS

DBT is generally considered as a model polyaromatic heterocyclic compound for BDS research because it is the most abundant thiophenic sulfur compound found in a variety of fossil fuels. Three primary pathways are known for DBT desulfurization (Klein *et al.* 1994). The first is a ring-destructive pathway in which one of the aromatic DBT rings is degraded by the cleavage of carbon-carbon bonds as a consequence of oxidative steps (the Kodama pathway), while the sulfur is not released (Kodama *et al.* 1973; Setti *et al.* 1992; Kropp and Fedorak 1998). The Kodama pathway has been presented in Fig. 1. HFBT is the end product of this pathway that accumulates in pure cultures and there are few reports on its fate (Bressler and Fedorak 2001).

The second pathway is also a completely destructive pathway in which the aromatic DBT ring is completely degraded to carbon dioxide, sulfite and water (Fig. 2) (Setti *et al.* 1999; Marcelis 2002). Both of the described pathways are undesirable for a process designed to selectively remove

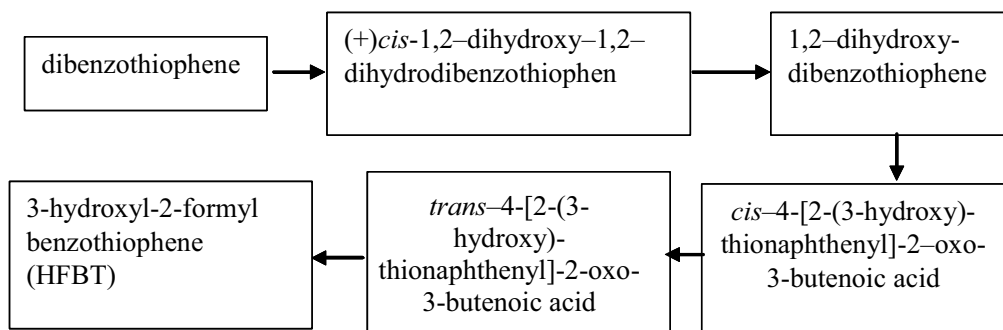


Fig. 1 Kodama pathway for BDS of DBT.

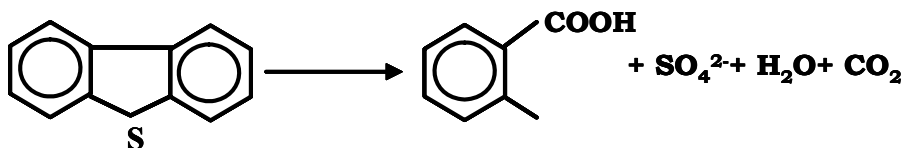


Fig. 2 Completely destructive pathway of DBT.

organosulfur compounds without oxidation of other aromatic compounds found in petroleum products or reducing the value of fuel. The efficiency of BDS depends on the biocatalyst's capacity to remove sulfur from organic compounds without altering the carbon skeleton of the molecule (Setti *et al.* 1999; Marcelis 2002).

The third pathway is non-destructive in which the carbon skeleton of DBT is not destroyed and initial catalysis is directed against the sulfur center; as a result only sulfur is removed from DBT. This pathway is a sulfur-specific metabolic pathway for DBT desulfurization and it is named the 4S pathway (McFarland 1999; Setti *et al.* 1999; Marcelis 2002).

AEROBIC BDS

Several aerobic microorganisms are found to desulfurize DBT and its derivatives via the 4S pathway. The first report on sulfur-selective desulfurization bacteria (*Rhodococcus erythropolis*), were published by Kilbane (1989). The results of several studies indicated that some strains could desulfurize DBT by resting cells, i.e. bacteria that are not in a growth phase. Also there are several reports on BDS via a sulfur-selective pathway at growing conditions. The number of organosulfur model compound BDSs by aerobic microorganisms in growing and resting cells is summarized in Tables 3 and 4, respectively.

THE SULFUR SPECIFIC PATHWAY (4S PATHWAY)

The sulfur-specific metabolic pathway has been called the 4S pathway because it involves four enzymatic steps (McFarland 1999 Marcelis 2002). These steps are shown in Fig. 3. The primary genes involved in DBT desulfurization, *dsz* or *sox*, have been sequenced and analyzed by Ohshiro and Izumi (1999). In *Rhodococcus* IGTS8, DBT is converted to 2-HBP by four enzymes, which were designated as DszA, DszB, DszC, which are encoded by the plasmid-located (*dsz*) operon (Xi *et al.* 1997), and DszD, a flavin mononucleotide (FMN)-dependent reduced pyridine nucleotide (NADH) oxidoreductase (NADH-FMN) which is located on the genome (Denome *et al.* 1993; Xi *et al.* 1997; Marcelis 2002). In the mechanism proposed for DBT desulfurization, a monooxygenase (DszC) catalyzes the conversion of DBT into DBT-sulfone (DBT-S, 5-dioxide; (DBTO₂)) via the two-step S-oxidation of DBT, then the second monooxygenase (DszA) catalyzes the conversion of DBTO₂ to 2-hydroxybiphenyl-2-sulfinate (HBPS). In the final step, DszB, which is a desulfinase, catalyzes the conversion of HBPS into 2-hydroxybiphenyl (2-HBP) and sulfite as the end products (Denome *et al.* 1993; McFarland 1999; Marcelis 2002). Both of the DszA and DszC enzymes require a cofactor (FMNH₂) for performing the catalytic activity. Then a flavin-oxidoreductase enzyme (DszD) causes

the regeneration of FMNH₂, and this process requires another cofactor, NADH (Denome *et al.* 1993; Gray *et al.* 1996; Marcelis 2002). The essential role of NADH as a cofactor in the mechanism of enzymatic reactions of DBT desulfurization has been studied on *R. erythropolis* D-1 (Izumi *et al.* 1994; Ohshiro *et al.* 1994; Ohshiro and Izumi 1999; Marcelis 2002). Gray *et al.* (1996) reported the desulfination of HPBS to 2-HBP catalyzed by DszB, which was found to be the rate-limiting step of the 4S pathway. The end product of this pathway is sulfite that is released into the cytoplasm and absorbed (Gray *et al.* 1996).

ANAEROBIC BDS

BDS with sulfate-reducing bacteria was first proposed in the 1950s by Zobell. The significant conversion of various model compounds was reported under anaerobic conditions by Kim *et al.* (1995). *Desulfovibrio desulfuricans* M6, which is a sulfate-reducing bacterium isolated from soil has been selected for its high hydrogenase activity. This bacterium is able to reductively convert DBT to biphenyl as the major reaction product and H₂S. The reaction pathway of this anaerobic desulfurization bacterium on DBT is shown in Fig. 4. In this pathway, DBT is used as the sole electron acceptor and sulfur is selectively removed by *D. desulfuricans* M6 (Kim *et al.* 1990a, 1990b).

Lizama *et al.* (1995) cultivated *Desulfotomaculum orientis*, *D. desulfuricans* and *Thermodesulfobacterium commune* with a carbon source and DBT as a sole sulfur source. Sulfide formation was demonstrated without producing biphenyl. Bahrami *et al.* (2001) reported on the anaerobic degradation of DBT but the final product was not detected.

COMPARISON OF AEROBIC AND ANAEROBIC DESULFURIZATION

In aerobic desulfurization, oxygen molecules are added to the hydrocarbon skeleton, and then 2-hydroxy biphenyl (2-HBP) and sulfate are formed as the end products of this route (McFarland 1999; Monticello 2000). The production of 2-HBP is not desirable; because 2-HBP is involved in the formation of viscous oil sludge (gum) in fuel and increasing 2-HBP might play the role of inhibitor in the culture (Nekodzuka *et al.* 1997). On the other hand, produced sulfate must be removed from the fuel (McFarland 1999). Aerobic microorganisms use 50% of total produced energy for growth, while anaerobic bacteria use approximately 10% of it (Marcelis 2002).

Under anaerobic conditions; H₂S and biphenyl are formed as the end product but H₂S can be treated with existing refinery desulfurization plants e.g. Claus process (Baglio *et al.* 1982; Pujare *et al.* 1989). This product is used in most oil production facilities and all refineries are equipped to handle it (McFarland 1999). On the other hand, the absence of oxygen prevents the production of the nonspecific oxida-

Table 3 An overview of reported sulfur selective aerobic desulfurization in growing conditions.

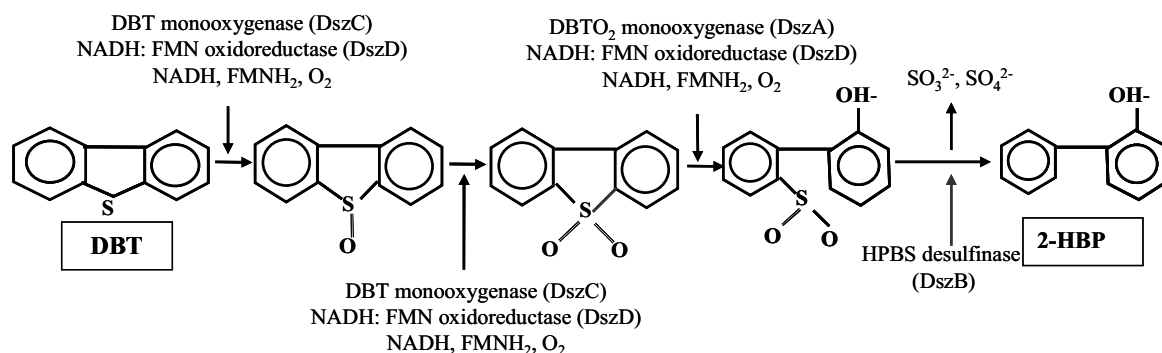
Strain/microorganism	Targeted organic sulfur compounds	References
<i>Paenibacillus</i> sp. A11-2 thermophile at from 45°C to 55°C	DBT alkylated DBT's BT analogous BT (more than DBT)	Konishi <i>et al.</i> 1997 Konishi <i>et al.</i> 1997; Onaka <i>et al.</i> 2001b Ishii <i>et al.</i> 2000 Konishi <i>et al.</i> 2000
<i>Rhodococcus</i> strain T09	BT and alkylated BT's (but not DBT)	Matsui <i>et al.</i> 2000
<i>Rhodococcus</i> strain T09 (recombinant)	Alkylated BT's and DBT's	Matsui <i>et al.</i> 2001a, 2001b
<i>Rhodococcus</i> strain ECRD-1	alkylated DBT's (distillates of diesel)	Lee <i>et al.</i> 1995; Grossman <i>et al.</i> 1999, 2001
<i>Nocardia</i> strain CYKS2	DBT in diesel oil	Chang <i>et al.</i> 1998
<i>Gordona</i> sp. strain 213E	BT (but not DBT)	Gilbert <i>et al.</i> 1998
<i>Rhodococcus erythropolis</i> N1-36	DBT (batch system) DBT (continuous system)	Wang <i>et al.</i> 1996b Wang <i>et al.</i> 1996a
<i>Corynebacterium</i> SY1	DBT	Omori <i>et al.</i> 1992
<i>Rhodococcus</i> strain WU-K2R	Naphthothiophene (NTH); Benzothiophene (BT)	Kirimura <i>et al.</i> 2002
<i>Bacillus subtilis</i> WU-S2B; thermophile form 30-50°C	DBT and alkylated DBT	Kirimura <i>et al.</i> 2001
<i>Pseudomonas aeruginosa</i> (recombinant strain)	DBT and alkylated DBT	Watanabe <i>et al.</i> 2002
<i>Rhodococcus erythropolis</i> D1	DBT	Izumi <i>et al.</i> 1994
<i>Rhodococcus erythropolis</i> Xp	DBT and alkylated DBT's, BT and alkylated BT's; benzophthothiophene (BNT) for a model component	Yu <i>et al.</i> 2006
<i>Mycobacterium phlei</i> WU-F1; thermophile 50°C	DBT; 2,8-dimethylDBT; 4,6-dimethylDBT and 3,4- benzoDBT	Furuya <i>et al.</i> 2001
<i>Rhodococcus</i> sp. strain JVH1	pentafluorophenylpropyl sulfide (PFPS)	van Hamme <i>et al.</i> 2004
IMP-S02, IMP-S06, IMP-S24, IMP-S24	DBT and 4,6-DMDBT	Castorena <i>et al.</i> 2002
<i>Mycobacterium phlei</i> GTIS10, thermophile 30-52°C	DBT and BT	Kayser <i>et al.</i> 2002
<i>Xanthamonas</i> sp. RIPIS-81	DBT DBT and alkylated DBT's	Constanti <i>et al.</i> 1994 Rashidi <i>et al.</i> 2006

Abbreviations: BNT, benzophthothiophene, BT, benzothiophene, DBT, dibenzothiophene, NTH, naphthothiophene, PFPS, pentafluorophenylpropyl sulphide

Table 4 An overview of reported sulfur selective aerobic desulfurization in resting cells.

Strain/microorganism	Targeted organic sulfur compounds	References
<i>Pseudomonas aeruginosa</i> (recombinant strain)	Light gas oil (LGO)	Watanabe <i>et al.</i> 2002
<i>Rhodococcus erythropolis</i> KA2-5-1	DBT DBT and several alkylated DBT's alkylated BT's and DBT's DBT and alkylated DBT's	Kobayashi <i>et al.</i> 2001; Natio <i>et al.</i> 2001 Kobayashi <i>et al.</i> 2000, 2001; Onaka <i>et al.</i> 2001a Luo <i>et al.</i> 2003
<i>Pseudomonas Delafieldii</i> R-8	DBT	Kobayashi <i>et al.</i> 2001
<i>Rhodococcus erythropolis</i> rKA2-5-1 (genetically modified)	DBT and alkylated DBT	Hirasawa <i>et al.</i> 2001
<i>Bacillus subtilis</i> WU-S2B	DBT and alkylated DBT	Kirimura <i>et al.</i> 2001
<i>Rhodococcus rhodochrous</i> IGTS8	DBT	Kayser <i>et al.</i> 1993; Honda <i>et al.</i> 1998; Kaufman <i>et al.</i> 1998
<i>Rhodococcus erythropolis</i> H-2	DBT and alkylated DBT DBT	Ohshiro <i>et al.</i> 1996a Ohshiro <i>et al.</i> 1995, 1996b
<i>Rhodococcus erythropolis</i> Xp	BT, alkylated BT's and benzophthothiophene	Yu <i>et al.</i> 2006
<i>Mycobacterium</i> strain G3	DBT, alkylated DBT's	Okada <i>et al.</i> 2001, 2002
<i>Rhodococcus erythropolis</i> I-19	DBT, alkylated DBT's	Folsom <i>et al.</i> 1999
<i>Gordona</i> strain CYSK1	DBT and diesel fuel	Rhee <i>et al.</i> 1998
<i>Mycobacterium phlei</i> WU-F1	DBT, alkylated DBT's	Furuya <i>et al.</i> 2001
<i>Mycobacterium</i> sp. X78 (thermophile from 25 to 45°C)	DBT and its alkylated in diesel fuel	Li <i>et al.</i> 2003
<i>Mycobacterium</i> sp. NCIMB 10403 strain MR 65	4,6-Dipropylidibenzothiophene use sulfur from light gas oil	Noda <i>et al.</i> 2003
<i>Rhodococcus</i> strain P32C1	DBT Diesel oil	Maghsoudi <i>et al.</i> 2000, 2001
RIPIS-22	DBT	Rashtchi <i>et al.</i> 2006

Abbreviations: BT, benzothiophene; DBT, dibenzothiophene; LGO, light gas oil

**Fig. 3** The sulfur specific pathway (4S) for DBT desulfurization of *Rhodococcus* sp.

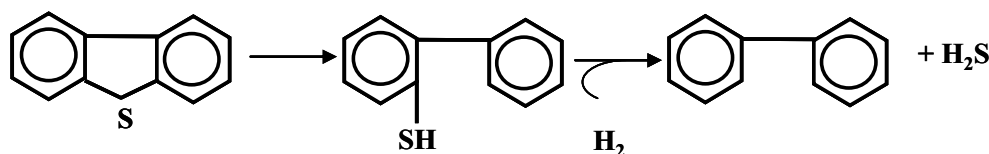


Fig. 4 Anaerobic desulfurization pathway of DBT in *Desulfotribrio* sp. in the presence of hydrogen molecules.

tion of hydrocarbons to colored, acidic or gum products (Setti *et al.* 1997). Under anaerobic conditions, the caloric value of the fuel is not reduced because of heavy oil composition before and after microbial treatment, and does not really differ, despite a significant decrease of organic sulfur percentage values (Setti *et al.* 1997; McFarland 1999).

Anaerobic desulfurization is considerable for industrial applications as the process is similar to HDS but in this process, anaerobic bacteria do not degrade aliphatic and aromatic compounds. Nevertheless, sulfur-reducing bacteria are not capable of desulfurizing BT and DBT derivatives, which are the most abundantly found in heavy oils and crude oils (Setti *et al.* 1997).

The growth rate of desulfurizing bacteria under anaerobic conditions is slower than aerobic ones. There are a few reports on the significant commercial application of anaerobic desulfurization. Aerobic microorganisms can remove a high level of organosulfur compounds. The limitations of low specificity under aerobic BDS could be overcome by using cell-free extracts. There are numerous aerobic bacteria for desulfurizing DBT and the most researches are performed on aerobic desulfurization because of their bioavailability (Setti *et al.* 1997).

THE PROCESS VARIABLES OF GROWTH AND ACTIVITY OF BDS IN MICROORGANISMS

Effect of carbon and sulfur sources and 2-hydroxybiphenyl (2-HBP) as product

Research on *Gordona* sp. CYKS1 (Rhee *et al.* 1998), *Rhodococcus rhodochrous* IGTS8 (Kayser *et al.* 1993; Kaufman *et al.* 1998), *R. erythropolis* N1-36 (Wang *et al.* 1996; Wang and Krawiec 1996) and *R. erythropolis* D-1 (Ohshiro *et al.* 1994) showed that sulfate can be consumed easily and increase cell growth. When sulfate was added to the culture DBT was not desulfurized and sulfate repressed the expression of DBT-desulfurizing activities. Therefore to achieve the sufficient desulfurization of crude oils, the development of new strains that are not susceptible to sulfate repression is proposed by Piddington *et al.* (1995). Also as DBT is applied as the sole sulfur source, 2-HBP inhibits growth (Kayser *et al.* 1993; Omorio *et al.* 1995). The presence of sulfate can moderate this inhibitory effect. To produce a high cell density a proper concentration of sulfate is necessary, following which the addition of DBT can activate the cells for BDS. The application of fed-batch feeding for carbon and nitrogen source is a successful strategy that results in high cell density cultivation. In *R. rhodochrous* IGTS8 it was shown that the application of glucose instead of succinate as a carbon source promoted growth and reduced the lag phase (Setti *et al.* 1999).

Inoculum percent, temperature and initial concentration of substrate

The effect of percentage inoculum, temperature and initial concentration of substrate has been studied to optimize the specific growth rate of *Rhodococcus*. In optimum conditions 40.2 and 27.1% of total sulfur and organic sulfur compounds of Mengen coal were deleted, respectively. Moreover, the initial concentration of DBT in the range of 0.05 to 0.5 mM did not significantly influence the growth rate of *Rhodococcus* sp. strain P32C1 (Maghsoudi *et al.* 2000, 2001).

Mixing and aeration

Culture of *Sulfolobus acidocaldarius* (Kargi and Robinson 1982) in a 4.5 L fermentor agitated at 600 rpm and aerated at 1 vol/vol/min results in a cell concentration of 0.4 g/L. At 700 rpm and 2 vol/vol/min aeration, this amount will reach 2 g/L. Highly dependence of growth on agitation and aeration suggests that the limitation of growth is related to the gas-liquid contact surface.

PROCESS VARIABLES AFFECTING GROWTH AND ACTIVITY OF MICROORGANISMS IN BDS

The volumetric ratio of organic to aqueous phase

The main aim in BDS is to apply highly effective cells for BDS of organic compounds in an organic medium. So it is important to evaluate BDS activity in the absence of water. The production rate of 2-HBP by *Rhodococcus* sp. strain P32C1 (Maghsoudi *et al.* 2000, 2001) was studied in three different volumetric ratios of organic to aqueous phase (25, 50 and 75% v/v). The results show that in all cases BDS was similar. Maximum growth rate for 2-HBP production is achieved in a 75% (v/v) ratio and 1 mM DBT.

DBT concentration

Total BDS behavior of P32C1 (Maghsoudi *et al.* 2000, 2001) in the presence of 1 and 24 mM DBT was the same. However in 24 mM the 2-HBP production rate was high but maximum production was low. Luo *et al.* (2003) reported that 14 mM (and more) DBT does not show any inhibitory effect on BDS by *Pseudomonas delafieldii* R-8.

Cell concentration

The results of studies on *P. delafieldii* R-8 (Luo *et al.* 2003) and *Rhodococcus* sp. strain. P32C1 (Maghsoudi *et al.* 2000, 2001) show that increasing cell density caused a decrease in the 2-HBP production rate but increased the maximum transformation rate, caused by a resistance to mass transfer, oxygen transfer for DBT oxidation and DBT bioavailability.

GENETIC ENGINEERING STRATEGIES FOR IMPROVEMENT OF BDS

There are many problems for the development of an efficient BDS. One of these problems is the low activity of naturally occurring bacterial cultures in comparison to the requirements of a commercial process. To overcome this problem, genetic engineering has been used to obtain a high desulfurization rate. Therefore, between 1990 and 1998, new recombinant biocatalysts were obtained and the activity of biocatalysts increased 200-fold (McFarland 1999; Pacheco *et al.* 1999). The highest desulfurization rates were obtained when a genetically modified strain of *R. erythropolis* KA2-5-1 achieved desulfurization activity from 50-250 $\mu\text{mol/g DCW/h}$ in medium containing DBT (Kobayashi *et al.* 2000; Hirasawa *et al.* 2001; Konishi *et al.* 2005). Also, genetic engineering has been used to improve the activity of DBT desulfurization of the IGTS8 strain, leading to a 200-fold BDS activity (Borgne and Quintero *et al.* 2003). As a result of this modification, the rate of DBT desulfurization achieved 20 $\mu\text{mol/min.g dry cells weight}$. By genetic manipulation, the DBT desulfurization (*dsz*) operon from *Rhodococcus erythropolis* IGTS8, which encodes three proteins, DszA, DszC, DszB has been isolated, cloned, mutated and

overexpressed to increase biocatalytic desulfurization, then multiple copies of *dszA*, *dszC*, *dszD* were cloned back into *R. erythropolis* to enhance enzyme production (Denome *et al.* 1993; Piddington *et al.* 1995). On the other hand, the number of *dsz* gene copies increased and the repression of sulfate was eliminated by changing the promoter and deleting the last gene in the metabolic pathway *dszB*. After elimination of the *DszB* gene, there was a subsequent decrease in the rate of the DSZ pathway; consequently hydroxybiphenyl sulfinate accumulated in the culture. The accumulation of hydroxybiphenyl sulfinate can be recovered from the aqueous phase and be used as a surfactant (Monticello 2000; Borgne and Quintero 2003).

As presented in studies of the expression of *dsz* genes in different hosts, it is clear that the contribution of the host is very important in the treatment of the desulfurization pathway and that there are a number of factors which are not yet understood in genetic manipulation.

On the other hand, mass transfer issues are the most important in BDS. A biocatalyst should be able to function optimally in the presence of a high concentration of hydrocarbons; therefore they must tolerate high concentrations of solvent (McFarland 1999; Kilbane 2006). The solvent tolerance of *Rhodococcus* is lower than *Pseudomonas*. *Rhodococcus* strains can take up very hydrophobic Cx-DBTs from oil whereas the DBT desulfurization rate can be increased when the *dsz* gene cassette is engineered into a rhamolipid-producing *Pseudomonas* (McFarland 1999).

THE COMMERCIAL ASPECTS OF BDS

The use of BDS as a commercial application is needed to understand the basis of BDS mechanisms and decreasing limiting factors for industrial application of BDS. The critical factor for application of BDS is a bioreactor design to achieve an efficient and adequate technique. The application of mixtures of biocatalysts is necessary for an efficient desulfurization of crude oil containing a wide range of sulfur compounds (Borgne and Quintero 2003). Also a cost-effective two-phase bioreactor system with requirements for oil-water separation, product recovery and recycling of the biocatalyst is essential. The conventional stirred tank bioreactor is applied in most studies of BDS (Monticello 1998) but it has been suggested that multiple-stage airlift reactors reduce mixing costs and promote mass transfer (Borgne and Quintero 2003; Mehrnia *et al.* 2004, 2005). It was reported that transfer of polycyclic aromatic sulfur heterocycle from the oil to the water and then from the water to the cells cause to limit the rate of its metabolism. A novel biodesulfurization technology was reported by Guobin *et al.* (2005). They assembled γ - Al_2O_3 nanoabsorbent which can selectively adsorb DBT from organic phase, on the surfaces of microbial cell and increase the rate of biodesulfurization (Guobin *et al.* 2005). Centrifugal methods have been used to conduct oil desulfurization reaction, break the emulsion and recycle the cells (Borgne and Quintero 2003). There are several process variables, which have a significant impact on BDS efficiency and ought to be known for bioreactor design. The oil/water volumetric ratio (with a maximum value of 1.25 ml/g (Monticello 1998)), oxygen availability, cofactors, regeneration, oil/water separation, diffusive mechanisms for interface of organic and aqueous phases and biocatalyst recovery are important parameters for designing a bioreactor (Borgne and Quintero 2003). The reported research on bioreactor design have been conducted in air lift, stirred tank reactors, emulsion phase contactor with free cells, and fluidized bed reactor with immobilized cells (Monticello 1998).

Other limitations of the commercial acceptance of BDS include the logistics of sanitary handling, shipment, storage and the sufficient longevity of the biocatalyst (McFarland 1999). BDS has not yet been cost-effective for heavy or middle distillates of petroleum oil. After distillation, dibenzothiophene (DBT) and alkylated DBT (Cx-DBTs) are accumulated in the middle distillate fractions and they may be

concentrated to 70% of the sulfur present in diesel oil (Monticello 1998). HDS technologies cannot reduce the sulfur content of diesel fuel to 30 ppm in the future but the combination of BDS and HDS technology has this potential. One of these problems is the low activity of naturally occurring bacterial cultures in comparison to the requirements of a commercial process. Application of mixtures of biocatalysts is necessary for an efficient desulfurization of crude oil containing a wide range of sulfur compounds.

CONCLUSION

BDS can be used as a complementary process, after the bulk sulfur is removed using HDS techniques. Desulfurization of sulfur compounds can be performed either with an aerobic or anaerobic mechanism but aerobic bacteria exhibit a higher activity.

The accumulation of 2-HBP inhibits growth and desulfurization; therefore mutants resistance to 2-HBP may be needed for commercial applications. Despite considerable progress in improving the expression of the key enzymes in the pathway, the flux throughout the system is still too low for widespread commercial applications. The problem is both the rate and extent of desulfurization. Sustained desulfurization rates over 20 $\mu\text{mol}/\text{min}\cdot\text{g}$ of catalyst are needed. The most important development in this area is the successful application of directed techniques to this system. The recombinant strain desulfurizes DBT more efficiently than the native one; recombinant strains also have increased stability and sulfur selectivity. Both directed evolution and gene shuffling cause increase rates of desulfurization and widen the sulfur substrate range. Recombination techniques can be applied to create new hybrid enzymes with high activities that meet the needs of refinery operations and go far beyond the needs of the bacteria to remove sulfur from oil.

Rhodococcus strains can take up very hydrophobic Cx-DBTs from the oil whereas DBT desulfurization rates can be increased when the *dsz* gene cassette is engineered into a rhamolipid-producing *Pseudomonas* (McFarland 1999).

Decreasing the limiting factors is necessary for the commercial application of BDS. The critical factor for the industrial application of BDS is a bioreactor design to achieve an efficient adequately technique. Application of mixtures of biocatalysts is necessary for an efficient desulfurization of crude oil containing a wide range of sulfur compounds.

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