

Review

Correspondence
Ghasemali Mohebali
mohebaligh@ripi.ir

Biocatalytic desulfurization (BDS) of petrodiesel fuels

Ghasemali Mohebali¹ and Andrew S. Ball²

¹Department of Petroleum Biotechnology, Biotechnology Research Center, Research Institute of Petroleum Industry, Tehran, Iran

²School of Biological Sciences, Flinders University of South Australia, Adelaide, SA 5001, Australia

Oil refineries are facing many challenges, including heavier crude oils, increased fuel quality standards, and a need to reduce air pollution emissions. Global society is stepping on the road to zero-sulfur fuel, with only differences in the starting point of sulfur level and rate reduction of sulfur content between different countries. Hydrodesulfurization (HDS) is the most common technology used by refineries to remove sulfur from intermediate streams. However, HDS has several disadvantages, in that it is energy intensive, costly to install and to operate, and does not work well on refractory organosulfur compounds. Recent research has therefore focused on improving HDS catalysts and processes and also on the development of alternative technologies. Among the new technologies one possible approach is biocatalytic desulfurization (BDS). The advantage of BDS is that it can be operated in conditions that require less energy and hydrogen. BDS operates at ambient temperature and pressure with high selectivity, resulting in decreased energy costs, low emission, and no generation of undesirable side products. Over the last two decades several research groups have attempted to isolate bacteria capable of efficient desulfurization of oil fractions. This review examines the developments in our knowledge of the application of bacteria in BDS processes, assesses the technical viability of this technology and examines its future challenges.

Introduction

Biotechnology is now accepted as an attractive means of improving the efficiency of many industrial processes, and resolving serious environmental problems. One of the reasons for this is the extraordinary metabolic capability that exists within the bacterial world. Microbial enzymes are capable of biotransforming a wide range of compounds, and the worldwide increase in attention being paid to this concept can be attributed to several factors, including the presence of a wide variety of catabolic enzymes and the ability of many microbial enzymes to transform a broad range of unnatural compounds (xenobiotics) as well as natural compounds. Biotransformation processes have several advantages compared with chemical processes, including: (i) microbial enzyme reactions are often more selective; (ii) biotransformation processes are often more energy-efficient; (iii) microbial enzymes are active under mild conditions; and (iv) microbial enzymes are

environment-friendly biocatalysts. Although many biotransformation processes have been described, only a few of these have been used as part of an industrial process. Many opportunities remain in this area.

Petroleum biotechnology is based on biotransformation processes. Petroleum microbiology research is advancing on many fronts, spurred on most recently by new knowledge of cellular structure and function gained through molecular and protein engineering techniques, combined with more conventional microbial methods. Current applied research on petroleum microbiology encompasses oil spill remediation, fermenter- and wetland-based hydrocarbon treatment, biofiltration of volatile hydrocarbons, enhanced oil recovery, oil and fuel biorefining, fine-chemical production, and microbial community-based site assessment (Van Hamme *et al.*, 2003).

Biorefining is a possible alternative to some of the current oil-refining processes. The major potential applications of biorefining are biodesulfurization, biodenitrogenation, biometallization, and biotransformation of heavy crude oils into lighter crude oils. The most advanced area is biodesulfurization, for which pilot plants exist (Le Borgne & Quintero, 2003). The results obtained for biodesulfurization may be generally applicable to other areas of biorefining.

Abbreviations: API, American Petroleum Institute; BDS, biocatalytic desulfurization; DBT, dibenzothiophene; C_x-DBT, alkylated dibenzothiophene; DBTO, DBT sulfoxide; DBTO₂, DBT sulfone; EBC, Energy BioSystems Corporation; HDS, hydrodesulfurization; 2-HBP, 2-hydroxybiphenyl; LGO, light gas oil; psig, p.s.i. gauge.

A supplementary table is available with the online version of this paper.

Sulfur level in oil fractions and legislative regulations

The sulfur content of crude oil can vary from 0.03 to 7.89 % (w/w) (Kilbane & Le Borgne, 2004). The API gravity of oil is decreasing and sulfur content is increasing (Swaty, 2005), resulting in an increase in the sulfur concentrations in finished petroleum products. Sulfur is preferentially associated with the higher molecular mass components of crude oils. When crude oil is refined the sulfur concentrates into the high molecular mass fractions.

A typical flue gas from the combustion of fossil fuels will contain quantities of NO_x, S and particulate matter. SO₂ gas at elevated levels can cause bronchial irritation and trigger asthma attacks in susceptible individuals. Potential health risks expand to a broader section of the public when the gas turns to particulate matter (see <http://www.epa.gov/acidrain/>). Long-term exposure to combustion-related fine particulate air pollution is an important environmental risk factor for cardiopulmonary and lung cancer mortality (Pope *et al.*, 2002). Many governments have recognized the problems and decided to reduce sulfur emissions through legislation. Increasingly strict environmental regulations in the developed world have forced car manufacturers and fuel refiners to produce vehicles and fuels that meet these legislative goals. The US Environmental Protection Agency (EPA) has taken steps to limit emissions of NO_x and SO₂ (<http://www.epa.gov/airmarkets/progsregs/arp/>).

The simplest way to decrease the amount of SO₂ emitted into the air is to limit the amount of sulfur in fuel. Legislative regulations in many countries call for the production and use of more environmentally friendly transportation fuels. Previous to 2003 the US EPA proposed that non-road diesel fuel sulfur be reduced from 3400 p.p.m. to 500 p.p.m. by 2007 (Song, 2003), and the European Union declared that the sulfur concentration in diesel fuel must reduce to <50 p.p.m. by 2005 (Marcelis, 2002) and <10 p.p.m. by 2009 (European Directive 2003/17/CE). The maximum allowable sulfur content for diesel in the USA is currently 15 p.p.m., with a target of 10 p.p.m. by 2010 (Kilbane, 2006; Song, 2003). New and more effective approaches are needed for producing affordable ultra-clean (ultralow-sulfur) transportation and non-road fuels, because meeting the new government sulfur regulations in 2006–2010 represents only a milestone (Song, 2003).

Petroleum refining

All crude oils are composed primarily of hydrocarbons of paraffinic, naphthenic and aromatic classes with a very broad range of molecular masses. Refining is the physical, thermal and chemical separation of crude oil into its major distillation fractions, which are then further processed through a series of separation and conversion steps into finished petroleum products. The primary products of the industry fall into three major categories: fuels (such as

gasoline, diesel oil, jet fuel and kerosene), finished non-fuel products (such as solvents, lubricating oils, greases, petroleum wax, asphalt and coke), and chemical industry feed stocks (such as naphtha, ethane, propane, butane, benzene, toluene and xylene) (EPA, 1995).

Hydrodesulfurization (HDS)

Conventional HDS is a high-pressure (150–200 psig) and high-temperature (200–450 °C) catalytic process that converts organic sulfur to hydrogen sulfide gas by reacting crude oil fractions with hydrogen in the presence of an efficient inorganic catalyst. The reactivity of organosulfur compounds varies widely depending on their structure and the local sulfur atom environment. The conditions depend upon the level of desulfurization required (Gupta *et al.*, 2005).

HDS of diesel feedstock for a low-sulfur product requires a larger reactor volume, longer processing times, and substantial hydrogen and energy inputs. Deep HDS technology results in various problems in the process, which limit its usefulness: (i) the application of extreme conditions to desulfurize refractory compounds results in the deposition of carbonaceous coke on the catalysts; (ii) exposure of crude oil fractions to severe conditions including temperatures above about 360 °C decreases the fuel value of treated product; (iii) deep HDS processes need large new capital investments and/or have higher operating costs; (iv) the H₂S that is generated poisons the catalysts and shortens their useful life; (v) deep HDS is affected by components in the reaction mixture such as organic heteroatoms and polyaromatic hydrocarbons (Egorova, 2003; Folsom *et al.*, 1999; Konishi *et al.*, 2000; Monticello, 1996).

Biocatalytic desulfurization (BDS)

BDS is often considered as a potential alternative to the conventional deep HDS processes used in refineries. In this process, bacteria remove organosulfur from petroleum fractions without degrading the carbon skeleton of the organosulfur compounds. During a BDS process, alkylated dibenzothiophenes (C_x-DBTs) are converted to non-sulfur compounds, for example 2-hydroxybiphenyl (2-HBP), and sulfate. BDS offers mild processing conditions and reduces the need for hydrogen. Both these features would lead to high energy savings in the refinery. Further, significant reductions in greenhouse gas emissions have also been predicted if BDS is used (Linguist & Pacheco, 1999).

BDS as a complementary technology

As already mentioned, HDS is not equally effective in desulfurizing all classes of sulfur compounds present in fossil fuels. The BDS process, on the other hand, is effective regardless of the position of alkyl substitutions (Pacheco, 1999). However, the HDS process conditions are sufficient

not only to desulfurize sensitive (labile) organosulfur compounds, but also to (i) remove nitrogen and metals from organic compounds, (ii) induce saturation of at least some carbon-carbon double bonds, (iii) remove substances having an unpleasant smell or colour, (iv) clarify the product by drying it, and (v) improve the cracking characteristics of the material (McFarland, 1999; Monticello, 1996; Swaty, 2005). Therefore, with respect to these advantages, placing the BDS unit downstream of an HDS unit as a complementary technology to achieve ultradeep desulfurization, rather than as a replacement, should also be considered. Monticello (1996) suggested a multistage process for desulfurization of fossil fuels. This method was based on subjecting vacuum gas oil to HDS prior to BDS in defined conditions. Pacheco (1999) reported that the Energy BioSystems Corporation (EBC) used BDS downstream of HDS. Fang *et al.* (2006) also showed that combination of HDS and BDS could reduce the sulfur content of catalytic diesel oil from 3358 to $<20 \mu\text{g g}^{-1}$.

Model compounds in BDS studies

The organosulfur compounds found in crude oil are generally classified into two types: non-heterocyclics and heterocyclics. The former comprise thiols, sulfides and disulfides. Cyclic or condensed multicyclic organosulfur compounds are referred to as sulfur heterocyclics. Thiophenic sulfur is present in many types of these compounds.

The most abundant form of sulfur in petroleum is usually thiophenic. Thiophenic sulfur often constitutes 50–95 % of the sulfur in crude oil and its fractions, and alkylated derivatives of dibenzothiophene (DBT) are the most common organosulfur compounds typically found in crude oil and fractions used to produce diesel (Kilbane & Le Borgne, 2004). DBT and its derivatives can account for a significant percentage of the total sulfur content of particular crude oils. In the middle distillate (diesel range) fractions, the sulfur that remains after conventional HDS treatment is typically in the form of C_x -DBT compounds.

It is therefore reasonable to infer that the development of BDS processes is dependent on the establishment of a microbial system with the potential to desulfurize a broad range of organosulfur compounds present in crude oil fractions. However, there is no common model compound that can be used for all the various crude oil fractions. Although middle-distillate fractions contain a complex mixture of sulfur compounds, they can be represented by two general chemical types: thiophenes and aliphatic sulfides. DBT has been used as a model thiophene, and benzylsulfide as a model aliphatic sulfide, in the study of BDS (McFarland, 1999). DBT is one of many tens of thousands of polyaromatic sulfur heterocyclics found in hydrotreated diesel samples, and the alkyl side chains generate all these isomers (Monticello, 1998).

Susceptibility of DBT to microbial attack

Micro-organisms have evolved diverse biochemical pathways in order to use sulfur, which is an essential element. In terms of DBT utilization, two main pathways have been reported: ring-destructive (degradation) and sulfur-specific (desulfurization) pathways. To date, two ring-destructive pathways for metabolism of DBT have been recognized.

The most common pathway of DBT degradation, known as the 'Kodama pathway', is analogous to that of naphthalene degradation (Kodama *et al.*, 1973). In this pathway initial dioxygenation is carried out at the peripheral aromatic ring of DBT, followed by cleavage of the ring (Fig. 1). This process leads to the accumulation of 3-hydroxy-2-formylbenzothiophene as a water-soluble end product, with a lower carbon content than DBT. In this pathway no desulfurization of the organosulfur substrate occurs. Denome *et al.* (1993b) cloned and sequenced a 9.8 kb

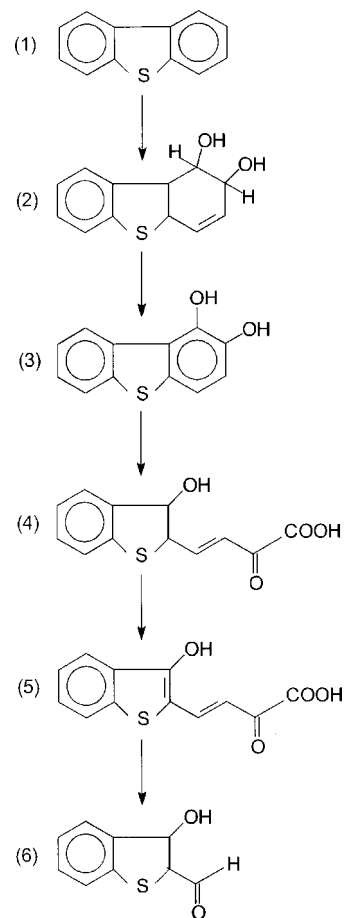


Fig. 1. The Kodama pathway for the degradation of DBT (McFarland, 1999; Soleimani *et al.*, 2007). 1, dibenzothiophene; 2, *cis*-1,2-dihydroxy-1,2-dihydrodibenzothiophene; 3, 1,2-dihydroxydibenzothiophene; 4, *cis*-4-[2-(3-hydroxy)thionaphthenyl]-2-oxo-3-butenoate; 5, *trans*-4-[2-(3-hydroxy)thionaphthenyl]-2-oxo-3-butenoate; 6, 3-hydroxy-2-formylbenzothiophene.

DNA fragment from *Pseudomonas* strain C18 that encoded DBT-degrading enzymes. Nine ORFs were identified and designated *doxABDEFGHIJ*; collectively, these genes were referred to as the DOX (DBT oxidation) pathway. The results indicated that a single genetic pathway controls the metabolism of DBT, naphthalene and phenanthrene in strain C18.

Another ring-destructive pathway that results in mineralization of DBT is that described by Van Afferden *et al.* (1993). They isolated *Brevibacterium* sp. DO, capable of using DBT for growth as the sole source of carbon, sulfur and energy. During DBT mineralization three metabolites were identified: DBT sulfoxide (DBTO), DBT sulfone (DBTO₂) and benzoate. This pathway resulted in the complete mineralization of DBT, with the release of the sulfur atom as sulfite stoichiometrically, which then oxidized to sulfate. There are no detailed studies of the enzymology or molecular biology of DBT degradation by this strain. This ring-destructive pathway may be valuable in biodegradation of DBT in the environment.

The above ring-destructive pathways are not commercially useful for the petroleum industry because use of the carbon skeleton of sulfur compounds by the bacteria reduces the fuel's calorific value.

DBT desulfurization pathways

Several investigators have reported that a sulfur-specific pathway could be carried out by anaerobic and aerobic bacteria. Sulfate-reducing bacteria have been reported to desulfurize model compounds and fossil fuels (Kim *et al.*, 1995; Lizama *et al.*, 1995). However, under well-controlled sulfate-reducing anaerobic conditions, no significant reductions in the sulfur content of DBT or in the total sulfur content of vacuum gas oil, deasphalted oil or bitumen were observed (Armstrong *et al.*, 1995, 1997). Since there is currently little evidence for the potential for a commercially significant anaerobic desulfurization, aerobic biodesulfurization has been the focus of most research.

The 4S pathway is a specific desulfurization pathway in which DBT is desulfurized and converted to 2-HBP (Fig. 2). Through this pathway the carbon skeleton of DBT is released intact and thus the calorific value of the fuel is not lost. This pathway is energetically expensive because the carbon skeleton is not mineralized in order to get back the energy invested. The use of this pathway has been proposed for the desulfurization of petroleum in production fields and also refineries (McFarland, 1999; Monticello, 1998). Recently, a new desulfurization pathway has been found that is different from the 4S pathway. *Rhodococcus* sp. WU-K2R was reported to grow on naphtho[2,1-*b*]thiophene (NTH) as the sole sulfur source but not as the sole carbon source. NTH is an asymmetric structural isomer of DBT. Strain WU-K2R could not utilize DBT, DBTO₂ or 4,6-DMDBT (Kirimura *et al.*, 2002).

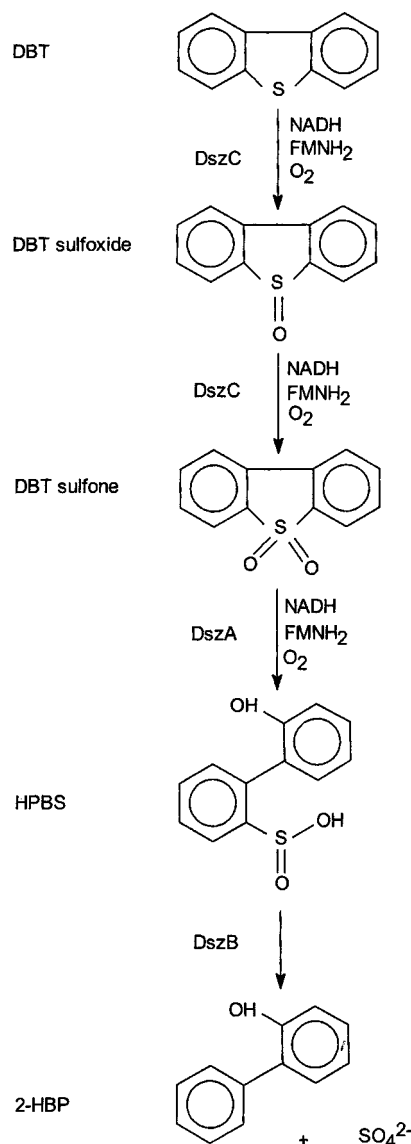


Fig. 2. The 4S pathway for the biocatalytic desulfurization of DBT and its derivatives. DBT, dibenzothiophene; HPBS, hydroxyphenylbenzene sulfonate; 2-HBP, 2-hydroxybiphenyl; DszA, DBT sulfone monooxygenase; DszB, HPBS desulfinase; DszC, DBT monooxygenase.

The genetics of biodesulfurization

In 1985, Isbister & Koblynski described a strain of *Pseudomonas* sp., CB-1, that could accomplish sulfur-specific metabolism of DBT (Gallagher *et al.*, 1993). The intermediates were DBTO and DBTO₂ and the end product was dihydroxybiphenyl; unfortunately this strain was lost before the metabolic pathway could be fully characterized (Gallagher *et al.*, 1993). Isolation of a suitable bacterium was achieved after 40 years of research effort when Kilbane (1990) at the Gas Technology Institute (formerly Institute of Gas Technology), USA, isolated

Rhodococcus erythropolis IGTS8. Since then, many researchers have isolated bacteria capable of desulfurizing DBT and its alkylated forms via the 4S pathway (for a full list, see Supplementary Table S1, available with the online version of this paper).

The genes involved in DBT metabolism have been called *bds* (Ohshiro *et al.*, 2005), *dsz* (Piddington *et al.*, 1995), *tds* (Ishii *et al.*, 2000), *mds* (Nomura *et al.*, 2005) and *sox* (Denome *et al.*, 1994). Because several other unrelated genes have already been labelled *sox* and to avoid confusion with these other genes, the *sox* designation has been generally rejected. *Bds*, *Dsz*, *Tds* and *Mds* have all been accepted as gene products. *R. erythropolis* IGTS8 (Gallardo *et al.*, 1997; Gray *et al.*, 1996; Oldfield *et al.*, 1997; Piddington *et al.*, 1995) and *Rhodococcus* sp. X309 (Denis-Larose *et al.*, 1997) were among the first strains to be characterized at the molecular level. A gene cluster that could complement a desulfurization-negative mutant of IGTS8 has been cloned and sequenced (Denome *et al.*, 1993a, 1994; Piddington *et al.*, 1995), and found to comprise three ORFs, designated *dszA*, *B* and *C*. Subclone analyses revealed that the product of *dszC* converts DBT directly to DBTO₂ and that the products of *dszA* and *dszB* act in concert to convert DBTO₂ to 2-HBP. The three genes are clustered on a 120 kb linear plasmid of strain IGTS8 (Denome *et al.*, 1994). The desulfurization (*Dsz*) phenotype was conferred by a 4 kb gene locus on the large plasmid (Denis-Larose *et al.*, 1997; Gray *et al.*, 1998; Oldfield *et al.*, 1997; Rambossek *et al.*, 1999). The desulfurization ability of *Rhodococcus* sp. ECRD-1 appeared to be an exclusive property of a 4 kb gene locus on a large plasmid (Prince & Grossman, 2003). In *Rhodococcus* the *dsz* genes are located near insertion sequences (Denis-Larose *et al.*, 1997; Kilbane & Le Borgne, 2004). Therefore, the desulfurization system is organized as one operon with three genes (*dszA*, *dszB*, *dszC*) transcribed in the same direction and under the control of a single promoter (Gray *et al.*, 1998; Oldfield *et al.*, 1997).

In BDS research, two monooxygenases, *DszA* and *DszC*, have been shown to be active in the desulfurization reaction with the aid of an oxidoreductase encoded by *dszD*. The *dszD* gene was cloned and sequenced from *R. erythropolis* IGTS8. The gene encoded a protein of ~20 kDa and was present on the chromosome of IGTS8 rather than on the plasmid that contains *dszABC* (Gray *et al.*, 2003). Matsubara *et al.* (2001) purified and characterized the flavin reductase from *R. erythropolis* D-1. The N-terminal amino acid sequence of the purified flavin reductase was identical to that of *DszD* of *R. erythropolis* IGTS8. The gene (*frm*) encoding flavin reductase from *Mycobacterium phlei* WU-F1 has been cloned and the cloned enzyme has been purified and characterized. The deduced amino acid sequence of the *frm* product shared only 33% identity with that of the flavin reductase gene (*dszD*) from *R. erythropolis* IGTS8 (Furuya *et al.*, 2005). Ishii *et al.* (2000) isolated and cloned the gene *tdsD* encoding the flavin reductase which coupled with the monooxygenases of

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

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

A recent finding is that bacterial cultures that possess identical *dsz* gene sequences can have very different *Dsz* phenotypes. This was clearly illustrated by examining the desulfurization activity of *Mycobacterium phlei* GTIS10 having *dszABC* gene sequences identical to *R. erythropolis* IGTS8; the temperature at which maximum desulfurization activity was detected in the cultures was about 50 °C and 30 °C, respectively (Kayser *et al.*, 2002; Kilbane, 2006). Characterization of four bacterial cultures capable of utilizing DBT as the sole source of sulfur revealed that these cultures had identical *dsz* genes, but the cultures differed significantly with regard to their substrate range, desulfurization activity and yield of metabolites (Abbad-Andaloussi *et al.*, 2003). A comparative study of *M. phlei* SM120-1 and *M. phlei* GTIS10 aimed at broadening the understanding of the *Dsz* trait at intra-species level revealed considerable differences in the phenotypic and genotypic characteristics of these two desulfurizing strains (Srinivasaraghavan *et al.*, 2006). The range of *Dsz* phenotypes observed in different cultures may reflect the ability of each bacterial species or strain to provide cofactors and reaction substrates under the conditions tested. The transport of substrates and products might also contribute to desulfurization activity, as demonstrated by the fact that cell-free lysates of desulfurization cultures can exhibit a broader substrate range than the intact cell culture (Kilbane, 2006).

Regulation and repression of *dsz* genes

Inorganic sulfate is the preferred sulfur source for growth of most micro-organisms. Under sulfate-limited conditions

several species of bacteria synthesize a set of extra proteins which are required for metabolism of alternative sulfur sources. These so-called sulfate-starvation-induced proteins are synthesized only in the absence of 'preferred sulfur sources' including sulfate. These proteins may be enzymes and transport systems involved in scavenging and metabolizing alternative sulfur sources from the environment (Kertesz, 2000). The Dsz enzymes can probably be considered in this category.

Several investigators have reported that the desulfurization activity in various bacteria was completely repressed by sulfate or other readily bioavailable sulfur sources, including methionine, cysteine, taurine, methanesulfonic acid and Casamino acids (Gunam *et al.*, 2006; Kertesz, 2000; Lee *et al.*, 1995; Li *et al.*, 1996; Matsui *et al.*, 2002; Noda *et al.*, 2002; Ohshiro *et al.*, 1996b; Piddington *et al.*, 1995; Rhee *et al.*, 1998; Serbolisca *et al.*, 1999; Wang & Krawiec, 1996). Sulfate had no effect on the activity of the desulfurizing enzymes (Li *et al.*, 1996; Wang & Krawiec, 1996). There is no evidence of feedback inhibition of the 4S pathway enzymes themselves, as the presence of sulfate in growth media represses expression of desulfurization activity, but addition of sulfate to resting (non-proliferating) cells grown on DBT does not inhibit desulfurization activity (Guobin *et al.*, 2006; Li *et al.*, 1996; Wang & Krawiec, 1996).

The production of DBT desulfurizing enzymes has been shown to be inhibited by inorganic sulfate (Alves *et al.*, 2005; Gunam *et al.*, 2006; Lee *et al.*, 1995; Li *et al.*, 1996; Mohebbali *et al.*, 2007a; Noda *et al.*, 2002; Ohshiro *et al.*, 1996b; Rhee *et al.*, 1998; Wang & Krawiec, 1996). No radiolabelled sulfite was produced in incubations of sulfate-grown *R. erythropolis* IGTS8 resting cells with [³⁵S]DBT and no detectable 2-HBP was produced (Oldfield *et al.*, 1997). Li *et al.* (1996) found that transcription from the *dsz* promoter was strongly repressed by SO₄²⁻, cysteine or Casamino acids, indicating that there could be a Dsz repressor in strain IGTS8. Their studies showed that each deletion that retained *dsz* promoter activity was fully repressible, indicating that if there is a Dsz repressor protein, it binds to a site that overlaps the promoter. Promoter deletion and replacement strategies alleviated sulfur repression (Gallardo *et al.*, 1997; Gray *et al.*, 1998; Serbolisca *et al.*, 1999).

Overcoming the repression of *dsz* genes

The 4S pathway is a complex enzyme system and its cofactor requirements prohibit the use of purified enzyme systems rather than whole cells for a practical BDS process (Kilbane & Le Borgne, 2004). Moreover, cell-free extracts exhibit a lower desulfurizing activity (Gupta *et al.*, 2005). Implementation of the BDS process consists of several stages, including (i) growing the selected strain in a suitable medium so as to obtain cells which exhibit the highest possible desulfurizing activity and (ii) harvesting these active cells and using them in the form of resting cells (biocatalysts)

in a BDS process. The formulation of the growth medium is important for production of a high density of resting cells, which express the highest level of desulfurization activity. In this context, sulfate contamination of the growth medium is the main barrier to the expression because the Dsz phenotype is repressed by sulfate.

Several researchers have studied the expression of the *dsz* genes in a heterologous host as a means of decreasing the problem of repression of the *dsz* genes. It has been reported that the *dsz* cluster from *R. erythropolis* IGTS8 could be engineered as a DNA cassette under the control of heterologous regulatory signals to increase the ability of *Pseudomonas* strains to efficiently desulfurize DBT (Gallardo *et al.*, 1997). Promoter replacement has also been examined as a possible alternative to expression of the *dsz* genes in a heterologous host as a means of alleviating the repression problem (Serbolisca *et al.*, 1999; Matsui *et al.*, 2002).

To obtain large-scale production of desulfurizing resting cells, repression can also be avoided by various microbiological procedures, including lowering the repressor (sulfate) content of growth medium, substitution of sulfate by an alternative sulfur source, and production of resting cell biomass on sulfate or another suitable sulfur source followed by induction of the Dsz phenotype in the resting cells using DBT (Chang *et al.*, 2001; Honda *et al.*, 1998; Ma *et al.*, 2006a; Mohebbali *et al.*, 2008).

Because *dsz* genes are repressed by inorganic sulfate, most researchers have used synthetic media containing DBT as the sulfur compound for growth. Desulfurizing activity has been shown to be enhanced by restricting the amount of DBT added to the medium (Yoshikawa *et al.*, 2002). Mass production of biocatalyst using DBT has been considered to be commercially impractical as a result of its high price and low water solubility, and growth inhibition by 2-HBP. Researchers have therefore tried to find a suitable alternative to DBT as a sulfur source for growing cells, for example 2-aminoethanesulfonic acid (Yoshikawa *et al.*, 2002) and dimethyl sulfoxide (Mohebbali *et al.*, 2008).

Recombinant biocatalysts

The desulfurization activity of naturally occurring bacterial cultures is low in comparison to the requirements of a commercial process. It has been estimated that a successful commercial process would require a biocatalyst with desulfurization activity of 1.2–3 mM DBT (g dry cell weight)⁻¹ h⁻¹ as measured with petroleum products. To achieve this, the currently available biocatalysts require an increase in desulfurization rate of about 500-fold (Kilbane, 2006). The key to increasing the bacterial desulfurization rate is to identify the genes responsible for C–S bond cleavage in organic sulfur compounds and manipulate the system through genetic engineering techniques. Therefore, future BDS studies will focus on development of this promising research area. To develop an efficient biocatalyst many investigators have constructed recombinant biocatalysts. For

example, the *dsz* genes from *R. erythropolis* DS-3 were successfully integrated into the chromosomes of *Bacillus subtilis* ATCC 21332 and UV1, yielding two recombinant strains, *B. subtilis* M29 and M28, in which the integrated *dsz* genes were expressed efficiently under control of the promoter Pspac. The DBT desulfurization efficiency of M29 was 16.2 mg DBT l⁻¹ h⁻¹ at 36 h, significantly higher than that of *R. erythropolis* DS-3, and also showed no product inhibition (Ma *et al.*, 2006b). Tao *et al.* (2006) constructed a solvent-tolerant desulfurizing bacterium by introducing the gene cluster *dszABCD* from *R. erythropolis* XP into the solvent-tolerant strain *Pseudomonas putida* Idaho. The recombinant strain had the same substrate range as *R. erythropolis* XP and could desulfurize DBT in the presence of *p*-xylene and many other organic solvents at a concentration of 10% (v/v). Li *et al.* (2007a) improved the DBT desulfurization activity of *R. erythropolis* DR-1 by removing the gene overlap in the *dsz* operon. Desulfurization activity of the redesigned strain was about fivefold higher than that of strain DR-1.

An improvement in the uptake of sulfur compounds in oil fractions should be effective in enhancing the biodesulfurization activity. Watanabe *et al.* (2003) transferred the *dsz* gene cluster from *R. erythropolis* KA2-5-1 into *R. erythropolis* MC1109, which was unable to desulfurize light gas oil (LGO). Resting cells of the resultant recombinant strain, named MC0203, decreased the sulfur concentration of LGO from 120 mg l⁻¹ to 70 mg l⁻¹ in 2 h. The LGO-desulfurization activity of this strain was about twice that of strain KA2-5-1.

Matsui *et al.* (2001b) introduced *dsz* genes into *Rhodococcus* sp. T09, a strain capable of desulfurizing benzothiophene (BT). The resulting recombinant strain grew with both DBT and BT as the sole sulfur sources. The recombinant cells desulfurized not only alkylated BTs, but also various C_x-DBTs, producing alkylated hydroxyphenyls as the end products. Coco *et al.* (2001) used the DNA-shuffling method to generate recombined genes and evolved enzymes. An increase in steady-state DBT sulfone concentration of at least 16-fold was observed in pathways containing shuffled *dszC* genes.

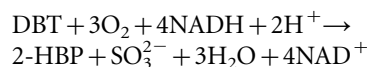
FMN reductase is among the four key enzymes of the 4S pathway. When flavin reductase, FMN reductase or various oxidoreductases were added to the reaction mixture, or overexpressed in recombinant constructs, the desulfurization rate increased (Gray *et al.*, 1996, 1998; McFarland, 1999; Ohshiro *et al.*, 1995; Rambossek *et al.*, 1999; Squires *et al.*, 1998, 1999). The use of the flavoprotein could result in an approximately 100-fold improvement in the rate of reaction compared with a system where no flavoprotein was added (Squires *et al.*, 1998). Several research groups have focused on overexpression of this enzyme. FMN reductase can be overexpressed in desulfurizing microorganisms via mutagenesis. The DNA encoding flavin reductase can be transferred into desulfurizing microorganisms, can be simultaneously or independently

transferred into a desired host cell with the DNA encoding the Dsz enzymes, and can be under the control of the same or a different promoter as the DNA encoding the Dsz enzymes (Squires *et al.*, 1999). Matsubara *et al.* (2001) amplified the flavin reductase gene with primers designed by using *dszD* of *R. erythropolis* IGTS8; the enzyme was overexpressed in *Escherichia coli*. The specific activity in crude extracts of the overexpressing strain was about 275-fold that of wild-type strain, *R. erythropolis* strain D-1.

As mentioned above, a popular strategy in metabolic engineering is to change the host strain for the *dsz* genes entirely, perhaps to take advantage of another strain's growth properties, physical properties (for mixing and separations), or higher intrinsic metabolic rate. An interesting feature of genetic experiments involving the *dsz* genes is that the level of desulfurization activity achieved by genetic manipulation appears to be limited by factors that are not yet understood. In order to achieve very high levels of expression of the desulfurization pathway, a better understanding of the host factors that contribute to the functioning of the pathway is needed (Kilbane, 2006). Therefore, in general, it has been preferred to use the original strain for technical (e.g. gene expression and codon usage preferences) and regulatory reasons (Monticello, 1998). In addition, self-cloning is generally regarded as more effective than heterologous recombination, as gene expression and DBT permeation are readily achieved (Hirasawa *et al.*, 2001; Li *et al.*, 1996; Matsui *et al.*, 2001a).

Dsz enzymes

The 4S pathway (Fig. 2) involves sequential oxidation of the sulfur moiety and cleaving of the C–S bonds. In the sequential oxidation, four key enzymes are involved, two monooxygenases, one desulfinase, and one NADH:FMN oxidoreductase. The latter supplies the two monooxygenases with reduced flavin. In *R. erythropolis* IGTS8 the pathway proceeds via two monooxygenases (DszC and DszA), supported by an FMN:NADH oxidoreductase (DszD), and a sulfinate (DszB). In this strain each of the enzymes has been purified and characterized. The desulfurization pathway needs cellular metabolism to produce reducing equivalents and is an energy-intensive process, with ~4 mol NADH required per mol DBT desulfurized (Oldfield *et al.*, 1997). DszC and DszA do not use NADH directly, but use FMNH₂ from DszD. DszD couples the oxidation of NADH with substrate oxidation by DszA and DszC. The products of the reactions (the 4S pathway) are 2-HBP and sulfite. The overall reaction (for neutral pH) is as follows:



The DszC enzyme

DszC, a ~45 kDa protein, catalyses the sequential conversion of DBT→DBTO→DBTO₂. The two catabolic steps

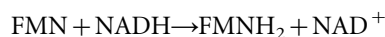
require oxygen and FMNH₂ for activity (Denome *et al.*, 1994; Gray *et al.*, 1996; Oldfield *et al.*, 1997; Xi *et al.*, 1997). DszC seems exceptional because it catalyses two consecutive flavomonooxygenase reactions (Lei & Tu, 1996).

The DszA enzyme

DszA, a ~50 kDa protein, catalyses the transformation of the sulfone to a sulfinate [2-(2'-hydroxyphenyl)benzene sulfinate, HBPS], also utilizing FMNH₂ as a co-substrate (Denome *et al.*, 1994; Oldfield *et al.*, 1997; Xi *et al.*, 1997), with a reaction rate 5–10-fold higher than DszC (Gray *et al.*, 1996). The reaction required oxygen and is NADH-dependent. The oxygen atoms are derived from molecular oxygen (Lei & Tu, 1996; Oldfield *et al.*, 1997). Gray *et al.* (1996) showed that in *R. erythropolis* IGTS8, DszA, like DszC, utilizes FMNH₂ as a co-substrate, apparently derived from the DszD reaction. This molecule would attack a C–S bond of the substrate (DBTO₂), leading to its cleavage by the expulsion of sulfinate.

The DszD enzyme

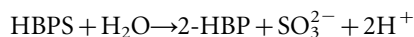
Ohshiro *et al.* (1994) studied DBT desulfurization using a dialysed cell-free extract of *R. erythropolis* D-1 grown on DBT as the sole sulfur source. NADH was an absolute requirement for DBT desulfurization, and desulfurization activity of the cell-free extract was stimulated by an increase in the NADH concentration. Matsubara *et al.* (2001) purified and characterized the flavin reductase from *R. erythropolis* D-1 grown in a medium containing DBT as the sole sulfur source. The specific activity of the purified enzyme and the N-terminal amino acid sequence were similar or identical to those of Dsz from *R. erythropolis* IGTS8. Xi *et al.* (1997) demonstrated that an FMN:NADPH oxidoreductase from *Vibrio harveyi* complemented the activities of purified DszA and DszC. Squires *et al.* (1998) showed that expression of the Dsz phenotype in *E. coli* was dependent on flavin reductase and was significantly enhanced by the flavin reductase protein. Gray *et al.* (1998) isolated, purified and analysed amino acid sequences of DszD from strain IGTS8. The flavin reductase was not a flavoprotein because it did not include a tightly associated FMN cofactor; instead this enzyme appeared to utilize FMN as a dissociable substrate. The enzyme was specific for FMN and NADH and did not accept NADPH and FDN as substrates. DszD was not encoded by the *dsz* operon (Gray *et al.*, 1996; Lei & Tu, 1996; Xi *et al.*, 1997). It was believed that this enzyme serves to couple the oxidation of NADH to substrate oxidation by catalysing the following reaction:



The DszB enzyme

DszB is a ~40 kDa protein which catalyses the conversion of HBPS to 2-HBP (Denome *et al.*, 1994). On this basis

DszB is classified as an aromatic sulfinic acid hydrolase catalysing the following reaction (Oldfield *et al.*, 1997):



It has been reported that DszB uses a nucleophilic attack of a base-activated water molecule on the sulfinate sulfur to form 2-HBP in a rate-limiting reaction (McFarland, 1999; Ohshiro & Izumi, 1999). HBPS is the penultimate intermediate in the 4S pathway since HBPS→2-HBP would only require the cleavage of a single C–S bond. DszB is an intracellular enzyme (Oldfield *et al.*, 1997) and although the 4S pathway genes are expressed as an operon, DszB is present at concentrations several-fold less in the cytoplasm compared with DszA and DszC (Gray *et al.*, 1996; Li *et al.*, 1996).

Cellular location of desulfurization activity

Several researchers, including Oldfield *et al.* (1997) and Monticello (2000), have reported that the desulfurization reactions occur within the cells. In *R. erythropolis* IGTS8, Dsz enzymes are soluble and presumably found in the cytoplasm (Gray *et al.*, 1996). While the intracellular metabolism of DBT by strain IGTS8 has been reported, there is no evidence that DBT is actively transported into the cell (Monticello, 2000), nor is there evidence for mass transfer limitations in DBT metabolism (Kilbane & Le Borgne, 2004). It has been reported that in strain IGTS8, desulfurization activity was associated with the external surface of the cells (Kayser *et al.*, 1993; Kilbane, 1991; Patel *et al.*, 1997). A substantial proportion (70%) of the total desulfurization activity expressed by this strain was found in the cell debris fraction, which contains external cell membrane and cell-wall fragments; thus, in this organism, the enzyme biocatalyst responsible for desulfurization was reported as being a component of the cell envelope (Monticello, 1996). There is no evidence that Dsz enzymes are excreted from IGTS8 cells, but the size of substrates metabolized and the ability of other bacterial species to successfully compete for sulfur liberated from organosulfur substrates by strain IGTS8 make it likely that desulfurization does not occur intracellularly, but in association with the external surface of cells (Kilbane & Le Borgne, 2004). Therefore, it is believed that the desulfurization pathway may function in association with the cell membrane, such that extracellular substrates and intracellular cofactors can both be accessed.

Desulfurization of alkylated DBTs (C_x-DBTs)

Alkylated DBTs are major sulfur components in HDS-treated oil fractions because they are highly recalcitrant to chemical catalysis. C_x-DBTs bearing alkyl substitutions adjacent to the sulfur atom are sterically hindered with regard to access to the sulfur moiety. C_x-DBTs with alkyl substitutions in positions 4 and 6 on the DBT rings are the most resistant. These refractory compounds prevent the

HDS technology from achieving acceptably low sulfur levels in heavier fuels, and they, rather than DBT, are the main target molecules to desulfurize when extremely low sulfur levels (<15 p.p.m.) are desired (Le Borgne & Quintero, 2003). Therefore, C_x-DBTs are important targets for BDS technology.

Biodesulfurization of various C_x-DBTs has been investigated by many research groups (Table 1). Desulfurization studies have revealed that all DBT-desulfurizing bacteria can desulfurize C_x-DBT via the 4S pathway. The manner of the attack on the C_x-DBTs is affected by not only the position but also the number and length of the alkyl substituents (Onaka *et al.*, 2001). In general, increasing alkylation decreases the reactivity. Konishi *et al.* (1999) reported that the degree of growth at the expense of the sulfur substrates was control < 4,6-DMDBT < 3,4,6-TMDBT < 4-MDBT < 2,8-DMDBT; various hydroxylated compounds corresponding to 2-HBP were formed. The preferential attack of desulfurizing bacteria on DBT and its derivatives has been investigated (Folsom *et al.*, 1999; Grossman *et al.*, 2001; Lee *et al.*, 1995; Mingfang *et al.*, 2003). Studies on the preference of any selected desulfurizing system are required and will be useful in predicting the system's activity on a mixture of substrates, i.e. oil fractions.

The main advantage of BDS versus HDS is the possibility to achieve higher activity on C_x-DBTs (specially 4,6-dimethyl DBT) than on DBT. The alkyl-substituted hydroxyphenylbenzene sulfonate (HPBS) compounds 3,3'-dimethyl-2-hydroxybiphenyl-2'-sulfinate and 5,5'-dimethyl-2-hydroxybiphenyl-2'-sulfinate, which are derived from the action of the two Dsz monooxygenases

on 4,6-dimethyl DBT and 2,8-dimethyl DBT, respectively, are eight times more reactive with the desulfinase than is HPBS (Gray *et al.*, 2003).

Biodesulfurization of crude oil and its fractions

Several researchers have studied the BDS process in the presence of synthetic oil fractions or pure hydrocarbons (Caro *et al.*, 2007; Finnerty, 1993; Jia *et al.*, 2006; Li *et al.*, 2007b, c; Ohshiro & Izumi, 1999; Rhee *et al.*, 1998). Clearly, the investigation of the biodesulfurization of organosulfur compounds in the presence of pure hydrocarbon provides a valuable background to the study of the biodesulfurization of crude oil fractions.

Biodesulfurization of oil fractions has also been reported (Table 2). Rhee *et al.* (1998) studied the desulfurization of MDUF (middle distillate unit feed) and LGO (light gas oil) by resting cells of *Gordona* strain CYKS1. Specific desulfurization rates of MDUF and LGO were 5.3 and 4.7 μM S (g dry cell weight)⁻¹ h⁻¹, respectively. Folsom *et al.* (1999), using resting cells of *R. erythropolis* I-19, investigated the desulfurization of C_x-DBT present in HDS-treated-middle-distillate petroleum containing the organosulfur compounds (>95%) as thiophenic compounds. The initial desulfurization rate of the oil fraction was 2.5 μM (g dry cell weight)⁻¹ min⁻¹, based on the change in total sulfur content in the oil phase. The authors concluded that reactivities were not equivalent for all organosulfur compounds. Grossman *et al.* (1999) evaluated *Rhodococcus* sp. ECRD-1 for its ability to desulfurize a middle-distillate (diesel range) fraction (232–343 °C) of Oregon Basin crude oil. Overall, about 30% of the sulfur

Table 1. Desulfurization of alkylated DBTs (C_x-DBT) by some bacterial strains

Bacterium	Sulfur substrate(s)	End product(s)	Reference(s)
<i>Rhodococcus erythropolis</i> H-2	2,8-DMDBT, 4,6-DMDBT	Corresponding hydroxylated biphenyl	Ohshiro <i>et al.</i> (1996a)
Bacterial strain RIPI-S81	3,4-Benzo-DBT	α-Hydroxy-β-phenylnaphthalene	Rashidi <i>et al.</i> (2006)
	4-MDBT	2-Hydroxy-3'-methylbiphenyl, 2-hydroxy-3-methylbiphenyl	
	4,6-DMDBT	2-Hydroxydimethylbiphenyl	Kirimura <i>et al.</i> (2001)
<i>Bacillus subtilis</i> WU-S2B	2,8-DMDBT, 4,6-DMDBT, 3,4-benzo-DBT	Corresponding hydroxylated biphenyls	
<i>Paenibacillus</i> sp. A11-2	Methyl, ethyl, dimethyl, trimethyl and propyl DBTs	Corresponding hydroxylated biphenyls	Konishi <i>et al.</i> (1997); Konishi <i>et al.</i> (1999); Onaka <i>et al.</i> (2001)
<i>Rhodococcus</i> sp. ECRD-1	4,6-DEDBT	Hydroxydimethylbiphenyl	Lee <i>et al.</i> (1995)
<i>Rhodococcus erythropolis</i> KA2-5-1	Alkylated DBTs (C ₂ -DBTs and C ₃ -DBTs)	Corresponding hydroxylated biphenyls	Kobayashi <i>et al.</i> (2000); Onaka <i>et al.</i> (2001)
<i>Rhodococcus erythropolis</i> XP	4-MDBT	2-Hydroxy-3'-methylbiphenyl, 2-hydroxy-3-methylbiphenyl	Yu <i>et al.</i> (2006)
	4,6-DMDBT	2-Hydroxy-3,3'-dimethylbiphenyl	Okada <i>et al.</i> (2002); Nomura <i>et al.</i> (2005)
	Benzenaphthothiophene	α-Hydroxy-β-phenylnaphthalene	
<i>Mycobacterium</i> sp. G3	4,6-Dibutyl DBT, 4,6-dipentyl DBT, 4,6-DMDBT, 4,6-DEDBT	C-S bond cleavage products	

Table 2. Desulfurization of crude oil fractions by some bacterial strains

Bacterium	Crude oil fraction† and its sulfur content	Total sulfur reduction (%)	References
<i>Gordona</i> * sp. CYKS1 (resting cells)	MDUF (1500 p.p.m.)	70	Rhee <i>et al.</i> (1998)
	LGO (3000 p.p.m.)	50	
<i>Gordonia</i> * sp. SYKS1 (resting cells)	HDS-untreated LGO (3000 p.p.m.)	35	Chang <i>et al.</i> (2000)
	HDS-untreated MDUF	60	
	Diluted with hexadecane (1500 p.p.m.)		
<i>Gordonia</i> * sp. SYKS1 (resting cells)	Diesel oil (250 p.p.m.)	76	Chang <i>et al.</i> (2001)
<i>Rhodococcus erythropolis</i> XP	HDS-treated diesel oil (259 p.p.m.)	94.5	Yu <i>et al.</i> (2006)
<i>Rhodococcus</i> sp. ECRD-1 (growing cells)	Middle-distillate (diesel range) fraction of Oregon Basin crude oil (20000 p.p.m.)	8.1	Grossman <i>et al.</i> (1999)
<i>Rhodococcus</i> sp. ECRD-1 (growing cells)	Catalytic cracker middle-distillate light cycle oil (LCO) (669 p.p.m.)	92	Grossman <i>et al.</i> (2001)
<i>Rhodococcus</i> sp. P32C1 (resting cells)	HDS-treated light diesel oil (303 p.p.m.)	48.5	Maghsoudi <i>et al.</i> (2001)
<i>Rhodococcus erythropolis</i> I-19 (resting cells)	HDS-treated-middle distillate (1850 p.p.m.)	67	Folsom <i>et al.</i> (1999)
<i>Sphingomonas subarctica</i> T7b (growing cells)	HDS-treated LGO (280 p.p.m.)	59	Gunam <i>et al.</i> (2006)
<i>Mycobacterium phlei</i> WU-0103 (growing cells)	12-fold-diluted straight-run LGO (1000 p.p.m.)	52	Ishii <i>et al.</i> (2005)
<i>Mycobacterium</i> sp. X7B (resting cells)	HDS-treated diesel oil (535 p.p.m.)	86	Li <i>et al.</i> (2003)
<i>Pseudomonas delafieldii</i> R-8 (resting cells)	HDS-treated diesel oil (591 p.p.m.)	90.5	Guobin <i>et al.</i> (2005)
<i>Pseudomonas delafieldii</i> R-8 (growing cells)	HDS treated diesel oil (591 p.p.m.)	47	Guobin <i>et al.</i> (2006)

*Names as used in the original papers; the genus *Gordona* was renamed *Gordonia* in 1997 (Arenskötter *et al.*, 2004).

†LGO, light gas oil; MDUF, middle distillate unit feed.

was removed, and 50% of the remaining sulfur was oxidized to a chemical state most similar to that of DBTO₂ and HBP-sultine. Biodesulfurization reduced the number of sulfur compounds present in the oil and also reduced its sulfur content by 92%. With growing cells of *Mycobacterium phlei* WU-0103, total sulfur content in 12-fold-diluted straight-run LGO was reduced from 1000 to 475 p.p.m. S at 45 °C (Ishii *et al.*, 2005). In another study, the sulfur content of straight-run diesel oil was reduced from 1807 to 741 p.p.m. by resting cells of *Nocardia globerula* R-9, at a mean rate of 5.1 mmol S kg⁻¹ h⁻¹ (Mingfang *et al.*, 2003).

Biodesulfurization of different crude oils has also been reported (Kaufman *et al.*, 1999; Li *et al.*, 2007c).

Thermophilic biodesulfurization

It is presumed that most sulfur compounds will be desulfurized by the HDS process in petroleum refining, after which the BDS process will be applied to desulfurize the more recalcitrant sulfur compounds. In a petroleum refinery process, fractional distillation and desulfurization reactions are carried out at high temperatures. To incorporate a BDS step into the petroleum refining process it is desirable that the BDS reaction also be carried out at a high temperature. Better understanding of thermophilic biodesulfurization will help to optimize the integration of BDS process into refineries.

Most of the DBT-desulfurizing micro-organisms so far reported are mesophiles. For high-temperature

desulfurization it is preferable to use a micro-organism capable of both growing and desulfurizing organic sulfur compounds at high temperatures. Several research groups have been working to isolate and characterize thermophilic desulfurizing bacteria (Bahrami *et al.*, 2001; Ishii *et al.*, 2005; Kayser *et al.*, 2002; Kirimura *et al.*, 2001; Konishi *et al.*, 1997; Li *et al.*, 2003, 2007c; Ohshiro *et al.*, 2005). Elevated temperatures provide several advantages: higher temperature decreases oil viscosity, makes molecular displacement easier, improves enzymic rates, and decreases bacterial contamination (Gray *et al.*, 2003; Soleimani *et al.*, 2007).

Improved thermostability of the desulfurization enzymes is also needed. The more rapid thermal inactivation of purified desulfurization enzymes as compared with desulfurization activity detected in whole cells requires further investigation. Through the use of mutagenesis combined with natural selection, directed evolution can be employed to obtain thermostable derivatives of the desulfurization enzymes.

Challenges for BDS

In order to make a BDS process competitive with deep HDS a five-step process is needed: (i) production of active resting cells (biocatalysts) with a high specific activity; (ii) preparation of a biphasic system containing oil fraction, aqueous phase and biocatalyst; (iii) biodesulfurization of a wide range of organic sulfur compounds at a suitable rate; (iv) separation of desulfurized oil fraction, recovery of the biocatalyst and its return to the bioreactor; and (v) efficient

wastewater treatment. Each step is affected by a number of factors. In this section the limitations and solutions will be discussed.

Biocatalyst activity improvement

The development of commercial biorefining processes will depend on significant improvements in the cheap and abundant production of highly active and stable biocatalysts adapted to the extreme conditions encountered in petroleum refining (Le Borgne & Quintero, 2003). The desulfurization rate obtained with crude oil or a petroleum product is an important measurement in determining the suitability of a biocatalyst for an industrial BDS process. The currently available biocatalysts require an increase in desulfurization rate of about 500-fold (Kilbane, 2006). Since 1990 researchers at EBC have increased the biocatalyst activity level more than 200-fold in experiments using a model oil (0.6 %, w/w, DBT dissolved in pure hexadecane). Improvements in biocatalyst activity have been achieved in several steps, including optimization of biocatalyst production conditions, increasing Dsz enzyme concentrations through genetic engineering, recognition of need for high levels of DszD enzyme, optimization of the catalyst design with all four enzymes, removal of the DszB enzyme, and optimization of media used in the bioreactor itself (Pacheco, 1999).

Biocatalyst longevity improvement

Another barrier to commercial acceptance of BDS involves the logistics of sanitary handling, shipment, storage and use of living bacterial cells within the refinery environment. The biocatalyst can regenerate enzymes destroyed or lost during the reaction process; it can reproduce itself. The EBC's original BDS process had unacceptable catalyst longevity of only 1–2 days. The next design included the production and regeneration of the biocatalyst within the BDS process, with biocatalyst longevity in the range of 200–400 h (McFarland, 1999). Naito *et al.* (2001) did experiments using immobilized *R. erythropolis* KA2-5-1 in DBT-containing *n*-tetradecane as a model oil. The cells were immobilized by entrapping them with the photocrosslinkable resin prepolymer ENT-4000. ENT-4000-immobilized cells could catalyse BDS repeatedly in this system for more than 900 h with reactivation, much longer than the longest longevity previously reported at that time of 150–192 h (Soleimani *et al.*, 2007).

Phase contact and separation

Most bacterial species are sensitive to organic solvents. The development of micro-organisms that are stable and active in the presence of non-aqueous solvents is desirable in the biocatalytic upgrading of crude oil fractions. In the BDS bioreactor, a limiting factor is the transport rate of the sulfur compounds from the oil phase to the bacterial cell membrane. Efficiency of sulfur removal is likely to be related to oil droplet size (Shennan, 1996). Therefore, access

to organic sulfur by resting cells requires the costly dispersal of the oil fraction in the aqueous phase. The effects of surfactants on bacterial desulfurization of DBT have been investigated in biphasic (oil–water) systems; biodesulfurization has been enhanced by addition of surfactants. It has been suggested that these conditions favoured more effective contact between the biocatalyst and the hydrophobic substrate (Li *et al.*, 2006). One problem, which has yet to be resolved, is whether the chemical surfactants would be toxic to the process organisms or act against the characteristic adhesion mechanisms of the bacteria to oil droplet surfaces (Shennan, 1996).

The main mass transfer resistance is in the aqueous phase, influencing DBT transport from the oil–water interface to the bacterium (Marcelis *et al.*, 2003). The hydrophobic nature of several desulfurizing bacteria, e.g. *R. erythropolis* IGTS8 (Monticello, 2000) and *Gordonia alkanivorans* RPI90A (Mohebbali *et al.*, 2007b), makes the resting cells adhere preferentially to the oil–water interface in oil–water systems. This property is advantageous, as the sulfur compounds are directly transferred from the oil into the cells, decreasing process mass transfer limitations. In some bioreactors, such as mechanically mixed reactors and electro-spray reactors, emulsions are created. In these conditions emulsion stabilization can help prolong the longevity of the emulsion created. The resting cells may stabilize oil–water emulsions (Mohebbali *et al.*, 2007b). Under these conditions the maximum surface exposure is provided and therefore the mass transfer from oil droplets to the resting cells is more rapid.

As stabilized emulsions are formed, there may be a difficulty associated with separation. Several solutions to this problem have been suggested, including: (i) avoiding the formation of a stable water-in-oil emulsion, in order to facilitate oil recovery; (ii) the use of emulsion-destabilizing chemical agents; and (iii) a cell-immobilized BDS process (Chang *et al.*, 2000; Lee *et al.*, 2005; Naito *et al.*, 2001). In order to overcome the separation problems a complementary step is needed as mentioned below.

A significant decrease occurs in the emulsion-stabilizing activity in resting cells of *G. alkanivorans* RPI90A harvested at the time of the transition from the late exponential growth phase to the stationary phase (Mohebbali *et al.*, 2007b). The reason(s) for this phenomenon requires further investigation, but it will be helpful in de-emulsifying the treated oil product (desulfurized oil fraction). One possible process rationale would be to prepare a stable emulsion which has a more efficient mass transfer and in the next step, to facilitate oil and cell recovery, to change the reactor conditions to provide stationary-phase conditions in order to break the particle-stabilized emulsions (Mohebbali *et al.*, 2007b).

Process engineering research

There are very few reports on BDS process designs and cost analysis. In order to ensure that capital and operating costs

for BDS will be lower than for HDS it is necessary to design a suitable biocatalytic process (Monticello, 2000). The cost of building a bioreactor can be reduced by changing from a mechanically agitated reactor to air-lift designs. An air-lift reactor was used at EBC to minimize energy costs (Monticello, 2000; Pacheco, 1999). However, specific details about the EBC process and the results achieved were not published (Kilbane & Le Borgne, 2004). Lee *et al.* (2005) investigated diesel oil desulfurization in a combination of air-lift/stirred-tank reactor using immobilized cells of *Gordonia nitida* CYKS1 (Soleimani *et al.*, 2007).

Process-engineering research can decrease the volumetric reaction rate (water/oil ratio). The water/oil volume ratio is among the most important technical bottlenecks in the development of petroleum biotechnological processes, and in order to reduce operational costs associated with handling, separation and disposal of water, ideally the volume ratios of water/oil should be minimized. The use of immobilized biocatalysts was considered to be a potential alternative (Chang *et al.*, 2000; Naito *et al.*, 2001).

Conclusion

It is not cost-effective to remove the organosulfur compounds present in middle and heavy distillate fossil fuels using ultradeep HDS processes. The high cost is relative and can be dependent on the extent of desulfurization. Instead, recent research has focused on the improvement of HDS catalysts and processes and also on the development of alternative technologies for upgrading the quality of fossil fuels.

In order to develop BDS as a complementary process the interdisciplinary participation of experts in biotechnology, biochemistry, refining processes and engineering will be essential. Over the last two decades several research groups have attempted to isolate and characterize bacteria capable of desulfurizing oil fractions. Further research into BDS development is required before realistic assessments in pilot-plant studies can be made. It will be difficult to be competitive with chemical desulfurization, which has also made a lot of progress recently.

For any process to be viable in the petroleum industry it must not only be capable of treating the complex mixture of chemicals that constitute petroleum but it must also treat very large volumes in a cost-effective way. The two main steps to the commercial success of BDS are (i) to continue making rapid technical progress, and (ii) to find optimum ways to integrate biotechnology into the refineries. Integrating a BDS process into a refinery is the only way to treat diesel, but this requires a substantial modification of current refinery operations. In addition, the BDS process must operate at the same speed and reliability as other refinery processes so as not to disrupt normal refining operations. Thus despite the great interest and potential of BDS, it will be challenging to develop it to a stage where it can be practically implemented in refineries.

Acknowledgements

We would like to thank the Research Institute of Petroleum Industry (RIPI), Tehran, Iran, for its financial support of our study. Many thanks also to many investigators working in the microbiology and engineering areas, particularly those working in BDS research, for discussions of petroleum biotechnology concepts.

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