Microbial Desulfurization of Diesel Oils by Selected Bacterial Strains

S. Abbad-Andaloussi[1*], M. Warzywoda[1] and F. Monot[1]

1 Institut français du pétrole, 1 et 4, avenue de Bois-Préau, 92852 Rueil-Malmaison Cedex - France
e-mail: abbad@univ-paris12.fr - michel.warzywoda@ifp.fr - frederic.monot@ifp.fr
Present address:
Laboratoire de biologie des sols et des eaux, UMR-IRD-Paris XII, 61, avenue du Général-de-Gaulle, 94010 Créteil - France

Résumé — **Désulfuration microbiologique de gazoles par des souches bactériennes sélectionnées** — En raison du durcissement progressif des normes sur la teneur en soufre des carburants automobiles, la désulfuration biologique est souvent envisagée comme une alternative possible aux procédés d'hydro-désulfuration (HDS) profonde utilisés dans l'industrie du raffinage. La première souche microbienne capable d'oxyder spécifiquement le soufre d'une molécule comme le dibenzothiophène (DBT) sans affecter son contenu carboné, *Rhodococcus erythropolis* IGTS8, a été isolée il y a plus de dix ans. La voie métabolique suivie (voie des 4S) a été élucidée et les gènes responsables caractérisés et séquencés. La présente étude vise à tirer profit de la biodiversité microbienne pour sélectionner, sur la base de critères importants du point de vue industriel, de nouvelles souches potentiellement intéressantes pour une désulfuration ultraprofonde des gazoles. Dans un premier temps, 15 souches pures capables d'utiliser le DBT comme seule source de soufre et de le convertir en 2-hydroxybiphényle (HBP) ont été isolées à partir de différents sols. Dans un second temps, 5 isolats appartenant au cluster *Rhodococcus/Gordonia*, ayant une bonne croissance et de fortes activités de désulfuration aussi bien en milieu aqueux qu'en milieu organique ont été sélectionnés. L'action des cellules non proliférantes obtenues à partir de ces souches vis-à-vis de différents types de gazole a été déterminée afin de mieux évaluer les potentialités de la biodésulfuration comme étape de finition d'une hydrodésulfuration profonde. En fait, malgré leur forte similitude taxonomique, les 5 souches ont des activités différentes sur les gazoles testés. Le taux de désulfuration dépend également du type de gazole, en particulier de sa teneur en soufre. Certains composés récalcitrants à l'HDS, comme le 4,6-diméthyl dibenzothiophène, peuvent être totalement désulfurés, mais les dibenzothiophènes fortement alkylés sont résistants à l'action des biocatalyseurs.

Abstract — *Microbial Desulfurization of Diesel Oils by Selected Bacterial Strains* — *Because of increasingly stringent regulations concerning the sulfur content of motor fuels, sulfur removal by biocatalytic means is often considered as a potential alternative to conventional deep hydro-desulfurization processes used in the refinery industry. The first microbial strain able to selectively oxidize sulfur of molecules such as dibenzothiophene (DBT) without altering its carbon content,* Rhodococcus erythropolis *IGTS8, was isolated more than ten years ago. The metabolic pathway (4S pathway) was elucidated and the genes involved characterized and sequenced. The present study aimed at exploiting microbial diversity to select new strains potentially interesting for ultradeep desulfurization of diesel oils, taking into account industrially important criteria. In a first step, 15 pure strains able to use DBT as a sole sulfur source and to convert it to 2-hydroxybiphenyl (HBP) were obtained from different soils. In a second step, 5 isolates belonging to the* Rhodococcus/Gordonia *cluster and exhibiting*

good growth characteristics and high biodesulfurization activities in both aqueous and organic media were selected. The action of resting cells from these strains towards different types of diesel oils was also determined in order to better assess the potentiality of biodesulfurization, especially as a finishing step complementary to deep hydrodesulfurization (HDS). Actually, in spite of their taxonomic similarity, the 5 strains displayed different activities towards the diesels oil tested. Biodesulfurization yield was also dependent upon the diesel oil used, especially its sulfur content. Some HDS-recalcitrant compounds such as 4,6-dimethyl dibenzothiophene, could be completely removed, but highly-alkylated dibenzothiophenes were resistant to the action of the biocatalysts.

INTRODUCTION

The combustion of sulfur-containing fuels yields sulfur oxides (SOx) which are air pollutants. Environmental agencies are imposing more and more stringent regulations standards on the sulfur content of petroleum products such as gasoline and diesel oil. For example, the sulfur content in diesel oil will probably be less than 10 or 15 ppm (w/w) in United States and Europe by 2010. The sulfur content of crude oil ranges from 0.04 to 6% (w/w) according to its origin. After distillation, dibenzothiophene (DBT) and alkylated dibenzothiophenes (C_x-DBTs) are concentrated in the middle distillate fractions and they may represent up to 70% of the sulfur present in diesel oil. Conventional chemical hydrodesulfurization (HDS) processes used in the refinery industry cannot completely remove these heterocyclic organosulfur compounds and biodesulfurization (BDS) has been considered as an alternative to ultradeep HDS [1, 2].

Several aerobic bacterial strains able to selectively oxidize the sulfur atom of DBT without degrading its carbon skeleton have been isolated [2, 3]. The final products of DBT conversion are 2-hydroxybiphenyl (HBP) and sulfite. This pathway, often named the 4S pathway, has been elucidated using the first strain found able to use DBT as a sole sulfur source, *Rhodococcus erythropolis* IGTS8 [4, 5] *(Fig. 1)*. The four genes involved have been isolated and sequenced [3, 6]. The first two enzymes, DszC and DszA, are $FMNH_2$-dependent monooxygenases catalysing the successive oxidations of DBT to DBT-sulfone and then to 2-hydroxybiphenyl-2'-sulfinic acid [7]. The $FMNH_2$ required is supplied by DszD, a NADH-flavin mononucleotide oxidoreductase. Finally, a desulfinase (DszB) is responsible for the formation of HBP from HBP-sulfinate. The multi-enzymatic nature of the 4S pathway and the requirement for cofactors make the use of pure enzymes inappropriate from a process point of view [8]. Most mesophilic strains possessing the 4S pathway belong to the *Rhodococcus* genus and the *dsz* genotype seems to be well preserved among them [9, 10].

The present study aimed at selecting new efficient strains, taking into account several properties which could be crucial for the implementation of a BDS process, such as activity and stability of resting cells in aqueous or organic media,

activity on alkylated DBTs. The biodesulfurization activity of the selected strains on different types of diesel oils was also investigated and their action on the different structural classes of DBT derivatives determined.

1 MATERIALS AND METHODS

1.1 Isolation of Strains

Soil samples from different sites (coal storage, polluted soil) were used to isolate biodesulfurizing strains. A standard minimum synthetic sulfate-free (SMM) liquid culture medium containing 25 mg·l⁻¹ DBT as a sole sulfur source and a mixture of glucose, glycerol and sodium succinate as carbon sources was used for enrichment cultures [11]. After at least four successive transfers in liquid media, isolated colonies could be obtained by streaking on solid SMM medium. The purity of the strains obtained was checked on Tryptone Soy Agar and they were cultivated on a modified liquid SMM containing only glycerol and succinate as carbon sources (MSMM). In some cases, 10 mg·l⁻¹ dimethyl-sulfoxyde (DMSO) was used instead of DBT. Strains were identified according to their phenotypic characteristics and their 16S rDNA sequence [11].

1.2 Conversion of DBT

Cells previously cultivated on MSMM and washed with 0.1 M potassium phosphate buffer (pH 7.0) were used as resting cells for conversion of DBT or diesel oil. The DBT conversion medium contained 2 g·l⁻¹ cell dry weight in 0.1 M phosphate buffer (pH 7.0) and 25 mg·l⁻¹ DBT. The cells used were frozen at –80°C (freezing did not change the activity of cells). The reaction was carried out at 30°C and was followed for 24 h. The reaction was stopped by addition of a known amount of acetonitrile. The activity of the cells was determined from the rate of DBT consumption or HBP formation during the first 2 h. Two control experiments (one without cells, one without addition of DBT) were performed under the same conditions. In the case of conversions in the presence of an organic phase (hexadecane or diesel oil), the concentrations of sulfur compounds and their metabolites were measured in the organic phase.
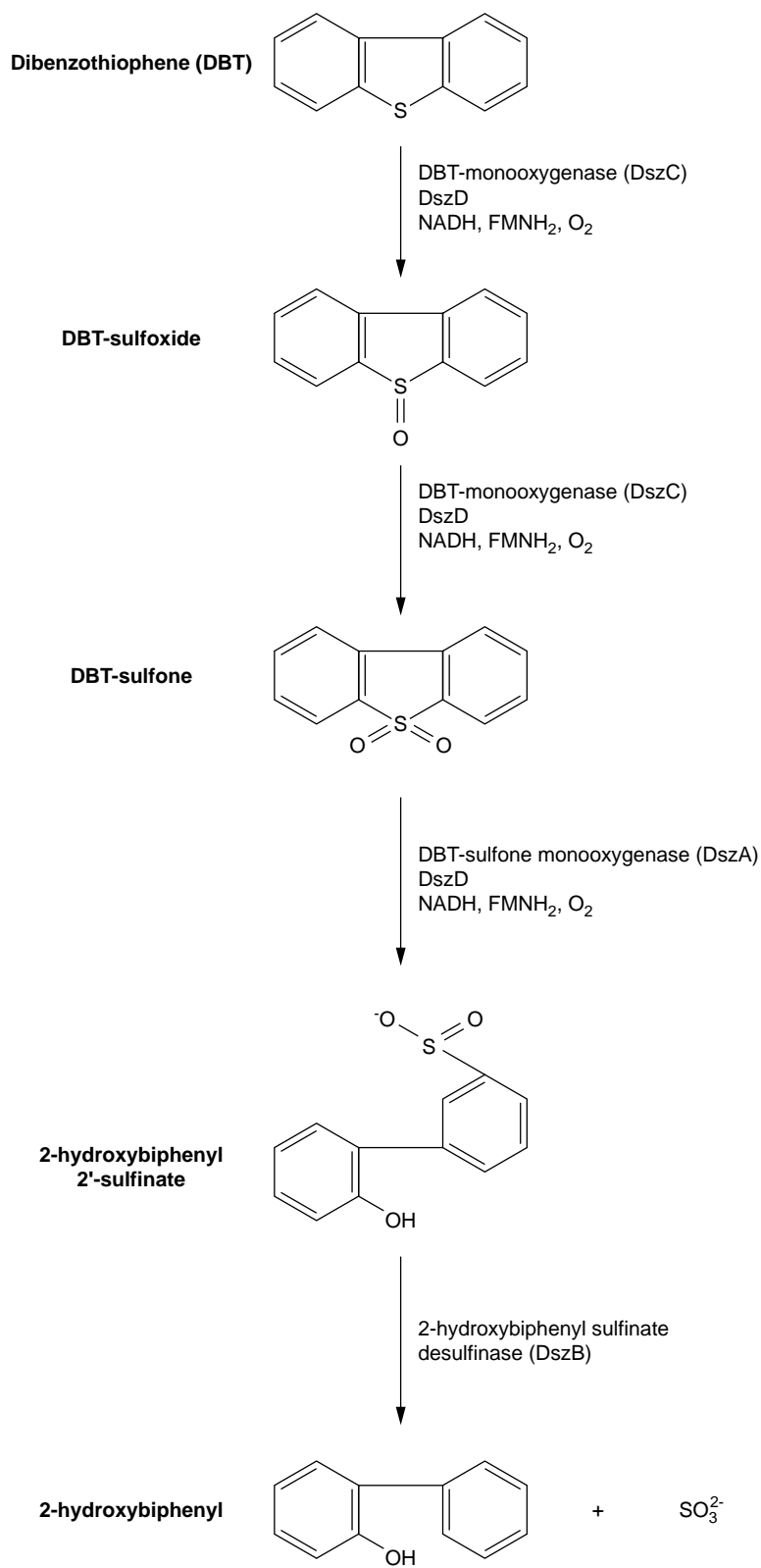
Figure 1

DBT desulfurization pathway by *Rhodococcus* IGTS8 (4S pathway).

1.3 Conversion of Diesel Oils

Biodesulfurization of diesel oils was carried out in shaken Fernbach flasks containing 200 ml 0.1 M phosphate buffer (pH 7) and 40 ml diesel oil (oil/water ratio = 0.2). The concentration of resting cells was 2 g·l^{-1} (dry weight). Incubation time and temperature were 96 h and 30°C, respectively. Abiotic controls were performed in the same conditions without biocatalyst. The sulfur content and sulfur composition of diesel oil were determined using the organic phase. Desulfurization yields were calculated taking the sulfur content of the abiotic controls as the initial value. The characteristics of the different types of diesel oils used are indicated in Table 1.

TABLE 1

Main characteristics of the diesel oils used

Diesel oil	Origin	Hydro-desulfurization	Sulfur content (mg·kg^{-1})
3990	SR[a]	No	440*
103	SR	Moderate	535
104	SR	Deep	45
101	(SR + LCO[b]) (0.5/0.5)	Moderate	660
690A	(SR + LCO) (0.5/0.5)	Deep	29
5290B	(SR + LCO) (0.5/0.5)	Deep	185

a: straight-run.
b: light cycle oil (gas-oil type fraction from catalytic cracking units).
*: original sulfur content: 1.5% (diluted to 440 mg·kg^{-1} in hexadecane).

1.4 Analyses

DBT and its degradation products were quantified by reversed phase HPLC coupled to UV detection [11]. Samples of bioconversion supernatants were mixed with acetonitrile before injection. The metabolites of DBT and of 4,6-dimethyl DBT were identified by GC/MS after extraction in dichloromethane. The total sulfur content of diesel oil was evaluated by X-ray fluorescence and the composition of the organo-sulfur compounds of diesel oil was determined by gas phase chromatography coupled to sulfur-specific chemiluminescence detection (SCD).

16S rDNA sequencing was carried out after DNA extraction and amplification by PCR using primers usually used for amplification of 16S rDNA of Procaryotes [12]. Sequencing was performed by *ESGS* (Evry, France) and sequences were compared using the BLAST 2 software *(National Center for Biotechnology Information, NCBI).*

2 RESULTS

2.1 Selection and Properties of Efficient DBT-Desulfurizing Strains

Fifteen pure strains able to use DBT as a sole sulfur source were isolated. DBT was converted to HBP in each case. The ability of these strains to maintain a DBT-desulfurizing activity after growth on dimethyl-sulfoxide (DMSO) was tested *(Table 2)*. Sulfate, which is the usual sulfur source of bacteria, was not used since it is known to repress the expression of the *dsz* genes [13]. Eight strains could be selected based on this criterion. A classical phenotypic analysis showed that they all belonged to the *Rhodococcus* genus.

The DBT-desulfurizing activity of the resting cells obtained from the different strains precultivated in identical conditions was measured in both aqueous and organic media *(Table 3)*. The decrease of activity caused by the presence of

TABLE 2

Biodesulfurization activity after growth on DMSO as a sulfur source
(+: production of 2-hydroxybiphenyl; –: no production of 2-hydroxybiphenyl)

No. sample	MK2				MK2-T3			MK3			MK5		MK7		
Strain	3	4	5	8	C1	C2	C3	C2	C3	T	C1	C2	C1	C2	C3
Activity	+	+	+	+	–	–	–	–	–	–	+	–	+	+	+

TABLE 3

Biodesulfurization activity in aqueous and organic (90% hexadecane) media (mg DBT consumed·h^{-1}·g^{-1} cell dry weight)

	MK2-3	MK2-4	MK2-5	MK2-8	MK5-C1	MK7-C1	MK7-C2	MK7-C3
Aqueous medium	3.9	2.8	3.2	2.2	2.2	3.9	1.9	2.8
Organic medium	0.89	1.2	0.7	0.6	0.9	1.7	0	1.4
Ratio of activity (organic/aqueous)	0.23	0.41	0.20	0.28	0.40	0.43	0	0.50

hexadecane was determined to select the 5 strains that were the least sensitive to the presence of an organic solvent. The ratio of the activities in the 2 types of medium was taken into account rather than the value of activity itself. The selected strains were identified by 16S rDNA sequencing as *Rhodococcus erythropolis* or *Gordonia* sp. strains *(Table 4)*. The strain MK7-C1 showed the 16S rDNA highest similarity with *R. erythropolis* IGTS8 (99.1%). The stability of the DBT-desulfurizing activity of the resting cells and their activity on 4,6-dimethyl DBT varied according to the strain used [11]. For instance, the resting cells from strain MK5-C1 were inactive on 4,6-dimethyl DBT whereas those from MK7-C1 were more active on this alkylated DBT than on DBT. These strains were deposited at the *Collection nationale de cultures de microorganismes* (*CNCM, Institut Pasteur*, France).

2.2 Desulfurization Activity of the Selected Strains on Different Types of Diesel Oil

The desulfurization yields calculated from the total sulfur content of various types of diesel oil after 96 h incubation in the presence of resting cells from the selected strains are shown in Figure 2. The results obtained using strain MK5-C1 are not shown because the desulfurization yield was always around 0.

The other strains exhibited a desulfurization activity on all types of diesel oil tested. Using strain MK7-C1 and diesel oil 690A, the total desulfurization yield was close to 50%. However, the final desulfurization yield was highly dependent on the diesel oil used. The higher was the sulfur content, the lower was the desulfurization yield. The highest yields were obtained with the diesel oil having the lowest
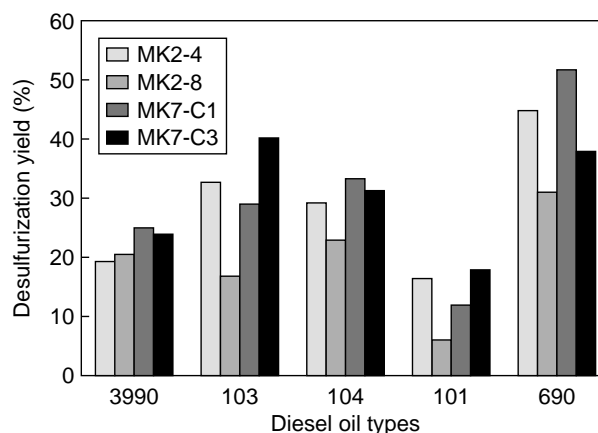

Figure 2

Action of the selected strains on the different diesel oils.

initial sulfur content (around 30 mg·kg^{-1}), although it was a mixture of straight-run middle distillate and LCO. Besides, it is noteworthy that the desulfurization activity was also dependent on the strain used. Apart from strain MK5-C1, the least active one was MK2-8. The other strains gave similar results.

2.3 Modification of the Composition in Organo-Sulfur Compounds of the Diesel Oils

The detailed analysis of the sulfur components was carried out on some samples to better investigate the desulfurizing action of the biocatalysts tested, especially on alkylated dibenzothiophenes (C_x-DBTs). Some of these compounds, *i.e.* DBT, C_1-DBT and C_2-DBTs, could be identified.

TABLE 4

Identification of the 5 strains selected

Strain (N° CNCM)	Identification by		
	Classical microbiological analyses	Cell wall and total protein analysis	16S rDNA sequence with highest similarity
MK2-4 (I-2204)	*Rhodococcus equi*	*Rhodococcus erythropolis*	*Rhodococcus* sp. or *R. erythropolis* (100%)
MK2-8 (I-2205)	*Rhodococcus equi*	*Rhodococcus erythropolis*	*Rhodococcus* sp. or *R. erythropolis* (100%)
MK5-C1 (I-2206)	*Rhodococcus equi*	*Rhodococcus rhodnii/Gordonia*	*Gordonia* sp. (97.0%)
MK7-C1 (I-2207)	*Rhodococcus* sp.	*Rhodococcus erythropolis*	*Rhodococcus* sp. or *R. erythropolis* (99.99%)
MK7-C3 (I-2208)	*Rhodococcus* sp.	*Rhodococcus erythropolis*	*Rhodococcus* sp. or *R. erythropolis* (99.99%)

The middle distillate S3990 diluted in hexadecane was the sole fraction tested containing benzothiophenes (BTs). The action of MK2-4 and MK7-C1 which were the least active and the most active strains, respectively, is shown in Table 5. In addition to dibenzothiophenes, benzothiophenes were attacked by both biocatalysts. However, the desulfurization yields remained limited.

TABLE 5

Desulfurization yields (ΔS) of the families
of organo-sulfur compounds after the action of different biocatalysts
(diesel oil S3990)

Sulfur compound	Abiotic Sulfur concentration ($mg \cdot kg^{-1}$)[a]	MK2-4 ΔS (%)[b]	MK7-C1 ΔS (%)[b]
C_1 BTs	4	36.6	50.0
C_2 BTs	12	38.9	42.4
C_3 BTs	30	43.6	48.3
C_4 BTs	66	23.5	30.5
BTs (total)	**112**	**31.0**	**37.3**
DBT	8	18.8	25.9
Total C_1 DBTs	**76**	**10.8**	**18.2**
Total C_2 DBTs	**59**	**24.7**	**30.6**
Total C_3 DBTs	**183**	**11.9**	**18.3**

a: sulfur concentration of each family of compounds.
b: sulfur removal with respect to the abiotic control.

The desulfurization capacities of the 4 strains towards the different sulfur species of a moderately-hydrodesulfurized diesel oil (sample 103) were compared *(Table 6 and Fig. 3)*. This diesel oil sample contained around 500 $mg \cdot kg^{-1}$ sulfur. Sulfur constituents were only alkylated DBTs. Significant differences between strains could be observed, especially when considering the lower action of strain MK2-8 on 4,6-dimethyl DBT, 4-methyl-6-ethyl DBT and 2,4,6-methyl DBT. C_2-DBTs were highly attacked by the biocacalysts. The most recalcitrant compounds were probably C_x-DBTs with an alkylation degree greater than 3.
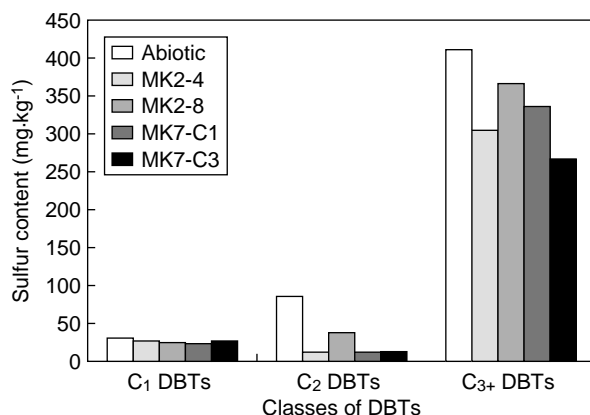

Figure 3

Alkylated dibenzothiophene content change after treatment of a moderately-hydrodesulfurized diesel oil (sample 103). C_3+ DBTs = C_3 and higher alkylated C_x-DBTs.

The action of strains MK2-4 and MK7-C1 towards diesel oil samples containing very low concentrations of sulfur was investigated using diesel oil 104 which was a deeply-hydrodesulfurized straight-run middle distillate and diesel oil 690A which contained 50% deeply-hydrodesulfurized middle distillate and 50% LCO. Results are presented in Figure 4 and Table 7 for sample 104 and in Figure 5 and Table 8 for sample 690A. The action of the 2 strains was similar in both cases. The desulfurization yield was higher with the mixture SR-LCO (690A). C_2-DBTs were completely removed. For instance, 4,6-dimethyl DBT, which was the predominant sulfur compound present after ultra-deep hydrodesulfurization, was not detected after the action of the biocatalysts. Concerning diesel oil 690A, other compounds such as 4-methyl DBT, 4-ethyl DBT, 1,3- 3,4- and 3,6-dimethyl DBT were also completely desulfurized. Some C_3-DBTs (4-methyl-6-ethyl DBT and 2,4,6-methyl DBT) were removed to more than 90%. However, the activity of the biocatalysts was lower when the degree of

TABLE 6

Removal (ΔS) of the predominant sulfur compounds
of a moderately-hydrodesulfurized diesel oil (sample 103)

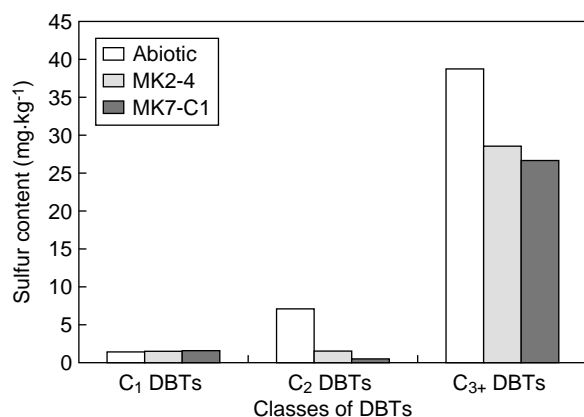| Sulfur compound	Initial sulfur content ($mg \cdot kg^{-1}$)	Sulfur removal (ΔS) by strain (%)			
		MK2-4 (ΔS)	MK2-8 (ΔS)	MK7-C1 (ΔS)	MK7-C3 (ΔS)
4,6-Me DBT	55.4	97	66	97	97
4-Me-6-Et DBT	35.6	64	22	66	78
2,4,6-Me DBT	35.5	87	28	82	100
C3 DBT (unknown 1)	27.5	86	33	85	90
C3 DBT (unknown 2)	35.8	55	26	53	65

Figure 4

Alkylated dibenzothiophene content change after treatment of a deeply-hydrodesulfurized straight-run diesel oil (sample 104). C_3+ DBTs = C_3 and higher alkylated C_x-DBTs.
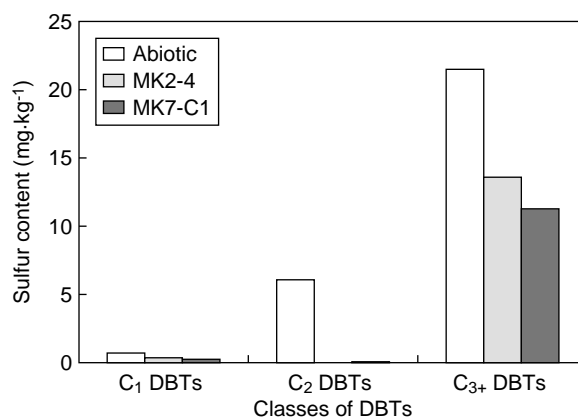

Figure 5

Alkylated dibenzothiophenes content change after treatment of a mixture of deeply-hydrodesulfurized straight-run and LCO diesel oils (sample 690A). C_3+ DBTs = C_3 and higher alkylated C_x-DBTs.

alkylation of DBT became higher than 3. It is also worth mentioning that strain MK7-C1 was more efficient than strain MK2-4.

TABLE 7

Removal (ΔS) of the predominant sulfur compounds of a deeply-hydrodesulfurized straight-run diesel oil (sample 104)

Sulfur compound	Initial sulfur content ($mg \cdot kg^{-1}$)	Sulfur removal (ΔS) by strain (%)	
		MK2-4 (ΔS)	MK7-C1 (ΔS)
4,6-Me DBT	6.3	92	100
4-Me-6-Et DBT	4.6	86	89
2,4,6-Me DBT	2.1	65	83
C3 DBT (unknown 1)	3.6	92	96
C3 DBT (unknown 2)	3.5	39	49

TABLE 8

Removal (ΔS) of the predominant sulfur compounds of a mixture of deeply-hydrodesulfurized straight-run and LCO diesel oils (sample 690A)

Sulfur compound	Initial sulfur content ($mg \cdot kg^{-1}$)	Sulfur removal (ΔS) by strain (%)	
		MK2-4 (ΔS)	MK7-C1 (ΔS)
4,6-Me DBT	4.9	100	99
4-Me-6-Et DBT	2.5	83	90
2,4,6-Me DBT	2.6	91	95
C3 DBT (unknown 1)	1.8	92	93
C3 DBT (unknown 2)	2.2	39	48

2.4 Kinetics of Diesel Oil Desulfurization

The time-course of desulfurization of diesel oil was studied using a mixture of deeply-hydrodesulfurized straight-run diesel oil and LCO and resting cells from strain MK7-C1. The diesel oil sample contained only C_x-DBTs as sulfur compounds. C_3-DBTs could be separated from more alkylated DBTs by GC. The results are presented on Figure 6. The total sulfur removal after 48 h was 76%. Sulfur from C_1- and C_2-DBTs was completely removed after 10 h and 70% of the C_3-DBTs could be desulfurized. C_x-DBTs with a higher alkylation degree were not attacked. The initial sulfur removal rate was equal to 1.86 $mg \cdot g^{-1} \cdot h^{-1}$.
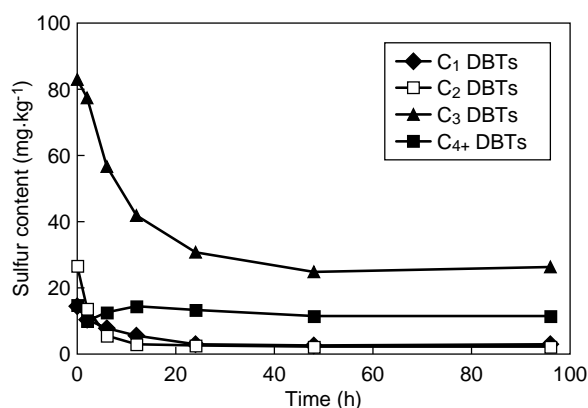

Figure 6

Time-course of the concentrations of alkyl-DBTs of a mixture of deeply-hydrodesulfurized and LCO diesel oils (sample 5290B) by cells of strain MK7-C1. Initial sulfur content: 185 $mg \cdot kg^{-1}$; cell concentration: 2$g \cdot l^{-1}$; oil/water ratio: 0.23; temperature: 30°C. C_4+ DBTs = C_4 and higher alkylated C_x-DBTs.

CONCLUSIONS

Some of the strains selected in the present work were able to efficiently desulfurize DBT and some alkylated DBTs. They all belonged to the *Rhodococcus* genus like many other desulfurizing strains [9, 10]. Despite their phylogenetic similarity, they exhibited different properties. It was shown that these discrepancies could not be ascribed to a difference in the sequence of the *dsz* genes and thereby to the structure of the desulfurization enzymes [11]. The phenomena involved in biodesulfurization are complex. In addition to the intracellular desulfurization itself, they include transport of hydrophobic substrates (DBTs) and inhibitory products (biphenyls) and supply of cofactors such as NADH [2]. These phenomena remain poorly documented. From an applied point of view, some other aspects which are crucial for the implementation of a biodesulfurization process in a refining scheme, *e.g.* stability of resting cells, activity in the presence of high levels of organic phase, have been studied here using several strains. These criteria were included in the selection of the best strains.

The present study also shows that some strains are highly active on diesel oil. However, the biodesulfurization activity and extent were dependent on both the type of diesel oil and on the strain used. For instance, strain MK5-C1, which is quite stable and active on DBT in organic media, was not able to desulfurize hydrodesulfurized middle distillate. This result can be explained by the narrow substrate specificity of this biocatalyst which could not oxidize 4,6-dimethyl DBT, a major constituent of hydrodesulfurized diesel oils. Previous studies devoted to the biodesulfurization of petroleum distillates did not present any comparison between strains and types of diesel oil [14-16]. We observed that biodesulfurization was more efficient on diesel oils with a low sulfur content and that biocatalysts were also active on LCO. This result suggests that inhibitory or toxic effects by certain sulfur compounds may occur. A maximum biodesulfurization yield of 76% was obtained and it was possible to decrease the sulfur content of a previously deeply-hydrodesulfurized diesel oil down to 14 mg·kg^{-1}. In addition, the sulfur removal rate was high. Furthermore, some HDS-recalcitrant compounds such as 4,6-dimethyl DBT could be totally desulfurized. The desulfurization kinetics were affected by the number and/or lengths of alkyl groups attached to the ring structure of DBT. From a technical point of view, these results show that biodesulfurization could be quite suitable as a finishing step complementary to deep HDS because of its high selectivity. However, the action of the biocatalysts on the highly-alkylated DBTs remained low and these compounds would also have to be removed to decrease the sulfur content of diesel oil to levels as low as 10 mg·kg^{-1}. That means that it would be necessary now to find biocatalysts that are active towards these target compounds. New strategies involving selection of new strains either by classical microbiological techniques or by directed molecular evolution need to be combined to construct biocatalysts possessing a larger substrate specificity [17].

ACKNOWLEDGEMENTS

REFERENCES

1 McFarland, B.L. (1999) Biodesulfurization. *Curr. Op. Microbiol.*, **2**, 257-264.

2 Monticello, D.J. (2000) Biodesulfurization and the Upgrading of Petroleum Distillates. *Curr. Op. Biotechnol.*, **11**, 540-546.

3 Ohshiro, T. and Izumi, Y. (1999) Microbial Desulfurization of Organic Sulfur Compounds in Petroleum. *Biosci. Biotechnol. Biochem.*, **63**, 1-9.

4 Kayser, K.J., Bielaga-Jones, B.A., Jackowski, K., Odusan, O. and Kilbane, J.J. (1993) Utilization of Organosulphur Compounds by Axenic and Mixed Cultures of *Rhodococcus rhodochrous* IGTS8. *J. Gen. Microbiol.*, **139**, 3123-3129.

5 Oldfield, C., Pogrebinsky, O., Simmonds, J., Olson, E.S. and Kulpa, C.F. (1997) Elucidation of the Metabolic Pathway for Dibenzothiophene Desulphurization by *Rhodococcus* sp. Strain IGTS8 (ATCC 53968). *Microbiology*, **143**, 2961-2973.

6 Denome, S.A., Oldfield, C., Nash, L.J. and Young, K.D. (1994) Characterization of the Desulfurization Genes from *Rhodococcus* sp. strain IGTS8. *J. Bacteriol.*, **176**, 6707-6716.

7 Gray, K.A., Pogrebinsky, O.S., Mrachko, G.T., Xi, L., Monticello, D.J. and Squires, C.H. (1996) Molecular Mechanisms of Biocatalytic Desulfurization of Fossil Fuels. *Nature Biotechnol.*, **14**, 1705-1709.

8 Monticello, D.J. (1998) Riding the Fossil Fuel Bio-desulfurization wave. *Chemtech*, **28**, 38-45.

9 Denis-Larose, C., Labbé, D., Bergeron, H., Jones, A.M., Greer, C.W., Al-Hawari, J., Grossman, M.J., Sankey, B.M. and Lau, P.C.K. (1997) Conservation of Plasmid-Encoded Dibenzothiophene Desulfurization Genes in Several *Rhodococci*. *Appl. Environ. Microbiol.*, **63**, 2915-2919.

10 Duarte, G.F., Rosado, A.S., Seldin, L., de Araujo, W. and van Elsas, J.D. (2001) Analysis of Bacterial Community Structure in Sulfurous-Oil-Containing Soils and Detection of Species Carrying Dibenzothiophene Desulfurization (*dsz*) genes. *Appl. Environ. Microbiol.*, **67**, 1052-1062.

11 Abbad-Andaloussi, S., Lagnel, C., Warzywoda, M. and Monot, F. (2003) Multi-Criteria Comparison of Resting Cell Activities of Bacterial Strains Selected for Biodesulfurization of Petroleum Compounds. *Enzyme Microb. Technol.*, **32**, 446-454.

12 Weisburg, W.G., Barns, S.M., Pelletier, D.A. and Lane, D.J. (1991) 16S Ribosomal DNA Amplification for Phylogenetic Study. *J. Bacteriol.*, **173**, 697-703.

13 Li, M.Z., Squires, C.H., Monticello, D.J. and Childs, J.D. (1996) Genetic Analysis of the *dsz* Promoter and Associated Regulatory Regions of *Rhodococcus erythropolis* IGTS8. *J. Bacteriol.*, **178**, 6409-6418.

14 Folsom, B.R., Schieche, D.R., Digrazia, P.M., Werner, J. and Palmer, S. (1999) Microbial Desulfurization of Alkylated Dibenzothiophenes from a Hydrodesulfurized Middle

Distillate by *Rhodococcus erythropolis* I-19. *Appl. Environ. Microbiol.*, **65**, 4967-4972.

15 Grossman, M.J., Lee, M.K., Prince, R.C., Minak-Bernero, V., George, G.N. and Pickering, I.J. (2001) Deep Desulfurization of Extensively Hydrodesulfurized Middle Distillate Oil by *Rhodococcus* sp. Strain ECRD-1. *Appl. Environ. Microbiol.*, **67**, 1949-1952.

16 Kobayashi, M., Horiuchi, K., Yoshikawa, O., Hirasawa, K., Ishii, Y., Fujino, K., Sugiyama, H. and Maruhashi, K. (2001) Kinetic Analysis of Microbial Desulfurization of Model and Light Gas Oils Containing Multiple Alkyl Dibenzo-thiophenes. *Biosci. Biotechnol. Biochem.*, **65**, 298-304.

17 Coco, W.M., Levinson, W.E., Crist, M.J., Hektor, H.J., Darzins, A., Pienkos, P.T., Squires, C.H. and Monticello, D.J. (2001) DNA Shuffling Method for Generating Highly Recombined Genes and Evolved Enzymes. *Nature Biotechnol.*, **19**, 354-359.