Choosing the best platform for the biotransformation of hydrophobic molecules

Philip T. Pienkos

Energy BioSystems Corp., 4200 Research Forest Dr., The Woodlands, TX 77381, USA

ABSTRACT

A process for the biological desulfurization of petroleum is being developed by Energy BioSystems Corporation. This process is based upon the metabolic capability of *Rhodococcus erythropolis* strain IGTS8 to transform hydrophobic organosulfur compounds such as dibenzothiophene to 2-hydroxybiphenyl and sulfite. The genes necessary for desulfurization have been cloned from IGTS8 and expressed in a wide range of bacterial species. A careful evaluation of process demands led to the identification of critical biocatalyst characteristics such as growth rate, metabolic activity, solvent tolerance, and affinity for hydrophobic substrates. These were used for the development of a battery of tests used for the classification of potential diesel desulfurization biocatalysts. In measures of growth rate, expression level, and model system specific activity, recombinant strains of *Pseudomonas* were superior to *Rhodococcus*. In the deep desulfurization of real diesel fuel, however, *Pseudomonas* was not as effective as *Rhodococcus*.

METHODS

Bacterial Strains

R. erythropolis strain RA-18 is a variant of strain IGTS8 (8) with a tandem duplication of the *dsz*ABC cluster. It was constructed by transformation with a suicide plasmid with selection for chloramphenicol resistance.

Various Gram negative isolates to be evaluated as hosts were obtained from culture collections around the world. They were made Dsz^+ by transformation or triparental mating using plasmids based on the broad host range vector RS1010 (Darzins, A., L. Xi, K. K. Zscheck, and C. H. Squires, Expression of the desulfurization (dsz) genes from *Rhodococcus* erythropolis IGTS8 in heterologous Bacterial Hosts, 97TH General Meeting, American Society for Microbiology, Palm Beach, FL, May 4-8, 1997).

Growth of R. erythropolis *strain RA-18*

RA-18 was grown in mineral salts medium (BSM2) containing DMSO as sulfur source, NH_rNO_3 as nitrogen source, and succinate, glycerol, and ethanol as carbon and energy source (11). Cultures were grown for ~ 72 at 30^{o}C with aeration and agitation. pH was controlled with NaOH and H_3PO_4. Cells were harvested via centrifugation.

Growth of Recombinant Strains

Recombinant strains were grown in glucose/ammonia mineral salts medium containing tetracycline in a fed batch process with pH controlled with NH_4OH and HCl. Cultures were

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generally grown for ~40 hours at 30°C to target OD's of ~50, with adjustments of agitation and aeration to avoid oxygen limitation. Sodium salicylate was added to induce expression of the dsz genes and cultures were harvested via centrifugation from 2 to 5 hours post-induction.

DBT Desulfurization Specific Activity Screen

Two phase desulfurization were carried out in 250 ml baffle flasks using 5 mg dry cell weight of cell paste resuspended with 30 ml 156 mM of phosphate buffer containing 1% glucose. After the cell suspension was well mixed in a 30°C shaking incubator, 10 ml of 25 mM DBT in hexadecane stock was added, and each flask was incubated for 1 hour at 30°C with shaking. Emulsions were then separated by centrifugation, the oil phase was analyzed for DBT and HBP via gas chromatography, and the aqueous phase was analyzed for HPBS via liquid chromatography. A desulfurization unit is defined as 1 μmole product per min per gram dry cell weight.

DBT Desulfurization Extent Screen

The extent assay was identical to the specific activity assay except that 500 mg dry cell weight of cell paste was used and the assay was carried out for 24 hours.

Diesel Fuel Desulfurization Extent Screen

The diesel fuel extent assay was identical to the DBT extent assay except that diesel oil containing 1850 ppm sulfur obtained from Total Raffinage was used in place of DBT in hexadecane. After 24 hours, the oil phase was recovered and analyzed for total sulfur via energy dispersive x-ray fluorescence to determine the extent of desulfurization.

Analytical Methods

Analysis of 2-(2-hydroxyphenyl)benzenesulfonate (HPBS) was performed using ion-pair reverse-phase HPLC (phenyl column 4.6 mm i.d. x 100 mm, tetraheptylammonium bromide as the ion pair reagent) with UV detection at 235nm. Analysis of dibenzothiophene (DBT) and hydroxybiphenyl (HBP) was performed using GC (DB-5 column 0.53 mm i.d x 15 m) with an FID. Diesel oil samples were analyzed by gas chromatography with a DB-5 column 0.53 mm i.d. x 15 m using a sulfur chemiluminescence detector and for total sulfur was performed using an energy dispersive x-ray fluorescence (EDXRF) instrument.

Introduction

Polyaromatic sulfur containing hydrocarbons (PASH's) are prevalent in fossil fuels. Combustion of these fuels leads to the production of oxidized sulfur species such as SO_2, a major environmental concern because of their contribution to acid rain (12). Environmental agencies around the world have responded to this problem by enacting more and more stringent regulations, demanding refiners to produce petroleum products such as diesel fuel and gasoline with lower sulfur levels. At the same time, the availability of low sulfur crude oils is declining, forcing refiners to use higher sulfur crudes as feedstock (1). The traditional technology used to remove sulfur from petroleum streams is a reductive desulfurization process known as hydrotreating, but this process is reaching the limits of its cost and technical effectiveness, and refiners are beginning to look increasingly at novel technologies to achieve the low levels of sulfur mandated by law.

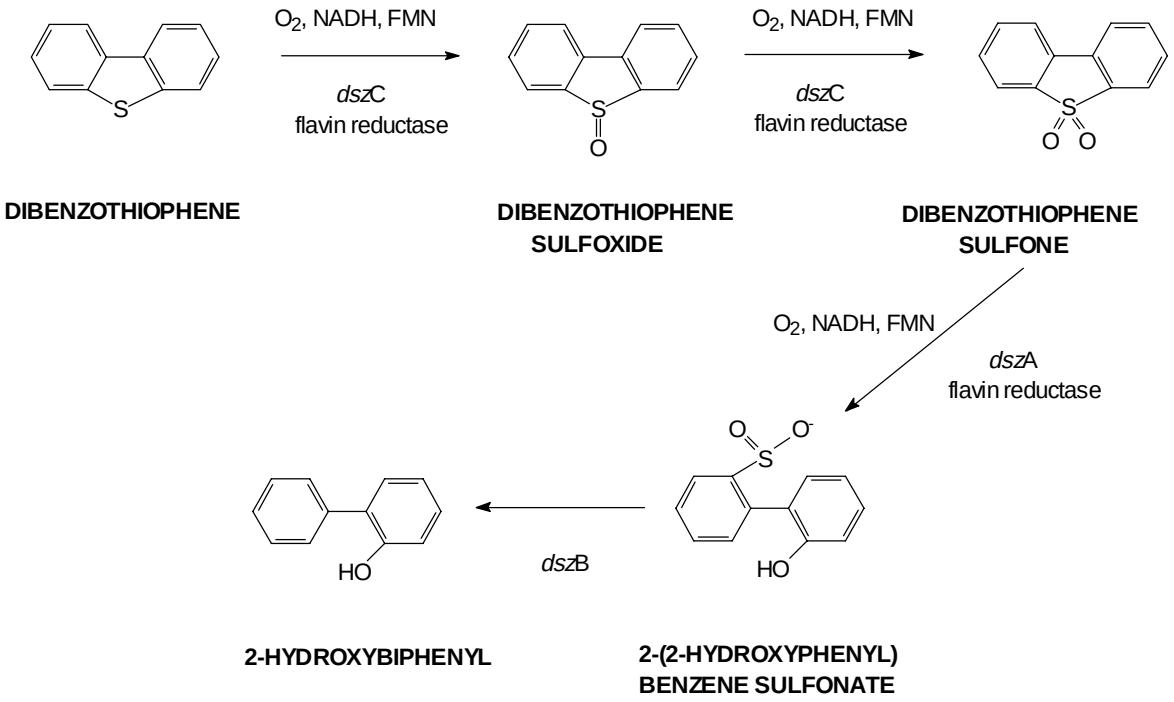

Figure 1. Pathway of Biological Desulfurization of Dibenzothiophene. DBT is converted to a sulfoxide and then a sulfone through the action of the DBT monooxygenase encoded by the gene *dsz*C. $DBTO_2$ is converted to 2-(2-hydroxyphenyl)benzenesulfonate through the action of $DBTO_2$ monooxygenase encoded by the gene *dsz*A. Both monooxygenases require $FMNH_2$ for activity which is supplied via a NAD(P)H-flavin oxidoreductase which transfers electrons from NAD(P)H to FMN. The final step in desulfurization involves a desulfinase encoded by the *dsz*B gene which cleaves the second carbon- sulfur bond, releasing sulfite and 2-hydroxybiphenyl.

One such novel technology is being developed by Energy BioSystems Corporation (EBC). This technology exploits a metabolic pathway first observed in *Rhodococcus erythropolis* strain IGTS8 in which the sulfur atom in hydrophobic molecules such as dibenzothiophene (DBT) is attacked specifically allowing for the release of sulfur with no degradation of the aromatic ring (9). The genetics (4,5) and biochemistry (7,16) of the IGTS8 desulfurization pathway have been elucidated, and are summarized in Figure 1. DBT is converted to a sulfoxide (DBTO) and then a sulfone ($DBTO_2$) through the action of the DBT monooxygenase (DBT MO) encoded by the gene *dsz*C. The first of the carbon-sulfur bonds is broken as $DBTO_2$ is converted to 2-(2-hydroxyphenyl)benzenesulfonate (HPBS) through the action of $DBTO_2$ monooxygenase (DBTO2 MO) encoded by the gene *dsz*A. Both monooxygenases require $FMNH_2$ for activity. This is supplied *in vivo* by the action of a NAD(P)H-flavin oxidoreductase which transfers electrons from NAD(P)H to FMN. The final step in desulfurization involves a desulfinase encoded by the *dsz*B gene which cleaves the second carbon-sulfur bond, releasing sulfite (which is abiotically oxidized to sulfate) and 2-hydroxybiphenyl (HBP).

The biodesulfurization process exploits this pathway by using live bacterial cells as biocatalyst in a slurry of water and a petroleum stream containing a mixture PASH's including DBT and alkyl-substituted DBT's. The sulfite and sulfate produced can be partially assimilated into biomass, but at the rates needed for a commercial process, it will

be accumulated in the aqueous phase while the HBP and alkyl-substituted HBP's are extracted back into the petroleum stream.

In order for biodesulfurization to be economically viable, it is critical to minimize the cost contribution that biocatalyst adds to the overall process. This paper outlines some of the efforts at EBC to improve the economics of biodesulfurization by evaluating recombinant strains carrying the *dsz* genes for their biocatalytic performance.

Results

Recently, developments in the understanding of the biochemistry of desulfurization led to the identification of the role of NAD(P)H-flavin oxidoreductase (sometimes referred to as "fourth factor" and "*dsz*D") in the manifestation of monooxygenase activities (16). The actual gene used by IGTS8 to code for NAD(P)H-flavin oxidoreductase was not yet in hand, but it had been shown that other NAD(P)H-flavin oxidoreductases could support DBT MO and DBTO2 MO activity *in vitro*. For our cloning efforts we employed the *fre* gene which was isolated from *Vibrio fischerii* (18).

Using the broad host-range vector RSF1010, we constructed plasmid pEX1087 which contained all of the genes necessary for desulfurization (*dsz*ABC from *R. erythropolis* and *fre* from *V. fischerii*). These genes were under the control of the *nah*R gene and expression could be induced by addition of salicylate. *Pseudomonas* strains were considered to be the ideal hosts to test because of rapid growth rate, availability of genetic techniques, metabolic diversity, and well-documented ability to carry out biotransformations in two phase systems. *P. putida* strain KT2440 was the first strain to receive pEX1087, and it was immediately clear that the resulting recombinant had higher desulfurization activity than anything seen previously in *Rhodococcus*, converting DBT at rates of 10-15 μmole/min/gdcw compared to the average activity in RA-18 of approximately 0.8 μmole/min/gdcw.

Poor expression of dszB in P. putida KT2440 resulted in accumulation of HPBS as the major product with only about 10% of the product appearing as HBP. Attempts were made to improve the level of desulfinase activity, but at the same time we realized that the conversion of DBT to HPBS was an acceptable process option because HPBS, unlike the hydrophobic DBT, is highly water soluble. The extraction of HPBS into the water phase is thus a different solution to the primary goal of biodesulfurization--the removal of sulfur from oil. This concept was extended by the construction of plasmid pEX1079, a derivative of RSF1010 carrying *dsz*A, *dsz*C and *fre* but not the *dsz*B gene.

A strain evaluation program was initiated and dozens of Gram negative strains were tested for the ability to convert DBT to HPBS as well as the ability to desulfurize diesel. The best strain tested was *P. fluorescens* NCIB11764 (pEX1079). This strain, not only grew faster and to higher cell density than RA-18 (Figure 2), but was also a superior desulfurization biocatalyst (Figures 3-5). In experiments carried out in batch stirred reactors, it was clear that *P. fluorescens* had a higher initial rate and longer extent of desulfurization than RA-18 in both the model system with DBT in hexadecane (Figure 6) and in middle distillate (Figure 7). Another apparent superiority of *P. fluorescens* was observed in its inability to form stable emulsions. The rapid separation of the phases is an important element of the processing cost of biodesulfurization. Samples of cells, oil, and water removed from biodesulfurization reactors separate into individual components quickly

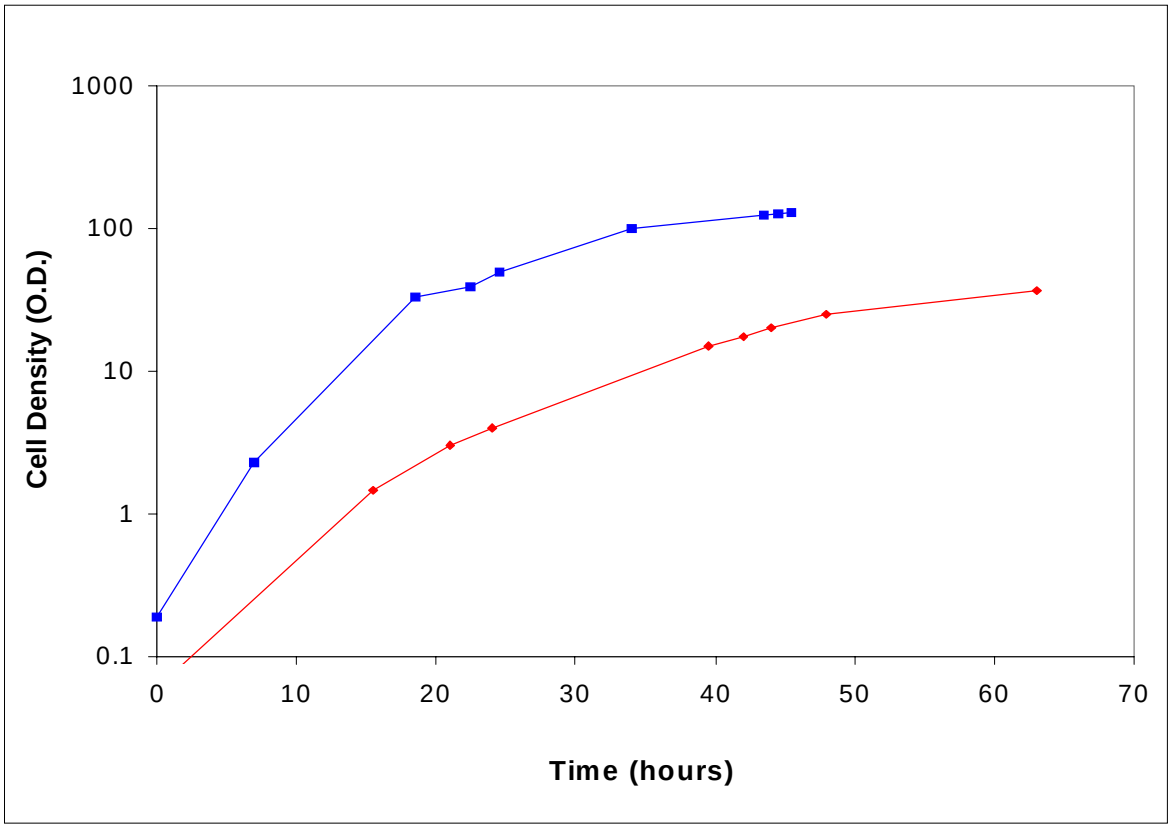

Figure 2. Fed-batch Fermentation of Biodesulfurization Biocatalysts. *R. erythropolis* strain RA-18 (red diamonds) was grown in minimal medium with a mixture of succinate, glycerol, and ethanol as carbon source (11). *P. fluorescens* (blue squares) was grown in minimal medium with glucose as sole carbon source. Cell density was monitored spectrophotometrically at 600 nm.

via gravity separation when *P. fluorescens* is used, but not when RA-18 was used (data not shown).

When more stringent sulfur regulations for diesel went into effect in Europe refiners were forced to carry out hydrodesulfurization at higher temperatures and pressures to achieve deeper desulfurization. *P. fluorescens* was tested with lower sulfur oil (650 ppm S in contrast to the 1850 ppm S used in the experiment captured in Figure 7), and we observed that *P. fluorescens* had virtually no activity with this oil, whereas RA-18 was able to reduce the sulfur in this oil down to approximately 200 ppm S within the same time frame. Oils recovered from these experiments was analyzed by gas chromatography with a sulfur chemiluminescence detector to give profiles of the PASH's remaining. It is clear from Figure 8 that *P. fluorescens* was only able to transform PASH's with short retention times (corresponding mainly to DBT's with low degrees of alkylation) whereas RA-18 was capable of transforming PASH's with a wide range of retention times and higher levels of alkylation. Further investigation demonstrated that the various Gram negative strains, unlike RA-18, had a low affinity for DBT in the oil phase, and that volumetric productivity could be improved by increasing the surface area of the oil droplets in the reactor. In addition, higher molecular weight compounds such a benzonaphthothiophene, which were reasonable desulfurization substrates for RA-18 and other desulfurizing strains of Rhodococcus (14) could not be transformed by recombinant *P. fluorescens* (Schneider, J.

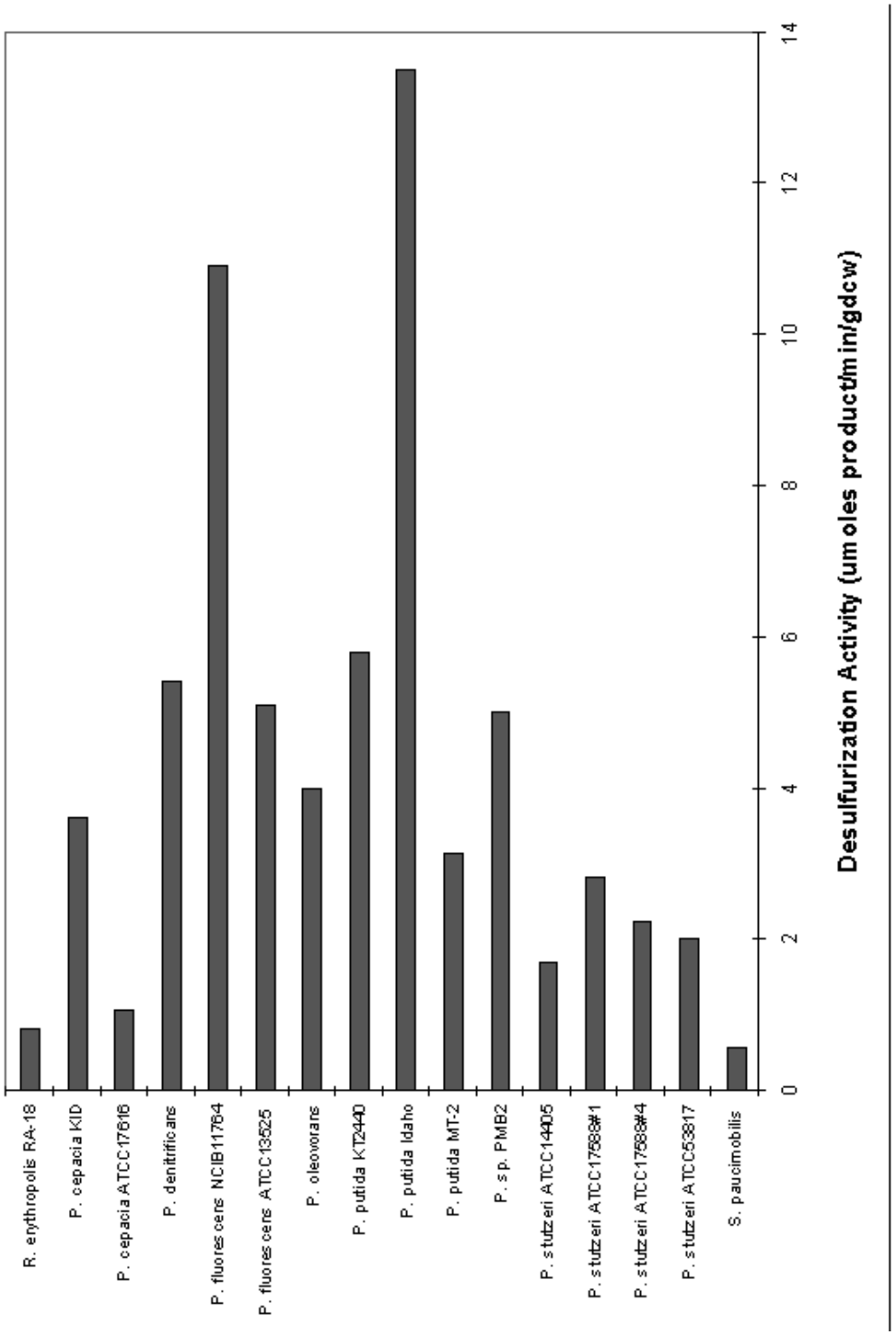

Figure 3. DBT Desulfurization Specific Activity Screen. Cells were grown in fed-batch fermentation cultures and harvested by centrifugation. Two phase desulfurization were carried out in 250 ml baffle flasks using 5 mg dry cell weight of cell paste resuspended with 30 ml 156 mM of phosphate 1 buffer containing % glucose. Cells were preincubated with shaking at 30°C for 30 minutes and then given 10 ml of 25 mM DBT in hexadecane. Each flask was incubated with substrate for 60 minutes at 30°C with shaking. The phases were separated, and the aqueous phase was analyzed for HPBS via liquid chromatography.

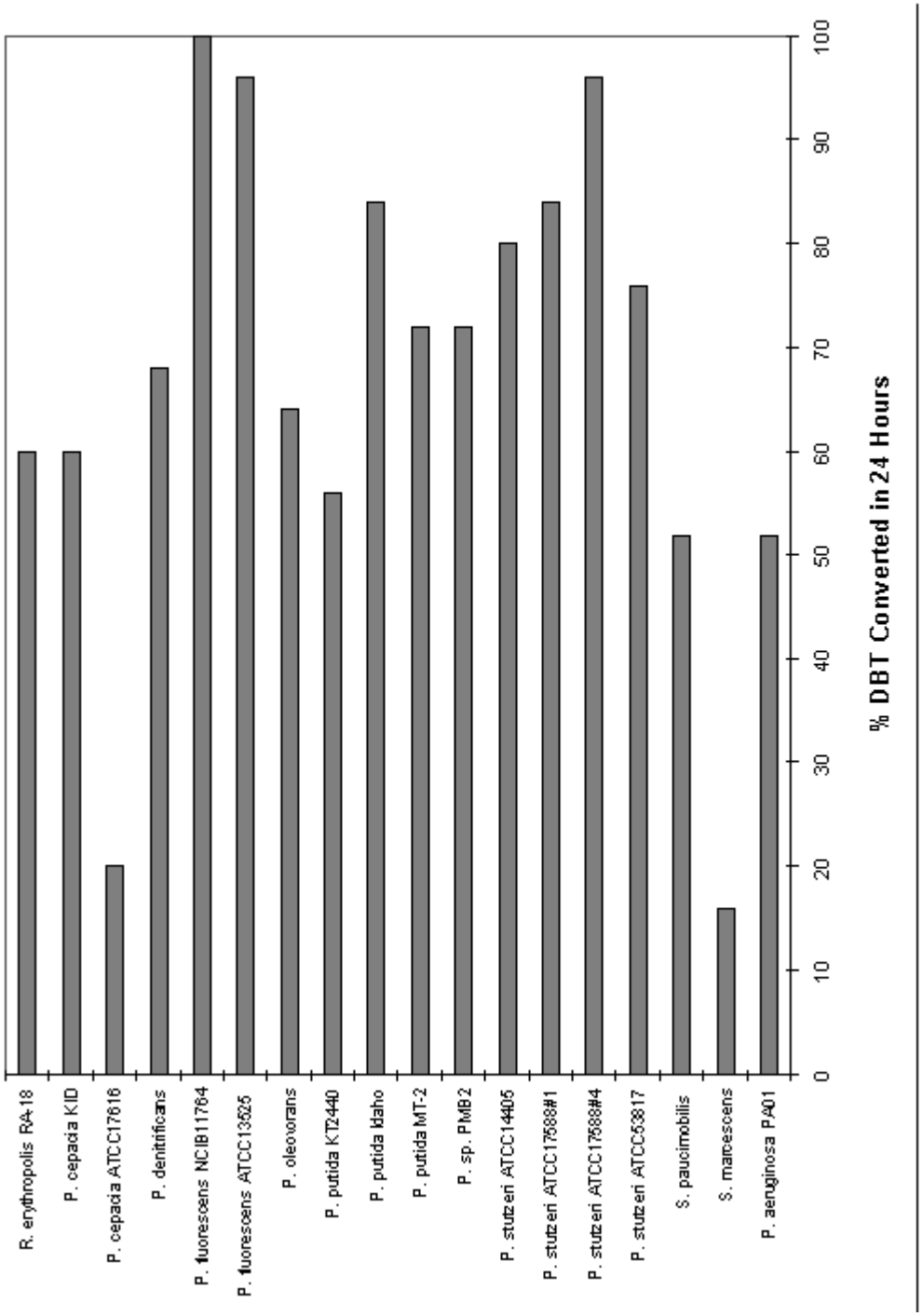

Figure 4. DBT Desulfurization Extent Screen. Cells were grown and assayed as described in the legend of Figure 3 except that 500 mg dry cell weight of cell paste was used in the reaction. After 24 hours of incubation, the phases were separated, and the DBT concentration in the organic phase was determined by GC-FID.

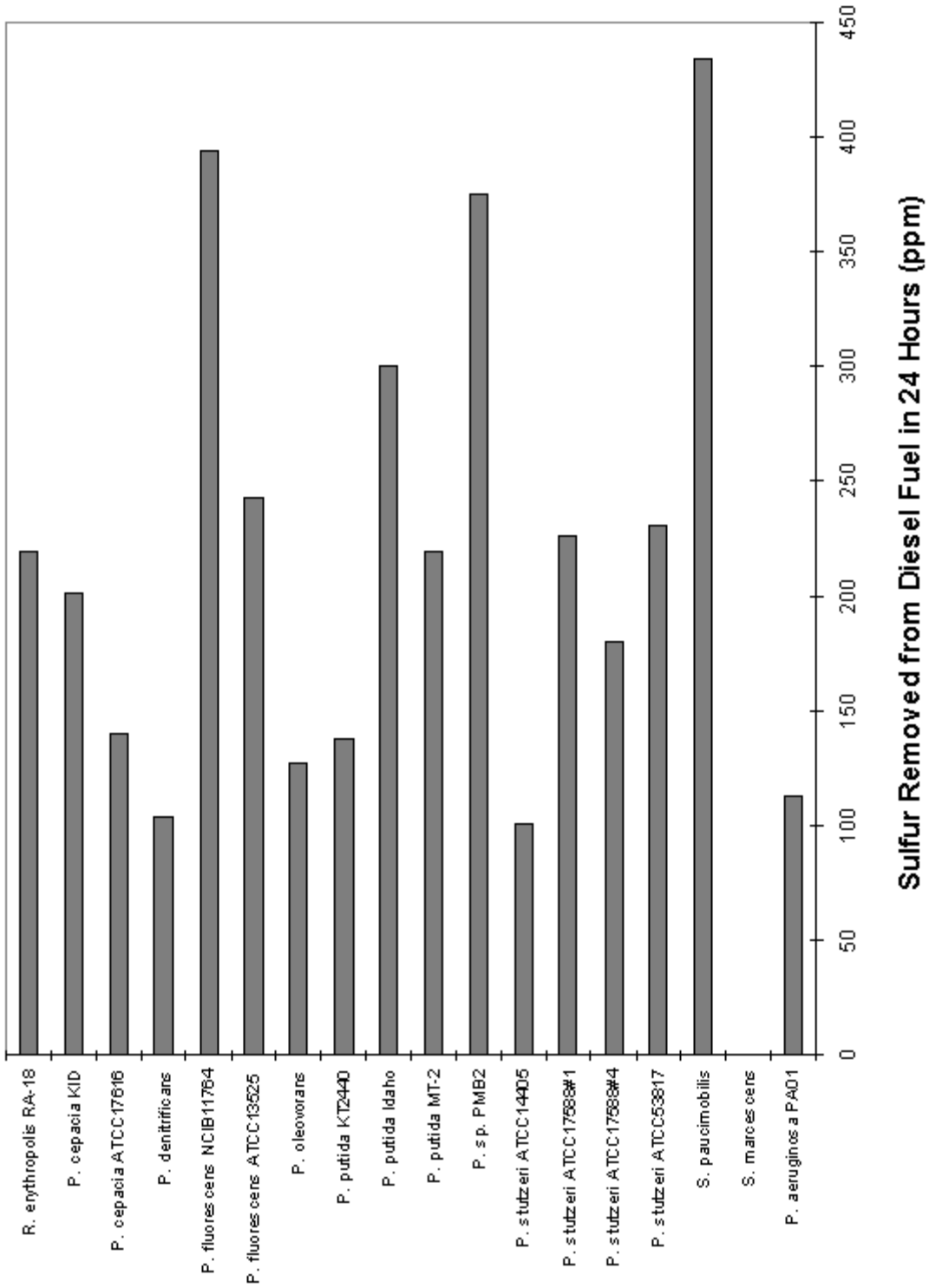

Figure 5. Diesel Desulfurization Extent Screen. Cells were grown and assayed as described in the legend of Figure 4 except that the organic phase used was diesel oil containing 1850 ppm sulfur. After 24 hours of incubation, the phases were separated, and the sulfur concentration in the organic phase was determined by energy dispersive x-ray fluorescence.

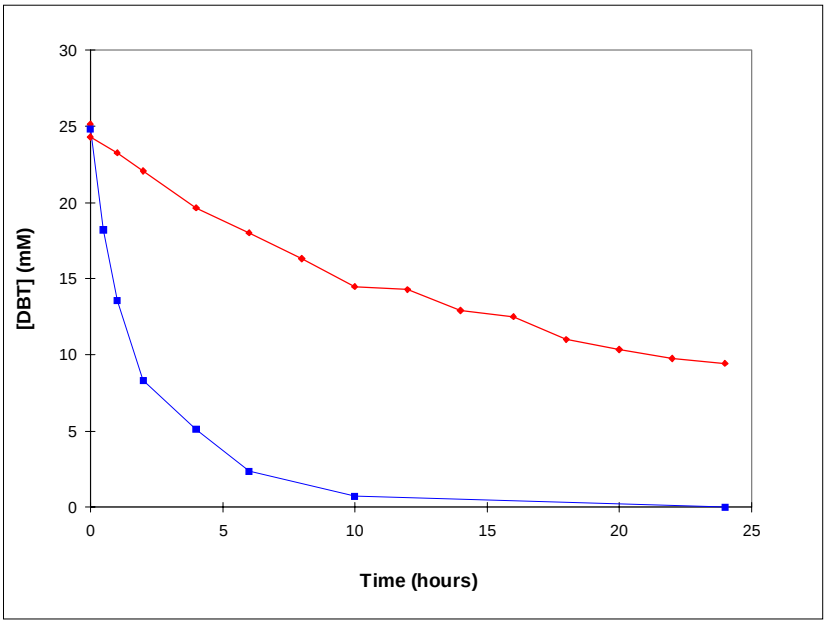

Figure 6. Desulfurization of DBT. Cells of *R. erythropolis* RA-18 (red diamonds) and *P. fluorescens* NCIB11764 (blue squares) were used in batch stirred reactors containing 12.5 g dry cell weight cell paste, 750 ml 156 mM of phosphate 1 buffer containing % glucose. And 250 ml of 25 mM DBT in hexadecane. The phases were mixed by agitation at 1000 rpm and aerated by sparging with air at 0.5 vvm. Samples were removed periodically and analyzed for DBT by GC-FID.

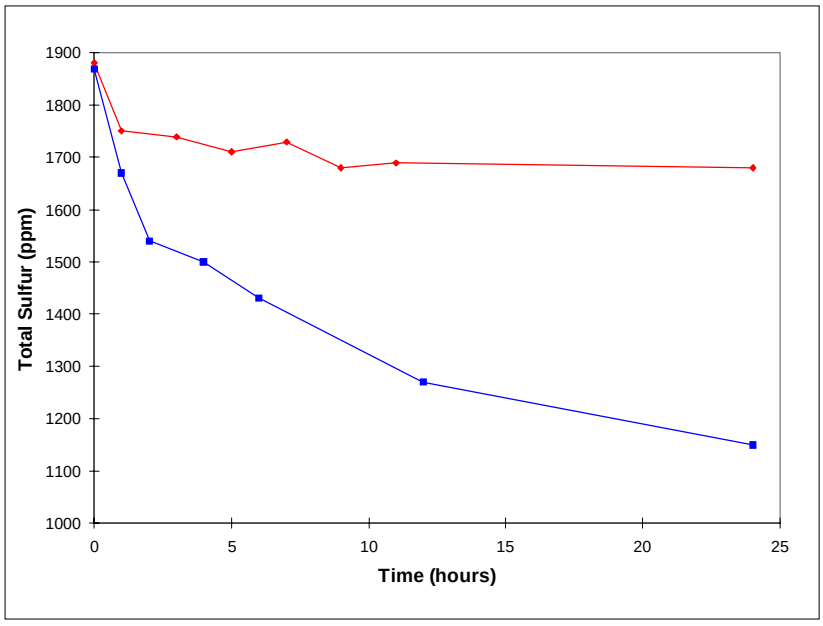

Figure 7. Desulfurization of Diesel Oil. Cells of *R. erythropolis* RA-18 (red diamonds) and *P. fluorescens* NCIB11764 (blue squares) were used in batch stirred reactors as described in the legend of Figure 6 except that the organic phase consisted of diesel oil containing 1850 ppm sulfur. Samples were removed periodically and the sulfur concentration in the oil phase was determined by energy dispersive x-ray fluorescence.

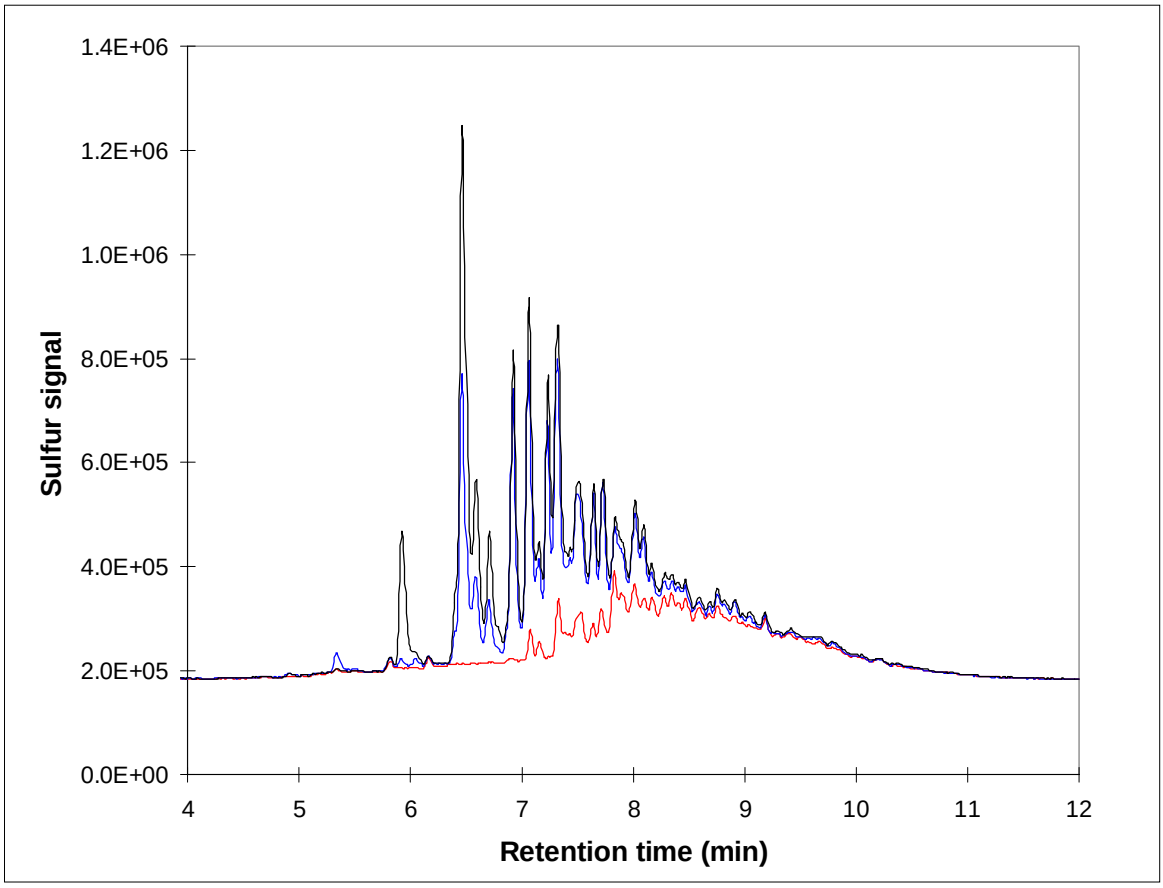

Figure 8. GC-SCD Chromatographs of Low Sulfur Diesel Before and After Biodesulfurization. Diesel oil containing 650 ppm sulfur was desulfurized with cells of *R. erythropolis* RA-18 (red trace) and *P. fluorescens* NCIB11764 (blue trace) in batch stirred reactors as described in the legend of Figure 7 except that the reactor contained 900 ml PO4 buffer and 100 ml oil. Untreated control is shown in the black trace. After 24 hours, the oil phases were analyzed by GC-SCD.

C., C. O'Chery, and S. E. Lantz, Mutant of *Pseudomonas fluorescens* with increased affinity for benzonaphthothiophene, 97TH General Meeting, American Society for Microbiology, Palm Beach, FL, May 4-8, 1997). We concluded that all of the Gram negative strains tested suffered from mass transfer limitations that were not seen in RA-18. The stable emulsions formed by RA-18 were an indication of its ability to adhere to the oil droplets. This affinity of *Rhodococcus* species for hydrophobic surfaces is well documented (10,13,17) has been confirmed in our microscopic observations of RA-18 attached to oil droplets. For cells that cannot adhere to oil droplets, hydrophobic PASH's must travel through the bulk aqueous phase before entering the cell for desulfurization, and therefore desulfurization activity is limited both by the concentration of the PASH's in the oil phase and by their partition coefficient from oil into water. Other examples of microorganisms capable of metabolizing hydrophobic compounds present in oil droplets either via mass transfer through the bulk aqueous phase or directly from the organic phase have been described (2,3,6,15).

The superiority of RA-18 in the ability to access the higher molecular weight PASH's predominant in low sulfur diesel led to renewed interest in *Rhodococcus* as biocatalyst.

Improvements in our understanding of molecular biology in *Rhodococcus* (11) and the isolation of a *Rhodococcus* NAD(P)H-flavin oxidoreductase (encoded by the *dsz*D gene) (16) offered opportunities to increase the specific activity beyond that of RA-18. A new IGTS8 derivative, I19, has higher levels of the *dsz* proteins, and demonstrates specific activities in model systems as high as 6 umoles/min/gdcw. When I19 was tested in batch stirred reactors with DBT in hexadecane (Figure 9) it was found to be vastly superior to RA-18 and comparable to *P. fluorescens* in terms of specific activity. I19 was also superior to RA-18 in reactor tests with diesel containing 1850 ppm sulfur (Figure 10) and superior to *P. fluorescens* in both rate of desulfurization and in catalytic longevity.

Discussion

The commercialization of the biodesulfurization process demands improvements in four critical success factors: biocatalyst production cost, specific activity, biocatalyst longevity, and extent of desulfurization. All of these factors address the issue of the contribution of biocatalyst to the overall process economics. In addition, the specific activity addresses the capital cost by dictating the size of the reactor vessel which is determined by the daily output of desulfurized diesel.

From an initial set of observations, it would appear that the switch from IGTS8 (and it's improved derivative RA-18) to recombinant *Pseudomonas* strains (especially *P. fluorescens* NCIB11764) resulted in a significant advance in all of the critical success factors. The

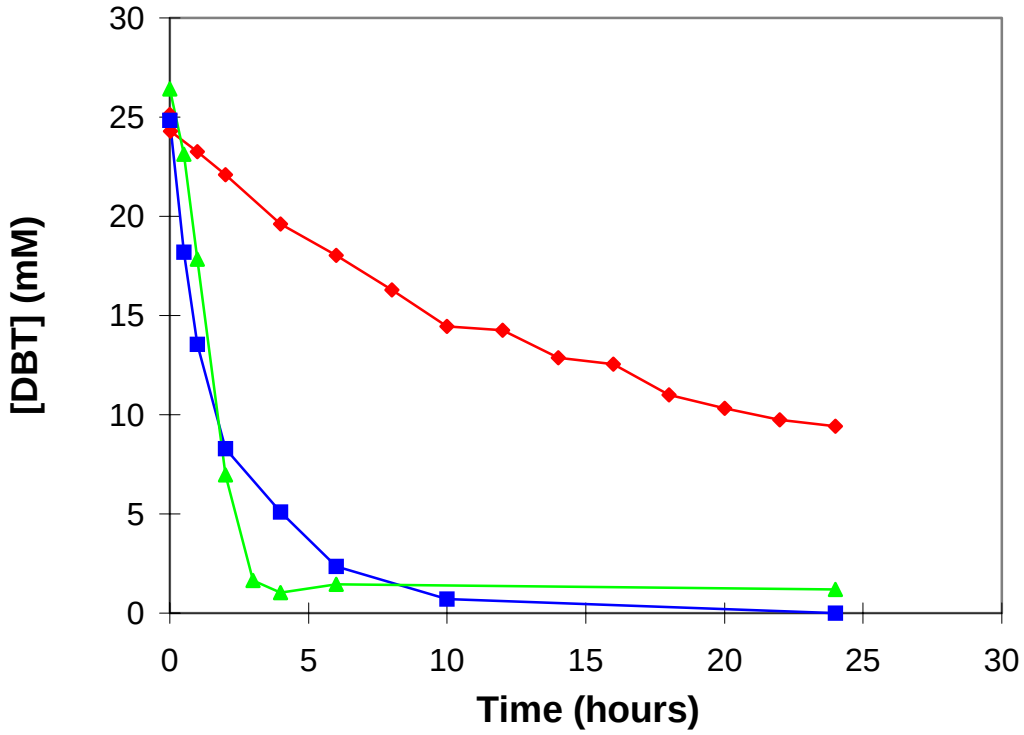

Figure 9. Desulfurization of DBT. Experiment carried out exactly as described in legend to Figure 6. Cells of *R. erythropolis* RA-18 (red diamonds), *R. erythropolis* I19 (green triangles) and *P. fluorescens* NCIB11764 (blue squares) were used in batch stirred reactors containing 25 mM DBT in hexadecane as the oil phase. Samples were removed periodically and analyzed for DBT by GC-FID.

growth rate and harvest yield of *P. fluorescens* are much greater than those of RA-18 (Figure 2), reducing the biocatalyst production costs. The specific activity in *P. fluorescens* is an order of magnitude higher than that of RA-18 (Figures 2 and 6), and the biocatalytic longevity (Figures 3, 6, and 7) and extent of desulfurization in high sulfur diesel (Figure 7) were superior as well.

The true issue of extent of desulfurization lie in the ability of biocatalyst to remove the more hydrophobic, the more highly alkylated high molecular weight PASH's from the oil, in order to achieve the lower sulfur regulations that are already in place as well as the increasingly stringent regulations that will continue to be implemented. On this issue *P. fluorescens* and other Gram negative strains could not compete with RA-18 because of mass transfer limitations. Attempts were made to isolate *Pseudomonas* strains with increased hydrophobicity (data not shown) or with a higher affinity for benzonaphthothiophene, but no improvements in desulfurization of low sulfur diesel were obtained (Schneider, J. C., C. O'Chery, and S. E. Lantz, Mutant of Pseudomonas fluorescens with increased affinity for benzonaphthothiophene, 97TH General Meeting, American Society for Microbiology, Palm Beach, FL, May 4-8, 1997), and our focus

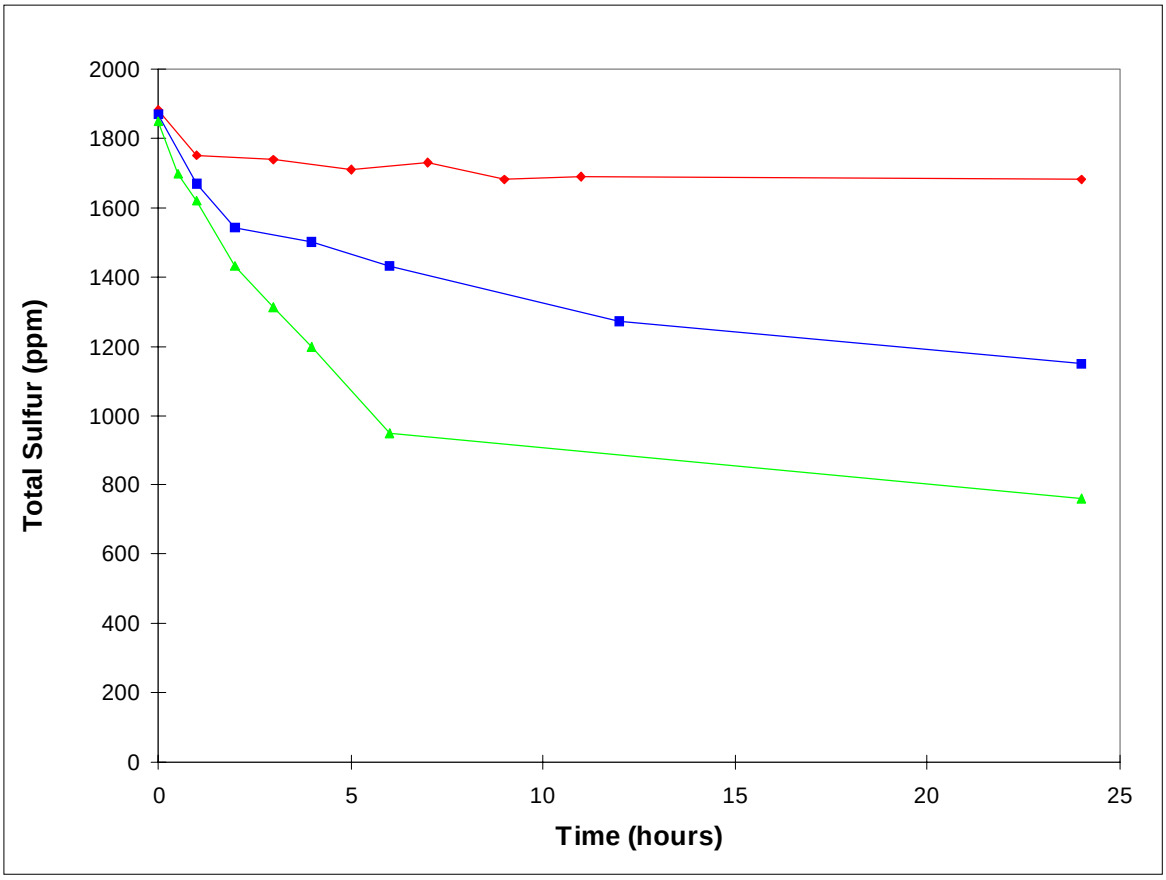

Figure 10. Desulfurization ofDiesel. Experiment carried out exactly as described in legend to Figure 7. Cells of *R. erythropolis* RA-18 (red diamonds), *R. erythropolis* I19 (green triangles) and *P. fluorescens* NCIB11764 (blue squares) were used in batch stirred reactors containing diesel oil with 1850 ppm sulfur. Samples were removed periodically and analyzed for total sulfur by energy dispersive x-ray fluorescence.

returned to solving the critical success factors in *Rhodococcus*. Manipulation of the *dsz* gene expression level led to the construction of the IGTS8 derivative I19. This strain manifests a specific activity and longevity that are at least as good as those of *P. fluoresescens* (Figures 9 and 10) with a vastly superior capability of removing alkylated DBT's from diesel (Figure 8). The cost of biocatalyst production remains an issue as the growth rate of I19 is slower than that of *P. fluorescens*, but a combination of strain improvements and fermentation process improvements have made contributions to reducing this critical success factor.

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Strain construction by Al Darzins, Lei Xi, John Childs, Mamie Li, and Ken Mitchell. Fermentation and screening by Mark Bauer, Caroline Cox, Xuping Fu, and Christine McDonald. Reactor studies by Brian Folsom, David Schieche, Li-Qun Yu, and Melanie Leenhouts.

References

1. Absi-Halabi, M. , A. Stanislaus, and H. Qabazard (1997) Trends in catalysis research to meet future refining needs. Hydrocarbon Processing, February, pp-45-55
2. Bouchez, M., D. Blanchet, and J.-P. Vandecasteele (1997) An interfacial uptake mechanism for the degradation of pyrene by a *Rhodococcus* strain. Microbiol 143:1087-1093.
3. Bredholt, H., K. Josefsen, A. Vatland, P. Bruheim, and K. Eimhjellen (1998) Emulsification of crude oil by an alkane-oxidizing *Rhodococcus* species isolated from seawater. Can. J. Microbiol 44:330-340.
4. Denome , S. A., E. S. Olson, and K. D. Young (1993) Identification and cloning of genes involved in specific desulfurization of dibenzothiophene by *Rhodococcus* sp. strain IGTS8. Appl and Environ Microbiol 59:2837-2843.
5. Denome, S. A., C. Oldfield, L. J. Nash, and K. D. Young (1994) Characterization of the desulfurization genes from *Rhodococcus* sp. strain IGTS8. J Bacteriol 176:6707-6706 (1994).
6. Efroymson, R. A. and M. Alexander (1994) Role of partitioning in biodegradation of phenanthrene dissolved in nonaqueous-phase liquids. Environ Sci Technol 28:1172-1179.
7. Gray, K. A., O. S. Pogrebinsky, G. T. Mrachko, L. Xi, D. J. Monticello, and C. H. Squires (1996) Molecular mechanisms of biocatalytic desulfurization of fossil fuels. Nature Biotechnol 14:1705-1709
8. Kilbane, J. J. and B. A. Bielaga Genetic study of biodesulfurization *In* S. Yunker and K. Rhee (eds.), Proceedings: 1990 First International Symposium of the Biological Processing of Coal. Electric Power Research Institute, Inc., Palo Alto, Calif. ,pp. 2/15-2/32 (1990).
9. Kilbane, J. J. and K. Jackowski (1992) Biodesulfurization of water-soluble coal-derived material by *Rhodococcus rhodochrous* IGTS8. Biotechnol Bioeng 40:1107-1114.
10. Lemmer, H., G. Lind, M. Schade, and B. Ziegelmayer (1998) Autecology of scum producing bacteria. Wat Sci Tech 37:527-530.

11. Li, M. Z., C. H. Squires, D. J. Monticello, and J. D. Childs (1976) Genetic analysis of the *dsz* promoter and associated regulatory regions of *Rhodococcus erythropolis* IGTS8. J Bacteriol 178:6409-6418
12. Monticello, D. J., and W. R. Finnerty (1985) Microbial desulfurization of fossil fuels. Annu Rev Microbiol 39:371-389.
13. Neu, T. R. And K. Poralla (1988) An amphiphilic polysaccharide from an adhesive *Rhodococcus* strain. FEMS Microbiol Lett 49:389-392.
14. Ohshiro, T., T. Hirata, and Y. Izumi (1996) Desulfurization of dibenzothiophene derivatives by whole cells of *Rhodococcus erythropolis* H-2. FEMS Microbiol Lett 142:65-70.
15. Ortego-Calvo, J.-J. and M. Alexander (1994) Roles of bacterial attachment and spontaneous partitioning in the biodegradation of naphthalene initially present in nonaqueous-phase liquids. Appl and Environ Microbiol 60:2643-2646.
16. Xi, L., C. H. Squires, D. J. Monticello, and J. D. Childs (1997) A flavin reductase stimulates DszA and Dsz C Proteins of *Rhodococcus erythropolis* IGTS8 in vitro. Biochem Biophys Res Commun 230:73-75
17. Stratton, H., B. Seviour, and P. Brooks (1998) Activated sludge foaming: what causes hydrophobicity and can it be manipulated to control foaming? Wat Sci Tech 37:503-509.
18. Zenno, S. and K. Asigo (1994) Identification of the genes encoding NAD(P)H-flavin oxidoreductases that are similar in sequence to *Escherichia coli* Fre in four species of luminous bacteria: *Photorhabdus luminescens, Vibrio fischeri, Vibrio harveyi, and Vibrio orientalis*. J Bacteriol 176:3544-3551.