FINAL Project Report

Improving Enzyme Activity and Broadening Selectivity for Biological Desulfurization and Upgrading of Petroleum Feedstocks

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PROJECT SCOPE

The objective of this project was to develop improved biocatalysts for desulfurization and upgrading of petroleum feedstocks. The goal was to improve the activity and broaden the selectivity of desulfurization enzymes using directed evolution as a tool as well as to explore the impact of ring-opening on biological desulfurization.

PROJECT SUMMARY

Biological desulfurization is an attractive alternative to hydrodesulfurization due to its mild operating conditions. However, there are several bottlenecks limiting commercialization of the process. These include biocatalyst stability, specificity, desulfurization rate, need for a carbon source to regenerate co-factors, oil-water separation and product recovery. This project was aimed at some of these bottlenecks. First was the biocatalyst's rate of desulfurization and the second was its specificity. The limiting factor in the overall rate of desulfurization is due the enzyme (desulfinase) responsible for breaking the carbon-sulfur bonds of the sulfonic acid intermediate in the desulfurization pathway. The desulfinase gene, *dszB*, was therefore, targeted for improvement using directed evolution.

Results showed up to a four-fold increase in the desulfurization rate in mutants obtained in the first generation. Investigations into the specificity of desulfurization by the Dsz enzymes in the parent strain Rhodococcus IGTS8 revealed that it could not desulfurize alkyl aryl sulfides and thiophenes. A third goal of this project was to study the effect of biological ring opening (biocracking) on biodesulfurization. Results conducted during the first two years of the project were not conclusive and therefore additional work is necessary to demonstrate the advantage of this process. This project was a collaborative effort between the University of Tennessee-Knoxville, Oak Ridge National Laboratory and Industrial partners, ChevronTexaco and ExxonMobil. ChevronTexaco was the lead in developing the ring-opening catalysts, while ExxonMobil assisted the team in genetic engineering of the dsz enzymes.

TECHNICAL REPORT

Activity improvement via directed evolution

Development of an improved desulfinase enzyme was investigated via the use of point mutagenesis of the *dszB* gene. The mutations were introduced by error-prone PCR (polymerase chain reaction). An *E. coli* strain containing a plasmid pESOX1, harboring the dsz gene was kindly provided by Dr. Eduardo Diaz, Centro de Investigaciones Biologicas (CIB)-CSIC, Madrid, Spain. PCR primers were designed using restriction enzyme sites: *NheI* (forward) and *SexAI* (reverse) as shown in Figure 1. In addition, two other primers with restriction sites *NotI* and *NcoI*, were also prepared as reverse primers.

The PCR product was ligated into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) and transformed into *E. coli* Top10F'. Plasmid DNA (pACKI) isolated from the transformed cells was digested with the appropriate restriction enzymes to release the *dszB* containing DNA fragment generated from error prone PCR. This fragment was then ligated back into the vector portion of pESOX1. These mutagenized plasmids (pACKD) were then transformed into *E. coli* JM109. Transformant cells were then screened for desulfinase activity. About 800 colonies were

created in the first generation and screened in 96-well microplates. The analysis of the product, 2-HBP, was conducted by HPLC (5-min run per sample). An example of the activity results is shown in Figure 2.

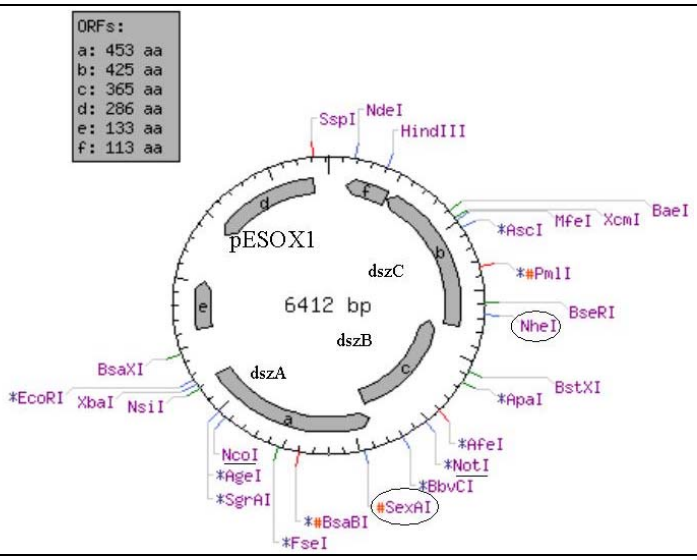


Figure 1. Restriction sites for pESOX1 plasmid carrying the dsz cassette.

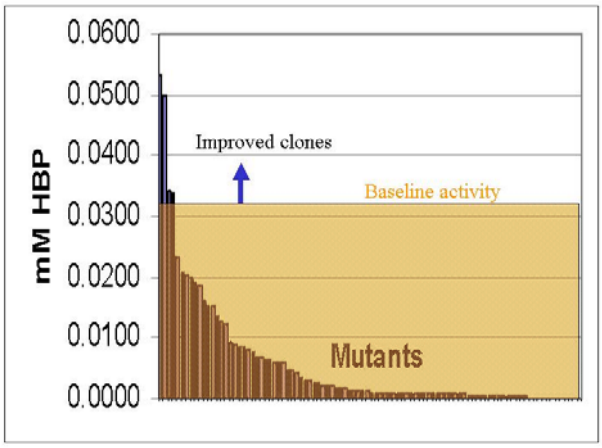


Figure 2. **Activity of desulfinase mutants generated by error-prone PCR. Baseline activity was around 0.034. In this set (96 mutants shown), three mutants show higher than baseline (native enzyme strain) activity.**

Selected mutants with improved activity were tested in flask assays (20 mL) to confirm the activity improvement. The experiment was run in duplicate. The results showed some mutants to have higher activity compared to the native strain. Flask experiments with resting cell cultures were conducted to determine the exact activity of the mutant strains. The mutants 8B1 and 6A10 showed about 2.5 and 4.0 times higher activity than the native strain. The activities were confirmed through repeat experiments. Sequencing of the *dszB* DNA isolated from the improved strains was conducted to find if this was due to a change in the gene sequence. No change was observed from the parent sequence, however, the possibility that a mutation in the regulatory portion of the DNA sequence may be responsible for the improved activity cannot be ruled out. Another possible reason for the observed increase in activity may be the presence of a different

copy number of the plasmid carrying the *dszB* gene in different strains. Whatever the reason, the two strains gave consistently higher activities over multiple runs and over several generations. Thus, further investigations are necessary to explain the improvements in desulfurization activities.

Substrate specificity studies

In order to investigate the specificity of the biocatalysts, model compounds as well as real feedstocks (diesel) were used. The model compounds consisted of non-oxygen containing organosulfur compounds as well as oxygen-containing (sulfonate) compounds. The purpose of the first class of compounds was to determine the specificity of the DBT monooxygenases, while the sulfonates were used to determine the specificity of the desulfinase enzyme.

Model compounds

To determine the specificity of the biocatalyst *Rhodococcus* sp. IGTS8, flask experiments were conducted with dimethyl thiophene, 2-phenyl thiophene, benzylphenylsulfide and phenylsulfide. The experiments were also conducted with dibenzothiophene as a positive control. A negative control was done without the biocatalyst. Analysis was conducted by HPLC. The results indicated no conversion of any of the compounds (except DBT). These compounds were thus recalcitrant and can be used as models to broaden the substrate specificity of the enzyme.

A second set of experiments was done using five aryl alkyl sulfonate compounds as substrates for the desulfinase enzyme. The compounds are listed in Table 1. For this experiment, the biocatalyst *Rhodococcus*, sp. IGTS8, was grown using DMSO as sulfur source (to induce the genes to produce the desulfurization enzymes), and the test compounds were added after a one-hour induction period. The experiments were conducted using whole cells in 5 mL resting cultures. Additionally, two controls, one with no cells and the other with dibenzothiophene (DBT) at a concentration of 0.02 mM in addition to the sulfonate (0.2 mM) were also run. The results indicated disappearance of 2-methylbutyl p-toluenesulfonate and 2,3-xylyl benzenesulfonate in the presence of the *Rhodococcus* biocatalyst, however, no new product peaks were observed. Additionally, the standard deviation between the triplicate was relatively high. Thus, there is evidence that some of the compounds may be converted by IGTS8, but no firm conclusions can be drawn at this time and more research is necessary.

Table 1: Aryl alkyl sulfonate compounds used for specificity studies.

	Sulfonate substrate	Analysis method	Conversion
1	2-methylbutly p-toluenesulfonate	HPLC	-
2	2,3-xylyl benzenesulfonate	HPLC	-
3	2-methylbutyl methanesulfonate	GC-FID	-
4	2,2-Dimethyl-1,3-dioxolan-4-lymethyl p-toluenesulfonate	GC-FID	-
5	2,3-diphenylpropyl p-toluenesulfonate	GC-FID	-

Diesel experiments

Two types of diesel feedstocks were used in these experiments, a straight-run (non-hydrotreated) diesel from ChevronTexaco and a severely hydrotreated diesel provided by ExxonMobil. In addition to using *Rhodococcus* IGTS8 as the biocatalyst, two other strains: *Pseudomonas putida* pESOX3 (also provided by Dr. Eduardo Diaz) and *E. coli* pESOX1 were also used. The reason for using these other strains was to evaluate whether the Dsz biocatalysts could desulfurize the range of compounds that they can transform in the native *Rhodococcus* host. The results are shown in Figure 3.

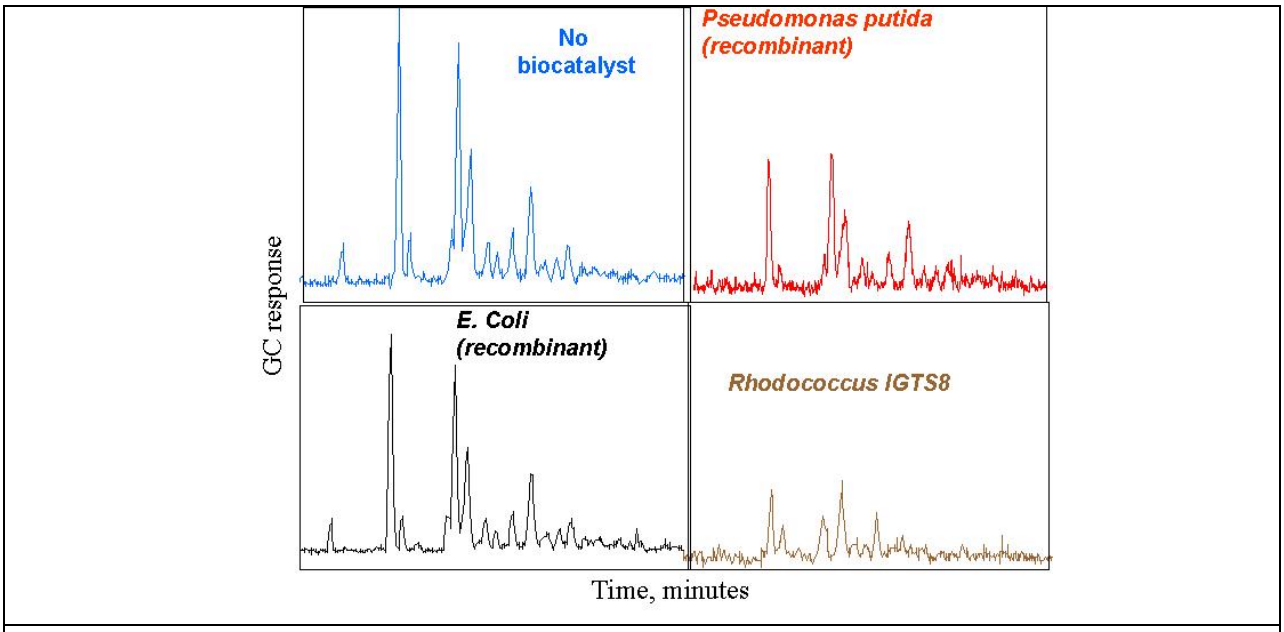


Figure 3. Biodesulfurization of severely hydrotreated diesel with three biocatalyst strains harboring the dsz gene, under similar experimental conditions.

Results from specificity experiments with the non-hydrotreated diesel were similar to the severely hydrotreated diesel in terms of the biocatalyst host effect. The removal of sulfur from the non-hydrotreated diesel by the *Rhodococcus* strain is shown in Figure 4 and comparison with other strains is shown in figure 5. It is clear that biodesuflurization of diesel is significantly greater with the Dsz system when it is expressed using *Rhodococcus* as the host organism. The *Psuedomonas putida* host appears somewhat better than the *E. coli* host judging from the disappearence of sulfur containing peaks in the chromatograms as shown in Fig 3, however the difference is slight.

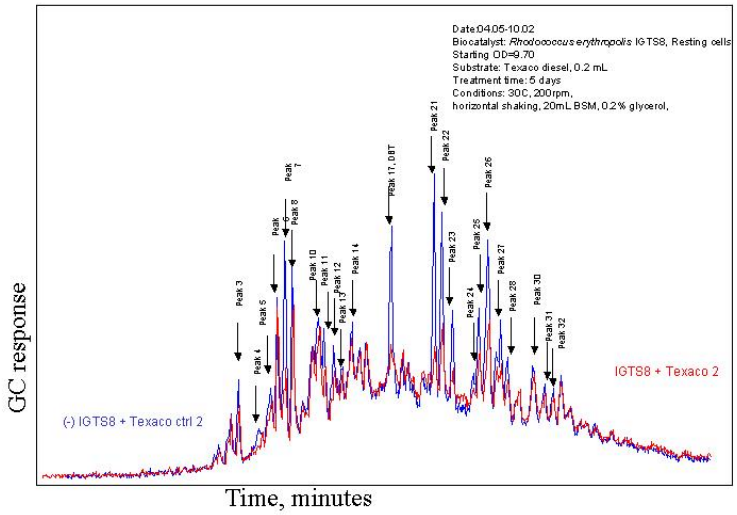


Figure 4. Biodesulfurization of non-hydrotreated diesel by *Rhodococcus* IGTS8. The arrows point to the peaks that showed a decrease after the biotreatment.

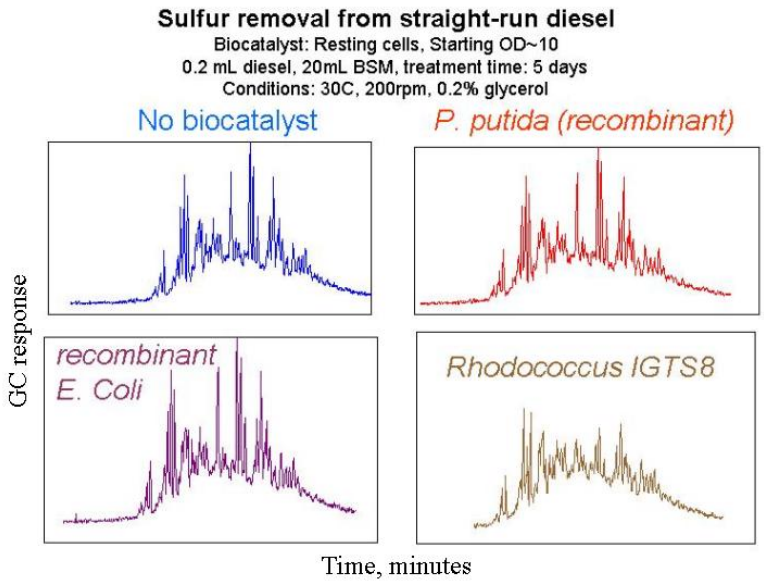


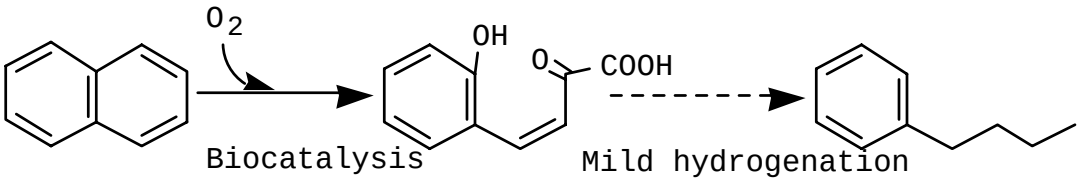
Figure 5. Comparison of sulfur removal from straight-run (non-hydrotreated) diesel by three biocatalyst strains harboring the dsz gene, under similar experimental conditions. *E. coli* and *Pseudomonas* strains showed very low conversion as compared to *Rhodococcus*.

The difference in the conversion by different strains harboring the same enzyme system indicates that factors other than kinetics are controlling the transformation of sulfur substrates. Several factors may be responsible for this behavior. These include substrate mass transfer through the cell wall and/or inhibition of activity by components of diesel. *Rhodococcus* IGTS8 has been reported to have significant affinity for the organic phase, as compared to the *E. coli* cells and our results would support this. These results therefore suggest that *E. coli* may not be a suitable host for studying substrate specificity of the DszC enzyme (the first enzyme in the pathway), especially for molecules larger than DBT. Other alternatives, such as the use of *Rhodococcus-E.*

coli shuttle vector to transfer the mutated genes created in *E. coli* into the *Rhodococcus* host may be helpful.

Development of the ring-opening biocatalysts

ChevronTexaco developed a biocatalyst for ring-opening of polyaromatic hydrocarbons. A non-proprietary description of the biocatalyst produced is provided. A stable mutant of *Pseudomonas fluorescens* was created and shown to catalyze ring cleavage of various aromatic hydrocarbons and heterocycles commonly found in petroleum distillates. This mutant accumulates the predicted ring cleavage products from two- and three-ring aromatics (naphthalene and phenanthrene) as well as many alkyl-substituted homologues, but does not oxidize aliphatic hydrocarbons. It can be pre-grown to high density in rich broth, induced to high activity, then used as a non-growing biocatalyst in an aqueous suspension. The ring cleavage metabolites it produces are water-soluble and can be recovered from the aqueous phase by solvent extraction. While initial studies were performed with pure substrates, the mutant has been shown to open aromatic substrates present in a variety of authentic middle distillates, a diesel cut, and a Texas crude oil, without affecting the saturated fraction. We have quantified biotransformation of the model substrates phenanthrene and dibenzothiophene dissolved in authentic distillates or their aliphatic and aromatic fractions. After treatment of the petroleum feedstocks with this catalyst, a mild hydrogenation step as shown below is suggested for improving the quality of the product.



Synergistic biocracking-biodesulfurization studies

Sand Flat crude oil was treated with the ring opening catalyst developed by ChevronTexaco and shipped to UTK for the BDS treatment. The ring opening treatment did not show a pronounced effect on the sulfur compounds, as observed from the GC chromatograms (data not shown). Due to the presence of a multitude of sulfur compounds in the oil, few are resolvable by GC alone. It is therefore not surprising that a significant effect was not observed even if it occurred.

The oil treated with ring opening catalyst was then subjected to a treatment with *Rhodococcus* IGTS8 catalyst. The goal of the experiment was to see if any improvement in sulfur removal occurs due to biological ring opening. The results from the first experiment conducted so far are shown in the Figure 6. The panel 6-A shows results from biodesulfurization of the oil without any ring-opening catalyst treatment. The oil was extracted with methylene chloride after the biocatalytic treatment. The results from the experiment with the ring-opening treatment are shown in Figure 6-B. The chromatograms in panels B1 and B2 represent the sulfur level in the ethyl acetate and the methylene chloride extract, respectively, for the experimental and the control reactors. The control contained autoclaved biocatalyst (i.e., *Rhodococcus* sp. IGTS8). The oil-water mixtures obtained from the biocatalytic treatment were extracted with ethyl acetate in addition to methylene chloride in order to recover the partially oxidized components of the oil that partition into the aqueous phase. Since the sulfur compounds are divided into the two solvent extracts, it is difficult to demonstrate the effect of ring opening catalyst from the results

of this experiment. Although further experiments were planned, this could not be done due to termination of the project after the second year. Thus additional experiments are needed with modified extraction procedures to better understand the effect of the pretreatment with ring-opening catalysts.

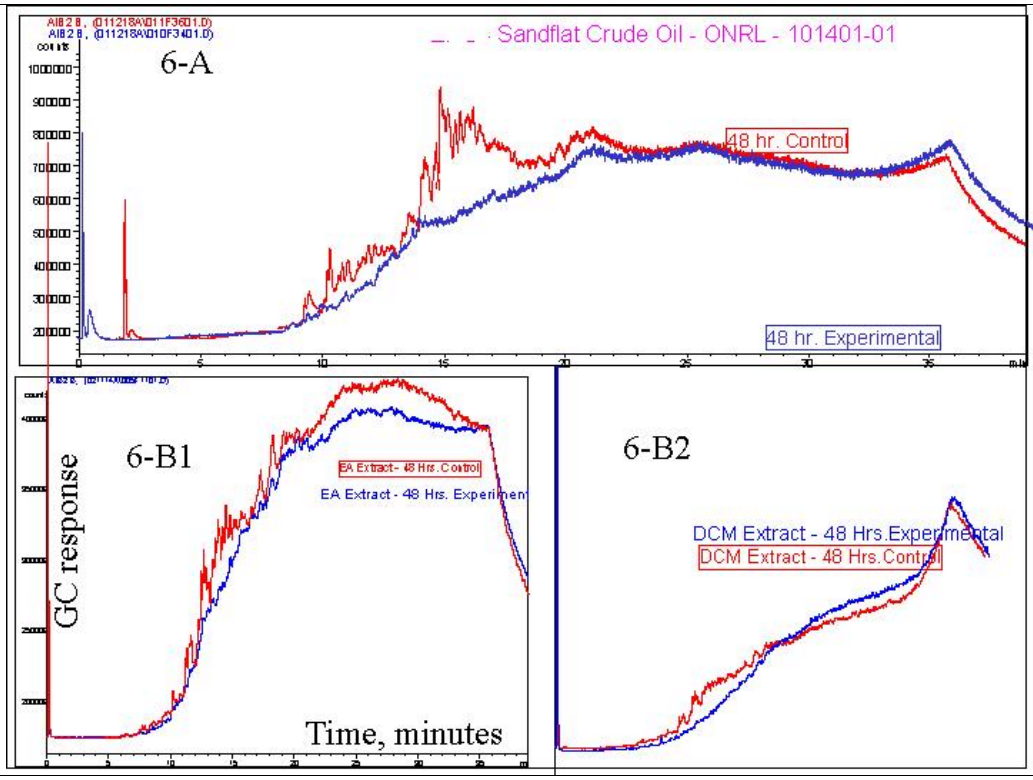


Figure 6. Results from biological treatment of Sand Flat crude oil with ring opening and biodesulfurization catalysts. Figure 6-A is the chromatogram representing sulfur content of the oil with the *Rhodococcus* catalyst treatment (experimental). The control experiment was conducted using autoclaved biocatalyst. This experiment (A) was done without previous treatment with ring-opening catalyst, where as the second experiment (B) was done by performing the ring opening treatment first, followed by the biodesulfurization treatment. Figure 6-B1 is the chromatogram of the ethyl acetate extract and 6-B2 is the methylene chloride extract of oil treated with ring opening as well as *Rhodococcus* catalyst.

Publications and presentations:

Borole, A P and Hamilton, C Y; *'Enzyme specificity and host effect during biodesulfurization of petroleum feedstocks'* a poster presented at the 24th Symposium on Biotechnology for Fuels and Chemicals, Gatlinburg, TN, May 2002.

Borole, A. P., '*Potential for Biocatalysis in Petroleum and Petrochemical Industry: Related Issues and Challenges*', Meriden, NH, July 2002.

Borole A. P., Eric N. Kaufman, M. J. Grossman, V. Minak-Bernero, R. Bare, M. K. Lee; '*Emulsion formation and breakage characteristics of Rhodococcus erythropolis and Echereshia coli hosting biodesulfurization genes,* Biotechnology Progress, 2002, 18, No. 1, 88-93.