Task 1.10 - Biodesulfurization Year 2

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TASK 1.10 - BIODESULFURIZATION YEAR II

1.0 INTRODUCTION

Conventional catalytic hydrodesulfurization involves high costs largely due to heavy metal deactivation of the catalysts. A potential lower-cost treatment is a microbiological or enzymatic desulfurization. Recent advances at the Energy & Environmental Research Center (EERC) at the University of North Dakota have improved our understanding of sulfur-specific microbial desulfurization pathways in *Rhodococcus* bacteria (1, 2), but further work is needed to develop a technology based on biodesulfurization.

2.0 OBJECTIVES

The main focus in this task is the application of desulfurization enzymes to remove sulfur from crude oil, diesel fuel, or resid precursors for needle cokes. The most important question to be answered is how to utilize the *Rhodococcus* desulfurization complex in a nonaqueous solvent or medium, such as the oil itself. Successful application of nonaqueous enzymology to this problem will involve finding ways to stabilize the active conformations of the enzymes and provide for easy recovery of the enzymes, perhaps in an immobilized-enzyme packed-bed reactor. A second objective is to explore biological systems that can catalyze the cleavage of organometallics in fossil fuels, such as metal porphyrins. A biometallation function could greatly aid in the refining of high-metal, high-sulfur crudes and bitumens.

The work will involve isolation and immobilization of the bacterial (*Rhodococcus*) enzyme systems that excise sulfur from thiophenic structures. Sulfur-specific microbial desulfurization has been shown to be an inducible trait that probably resides on the cell surface as a multienzyme cluster. Dead and broken cells exhibit the desulfurization activity, thus an immobilized cell-free system is plausible. Methods for dispersion and immobilization of enzyme clusters will be investigated. Immobilization is desired for ease in recovery and reuse and preparing packed-column reactors for continuous processing. The critical task is to develop a system that is active in a nonaqueous solvent so that the reaction is not limited by the diffusion of sulfur compounds from the oil phase into an aqueous phase and so that phase separations at the end of the processing are eliminated. Attachment of stabilizing groups to the support matrix or to the enzymes may be required to achieve activity in the nonaqueous solvents. Both nonpolar and polar solvents will be investigated. It is also essential that we determine whether cofactors or other regulatory species are needed for functioning of the cell-free enzymes. Further information will be obtained on the nature of the pathways and desulfurization mechanisms and the inducers and inhibitors of the microbial activity via experiments with alternative sulfur substrates and inducers and with labeled substrates.

3.0 WORK PERFORMED

Work in the previous year was not successful in breaking *Rhodococcus* cell walls and recovering the desulfurization activity. A number of experiments were initiated that will lead us to a better understanding of how to accomplish this task.

Work was initiated to develop new rapid and highly quantitative assays for specific desulfurization activities in isolated and separated enzyme fractions. The present methods will work, but will be extremely time-consuming. We also need to develop methods for assay in nonaqueous systems. These methods are based on reactions of nitrobenzenesulfonyl, nitrophenylthio, and similar substrates that either develop color on reaction with one or more enzymes in the pathway or lose color. These methods can also be used in screening new bacterial stains or mutants.

The preparation of water-soluble chlorophyllin was carried out for use in assaying for demetallation activity in microorganisms. Cleavage of the dark green porphyrin system in the chlorophyllin will be a indicator of activity that may be useful for cleavage of other metal porphyrins in crudes and bitumens.

4.0 RESULTS/CONCLUSIONS

A number of batches of *Rhodococcus* were grown up and frozen for use in later cell destruction and enzyme isolation experiments. In addition to French pressure cells, other rupturing systems have been considered, and one of these may need to be utilized. Ultrasonication may be reexamined with more careful experiments to reconstitute the active systems. The active systems may include more than one reducing cofactor, depending on which of the three enzymes in the desulfurization pathway is being assayed.

Experiments were performed with whole cells using the substrate 4-nitrobenzenesulfonic acid. The cells active for desulfurization showed the development of a yellow color resulting from the formation of 4-nitrophenolate ion. This reaction then tests for the second enzyme in the pathway, which is a sulfone/sulfonate oxydesulfonase that substitutes a hydroxy group for the sulfonate group.

Another new substrate, 4-nitrophenyldisulfide, was used to test for desulfurization activity. In this case, the sulfide would have to be oxidized first to the sulfonyl group by the first enzyme in the pathway, the sulfide monooxygenase. Then the second enzyme would cleave the C-S linkage and form the yellow 4-nitrophenolate ion. In this case, no yellow actually developed in the cultures. Thus the disulfide must not be a good substrate for one or both of the enzymes. The 4-nitrophenylsulfide is currently being prepared for testing.

A batch of dark green chlorophyllin was prepared by hydrolysis of chlorophyll. The sodium salt was purified by dissolution in water and precipitation with ethanol. The product is highly water-soluble and should be useful for testing for porphyrin cleavage activity.

5.0 FUTURE WORK

The main focus will be in splitting open the bacterial cells and demonstrating activity in various separated and reconstituted fractions. Some further assay methods will be developed for the first and third enzymes in the 4S pathway. Chlorophyllin will be utilized in screening for demetallation activity.

6.0 REFERENCES

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