Task 1.10 - Biodesulfurization Year 2

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TABLE OF CONTENTS

1.0	INTRODUCTION	1
2.0	OBJECTIVES	1
3.0	WORK PERFORMED	2
4.0	ACCOMPLISHMENTS	2
5.0	REFERENCES	4

TASK 1.10 - BIODESULFURIZATION YEAR 2

1.0 INTRODUCTION

Conventional catalytic hydrodesulfurization involves high costs, largely due to heavy metal deactivation of the catalysts. A potentially 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 involves 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 to 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

The production of inactive *Rhodococcus* cells for biodesulfurization of dibenzothiophene continues to be a problem. Several experiments have been performed to understand how to obtain cells with maximum activity. To develop this understanding, the cells that are active for dibenzothiophene are being used in experiments to determine their activity in solvent and other systems. Reactions were performed in the presence of organic solvents to determine their effects on the desulfurization of dibenzothiophene. The addition of anion exchangers was also studied to determine whether scavenging the sulfate and sulfur-containing intermediates would initiate greater desulfurization activity in the organism.

Several experiments were performed to demonstrate that the *Rhodococcus* enzyme complex will be able to desulfurize the heterocyclic components of fossil fuels besides dibenzothiophene. Some methylated, carboxylated, and chlorinated dibenzothiophenes were obtained and used in experiments with both stationary-phase and growing cells. These substrates include the following sulfur compounds: 1-, 2-, 3-, and 4-methyldibenzothiophene, 2-bromodibenzothiophene, and 4-chlorophenyl phenyl sulfone.

4.0 ACCOMPLISHMENTS

Several substituted dibenzothiophenes were demonstrated to undergo the bacterial desulfurization. The 4-methyldibenzothiophene was converted to a mixture of 3-methyl-2-hydroxybiphenyl and 3'-methyl-2-hydroxybiphenyl, but only one of the isomers predominated in the product mixture. Thus, there appears to be a large regioselectivity factor operating in the enzyme-catalyzed thiophene ring-opening reaction. By comparison with the gas chromatography (GC) retention time of products formed from the 2-methyldibenzothiophene (see below), the large peak at 23.18 min is the 3-methyl-2-hydroxybiphenyl, and the very small peak at 23.60 min is the 3'-methyl-2-hydroxybiphenyl. The ratio of the peak areas is 16:1. Work is currently in progress to determine which isomer was formed by synthesis of some the methyl-2-hydroxybiphenyls. Other products were also isolated from the bacterial desulfurization reaction. These include a fairly large peak corresponding to the sulfoxide or sulfone and three smaller peaks corresponding to the various sultine and sultone intermediates.

The 2-methyldibenzothiophene was desulfurized to a mixture of two isomeric methylhydroxybiphenyls: 5-methyl-2-hydroxybiphenyl (at 23.90 min) and 3'-methyl-2-hydroxybiphenyl (at 23.63 min). In this reaction, the 5-methyl-2-hydroxybiphenyl product was larger than the 3'-methyl-2-hydroxybiphenyl by a factor of 2.4. Thus the major product from both of the methyldibenzothiophenes is the isomer with the hydroxyl group in the same ring as the methyl. Since the product isomer ratio (regioselectivity factor) is quite different for the two methyldibenzothiophene substrates, the main directing factor is likely to be steric rather than electronic. Furthermore, the steric factor is more important in the 4-methyl isomer since this position is closer to the site of ring-opening attack.

Dibenzothiophene was also present as a substrate in the reaction of 2-methyldibenzothiophene, so that a competitive rate could be determined. The ratio of the hydroxybiphenyl product to the sum of the methylhydroxybiphenyl products was the same as the ratio of the dibenzothiophene to 2-methyldibenzothiophene. Thus the two substrates are degraded at the same rate. This is consistent with the weakness of the electronic effect of the methyl substituent and the lesser importance of the steric effect from the 2- position on the dibenzothiophene.

A mixture of 1-methyldibenzothiophene and 3-methyldibenzothiophene was reacted with the *Rhodococcus* system. Only three peaks corresponding to methylhydroxybiphenyls were observed in the chromatogram of the products. The four possible products are 6-methyl-2-hydroxybiphenyl and 2'-methyl-2-hydroxybiphenyl from 1-methyldibenzothiophene and 4-methyl-2-hydroxybiphenyl and 4'-methyl-2-hydroxybiphenyl from the 3-methyldibenzothiophene. It is likely that the 4-methyl-2-hydroxybiphenyl product is present, based on the similarity in retention time to that of the 5-methyl-2-hydroxybiphenyl, but identification of the other products is in doubt. Nevertheless, it is clear that both isomers reacted, but one product is missing owing to a large regioselectivity factor.

Several experiments were performed with stationary-phase cells to determine whether the yields of hydroxybiphenyl could be increased if the intermediates in the pathway were captured. This approach is based on the hypothesis that the bacteria would need to increase the efficiency of the desulfurization activity to compensate for the lower levels of usable sulfate being generated by the desulfurization system as a result of the loss of intermediates. Since the enzymes are already present in the cells, the lack of sulfate will probably affect only the regulation of existing enzymes. Thus, anion exchange materials were added to the media to trap the sulfinate and sulfonate ions that diffuse into the media from the cell interiors. Previous work showed that these ions are trapped by the ion exchanger and also that these ions can diffuse through the membrane both ways. Cells from the same batch were used with an organic ion exchanger resin (two concentrations), an inorganic ion exchanger, and the control with no exchanger.

The results showed that a lower yield of hydroxybiphenyl was actually obtained in the reactions that contained the anion-exchange resin. The reactions that contained 0.1 g of the ion-exchange resin gave yields of hydroxybiphenyl similar to those obtained with the reaction containing 1.0 g of resin, and these were much lower than the yields with no resin. The negative effect exerted by the resin is presently not understood. It could result from association of the resin with the cells that inhibit the metabolism or the transport of ions or organic species. It is possible that the resin competitively blocks dibenzothiophene adsorption sites on the cell surface. The addition of the inorganic anion-exchange material, a magnesium aluminum hydrotalcite, inhibited the formation of hydroxybiphenyl severely, such that only a very small amount formed. To investigate this phenomenon further, cells recovered from this type of experiment will be cleaned of ion exchanger and reinnoculated with dibenzothiophene to determine whether the loss of activity can be reversed.

The effects of organic solvents on the desulfurization of dibenzothiophene were also briefly studied. Addition of hexane resulted in no desulfurization of dibenzothiophene to

hydroxybiphenyl or other intermediates. Similar results were obtained with tetralin. These results indicate that the bacteria, or at least the whole cell suspensions, are not very selective for dibenzothiophene. The addition of ion-exchange resin to the hexane addition experiment and to the tetralin addition experiment both yielded small amounts of hydroxybiphenyl. We have no explanation for this behavior. Further experiments with smaller amounts of added organics are needed to further elucidate this phenomenon. When broken cells with good activities are available, the experiments will be repeated to determine whether the enzymes are really highly selective for the dibenzothiophenes or are actually inhibited by the organic materials.

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