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# SUMMARY

This experimental research project investigated protein-based biocatalysis in supercritical fluid solvents as an integrated process approach to catalyze the removal of sulfur atoms from crude oils and fuels. The work focused on the oxidation of model sulfur-containing compounds in supercritical reaction media and included three major tasks: microbiological induction experiments, protein-catalyzed biooxidation in supercritical solvents, and a work-in-kind cooperative research and development agreement (CRADA). This work demonstrated that the biooxidation reaction could be improved by an order-of-magnitude by carrying out the reaction in emulsions in supercritical fluids.

Several catalysts were evaluated for this application including microbial enzymes, commercially available enzymes, and protein catalysts. Ultimately, hemoproteins were chosen as biocatalysts for their ability to oxidize sulfides, thioanisoles, thiophenes and dibenzothiophenes (DBT) to their sulfoxide/sulfone products. DBT was chosen for the reaction studies as a compound typical of organosulfur compounds in crude oils and fuels. Protein catalysts used in the current study included horseradish peroxidase (HRP), hemoglobin (Hb), Cytochrome c (Cyt c), and soybean peroxidase (SBP). Supercritical fluids explored included carbon dioxide, methane, ethane, propane and trifluoromethane.

Microbiological experiments were conducted to evaluate the possibility of producing enzymes from previously isolated strains of microorganisms for evaluation to compliment commercially available material. It was deemed that commercially available enzymes and proteins would be more convenient as well as economically favorable for experimental application to demonstrate the feasibility of utilizing SCF technology in combination with biocatalysis for heavy oil upgrading.

The supercritical fluid protein-catalyzed studies focused on the biooxidation of model sulfur containing compounds. The supercritical fluid efforts included the following studies:

- Solubility of substrates and co-factors
- Bench-mark liquid-phase biooxidation in aqueous, organic-solvent modified aqueous, and pure organic solvents
- Biooxidation in SCFs and modified SCFs
- Protein immobilization
- Conjugated protein and
- Reverse micelles, microemulsions, and emulsions in supercritical fluids.

A work-in-kind CRADA was established with UOP to enhance research and to provide a gateway to potential technology users.

The supercritical studies found that significantly higher solubilities could be obtained at SCF conditions compared to the aqueous phase. The solubility increase of three orders-of-magnitude suggested that much higher reaction rates could be obtained. However, biooxidation in pure and modified SCFs using proteins in there free, lyophilized, PEG-conjugate forms, and immobilized on

glass beads, alumina based support and agarose beads was not effective and resulted in only very small amounts of conversion to product (<1 wt.%). It was determined that a polar environment around the protein is needed for reaction in organic SCFs. Biooxidation of DBT in emulsions in supercritical fluids resulted in conversions to product that increased product yield by more than an order of magnitude compared to the aqueous phase reaction.

This project developed a new route to biooxidation based on the ability of proteins to retain activity within the water pool of the reverse micelles and emulsions formed in supercritical fluids. In macroemulsions, conversions up to 4.1 % were obtained. Due to the significantly higher solubility of DBT in the SCF media, this resulted in a 12-fold increase in product yield over aqueous based bioconversion. Although this project demonstrated an order-of-magnitude increase in biooxidation rates, further developments are necessary before biodesulfurization can become a commercially viable technology.

## ACKNOWLEDGMENTS

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# BACKGROUND

Significant volumes of crude oil are produced that are rich in complex hetero-atomic molecules that result in increased environmental concerns and reduced monetary value. In general terms, the heterocyclic structures that are problematic contain sulfur, nitrogen, and metal complexes. For example, feedstocks available to refineries are becoming heavier (decrease in API gravity of 0.17 per year) with an increase in sulfur content of about 0.027 %/year.[1] Sulfur moieties of concern are typically organosulfur compounds. Production and utilization of oils with appreciable sulfur content results in increased environmental pressures and costs to producers, refiners, and end users. Environmentally, utilization of high sulfur fuel products results in production of sulfur oxides that are believed to be partially responsible for "acid rain." Even small amounts of sulfur in transportation fuels interfere with the operation of complex emissions control systems that are being developed to meet ever tightening emission standards. Economically, organosulfur complexes represent a cost estimated at \$0.42/wt% of sulfur/bbl ± \$0.06.[2]

Biological removal of sulfur from oil (biodesulfurization) is a potentially attractive alternative to conventional refinery processes such as hydrotreating.[3] Biodesulfurization processes offer the potential of lower capital and operating costs compared to traditional refinery processes and the potential to overcome many of the steric limitations of traditional catalytic methods. Biodesulfurization may occur via oxidative or reductive pathways. Reductive reactions are accompanied by hydrogen sulfide byproduct formation, which can be dealt with using standard refinery processes. Oxidized products can be removed either by distillation or by heating above 300 °C to eliminate sulfur dioxide.

Although technologies such as hydrotreating are effective, novel upstream technologies are desired to adequately address the problems associated with the production, transportation, and utilization of heavy oils. Microorganisms with the ability to remove sulfur have been isolated and desulfurization processes based on this selectivity have been evaluated. Microorganisms with the ability to remove sulfur have been isolated, and desulfurization processes based on this selectivity have been evaluated. There are 103 bacteria and 163 filamentous fungi or yeast listed in the American Type Culture Collection (ATCC) with the ability to metabolize sulfur. Specific examples of organisms include the thermoacidophilic *Sulfolobus* which oxidizes sulfur, sulfide, and tetrathionate for the production of energy as well as the Thermoproteales-type bacteria (extremely thermophilic organisms that derive energy from sulfur such as *Thermoproteus*, *Pyrodictium*, *Staphylothermus*, and *Pyrobaculum*).

Hemoproteins have also been explored as biocatalysts for their ability to oxidize sulfides, thioanisoles, thiophenes and dibenzothiophenes (DBT) to their sulfoxide/sulfone products. Oxidation of DBT is well known in aqueous buffer/organic solvents and organic solvents.[4] Conversion up to 99% has been reported when the reaction was catalyzed by hemoglobin in aqueous buffer/organic solvents (75/25 vol.%) and 28 % conversion when the reaction was performed in 99 vol.% ethanol.

However, practical limitations for bioprocessing heavy oils are recognized and include low hydrocarbon solubility in water and mass-transfer limitations across oil-water interfaces, relatively slow reaction rate kinetics, catalyst recycle, requisite separation technologies to recover desired products, and maintenance of biological integrity. Proteins and whole cells have been shown to be active in organic solvents.[5] Bioprocessing in liquid organic solvents overcomes solubility and mass-transfer limitations, but organic solvents can be expensive, and separation or recycle from the reaction mixture can be difficult. These issues pose significant practical problems for scale-up and continuous operation of most bioprocessing technologies.

Supercritical fluid (SCF) systems have been demonstrated to offer several advantages over conventional water and liquid organic solvents as a reaction media.[6] In the simplest of terms, a supercritical fluid is a material that has been heated above the temperature at which it cannot be

compressed into a liquid form. Supercritical fluids provide high solubility of organic compounds, enhance mass-transfer rates due to the elimination of liquid/liquid interfaces, control feed and product solubility with mild variations in temperature and pressure, enhance reaction kinetics and the reaction approach to equilibrium, and are more amenable to energy efficient separation of end products. Supercritical fluids are already in use throughout the petroleum industry, e.g., enhanced oil recovery and refining. Enzymatic activity in SCFs has been proven and well documented.[7] Limiting factors which may affect enzymatic activity in SCF solvent systems have been identified and are well characterized.

The application of supercritical fluid technology to enzymatic transformations has the potential to provide a simple, highly efficient, low maintenance reaction system not limited by substrate bioavailability, mass-transfer limitations, slow reaction rate kinetics, catalyst recycle, or maintenance of biological integrity. This approach could result in a robust, continuous-reaction processes useful as a "mid-stream" upgrading process for crude oil.

# RESEARCH PROGRAM

This experimental research project investigated protein-based biocatalysis in supercritical fluid solvents as an integrated process approach to catalyze the removal of sulfur atoms. The work focused on the oxidation of model sulfur containing compounds in supercritical reaction media. The project included three major tasks: microbiological induction experiments, protein-catalyzed biooxidation in supercritical solvents, and a work-in-kind cooperative research and development agreement (CRADA).

Microbiological experiments were conducted to evaluate the possibility of producing enzymes from previously isolated strains of microorganisms for evaluation to compliment commercially available material. This approach was logical given that organisms capable of transforming organosulfur moieties have been investigated for nearly 50 years. Additionally, both the oxidative and reductive pathways, including enzymes and intermediates, have been extensively studied using model organosulfur compounds. These efforts have been inclusive of the genetics of the organodesulfurization process. Several investigators report biodesulfurization by both whole-cells and cell-free extracts while others note that enzyme systems out perform whole-cell cultures.

The supercritical fluid protein-catalyzed studies focused on the biooxidation of model sulfur containing compounds. The majority of the work studied dibenzothiophene (DBT) as the model compound, while a smaller body of work explored the oxidation of thioanisol. The supercritical fluid efforts included the following studies:

- Solubility of substrates and co-factors
- Bench-mark liquid-phase biooxidation in aqueous, organic-solvent modified aqueous, and pure organic solvents
- Biooxidation in SCFs and modified SCFs
- Protein immobilization
- Conjugated protein and
- Reverse micelles, microemulsions, and emulsions in supercritical fluids.

A work-in-kind CRADA was established with UOP to enhance research and to provide a gateway to potential technology users. UOP provided technical guidance on refining parameters, samples of catalytic support materials for reactor evaluation, and consultation on existing reductive and oxidative desulfurization technologies for performance benchmarking. Additional efforts to establish a CRADA with Texaco were not completed during the course of the project. However, valuable input was provided concerning oil chemistries, refining economics, and industry needs.

The supercritical studies found that significantly higher solubilities could be obtained at SCF conditions compared to the aqueous phase. Biooxidation in pure and modified SCFs using proteins in there free, lyophilized, PEG-conjugate forms, and immobilized on glass beads, alumina based support and agarose beads resulted in only very small amounts of conversion to product (<1 wt.%). However, biooxidation of DBT in emulsions in supercritical fluids resulted in conversions of product that increased product yield by more than an order of magnitude compared to the aqueous phase reaction.

## Experimental Procedures and Results

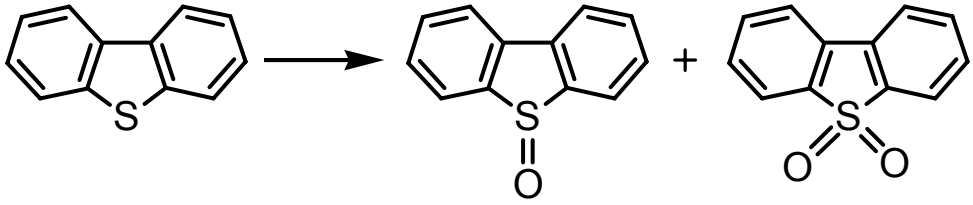
The experimental procedure and results are presented below for the SCF and microbial evaluation studies performed in this project. This section has been organized to provide general procedure information for the SCF experimental studies, followed by the solubility and stability studies and the biooxidation in reverse micelles and emulsions studies. These studies are presented initially since they provide the significant results obtained in this research project. This section then presents the initial project work that evaluated microbial enzyme production and protein catalyzed biooxidation in simple

SCF solvent systems. These efforts were largely unsuccessful, but are included to provide a complete coverage of the project work and to document the motivation to explore the emulsion environments.

**SCF Experimental Studies:**

The SCF experimental efforts focused on obtaining underlying solubility measurements, benchmark data points, and exploring protein-catalyzed biooxidation in SCFs under a variety of conditions. Results from this work demonstrated that solubilities of organic sulfur compounds in SCFs are significantly higher than in aqueous buffers. The increased solubility can allow for higher product yields; however, non-polar liquid or SCF reaction environments were undesirable for the protein-catalyzed biooxidation. In this work, it was determined that emulsions in SCFs provided the desired higher substrate solubility and appropriate environment for improved product yields from the protein-catalyzed reaction.

The SCF work focused primarily on the oxidation of DBT to it's oxide products, dibenzothiophene sulfoxide (DBTO), and dibenzothiophene sulfone ($DBTO_2$).



| Dibenzothiophene | Dibenzothiophene sulfoxide | Dibenzothiophene sulfone |
|---|---|---|
| **DBT** | **DBTO** | **$DBTO_2$** |

Experimental reaction conditions explored temperatures from 25 °C to 65 °C, pressures from 2,000 psi to 4,500 psi for SCFs and atmospheric for liquids, and reaction times from 2 to 15 hours. Biocatalysts included horseradish peroxidase (HRP), hemoglobin (Hb), cytochrome c (Cyt c), and soybean peroxidase (SBP). The cofactor for most oxidation reactions was hydrogen peroxide ($H_2O_2$). SCFs explored were carbon dioxide ($CO_2$), methane ($CH_4$), ethane ($C_2H_6$), propane ($C_3H_8$) and trifluoromethane ($CHF_3$). The SCFs were occasionally modified with methanol, ethanol and water.

**Experimental Setup:**

A general schematic of the experimental system is shown below in Figure 1:

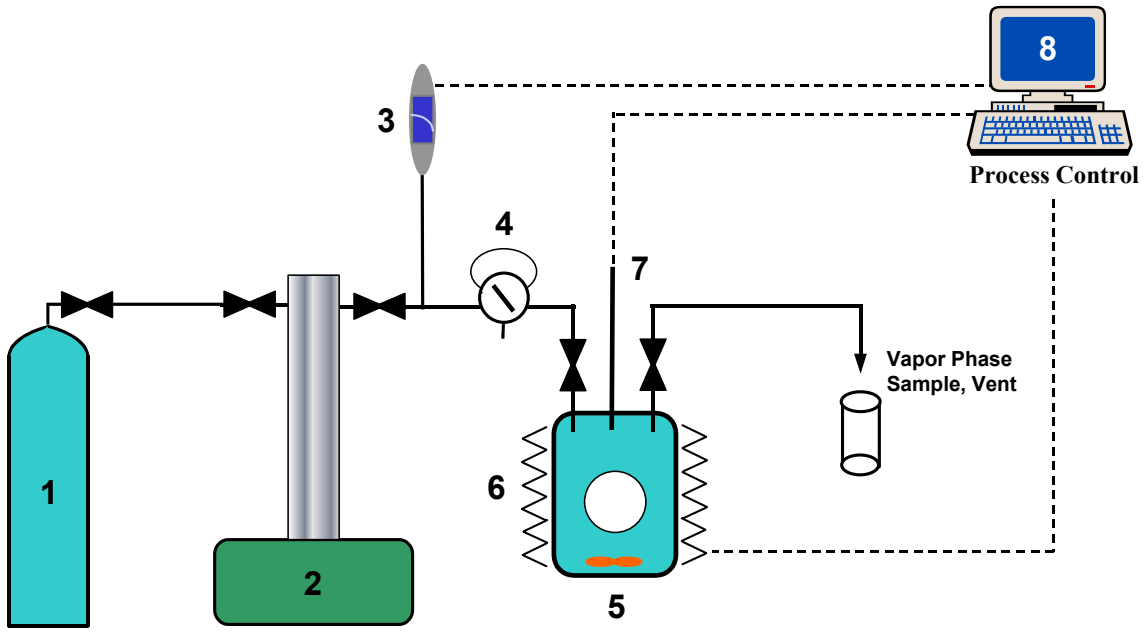



Figure 1. Schematic of the high-pressure reaction system. 1- SCF Supply, 2 - ISCO pump, 3 - pressure transducer, 4 - HPLC injection valve, 5 - high pressure reactor, 6 - heating element, 7 - thermocouple, 8 - process control.

The high-pressure apparatus consisted of an ISCO high-pressure syringe pump (Model 260D), an HPLC injector, a pressure and temperature control system (Omega) and a stainless steel reaction vessel. Two types of reaction vessels were used, either a 100 ml autoclave (Autoclave Engineers*)* or a 4.1 ml optical cell equipped with quartz windows. Heat was supplied to the reaction vessel by a water bath or electrical resistance heating. The components of the system were connected via high pressure valves and fittings. A 5890 Series II HP gas chromatograph with a flame ionization detector (FID) and a 5890 Series II HP gas chromatograph/mass selective (MS) detector was used to analyze the product mixture.

**Solubility and Stability Studies:**

The solubility of DBT in water, measured at 6 □M,[8] is very poor and limits bioavailability. The use of SCFs can increase the solubility of organic sulfur compounds by several orders-of-magnitude. DBT solublity in SC $CO_2$ was examined as a function of temperature and pressure. Additionally, the solubility and stability of the cofactor, $H_2O_2$, was also determined.

**Solubility Procedures**. Excess DBT was placed in the autoclave reactor. The reactor was heated to the desired temperature and filled with $CO_2$ (99.5 % purity) to the lowest experimental pressure. After three hours of stirring, the system was allowed to equilibrate unstirred overnight. A 0.5 ml sample was collected using two HPLC valves and vented into 30 ml of methylene chloride. The HPLC loop was then washed with 5 ml methylene chloride and flushed with high purity nitrogen. The reactor pressure was increased, the mixture stirred for 30 minutes and equilibrated (unstirred) for 90 minutes. Another sample was obtained. The operation was repeated until the maximum desired pressure was reached. Samples were analyzed by GC/FID.

Solubility of hydrogen peroxide in $CO_2$ was determined by UV-VIS on a Shimadzu spectrometer at 240 nm. A 5 μl 30% $H_2O_2$ solution was added to an optical stainless steel cell (3.7 ml volume, 1.6 cm pathlength). The cell was heated to 40 °C and then pressurized with $CO_2$. The contents of the cell were stirred in some cases. An absorption spectrum was recorded from 190 nm to 400 nm.

The stability of hydrogen peroxide in the stainless steel reactor with $CO_2$ pressure was determined by UV-Vis at 240 nm. A 100 □L 30% $H_2O_2$ sample was placed in the reactor to which 25 ml water was added. The reactor was closed, heated to 40 °C and pressurized with $CO_2$. The mixture was then stirred at 1200 rpm for 19.5 hours. The reactor and its contents were cooled to room temperature and the $CO_2$ slowly vented. The reactor was then opened and the liquid solution at the bottom of the reactor collected. The absorption spectrum of the resulting solution was recorded.

**Solubility and Stability Results**. DBT solubility was measured at 35, 40 and 45 °C from 900 to 2,900 psi. Solubility data, shown in Figure 2, was in good agreement with published data.[9,10] The solubility of DBT in $CO_2$ at 45 °C and 2900 psi is 5.7 mg/ml, or about 5,000 times higher than the solubility of DBT in water. This high solubility provides a promising media for biooxidation since higher concentrations can lead to ostensibly higher reaction rates. Solubility of DBT varied 300 fold by adjusting temperature and pressure, which could potentially be used to control inhibition phenomena. Similar increases in solubility could also be expected for other organic sulfur compounds found in crude oil.

The solubility of hydrogen peroxide, shown in Figure 3, was measured at 40 °C between 800 and 2,300 psig. A solubility of 4.5 mM was measured at 2300 psi. Although lower than in water, this solubility represents a significant excess of co-factor requirement for the conditions under investigations. This work also found that the addition of small amounts of methanol, used to modify the SCF, increased hydrogen peroxide solubility by nearly two-fold.

Hydrogen peroxide stability was measured at 40 °C exposed to carbon dioxide at 2,300 psi. At atmospheric pressure in a glass vial, there was no measurable decomposition of hydrogen peroxide after 19 hours. However, hydrogen peroxide decomposition in the autoclave reactor under $CO_2$ pressure after 19 hours was measured to be 46%. It was determined that the stainless steel in the reactor was responsible for 25 % decomposition while the pressurized carbon dioxide was responsible for 21 % decomposition. Based on the decomposition, and the solubility, it was determined that there was still sufficient excess of hydrogen peroxide for the biooxidation reaction.

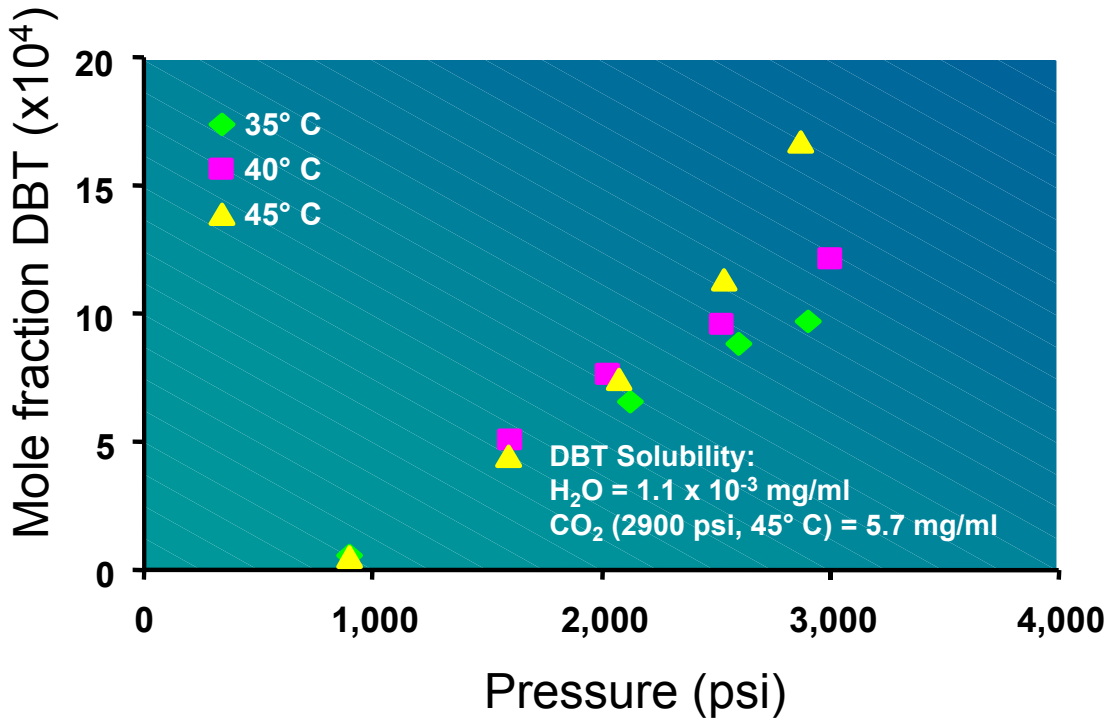



Figure 2: Solubility of DBT in supercritical carbon dioxide.

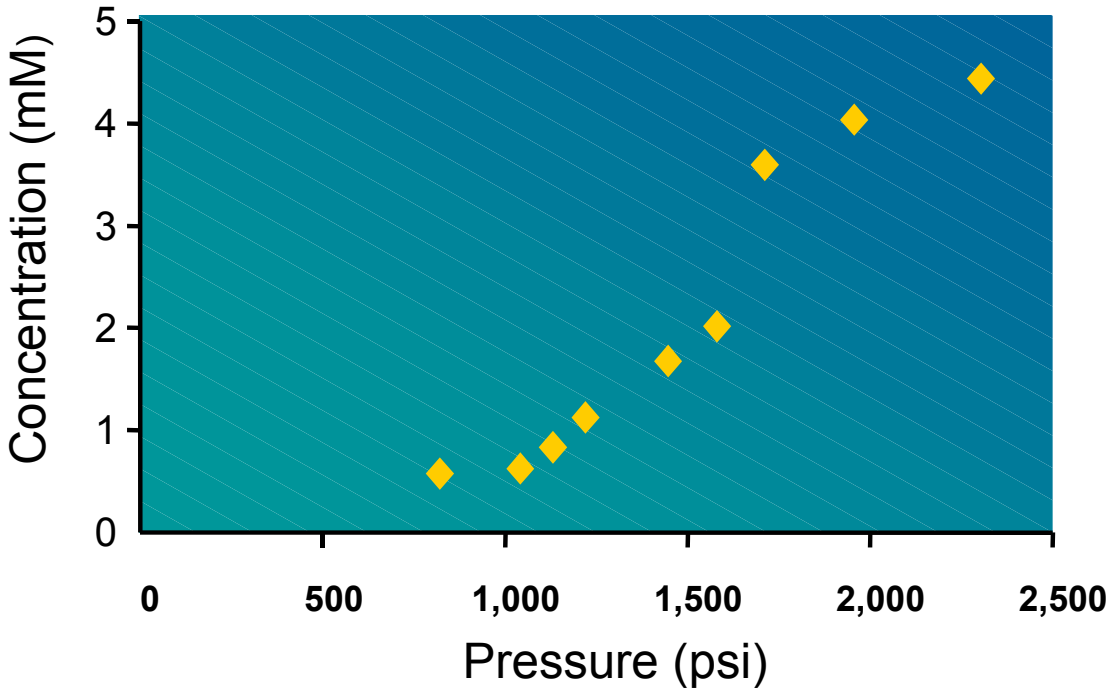



Figure 3: Solubility of hydrogen peroxide in supercritical carbon dioxide at 40 °C.

**Biooxidation in Reverse Micelles and Emulsions:**

An aqueous phase buffer biooxidation reaction was run as a base case to compare results obtained in SCFs. The reaction was run using Hb in acetate buffer, pH=5.0, at concentrations of 3 □M DBT, 1 mM $H_2O_2$ and 12.6 mM enzyme. The mixture was stirred at 40 °C for 2 hours. Conversion to product in the liquid buffer was 30.2 wt.%. Due to the low solubility of DBT in the buffer solution, this conversion represents a product yield of 0.9 □M.

We previously studied the protein biooxidation of DBT in pure and modified SCFs. Hb and Cyt c were used in most of the cases since they are known to be more active towards the oxidation of DBT than HRP[7]. Proteins were studied in their free, lyophilized, PEG-conjugate forms, and immobilized on glass beads, alumina based support and agarose beads. In all cases, only very small amounts of product were formed (<1 %). Control experiments showed that the low yield was due to protein inactivation caused by high localized concentrations of hydrogen peroxide and that a more hydrated environment was needed for the protein. The water pool of a reverse micelle appeared to be a promising media to provide the desired environment.

Reverse micelles and microemulsions consisting of water and SCFs can simultaneously disperse high concentrations of both hydrophilic molecules such as proteins and hydrophobic compounds. Larger amounts of water may be dispersed in SCFs by forming emulsions, allowing more hydrophilic materials to be solubilized in the fluid. Several research groups have demonstrated that stable reverse micelles, microemulsions and emulsions based on perfluoropolyetherammonium carboxylate (PFPE) ($CF_3O(CF_2CF(CF_3)O)_nCF_2COO^-NH_4^+$) surfactant can be formed in supercritical carbon dioxide.[11,12] These emulsions are easily broken by decreasing pressure, allowing for simple catalyst recovery and energy efficient product separation.

**General procedure for biooxidation of DBT in emulsions in supercritical fluids.** DBT, surfactant, buffer and solid protein were loaded into the open reactor. The vessel was closed and sealed. The vessel and its contents were heated to 40 °C for 1.5 hr and then pressurized to 1000 psi with the selected SCF. The HPLC injector valve was used to add a 0.5 ml dilute solution of $H_2O_2$ with the final volume of SCF that was used to bring the reactor to its final pressure. Final concentrations were: 0.28 mM DBT, 1 – 25 □M protein, 1.4 wt% surfactant, and 4.48 mM hydrogen peroxide ($H_2O_2$). The contents of the vessel were stirred in the absence of light for 15 hours. Gas was slowly vented and bubbled through a solution of methylene chloride and methanol. The reactor was opened. Methylene chloride and methanol were used to wash and transfer the contents of the cell and the stirring shaft. The combined methylene chloride and methanol washes were evaporated by rotary evaporation at low temperature, followed by the evaporation of water at 60 °C for 1.5-2 hours. The resulting mixture was then redissolved in a known volume of methylene chloride and analyzed by GC/FID or GC/MS.

**General procedure for biooxidation of DBT in reverse micelles/microemulsions in supercritical fluids** was the same as for biooxidation in emulsions, except for the following. The vessel was pressurized with the desired fluid to 90 % of the final pressure. The contents of the vessel were stirred for 45 minutes to allow micelle formation. Micelles were visually detected by the appearance of a single phase. A buffered hydrogen peroxide solution was then added simultaneously with the remaining fluid via an HPLC injector.

PFPE was prepared according to a literature procedure[13] by reacting perfluoropolyether carboxylic acid (Fluorolink 7004, average MW = 615, Lot BL5420, Ausimont) with an excess of ammonium hydroxide solution, except for the final drying step. In this work, final drying was begun at 25 °C and gradually increased to 60 °C.

The biooxidation of DBT was explored using the proteins HRP, Hb, Cyt c and SBP with the surfactants sodium bis (2-ethyl 1–hexyl)-sulfosuccinate (AOT), cetyl trimethyl ammonium bromide (CTAB), and perfluoropolyetherammonium carboxylate (PFPE). Supercritical fluids studied were carbon dioxide and ethane. The AOT surfactant requires co-solvents such as octanol and hexanol in order to dissolve reasonable quantities of water in SC $CO_2$. These co-solvents were found to suppress the biooxidation of DBT by Cyt c. Further it was determined that AOT or a hydrolysis product formed in the presence of AOT suppressed the biooxidation by Hb when the reaction was conducted in SC ethane.

Biooxidation of DBT in PFPE reverse micelles in carbon dioxide was explored as a potential desired reaction media. Since carbonic acid with a pH of about 3 forms when aqueous solutions are in contact with $CO_2$ at high pressures, 1M buffers (potassium phosphate, pH = 7, Tricine, pH = 8.5 and sodium acetate, pH = 6.83) were employed. When 8.4 μM Hb was added with a 1 M potassium phosphate buffered solution, micelles were formed after 25 min. of stirring, as detected by the observation of a single phase. However no product was formed. Additional reverse micelle studies also failed to result in product formation.

It became clear that solution pH, driven by the presence of SC $CO_2$, negatively impacted enzyme activity and function. Therefore, buffer capacity played an important role. To explore this effect, control experiments were conducted using a 40 vol.% pool of liquid in the reactor exposed to an upper bed of SCF. Potassium phosphate buffer at 0.025 M and 1 M, pH = 7, were used in the hemoglobin-catalyzed oxidation of DBT in an aqueous buffer/ethanol (80/20 vol.%) solution exposed to $CO_2$ at 3,000 psi. Less than 1 % product was formed at the high buffer concentration and no product was formed at lower buffer concentrations. When similar solutions were exposed to a 3,000 psi bed of ethane, the reaction resulted in a 26 % product yield, even at the low buffer concentration. This data suggests that the acidity caused by presence of SC $CO_2$ negatively impacted the activity of the protein.

Surfactant – protein interactions were explored by comparing product formation for the protein-catalyzed oxidation of dibenzothiophene (DBT) in the presence and absence of surfactant. Comparisons were carried out in 80 % aqueous buffer – 20 % ethanol solutions at 40 °C for two hours. Interactions were explored between surfactants AOT, CTAB, and PFPE and the proteins, HRP, Hb and SBP. The protein SBP and the surfactant PFPE appeared promising. SBP is active at low pH which is compatible with oxidation in organized media using supercritical (SC) carbon dioxide with the PFPE surfactant.

Soybean peroxidase (SBP) is a commercially available, relatively inexpensive enzyme that exhibits high stability at acidic pHs and higher temperatures.[14,15] SBP is known to be active towards oxidation of methyl phenyl sulfide in glycine buffer.[16] We found that SBP exhibited similar activity towards the oxidation of DBT in an ethanol/glycine buffer (20/80 vol.%) solution at pHs from 2.4 to 3.5. We carried out the SBP-catalyzed oxidation of DBT in a 50/50 wt.% $CO_2$/glycine buffer emulsion using 0.02 mM glycine buffer, pH = 2.4, at 3,000 psi and at two different temperatures, 45 °C and 65 °C, for 15 hours. The protein was more active at 65 °C. At 45 °C, product yield was less than 1 %, but increased to 4.1 % at 65 °C. At the higher DBT solubility obtained in the emulsion, the 4.1% conversion represents a product yield of 11.4 □M; 12 fold higher than that obtained in the aqueous base case.

These results were consistent with SBP catalyzed biooxidation of methyl p-tolyl sulfide which showed an enhanced yield at the optimum temperature (65 °C). Therefore, we studied the SBP-catalyzed oxidation of DBT in $CO_2$ – glycine buffer emulsions and microemulsions, pH = 2.4, at 2,000 and 3,000 psi and at various water contents present in the reaction. Results are presented in Table 1.

Table 1. SBP-Catalyzed Oxidation of DBT in $CO_2$/Glycine Buffer Emulsions for 15 hours. (Reaction conditions: 0.02 mM glycine buffer, pH=2.4, 0.28 mM DBT, 1 mg/ml SBP, 1.4 wt% PFPE, and 4.48 mM $H_2O_2$.)

| Buffer (wt.%) | $CO_2$ (wt.%) | Pressure (psi) | Temperature (°C) | Conversion to Product (wt.%) | Product Yield (mM) |
|---|---|---|---|---|---|
| 0.75* | 99.25 | 3000 | 65 | 0.5 | 0.001 |
| 30 | 70 | 3000 | 65 | 2.8 | 0.0078 |
| 50 | 50 | 3000 | 65 | 4.1 | 0.0114 |
| 50 | 50 | 2000 | 65 | Trace | - |
| 50 | 50 | 3000 | 45 | Trace | - |

*microemulsion

Product yield increased with the amount of aqueous buffer. In the microemulsion, with less than 1 wt.% buffer solution, the hydrogen peroxide may be localized in the layer of hydration surrounding the protein. As aqueous buffer quantity increased, the concentrations of hydrogen peroxide decreased, providing a more suitable environment for protein activity. At the lower pressure of 2,000 psi, only a trace of product was observed. It was assumed that this pressure was not sufficient to provide sufficient solubility for the aqueous emulsion phase. No product was formed in the absence of surfactant, or protein or hydrogen peroxide, suggesting that the reaction took place in the emulsion. However, the emulsion was not stable and required continual mechanical agitation. Even with constant stirring, the emulsion broke somewhat during the course of the reaction and by the end of the experiment, the cell contained a lower emulsion phase and an upper $CO_2$ phase. More aggressive stirring may have been necessary and may have increased conversion to product. Despite the modest conversions, total product yield was more than an order of magnitude greater in the SCF emulsion than in the aqueous phase reaction.

## Initial Microbial Evaluation and SCF Biooxidation Studies:

The initial project work evaluated microbial enzyme production and protein catalyzed biooxidation in simple SCF solvent systems. These efforts were largely unsuccessful, but are discussed in detail below to provide a complete coverage of the project work and to demonstrate the motivation to explore the emulsion environments. Microbiological experiments were conducted to evaluate the possibility of producing enzymes from previously isolated strains of microorganisms for evaluation to compliment commercially available material. Initial SCF studies were carried out to determine the feasibility of carrying out the biooxidation reaction in simple SCF systems. Theses efforts explored the protein catalyzed reaction in pure SCFs, modified SCFs, SCF-liquid systems. Proteins were studied in their free, lyophilized, PEG-conjugate forms, and immobilized on glass beads, alumina based support and agarose beads.

## Evaluation of Microbial Enzyme Production:

The American Type Culture Collection (ATCC) catalog alone contains 103 bacteria and 163 filamentous fungi capable of sulfur transformation. Of those available, four cultures were selected (Table 2) for amplification and induction. A typical growth experiment consisted of growing 1 liter of cells in a 2 liter erlenmeyer flask. An overnight stock was prepared by defrosting a frozen stock of the organism(s) and transferring it into fresh nutrient broth. The nutrient broth culture was incubated at 30 °C overnight. A 5 % volume of the overnight culture was used for inoculum to 1 liter of growth medium. The growth medium was incubated at 30°C and agitated at 150 rpm for 40 hours. The cells were removed by centrifugation, (10 °C, 15 minutes, 6,812 x g) and washed twice with 67 mM phosphate

buffer. After decanting the buffer, a wet weight of the cells was obtained. Cell weights ranged from 3-7 g. The wet cells were stored overnight at –4 °C.

Table 2. Organisms selected for evaluation

| ORGANISM | ATCC NUMBER | DESIGNATION | DEPOSITOR |
|---|---|---|---|
| 1. Rhodococcus rhodochrous | 53968 | IGTS8 | Inst. Gas Tech. |
| 2. Comamonas testosteroni | 39523 | DBT2 | W.R. Grace Co. |
| 3. Pseudomonas stutzeri | 39524 | DBT3 | W.R. Grace Co. |
| 4. Unidentified mixed culture | 39327 | - | Stevens, Burgess |

An induction experiment consisted of removing the cells from cold storage and adding them to 100 mls of induction medium in a 500 ml erlenmeyer flask containing 0.6 mM dibenzothiopohene suspended in ethanol. The cells were "induced" for 5 hours at 30°C, 150 rpm according to the protocol of Honda, et al.[17] This technique amplifies biomass with a robust growth media and then induces the enzymes responsible for transformation of organosulfur compounds with 0.6 mM DBT. This technique theoretically compresses experiments from 300 hrs to 40 hrs. Samples (4-5 ml) were removed from the culture broth at 0 and 5 hours and acidified with 6N HCl. At the termination of the experiment, the induced cells were removed by centrifugation (10 °C, 15 minutes, 6,812 x g), resuspended in 100 mM phosphate buffer and sonicated (4 cycles, 1 cycle = 30 sec sonication; 30 sec cooling on ice). The cellular debris was removed by centrifugation and the supernatant was collected. Dibenzothiophene and 2-hydroxybiphenylwere extracted from the 1) suspension of sonicated cells, 2) sonicated supernatant, and 3) the 0 and 5 hour samples by adding an equal volume of methylene chloride. The methylene chloride layer was removed and used for GC analysis.

In general, DBT concentration in the culture was depleted by the presence of cells. However, this apparent depletion was recovered from the cell pellet, and was not transformed to 2-hydroxybiphenyl, indicating the enzymes had not been induced. Separation was by GC with mass spectroscopy and flame ionization detection. Analytical concentration range was 0 - 1 mM with an $R^2$ of 0.9937 for DBT and 0.9938 for HBP. It was originally suspected that repression occurred from exogenous sulfur compounds in the DBT (non- recrystalized in these experiments). Subsequent analysis of the growth and induction medium for sulfate, a known repressor of DBT desulfurization, resulted in finding no sulfate in the induction medium and 1,347.5 ppm in the growth medium.

At this point it was deemed that commercially available enzymes and proteins would be more convenient as well as economically favorable for experimental application to demonstrate the feasibility of utilizing SCF technology in combination with biocatalysis for heavy oil upgrading.

**Biooxidation in Liquid and SCF Solvents:**

Biooxidation of DBT was carried out in simple liquid and SCF solvent systems. Reactions were carried out in liquid phase solvents to develop a base case for comparing results obtained in SCFs. Liquid phase reactions were run in an aqueous buffer, ethanol, ethanol/aqueous buffer mixture and hexane at atmospheric pressure. One volume percent water was added to the nonaqueous solvents. Reactions were run for 2 hours at 40 °C. Reaction concentrations were 0.23 mM DBT, 10 mM $H_2O_2$, and 25 □M protein. Due to the low solublility of DBT in water, the aqueous reaction was performed using Hb in acetate buffer, pH=5.0, at concentrations of 3 □M DBT, 1 mM $H_2O_2$ and 12.6 mM enzyme.

SCF experiments employed carbon dioxide, methane and ethane. Reactor concentrations were the same as those used in the non-aqueous solvents (0.23 mM DBT, 10 mM $H_2O_2$, and 25 □M protein).

The reactions were run at 40 °C for up to 6 hours. SCF reaction pressures were between 2,100 psi and 2,300 psi.

Results for the aqueous, non-aqueous liquid and SCF experiments are shown below in Table 3. In the aqueous phase, a reasonable conversion to product (30 wt.%) was obtained. However, since the quantity of DBT reacted was quite small, as limited by solubility, the overall product yield was extremely low. By carrying out the reaction in ethanol, where DBT solubility was not limiting, a similar conversion resulted in a 75 fold increase in product yield. The conversion and product yield were increased further by increasing the water content in the ethanol from 1 vol.% to about 12 vol.% resulting in over two orders of magnitude improvement in product yield. In contrast, no conversion to product was observed when the reaction was explored in non-polar solvents, in both liquid and SCF phases.

Attempts were made to improve the polar environment by adding modifiers to the SCF phase. Both methanol and ethanol were individually added to SC ethane at a mole fraction of 0.02. At reaction temperatures of 40 °C and pressure ranging from 2,200 to 2,500 psi, conversions were typically less than 1 wt.% after two hours. These studies suggested that a more polar or hydrated environment was needed for the protein.

Table 3. Biooxidation of DBT in simple liquid and SCF systems at 40 °C.

| Solvent[1] | Phase | Protein | Conversion to Product (wt.%) | DBT Conc. (M) | Product Yield (mM) |
|---|---|---|---|---|---|
| Buffer[2] | Liquid | Hb | 30.2 | $3x10^{-6}$ | 0.0009 |
| Ethanol | Liquid | HRP | 2.4 | $2.3x10^{-4}$ | 0.0055 |
| Ethanol | Liquid | Hb | 29.6 | " | 0.068 |
| Ethanol/Aq. Buffer (7.6/1) | Liquid | Hb | 70.0 | " | 0.161 |
| Hexane | Liquid | HRP, Hb | None | " | - |
| Carbon Dioxide | SCF | HRP | None | " | - |
| Methane | SCF | Hb | None | " | - |
| Ethane | SCF | Hb | None | " | - |

1. 1 vol.% water used in non-aqueous solvents.
2. Acetate buffer, pH 5.0.
3. Acetate phosphate buffer, pH 5.2.

Several fundamental experiments were performed to explore biocatalyst in the SCF reactor. Both DBT and the protein were placed in the liquid phase while a layer of supercritical fluid was maintained in the top of high pressure reactor. Methane and ethane were employed as the SCFs at pressures from 2,200 to 2,300 psi using ethanol or methanol with 1 vol.% water as the liquid phase. Conversions to product using Hb ranged from 26 to 30 wt.%. This study showed that protein was active in the high-pressure environments.

A similar study was performed where the protein was placed in the liquid phase while the DBT was placed in a porous container suspended in the SCF phase. This study was performed to determine if the SCF could be used to solubilize and transport the DBT to the active protein environment. The liquid phase was composed of ethanol with 0.45 vol.% water and represented 10 vol.% of the reaction mixture. Supercritical fluids included carbon dioxide, methane, and ethane. The reaction was run for 2 hours at a temperature of 40 °C and pressures from 2,200 to 2,300 psi. Results are shown below in Table 4. The lack of conversion to product in the methane was attributed to the poor solubility and transport of DBT in that fluid. Conversion using ethane and carbon dioxide were still low.

Table 4. Two Phase Reaction with Hb Suspended in a Liquid Layer

| SCF | Liquid[1] | Pressure (psi) | Temperature (°C) | Conversion to Product (wt.%) | Product Yield (mM) |
|---|---|---|---|---|---|
| $CH_4$ | EtOH | 2310 | 39 | None | - |
| $C_2H_6$ | EtOH | 2220 | 41 | 0.7 | 0.002 |
| $CO_2$ | EtOH | 2220 | 41 | 1.2 | 0.003 |

1. 10 vol.% liquid containing 0.45 vol.% water.

Additional work was performed with the protein in the liquid phase and the DBT suspended in the SCF phase. This effort evaluated the nature of the liquid phase. The SCF explored was ethane, the biocatalyst hemoglobin (Hb). The liquid phase was a mixture of ethanol and aqueous buffer, where the ethanol to aqueous buffer volumetric ratio was varied between 1:4.25 to 20:1. Conversion of DBT to its sulfone product ($DBTO_2$) was only observed at the highest concentration of ethanol and ranged from approximately 1 to 2 %. Control experiments suggest that significantly higher levels of conversion were attainable but transport of the DBT from the SCF to liquid phase limits the overall extent of transformation.

Since the proteins explored exhibited little to no transformation in non-polar fluids, we chose to explore a protein designed to operate in organic solvents. Oak Ridge National Laboratory donated a sample of a polyethyleneglycol (PEG) – cytochrome c (Cyt c) conjugate. Initial efforts explored the oxidation of thioanisole. Reaction conditions were 1 mM thioanisole, 1 mM $H_2O_2$, 0.3 g/L PEG-Cyt c at 40 °C for two hours. Low levels of conversion to product were observed in liquid aqueous buffer solution and in benzene. Studies in supercritical solvents found no conversion to product in SC ethane, but minor amounts of conversion in SC carbon dioxide and SC trifluoromethane. By modifying the SC trifluoromethane with 2 vol.% ethanol and operating at a pressure of 2240 psi, up to 5 wt.% sulfoxide product was observed.

The substrate was changed to DBT. In an acetate phosphate buffer (pH =5.2)/ethanol solution at a volumetric ratio of 80/20, transformation of DBT to products at room temperature was measured to be 58 wt.%. No transformation to product was observed in liquid toluene or SC trifluoromethane with 2 vol.% ethanol.

**Protein Immobilization**:

The biooxidation experiments using supercritical fluids with and without liquid reaction layers demonstrated that hydrated environments were necessary for protein activity along with high levels of substrate solubility and mass-transfer. In an attempt to provide these conditions, protein immobilization was explored. By maintaining a layer of hydration around the protein and by providing high surface area from the support, it was anticipated that the conversion to product could be improved. Solid supports investigated included glass beads, agarose, and a proprietary support provided by UOP. In all cases, only very small amounts of product were formed (<1 %). Control experiments showed that the low yield was due to protein inactivation caused by high localized concentrations of hydrogen peroxide and that a more hydrated environment was needed for the protein. The water pool of a reverse micelle appeared to be a promising media to provide the desired environment.

## Technology/Information Transfer

The scientific information from this research project was disseminated to the technical community through the following publications and presentations:

## Publications:

Marina A. Stanescu, Daniel M. Ginosar, Gregory A. Bala and Raymond P. Anderson, "Biocatalytic Treatment Of Organosulfur Compounds In Emulsions In Supercritical Fluids" submitted to *Fuel Chemistry Preprints, ACS*, March 2002.

## Presentations:

Marina A. Stanescu, Daniel M. Ginosar, Gregory A. Bala and Raymond P. Anderson, "Biocatalytic Treatment Of Organosulfur Compounds In Emulsions In Supercritical Fluids" submitted to the 224th ACS National Meeting, Division of Fuel Chemistry, Boston, MA, August 18-22, 2002.

M.A. Hauck, D.M. Ginosar, G.A. Bala, R.P. Anderson, "Hemoproteins-catalyzed oxidations of organosulfur compounds in reverse micelles, microemulsions, and emulsions in supercritical fluids", 222nd American Chemical Society National Meeting, Division of Organic Chemistry, paper 487, Chicago, IL, August 26-30, 2001.

Raymond P. Anderson, Gregory A. Bala, Daniel M. Ginosar and Marina A. Hauck, "Bioprocessing of High Sulfur Crudes via Application of Critical Fluid Biocatalysis", DOE-FE 2000 Petroleum Technology Contractor Review, paper EPTA-04, Denver, CO, June 27-29, 2000.

M. A. Hauck, D. M. Ginosar, G. A. Bala and C. Rae, "Hemoprotein-Catalyzed Oxidation of DBT in Supercritical Fluids", 2000 Northwest & Rocky Mountain Joint Regional Meeting, Paper 150, Idaho Falls, ID, June 15-17, 2000.

## Conclusions

This research effort demonstrated that the solubility of organic sulfur compounds could be enhanced 5,000 fold by employing supercritical fluids. Increased solubility over levels in aqueous liquids is an essential element necessary for amplifying biooxidation reaction rates. Providing an appropriate environment for high protein activity is also required. Biooxidation of DBT in emulsions in carbon dioxide appears to be promising and can be extended to organosulfur compounds in general. We found an order of magnitude increase in product yields in water/$CO_2$ emulsion systems compared to aqueous systems. Future efforts that can improve the effective conversion to product while maintaining high levels of product and substrate solubility could potentially result in reaction rate improvements from 2 to 4 orders of magnitude.

# REFERENCES


1. Swain, E.J. "U.S. Refiners Face Declining Crude Quality, Insufficient Price Spread." Oil and Gas J., March 1993, 62-65.

2. Shong, R. Personal Communication, Texaco Co., September 1998.

3. Monticello, D.J. *Curr. Oppin. Biotech.* **2000**, *11*, 540.

4. Klyachko, N.L.; Klibanov, A.M. *Appl. Biochem. Biotech.* **1992**, *37*, 53.

5. Klibanov, A.M. *Nature*, **2001**, *409*, 241.

6. Hutchenson, K.W.; Foster, N.R. In *Innovations in Supercritical Fluids, ACS Symposium Series*; Hutchenson, K.W., Foster, N.R., Eds.; American Chemical Society: Washington, DC, 1995; Vol. 608, p 1.

7. Mesiano, A.J.; Beckman, E.J.; Russell, A.J. *Chem Rev*. **1999**, *99*, 623.

8. Dordick, J. S.; Ryu, K.; McEldoon, J. P. *Resources, Conservation and Recycling*, **1991**, *5*, 195.

9. Mitra, S.; Chen, J. W. *J. Chem. Eng. Data* **1988**, *33*, 35.

10. Ta-Chang, L., Ph.D. Dissertation, Southern Illinois University at Carbondale, 1988.

11. Johnston, K.P.; Harrison, K.L.; Clarke, M.J.; Howdle, S.M.; Heitz, M.P.; Bright, F.V.; Carlier, C.; Randolph, T.W.; *Science*, **1996,** *271*, 624.

12. Lee, C.T.; Psathas, P.A.; Johnston, K.P.; deGrazia, J.; Randolph, T.W. *Langmuir*, **1999,** *15*, 6781.

13. Chittofrati, A.; Lenti, D.; Sanguineti, A.; Visca, M.; Gambi, C.; Senatra, D.; Zhou, Z. *Progr. Colloid Polym. Sci.* **1989**, *76*, 218.

14. Kraus, J.J.; Munir, I.Z.; McEldoon, J.P.; Clark, D.S.; Dordick, J.S. *Appl. Biochem. Biotech.* **1999**, *80*, 221.

15. McEldoon, J.P.; Dordick, J.S. *Biotechnol. Prog.* **1996**, *12*, 555.

16. Johnson, M.A.; Pokora, A.R.; Cyrus, J.; William, L. U.S. Patent 5,391,488, 1985.

17. Honda, H.; Sugiyama, H.; Saito, I.; Kobayashi, T. "High Cell Density Culture of Rhodococcus rhodochrous by pH-Stat Feeding and Dibenzothiophene Degradation," 1998. J. Ferment. Bioeng., **1998**, *85*, 334.