UTILIZATION OF CARBON DIOXIDE FROM FOSSIL FUEL-BURNING POWER PLANTS WITH BIOLOGICAL SYSTEMS

John R. Benemann and David M. Tillett*
1212 Kelley Ct., Pinole, California, 94564; * SeaAg, Inc.,
5220 Old Dixie Hwy., Ft. Pierce, Florida, 34946

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ABSTRACT

Currently available physical-chemical processes for CO_2 capture and disposal from fossil fuel-fired power plant flue-gases are very expensive. Biological systems for CO_2 utilization involve plant photosynthesis and conversion of the biomass produced to fuels that can substitute for fossil fuels. Photosynthesis by many plants increases with higher CO_2 levels suggesting that flue gas fertilized greenhouses or even flue gas dispersal into open plant stands could increase biomass production. However, such systems are neither effective nor practical. Only for submerged aquatic plants - microalgae, seaweeds, and some higher plants - does a high concentration of CO_2 as present in flue-gases, result in large increases in productivity. Microalgae have the potential for high productivities and ready conversion to gaseous and liquid fuels. A cost-analysis of such a process suggests that if high productivities are indeed achievable, overall costs could be much lower than currently available methods for CO_2 flue gas capture and disposal. Limitations are the relatively large land areas required, a maximal reduction in CO_2 outputs of only 25 to 30% of total emissions, and the relatively undeveloped state of this technology.

INTRODUCTION

Reducing CO_2 loads on the atmosphere is required to forestall potentially catastrophic consequences of the greenhouse effect. Although the possible consequences are highly uncertain, reducing the current and projected rise in CO_2 concentration appears to be a prudent course of action. Several European countries and Japan are proposing to reduce current levels of CO_2 emissions from fossil fuels by 20% to 25%. Natural processes already remove 50 to 60% of anthropogenic CO_2 emissions, thus a 25% reduction would actually slow atmospheric CO_2 increases by between 40% to 50%, assuming current conditions continue. This would significantly reduce the probabilities of the catastrophic consequences of the greenhouse effect (Benemann, 1992). Fossil fuel-burning power plants generate about 25% of all fossil fuel derived atmospheric CO_2 inputs. Thus they are a major target in plans to reduce CO_2 accumulation in the atmosphere.

Reducing the CO_2 outputs of fossil fuel-fired power plants could be accomplished through a number of methods, such as increasing efficiency in fossil fuel utilization, substitution of fossil fuels with energy sources that do not produce net CO_2 emissions, establishing remotely sited reforestation projects that would sequester CO_2 into standing biomass (Marland, 1988), and recovery and subsequent sequestration of CO_2 directly from flue (stack) gases of fossil fuel-burning power plants. The latter options, CO_2 sequestration from flue gases using presently available chemical scrubbing systems, appear to be much more expensive than the former. Recent cost-analysis of the costs of

CO_2 removal and concentration from stack gases suggests that this would essentially double current electricity costs, and the amount of fossil fuel used. This does not include ultimate disposal of the sequestrated CO_2 (in the ocean depths, depleted oil and gas wells) which add to costs and uncertainties (Herzog et al., 1991; Fluor Daniels, 1991).

BIOLOGICAL CO_2 MITIGATION OPTIONS

A variety of microbes utilize CO_2. But all, except for the microalgae, require some inorganic reducing agent (H_2, H_2S, NH_3, pyrites, etc.). Such substrates are unlikely to be available in the quantities required for CO_2 removal from power plants. If nuclear or solar (photovoltaics) electricity generation were to allow economical H_2 production, it could be plausibly converted to a C-based fuel using CO_2. The methanogenic bacteria are able to convert H_2 and CO_2 into CH_4. However, it is likely that methanol would be the preferred product, as it would be useable as a liquid fuel, favoring chemosynthetic processes. Thus, only photosynthetic processes based on water as the electron source for CO_2 reduction are likely applicable for CO_2 mitigation.

Photosynthetic processes are able to convert CO_2 into biomass, which can be used or in turn converted to biomass fuels that can replace fossil fuels, either for electricity production or in other sectors of the economy (e.g. transportation) (Benemann, 1980). Plant photosynthesis is already a major world-wide source of fuels, with biomass fuels representing about 15% of all primary energy consumption (Scurlock and Hall, 1987). Biomass fuels could displace a major fraction of current fossil fuel consumption, particularly if CO_2 mitigation were to become a policy and economic goal.

Biomass production takes place in the presence of atmospheric levels of CO_2, the concentrated CO_2 present in flue gases is not required. Nevertheless, it is well known that plants exhibit higher productivities under elevated levels of CO_2. In greenhouses, elevated levels of CO_2 are routinely used to increase plant production. The cultivation of algae, both the seaweeds and the smaller microalgae, requires an enriched source of CO_2, as the transport of CO_2 from the atmosphere into the growth ponds is not sufficient to support their growth. These possibilities were reviewed in the present evaluation of biological systems for direct utilization and mitigation of stack-gas CO_2 sources.

PHOTOSYNTHETIC PROCESSES FOR FLUE GAS CO_2 UTILIZATION

The use of higher plants, either in greenhouses or in the open air, for the utilization of flue gas CO_2 has been proposed (Bassham, 1977) but does not appear practical nor feasible. CO_2 fertilization can increase plant productivities by a significant factor (20 to 30% are typical enhancements, although higher values are reported, see Benemann, 1982, for references). However, comparisons with open air cultivation are not as favorable because of the reduction in light intensities in greenhouses due to glazing - which typically are in the same range as the CO_2 fertilization effects. Thus, overall, greenhouse agriculture is, in principle, not significantly more productivity than open air systems. However, greenhouse agriculture does exhibit high productivities (Wilson et al., 1992), but for other reasons: greater control over water supply and fertilizers, higher management inputs, and, of course, temperature control to overcome low temperatures in unfavorable climates. However, greenhouse crops cost typically over ten times more to produce than open air crops. Thus, greenhouses would not be a suitable method for biomass fuels production, the objective of any CO_2 mitigation program.

An alternative possibility would be to fertilize open air stands of plants (trees, row crops) with flue-gas CO_2, dispersed through distribution pipes. The major factors to consider are the effects of wind, turbulence, etc. on the dispersal plume and the effects of highly variable CO_2 concentrations on plant productivity. Experimental systems are being operated, to study the effects of CO_2 on natural stands (e.g. outside of greenhouses) in which dispersal is through distribution pipes which discharge CO_2 from various points in a stand, computer controlled to adjust for variations in wind direction, intensity, daytime, etc. In a large stand, encompassing many square miles, such systems may indeed achieve a relatively good dispersal. However, the incremental productivities, estimated at 20 to 30% from those observed without CO_2 supplementation, and most likely only half those, would not likely justify the extensive distribution piping and control systems required. Also, the actual utilization factor for the CO_2 is likely to be low. This preliminary analysis suggests that such a process could not be justified.

This leaves the submerged plants - microalgae, seaweeds and some higher plants - as the only biological systems which could benefit from the use of flue gas levels of CO_2 (typically about 10% by volume). The transfer of CO_2 from the atmosphere into a pond, assuming essentially zero CO_2 in the ponds, would only support 1 to 2 g of biomass production/m^2 per day, a small fraction (< 5%) of potential productivity. Energetically it is not feasible to supply CO_2 by bubbling air through the cultures, to provide CO_2 (Steinberg, 1991). Only a highly enriched source of CO_2, e.g. flue-gas, could supply submerged plants with the CO_2 required. Thus, among biological systems only submerged plants could make use of flue gas CO_2 sources.

SUBMERGED HIGHER PLANTS AND SEAWEEDS

Of the three alternatives - microalgae, seaweeds, and higher plants - the latter two have significant limitations. Higher submerged plants exhibit relatively low productivities, even under optimal conditions of nutrient and CO_2 supply (Murry and Benemann, 1980). The reasons for this is primarily due to hydrodynamic factors; it is difficult to get good water exchange in dense stands of such submerged plants. In water CO_2 diffusion is over 1,000 times slower than in air, and thus not only the transfer into the ponds but also from the water phase to the leaves is a major limitation in such systems. Creation of sufficient turbulence to overcome diffusion limitations does not appear practical.

Seaweeds exhibit relatively higher productivities than submerged higher plants, and have been produced commercially in near-shore, shallow ocean systems and have been considered for energy production (See Bird and Benson, 1987, for reviews). In such systems the C required is provided from seawater, and the relatively high water exchange in such open systems. Seawater, at pH 8.2 and 2.3 meq. l^{-1} alkalinity, contains almost 40 mg of available CO_2 (assuming an upper pH of about 10 for seaweed growth). A 1 m deep culture system thus would be able to support a production of about 20 g/m^2 of biomass (organic dry weight assuming 50% C), suggesting a once to twice a day water turn-over to supply the required C. Thus, seaweed culture, as currently practiced in near-shore environments, would not be limited by C, and would not require flue gas CO_2 injection.

In on-shore, shallow ponds, water exchange would become a limiting factor, depending on lift (head losses). From the above, it would require approximately 50,000 m^3 of seawater to supply the C required for one ton of biomass, which would make seawater an uneconomical source for this nutrient for on-shore systems. The advantages of on-shore,

vs. near-shore, cultivation is that a higher control over cultivation conditions is feasible, including predations, competing algae, diseases, etc. Also, other nutrients (N, P, Fe, etc.) can be supplied without the large losses experienced in near-shore farming techniques. And, perhaps most important, the losses experienced due to storms would be minimized. However, seaweed culture in open ponds has not been developed except on an experimental basis. The reason for this are the hydrodynamic constraints on such cultures: the rather dense seaweed cultures require considerable mixing and turbulence to allow effective transfer of nutrients (particularly CO_2) to the plants (Wheeler, 1988). Such mixing and turbulence requires considerable energy inputs, which would make such systems impractical, at least for the production of fuels. Although preliminary, this evaluation suggests that on-shore seaweed cultivation is not a favorable approach to flue gas CO_2 utilization. These mixing limitations do not apply to the smaller microalgae.

MICROALGAE FOR CO_2 MITIGATION

Microalgae culture technology has been developed for over 40 years (Burlew, 1953). The concept of using the CO_2 in power plant flue gases for producing microalgae and converting the biomass to fuel, was first studied over thirty years ago by Oswald and Golueke at the University of California Berkeley. They proposed using municipal sewage to grow algae in large open ponds into which flue gas would be injected, harvesting the biomass by settling, and digesting it to methane gas, which would be used by the power plant. The digester residues (containing the nutrients and residual organic and inorganic C) and water would be recycled, allowing system size expansion well beyond that feasible from the production of algae for waste water treatment alone. A laboratory-scale system, involving algae growth, digestion to methane, and recycle of the nutrients, was successfully demonstrated (Golueke and Oswald, 1959). A preliminary analysis concluded that with favorable assumptions this process could be economically competitive with nuclear power (Oswald and Golueke, 1960). This concept was further refined by Oswald and colleagues and others during the 1970's, with research sponsored in large part by the U.S. Department of Energy (U.S. DOE). Conceptual engineering designs and cost estimates (Benemann et al., 1982) supported the conclusion that, in principle, algal biomass cultivation in open ponds could be relatively inexpensive.

About a decade ago, the "Aquatic Species Program" (ASP), was initiated at the Solar Energy Research Institute (SERI, now NREL, National Renewable Energy Laboratory, a U.S. DOE facility). The ASP emphasized the development of algae systems for the production of liquid transportation fuels (specifically vegetable oils) (Neenan et al., 1986). This program, supported many basic research projects, including isolation of a large number of algal strains and investigation of biochemical and genetic aspects of lipid production in microalgae. Three outdoor algal production facilities were supported by the ASP, in California Hawaii and New Mexico. These outdoor projects, together with an updated engineering and cost feasibility analysis (Weissman and Goebel, 1987), and the considerable experience from commercial operations for microalgae production for food supplements (Benemann et al., 1987), and use of microalgae in waste water treatment (Oswald and Benemann, 1980), support the conclusion that, in principle, it is possible to produce microalgae in large-scale outdoor ponds at both high productivity and at relatively low cost. Microalgae are now being studied for CO_2 mitigation in the U.S. and Japan (Laws and Berning, 1991, Negoro et al., 1991, for examples). However, considerable R&D is still required and many aspects of this technology remain to be demonstrated, from species control and stability, to harvesting and algal processing.

COST ANALYSIS OF MICROALGAE CO_2 MITIGATION

Table 1 summarizes the overall cost estimates for a large-scale (appx. 1,000 ha) microalgae production system for liquid-fuels using flue gas CO_2 from a power plant. Using a "CO_2 mitigation credit" of \$16/tCO_2, fuel costs of about \$40/barrel are projected. These cost estimates are based on prior studies (Benemann et al., 1982; Weissman and Goebel, 1987), and reflect numerous favorable assumptions about both the engineering and biological aspects of such a system. For example, the individual growth ponds would be "raceway" designs, with a single central baffle and 10 ha in size, over ten times larger than any operated previously, and mixed with paddle wheels. For economy, the ponds would be earthwork construction without plastic liners, with a clay sealer to minimize percolation. The water source (such as seawater) must containing sufficient alkalinity to allow some CO_2 storage. CO_2 would be supplied via diffusers and sumps. Major design factors are the depth of the sumps (which determines transfer efficiency), the mixing velocities (typically 20 - 30 cm/sec), the number of carbonation stations (which depend on the CO_2 storage, pH range for operations and outgasing rates), the depth of the pond culture (typically 20 to 30 cm). These factors are interactive and must be optimized.

Many aspects of this process require R&D. Harvesting involves "bioflocculation", in which the algae spontaneously flocculate and sediment in settling ponds. Although a well known natural process which has been demonstrated in waste grown algae (Benemann et al., 1980), its applicability to large-scale systems needs to be demonstrated. The extraction and processing of the vegetable oils from the algal biomass was cost mated based on soybean processing as no relevant data for algal biomass is available. The algal oils would be produced by limiting the algal cultures for nitrogen, which has been demonstrated at the laboratory scale (Benemann and Tillett, 1987) but not yet in open ponds. The residue from the oil extraction would be fermented to produce methane gas.

Most important, in Table 1 two different productivities were assumed, about 109 and 219 metric tons/ha/yr, corresponding to about 5 and 10% solar energy conversion efficiencies, for favorable sites in the U.S.. The lower productivity is based on present experience. The higher productivity will require the development of algal strains that have a lower pigment content, allowing better overall light utilization in dense cultures (Benemann, 1990). If the higher productivities are indeed achievable, and the other assumptions on which this cost estimate is based are verified, then such a process could produce biomass fuels using flue gas CO_2, with a relatively modest CO_2 mitigation credit (\$16/t CO_2), within the range of those presently discussed (Lashoff and Tirpak, 1989).

A major constraint on such systems, besides the R&D issues, is the availability of sufficient land and water near the power plant. A 1,000 MWe power plant would require as few as 6,000 ha (Table 2). Also, only about 30% of the CO_2 emissions from the power plant could be captured, as the system would be sized to utilize most of the CO_2 produced during peak summer daytime utilization, wasting night and much of the winter CO_2 outputs. However, as pointed out in the introduction, such a rate of CO_2 capture would mitigate most of the potential adverse effects of CO_2 released from such a power plant. And the land area required is a small fraction, less than one tenth, that required for other biomass systems (tree farms). Perhaps most important, such systems would provide over 3 million barrels of fuel per year. Although much R&D is still needed, no insurmountable problems are apparent and no "breakthroughs" are required. Microalgae systems could become an affordable process for CO_2 removal from flue gases.

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TABLE 1. MICROALGAE SYSTEM CAPITAL AND OPERATING COSTS

PRODUCTIVITY ASSUMED: Average Daily: (ash-free dry weight) Annual:	30 g/m^2/d 109 mt/ha/yr	60 g/m^2/d 219 mt/ha/yr
CAPITAL COSTS ($/ha):		
Ponds (earthworks, CO_2 sumps, mixing)	27,500	33,000
Harvesting (settling ponds, centrifuges)	12,500	17,000
System-wide Costs (water, CO_2 supply, etc.)	30,000	40,000
Processing (oil extraction, digestion)	10,000	20,000
Engineering, Contingencise (25% of above)	20,000	27,500
TOTAL CAPITAL COSTS ($/ha)	100,000	137,500
Capital Costs $/t-yr	920	630
Barrels of Oil/y (@ 3.5 bar./t)	380	760
CAPITAL COSTS $/Barrel/y	260	180
OPERATING COSTS ($/ha/yr):		
Power, nutrients, labor, overheads, etc.	10,000	15,500
Credit for methane	- 3,000	- 6,000
Net Operating Costs $/ha/yr	7,000	9,500
Net Operating Costs $/barrel oil	18	13
CO_2 Mitigation Credits ($16/t$CO_2$)	-10	-10
Annualized Capital Costs (0.2 x Capital)	52	36
TOTAL COSTS $/BARREL	60	39

TABLE 2. LAND REQUIREMENTS FOR ALGAE CO_2 UTILIZATION

Assumptions: 30% CO_2 average annual CO_2 utilization
1,000 MW power plant, 0.88 kgCO_2/kwh (Herzog et al., 1991).
Composition: 50% lipid, 25% carbohydrate, 25% protein.
Heat of Combustion: 7.5 Kcal/g (60% C in biomass).
Avg. Annual Solar Insolation: 500 Langleys, 45% visible.
Production: 1.05 x 10^6 mt/yr biomass; 3.7 x 10^6/yr barrels oil.

PRODUCTIVITY ASSUMPTIONS:		
Avg. Ash free dry weight g/m^2/d	30	60
Annual Productivity mt/ha/yr	109	219
Lipid fuels barrels/ha/yr	380	760
Solar Conversion Efficiency (appx.)	5	10
Fixation C mt/ha/yr	66	131
Fixation CO_2 mt/ha/yr	241	482
LAND AREA REQUIREMENTS:		
,000 Ha required growth ponds area	9.6	4.8
,000 Ha total area (ponds x 1.25)	12	6

that *T. denitrificans* may be readily cultured aerobically and anaerobically in batch and continuous reactors on gaseous H_2S under sulfide-limiting conditions. A microbial process for the removal of H_2S from gases have been proposed based on contact of the gas with a culture of *T. denitrificans* [3]. Sublette and Sylvester [3,4] have shown that sulfide concentrations as low as 100 to 200 μM inhibit the growth of the wild-type strain of *T. denitrificans* (ATCC 23642) on thiosulfate. Complete inhibition was observed at initial sulfide concentrations of 1 mM. However, a sulfide- and glutaraldehyde-resistant strain (strain F) of *T. denitrificans* has been isolated by enrichment from cultures of the wild-type [7]. This strain grows at inorganic sulfide concentrations in excess of 1000 μM and glutaraldehyde concentrations of 25 to 40 ppm. These concentrations are lethal to the wild-type.

T. denitrificans strain F has been successfully grown in co-culture with the sulfate-reducing bacterium, *Desulfovibrio desulfuricans*, both in liquid culture and through Berea sandstone cores without the accumulation of sulfide [8]. The presence of the sulfide-resistant strain F also controlled microbial sulfide production in an enrichment from an oil field brine. The effectiveness of strain F is due to its ability to grow and use sulfide at levels which are inhibitory to the wild-type strain of *T. denitrificans*.

The ability of *Thiobacillus denitrificans* strain F to control H_2S production in an experimental system using cores and formation water from a gas storage facility was investigated. Strain F and nitrate were added to nutrient amended formation water and injected into the core system. It is important to note that the objective was not to control the concentration of sulfate-reducing bacteria. Strain F does not inhibit the growth of sulfate-reducing bacteria; it simply removes the unwanted product of sulfate reduction, sulfide [8]. The test was therefore considered successful if the sulfide concentration of the effluent of the core treated with strain F was lower than that found before strain F treatment.

MATERIALS AND METHODS

Formation Water

Formation water was collected daily from well Davis-6 of the Northern Natural Gas Co. gas storage in Redfield, Iowa. The chemical composition of the water was as follows (in mg/L): iron (0.6), sulfide (9), chloride (420), sulfate (450), phosphate (1.8), hardness (960), alkalinity (660), and total dissolved solids (718).

Core System

The core system used in these experiments was assembled by Bioindustrial Technologies, Inc. (BTI, Grafton, NY) and was previously used to test the effectiveness of biocide formulations in controlling sulfide production by sulfate-reducing bacteria in the cores. BTI operated the core system with a feed of Davis-6 formation water for approximately ten weeks. Following the completion of the BTI studies, the core system was flushed with formation water at approximately 75 mL/hr in the treated core (see below) and at approximately 14 mL/hr in the control core for seven days before the experiments described here were initiated.

The core system contained cylindrical cores of St. Peter sandstone with dimensions of about 2.5 cm diameter and 7.5 cm length, each of which was mounted in polyvinyl chloride (PVC) tubing. Two sets of three cores each were connected in series using stainless steel tubing and compression fittings. Each set of cores had its own feed pump to inject fluids. The intake line of each core system had a course 5 μm membrane

filter to remove suspended solids (iron sulfides) from the fluid before injection into the cores. A sampling port was located at the inlet side of each core in the set. The porosity of the St. Peter sandstone was 30%. From the porosity of the cores and the volume of the tubing, the liquid volume of the core system was estimated to be 240 mL.

As noted above, one set of cores had previously been treated with biocides while the other set of core set served as the control. The injectivity of the control set of cores was much lower than that of the biocide treated set. Because of this, the flow rates of the two core sets were very different; the flow rate of the control core set was 14 mL/h while that of the treated core set was 75 mL/h. These were the maximum flow rates that could be obtained without leakage due to an excessive pressure. The hydraulic retention times were 3.2 and 16.7 h for the test and control cores systems, respectively.

Stock Cultures

Stock cultures of *Thiobacillus denitrificans* strain F were maintained anaerobically in thiosulfate medium described previously [4]. In this medium, thiosulfate is the energy source, nitrate is the terminal electron acceptor, bicarbonate is the source of carbon and ammonium ion is a source of reduced nitrogen. Stock cultures were transfered every 30 days and stored at 4°C until used.

Growth of Cells for Core Injection

T. denitrificans strain F cells were grown anaerobically in thiosulfate medium in 2-L cultures in a B. Braun Biostat M at 30°C and pH 7.0. The culture received a gas feed consisting of 30 mL/min of a gas mixture containing 5% CO_2, with the balance being N_2 to ensure that the culture did not become carbon limited. When the OD_{460} of the culture medium reached approximately 1.0 (about 10^9 cells/mL), the cells were harvestesd by centrifugation at 5000 x *g* and 25°C. The cells were then washed with 15 mM phosphate buffer, pH 7.0, and shipped as a wet pellet by overnight delivery service to the test site. Sufficient medium (without thiosulfate) was used to resuspend the pellet in a five-liter beaker so that the suspension was only slightly turbid. The viable cell concentration of the suspension was estimated by end-point dilution method using the above medium with thiosulfate. Because of the lack of facilities on site, medium that was injected into the core system was not sterilized.

Core Experiments

The objective of the first experiment (E1) was to determine whether indigenous microbial populations capable of oxidizing sulfide and using nitrate as the electron acceptor were present in the core system. Formation water supplemented with 40 mM sodium nitrate was injected into the test core set only for 24 h. Formation water with sodium nitrate was then injected into both sets of cores for another 24 h. After each 24 h period, a sample was collected from the sample port located upstream of the first core of each set and from the tubing exiting each core set. The samples were immediately analyzed to determine the concentrations of sulfide, sulfate-reducing bacteria, acid-producing bacteria, and strain F. The remainder of each sample was frozen and analyzed for nitrate, nitrite, sulfate, and sulfite at a later date.

In a second experiment (E2), *T. denitrificans* growth medium without thiosulfate was injected into each core set for 40 h to determine whether the addition of nutrients would stimulate the production of sulfide in the core system. Samples for chemical and microbiological analyses were taken after 24 and 40 h of medium injection.

In a third experiment (E3), the test core was inoculated with strain F to determine the effectiveness of this organism in preventing the production of sulfide in a continuous flow system. Approximately 10^5 viable cells/mL of strain F suspended in growth medium (without thiosulfate) was injected into the test set of cores for 6 h (about 0.5 L). This was followed by the injection of growth medium without thiosulfate for 24 h. This cell inoculation procedure was repeated once. During the inoculation procedure, growth medium without thiosulfate was injected into the control core set. Samples for chemical and microbiological analyses were taken every 24 h.

In a fourth experiment (E4), a mixture of growth medium and formation water starting with 90% (vol/vol) growth medium without thiosulfate and 10% (vol/vol) formation water with 40 mM sodium nitrate was injected into both sets of cores. Every 12 h, the fraction of formation water with nitrate injected into the core system was increased by 10% until only formation water with nitrate was injected into the core system. When the fraction of formation water injected into the core was 30, 60 and 80%, the test core was again treated with a cell suspension of strain F in growth medium without thiosulfate for a period of six hours, followed by a 6 h treatment of the appropriate combination of formation water with 40 mM sodium nitrate. When the percentage of formation water with nitrate reached 100%, the amended formation water (with 40 mM nitrate) was injected into both cores sets for 24 h. Samples for chemical and microbiological analyses were taken every 12 h.

During the time that 100% amended formation water was injected into the core systems, samples from both sets of cores contained a compound that interfered with the detection of sulfide. This suggested that a nutrient may be limiting the growth of strain F which would result in the incomplete oxidation of sulfide or the incomplete reduction of nitrate. Because of this problem and after receiving 100% amended formation water for 24 h, the test core was treated with growth medium (without thiosulfate) for 6 h and then with formation water with 10 mM sodium nitrate containing (in g/l) KH_2PO_4 (1.8), $MgSO_4 \cdot 7H_2O$ (0.4), NH_4Cl (0.5), $CaCl_2$ (0.03), $NaHCO_3$ (1.0). In experiment E5, this nutrient amended formation water with the lower nitrate concentration was injected into both sets of cores for 32 h, after which time the fluid flow to both sets of cores was stopped. After 12 h of incubation without fluid flow, the injection of nutrient-amended formation water with 10 mM nitrate was reinitiated. Samples for chemical and microbiological analyses were periodically taken during this treatment.

Twelve hours after fluid flow was reinitiated, the fluid injected into the control set of cores was changed to formation water without any nutrient or nitrate amendments. The core system was operated in this manner, i.e., with nutrient-amended formation water with 10 mM nitrate injected into the test set of cores and formation water only injected into the control set of cores for an additional 48 h.

Microbiological and Chemical Analyses

Concentrations of *T. denitrificans* strain F, sulfate-reducing bacteria, and acid-producing bacteria were estimated used the end-point dilution method. One milliliter of the sample was diluted in the respective growth medium. The inoculated bottles were then incubated at 30°C and checked for growth on a daily basis.

Strain F was enumerated using the growth medium given previously described [4]. Sulfate-reducing bacteria and acid-producing bacteria were enumerated using BTI-SRB medium and BTI-APB medium (Bioindustrial Technologies, Inc., Grafton, NY).

Samples were analyzed for sulfide immediately by the methylene blue method using Hach Chemical (Loveland, CO) field kits. Sulfate, nitrate, and nitrite were determined by high pressure liquid chromatography (HPLC) using anion exchange column and a conductivity detector as previously described [8].

RESULTS

The addition of nitrate alone to the formation water injected into the core systems resulted in lower effluent sulfide levels (Table I). Concomitant with the reduction of sulfide was the decrease in nitrate concentrations in the core effluent, suggesting the presence of indigenous microbial populations capable of oxidizing sulfide using nitrate as the electron acceptor. However, in an earlier study of microbial activities in the subsurface at this site, BTI did not identify any indigenous organisms capable of sulfide oxidation [9]. The addition of nitrate did not affect the numbers of sulfate-reducing bacteria and acid-producing bacteria. Strain F-like organisms were not detected in the core effluents. In the test core system, where the flow rates were about five times faster than the control core system, the sulfide levels were reduced by about 40%, while in the control core system sulfide levels were decreased by 98%. The efficacy of the nitrate treatment clearly depended on the residence time of the liquids in the core system. It is interesting to note that the sulfide levels in the influent and the effluent before treatments began were similar. This suggested that little or no sulfide production occurred within the core system. (No organic nutrients were added to the formation water to support the growth of sulfate-reducing bacteria). In earlier studies at this site, BTI personnel observed that a change in microbial activities occurred when nitrate was detected in the produced water [9]. These investigators found that nitrate can be used as an electron acceptor by the majority of the community members. As a consequence, a reduction in sulfide concentration in the formation water was observed when nitrate was present [9].

The injection of nutrients for *T. denitrificans* did not stimulate sulfide production in the core systems. Although the numbers of sulfate-reducing bacteria were not affected, the influent and the effluent sulfide levels were low when only medium was injected into the core systems. This again suggested that little or no sulfide production actually occurred within the core system. Significant numbers of strain F cells were detected in the first two cores of the test core system after the first treatment with strain F. The number of strain F cells increased with the subsequent treatment with cells followed with medium injection. Thus, cells of strain F were maintained in the test core system when growth medium was used.

Preliminary studies suggested that the formation water contained a compound inhibitory to the growth of strain F (data not shown). Therefore, as noted above, the fraction of formation water injected into the core was increased in steps in order to acclimate strain F (experiment E4). Relatively high concentrations of strain F were detected in samples of the effluent and from each intermediate sampling port even when the influent contained 80% formation water with nitrate. When the influent was 100% formation water with nitrate, the levels of strain F decreased, but complete washout of strain F was not obsesrved. During these experiments the effluent from the control core system contained a compound that interfered with the detection of sulfide. This problem plus the fact that the control core and the test core systems operated at different flow rates made definitive comparisons between the two cores systems difficult. However, throughout this period, the concentration of sulfide in the effluent of the test core system was consistently lower than the influent concentration. There was also a concomitant reduction in nitrate levels in the test core system suggesting that these two processes were linked. Interestingly, the sulfate concentrations in the effluent relative to the

influent concentration of the test core system increased after strain F inoculation. This was not observed in the control core system and suggested that strain F was oxidizing endogenous sulfur compounds (such as iron sulfides) that had accumulated in the core system. This would explain why the concentration of nitrate in the effluent of the test core system was much lower than expected if the sulfide present in the influent was the only source of electrons for nitrate reduction.

During this experiment, strain F was consistently detected in all cores of the test core system. This suggested that strain F was active and growing in the test core system. However, when the influent was shifted completely to formation water, the concentration of strain F in the test core system decreased substantially and interferences in effluent sulfide analyses were observed. This suggested that some essential nutrient may be limiting the growth of strain F which would result in the incomplete oxidation of sulfide or in the incomplete reduction of nitrate. In subsequent treatments, the concentration of nitrate was decreased from 40 mM to 10 mM and nutrients were added to the formation water as noted in the Materials and Methods section.

The treatment of the test core system with strain F and the subsequent injection of formation water with reduced nitrate concentration and nutrient amendments resulted in the reestablishment of strain F in the test core system. Concomitant with the increase in strain F was the disappearance of the interfering substance from the effluent of the test core. A reduction in sulfide concentration in the effluent compared to the influent concentrations in the test core was also observed. The levels of sulfide in the effluent of the test core compared to the influent concentration were reduced by 84 to 99%. There was a substantial reduction in the levels of nitrate and a substantial increase in the levels of sulfate in the effluent compared to the influent of the test core system. This suggests that, in the core system, strain F was oxidizing the sulfide present in the formation water to sulfate using nitrate as the electron acceptor. However, the amount of sulfate detected in the effluent of the test core system was much higher than that expected if strain F completely oxidized only the sulfide present in the formation water. As noted above, this suggests that strain F may have metabolized sulfur compounds that had accumulated within the core system. These sulfur compounds may have been iron sulfides or other sulfide precipitates which accumulated in the core sections during previous experiments. Strain F has been observed to utilize as an energy source iron sulfide precipitates produced by sulfate-reducing bacteria in media containing Fe^{3+} [8].

DISCUSSION

Several lines of evidence support the conclusion that treating the test core system with *Thiobacillus denitrificans* strain F, nitrate, and certain inorganic nutrients was effective in controlling sulfide production. After the strain F treatments, the effluent sulfide concentration in the test core system was 84 - 99% lower than the influent concentration. Also, after strain F treatment, the effluent sulfide concentration in the test core system was 90 to 99% lower than the effluent sulfide concentrations before the test began. And lastly, following strain F treatment the sulfide concentration in the test core effluent was 86 to 97% lower than when the test core system was treated with formation water plus 40 mM nitrate. Since the control core system had a much slower flow rate and samples from this core system contained compounds that interfered with the detection of sulfide, direct comparisons between the test and control systems are not possible. However, it is clear that less sulfide was detected in the effluent samples of the test core system after strain F treatment.

High concentrations of strain F were observed in the effluent and at each sampling port in the test core system after inoculation and injection of nutrient supplemented formation

water with 10 mM nitrate. This indicates that strain F was able to colonize the core system and successfully compete with the indigenous microbial populations over a long period of time. The growth of strain F in the core did not result in any significant increase in the pressure drop through the system. The presence of high levels of strain F at the time when effluent concentrations of sulfide and nitrate decreased, and sulfate increased suggests not only that strain F was maintained in the system, but that it was metabolically active. Strain F-like organisms were not detected in samples from the control core system suggesting that the changes observed in the test core system were the result of strain F treatment.

Concomitant with the reduction of sulfide in the effluent was a decrease in the effluent concentration of nitrate and an increase in the effluent concentration of sulfate in the test core system. These changes suggest that as sulfide was used, nitrate was reduced and sulfate was produced. Little or no change was observed in the effluent concentrations of sulfate and nitrate compared to the influent concentrations of these compounds in the control core system. Since the control core system was not inoculated with strain F and strain F-like organisms were not detected in the control core system, this suggests that the changes in the effluent concentrations of nitrate and sulfate observed in the test core system were the result of the activity of strain F.

The addition of nitrate alone to the formation water did result in the reduction of sulfide in the core system. This was most pronounced in the control core system where little or no sulfide was detected in the effluent after nitrate treatment. However, the effectiveness of this treatment is difficult to determine since the samples from the control core system contained a compound that interfered with detection of sulfide. In the test core system, the addition of nitrate alone was not as effective in reducing sulfide concentrations compared to that observed in the control core system. These data suggest that the efficacy of nitrate addition clearly depended on the retention time of liquids in the system. At the shorter retention times that occurred in the test core system, the addition of nitrate was not as effective in reducing sulfide concentrations as was the strain F treatment.

The fact that the effluent sulfate concentration of the test core system after strain F treatment was almost twice the influent core system suggests that sulfur-containing compounds had accumulated within the test core system and were being oxidized to sulfate by strain F. This would explain why the such a large decrease was observed in the effluent concentrations of nitrate after strain F treatment. One possible source of endogenous sulfur compounds may have been iron sulfide precipitates. Iron sulfide precipitates were clearly visible in the formation water and the tubing entering the core systems. Montgomery *et al* [8] showed that strain F can metabolize sulfide in the form of iron sulfide resulting in a clearing of the medium and removal of blackened areas in sandstone cores. Iron sulfide precipitates that form as a consequence of sulfide production can plug pores in porous rock and lead to the loss of injectivity. The fact that strain F is able to use these precipitates should increase the permeability and injectivity of oil and gas wells.

CONCLUSIONS

Once inoculated into the test core system, *Thiobacillus denitrificans* strain F was maintained for a long period of time. After inoculation with strain F, a 84 to 99% reduction in the sulfide levels in the effluent compared to the influent concentration of the test core system was observed. Effluent sulfide levels of the test core system were much lower than those observed before the test began or after treating the core with nitrate amended formation water. Concomitant increases in effluent sulfate levels and

decreases in effluent nitrate levels suggest that strain F metabolized sulfide to sulfate while using nitrate. These data will support the conclusion that strain F was metabolically active and effective in controlling the level of sulfide in the test core system.

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TABLE I. Summary of the effects of strain F inoculation and nitrate addition on sulfide production in test core system.

TREATMENT	EFFLUENT SULFIDE (μM)	CELL CONCENTRATIONS (CELLS/ML)		
		SRB	APB	STRAIN F
None	160	10^5	10^5	0
Nitrate	110	10^5	10^7	0
Nitrate, strain F, and nutrients	3-16	10^7	10^7	10^7

MICROBIAL CONTROL OF HYDROGEN SULFIDE PRODUCTION

Michael J. McInerney
Vishvesh K. Bhupathiraju
Department of Botany and Microbiology
University of Oklahoma
770 Van Vleet Oval
Norman, OK 73019

Kerry L. Sublette
Center for Environmental Research and Technology
University of Tulsa
600 S. College Ave.
Tulsa, OK 74104

Key words: souring, petroleum, natural gas, desulfurization

INTRODUCTION

Microbial Souring of Oil and Gas Reservoirs

Hydrogen sulfide is a toxic and corrosive gas that greatly increases the cost of recovery of oil and natural gas. A major mechanism for hydrogen sulfide production in petroleum/gas reservoirs below 80°C is microbial sulfide production [1]. Because of their diverse metabolic properties and widespread occurrence, sulfate-reducing bacteria were once thought to be only microorganisms responsible for microbially induced souring. However, sulfate reducers are not the only organisms found in oil/gas reservoirs that produce sulfide [2]. In fact, the most commonly detected sulfide-producing bacteria (such as *Shewanella putrefaciens*) do not use sulfate as an electron acceptor, but use other sulfur oxyanions. Thus, methods to detect or control souring based solely to the detection or control of sulfate-reducing bacteria may not be effective in actual field situations.

The detrimental activities of sulfide-producing bacteria can be controlled by the effective use of biocides. This type of remediation strategy is most successful in controlling unwanted activities in surface facilities. However, the control of these activities in the reservoir through the use of biocides is often difficult and expensive. Our approach is to manipulate the ecology of the system so that the terminal electron-accepting process is changed from sulfate reduction to nitrate reduction. Thus, even if sulfate reducers are present in the reservoir, the accumulation of the unwanted product of their metabolism, sulfide, is prevented. This is done by the addition of specialized strain of *Thiobacillus denitrificans* that can oxidize sulfide to sulfate by reducing nitrate to nitrogen gas.

Thiobacillus denitrificans

Thiobacillus denitrificans is an obligate autotroph and facultative anaerobe which can utilize reduced sulfur compounds as energy sources and oxidize them to sulfate. Under anaerobic conditions, nitrate is used as a terminal electron acceptor and is reduced to elemental nitrogen. Sublette and Sylvester [3-5] and Sublette [6] have demonstrated

CYTOCHROME P-450 OF *STREPTOMYCES GRISEUS* AND XENOBIOTIC METABOLISM

F. Sima Sariaslani*; M. K. Trower; M. Ramachandra; S. E. Buchholz; Ralph Stahl and C. A. Omer
Central Research & Development, E. I. duPont de Nemours & Co.,Wilmington, DE 19880-0228, USA
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INTRODUCTION

Streptomyces are aerobic prokaryotic microorganisms that are present in terrestrial and aquatic environments.[1] These organisms have long been exploited as producers of antibioitcs and enzymes. In addition, because of their remarkable versatility and metabolic capability *Streptomyces* are instrumental in the breakdown of organic matter in the environment and therefore in recycling carbon in nature.[1]

Streptomyces griseus

We have been studying the enzymatic system employed by *Streptomyces griseus* for oxidation of a diverse array of xenobiotics. We have shown that growth in a medium enriched with soybean flour induces a multicomponent cytochrome P-450 ($P\text{-}450_{soy}$) enzyme system in *S. griseus*.[2,3] $P\text{-}450_{soy}$ resembles its mammalian counterparts in its broad substrate specificity. The reactions performed by $P\text{-}450_{soy}$ include, but are not limited to, aromatic and alicyclic hydroxylations (biphenyl, phenol, benzene, chlorobenzene, Benzo(a)pyrene, cyclohexane), *O*-dealkylation (7-ethoxycoumarin), epoxidation (precocene II) and *N*-acetylation (aniline), and *N*-oxidation (pyridine).[4]

S. griseus cells enriched in $P\text{-}450_{soy}$ exhibit lack of stereo- and regioselectivity during camphor oxidation.[5,6] This is in contrast to camphor oxidation by $P\text{-}450_{cam}$ of *Pseudomonas putida* which shows a high degree of regio- and stereospecificity resulting in the production of 5-*exo*-hydroxycamphor as the sole reaction product.[5,7] The strict specificity of $P\text{-}450_{cam}$ has been attributed to the presence

of tyrosine-96 in the structure of this enzyme.[8,9] The major product formed during oxidation of camphor by *S. griseus* cells containing P-450$_{soy}$ is 6-*endo*-hydroxycamphor together with 5-*endo*-, 3-*endo*- and 5-*exo*-hydroxy derivatives as minor products.[6] Multiple hydroxycamphor product formation is also observed in reconstituted assays containing homogeneous preparations of P-450$_{soy}$.[10] Alignment of the amino acid sequence of P-450$_{soy}$ with the *P. putida* enzyme indicates that tyrosine-96 is not conserved in P-450$_{soy}$. We have proposed that lack of this residue in P-450$_{soy}$ contributes to its lack of specificity during camphor oxidation.[10]

In search for the other ancillary proteins of P-450$_{soy}$ system we have identified a soybean flour-inducible, 14,000 molecular weight 7Fe ferredoxin (*S. griseus* 7Fe ferredoxin) in crude extracts of *S. griseus*.[11] This ferredoxin couples electron flow between spinach ferredoxin reductase and cytochrome P-450$_{soy}$ for NADPH-dependent substrate oxidation. We have determined the primary structure of this ferredoxin and have shown that it contains a [3Fe-4S] and a [4Fe-4S] cluster.[12] The amino acid sequence of this protein, which consists of 105 amino acids with a calculated molecular weight of 12,291, shows high homology to the other reported 7Fe ferredoxins.[12]

We have recently isolated an FAD-containing and highly unstable soybean flour-inducible ferredoxin reductase (*S. griseus* ferredoxin reductase) from *S. griseus* crude extracts.[13] This 60,000 molecular weight protein requires the presence of 20% glycerol and 5mM dithiothreitol for stabilization. *S. griseus* ferredoxin reductase is an NADH-dependent flavoprotein which requires Mg^{2+} for *in vitro* activity. A consensus FAD binding sequence, which possesses a high degree of homology to the other FAD-containing ferredoxin reductases, starts at residue 7 of the N-terminus of this flavoprotein. In reconstituted assays, the *S. griseus* ferredoxin reductase can use a variety of ferredoxins such as *Clostridium pasteurianum* ferredoxin, spinach ferredoxin and adrenodoxin for coupling electron transfer from NADH to cytochrome P-450$_{soy}$. We have observed catalytic activities of 20-40 nmol product formed/min/mg P-450$_{soy}$ when the *S. griseus* 7Fe ferredoxin and the ferredoxin reductase were used in reconstituted assays.

P-450$_{soy}$ is encoded by *soyC* (CYP105D) gene. This gene encodes a 413 amino acid protein with a molecular weight of 45,400.[10] Five base pairs downstream of the stop codon of *soyC* is a translationally coupled open reading frame for a ferredoxin-like

protein of 6,600 molecular weight. The amino acid sequence of this ferredoxin-like protein is dissimilar to the soybean flour-inducible *S. griseus* 7Fe ferredoxin. Due to its proximity to the *soyC* gene we think that this open reading frame probably encodes the *in vivo* ancillary ferredoxin for P-450$_{soy}$ and have therefore named this gene *soyB*.

To date we have shown that a recombinant *S. lividans* strain which contains the *soyC,B* gene oxidizes 7-ethoxycoumarin and precocene II. We are currently investigating metabolism of a wide array of xenobiotics by this recombinant strain. In addition, we are involved in over-expression of *soyC,B* genes which would allow isolation and characterization of the *soyB* gene product.

We have exploited the broad substrate specificity of P-450$_{soy}$ and its resemblance to its mammalian counterparts to activate promutagenic chemicals.[14] In a modified version of the *Salmonella*/gene mutation (Ames) assay, *Salmonella typhimurium* strains TA98 and TA1538 were reverted by mutagenic metabolites that were produced by *S. griseus* cells enriched in cytochrome P-450$_{soy}$. Promutagens that were activated included a variety of aromatic amines (benzidine; 2,4-diaminotoluene; 4-chloro-2-nitroaniline), polycyclic aromatics (benzo(a)pyrene), and small aliphatics (chloropicrin).

In another test system we have used two genetically engineered strains of *S. griseus* which can activate promutagenic chemicals and detect the presence of their mutagenic metabolites.[15] One recombinant strain detects point mutations, while the other strain is sensitive to frame shift mutations. To our knowledge this is the first reported single-organism test system in which the enzymatic machinery for activation of promutagens has been linked to a detection system within the same organism.

We have been studying the feasibility of using artificial electron donors for transfer of reducing power from NADH to the P-450$_{soy}$ component. We have shown that, in reconstiuted assays, redox mediators such as phenazine methosulfate (PMS) and phenazine ethosulfate (PES) effectively replace the ferredoxin reductase and the ferredoxin and affect the NADH-dependent substrate oxidation by P-450$_{soy}$.[16] We have shown reduction of P-450$_{soy}$, O-dealkylation of 7-ethoxycoumarin and epxodiation of precocene II by the NADH/PMS/ or NADH/PMS/P-450$_{soy}$ system.[16]

As summarized above, P-450$_{soy}$ of *Streptomyces griseus* mimics its mammalian counterparts in its broad substrate specificity

and the ability to activate promutagenic chemicals. Biochemical and molecular studies are currently underway to unravel the mechanism of the action of P-450$_{soy}$ and the nature of the active site of this interesting enzyme.

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BIOMIMETIC CATALYSTS: APPLICATION OF COORDINATING COMPLEXES CONTAINING AN ASYMMETRIC COORDINATING LIGAND

M.W. Droege; J.H. Satcher, Jr.; R.A. Reibold; T.J.R. Weakely[1], L. Chauffe[2], and B.E. Watkins

Lawrence Livermore National Laboratory, PO Box 808, Livermore, CA., 94550; [1]Dept of Chemistry-University of Oregon, Eugene, OR., 97403, [2]Dept. of Chemistry, Cal State Hayward, Hayward, CA. 94542

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INTRODUCTION

The catalytic conversion of methane from natural gas into a readily transportable liquid fuel is a research area that is currently attracting considerable attention. This is a difficult transformation from a thermodynamic viewpoint since methane is a relatively unreactive substance and chemicals derived from methane are substantially more reactive than methane itself. Thus, no industrial process to date has been able to convert methane to liquid products in high yield.

One of the more studied process is the oxidative coupling of methane to higher hydrocarbons and olefins. To date no laboratory processes have come close to a yield necessary for an economically viable process. Modeling studies suggest that a theoretical ceiling in overall yield exists as the rate constant of a catalyst is increased[1]. The rate constant of the catalyst must be sufficient to overwhelm the non-specific gas phase reactions but low enough to prevent over oxidation. In theory this limits the yield to about 35%.

Because of this perceived limitation in heterogeneous catalysts, we undertook a program to take advantage of the selectivity already known to exist in a bacterial enzyme system. A group of aerobic soil/water bacteria called methanotrophs can efficiently and selectively utilize methane as the sole source of energy and carbon for cellular growth.[2] The first reaction in this metabolic pathway is the conversion of methane to methanol and is catalyzed by the enzyme methane monooxygenase[3] (MMO). (Figure 1) Of the many liquid products available directly from methane, methanol is one of the more technologically important. It can be easily converted to liquid hydrocarbon transportation fuels, used directly as a liquid fuel itself, or serve as a feed stock for fine chemicals production.

Microorganisms can produce MMO in two distinct forms: a membrane-bound particulate form or a discrete soluble form. The soluble form contains an oxygenase subunit, whose active site includes a binuclear iron center.[4,5,6] The

complete details of the structure of the active site are not known. However, the general description of the iron site in the soluble form is a binuclear cluster containing some type of μ-oxo ligand between the iron atoms. The remaining ligands (derived from adjacent amino acid residues) coordinate to the metals through nitrogen or oxygen and the Fe-Fe distance is 3.4 Å. The best description of the amino acids coordinating the binuclear iron center is surmised by the close amino acid sequence homology between MMO and ribonucleotide reductase[7], an enzyme containing a binuclear iron center whose X-ray crystal structure has been determined[8].

Compared to the soluble form of MMO the particulate form is poorly characterized and is thought to contain copper at the active site. This form is also active in methane oxidation in the biological system, and may be selectively produced by manipulating the bacterial growth conditions.[9]

Our work centers on the characterization of the structure/activity relationships of the particulate form of MMO and synthesis and characterization of inorganic/organic chemical models of MMO (both particulate and soluble). We have focused on the synthesis of an asymmetrical, binuclear chelating ligand possessing an alkoxo group that can serve as a bridging μ-oxo ligand. The advantage of such a ligand system is twofold: (a) metal complexes of an asymmetric binuclear ligand will provide coordinate unsaturation at only one metal resulting in focused substrate reactivity at that site and (b) a single ligand with binuclear coordination provides a more robust environment for metal oxidation state changes and accompanying chemical reactions. These complexes are being evaluated for their ability to oxidize methane and other hydrocarbons.

EXPERIMENTAL

The synthesis of the chelating ligand HMeL obtained by a five step procedure in ~35% overall yield is outlined in Scheme 1. Mono and binuclear copper complexes of HMeL were prepared by dissolving 100 or 200 mole% of cupric perchlorate with 100 mole% HMeL and sodium acetate in methanol, as shown in Scheme 2. The crude materials were recrystallized by vapor diffusion of ethyl ether in acetonitrile and chemically characterized, including single crystal X-ray crystal structure[10].

Catalytic reactivity was determined under atmospheric pressure and ambient temperature. Cyclohexane as the hydrocarbon substrate, hydrogen peroxide the oxidant, and a metal complex were employed in ratios of 1000:100:1 respectively with final catalyst concentration 9.0×10^{-4}M. $Cu(BF_4)_2$, $Cu[HMeL][ClO_4]_2$, or $[Cu_2\text{-MeL-OAc}][ClO_4]_2$ (4.5×10^{-5} moles) were dissolved in acetonitrile and placed Fisher-Porter reaction bottle. A mixture of cyclohexane (4.5×10^{-2} moles) and 30% hydrogen peroxide (4.5×10^{-3} moles) in acetonitrile was added to the catalyst solution with vigorous stirring at T=0. Aliquots (1mL) were removed periodically and a portion was analyzed by GC/MS for

cyclohexanol/cyclohexanone content with the remainder being titrated with a standard solution of potassium permanganate to determine hydrogen peroxide conversion.

RESULTS

Scheme 1 shows the synthetic route for the prototype binuclear chelating ligand, HMeL. Elemental analysis and NMR studies confirm the composition and structure of the ligand. It possesses a hydroxyl functionality that could serve as a bridging alkoxo group and aliphatic and aromatic nitrogen coordination groups (benzimidazole). We expected that one metal ion would be coordinated by one aliphatic nitrogen and two imidazole nitrogens and bridged by the hydroxy group to another metal coordinated by one aliphatic nitrogen and one imidazole nitrogen. This structure establishes a basis for coordinate asymmetry. Additional ligands to fulfill the coordination requirements of the metals would be available from the solvent or by the addition of acetate.

HMeL will readily binds to one mole of Cu(II) to produce a mononuclear complex (Scheme 2). Addition of two moles of copper does not alter the resulting product, as formation of the mononuclear complex appears to be independent of copper concentration. Addition of 200 mole% of copper to HMeL in the presence of 200 mole% of acetate gives rise to a binuclear complex. One mole of the acetate is necessary to fulfill the coordination requirements of Cu(II) and he other to act as a Lewis base to assist in removing the hydroxyl proton on HMeL. The mononuclear complex may be readily converted to the binuclear complex by the addition of copper and acetate. This suggests the possibility that asymmetric binuclear complexes may be generated with different metals in specific positions.

One coordination site at one copper center of the binuclear Cu(II) complex is available to bind with extraneous ligands. This was shown by forming a stable complex between the binuclear Cu(II) complex and azide ion, whose structure was confirmed by solving the X-ray crystal structure. We expect that in a catalytic system that the vacant coordination site will be available to bind and activate oxygen, hydrogen peroxide, or other oxidizing agents.

These complexes were evaluated for their ability to oxidize cyclohexane and to disproportionate hydrogen peroxide and compared to unchelated Cu(II) under the same conditions. The results are presented in Table 1. Neither the unchelated Cu(II) or the mononuclear complex is capable of either disproportionating hydrogen peroxide or oxidizing cyclohexane. However, the binuclear Cu(II) complex is capable of oxidizing cyclohexane to a mixture of cyclohexanol and cyclohexanone, as well as disproportionating hydrogen peroxide. It is not clear if only the vacant binding (peroxide binding) site in sufficient for peroxide activation or if the coupled copper ions possess unique redox properties. These data show the proof-of-principal that these coordinately

asymmetric complexes will function as biomimetic catalysts and that they have the ability to oxidize hydrocarbons.

CONCLUSIONS

Our catalyst development effort has gone one full cycle in the design, synthesis and evaluation of a binuclear, coordinately-asymmetric coordination complex. Unchelated and a mononuclear copper complex do not show catalytic properties to activate hydrogen peroxide or to oxidize cyclohexane. However, the binuclear copper complex tested was capable of activation hydrogen peroxide and the presumed oxo-copper intermediate was able to oxidize cyclohexane. We are using this ligand as a starting point to synthesize new catalysts/ligands. Specifically, complexes with other metals (iron) and complexes with additional ligand groups and with varying oxygen to nitrogen contact atoms will be prepared.

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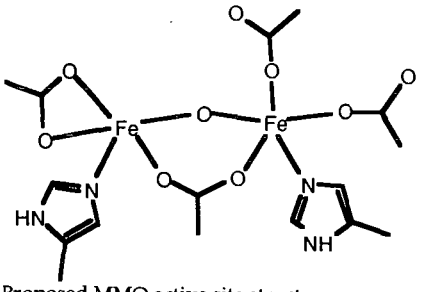
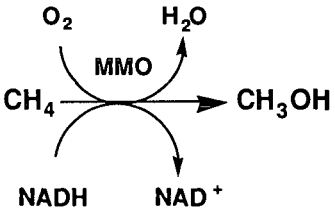
Table 1. Oxidation of Cyclohexane

Complex	% H_2O_2 Converted	Products	Turnover
$Cu(BF_4)_2 \cdot 6\ H_2O$	0 (24 hrs)	--	--
$[CuMEL][ClO_4]_2$	0 (48 hrs)	--	--
$[Cu_2MELOAc][ClO_4]_2$	40 (48 hrs)	cyclohexanol cyclohexanone	3

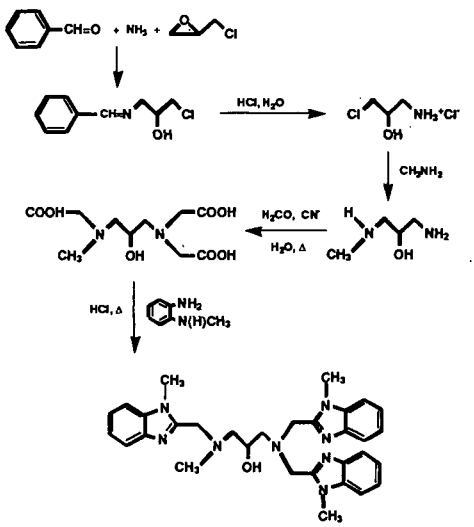


Proposed MMO active site structure

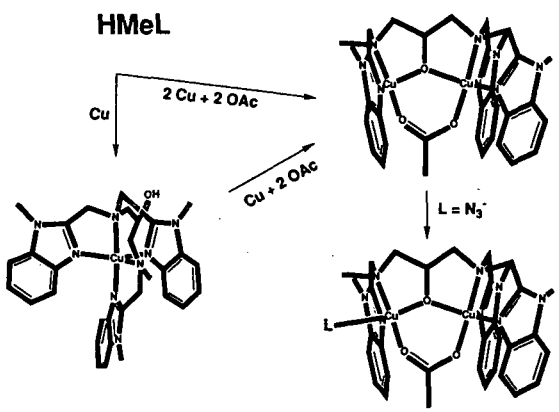


MMO Function

Figure 1. Structure/Function of MMO enzyme



Scheme 1. Synthesis of HMeL



Scheme 2. Copper Chelation by HMeL

ELECTRICAL ENHANCEMENT OF BIOCIDE ACTION FOR IMPROVED BIOFOULING AND BIOCORROSION CONTROL

S.A. Blenkinsopp, C.P. Anderson, A.E. Khoury*, J.W. Costerton
NSERC Biofilm Group, Biological Sciences, University of Calgary, Calgary, AB, Canada; *Urology Division, Hospital for Sick Children, University of Toronto, Toronto, Ontario, Canada

Keywords: Biocide, Bioelectric, Biofilm

ABSTRACT

Attached microbial communities or biofilms cause a significant proportion of the fouling and corrosion problems faced by industry. Unfortunately, bacteria within the exopolysaccharide matrix of a biofilm are very well protected from antimicrobial agents such as biocides. Biofilm bacteria are generally resistant to 50 to 500 times the biocide concentration sufficient to kill planktonic or free-floating cells of the same species. We have recently discovered that the application of a low strength electric field/current density can enhance the efficacy of three common industrial biocides to such an extent that biofilm bacteria are killed at biocide concentrations lower than the planktonic minimum inhibitory concentration (MIC). This enhancement of biocide efficacy in the presence of an electric field/current density is known as the "bioelectric effect". By exploiting this effect it may be possible to significantly reduce biocide levels and their impact on the environment, without sacrificing their performance.

INTRODUCTION

Microorganisms are commonly found attached to surfaces in aqueous industrial systems. When these attached or sessile microorganisms produce a blanketing layer of exopolysaccharide, they are termed a "biofilm" (3, 9). These biofilms are often responsible for the fouling (7, 11, 13) and corrosion (1, 2, 4, 5, 6, 18) problems faced by industry. It is also widely acknowledged that biofilms within water distribution systems may act as reservoirs for pathogenic bacteria (eg. *Legionella pneumophila*) that pose a threat to the general population (16, 17, 19).

In industrial systems, microorganisms may be found as free-floating or planktonic cells, which are quite susceptible to the action of killing agents. However, when these cells attach to surfaces and form biofilms, their resistance to biocides increases quite markedly (12, 15, 19). The practical consequence to this resistance is that it is absolutely essential to sample not only a bulk water sample to ensure planktonic cell kill, but also to sample the pipeline wall to ensure that the attached population has been eradicated. Unless this protocol is followed, the pipeline will be continuously re-inoculated by cells from the more resistant biofilm population.

Even with the above protocol in place, satisfactory control of biofilm populations may not be achievable if the costs of the necessary biocide applications are not economically feasible. Recent studies in the medical section of our laboratory have found that

antibiotics work more effectively when applied against biofilms in a low strength electric field/current density (10). We have discovered that the efficacy of the three common industrial biocides tested to date can similarly be dramatically enhanced against biofilm bacteria, when applied in the presence of a low strength electric field/current density.

In this paper, we will present representative findings for the biocide glutaraldehyde against an environmental *Pseudomonas aeruginosa* biofilm.

METHODS

Biofilms were grown by flowing an environmental isolate of *Pseudomonas aeruginosa* in M-56 nutrient medium (8) through parallel perspex chambers which contained sampling studs of known diameter (0.5 cm^2). These perspex chambers are called modified Robbins devices (MRDs)(14). One MRD was a non-electrified control and the other was electrified by connecting it, where stated, to a 3 V DC power source. Polarity was altered every 64 s, so that the electrodes alternated as anode and cathode.

The first experiment was performed to determine the effect of the electric field/current density on an established biofilm population. After a 24 h colonization period in the absence of an electric field/current density, the inoculum flask was replaced by one containing the nutrient medium M-56, and one MRD was electrified.

The second experiment was performed to determine whether biocide effectiveness was enhanced in the presence of an electric field/current density. The experimental protocol was identical, with the exception that the inoculum flask was replaced after a 24 h colonization period with one containing 5 ppm glutaraldehyde in M-56 nutrient medium.

Studs were sampled in duplicate from each device over the 24 h period following electrification. After sonication to remove the attached cells from the studs, the suspension was plated onto ½ Brain Heart Infusion plates. Results are reported as CFU/cm^2 after 48 h at 37°C.

RESULTS

The electric field/current density was of such a low strength that it did not have a significant effect on the established biofilm bacterial population when exposed in the absence of biocide. Viable cell counts were very similar between the control and electrified MRDs (Fig. 1).

Glutaraldehyde effectiveness was dramatically enhanced in the presence of the electric field/current density. The established biofilm bacterial population was killed within 24 hours (Fig. 2). This result was in sharp contrast to the non-electrified control, in which the level of glutaraldehyde employed had very little effect on the biofilm population over 24 hours (Fig. 2).

DISCUSSION

It is quite clear from these results that the biocidal action of glutaraldehyde can be significantly enhanced when applied in a low strength electric field/current density. It is also quite significant that this kill was achieved when using a glutaraldehyde concentration of only 5 ppm. This concentration is insufficient to kill the more sensitive planktonic bacterial cells.

This work and similar results from the two other common industrial biocides tested to date indicate that it may be possible to use this "bioelectric effect" to reduce the levels of biocide currently used, without sacrificing their effectiveness. This technology may be particularly attractive to industry if there are environmental impact concerns over the biocide concentrations employed at present. Further experiments are in progress to elucidate the mechanism of the effect.

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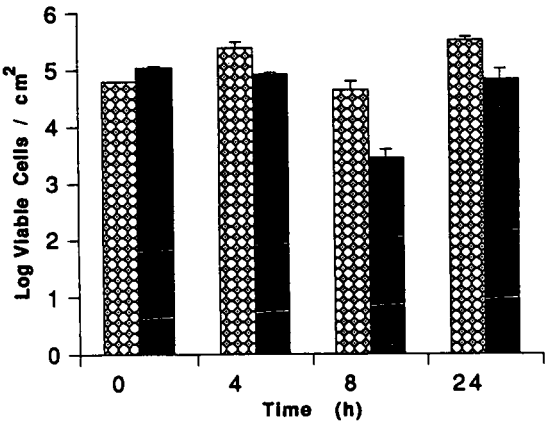

Figure 1 The effect of a low strength electric field/current density on an established *Pseudomonas aeruginosa* biofilm population. The control and electrified modified Robbins devices are represented by patterned and solid bars, respectively. (Mean ± 1 SE, n=2).

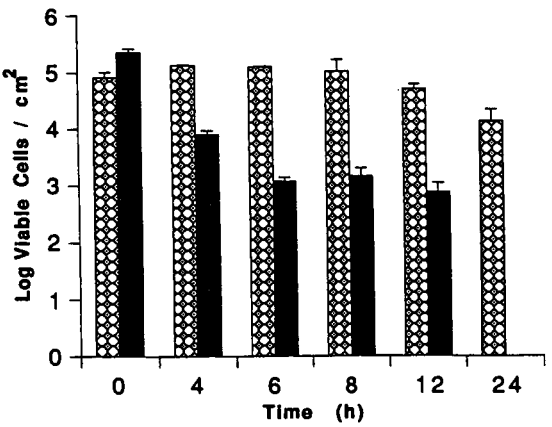

Figure 2 The effect of 5 ppm glutaraldehyde against an established *Pseudomonas aeruginosa* biofilm population in the absence (pattern) and presence (solid) of a low strength electric field/current density. (Mean ± 1 SE, n=2).