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Coal Solubilization by Enhanced Enzyme Activity in Organic Solvents

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ABSTRACT

Both oxidative and reductive enzymes can be utilized to enhance coal solubilization in aqueous and organic media. Aerobic solubilization was carried out with oxidases in a relatively polar medium, whereas solubilization in an anaerobic environment was conducted with reducing enzymes (dehydrogenase or hydrogenase) in aqueous and both polar and nonpolar organic media in the presence of hydrogen or a hydrogen donor. Solubilization of some enzymes in organic liquids was enhanced by complexation with polyethylene glycol. Enzyme concentration of 1–20 mg/mL was used, and most of the reactions occurred during the first 4 h, with up to 85% of the coal solubilized. There was some evidence of coal product inhibition and enzyme deactivation at higher temperatures.

Index Entries: Coal solubilization; enzymes; organic media; oxidases; hydrogenase; dehydrogenase.

INTRODUCTION

Progress continues to be made on the biological solubilization of coal. Most of the research is still associated with direct microbial action on low-ranked coal particulates in an aerobic environment in which the solubilization product is a complex aqueous solution of predominantly aromatic

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compounds that cover a wide molecular weight (1–5). It is expected that this is an extracellular process in which at least one of the mechanisms is associated with enzymatic action (*2,5*).

Earlier research has indicated that, under some circumstances, enzymes used in vitro can also affect the solubilization or liquefaction of coal (*3,5*). These previous results were very preliminary in nature and difficult to reproduce. However, such a bioprocessing approach is of great interest since both oxidative and reductive enzymes could potentially be used in either aerobic or anaerobic processes, thus allowing a variety of bioprocessing options. Furthermore, the use of an organic solvent instead of an aqueous medium, in conjunction with the enzyme solubilization process, may also be possible. Such a system may provide an environment that interacts more effectively with the coal particulates and is more conducive to a nonpolar solubilization product that would have properties that are similar to conventional thermal/chemical coal liquefaction products.

The use of isolated enzymes for coal solubilization has been further investigated with the emphasis on enhancing enzyme activity and determining the effects of operating parameters. Organic liquids, as well as aqueous solutions, were tested as the reaction media and a wide range of coals, varying from lignite to bituminous, was examined.

POSSIBLE ENZYMATIC INTERACTIONS

As previously discussed (*5*), solubilization or liquefaction of coal will require the transfer of hydrogen and/or oxygen between the coal and the reaction medium or portions of the coal itself. This should result in the disruption of the chemical bonding that makes the coal a stable, three-dimensional structure and in the modification of the resulting molecular fragments into more polar (oxidative process) or less polar (reductive process) constituents.

Enzymes that catalyze the exchange of H_2 and O_2 are categorized as oxidoreductases and classified as one of the six main groups of enzymes by the International Union of Biochemistry (*6*). These are the types of enzymes that would be expected to enhance chemical interactions with coal; however, there is a great variety in their functions and stability. The chemical system must also have an oxidation/reduction reagent that can be quite specific for a specific enzyme. Some oxidoreductases may be able to operate in either the oxidation or reduction mode, that is, as a reversible chemical reaction system. But, most enzymes will be much more effective as an oxidase or a reductase and are so designated.

Oxidative Processes

The aerobic processing of coal should result in an oxidized product in which additional oxygen has been introduced. Thus, enzymes that cata-

lyze the addition of oxygen would be expected to be effective in this environment. The two approaches that would seem appropriate for enzyme-enhanced, oxidative solubilization of coal are: the use of various oxidases that can transfer molecular oxygen,

$$\text{Coal} + O_2 - (\text{Oxidase}) \rightarrow \text{Oxidized Coal Fragments} \quad (1)$$

or those enzymes, such as the peroxidases, that can transfer oxygen from other oxidizing substances,

$$\text{Coal} + H_2O_2 - (\text{Peroxidase}) \rightarrow \text{Oxidized Coal Fragments} \quad (2)$$

The use of peroxide as the oxidizing agent may be more chemically effective than molecular oxygen, but it would result in a more expensive process.

Reductive Processes

Anaerobic enzymatic processes will require the addition of hydrogen to the coal matrix and the probable removal of some oxygen and other heteroatoms. Again, one can envision enzymes that can transfer the gaseous reagent (H_2)

$$\text{Coal} + H_2 - (\text{Hydrogenase}) \rightarrow \text{Reduced Coal Fragments} \quad (3)$$

or utilize a hydrogen donor, such as NADH, or even other enzymes, such as cytochrome *c*, that can serve as electron carriers

$$\text{Coal} + \text{NADH} - (\text{Dehydrogenase}) \rightarrow \text{Reduced Coal Fragments} \quad (4)$$

Some hydrogenases, such as one that can be isolated from *Clostridium pasteurianum* (*7*), are much more effective in producing molecular hydrogen from oxidized substrates rather than utilizing hydrogen to reduce a substrate, such as those enzymes isolated from *Proteus vulgaris, Desulfovibrio desulfuricans,* and *Chromatium* (*7,8*). Fortunately, the hydrogenases extracted from the latter types of microorganisms are much more stable and easy to use.

MATERIALS AND PROCEDURES

Enzymes

Some of the oxidoreductases were obtained from commercial sources with horseradish peroxidase from either the Sigma Chemical Co., St. Louis, MO (Type VI) or Calbiochem, Los Angeles, CA (E1A grade) and laccase (from *Pyricularia oryzae*), tyrosinase (mushroom), cytochrome *c* (Type IV from bovine heart), and alcohol dehydrogenase (ADH) (baker's yeast) from Sigma. There appeared to be significant variations in these enzymes, depending on the batch and source. A partially-purified hydrogenase was isolated from *Proteus vulgaris* using a modification of the method of Schengrund and Krasna (*9*).

The suggested techniques in the appropriate product bulletins from Sigma were used to determine enzyme activities for the peroxidase, laccase, tyrosinase, and dehydrogenase, and the technique of Errede et al. (*10*) was used to determine cytochrome activity. Hydrogenase activities were determined by the spectrophotometric method of Ballantine and Boxer (*11*).

Coal Samples

Three different types of coal were used in the solubilization tests, including a highly oxidized lignite or leonardite (American Colloid Company, Skokie, IL), Wyodak subbituminous (Amax Coal Company, Indianapolis, IN), and Illinois No. 6 bituminous (Illinois State Geologic Survey, Springfield, IL). In some cases, the subbituminous coal was preoxidized by treatment with 8*N* HNO_3 for 24 h at ambient temperature, followed by three successive water washes. The coal was sealed under nitrogen until ready for use, then processed in air prior to the solubilization tests. Coal samples were size-reduced by mortar and pestle and used in the size range of 100–170 mesh.

Organic Solvents and Chemicals

Several different organic solvents were used in the course of this research. These included pyridine (spectrophotometric grade; Burdick and Jackson Laboratories, Muskegon, MI), dioxane (spectrophotometric grade; MCB Reagents, Cincinnati, OH), and benzene (purified grade; Fisher Scientific Company, Fair Lawn, NJ). Polyethylene glycol (PEG) with a mol wt range of 6000–7000 daltons was obtained from J. T. Baker Chemical Co., Phillipsburg, NJ; methoxypolyethylene glycol activated with cyanuric chloride (average mol wt of 5000 daltons) and triazine dyes (Basilen Blue and Reactive Red 4) were obtained from Sigma. All other chemicals were reagent grade or highest purity available from several different chemical supply companies.

Coal Solubilization Tests

Two different types of coal solubilization tests were made. Most experiments were made by adding 0.1–0.2 g coal particulates to 50 mL temperature-controlled shake flasks, each of which typically contained 10–20 mL of a specific solubilization solution. Agitation was at 100 rpm, and either air or hydrogen was continuously added to the headspace of each flask. The gases were presaturated with the appropriate liquid so that there would be minimal solvent loss during the test. A few tests were made in a small fluidized-bed bioreactor with a volumetric capacity of 40 mL, in which 3 g coal particulates were suspended by 150 mL of circulating solubilization solution that was contacted with air in an external chamber (Fig. 1). The coal samples were initially dried in a vacuum oven at 100°C for 4 h and immediately weighed.

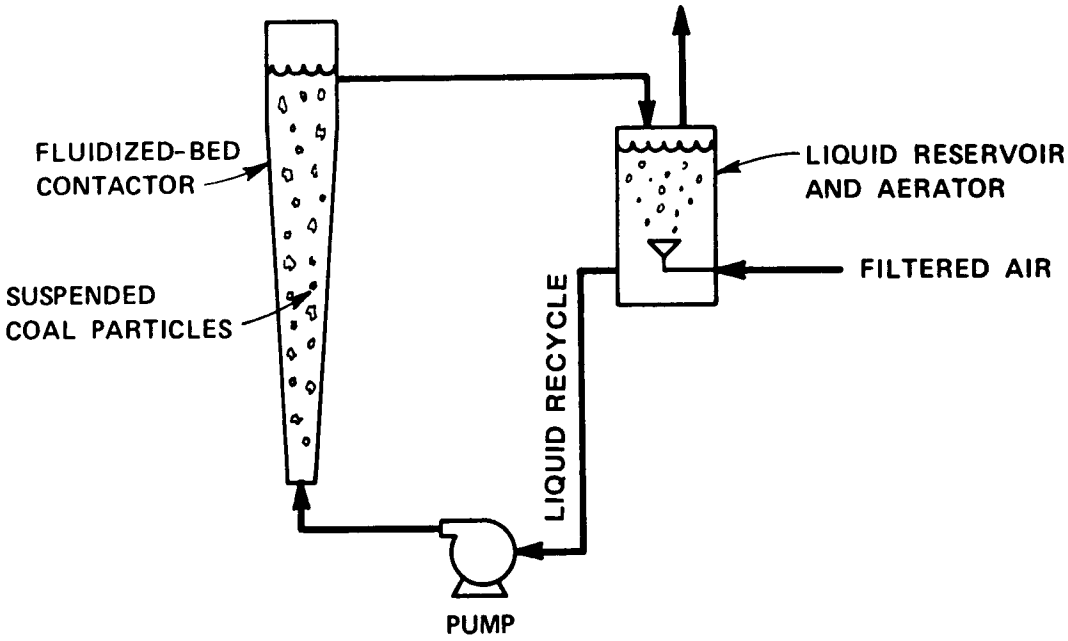

Fig. 1. Experimental system for coal solubilization in a small fluidized-bed bioreactor.

The course of solubilization was followed by two analytical methods. In most tests, at the end of the prescribed reaction time, the coal was removed from the solution mixture by centrifugation or filtration, washed three times with distilled/deionized water, and vacuum dried as above before weighing. When benzene was used as the solvent, the wash solution was acetone. In some cases, small samples of the reaction solution were periodically removed, and a spectrophotometric scan was made in the range of 200–600 nm. The increase in adsorbing species was an indication of coal solubilization.

Modifying Enzymes to Enhance Solubilization in Organic Solvents

It is desirable to enhance the solubilization of enzymes in organic solvents while maintaining high activity. The approach was to chemically modify the enzyme so they were more hydrophobic and, thus, more soluble in organic liquids.

Acylation by Palmitoyl Chloride

As previously suggested, palmitoyl chloride presumably could serve this purpose by acylating the protein materials to form a chemical complex (*12*). Several tests were made to further investigate this chemical interaction. The experimental approach was to solubilize the unmodified enzyme into an appropriate buffer solution and then add palmitoyl chloride at a level of 4 m*M*. This mixture was allowed to interact at ambient conditions

for 0.5 h. Thereafter, the mixture was added to the organic solvent, and any precipitate was removed by centrifugation. Light absorption measurement at 280 nm of the supernatant was used to determine the total amount of protein that remained in solution.

Attachment to Polyethylene Glycol by Triazine Dyes

A different approach has also been developed in which the enzyme is chemically bound to polyethylene glycol (PEG) by a bridging chemical that hopefully will not greatly affect activity. Triazine dyes are generally insoluble in organic solvents, but they are specific ligands with a general affinity for proteins. They have been used in affinity chromatography to remove enzymes from bulk protein solution (*13*). Similarly, triazine dyes can be bound to PEG, which is a water-soluble polymer that also has some solubility in organic solvents, especially aromatic compounds (*14*). The resulting PEG–dye–enzyme complex has now been shown to have enhanced organic solubility.

The dye was first attached to the PEG using a method that was a modification of that used by Johansson (*15,16*). Approximately 700 g/L PEG was dissolved in a 1.5*M* solution of NACl. The dye was then added to the solution, typically at 75 g/L, and the resulting solution was stirred at ambient temperature for 1 h. This resulted in a mixture of the PEG–dye complex and unreacted PEG and dye. After the reaction, approximately 60 mL of 0.5*M* NaH_2PO_4 and 2.5 L of 3*M* KCl was added per liter of mixture. The complex was then extracted with approximately 12.5 L of chloroform/L of mixture. The chloroform was removed by evaporation, leaving a crude PEG–dye product.

A further purification step was used for most tests. This included dissolving the crude product in distilled water using 20 mL water/g PEG and adding DEAE cellulose in 5 m*M* sodium phosphate buffer, pH 7.0, with an aqueous solution to polymer volumetric ratio of 0.35:1. This mixture was stirred at ambient temperature for 30 min and then filtered, followed by washing the filter cake with distilled water. The PEG–dye complex was then removed from the DEAE by an equal volume of 5 m*M* sodium phosphate buffer, pH 7.0, which also contained 2*M* KCl and again was extracted into an equal volume of chloroform. After evaporation of the chloroform, the solid PEG–dye complex was stored dry at ambient temperature until used. Various enzymes could then be attached to the PEG–dye complex by contact in a stirred aqueous solution at an ambient temperature at pH 6–7 for 0.5–2.0 h. The water was removed by evaporation, and the solid PEG–dye–enzyme complex was ready for use.

Attachment to PEG by Cyanuric Chloride

Methoxypolyethylene glycol will interact with cyanuric chloride, resulting in an activated PEG (PEG_1) that will react with the free amino acid moiety in proteins (*17*). The resulting PEG_1–enzyme complex will be much more hydrophobic and should be soluble in nonpolar organic sol-

vents. To carry out the chemical interaction, PEG_1 and the enzyme were dissolved in a 0.1*M* borate buffer, initially pH 8.0. The pH was then raised to 10 by the addition of 0.1*N* NaOH, and the reaction was allowed to progress at an ambient temperature (20±1°C) for 1 h. The reaction mixture was then "quenched" by reducing the pH to 8, by the addition of 0.1*N* HCl, ultrafiltered, and washed with 2 vol distilled water, lyophilized, and refrigerated until further use.

DISCUSSION

Additional tests have been made, with both oxidative and reductive enzyme systems, utilizing two primary approaches. In one case, the enzymes were dissolved in a buffered aqueous solution that contacted the coal particles in suspension, and in a second approach, the enzymes were first dissolved in an organic solvent, either a hydrous organic or a relatively nonpolar solvent, and then the solution mixtures were used to contact coal particles.

The processes being studied represent complex, heterogeneous chemical reactions with significant analytical problems. It is particularly difficult to accurately determine the degree of coal solubilization since the remaining coal particulates must be quantitatively recovered from a solution mixture that may also include precipitates and adsorbing macromolecules. At this stage of preliminary development, the trends established by the tests are considered to be valid, but specific solubilization rates are probably only qualitatively useful.

Enhanced Enzyme Solubilization in Organic Solvents

Three techniques of chemical modification were investigated to increase the concentration of active enzymes that can be incorporated into organic media.

Enzyme Reaction with Palmitoyl Chloride

Enzyme acylation by palmitoyl chloride was studied as a possible means of enhancing the solubilization of horseradish peroxidase (Sigma) in hydrous dioxane or pyridine. The former organic solvent had been previously used with the oxidase for other applications (*12*), and the latter has been frequently used for interaction with coal.

Palmitoyl chloride did enhance enzyme solubilization in both hydrous organic solvents. For example, the unmodified enzyme had a maximum concn. of 0.06 mg/mL in 95% dioxane, but this could be increased by as much as a factor of 10 after interaction with palmitoyl chloride (Table 1). Unfortunately, it was also discovered that apparent enzyme activity was significantly reduced when the enzyme was modified so that the total

Table 1
The Effect of Palmitoyl Chloride Modification on Peroxidase Solubilization in Hydrous Dioxane[a]

Form of enzyme	Concentration of enzyme, mg/mL[b]	
	Buffer	Organic
Unmodified	5.0	0.06
Unmodified	10.0	0.06
Unmodified	20.0	0.06
Modified	10.0	0.20
Modified	20.0	0.64

[a]All tests were made in shake flasks at 20°C under air with dioxane containing 5% of 0.1*M* phosphate buffer, pH 6.5. Horseradish peroxidase from Sigma was used.
[b]Equivalent concentration measured by absorbance at 280 nm after solids were removed by configuration.

Table 2
Effect of Palmitoyl Chloride Modification on Peroxidase Activity[a]

Form of enzyme	Hydrous solvent[b]	Enzyme activity, % of original
Unmodified	85% dioxane	0.1
Unmodified	85% pyridine	36.4
Modified	85% dioxane	<0.1
Modified	85% pyridine	40.7

[a]All tests were made in shake flasks under air at 20°C. Horseradish peroxidase from Calbiochem at an initial concentration of 2–5 mg/mL was used.
[b]A 0.1*M* phosphate buffer, pH 6.5, was used to initially solubilize the enzyme and as the aqueous phase in the hydrous organic solvent.

effect of palmitoyl chloride treatment was to decrease the catalytic effect in the organic (Table 2). However, there was some indication that solubilization in the organic provided a more stable enzyme since the peroxidase activity in the presence of dissolved coal after 4 h was higher in hydrous pyridine than aqueous buffer.

Enzyme Attachment to PEG by Triazine Dye

It was found that the resulting PEG–dye–enzyme complex did have increased solubility in relatively nonpolar solvents, such as benzene. Both peroxidase and ADH were attached to the PEG, and the dried complex was then dissolved into benzene. An aqueous buffer solution was then used to extract the PEG–dye–enzyme complex from benzene, after which enzyme activity was measured. Such a technique would imply enzyme

Table 3
Enhancement of Enzyme Activity in Benzene by the PEG–Dye Complex[a]

Enzyme	Concentration of PEG–dye complex in aqueous buffer, mg/mL	Enzyme activity, U/mL	
		Aqueous buffer before drying	Benzene after aqueous extraction
Alcohol dehydrogenase	0	113.0	0.00
	3	113.0	0.19
	6	83.7	0.02
Peroxidase	0	155.8	0.00
	1	161.8	0.76
	5	154.3	2.26
	10	190.3	3.06
Cytochrome[b]	0	2.0	<0.01
	5	2.0	0.60
	10	2.0	1.18
	20	2.0	2.10
Hydrogenase from *P. vulgaris*	0	0.12	<0.004
	5	0.12	<0.004
	10	0.12	0.006
	20	0.12	0.015

[a]All tests were made at 20–25°C in shake flasks under air for the oxidases and H_2 for the reductases. The concentration of the enzyme preparation prior to complexation was 1 g/mL for ADH and peroxidase, 2 mg/mL for cytochrome *c*, and 1.5 mg/mL for hydrogenase.
[b]Equivalent activity for cytochrome *c* is reported in equivalent mg/mL of the active enzyme.

activity in the organic. Activity of both peroxidase and ADH, dissolved in benzene, was increased well above control levels by use of this technique (Table 3). However, the ADH activity tended to decrease with increasing PEG–dye concentrations. This is probably because ADH is an NAD^+-dependent enzyme, and some triazine dyes are known to be competitive inhibitors of such enzymes (*18*). On the other hand, peroxidase activity was not inhibited by the PEG–dye, and benzene solubilization of the active enzyme increased with increasing PEG–dye concentration. The organic solubilization of hydrogenase also increased with increasing concentration of the PEG–dye complex.

Attachment to PEG by Cyanuric Chloride

Tests were also made on the complexation of hydrogenase with PEG_1. Such chemical changes had previously been made to suppress the immunological response of enzymes while maintaining enzyme activity (*19*). The resulting PEG_1–enzyme complex has also enhanced solubilization in

Table 4
Increase in Hydrogenase Solubilization
in Hydrous Benzene by Complexation with PEG_1[a]

Ratio of PEG_1 to enzyme concentration, g/g	Enzyme activity, U/mL		Solubility in hydrous benzene,[b] mg/mL
	in aqueous buffer	after PEG_1 modification	
0.0	1.20	--	<0.01
22.5	0.73	0.105	0.11
45.0	0.73	0.077	0.09
90.0	1.20	0.24	0.42

[a]Starting material was 4 mL of 1.8 mg/mL hydrogenase in a 0.1*M* phosphate buffer, pH 7.0. The resulting lyophilized material was contacted with 10 mL benzene at 30°C.
[b]Benzene was saturated with H_2O at 30°C.

organic solvents, such as hydrous (water-saturated) benzene. The aim was to utilize a sufficient quantity of PEG_1 to increase solubilization (tie up the free amino acid moiety) without significantly affecting enzyme activity (catalytic-active sites).

PEG_1 concentration ratios ranging from 22.5–90 mg PEG_1/mg hydrogenase were tested. It was found that the solubilization in hydrous benzene increased as the PEG_1 ratio increased, but there was also some loss of activity, especially at the higher concentrations of PEG_1 (Table 4). This enzyme modification technique has resulted in the highest level of enzyme solubilization in benzene.

Aerobic Enzyme Solubilization of Coal

Additional tests have now been made on enzymatic enhancement of coal solubilization in an aerobic environment, with emphasis on the use of horseradish peroxidase in hydrous organic solvents that are in contact with air.

Effect of Solubilized Coal on Peroxidase Activity

Peroxidase activity appears to decrease during extended contact with products from the oxidative solubilization of coal. Tests were made in shake flasks with air in the head space at 20°C with 85% pyridine, 20 m*M* H_2O_2 and 5 mg/mL of the enzyme in contact with 10 mg/mL subbituminous coal. After 1 h exposure, activity had decreased to 33% of the initial activity. This further decreased to 16% at 2 h and 11% at 4 h, and there was essentially no activity after 24 h. Some of this decrease may be owing to a decrease in pH (typically from an initial 6.0 to a final 5.7) during the course of the solubilization, but there should still be significant activity even at the lowest pH.

Effect of Temperature on Peroxidase Activity

Oxidative enzyme activity in hydrous organic solvents was found to be decreased by increasing temperature in tests similar to those described in the previous section. Peroxidase activity decreased during the coal solubilization tests at all temperatures tested, but activity was still 11% of the original after exposure for 4 h in the temperature range of 20–30 °C. There appeared to be complete deactivation at a temperature of 40 °C after only 2 h operation. This effect cannot be extrapolated to other enzyme systems and different operating environments, but obviously the temperature of the solubilization process will be an important factor.

Aerobic Solubilization of Coal

Several oxidative enzymes were studied for the solubilization of low-ranked coals (lignite and subbituminous) in both aqueous and hydrous organic media. Although horseradish peroxidase was the most thoroughly studied biocatalyst, scouting tests were also made with laccase and tyrosinase. Peroxidase requires hydrogen peroxide as the oxidizing agent, whereas the other two oxidases should be able to utilize molecular oxygen. As in previous tests (*5*), there was significant experimental variability, apparently owing to variations in the chemical environment (e.g., changes in pH during tests) and the enzyme preparation. Attempts were made to control the former by utilizing a relatively strong buffer (0.1*M*), but it was impossible to control the latter completely since the enzymes were obtained from commercial sources. These factors made it difficult to obtain consistent results, but it was possible to determine trends and overall effects.

In general, the peroxidase was much more effective than the other oxidases in enhancing coal solubilization. Although laccase and tyrosinase had apparent small solubilization enhancements (3–5%) in some tests with and without hydrous organic media, these results were difficult to reproduce, and the conclusion was that oxidases that utilize molecular oxygen are not effective biocatalysts for coal solubilization, at least under the range of conditions tested.

Peroxidase was shown to increase coal solubilization, especially for acid-oxidized subbituminous coal when used at a moderate temperature in both aqueous and hydrous organic media, with the latter being more effective (Table 5). The standard deviation for repetitive tests at the same conditions was typically 2–3%. In most tests, the enzyme enhancement was measurable, but relatively low; however, increasing enzyme and H_2O_2 concentrations tended to increase the solubilization enhancement by the peroxidase by as much as 44%. Hydrous dioxane and pyridine were more effective than aqueous buffer, with 85% pyridine giving the most significant enhancement.

Enzyme-enhanced solubilization was also confirmed in a small fluidized-bed bioreactor in which hydrous dioxane containing peroxidase and

Table 5
Enhancement of Aerobic Solubilization of Subbituminous Coal by Calbiochem Peroxidase in Aqueous Buffer and Hydrous Organic Solvents[a]

Type of coal	Reaction medium	Initial enzyme concen., mg/mL	Initial H_2O_2 concen., m*M*	Reaction time, h	Coal solubilization, % with enzyme[b]	Coal solubilization, % without enzyme
Preoxidized subbtiminous	Aqueous buffer	5	10	2	36.9	36.3
Preoxidized subbtiminous	95% dioxane	5	10	2	39.5	38.0
Preoxidized subbituminous	95% dioxane	5	10	4	60.2	43.2
Preoxidized subbituminous	95% dioxane	5	20	2	68.0	65.7
Preoxidized subbituminous	95% dioxane	20	20	48	82.5	71.3
Preoxidized subbtiminous	85% dioxane	5	10	2	50.0	41.7
Preoxidized subbituminous	85% dioxane	10	10	2	63.3	60.3
Preoxidized subbituminous	95% pyridine	5	10	2	50.4	42.6
Preoxidized subbituminous	85% pyridine	5	10	2	63.2	56.6
Preoxidized subbituminous	85% pyridine	20	10	2	64.2	44.6
Untreated subbituminous	85% dioxane	5	10	48	19.5	16.5
Untreated subbituminous	85% pyridine	5	10	48	21.1	16.7

[a] All tests were made at 20°C in contact with air in shake flasks. A 0.1*M* phosphate buffer, pH 6.0, was used either alone or as the aqueous component of the hydrous organics.
[b] Some of the enzyme precipitated when contacted with the organic medium and was removed by centrifugation at 5000 *g* for 15 min.

H_2O_2 was circulated through a suspended bed of coal particulates. Liquid samples were taken periodically, and the absorbance at 400 nm was used to determine the amount of solubilization. As shown in Fig. 2, solubilization was relatively rapid, and the amount of absorbing material was greater when the enzyme was used. After 48 h, the degree of solubilization was 85% when the enzyme was used, compared to approximately 50% when only the hydrous organic solvent was used.

Anaerobic Enzyme Solubilization of Coal

The anaerobic solubilization of coal may be even more important than the oxidative interaction, especially if molecular H_2 can be used at moderate temperatures. A series of tests have been made with untreated bituminous and subbituminous coals in which reducing enzymes were used

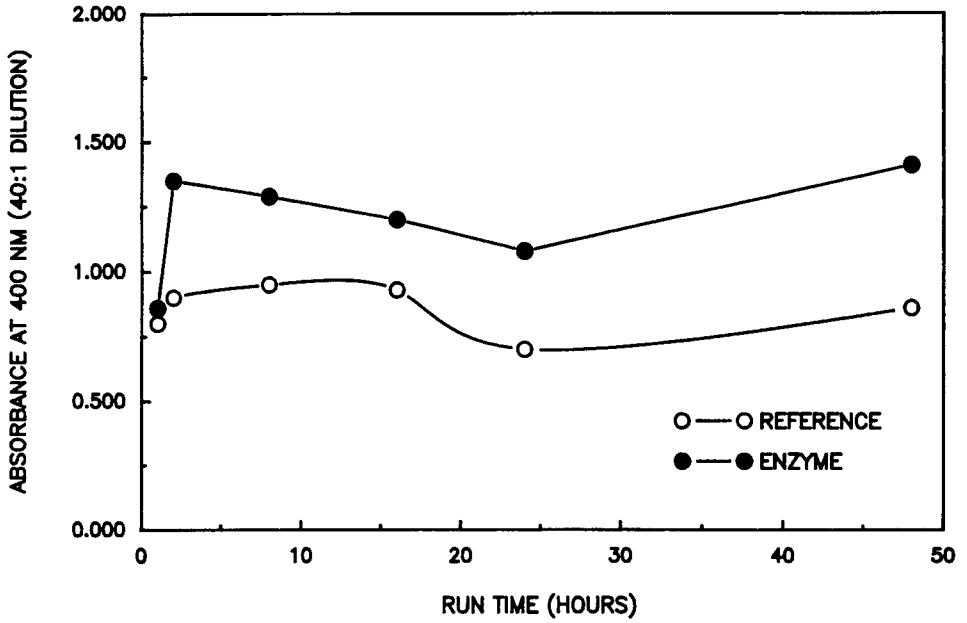

Fig. 2. Dissolution of preoxidized subbituminous coal in a fluidized-bed bioreactor at 20°C, with 1 g coal particulates contacted with 250 mL aerated dioxane, containing 5% 0.1*M* phosphate buffer, pH 6.0, with 10 mg/mL peroxidase and 10 m*M* H_2O_2.

in aqueous and organic media. Two primary approaches were taken: the use of a reducing enzyme that is also a reducing agent or can utilize a hydrogen donor and the use of a hydrogenase that could directly introduce molecular H_2 into the coal structure.

Hydrogen Donor Systems

Alcohol dehydrogenase and cytochrome *c* were tested for enhancing coal solubilization by catalyzing the transfer of hydrogen from a soluble reagent to the coal structure. All tests were made under a hydrogen atmosphere. ADH was tested over a wide range of operating parameters, but the highest enzyme activity appeared to be at a temperature of 20°C and a pH in the range of 7 to 8. It was necessary to utilize a buffer concentration of at least 0.1*M* in order to maintain the proper pH during the course of the tests. Most of the tests were made on untreated subbituminous coal, and a few tests were made with butiminous coal.

ADH was found to increase coal solubilization when used in an aqueous medium, especially when a reducing agent other than molecular hydrogen was used (Table 6). NADH was somewhat better than sodium dithionite, and the use of hydrous organic solvents as the solubilization solution resulted in higher rates. Cytochrome *c* in the reduced form also enhanced solubilization rates, and it was found that ADH increased bituminous coal solubilization when the enzyme was incorporated into the PEG-dye-enzyme complex and dissolved in benzene.

Table 6
The Effect of Reducing Enzymes Utilizing Hydrogen Donors for the Solubilization of Coal[a]

Type	Enzyme conc., mg/mL	Reducing agent		Reaction media	Reaction time, h	Coal solubilization, %	
		Type	Conc., mg/mL			with enzyme	without enzyme
ADH	20	NADH	0.0	Aqueous	1	13.1	11.8
	20	NADH	2.0	Aqueous	1	15.7	11.0
	40	NADH	4.0	Aqueous	1	18.2	17.0
	20	NADH	2.0	Aqueous	2	19.4	18.3
	20	NADH	2.0	85% pyridine	2	25.3	24.0
	20	NADH	2.0	85% dioxane	2	26.3	24.0
	20	Dithionite	2.0	Aqueous	1	14.7	11.4
	20	Dithionite	2.0	85% pyridine	2	20.8	23.0
	20	Dithionite	2.0	85% dioxane	2	22.5	22.7
Reduced cytochrome	20		0.0	Aqueous	2	20.0	13.2
PEG-dye reduced cytochrome	2		0.0	Benzene	2	6.7[b]	5.2[b]
	2		0.0	Benzene	4	15.8[b,c]	9.4[b,c]
PEG-dye-ADH	2		0.0	Benzene	2	9.2[b,c]	9.4[b,c]

[a]All tests were made with subbituminous coal, except where otherwise noted, in shake flasks operating under H_2 at 20°C. The aqueous component was 0.1*M* phosphate buffer at pH 8.0.
[b]Illinois #6 bituminous coal was used for these tests.
[c]Standard deviations vary from 0.3 to 1.0%.

Hydrogenase Enhancement of Coal Solubilization

The hydrogenase recovered from *P. vulgaris* has been shown to utilize molecular H_2 and be relatively stable, even when exposed to air for extended periods. Although there was apparently some variation in the different batches of hydrogenase that were isolated, especially in the specific activity, solubilization tests do show that there is slight enhancement of subbituminous coal dissolution during 4-h tests, even in an aqueous solution (Table 7). This effect appears to vanish at 40°C. Even more importantly, there appears to be an increase in bituminous coal solubilization when the hydrogenase is used in benzene via the PEG-dye-enzyme complex or when attached to PEG_1. The latter method of enzyme modification seems to be superior.

Liquid Product from Anaerobic Coal Dissolution

It was previously indicated that anaerobic coal solubilization resulted in a more polar product (*5*). Additional tests have now been made that tend to verify those results. At the end of an anaerobic solubilization test, where an organic solvent is used and in the absence of the PEG-dye com-

Table 7
The Effect of a Stable Hydrogenase
on the Solubilization of Subbituminous and Bituminous Coals[a]

Type of coal	Reaction media	Enzyme activity, U/mL	Temp., °C	Reaction time, h	Coal solubilization, % with enzymes	Coal solubilization, % without enzymes
Subbituminous	Aqueous	0.1	20	2	10.3	10.7
Subbituminous	Aqueous	0.1	20	4	20.4	19.2
Subbituminous	Aqueous	0.1	40	4	16.7	17.8
Bituminous	Peg-dye-enzyme[b]	0.16	30	4	7.7	7.4
Bituminous	PEG_1-enzyme[c]	0.13	30	4	19.7	15.9

[a]All tests were made in shake flasks operating under H_2. The aqueous phase was 0.1*N* phosphate buffer, pH 8.0.
[b]Ratio of PEG-dye to enzyme was 10 on a weight basis.
[c]Ratio of PEG_1 to enzyme was 80 on a weight basis.

Table 8
Characteristics of the Liquid Product
from Hydrogenase-Enhanced Solubilization of Bituminous Coal in Benzene[a]

Liquid product	Absorbance at 420 nm[b] Benzene fraction	Absorbance at 420 nm[b] Aqueous fraction
Benzene-PEG-dye	0.12	0.44
Benzene-PEG-dye-hydrogenase	0.57	<0.0

[a]The solubilization products from tests at 20°C for 2 h were contacted with an equal volume of 0.1*M* phosphate buffer, pH 7.0.
[b]The reference solutions for the two different samples were benzene and the aqueous buffer containing unreacted PEG-dye complex at the same concentration used in the solubilization tests.

plex, the color of the solution varies from a light yellow to a deep brown, depending on the degree of solubilization.

The product from hydrogenase-enhanced solubilization of bituminous coal in benzene was particularly interesting since no aqueous phase was used. Additional qualitative tests were made on the liquid product from this type of solubilization by contacting the organic phase with a 0.1*M* phosphate buffer, pH 7.0, to remove the PEG-dye-enzyme complex first and then making spectrophotometric measurements of the resulting liquids to infer the amounts of material dissolved in each phase, as previously demonstrated (2). It was found that a considerable amount of the solubilized coal remained in the benzene phase, especially when the hydrogenase was used (Table 8), thus indicating that the product had a significant con-

tent of polar substances. This material is obviously quite different from the product of the oxidative process that was shown to be minimally soluble in organic solvents (2).

CONCLUSIONS

Both oxidative and reductive enzymes can accelerate the solubilization of coal in aqueous and organic media. The reacting systems are very complex, with numerous important operating parameters. In many cases, the effects are measurable, but minimal, and cannot yet be the basis for process development until additional research is carried out. The use of a stable hydrogenase in an organic medium under anaerobic conditions for the solubilization of higher-ranked coals shows the most promise, with the resulting product being much less polar than the oxidative product.

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