ANAEROBIC LIQUEFACTION/SOLUBILIZATION OF COAL BY MICROORGANISMS AND ISOLATED ENZYMES*

Charles D. Scott
Brendlyn D. Faison
Charlene A. Woodward

Oak Ridge National Laboratory
Oak Ridge, Tennessee 37831

To be presented at the Liquefaction Contractors' Review Meeting, U.S. Department of Energy, Pittsburgh Energy Technology Center, Pittsburgh, Pennsylvania, September 3, 1991.

*Research supported by the Fossil Energy Advanced Research and Technology Program, part of which is managed by the Pittsburgh Energy Technology Center, U.S. Department of Energy, under contract DE-AC05-84OR21400 with Martin Marietta Energy Systems, Inc.

ANAEROBIC LIQUEFACTION/SOLUBILIZATION OF COAL BY MICROORGANISMS AND ISOLATED ENZYMES*

Charles D. Scott
Brendlyn D. Faison
Charlene A. Woodward

Oak Ridge National Laboratory
Oak Ridge, Tennessee 37831

ABSTRACT

Biocatalytic systems utilizing either living organisms or modified enzymes have been shown to enhance the liquefaction (products are liquid at ambient conditions) or solubilization of coal under anaerobic conditions. Microbial tests have been carried out in aqueous media with organisms isolated from outcropping of coal or from premium coal samples. Some of these isolates have been shown to grow on coal as the only carbon source and to produce small quantities of oxychemicals such as acetate or ethanol. Reducing enzymes, such as hydrogenase and cytochrome C, can be chemically modified to increase solubilization in organic solvents by attaching less polar chemicals, such as phenyl groups or polyethylene glycol, to the free amino groups on the enzymes. These biocatalysts have been shown to degrade model compounds and enhance the solubilization of coal in organic solvents under a hydrogen atmosphere. The resulting product is a relatively light hydrocarbon mixture with reasonably high volatility.

*Research supported by the Fossil Energy Advanced Research and Technology Program, part of which is managed by the Pittsburgh Energy Technology Center, U.S. Department of Energy, under contract DE-AC05-84OR21400 with Martin Marietta Energy Systems, Inc.

INTRODUCTION

Although thermal/chemical techniques have been developed to convert coal to liquids and gases, rather severe temperatures, pressures, and chemical environments must be used. Since biological processes operate at relatively mild conditions, they may offer attractive processing alternatives for coal conversion.

It has previously been shown that aerobic microbial systems and in vitro enzymes enhance the solubilization of low-rank coals in both aqueous and organic media.[1,2] Preliminary results have also indicated that under anaerobic conditions some microorganisms and enzymes also enhance coal interactions.[3,4] These investigations have been further extended with an emphasis on mixed cultures of microorganisms and the use of enzymes in organic solvents. New microbial consortia have been isolated from Argonne Premium Coal samples, and different approaches are under development for the chemical modification of enzymes to enhance solubilization in organic solvents.

MATERIALS AND METHODS

Microorganisms

Microorganisms recently investigated in this study include one strain that had been previously shown to interact with coal under aerobic conditions (*Candida* sp. ML13 isolated from a lignite outcropping by Prof. B. Ward, University of Mississippi) and those isolated at this laboratory from premium coal samples obtained from the Argonne collection. The isolates recovered from Argonne Premium Coal samples included one from lignite (L-1), one from subbituminous coal (S-1), and one from bituminous coal (B-1).

The culture from lignite grown in either acetate or benzoate contained Gram-positive nonsporulating rods, Gram-negative rods, and Gram-negative cocci. These organisms were tentatively identified as methanogens that exhibit Gram variability, and they probably include *Methanobacterium* and *Methanococcus* spp. The culture from subbituminous coals contained plump Gram-positive and

Gram-negative rods, some of which contained spores. Nonsporulating organisms were tentatively identified as methanogens, while the sporulating organisms were tentatively identified as members of the family *Bacillaceae*, which includes the genera *Clostridium, Bacillus, Sporolactobacillus, and Desulfotomaculus*. These genera are known to produce acids and alcohols from organic substrates.

The bituminous coal culture grown in an acetate medium contained Gram-negative rods and Gram-positive rods with spores tentatively identified as methanogens and bacilli, respectively. However, this culture grown in benzoate contained Gram-negative rods of irregular morphology (tentatively identified as members of the *Propionibacteriaceae* family), plus Gram-negative rods and cocci that were probably methanogens.

Chemicals

The primary chemical reactants used to modify enzymes were activated methoxypolyethylene glycol (PEG_1) and dinitrofluorobenzene (DNFB), both obtained from Sigma Chemical Co. Two model compounds used to simulate some of the chemical interactions necessary for coal solubilization were 1,2-bis(4-quinolyl)ethane (QE), supplied by Dr. M. Farcasiu of the Pittsburgh Energy Technology Center, and 1,2-bis(4-pyridyl)ethane (PE), obtained from Sigma Chemical Co. The model compounds have two identical aromatic components connected by an ethane group. Several expected breakdown products of these compounds were also obtained from Sigma Chemical Co. to be used as reference material for analysis by gas chromatography (GC). For QE these included quinoline (Q), the aromatic component, and lepidine (L), the aromatic component with a methyl group. Reference breakdown products for PE included pyridine (P), the primary aromatic component, picoline (PI), the aromatic component with a methyl group, and 4-ethyl pyridine (4-EP), the aromatic component with an ethyl group attached. Other chemicals used were reagent grade and obtained from Baker Chemical Co. The enzymes used for this study were hydrogenase, isolated from *Proteus vulgaris*, extracted and purified by a technique previously described,[3] and cytochrome C, available from Sigma Chemical Co.

Methods for Microbial Tests

The microbial isolates were cultured in free suspension in shake flasks first with media containing 1% glucose and then subcultured into 1% solutions of either sodium acetate or sodium benzoate as the sole carbon source. In later tests, various types of size-reduced (-100 mesh) coal were added to the cultures. All tests were carried out anaerobically, under nitrogen, at ambient temperature for periods of up to 42 d. The cultures were examined microscopically in order to establish a tentative characterization by Gram-staining reactions, and the liquid products were determined by GC, thin layer chromatography, and GC-mass spectrophotometry. In some of the tests, the methane content of the gas in the head space of the culturing flask was also determined by GC.

Methods for Enzyme Tests

Recovery of Membrane-Bound Hydrogenase. A major portion of the hydrogenase in *P. vulgaris* is apparently bound to the exterior membrane. In most of the tests, the enzyme was isolated from the membrane and further purified as an aqueous solution using a technique previously described.[3] In some tests, membrane fragments containing the enzyme were used. This crude hydrogenase mixture was prepared by harvesting of the cells during the exponential growth phase, sonication to disrupt the cells, and mild centrifugation at 500 *g* for 15 min to accumulate the membrane fragments. After washing with distilled water, the material was either lyophilized or suspended in a 0.05 *M* phosphate buffer at pH 7.0 for storage and later use.

Chemical Modification of Enzymes. Most aqueous soluble enzymes such as the purified hydrogenase and cytochrome C have unmeasurable solubilization in nonpolar organic solvents such as benzene. However, it has been shown that such enzymes can be chemically modified to increase the hydrophobicity and, thus, increase the solubilization in organics. These have been made with the addition of PEG_1 utilizing techniques previously developed[3] and a new approach in which

phenyl groups are added to the enzyme. In the latter case, DNFB is known to interact with "free" amino groups on proteins to form a stable chemical complex.[5] Typically, the reaction solution was 15 mL of 67% ethanol in distilled water into which 0.5 g of $NaHCO_3$ and 0.5 mL of DNFB were dissolved. Approximately 0.1 g of the enzyme (hydrogenase or cytochrome C) was then introduced to this mixture and allowed to react anaerobically in a shake flask for 1 h at ambient temperature. The resulting yellow precipitate was successively washed with 10 mL of an aqueous solution of sodium dithionite (5 mg/mL), to maintain the enzyme in a reduced state, 10 mL of ethanol, and 10 mL of diethyl ether. The solids were then dried under a N_2 atmosphere and stored in a stoppered container at 4°C. The solid product was the dinitrophenyl (DNP) derivative of the enzyme.

Enzyme Solubilization Tests. Tests for enzyme solubilization in organic solvents, primarily benzene, were made by mixing the enzyme preparation with benzene in shake flasks operating at 100 rpm for 1 h in the temperature range of ambient (~25°C) to 40°C under a N_2 or H_2 atmosphere. Equal volumes of the organic and enzyme preparation were used when the enzyme was in solution or suspension, while 5 to 20 mg of the dried enzyme preparation was added per mL of benzene. The amount of enzyme solubilized into the organic was determined either by weighting, after evaporation of the benzene, or by measuring the increase in light absorption at 280 nm.

Degradation of Model Compounds. Experiments were made with a nominal 1 mg/mL of each model compound dissolved in benzene and contacted with molecular hydrogen in a shake flask for up to 24 h at 30°C. Tests were carried out with and without the modified enzymes with each enzyme individually or in combination. Small liquid samples were taken periodically and analyzed for the model compound and potential breakdown products with a Waters DB5, 60 M capillary column in a Hewlett Packard 5890 GC with flame ionization detection.

Coal Solubilization Tests. Coal solubilization tests were made by adding 0.1 to 0.2 g of size-reduced coal (-100 mesh) to 10 mL of the benzene mixture in a 25-mL shake flask operating at 100 rpm. The flasks were temperature controlled in the range of 25° to 40°C, and a H_2 atmosphere was maintained during the course of the 24-h test. The coal was vacuum dried at 100°C for 4 h and weighted

prior to introduction to the benzene mixture, and, in some cases, the solid residue was similarly treated at the end of the test. Optical absorption spectra of the solubilizing solution were periodically measured during the course of each test for an indication of coal solubilization. Reference tests were made during each coal solubilization run in which identical chemical and physical conditions were maintained except for the inclusion of the enzyme.

RESULTS

A series of tests have been made with both anaerobic microbial systems and modified enzymes in vitro. Although the primary emphasis has been on understanding the underlying biological processes, preliminary tests have also been made on the enhancement of coal solubilization by these biological catalysts.

Microbial Interactions

Several of the microorganisms tested were shown to utilize either acetate or benzoate as the carbon source, while others actually produced trace quantities of oxychemicals with coal as the only carbon source.

Candida sp. ML13 was typical of several of the microbial isolates tested, because it was found to utilize various carbon sources even in an anaerobic environment. Small quantities of several oxychemicals were also detected during 14-d tests in which the only carbon source was coal (see Table 1). This *Candida* strain produced both ethanol and acetate when in contact with native lignite. However, when leached with water, there were no detectable quantities of either of these oxychemicals. Perhaps this indicates that only the water soluble fraction in lignite can be metabolized. Larger quantities of acetate were produced when subbituminous coal was used, even after leaching with water, although there was apparently no ethanol produced. *Candida* appeared to have little interaction with bituminous coal.

Table 1. The production of partially reduced products from coal by *Candida* ML13[a]

Coal substrate	Ethanol (mg/L)	Acetone (mg/L)
Cultures without added glucose		
North Dakota lignite	0	0
Washed lignite[b]	10	51
Wyodak subbituminous	0	104
Washed subbituminous[b]	0	83
Illinois No. 6 bituminous	0	0
Washed bituminous[b]	0	0
Cultures containing 1% glucose		
Reference (no coal)	415	374
North Dakota lignite	80	0
Washed lignite[b]	214	27
Wyodak subbituminous	1855	271
Washed subbituminous[b]	1743	649
Illinois No. 6 bituminous	2284	513
Washed bituminous[b]	1545	228

[a]All tests were made with 150 mL of a mineral salt medium in a 250-mL shake flask operating at 100 rpm under N_2 at 30°C. The flasks were inoculated to a microbial concentration of 106 cells/mL after which 2 g of size-reduced coal (-100 mesh) was added. All tests lasted for 14 d. Analysis of ethanol and acetone was by gas chromatography.

[b]The coal was washed by continuously passing distilled water through a bed of the particulates for 48 h at ambient temperature. The easily solubilized components of lignite inhibit the microbial process.

Similar tests were made but with the addition of 1% glucose. In this case, there was an apparent enhancement in the production of ethanol with the presence of subbituminous or bituminous coal, but an apparent inhibition in ethanol production when lignite was present. Perhaps this indicates that some of the easily solubilized components of lignite inhibit the microbial process.

Microbial cultures from Argonne Premium Coal Samples were found to produce methane, acetate, butanol, and CO_2 when in contact with coal in a 1% glucose nutrient solution (see Table 2). Trace amounts of isopropanol and butanol were detected, and a small amount of ethanol was also found.

Table 2. Product formation from glucose and coal by cultures associated with Argonne premium coal[a]

Culture/coal	Products (mg/mL)			
	CH_4	CO_2	Ethanol	Acetate
L-1/lignite[b]	4	0	11	2707
S-1/subbituminous[b]	32	37	0	2113
B-1/bituminous[c]	60	111	0	2921

[a]All tests were made with 150 mL of a mineral salts medium containing 1% glucose in a 250-mL shake flask operating at 100 rpm under N_2 at 30°C with the addition of 2 g of size-reduced (-100 mesh) North Dakota lignite, Wyodak subbituminous, or Illinois No. 6 bituminous coal. All tests lasted for 77 d. Analysis of the products was by gas chromatography.

[b]Trace quantities of isopropanol were detected.

[c]A trace amount of butanol was detected.

Membrane-Bound Hydrogenase

Two different types of tests were made to determine the solubilization in benzene of hydrogenase from *P. vulgaris* still attached to cell membrane fragments: (1) an aqueous slurry of the crude hydrogenase was contacted with benzene; and (2) freeze-dried particles of the crude hydrogenase were contacted with benzene. As indicated in Table 3, there was appreciable protein (enzyme) solubilization in the benzene as determined by absorbance measurements at 280 nm. Unfortunately, this crude enzyme product also contains large amounts of other proteins and membrane components. But, the apparent solubilization of the membrane-bound components could provide a useful basis for future approaches to enzyme modification.

Table 3. Benzene solubilization of membrane-bound hydrogenase[a]

Description of enzyme preparation	Absorbance (280 nm)	Approximate concentration (mg/mL)
Aqueous slurry[b]	0.81	0.7
Freeze-dried solids[c]	0.46	0.4

[a]Tests were made with 10 mL of benzene in a 25-mL shake flask operating at 100 rpm and ambient temperature under a N_2 atmosphere.

[b]10 mL of a slurry of the crude membrane-bound hydrogenase (20 mg/mL) in 0.05 *M* phosphate buffer (pH+7.0) contacted the benzene for 1 h followed by centrifugation at 500 *g* for 15 min and recovery of the benzene phase.

[c]120 mg of freeze-dried crude membrane-bound hydrogenase was added to the benzene for 1 h followed by centrifugation at 500 *g* for 15 min and recovery of the liquid.

Chemical Modification of Enzymes

Attachment of Polyethylene Glycol. *P. vulgaris* hydrogenase and cytochrome C were modified with PEG_1. Similar hydrogenases have about 80 free amino groups per molecule, while cytochrome C is known to have 20 free amino groups. Previous tests were carried out with a PEG_1 concentration equivalent to a molar ratio of 1:1 compared to the free amino groups. Now tests have been carried out over a range of PEG_1 concentrations equivalent to a molar ratio of 1:1 to 10:1 in order to determine the effect on enzyme solubilization in benzene. After solubilization in the organic, the modified enzyme was extracted from the benzene by contact with an equal volume of the phosphate buffer. Then the amount of enzyme activity was determined, thus giving a measurement of the active enzyme that was dissolved in benzene.

As shown in Figure 1, the activity of the modified enzymes in benzene initially increases as the relative PEG_1 concentration increases. Over the concentration range investigated, hydrogenase activity continued to increase with an increase in PEG. concentration; however, cytochrome activity reached a maximum at a molar ratio of 5:1, and thereafter it decreased. Apparently, additional chemical interactions with the PEG_1 affected the active sites in cytochrome.

Attachment of Phenyl Groups. It may be more appropriate to attach aromatic groups rather than polymeric hydrocarbons to cause the protein to be much less polar. To test this hypothesis, the reagent, DNFB, was used with both hydrogenase and cytochrome C. After conversion to the modified dinitrophenyl-enzyme (DNP-enzyme), 50 mg of each of the two enzymes was mixed with 10 mL of benzene and the absorption at 280 nm was measured. In both cases, the results were compared to a reference test in which the DNFB precipitate in the absence of protein was used. There was considerable solubilization of the modified protein with an estimate of >1.2 mg/mL for the DNP-hydrogenase and 1.8 mg/mL for the DNP-cytochrome. This promises to be an interesting new approach to enzyme solubilization in organics.

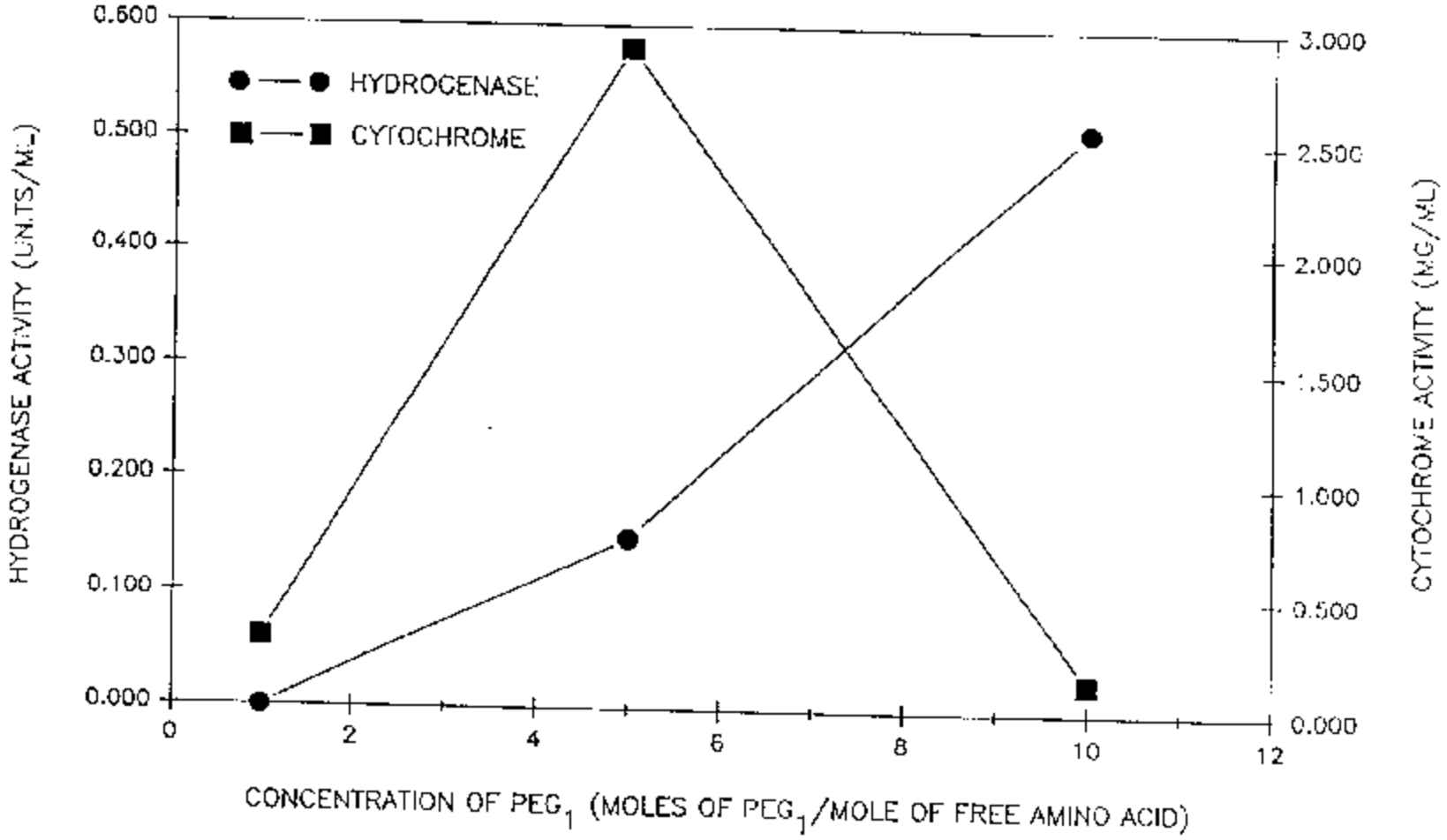

Fig 1. Effect of PEG_1 concentration on the solubilization and activity of modified enzyme in benzene.

Degradation of Model Compounds

Two model compounds [1,2,-bis(4-pyridyl) ethane (PE) and 1,2-bis(4-quinolyl) ethane (QE)] that have chemical structures somewhat similar to that of a portion of coal are being used to more clearly define enzyme interactions in benzene. Both enzymes chemically modified with PEG_3 were found to enhance the degradation of both model compounds. As shown in Figures 2 and 3, up to 20% of the model compounds were degraded over a period of 2 to 4 h. The optimum temperature of this type of conversion was found to be 25° to 30°C.

Each type of the DNP-enzyme derivatives was also tested for degradation of QE. Three different tests were made in which dissolved DNP-cytochrome, DNP-hydrogenase, and a mixture of the two modified enzymes in benzene were in contact with 1 mg/mL of QE under H_2 atmosphere at 30°C for 4 h. There was a definite enhancement of degradation of the model compound when the enzymes were present with the maximum effect occurring when both proteins were present (See Fig. 4). Greater than 50% of the QE disappeared during the first 12 h, with essentially no additional reaction occurring during the last 12 h of the 24-h test. Although there is indication of a small amount of the expected breakdown products, the major products have not yet been identified. This may indicate a more significant degradation than a simple cleavage at or in the ethane bridge.

Similar tests were also carried out with degradation of PE. The most significant results again were with mixed enzymes; however, the apparent degree of interaction was only about 20% (Fig. 4). As with the tests with QE, there were minimal amounts of the expected breakdown products, perhaps indicating a more significant alteration of the model compound.

Enzyme-Enhanced Coal Solubilization

Although the major research effort continues to be the enhancement of enzyme solubilization in organic solvents, periodic tests have also been made on the solubilization of coal, particularly bituminous coal (Illinois No. 6). As with the

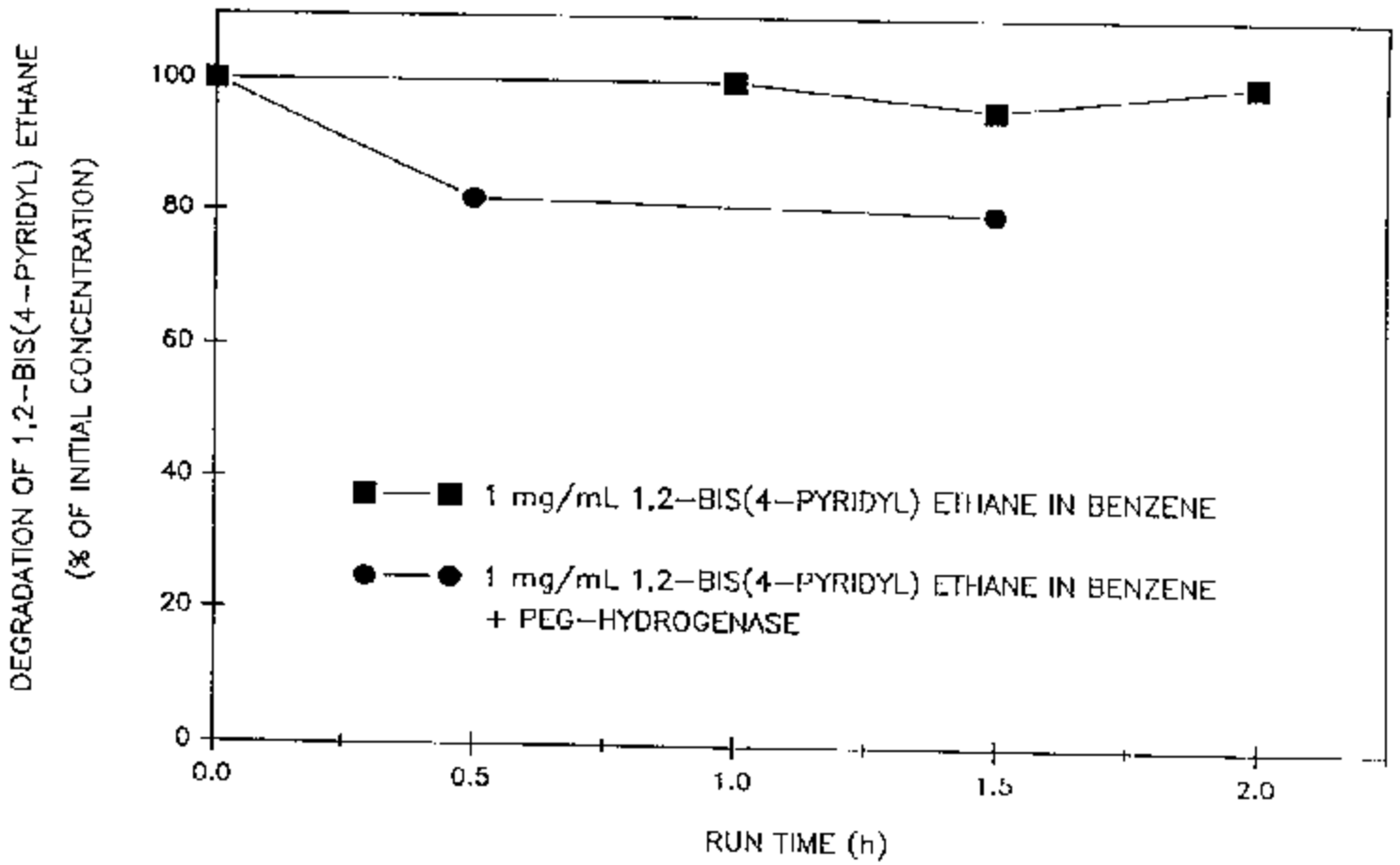

Fig 2. Enhanced degradation of 1,2-bis(4-pyridyl) ethane in benzene by the addition of PEG_1-modified hydrogenase.

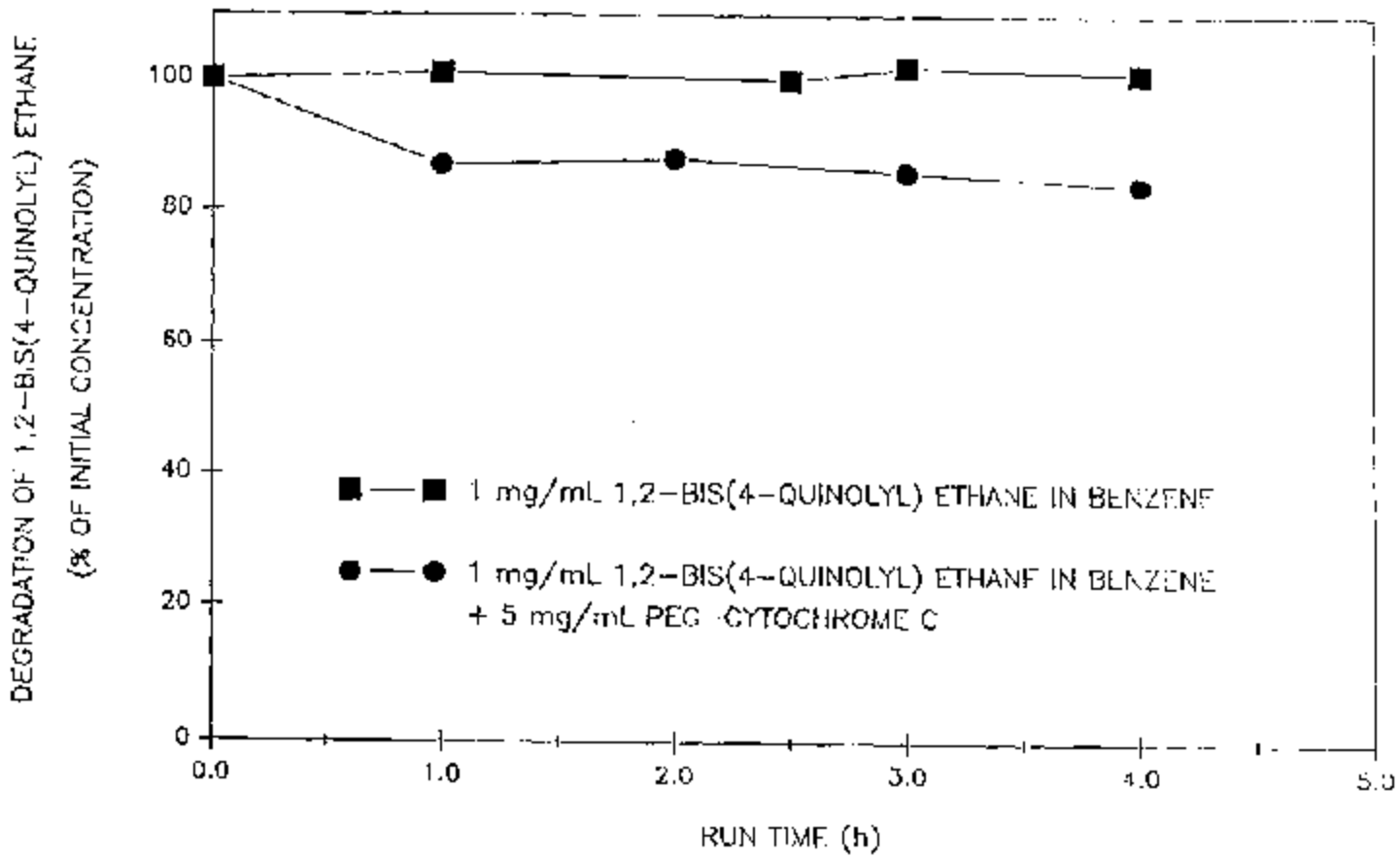

Fig 3. Enhanced degradation of 1,2-bis(4-quinolyl) ethane in benzene by the addition of PEG_1-modified cytochrome C.

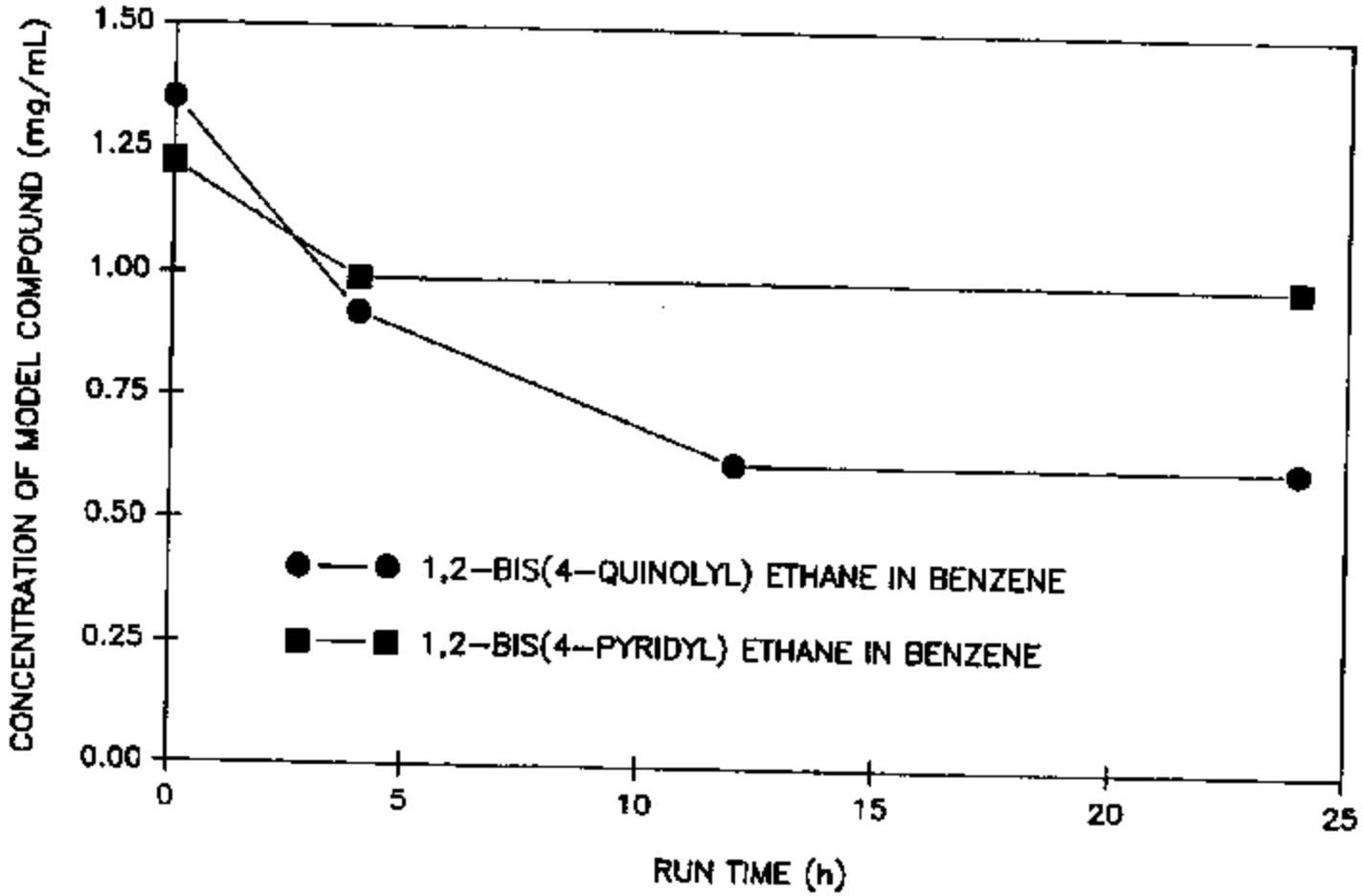

Fig 4. Decrease in the concentration of model compounds in benzene at 30°C under a hydrogen atmosphere in the presence of both DNB hydrogenase and DNB-cytochrome C.

case of PEG-enzymes previously reported,[3] it was also shown that the modified enzymes utilizing new techniques for benzene solubilization also enhanced the dissolution or conversion of coal in benzene when carried out at 30°C under a hydrogen atmosphere. This was true for the membrane-bound hydrogenase, DNP-hydrogenase, DNP-cytochrome C, and a combination of both modified enzymes (Table 4). The most significant effect was again when both hydrogenase and cytochrome C were present. Material balances of the coal are still difficult to make, but a very conservative estimate based on loss of weight of the coal in the test with enzymes indicated greater than 5% coal solubilization. There was indication of weight gain in the reference test without enzymes. (It is very difficult to remove some of the known precipitates that occur, so there was likely a much greater degree of solubilization.) Fractional distillation tests indicate a much greater degree of coal solubilization.

Table 4. Enzyme-enhanced solubilization of Illinois No. 6 bituminous coal in benzene at 30°C under a hydrogen atmosphere*

Enzyme	Increase in absorbance (280 nm)
Reference with no enzyme	0.08
Membrane-bound hydrogenase	0.32
DNP-hydrogenase	0.22
DNP-cytochrome C	0.48
DNP-hydrogenase + DNP-cytochrome	1.68

*All tests were made with 50 mg of -100 mesh coal in 10 mL of fluid in a 25-mL shake flask operating at 100 rpm. Enzyme concentrations in the benzene were the same as those shown in Tables 1 and 2.

Characterization of the Liquid Product

The product from liquefaction of bituminous coal in benzene that was enhanced with hydrogenase modified with PEG_1 was shown to be much less polar than the earlier product from aerobic solubilization of low-rank coal. When the organic phase with product was contacted with an aqueous phase of 0.1 *M* phosphate buffer at pH 7.0, the product tended to distribute between the two phases allowing a determination of the amount of product in each phase by spectral measurements. It was found that greater than 98% of the liquefied coal remained in the benzene phase, thus, indicating that the product was very polar. A small quantity of this liquid product was also used to determine the volatility by fractional distillation (Fig. 5). Since most of the material was the benzene solvent, 90% of the product was distilled by a temperature of 100°C. As indicated by the optical density of the condensate fractions, the solubilized coal started distilling significantly at 160°C, with over 60% in the fraction between 160° and 300°C. There was still a small quantity of liquid that had not volatilized at 300°C, as well as a small quantity of material that had the appearance of a highly viscous tar.

Crude fractional distillation tests were also conducted on the liquid products from coal liquefaction/solubilization tests in benzene that were enhanced by mixed enzymes modified with DNFB. As shown in Table 5, much of the material was the low-boiling benzene solvent, but there was a significant increase in the heavier fraction in the test with the modified enzymes present. The resulting residue of the enzyme-enhanced liquefaction/solubilization, after accounting for the dissolved enzymes, indicated that there was essentially complete coal solubilization. Undoubtedly, the actual degree of coal liquefaction lies somewhere between 5% and 100%. Now that the experimental approach is beginning to produce somewhat reproducible results with the possibility of a definitive material balance, quantitation will be the primary goal.

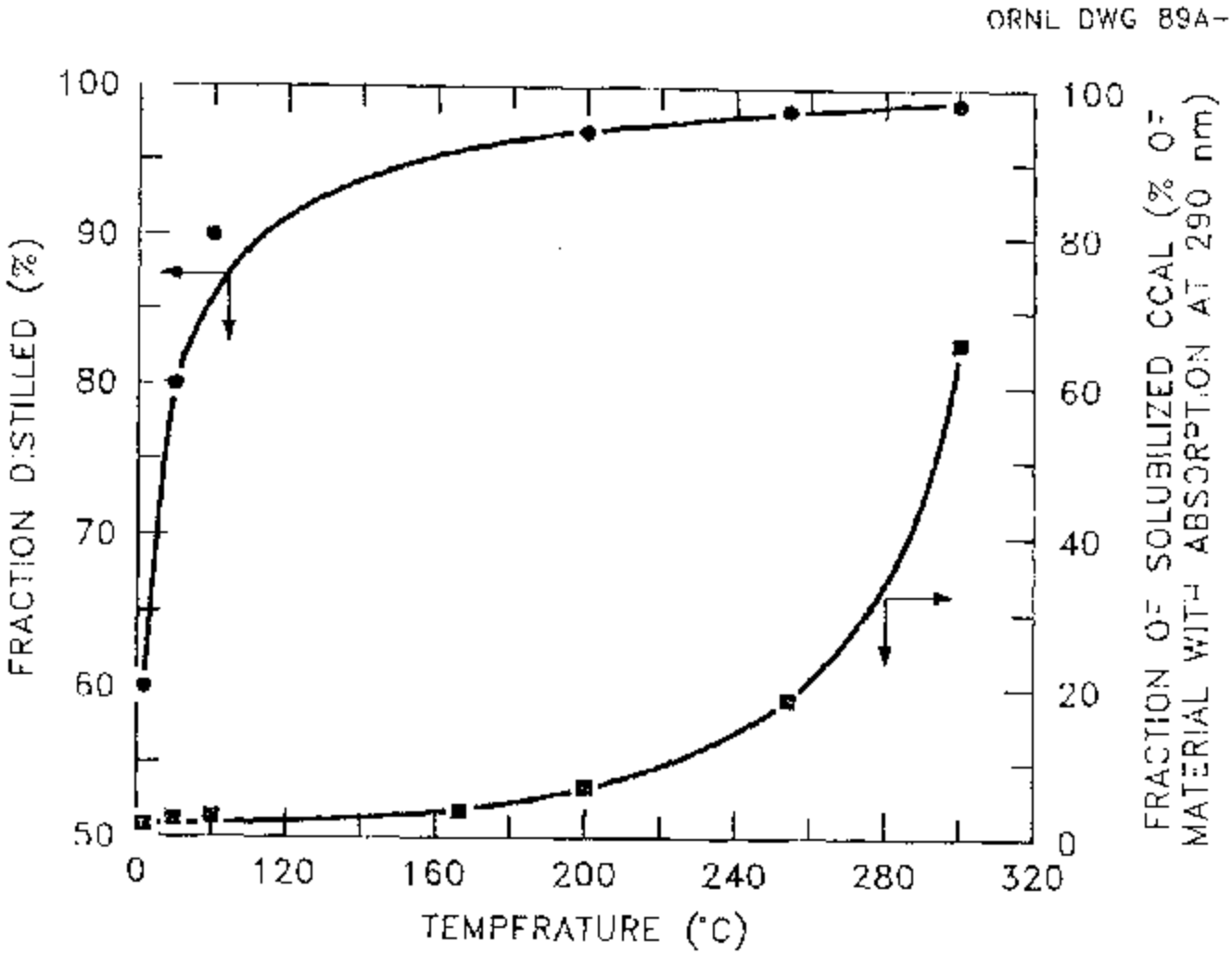

Fig 5. Volatility and liquified coal content as a function of temperature of the product from the PEG_1-hydrogenase enhanced conversion of Illinois No. 6 bituminous coal in benzene at 30°C in a hydrogen atmosphere for 24 h.

Table 5. Fractional distillation of the liquid product from the liquefaction/solubilization of Illinois No. 6 coal in benzene at 30°C under a hydrogen atmosphere with and without the presence of a mixture of DNP-hydrogenase and DNP-cytochrome C*

Description of test	<85°C	>85°C	Solid residue
Reference without enzymes	98	2	<1
With mixed enzymes	92	5	3

*Tests were carried out with about 5 mL of solution.

Possible Processing System

Although this processing concept has not been developed to the point where the parameters for process scale-up are available, it is interesting to consider what a simplified process flowsheet would look like. Quite likely, the primary bioconversion system would be a fluidized-bed bioreactor with continuous coal and solvent feed and recycle of the enzyme biocatalyst and hydrogen (Fig. 6). If possible, a process-derived light solvent would be used as the working fluid and the solid waste would be ash. As additional information is generated, the process flowsheet can be more completely defined.

CONCLUSIONS

Several types of microorganisms have been shown to directly interact with coal in an anaerobic environment with the production of small amounts of various oxychemicals. A new technique for chemically modifying enzymes, utilizing the addition of phenyl groups, enhances hydrophobicity and solubilization in an organic

ORNL DWG 89A-826

PROPOSED PROCESSING SYSTEM FOR ANAEROBIC COAL SOLUBILIZATION WITH HYDROGENASE IN A PROCESS-DERIVED LIQUID

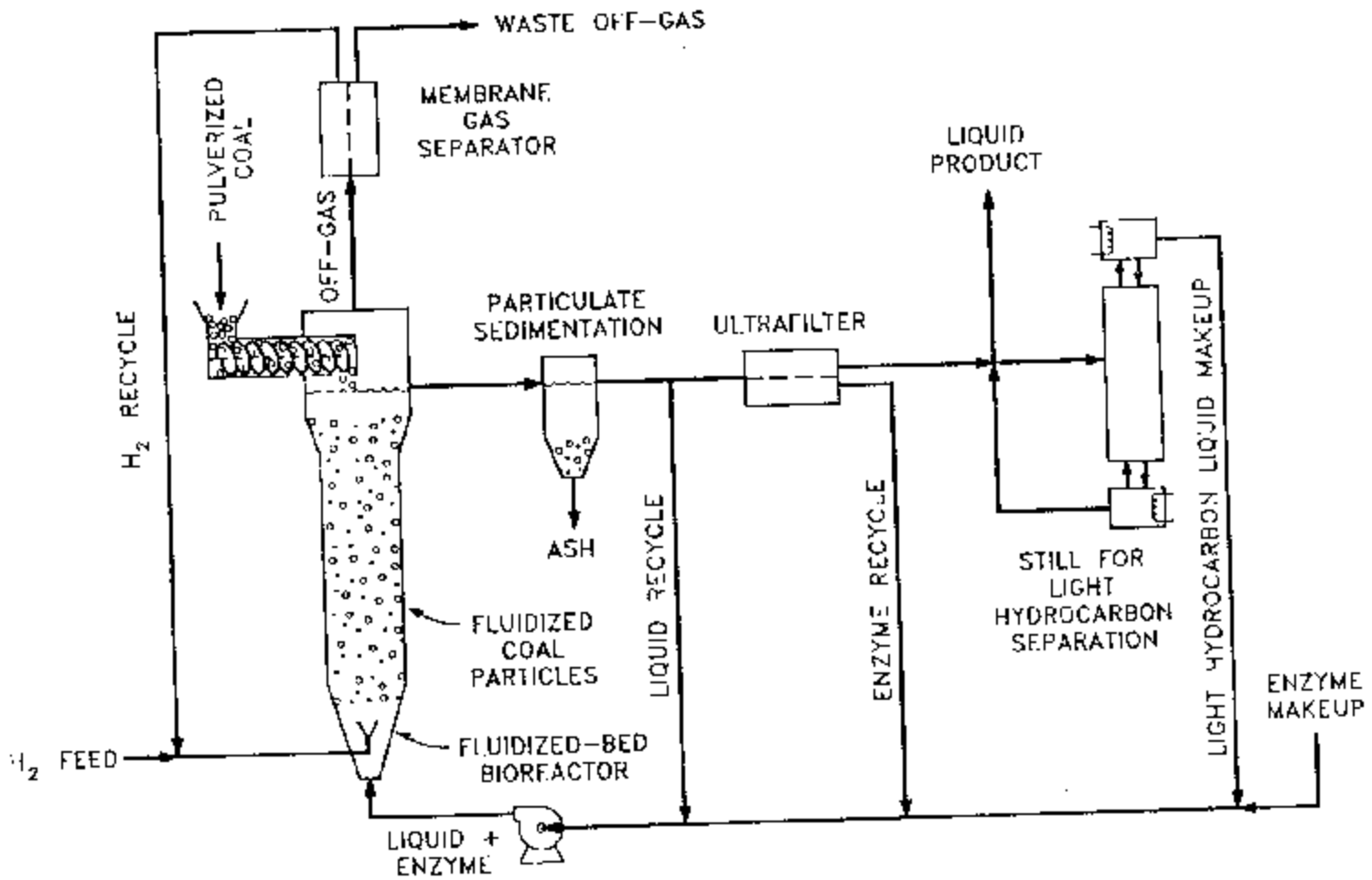

Fig 6. Simplified flowsheet for the enzyme-enhanced liquefaction of coal in a process-derived solvent utilizing molecular hydrogen.

solvent. This type of chemically modified biocatalyst, as well as membrane-bound enzymes, can be dissolved in organic solvents and used to catalytically degrade model compounds and enhance the solubilization of coal under anaerobic conditions.

The resulting product is relatively volatile and nonpolar. A processing system utilizing this technique would likely include a fluidized-bed bioreactor with a process-derived solvent.

REFERENCES

1. Scott, C. D., Strandberg, G. W., and Lewis, S. N. (1986), "Microbial Solubilization of Coal," *Biotechnol. Progress*, 2, 131.

2. Faison, B. D. and Lewis, S. N. (1989), "Production of Coal-Solubilizing Activity by Paecilomyces Sp. During Submerged Growth in Defined Liquid Media," *Appl. Biochem. Biotechnol.*, 20/21, 743.

3. Scott, C. D., Woodward, C. A., Thompson, J. E., and Blankinship, S. L. (1990), "Coal Solubilization by Enhanced Enzyme Activity in Organic Solvents," *Appl. Biochem. Biotechnol.*, 24/25, 799.

4. Faison, B. D. and Lewis, S. N. (1990), "Microbial Coal Solubilization in Defined Culture Systems: Biochemical and Physiological Studies," *Resour. Conserv. Recycle*, 3, 59.

5. Sanger, F. (1949), "The Terminal Peptides of Insulin," *Biochem. J.*, 45, 563.