DOE/PC/89772-T9

APPLICATIONS OF MICELLAR ENZYMOLOGY TO CLEAN COAL TECHNOLOGY

Third Quarterly Report

DOE/PC/89772--T9

DE92 016125

DOE Grant No. DE-FG22-89PC89772

2nd Qtr 1990

Submitted to:

Robert Dolence
Technical Project Officer
PM-20, MS 922-206
U.S. Department of Energy
Pittsburgh Energy Technology Center
P.O. Box 10940
Pittsburgh, PA 15236

Prepared by:

Carol T. Walsh, Ph.D.
Prinicipal Investigator
Boston University School of Medicine
80 E. Concord Street, L-603
Boston, MA 02118
617-638-4326

July 24, 1990

US/DOE Patent Clearance is not required prior to the publication of this document.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Section 1

INTRODUCTION

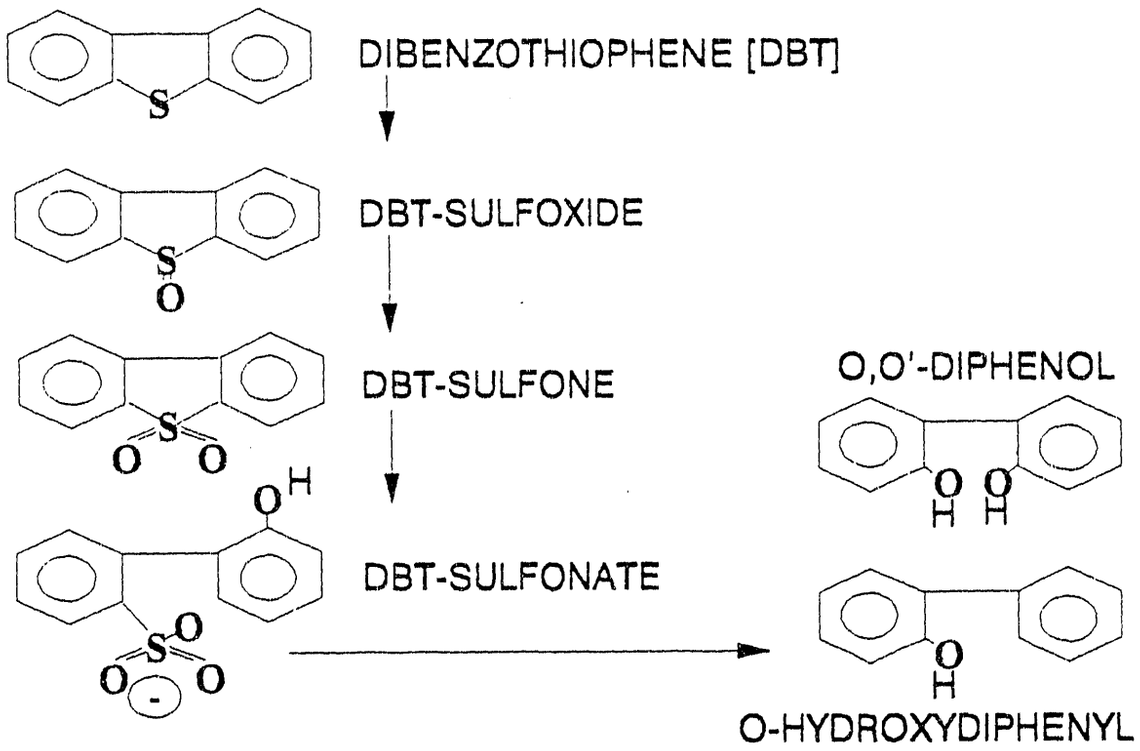
1.1 Program Overview

Full implementation of coal fuel sources will require more effective methods of providing "clean coal" as a fuel source. Methods must be developed to reduce the coal content of sulfur which significantly contributes to environmental pollution. This project is designed to develop methods for pre-combustion coal remediation by implementing recent advances in enzyme biochemistry. The novel approach of this study is incorporation of hydrophilic oxidative enzymes in reverse micelles in an organic solvent. Enzymes from commercial sources or microbial extracts are being investigated for their capacity to remove organic sulfur from coal by oxidation of the sulfur groups, splitting of C-S bonds and loss of sulfur as sulfuric acid (Figure 1). Dibenzothiophene (DBT) and ethylphenylsulfide (EPS) are serving as models of organic sulfur-containing components of coal in initial studies.

A goal of this project is to define a reverse micelle system that optimizes the catalytic activity of enzymes toward desulfurization of model compounds and ultimately coal samples. Among the variables which will be examined are the surfactant, the solvent, the water:surfactant ratio and the pH and ionic strength of the aqueous phase. Studies by several groups (Martinek et al., 1981; Kabanov et al., 1988; Martinek, 1989; Verhaert et al., 1990) have shown that the surfactant AOT over a broad concentration range in organic solvents produces micelles, comparatively uniform in diameter, which incorporate hydrophilic enzymes. The activity (kcat) of certain

FIGURE 1.

THE MODEL COMPOUNDS AND THEIR SULFUR OXIDATION PRODUCTS



enzymes in this system is higher than in aqueous solution. This surfactant is therefore being examined first, although the potential disadvantages of an SO_3 containing molecule have been recognized. Other surfactants to be tested include Tritons, Tweens and Brij 35.

1.2 Summary of Results

Goal Number 2 for this quarter, as defined in our second quarterly report, has been successfully carried out. Pyrocatechol has been shown to be an excellent substrate for demonstrating activity of laccase from Polyporus versicolor in a reverse micelle environment. We have not however demonstrated greater catalytic activity of this enzyme in reverse micelles than in an aqueous environment, possibly because we have been unable to obtain the extinction coefficients of the product in the two media.

Studies to date have not revealed activity of laccase towards DBT, DBT sulfoxide or DBT sulfone. The sulfoxide experiment was complicated by the presence of considerable sulfone impurity in the starting material and is being repeated. The sulfone experiment suggested no significant loss of starting materials; no dihydroxybiphenyl was detect which, based on the sensitivity of the HPLC procedure, would have been detectable at about 2% conversion of the sulfone.

Section 2

MATERIALS AND METHODS

2.1 Materials

Studies were carried out with laccase from Polyporus versicolor. The enzyme, 5 mg/ml 0.1M phosphate buffer (pH 6.0), was isolated by Dr. Bengt Reinhammer and obtained with his permission from Dr. Kent Kirk of Forest Laboratories, USDA. The enzyme was stored at -20°C. Substrates for laccase assays included pyrocatechol (Sigma Chemical. Co.), DBT (Aldrich Chemical Co.), DBT sulfoxide (K & K Laboratories), and DBT sulfone (Aldrich Chemical Co.). In addition to 2,2'-dihydroxybiphenol (Lancaster Synthesis Ltd.), the above metabolites of DBT were used as standards in HPLC chromatograms. Sodium acetate (Fisher Scientific), sodium phosphate (Fisher Scientific), isooctane (Optima, Fisher Scientific) and the surfactant AOT, di(2-ethylhexyl)sodium sulphosuccinate (Sigma Chemical Co.), were used to form reverse micelle solutions. AOT was used as obtained and not further purified; impurities in AOT from other sources, noted by Luisi et al. (1984) with UV spectroscopy, were not found in the AOT from Sigma.

2.2 Methods

2.2.1 Production of Reverse Micelle Solutions

AOT(MW 444) was dissolved in isooctane to produce a 0.1M solution. Aliquots of phosphate buffer (0.025-0.1M, pH 5-7) and acetate buffer (0.025M-0.15M, pH 4.65-

5.0), with or without laccase, were added to the organic solution and vortexed for 1 min to produce the following reverse micelle solutions:

System I: acetate (0.15M, pH 4.65): phosphate (0.1M, pH 6) (9:1), 3.2% aqueous phase

System 2: acetate buffer (0.025M, pH 4.65): phosphate buffer (0.1M, pH 6.0) (3:1). (pH adjusted to 5.0), 3.2% aqueous phase, H_2O:AOT of 18:1

System 3: acetate buffer (0.15M, pH 4.65): phosphate buffer (0.1M, pH 6.0) (1:1) (pH adjusted to 4.95), 2.3% aqueous phase, H_2O:AOT of 13:1

System 4: phosphate buffer (0.044M, pH 7.0), 3.2% aqueous phase.

For study of pyrocatechol oxidation, the substrate (1-25mM) was added to both aqueous and organic phases, systems 1-3. DBT, DBT sulfoxide and DBT sulfone were also incubated with laccase (20ug/ml) in reverse micelle solutions. DBT (2mM), the sulfoxide (0.02mM) and sulfone (0.02mM) were added to 0.1M AOT-isooctane prior to addition of the aqueous phase (DBT: System 2, pH 5 and System 4, pH 7; DBT sulfoxide and DBT sulfone: System 2, pH 5). Samples were incubated for 24 hr at room temperature with gentle shaking.

2.2.2 Assay of Pyrocatechol Oxidation in Aqueous and Reverse Micelle Solutions

The oxidative activity of laccase in aqueous and reverse micelle solutions was monitored by product formation (O-quinone) from pyrocatechol (Pshezhetskii et al, 1988; Martinek, 1989; Mozhaev et al., 1989).

For the aqueous assay, the laccase from Polyporus versicolor, 5 mg/ml in 0.1M phosphate buffer (pH 6.0), was diluted with acetate buffer to produce final

concentrations of 1-40 ug/ml. Pyrocatechol was dissolved in buffer solution to give a final concentration of 1mM - 20mM. The reaction was triggered by adding 40ul of the diluted laccase solution to 1.96 ml of the pyrocatechol solution. Solutions were vortexed for 10 sec., and absorbance readings were monitored at 400nm for 2-5min with a LKB Model 4050 spectrophotometer linked to an IBM compatible PC.

The micellar assay was performed by adding the desired amount of the diluted laccase in buffer to the organic phase and vortexing for 1 min. The activity of the enzyme was assesd by adding an equal volume of 0.1M AOT-isooctane, containing pyrocatechol(1-25mm) in both the aqueous and organic phases. The combined reverse micelle solutions were vortexed for 10 sec and absorbance monitored for 2-5 min. as described above.

2.2.3 Quantitative Analysis of DBT and Metabolites

HPLC Procedure

Samples were filtered and analyzed by HPLC as described in the Second Quarterly Report (2.2.2, p4, program rate as in "Experiment 2"). The reproducibility of the area values for standards are indicated in Table 1.

TLC Procedure

DBT-sulfoxide (4-20ug) and sulfone (1-2ug) in methylene chloride were chromatographed with silica gel plates -60F_{254} (Merck No. 5628) and benzene:methanol (97:3) as eluent. Spots were detected by UV lamp (Rf of sulfoxide = 0.35, Rf of sulfone = 0.81). DBT sulfoxide was removed from the plate and washed

TABLE 1. AREA OF HPLC CHROMATOGRAMS FOR DBT AND "4S" METABOLITES ALONE OR IN COMBINATION IN REVERSE MICELLE SOLUTIONS WITH AQUEOUS PHASE AT pH 5 OR 7.

	AREA	
STANDARD (ALONE)	pH 5	pH 7
DBT (2mM)	3,250,458[a] 3,171,220[c]	2,829,446[b]
DBT sulfoxide (0.02mM)	83,343 (45% sulfoxide) 89,529 (47% sulfoxide)	88,030 (46% sulfoxide)
DBT sulfone (0.02mM)	168,393 174,258	169,199
Dihydroxybiphenyl (0.02mM)	60, 907 55,062	49,339
STANDARDS (COMBINED)	pH 5	pH 7
DBT (2mM)	3,259,934[a] 3,093,863[b]	3,165,363[a] 3,068,835[b]
DBT sulfoxide (0.02mM)	83,636 46,754	65,303 88,642
DBT sulfone (0.02mM)	166,801* 164,877*	168,390* 168,888*
Dihydroxybiphenyl (0.02mM)	74,768 62,970	78,922 67,416

Note: * split peak a 6-11-90
b 6-12-90
c 6-13-90

twice with methylene chloride (10ml each). After a period of several days, the sulfoxide was retested for DBT sulfone impurity. Recovery of DBT sulfone from the plate using this wash procedure was calculated to be 106% based on absorbance readings of the recovered material (Molar extinction coefficient = 25, 627 cm^{-1}).

Section 3

RESULTS

3.1 Activity of Laccase in Aqueous and Reverse Micelle Solutions with Pyrocatechol as Substrate

Relationship between Pyrocatechol Concentration and its Oxidation by Laccase from Polyporus versicolor.

The oxidation of pyrocatechol by laccase (8.1 ug/ml) in aqueous and reverse micelle solutions (System 1) was examined as a function of substrate concentration. Although the number of data points was limited, K_m and V_{max} of this reaction were estimated from data presented in Figure 2 (aqueous assay) and Figure 3 (reverse micelle assay). The values were comparable; aqueous K_m=16.3mM, V_{max} = 0.34 abs u/min, micellar K_m=15.9mM, V_{max}=0.28. Further concentrations should be tested to vigorously compare these values. However, these initial data on the relatively high activity of laccase on reverse micelle solution were encouraging. The 20mM pyrocatechol concentration which in the reverse micelle experiments appeared to be saturating (but not inhibitory) substrate levels were used in subsequent studies to measure activity as a function of enzyme concentration.

FIGURE 2.

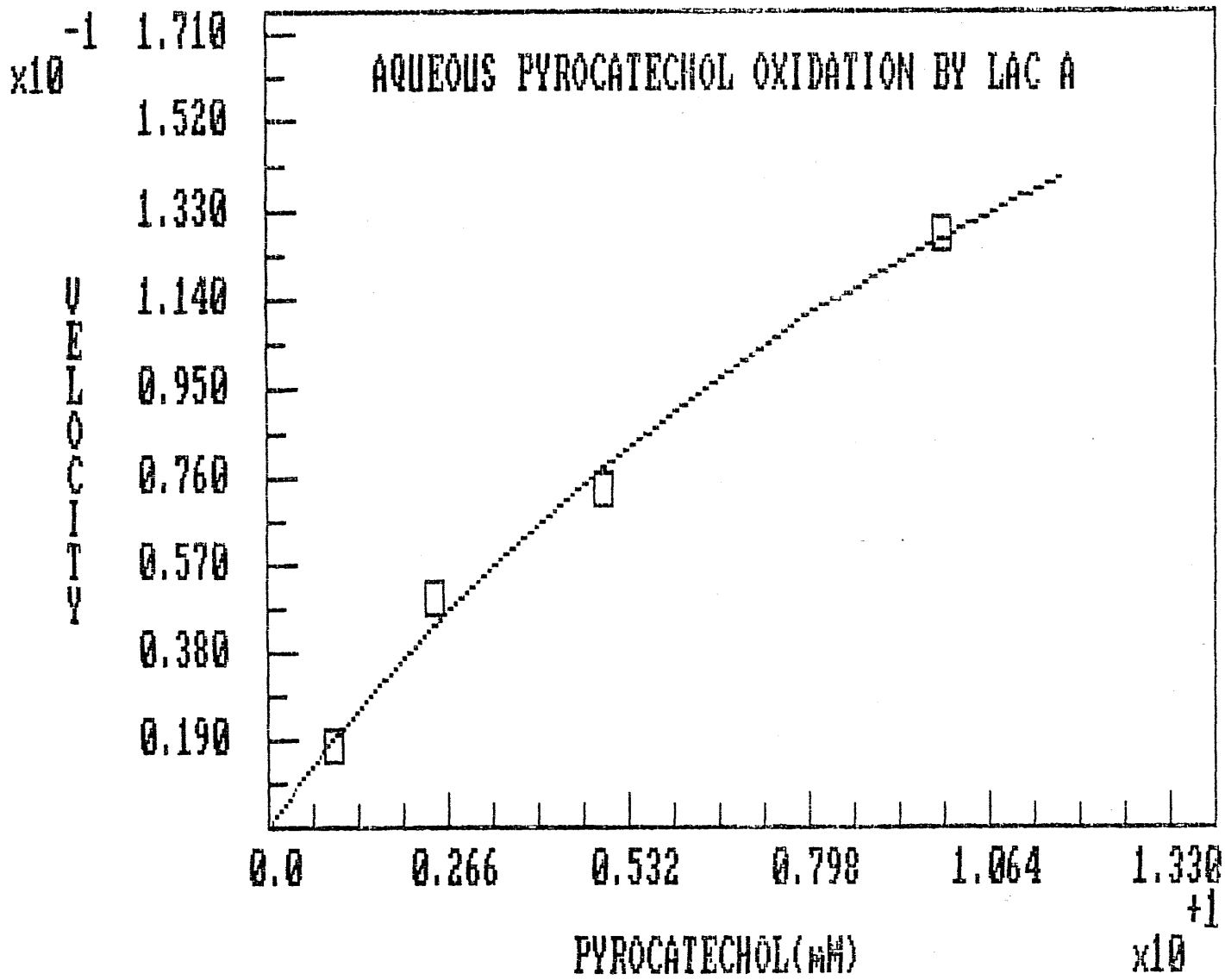
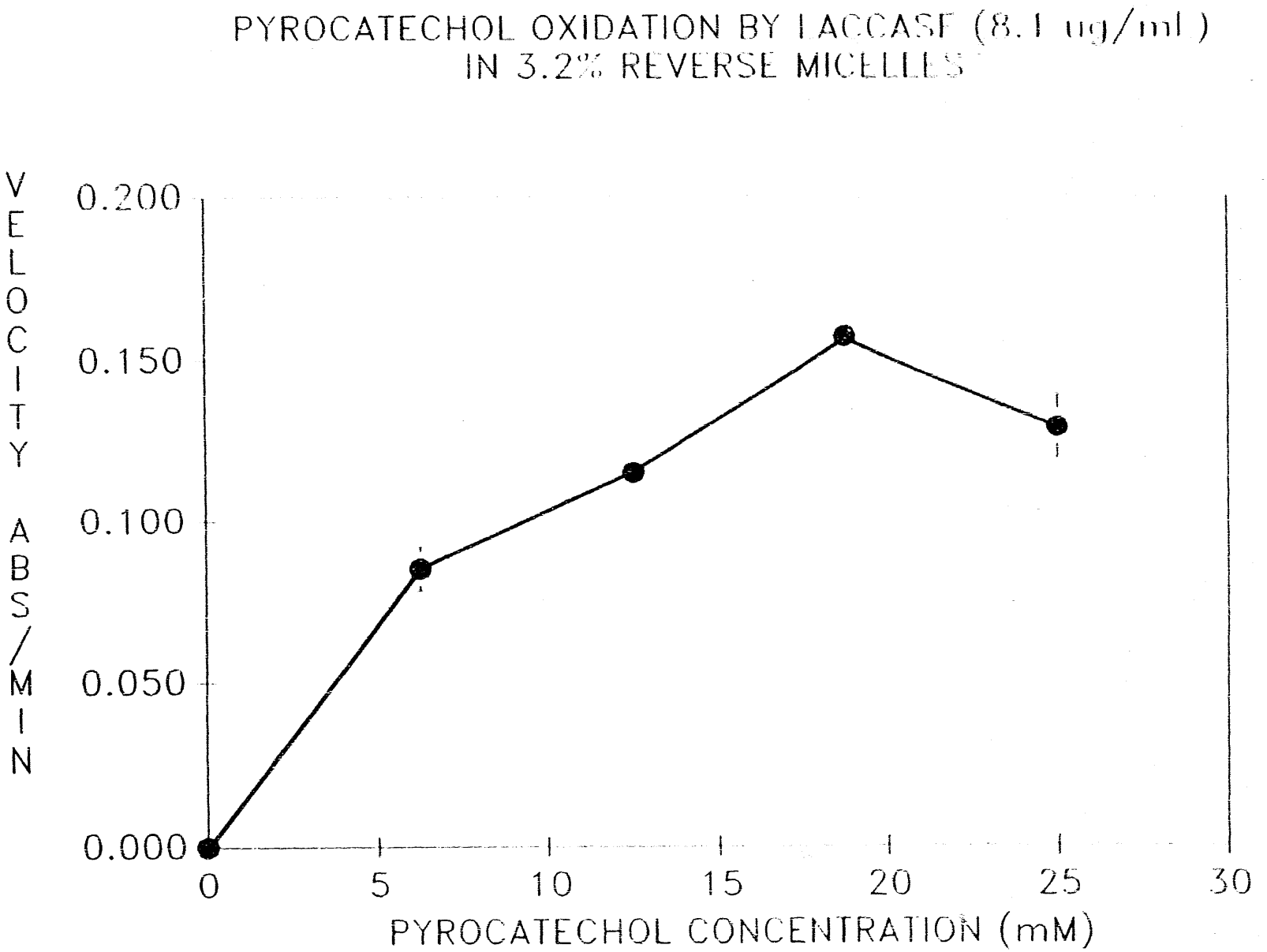

AQUEOUS PYROCATECHOL OXIDATION BY LAC A
x10 -1
1.710
1.520
1.330
1.140
0.950
0.760
0.570
0.380
0.190
VELOCITY
0.0
0.266
0.532
0.798
1.064
1.330
PYROCATECHOL(mM)
x10 +1

FIGURE 3.


PYROCATECHOL OXIDATION BY LACCASE (8.1 ug/ml)
IN 3.2% REVERSE MICELLES
VELOCITY ABS/MIN
0.200
0.150
0.100
0.050
0.000
0
5
10
15
20
25
30
PYROCATECHOL CONCENTRATION (mM)

Effect of Laccase Concentration on Pyrocatechol Oxidation by Laccase

Pyrocatechol oxidation in reverse micelles increased as a function of laccase concentration (Figure 4). The initial study suggested the relationship was not linear up to 40 ug/ml of enzyme. This experiment did not adequately control the ionic strength of the aqueous phase which was higher for lower concentrations of enzyme. This variable may have altered the configuration of micelles. The acetate buffer, 0.15M, orginally chosen for the assay based on aqueous studies of laccase (Fahraeus and Ljunggren, 1961), was found to exceed the level appropriate for formation of a clear micellar solution.

In a subsequent study (Figure 5) the reaction velocity was examined for laccase concentrations of 5-20 ug/ml in System 2. Pyrocatechol (20mM) oxidation increased linearly with enzyme concentration although the slope of the curve was less than that observed under aqueous conditions. With a smaller aqueous phase (2.3%), which corresponds to a H_2O:AOT ratio of 13 to 1, similar results were obtained (Figure 6). Higher H_2O:AOT ratios, such as 25:1 previously shown by Pshezhetsky (1988) to maximize kcat for laccase with respect to pyrocatechol oxidation, could not be achieved in this system with 20 ug/ml of this enzyme preparation, because the ionic strength of its phosphate buffer prevented micelle formation. Dialysis of the enzyme preparation would have to be carried out.

FIGURE 4.

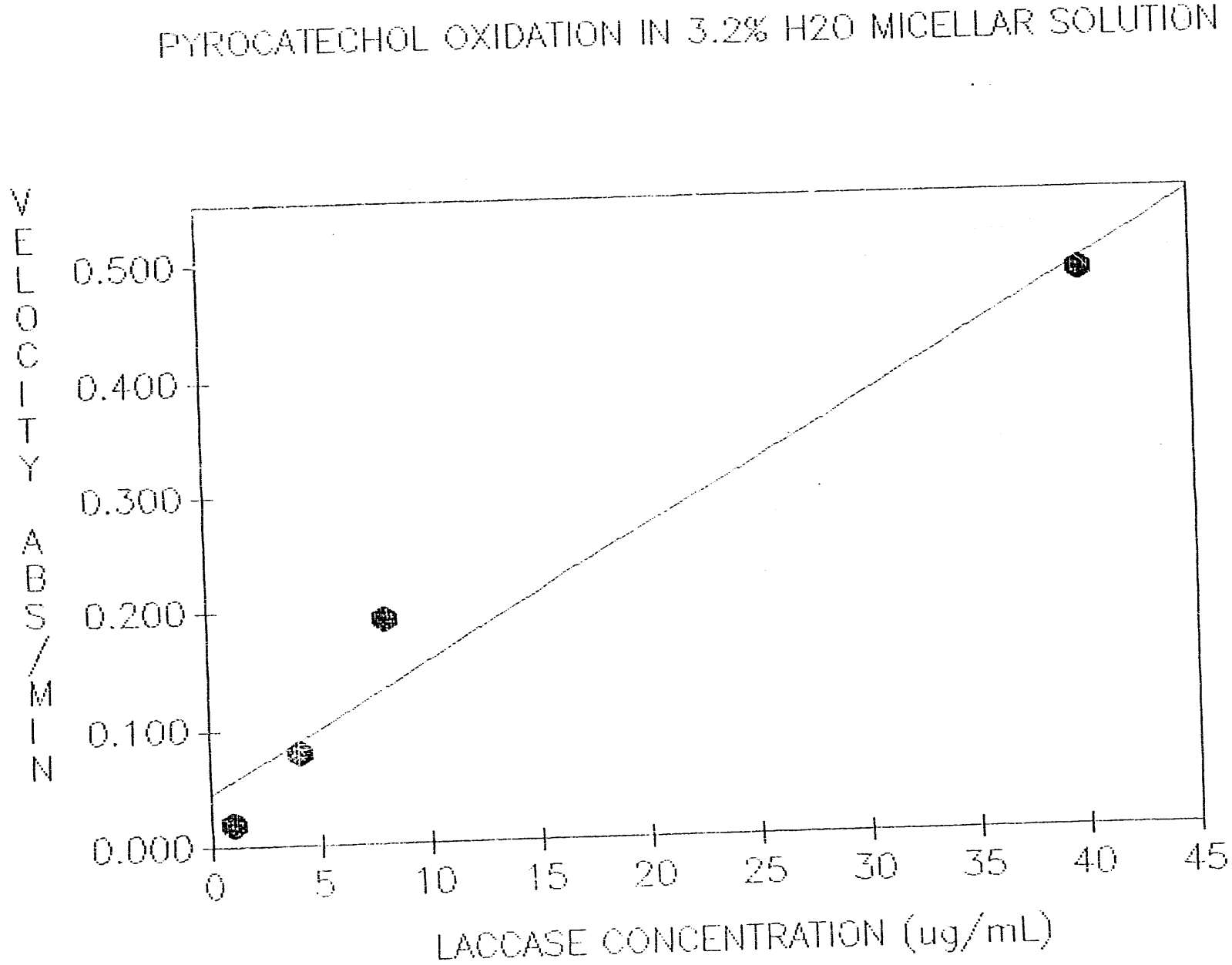

PYROCATECHOL OXIDATION IN 3.2% H2O MICELLAR SOLUTION
VELOCITY ABS/MIN
0.500
0.400
0.300
0.200
0.100
0.000
0
5
10
15
20
25
30
35
40
45
LACCASE CONCENTRATION (ug/mL)

FIGURE 5.

PYROCATECHOL OXIDATION IN REVERSE MICELLAR AND AQUEOUS SOLUTIONS

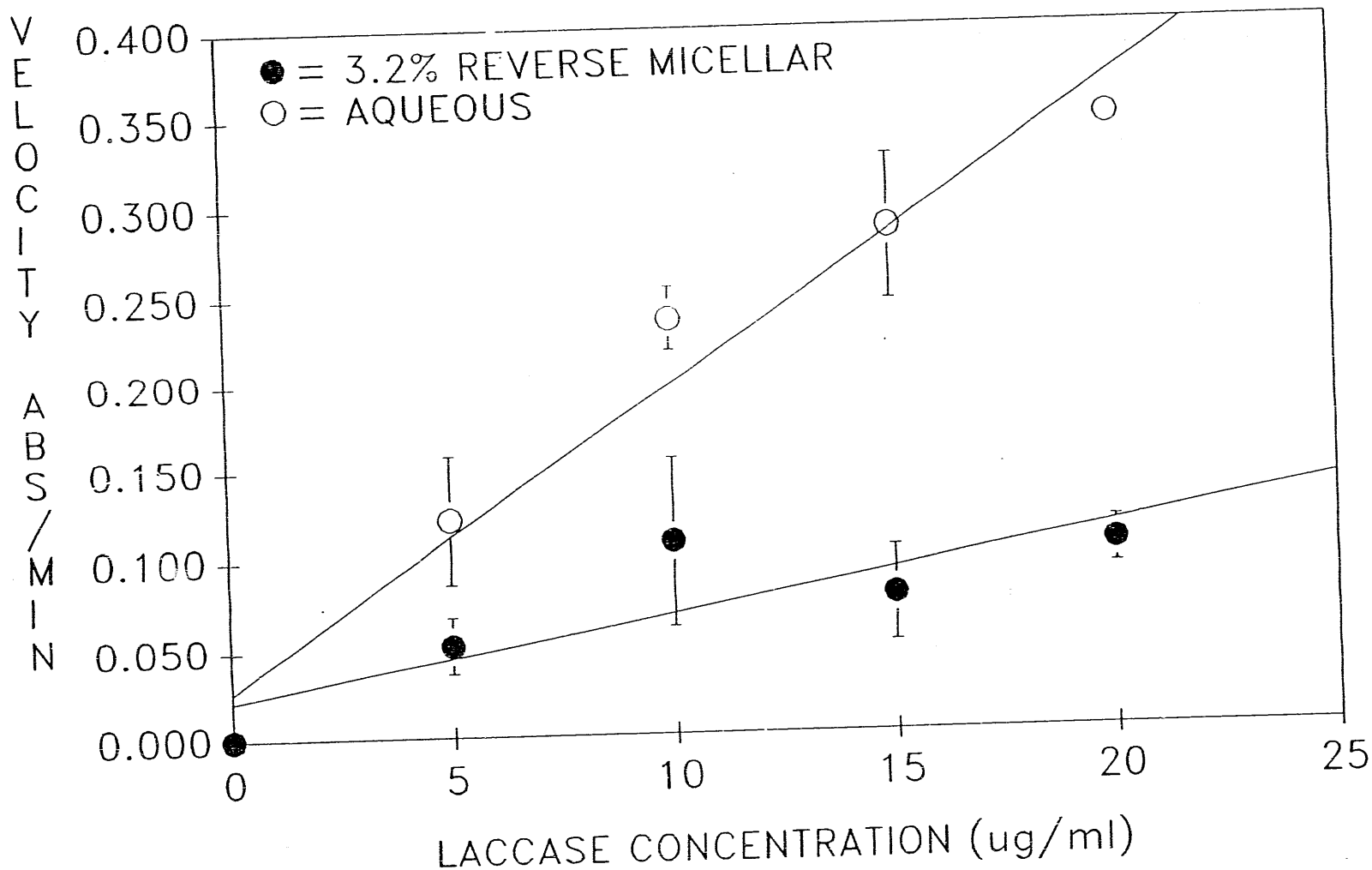
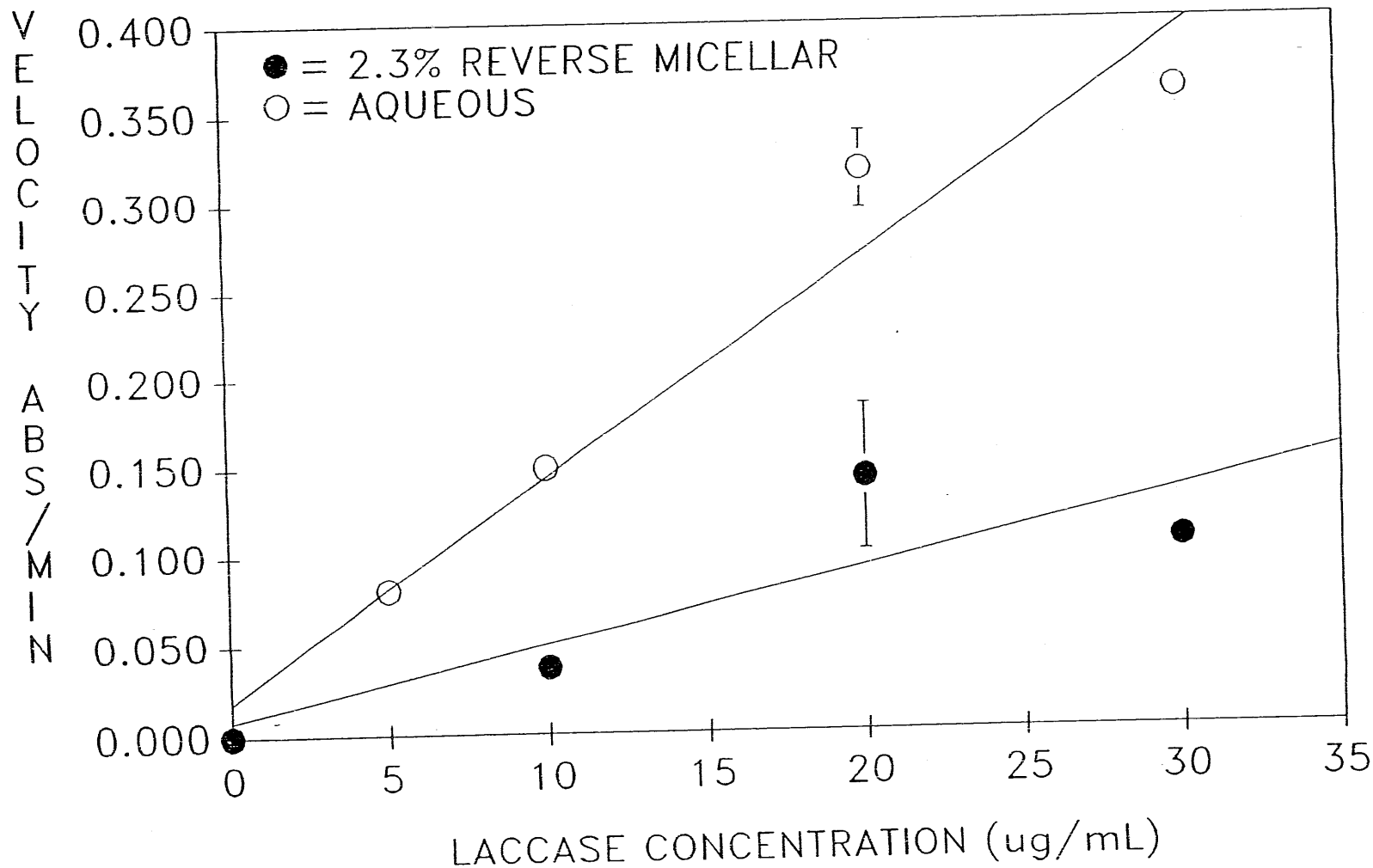


FIGURE 6.

PYROCATECHOL OXIDATION IN REVERSE MICELLAR AND AQUEOUS SOLUTIONS



Stability of Laccase Activity

Laccase (20 ug/ml) in aqueous or System 2 reverse micelle solution was tested in quadruplicate for oxidative activity towards pyrocatechol (20mM) after a 24 hour incubation at room temperature (Figure 7). Activity was present after the incubation although the reaction velocity declined to about 44 % of the zero-time value. Part of this change related to variability in day-to-day results for laccase activity in reverse micelles. No significant change in laccase activity occurred in aqueous solution and results were well replicated from day-to-day.

3.2 Activity of Laccase in Reverse Micelle Solutions with DBT, DBT Sulfoxide and DBT Sulfone as Substrates

DBT

Laccase from Polyporus versicolor in a reverse micelle solution did not significantly decrease the concentration of DBT (2mM) after a 24-hr incubation at room temperature (Table 2). No sulfone or dihydroxybiphenyl were detectable. A trace amount of sulfoxide was observed in one control (pH 7) and one enzyme-containing sample (pH 5).

DBT Sulfoxide

HPLC analysis indicated substantial (55%) contamination of sulfoxide with sulfone. A new supply of sulfoxide from K & K Laboratories was similarly contaminated as assessed by TLC analysis. Results from the laccase incubation experiment (System 2) with the impure DBT sulfoxide were complicated by extreme

FIGURE 7.

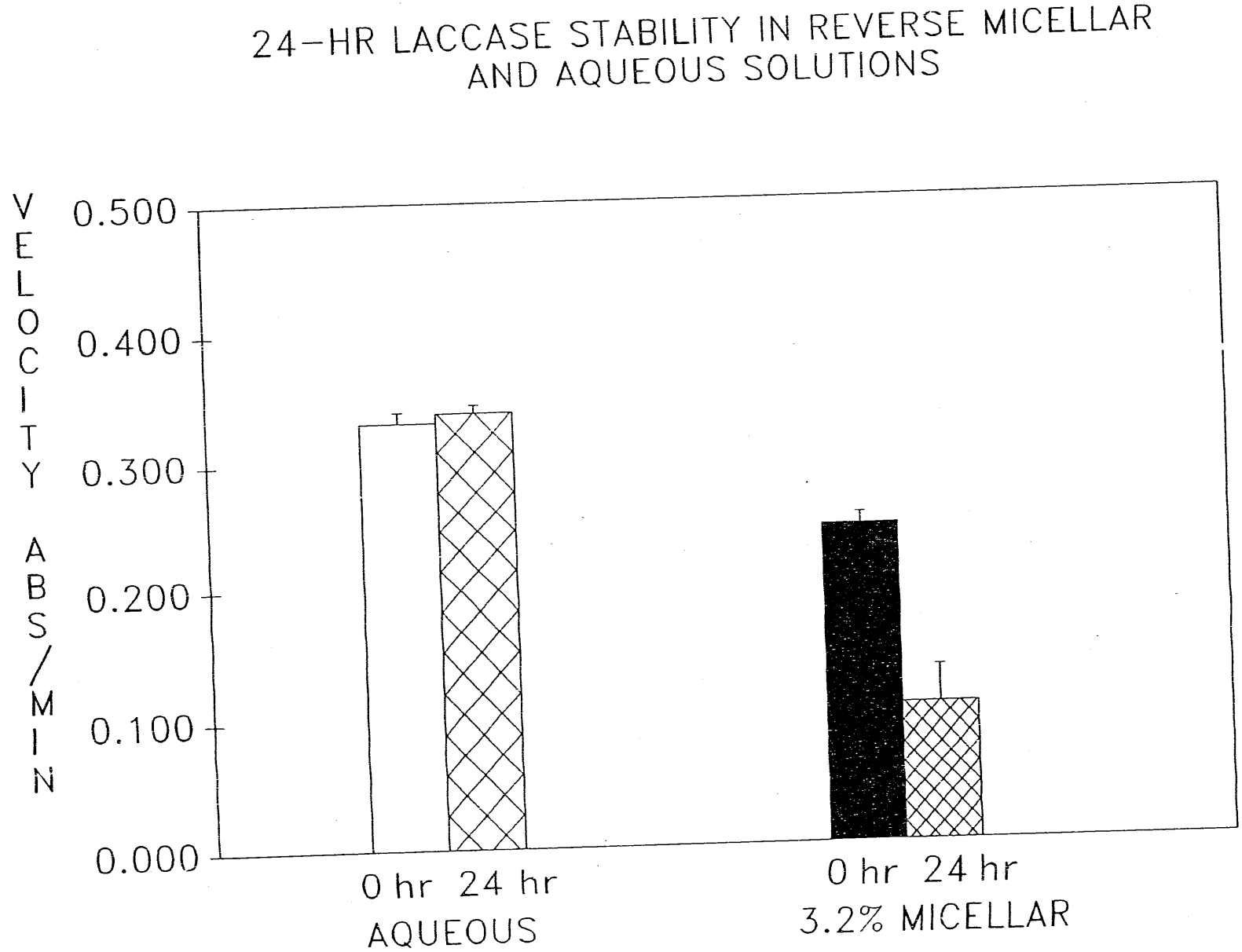

24-HR LACCASE STABILITY IN REVERSE MICELLAR AND AQUEOUS SOLUTIONS
VELOCITY ABS/MIN
0.500
0.400
0.300
0.200
0.100
0.000
0 hr 24 hr
AQUEOUS
0 hr 24 hr
3.2% MICELLAR

TABLE 2. Effect of Laccase from Polyporus versicolor on DBT (2.0mM) after 24 hrs. in a Reverse Micelle Solution.

	DBT (mM)	DBT-sulfoxide (mM)	DBT-sulfone (mM)	Dihydroxybiphenyl (mM)
Control:				
pH 5	2.06, 1.98	-,-	-,-	-,-
pH 7	1.94, 2.39	-, 0.0003	-,-	-,-
Laccase:				
pH 5	1.94, 2.06	0.0004,-	-,-	-,-
pH 7	2.08, 2.25	-,-	-,-	-,-

- none detected

variability in both control and enzyme-containing samples. This experiment in being replicated with DBT sulfoxide purified by TLC.

DBT Sulfone

Two experiments (Table 3) were carried out to study 0.02mM DBT sulfone incubations with laccase in reverse micelles (System 2). In experiment 1 the area values for both control and enzymes containing incubates were considerably lower than values for standards. However, no other peaks were detectable. The peaks of the sulfone were split in configuration which was not observed in the standard of sulfone alone but only in standards with sulfoxide in combination with sulfone. The possibility that sulfoxide and sulfone were converted to a compound that partitions similarly to sulfone but is chemically distinct was of interest.

In experiment 2, however, HPLC analysis resulted in single peaks for sulfone in both control and enzyme-containing incubations. No significant decrease in DBT sulfone concentration occurred and there was no evidence of peaks associated with possible oxidation products such as dihydroxybiphenyl.

TABLE 3. EFFECT OF LACCASE FROM POLYPORUS VERSICOLOR ON DBT SULFONE (0.02mM) AFTER 24HRS in REVERSE MICELLE SOLUTION

	CONCENTRATION OF DBT SULFONE AFTER 24 HRS (mM)	
	CONTROL	LACCASE[a]
Experiment 1	0.007±0.0000[b]	0.0066±0.0005
Experiment 2	0.2055±0.00062	0.02020±0.00045

a. 20ug/ml laccase in 0.1M AOT-isooctane with 3.2% aqueous phase (pH 5)

b. mean ± S.D. of triplicate incubations

Section 4

Plans for the Fourth Quarter

1. Study of the effect of a more active preparation of HRP (Type VI, RZ-3.1) in reverse micelles on DBT and EPS. Comparison of HRP activity will be made using micelles from the surfactants, AOT, Triton X-100 and CTAB.

2. Study of the DBT-metabolizing active preparation from GB-1, a bacteria isolated by Dr. Kitchell's group at DynaGen. Initial work will be carried out with the intracellular fraction from this organism incubated with DBT in reverse micelle solutions.

Section 5

BIBLIOGRAPHY

Fahraeus, G. and Ljunggren, H. Substrate specificity of a purified fungal laccase. Biochimica et Biophysica Acta 46: 22-32, 1961.

Luisi, P.L., Meier, P., Imre, V.E., et al. Reverse micelles: biological and technological relevance of amphiphilic structure in apolar media. P.L. Luisi and B.E. Straub, ed., Plenum, NY, 1984, p. 323.

Mozhaev, V., Khmelnitsky, Y., Sergeeva, M., et al. Catalytic activity and denaturation of enzymes in water/organic cosolvent mixtures. α-chymotrypsin and laccase in mixed water/alcohol, water/glycol and water/formamide solvents. Eur. J. Biochem. 184: 597-602, 1989.

Pshezhetsky, A.V., Merker, S., Pepanyan, G.S., et al. Modelling of the membrane environment of enzymes: superactivity of laccase entrapped into surfactants reversed micelles in organic solvents. Biokhimiya (Russ). 53: 1013, 1988.

DATE
FILMED
8/25/92