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Solvent hydrophobicity predicts biocatalytic behaviour of lignin peroxidase and cytochrome *c* in aqueous solution of water-miscible organic solvents

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Abstract

A new hydrophobicity parameter for water-organic solvent mixtures is proposed. This hydrophobicity concept (H) includes the thermodynamic activity of solvent and solvent polarity parameter from Dimroth-Reichardt transition energy [$E_T(30)$]. Solvent hydrophobicity (H) was a useful tool for the prediction of the threshold concentration C_{50} for biocatalytic oxidation of five hydrophobic substrates with lignin peroxidase and cytochrome *c*, in aqueous solutions of water-miscible organic solvents.

Keywords: Biocatalyst; Cytochrome *c*; Heme; Hydrophobicity; Lignin peroxidase; *Phanerochaete chrysosporium*; Organic solvent

1. Introduction

The use of organic solvents in biocatalysis induced a rapidly expanding biotechnological field (Zaks and Klibanov, 1988; Khmelnitsky et al., 1988; Dordick, 1989). A biocatalyst placed into non-aqueous medium is subjected to many factors that can alter its native, aqueous-based structure and function. Physical properties of pure solvents, such as logarithm of the partition coefficient octanol/water (log P), have been used to predict the enzyme behaviour in organic solvent (Laane et al., 1987; Halling, 1990; Manjon et al., 1992). Most of these studies were performed in pure organic solvents containing low amounts of water. However, the log P cannot be applied for aqueous mixtures containing water-miscible organic solvents.

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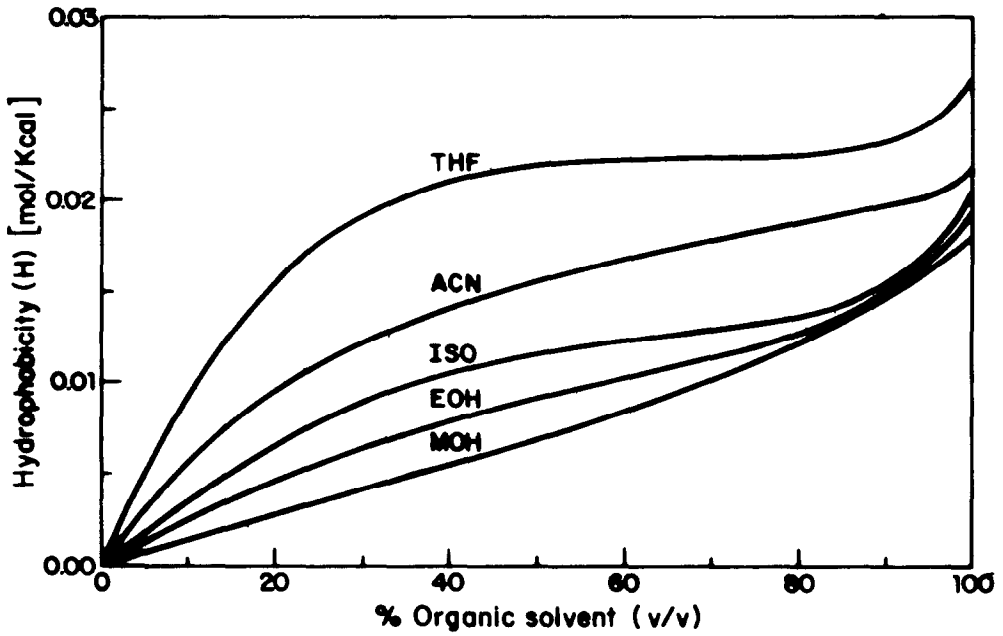

Fig. 1. Hydrophobicity parameter (H) for aqueous mixtures of water-miscible organic solvents. THF, tetrahydrofuran; ACN, acetonitrile; ISO, isopropanol; EOH, ethanol; MOH, methanol.

Increasing concentrations of water-miscible organic solvents into aqueous solutions results in a decrease of enzymatic activity. Threshold concentration (C_{50}) is the organic solvent concentration at which half-inactivation occurs (Khmelnitsky et al., 1988, 1991; Vazquez-Duhalt et al., 1993b). Inactivation at high organic solvent concentration has been correlated with changes of protein structure (Mozhaev et al., 1989; Ryu and Dordick, 1992). Disruption of non-covalent forces, including hydrogen bonding, and ionic, hydrophobic and van der Waals interactions, can lead to decrease in catalytic activity. A mathematical model describing the solvent effect on catalytic activity has been proposed (Khmelnitsky et al., 1991). However, several exceptions have been found, and extensive experimental work should be done to obtain confident thermodynamic predictions (Halling, 1994).

In this work, a new numeric concept for hydrophobicity of water-organic solvent solutions is proposed. This hydrophobicity parameter has correlated with the biocatalytic behaviour of lignin peroxidase and cytochrome *c*.

2. Materials and methods

2.1. Chemicals

Lignin peroxidase, partially purified, was obtained from Tienzyme Inc. (State College, PA). Cytochrome *c* from horse heart, yeast cytochrome *c*, chloroperoxidase from *Caldariomyces fumago*, human hemoglobin, and pinacyanol chloride were obtained from Sigma Chemical Co. (St. Louis, MO). Pyrene, thianthrene and diphenylamine were purchased from Aldrich (Milwaukee, WI). Bromophenol blue and protein reagent were obtained from Bio-Rad Laboratories (Richmond, CA). The high-performance liquid chromatography (HPLC)-grade solvents acetonitrile, isopropanol, ethanol, methanol and tetrahydrofuran were obtained from Merck (Darmstadt, Germany). Tetrahydrofuran was distilled in the presence of ferrous sulphate to eliminate peroxides. Buffer salts and hydrogen peroxide were purchased from J.T. Baker (Phillipsburg, NJ). Iso-1-cytochrome *c* variants were a gift from Dr. A. Grant Mauk (Department of Biochemistry and

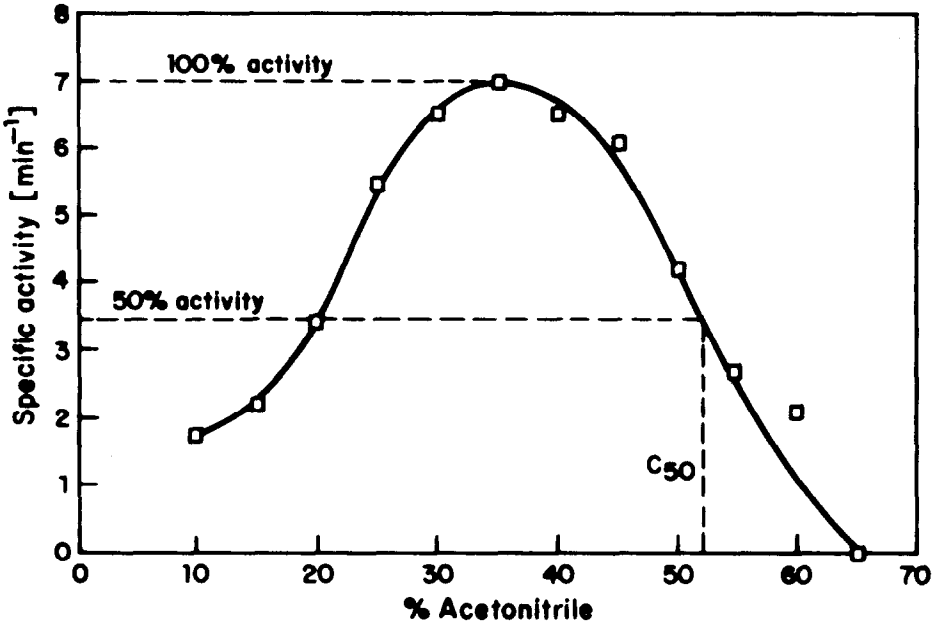

Fig. 2. Threshold concentration (C_{50}) of thianthrene oxidation in acetonitrile by horse heart cytochrome *c* in the presence of hydrogen peroxide.

Molecular Biology, University of British Columbia). Poly(ethylene)glycol-modified cytochrome *c* was prepared as previously reported (Vazquez-Duhalt et al., 1993b) by using activated poly(ethylene)glycol (MW 5000).

2.2. Protein determination

Protein concentrations were estimated by protein measurement with the Bio-Rad procedure and by spectrophotometry using the extinction coefficients, as previously reported (Torres et al., 1995).

2.3. Reaction conditions

Specific activities of lignin peroxidase from *Phanerochaete chrysosporium* were estimated in a 1-ml reaction mixture containing from 1.4 to 7.2 nM of enzyme and 20 μM substrate (pyrene, thianthrene, diphenylamine, or bromophenol blue) in 40 mM succinate buffer pH 4.0 with different concentrations of water-miscible organic solvent. When pinacyanol chloride was used the substrate concentration was 5 μg/ml. Reactions were started by adding 40 μM hydrogen peroxide.

Reactions with horse heart cytochrome *c* were performed using from 140 to 440 nM of cytochrome *c* in 60 mM phosphate buffer pH 6.1 with different concentrations of organic solvent. One ml cytochrome reactions were started by adding 1 mM hydrogen peroxide. For chloroperoxidase the buffer solution was 60 mM sodium acetate (pH 3.0). Reactions of all the other hemoproteins were carried out in 60 mM phosphate buffer (pH 6.1).

The reaction progress was monitored spectrophotometrically as a decrease in absorbance and by using the following extinction coefficient (mM^{-1} cm^{-1}): 32.6 for pyrene at 335 nm; 35.0 for thianthrene at 254 nm; 13.6 for diphenylamine at 279 nm; 82.35 for pinacyanol chloride at 603 nm; and 77.24 for bromophenol blue at 595 nm. The specific activity is defined as the number of substrate molecules transformed by 1 molecule of protein per time unit.

Table 1
Biocatalytic activity, threshold concentration (C_{50}), solvent (a_S) and water (a_W) activities, and mixture hydrophobicity at C_{50} concentrations (H_{50}) for the biocatalytic oxidation of different substrates by lignin peroxidase and hydrogen peroxide

Substrate/Solvent	Maximum activity (min^{-1})	C_{50} (%v/v)	a_S	a_W	H_{50} (μmol/cal)
Pyrene					
Tetrahydrofuran	17.5	14.7	0.48	0.97	12.8
Acetonitrile	61.2	19.1	0.43	0.94	9.2
Isopropanol	65.9	18.8	0.31	0.96	6.3
Ethanol	28.2	27.9	0.31	0.91	6.0
Methanol	57.3	21.7	0.17	0.90	2.9
Thianthrene					
Tetrahydrofuran	45.8	18.0	0.55	0.96	14.6
Acetonitrile	97.6	20.1	0.44	0.94	9.6
Isopropanol	60.5	19.6	0.32	0.96	6.5
Ethanol	55.0	26.2	0.31	0.92	5.9
Methanol	39.4	28.1	0.22	0.87	2.9
Diphenylamine					
Tetrahydrofuran	58.4	19.3	0.57	0.96	15.2
Acetonitrile	64.6	24.7	0.50	0.92	6.6
Isopropanol	59.5	25.6	0.39	0.94	8.0
Ethanol	54.3	27.1	0.31	0.99	5.9
Methanol	52.3	18.9	0.14	0.91	2.6
Pinacyanol					
Tetrahydrofuran	274.4	23.0	0.63	0.95	16.9
Acetonitrile	396.3	28.0	0.54	0.92	11.8
Isopropanol	364.1	30.0	0.43	0.93	8.9
Ethanol	304.0	29.3	0.33	0.91	6.3
Methanol	157.5	15.0	0.11	0.93	2.0

2.4. Analytical methods

Substrate hydrophobicity was estimated by HPLC on a Waters (Millipore) system with a UV detector. Substrate hydrophobicity was determined as the elution time on a reverse-phase column Resolve C_{18} 5 μ (3.9 × 150 mm). The elution solvent was acetonitrile-water (60:40 v/v) at a flow rate of 1 ml/min.

Ligand binding of substrate compounds to cytochrome *c* was detected with the spectrum change as previously reported (Vazquez-Duhalt et al., 1993a). Spectrum change was detected in a Beckman DU-650 spectrophotometer. The sample (1 ml) contained 5 μM cytochrome *c* and 40 μM substrate in phosphate buffer with different proportions of organic solvent. The blank consisted of 5 μM cytochrome in the same organic solvent concentration.

3. Results and discussion

Polarity of pure organic solvent can be estimated by using an empirical parameter described by Dimroth-Reichardt (Reichardt, 1979), $E_T(30)$, which represents the ability of different solvents to solvate polar fragments measured by solvatochromism. This parameter has been derived from the principle of linear relationship between free energies (LFE relationships). The $E_T(30)$ value has been introduced by Khmelnitsky et al. (1991) in a thermodynamic model for enzyme denaturation by organic solvents. However, with this model, which also includes log *P* value, several exceptions were found. Correlations have been found between $E_T(30)$ value and maximum specific activity of cytochrome *c* (Vazquez-Duhalt et al., 1993b), and a solvent effect parameter (Ryu and Dordick, 1992).

Table 2
Biocatalytic activity, threshold concentration (C_{50}), solvent (a_S) and water (a_W) activities, and mixture hydrophobicity at C_{50} concentrations (H_{50}) for the biocatalytic oxidation of different substrates by cytochrome *c* and hydrogen peroxide

Substrate/Solvent	Maximum activity (min^{-1})	C_{50} (%v/v)	a_S	a_W	H_{50} (μmol/cal)
Pyrene					
Tetrahydrofuran	1.0	19.1	0.57	0.96	15.1
Acetonitrile	1.1	28.0	0.54	0.92	11.7
Isopropanol	0.9	27.6	0.41	0.94	8.4
Ethanol	1.6	32.9	0.36	0.88	6.8
Methanol	1.7	27.8	0.21	0.86	3.8
Thianthrene					
Tetrahydrofuran	2.7	36.6	0.77	0.94	20.6
Acetonitrile	7.0	52.2	0.73	0.86	15.9
Isopropanol	6.3	57.8	0.59	0.88	12.2
Ethanol	5.8	60.0	0.53	0.81	10.3
Methanol	6.2	63.7	0.51	0.63	9.1
Diphenylamine					
Tetrahydrofuran	4.5	65.4	0.83	0.93	22.3
Acetonitrile	9.3	58.0	0.76	0.84	16.6
Isopropanol	8.3	61.5	0.60	0.88	12.4
Ethanol	6.7	70.0	0.59	0.76	11.4
Methanol	9.4	67.5	0.54	0.59	9.8
Bromophenol					
Tetrahydrofuran	2.9	62.0	0.83	0.93	22.3
Acetonitrile	4.7	62.5	0.78	0.83	17.0
Isopropanol	5.6	61.0	0.60	0.88	12.4
Ethanol	6.3	59.2	0.53	0.81	10.2
Methanol	4.3	64.9	0.52	0.61	9.3
Pinacyanol					
Tetrahydrofuran	14.1	65.2	0.83	0.93	22.3
Acetonitrile	26.9	73.6	0.84	0.79	18.2
Isopropanol	16.4	64.0	0.61	0.87	12.6
Ethanol	23.4	60.7	0.54	0.81	10.3
Methanol	15.4	75.0	0.62	0.51	11.1

We assume that the solvent polarity is inversely correlated with the solvent hydrophobicity and that, for a water-organic solvent mixture, the hydrophobicity is directly correlated with the organic solvent concentration. This may be expressed by the following equation:

$$H = \frac{a_S}{E_T(30)}$$

where H is the hydrophobicity parameter and a_S is the thermodynamic activity of organic solvent in the mixture. In contrast to concentrations, thermodynamic activities represent the available amount of organic solvent for the solvate process. The solvent activities (a_S), in aqueous solutions, were determined by using isothermal data for vapor-liquid equilibrium at 25°C, and calculated by NRTL equations (Gmehling et al., 1981). Thus, the hydrophobicity of an aqueous mixture of water-miscible organic solvent could be defined by the organic solvent activity (a_S) divided by the transition energy as Dimroth-Reichardt parameter. Fig. 1 shows the hydrophobicity parameter at different concentrations of organic solvent. In

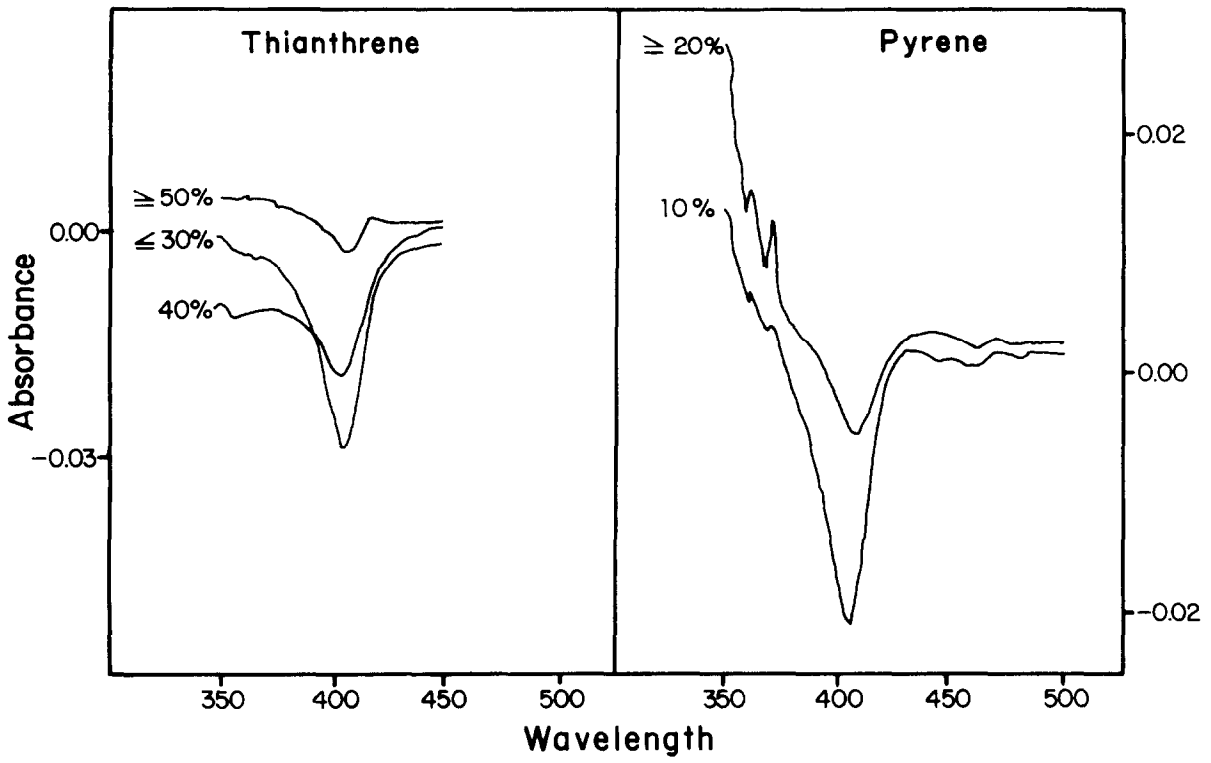

Fig. 3. Difference spectra of cytochrome *c* with thianthrene (A), and pyrene (B). The percentage values represent the tetrahydrofuran concentration in the binding reaction mixture.

contrast to the log *P*, the hydrophobicity parameter (*H*) is correlated with the capacity to dissolve hydrophobic compounds, such as polycyclic aromatic hydrocarbons (PAH) in aqueous solutions containing water-miscible organic solvents. For example, solutions containing acetonitrile (log $P = -0.33$) are able to dissolve larger amounts of PAH than isopropanol (log $P = 0.38$) solutions, beside the lower log *P* value. However, at any concentration, in volume basis, the acetonitrile solutions show higher hydrophobicity value (*H*) than the isopropanol ones (Fig. 1).

Lignin peroxidase and cytochrome *c* biocatalyses were carried out with the aim of testing our hydrophobicity parameter for the prediction of catalytic activity in mixtures containing water-miscible organic solvents. Biocatalytic oxidations of 5 hydrophobic substrates in 5 different water-miscible organic solvents were performed in the presence of hydrogen peroxide. Lignin peroxidase is able to oxidize hydrophobic substrates such as polycyclic aromatic hydrocarbons to form ketones and hydroxyles (Hammel et al., 1986; Vazquez-Duhalt et al., 1994). Cytochrome *c* showed peroxidase activity (Fujita et al., 1994), and was able to oxidize thiophenes and organosulfide compounds (Vazquez-Duhalt et al., 1993a; Klyachko and Klibanov, 1992) and polycyclic aromatic hydrocarbons, PAH (Torres et al., 1995). Pyrene, thianthrene, diphenylamine, pinacyanol chloride and bromophenol blue were used as substrates. Tetrahydrofuran, acetonitrile, isopropanol, ethanol and methanol were used as organic solvents. No oxidation was detected when biocatalyst or hydrogen peroxide were added alone.

Hydrophobic substrates showed a threshold behaviour (Fig. 2), in which at low organic solvent concentration, the mass action can limit the biocatalytic activity. When the concentration of organic solvent is increased, the mass transfer

limitation is reduced and the biocatalytic activity reaches its maximum value (100% activity). Significant enhancement of catalytic activity has also been found for some enzymes in systems containing low concentration of water-miscible organic solvents (Khmelnitsky et al., 1988; Batra and Gupta, 1994). Then, an increase of the organic solvent concentration induces a decrease of the biocatalytic activity. Threshold concentration (C_{50}) is defined as the specific organic solvent concentration at which half-inactivation occurs (Fig. 2).

Specific threshold concentrations were found for each substrate using both biocatalysts (Tables 1 and 2). Similarly, differences of C_{50} values were found for each substrate and the water-miscible organic solvent. In general, the lower the solvent hydrophobicity, the higher is the solvent concentration showing activity. On the other hand, the lower substrate hydrophobicity, the higher is the solvent concentration showing activity. Substrate hydrophobicity was determined as the elution time of the compound on HPLC system with a reverse-phase column (see Methods). This method has been used for polarity determination of hydrophobic compounds with $P > 1000$ values (Braun et al., 1986). The obtained values were: pyrene, 16.25; thianthrene, 12.57; diphenylamine, 5.64; bromophenol blue, 0.80; and pinacyanol chloride, 0.76. This behaviour was more conspicuous for cytochrome biocatalysis. Tables 1 and 2 also show the maximum specific activities, thermodynamic activities of solvent and water at C_{50} concentrations, and the system hydrophobicities at C_{50} concentration (H_{50}), for lignin peroxidase and cytochrome *c* respectively.

Table 3
Maximum specific activities and threshold concentrations (C_{50}) for pyrene oxidation in tetrahydrofuran-water mixtures with different hemoproteins

Hemoprotein	Maximum specific activity (min^{-1})	C_{50} (% vol.)
Chloroperoxidase	2291	19.9
Lignin peroxidase	17.5	14.7
Hemoglobin	1.13	22.1
Horse heart cytochrome *c*	1.02	21.8
PEG-cytochrome *c*	15.24	17.9
Yeast iso-1-cytochrome *c*	3.01	16.8
Phe82Gly variant	5.09	16.8
Lys79Ala variant	2.62	19.4

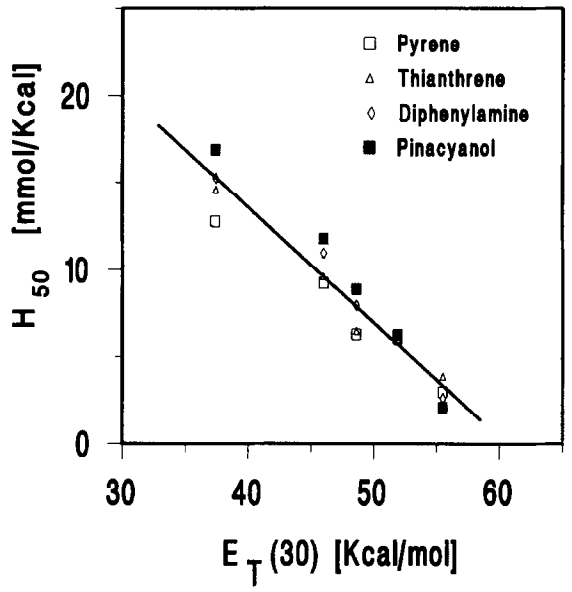

Fig. 4. Lignin peroxidase shows a linear correlation between the hydrophobicity of solvent mixture at C_{50} concentration (H_{50}) and the polarity parameter of pure solvent [$E_T(30)$]. Pure solvent polarities [$E_T(30)$] in kcal/mol are: tetrahydrofuran, 37.4; acetonitrile, 46.0; isopropanol, 48.6; ethanol, 51.9; methanol, 55.5; and water, 63.1 (from Reichardt, 1979).

From these results, it seems that the substrate properties are the main factor of the biocatalytic behaviour in organic solvent mixtures. This could be explained by the substrate partitioning between the active site (heme) and the bulk solvent. Substrates can interact with the heme group of cytochrome *c* as ligand (Vazquez-Duhalt et al., 1993a), detected by difference spectra. Fig. 3 shows the difference spectra of horse heart cytochrome *c* with pyrene and thianthrene in different THF concentrations. Pyrene showed a strong interaction at 10% THF and no interaction could be detected at 20% THF. Thianthrene showed an interaction with the heme prosthetic group at 40% THF, while no interaction has been detected at 50% THF. These substrate interactions are corre-

lated with the decrease of activity when the concentration of organic solvent is increased.

Ryu and Dordick (1992), working on phenols oxidation by horseradish peroxidase, suggested that the substrate partitioning into the enzyme's active site in non-aqueous media is solely governed by hydrophobic interactions. We have tried to modify the interaction behaviour by changing the heme pocket in the protein. This was done by using different hemoproteins, and by using site-directed variants of iso-1-cytochrome *c* from *Saccharomyces cerevisiae*. Three variants of yeast cytochrome, three hemoproteins, and two hemoenzymes were tested for threshold concentration of tetrahydrofuran in pyrene oxidation (Table 3). In spite of differences in the nature of residues placed close to heme group (active site pocket) for the different hemoproteins, only slight differences of solvent effect on catalytic activity have been found.

With the aim to find a mathematical model able to predict the solvent effect on biocatalysis, the H_{50} value was used and correlated with the solvent polarity [$E_T(30)$]. A linear correlation has been found for both biocatalysts, lignin peroxidase and cytochrome *c* (Figs. 4 and 5). This correlation can predict the H_{50} value for all solvents and all substrates, as follows:

$H_{50} = 39.9 - 0.66\ E_T(30)$ for lignin peroxidase

$H_{50} = 46.7 - 0.69\ E_T(30)$ for cytochrome *c*.

Moreover, in both cases the line projection to a hydrophobicity value of zero ($H = 0$), which corresponds to 100% water in the system ($a_S = 0$), matched with the $E_T(30)$ value for water (61.3 kcal/mol).

Thus, our hydrophobicity value (H) seems to be a good solvent parameter for aqueous mixtures containing water-miscible organic solvent. On the other hand, the substrate partitioning between active site and bulk solvent may determine the catalytic behaviour in systems containing organic solvents. This substrate partitioning may determine the C_{50} value before changes of protein structure leading to the inactivation. Biocatalytic oxidation of hydrophobic compounds cannot be envisaged without a favourable substrate partitioning between active site and bulk solvent.

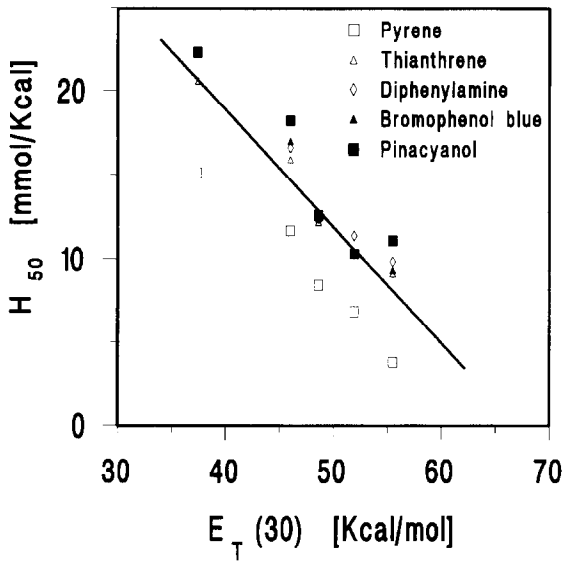

Fig. 5. Cytochrome *c* shows a linear correlation between the hydrophobicity of solvent mixture at C_{50} concentration (H_{50}) and the polarity parameter of pure solvent [$E_T(30)$]. Pure solvent polarities are shown in Fig. 4 legend.

Acknowledgements

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