0273-2289/98/70–72—0765$11.25

Biocatalytic Removal of Nickel and Vanadium from Petroporphyrins and Asphaltenes

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ABSTRACT

Asphaltenes from a crude oil rich in heavy metals (Castilla crude oil) were fractionated and partially characterized. Biocatalytic modifications of these fractionated asphaltenes by three different hemoproteins: chloroperoxidase (CPO), cytochrome C peroxidase (Cit-C), and lignin peroxidase (LPO) were evaluated in both aqueous buffer and organic solvents. The reactions were carried out in aqueous buffers, ternary systems of toluene:isopropanol:water, and aqueous-miscible organic solvent solutions with petroporphyrins as substrate. The petroporphyrins were more soluble in the ternary systems and aqueous miscible-organic solvent systems than in the aqueous buffer systems. However, only the CPO-mediated reactions were effective in eliminating the Soret peak in both aqueous and organic solvent systems. The effects of CPO-mediated reactions on the release of the metals complexed with the porphyrins and asphaltenes were also determined. Chloroperoxidase was able to alter components in the heavy fractions of petroleum and remove 53 and 27% of total heavy metals (Ni and V, respectively) from petroporphyrin-rich fractions and asphaltenes.

Index Entries: Asphaltenes; chloroperoxidase; biocatalytic modification; organic solvents; demetallation.

INTRODUCTION

Petroleum is a complex mixture containing a wide type of organic compounds. Among these are petroporhyrins, macromolecules of high molecular weights, associated with the pentane-insoluble fraction of petroleum known as asphaltenes *(1)*. Asphaltenes are very high-molecular-

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weight compounds containing aromatic and aliphatic constituents, heteroatoms (S,O, and N) *(1,2)* and heavy metals, like Ni and V *(3)*.

Their chemical and average molecular structures may vary considerably with their sizes *(4,5)*. In addition, high-molecular fractions of asphaltenes are more prone to forming methylene chloride-insoluble polymers on heating than are the low-molecular-weight fractions *(4)*. These differences in both chemical structures and chemical reactivities suggest that some fractions of asphaltenes are more susceptible to enzymatic or microbial attack than others *(6)*.

Microorganisms have been shown to associate with bitumens. Although there have been numerous reports demonstrating that microbial cells can degrade hydrocarbons such as polycyclic aromatic hydrocarbons (PAHs) *(7)* and sulfur heterocycles *(8)*, there is a little chemical or physical evidence that microorganism can modify asphaltenes *(9)*.

The data included in this paper are the result of the separation and partial characterization of asphaltenes from Colombian crude oil rich in heavy metals (Castilla crude oil) and its biocatalytic modification by three different hemoproteins in organic solvents. The investigations showed that only chloroperoxidase (CPO E.C. 1.11.1.10) in a buffer phosphate and organic solvent systems could eliminate the Soret peak in the petroporphyrin fraction of the asphaltenes under the experimental conditions studied. The effect of the CPO-mediated reactions on the fate of the metals complexed with the porphyrins and asphaltenes was also determined.

MATERIAL AND METHODS

Chemicals

Cytochrome C from horse heart was obtained from Sigma (St. Louis, MO). Lignin peroxidase, partially purified was obtained from Tienzyme (State College, PA). Chloroperoxidase from *Caldariomyces fumago* (*Rz* = 1.4) was a gift from Michael Pickard (Department of Microbiology, University of Alberta). The high-performance liquid chromatography (HPLC)-grade solvents isopropanol, acetonitrile, methylene chloride, chloroform, toluene, 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 Merck. Asphaltenes was obtained by n-hexane precipitation from Castilla crude oil.

Fractionation of Asphaltenes

Castilla crude oil is an oil with a 25% asphaltene content. Asphaltenes were fractioned by adsorption chromatography on silica gel using chloroform as eluent.

Analytical GPC

The fractions from the silica gel column were analyzed by HPLC using three 45 × 1-cm polystyrene/divinylbenzene columns (100, 500, and 10,000) in series with methylene chloride as eluant. The instrument used was a Perkin Elmer HPLC 410 UV LC-95 System with a UV detector at 254 nm.

Ultraviolet-Visible Spectrophotometry and Calculation of Porphyrin Concentrations

A Varian Cary 1E spectrophotometer was used to obtain UV-visible scans (200–600 nm) of asphaltene fractions dissolved in methylene chloride. The presence of petroporphyrins was detected by the prominent absorbance peak at approx 410 nm (Soret peak) and a weaker absorbance a 573 nm (α peak). Semiquantitative analysis of porphyrins was done according to Semple et al *(5)*. The concentration, in (μg/g) was estimated using an approximated molecular weight for porphyrins of 600 g/mol (Vanadium octaethylporphyrin).

Biocatalytic Modifications with Hemoproteins in Aqueous Buffer Solutions

The enzymatic reactions with hemoproteins in aqueous systems were carried out using petroporphyrins from fractionated asphaltenes as substrate.

Specific activities of lignin peroxidase (LPO) from *Phanerochaete chrysosporium* were estimated in a 2-mL reaction mixture containing 25 n*M* of enzyme and 50 μg of substrate in 40 m*M* succinate buffer pH 4.0 with KCl (9 m*M*). Reactions were started by adding 0.1 m*M* hydrogen peroxide.

Mixture reactions (2 mL) with horse heart cytochrome c (Cit-C) were carried out with 0.8 μ*M* of cytochrome C with 50 μg substrate in a 60 m*M* phosphate buffer pH 6.1 with KCl (9 m*M*). The reactions were started by adding 1 m*M* hydrogen peroxide.

For CPO, the reaction mixture (2 mL) contained: 50 μg petroporphyrin, 9 mM KCl in a 3m*M* KH_2PO_4 (pH 3.0) buffer. For enzyme reactions, the H_2O_2 was added first and mixed in the buffer and then CPO (10 μg/mL) was added to start the reaction. Six additions (6X) of CPO 5 μg/mL with 0.25 m*M* H_2O_2 were both effective in reducing the Soret peak of petroporphyrin *(6)*.

Methylene chloride (2 mL) was added to the small vial in order to stop the reaction and to extract the porphyrin for all hemoprotein-mediated reactions. This porphyrin layer was transfered into a cuvet to determine changes in the UV-visible spectrum.

In aqueous buffer systems, only CPO was effective in eliminating the Soret peak. For this reason the reactions in organic solvents were mainly

carried out with CPO so as to evaluate the biocatalyitic modifications and demetallation of petroporphyrin and asphaltenes.

CPO Reactions in Ternary Solvent Systems

The experiments used a solvent system of toluene:isopropanol:-water. Five different ternary systems were prepared using clear microemulsion (normal ternary system). All hemoproteins were evaluated in these systems. However, only CPO could eliminate the Soret peak from petroporphyrins

For all hemoprotein reactions, petroporphyrins were dissolved in toluene and added to the solvent mixture. The reactions were carried out with two different concentrations of petroporphyrins (12.5 and 25 μg/mL).

Aqueous portion for enzymatic reactions was: For LPO, 40 m*M* succinate buffer pH 4.0, for CPO, 3 m*M* KH_2PO_4 buffer, pH 3.0, and for Cit-C, 3 m*M* phosphate buffer pH 6.1. KCl at concentration of 20 m*M* was added to all buffers. Isopropanol was added at different concentrations (Table 4) and the mixtures were then shaken well to ensure the formation of a clear microemulsion.

The complete reaction mixture contained 0.5 m*M* H_2O_2 and 1.25 μg CPO/mL. For petroporphyrins, the disappearance of the Soret peak at 407 nm was followed. The maximum rate of the reaction was determined from the linear portion of the curve. The appearance and disappearance of the visible peak at 435 nm was also monitored.

CPO Reactions in Binary Solvent Systems (THF-Aqueous Buffer)

Several water-miscible organic solvents for dissolution of asphaltenes were evaluated (data not shown). The experiments of biocatalytic modifications were carried out with CPO, Cit-C, and LPO by using different tetrahydrofuran concentrations (range 5–30% v/v of THF). As in aqueous buffer and microemulsions experiments, only CPO showed biocatalytic modification on petroporphyrins indicated by changes in the UV-visible spectra (elimination of Soret peak).

For CPO reaction, the buffer solution was 3 m*M* KH_2PO_4 pH 3.0 and KCl 20 (m*M*). Petroporphyrins were dissolved in THF and then were added to the reaction mixture. Two different concentrations of petroporphyrins (25 and 50 μg/mL) were evaluated in THF. The complete reaction mixture contained 0.5 m*M* H_2O_2 and 1.25 μg CPO/mL. The biocatalytic reaction was stopped by adding THF (60% v/v). The reaction mixtures were then transfered to cuvets to quantify the disappearance of the visible peak at approx 410 nm (Soret peak).

Scale-Up of CPO-Mediated Reactions in Ternary Systems and Demetallation

Reactions in ternary systems with CPO were used for scale-up experiments. Petroporphyrins or asphaltenes dissolved in methylene chloride

were distributed into 500-mL Erlenmeyer flasks and the solvent was allowed to evaporate. The solid residue in the Erlenmeyer flask was then dissolved in toluene and appropriate volumes of isopropanol and buffer were added. Mixture 5 showed very good enzyme activity, and this was chosen for scale-up experiments. The H_2O_2 was added to give a final concentration of 0.5 m*M*, and the reaction was started by the addition of CPO to give a concentration of 1.25 μg of enzyme per mL. The progress of the reaction with petroporphyrins was followed by placing a portion of the reaction mixture into a cuvet and following the disappearance of the Soret peak or the appearance and subsequent reduction of the product peaks (A = 435 nm). Further additions of CPO and H_2O_2 were made at 10-min intervals (10X) in order to complete the reaction. An extraction blank, consisting of a ternary system without porphyrin (or asphaltene), and controls with CPO only or with H_2O_2 only were included with each experiment. At the end of the reaction, two volumes of water were added, thus yielding two phases and allowing for the extraction of the porphyrin material.

Analytical Methods

Petroporphyrins and asphaltenes were acid-extracted from the reaction mixtures in a separatory funnel. The organic phase was filtered through sodium sulfate to remove water. The sample was evaporated under a nitrogen atmosphere and analyzed by atomic absorption. The aqueous phase of the reaction was collected in an acid-washed beaker, concentrated more than 20-fold on a steam bath, and then dissolved in 2% HNO_3. The presence of released metals (Ni and V) was analyzed by atomic absorption spectroscopy in a Perkin Elmer 5100 spectrophotometer.

The enzyme concentrations were estimated by protein measurement with the Bio-Rad (Richmond, CA) procedure using bovine serum albumin (BSA) as standard and by spectrophotometry using an absorption coefficient of 168 mM^{-1}/cm at 409 nm for LPO *(10)*, 75.3 mM^{-1}/cm at 403 nm for chloroperoxidase *(11)* and 29.5 mM^{-1}/cm at 550 nm for cytochrome C peroxidase *(12)* reduced with sodium dithionite.

RESULTS AND DISCUSSION

Fractionation of Asphaltenes

Tables 1 and 2 show petroporphyrin contents and heavy metal (nickel and vanadium) composition in asphaltene fractions obtained by adsorption chromatography in silica gel. Petroporphyrins were measured by quantification of Soret peak at approx 410 nm. Almost 65% of the petroporphyrins contained in asphaltenes were concentrated in fractions 5 and 6, with molecular weights close to 600 g/moL (probably vanadium-porphyrins) *(5)*. These petroporphyrin fractions were used to evaluate biocatalytic

Table 1
Characterization of Asphaltene Fractions Obtained from Castilla Crude Oil

Fraction	Porphyrin content[a]		Distribution in asphaltenes (%)	MW[c](g/mol)
	μg/g	μmoles/g[b]		
1	290	0.48	5.0	940
2	270	0.45	6.6	765
3	450	0.75	7.0	600
4	605	1.01	7.0	540
5	4900	8.17	12.8	535
6	20650	34.42	51.3	520
No Fractionated[c]	295	0.49	10.3	N.D.

[a] Initial content is 1.53 μmoles/g asphaltene and 920 μg/g asphaltene.
[b] A MW of 600 is used for the porphyrins.
[c] No fractionated asphaltenes.
[d] MW obtained from maximum peak. Average MW of asphaltenes = 750 g/mol.
N.D.: Not determined.

Table 2
Nickel and Vanadium Contents in Asphaltene Fractions Using Adsorption Chromatography

Fraction	Metal content (μg/g)[a]		V in porphyrins[b]	
	Ni	V	μg/g	%[c]
1	543	2071	25	1.2
2	508	1907	23	1.2
3	513	1937	38	2.0
4	507	1845	51	2.8
5	458	1755	416	23.7
6	294	4155	1753	42.2
No Fractioned	N.D.	N.D.	(25)	(26.9)

[a] Metal contents in asphaltenes is 1686 and 43 μg/g for V and Ni, respectively.
[b] Determined from μmol/g porphyrin assuming 1 mol V complexed per porphyrin.
[c] % of V found that is associated with petroporphyrins. In asphaltenes is 4.4%. The value between parentheses was determined by difference.
N.D.: Not determined.

reactions with hemoproteins. This large fraction of vanadium porphyrins was confirmed with atomic absorption analysis. In these asphaltenes, higher concentrations of vanadium were complexed with porphyrins *(13,14)*. Table 2 displays Ni and V contents in fractionated porphyrins. The vanadium content varies with porphyrin concentrations, and this is

Table 3
Biocatalytic Modification of Petroporphyrin in Aqueous Buffer with Three Different Hemoproteins

Enzyme	Relative Activity (ΔAbs_{410}/min/μg HP)[a]	
	Petroporphyrin (12.5 μg/mL)	Petroporphyrin (25 μg/mL)
LPO	N.D.	N.D.
Cit-C	N.D.	N.D.
CPO	1.71×10^{-3}	3.14×10^{-3}

[a] HP = Hemoprotein.
N.D.: Not detected.

increased in rich-porphyrin fractions. However, nickel contents are decreased as asphaltenes are eluted from the column. These results indicate that vanadium is more associated to light fractions (vanadium porphyrins) *(13)*, whereas nickel appears at higher concentrations in heavy nonporphyrinic fractions. These results are in agreement with characterizations of porphyrinic and nonporphyrin metal complexes in asphaltenes from other oils *(3,13,15)*.

Reactions in Aqueous Buffer

Treatment of petroporphyrin with LPO, Cit-C, and CPO were evaluated in the presence of H_2O_2. Table 3 shows the results obtained in biocatalytic modification of petroporphyrins in aqueous buffer. Only CPO resulted in changes in the petroporphyrin UV-visible spectrum. The Soret peak at 410 nm, characteristic of vanadium-petroporphyrin molecules *(5,16)* was reduced in size (Fig. 1). There was no effect when this fraction was treated with either CPO or H_2O_2 alone. Moreover, chloride ions were required for biocatalytic modification of petroporphyrin in aqueous solutions *(6)*.

No biocatalytic modifications of asphaltenes was observed with Cit-C and LPO. It was not possible eliminate the Soret peak in all reaction mixtures. Although Cit-C and LPO can oxidize polyaromatic hydrocarbons (PAHs) *(17,18)* and sulfur asphaltenes as dibenzotiophene *(19)*, these hemoproteins were not effective in the elimination of the Soret peak.

In order to get most complete reduction of the Soret peak, it was better to use multiple additions of small amounts of enzyme and peroxide rather than a single addition. Successive additions of CPO and H_2O_2 produced a complete reduction of the Soret peak. The results obtained in the aqueous system in differents replicates showed that the reduction of the Soret peak reached a maximum of 80% under the experimental conditions. This incomplete peak removal is mainly caused by mass-transfer limita-

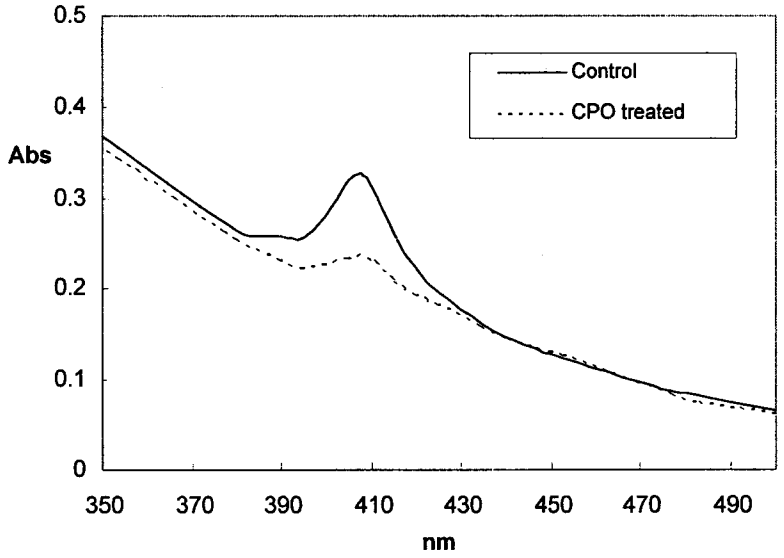

Fig. 1. UV-visible spectra of asphaltenes rich in petroporphyrins with and without CPO.

tions. The results were inconsistent due to the insolubility of petroporhyrin and because mass-transfer limitations were difficult to avoid.

CPO Reactions in Ternary Solvent Systems

Treatment of petroporphyrin in microemulsions with LPO, Cit-C, and CPO were evaluated in the presence of H_2O_2. However only CPO was able to eliminate the Soret peak. In order to eliminate mass-transfer limitations in the system and improve the action of CPO on petroporphyrins, the enzyme reactions were carried out in ternary systems of isopropanol:toluene:water *(6,20)*. Five different mixtures were used to evaluate biocatalytic modifications of petroporphyrin because they were effective in eliminating the Soret peak for nickel octaethylporphine (NiOEP) in presence of CPO and H_2O_2 *(6)* (Table 4). In this system, CPO showed a higher activity than in aqueous buffer. The rate of disappearance of the Soret peak of petroporphyrins in this ternary system was proportional to the amount of CPO used and the relative composition of compounds in the microemulsion.

The highest enzyme activity was observed in mixture 5. Reaction mixtures with a low water content (approx 15%) showed good results in removal of Soret peak. Some reactions stopped with the formation of a product with a maximum absorption peak of 435 nm. There was no loss of Soret peak when the reaction mixtures lacked CPO or H_2O_2 and chloride was absolutely required for activity of CPO on the petroporphyrins in the microemulsions to take place. These results suggest that free radical

Table 4
Biocatalytic Modification of Petroporphyrins in Normal Ternary Systems

Mixture	Toluene	Isopropanol	Aqueous	Relative Activity (ΔAbs_{410}/min/μg CPO) Petroporphyrin (12.5 μg/mL)	Relative Activity (ΔAbs_{410}/min/μg CPO) Petroporphyrin (25 μg/mL)
1	20.7 (0.11)	64.9 (0.46)	14.4 (0.43)	5.4×10^{-3}	10.1×10^{-3}
2	30 (0.14)	55 (0.39)	15 (0.46)	12.6×10^{-3}	23.8×10^{-3}
3	5 (0.02)	75 (0.46)	20 (0.52)	9.2×10^{-3}	20.0×10^{-3}
4	20 (0.09)	60 (0.38)	20 (0.53)	17.6×10^{-3}	34.8×10^{-3}
5	15 (0.06)	60 (0.34)	25 (0.60)	21.7×10^{-3}	43.3×10^{-3}

Percent composition by volume (mole fraction).

formation of ClO^- is necessary for the destruction of porphyrin in asphaltenes *(6)*. Free-radical production by some peroxidases has been demonstrated in monophasic organic solvents *(21,22)*.

Moreover, CPO is more selective to oxidize chloride than other hemoproteins *(23)*. Hemoproteins such as horseradish peroxidase, lactoperoxidase, and lignin peroxidase have been reported to catalyze the oxidation of iodide and bromide but only CPO has been shown to be effective in the oxidation of chloride *(23,24)*.

Microemulsions based on toluene or hexane have been used for enzyme reactions on water-insoluble substrates *(6,21)*. In this work, the ternary system of toluene:isopropanol:buffer enhanced the CPO-catalyzed modifications of water insoluble petroporphyrins.

CPO Reactions in Binary Solvent Systems (THF-Aqueous Buffer)

In these experiments, solvent systems containing THF were used because it was the only water-miscible organic solvent able to dissolve appreciable amounts of petroporphyrins and asphaltenes. The biocatalytic modification of petroporphyrins (a water-insoluble substrate) in an organic solvent system requires a good correlation between enzyme activity, stability, and the solubility of the substrate *(22)*.

Figure 2 shows the results obtained with CPO at different concentrations of THF. In this system CPO carried out similar modifications of the petroporphyrins as in microemulsions. However, the enzyme activity was dramatically decreased in the presence of high concentrations of THF. No reaction was observed in a mixture containing 25% of THF. This enzyme inactivation by high concentrations of THF and other aqueous-miscible organic solvents has been observed in other hemoproteins *(17,25,26)*. The enzymatic activity in these water-miscible systems with high organic solvent concentrations is suppressed because the organic solvent replaces water in the protein surface layer *(25,27)*.

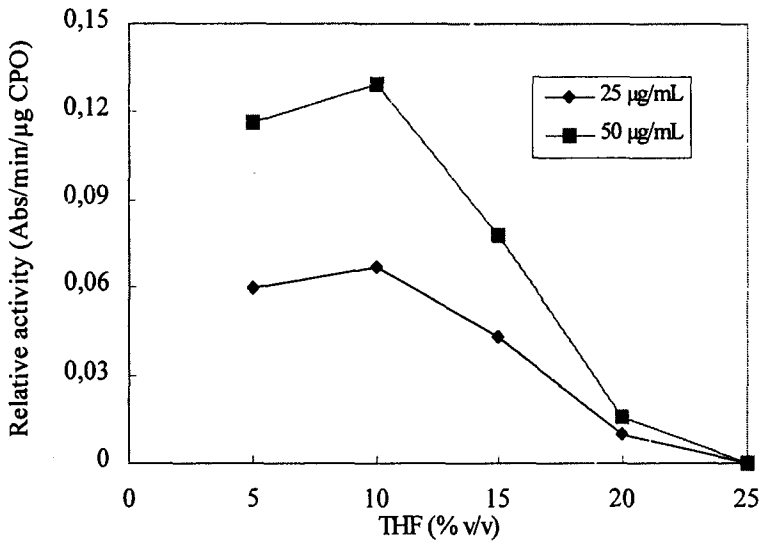

Fig. 2. Effect of THF concentration on the activity of CPO using petroporphyrin as substrate.

Demetallation of Petroporphyrins and Asphaltenes

The reactions were carried out with mixture 5, because it was the microemulsion that resulted in the best demetallation of Ni and V from petroporphyrins and asphaltenes. The enzyme reactions were worked with 12 mg of asphaltenes (or porphyirins) and an enzyme concentration of 1.25 µg mL (10X).

Tables 5 and 6 include the results obtained in the demetallation experiments using the ternary system (mixture n°5). Table 5 shows that the CPO reactions increased the amount of Ni and V in aqueous phase with a total heavy metal removal from petroporphyrins of 53%. Metal removal rates of heavy metals from petroporphyrins were 4.1 (mg Ni/g CPO/h) and 35.76 (mg V/g CPO/h).

Table 6 shows the results obtained with demetallation of asphaltenes. Metal removal rates were estimated from these results. Heavy metals removal from asphaltenes were lower than petroporphyrins. The following metal removal rates were reached: 2.4 (mg Ni/g CPO/h) and 15.5 (mg V/g CPO/h). These results confirm metal release from the petroporphyrins by biocatalytic activity with a total removal of 27% of both Ni and V.

Oxidative demetallation by chemical means has been carried out by using oxidating agents such as hypochlorite, chlorine, and sulfuryl chloride. The objective of such studies was the destruction of porphyrins with minor effects on petroleum *(28,29)*.

Treatment of asphaltenes with CPO is an alternative technology to remove heavy metals from crude oils and minimize problems related with poisoning catalysts. Ni and V from organometallic compounds are concen-

Table 5
Metal Analysis of Asphaltene Fraction Rich in Heavy Metals in Mixture 5. Experiences of Demetallation Using CPO (10×)[a]

	Amount of nickel				Amount of vanadium			
	Organic phase		Aqueous phase		Organic phase		Aqueous phase	
Sample	g	%R[b]	g	%F[c]	g	%R	g	%F
Control without CPO	3.0	0	0.6	20.0	28.4	0	0.2	0.7
Treated with CPO (10×)	1.3	56.7	1.7	53.1	13.5	52.5	4.5	15.8
Control without Asphaltenes	N.D.		0.3		N.D.		0.2	

[a] A sample of 12 mg of petroporphyrins was used, and this expected to contain approx 32.4 V and 3.67 μg Ni.
[b] %R is defined as percentage of metal removal from asphaltene. Based on Ni or V in organic phase control.
[c] %F is defined as percentage of metal found. Based on Ni or V in organic phase control.
N.D.: Not determined.

Table 6
Metal Analysis of Asphaltenes in Mixture 5. Experiences of Demetallation Using CPO (10×)[a]

	Amount of niquel				Amount of vanadium			
	Organic phase		Aqueous phase		Organic phase		Aqueous phase	
Sample	g	%R[b]	g	%F[c]	g	%R	g	%F
Control without CPO	4.3	0	0.1	2.3	18.7	0	<0.1	<0.5
Treatment with CPO (10×)	3.4	20.9	0.3	8.8	12.9	31.0	1.4	7.5
Control without Asphaltenes		N.D.		0.3		N.D.		<0.1

[a] A sample of 12 mg of asphaltenes was used, and this expected to contain approx 20.3 V and 5.2 μg Ni.
[b] %R is defined as percentage of metal removal from asphaltene. Based on Ni or V in organic phase control.
[c] %F is defined as percentage of metal found. Based on Ni or V in organic phase control.
N.D.: Not determined.

trated in the asphaltenes and affect hydrotreatment and cracking catalysts *(30)*. In this perspective, the application of biocatalysis in organic solvents can open new technological processes in petroleum upgrading.

ACKNOWLEDGMENTS

This work was supported by the Colombian Petroleum Institute, Ecopetrol, Colombia. Acknowledgment is made to Michael Pickard from

the University of Alberta, Canada for donation of chloroperoxidase vials. The advice of Rafael Vazquez-Duhalt from Biotechnology Institute, UNAM, Mexico and the review of the English version by Luis E. Ortiz from Environmental Department, ECOPETROL, Colombia is gratefully appreciated.

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