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# A Different Method of Measuring and Detecting Mono- and Dioxygenase Activities

*Key Enzymes in Hydrocarbon Biodegradation*

**Roberto Zazueta-Sandoval,* Vanesa Zazueta Novoa, Hortencia Silva Jiménez, and Roberto Cabrera Ortiz**

*Instituto de Investigación en Biología Experimental, Facultad de Química, Universidad de Guanajuato, Noria Alta s/n, Apartado Postal 187, Guanajuato, Gto. 36000, México, E-mail: zazueta@quijote.ugto.mx.*

## Abstract

A spectrophotometric method of measuring oxygenase activity in cell extracts or in zymograms was developed. It is an easy and cheap method that allows spectrophotometric measurement of activity by a colored reaction and reveals activity bands in a polyacrylamide gel electrophoresis (PAGE) gel as brown bands. To prove its usefulness, we report on a study with the oxygenase present in strain YR-1, isolated from petroleum-contaminated soils, that uses hydrocarbons as its sole carbon source. Soluble oxygenase activity was detected (under our conditions of cellular homogenization) in the mycelium of a filamentous fungus strain named YR-1. Oxygenase activity from aerobically grown mycelium was detected in growth medium containing the hydrocarbons decane or hexadecane; the enzyme activity exhibited similar optimum pH for the hydroxylation of different aliphatic or aromatic substrates (decane, hexadecane, benzene, and naphthalene) to the corresponding alcohols. Zymogram analysis conducted with partially purified fractions from cell extracts from the aerobic mycelium of the YR-1 strain indicated the existence of only one oxygenase enzyme. Partially purified samples of enzyme, analyzed by sodium dodecyl sulfate PAGE, indicated the presence of one major protein band with a mol wt of 56 kDa that can be a constituent of the native enzyme. In samples of the enzyme, the 56-kDa protein gave a positive reaction in immunodetection experiments with antibodies directed against oxygenase from

*Author to whom all correspondence and reprint requests should be addressed.

soybean. The partially purified enzyme oxidized different substrates, although higher activity was displayed with benzene. $K_m$ values obtained for benzene and decane indicated a higher affinity for the latter.

**Index Entries:** Oxygenases; filamentous fungi; hydrocarbon biodegradation; petroleum contamination.

## Introduction

Hydrocarbons represent an enormous energy resource and are mainly exploited as fossil fuels. As well as being the predominant energy source in most countries, hydrocarbons are an important feedstock for the chemical industry. Their potential impact on biotechnology is enormous, but as yet their exploitation has been limited. The chemical nature of these compounds is very diverse, varying from simple saturated aliphatic alkanes to complex polycyclic aromatic compounds. Such a range of carbon substrates can support the growth of many microorganisms using diverse and often not well-understood metabolic pathways. The potential for innovative industrial application is therefore high. The absence of a basic understanding of the biochemical nature of hydrocarbon metabolism has slowed down and in some cases halted many research projects. In addition, a variety of problems associated with the development of high-productivity fermentation strategies for insoluble high-energy status substrates necessitate an approach somewhat different from sugar-based fermentation technology *(1)*.

Several possible biochemical pathways are involved in hydrocarbon biodegradation. The first step in hydrocarbon biodegradation is hydroxylation, which is catalyzed by the cytochrome P-450 protein complex *(2)*. Two mechanisms have been proposed to explain the incorporation of molecular oxygen into the hydroxylated product: one atom in the case of monooxygenases *(3)*, and two atoms in the bacterial dioxygenase case *(4)*. Cytochrome P-450 is capable of using a wide range of xenobiotic compounds as substrates and, by means of many types of chemical transformations, leads to the production of alcohols found in microorganisms as well as in plants and animals *(5)*.

In the present article, we describe an easy and useful spectrophotometer method to measure the oxygenase activity in cell-free extracts and a variation of the same method for the detection of this activity in zymograms. We also present some biophysical properties of the oxygenase activity present in cell-free extracts of strain YR-1 filamentous fungi isolated from petroleum-contaminated soils.

## Materials and Methods

### *Reagents and Chemicals*

Molecular weights standards, phenylmethylsulfonyl fluoride (PMSF), and yeast AO were purchased from Sigma (St. Louis, MO). The alcohol substrates were from J.T. Baker (Phillipsburg, NJ) All other reagents were

of the highest purity commercially available. Protein was measured by the method of Lowry *(11)*, with bovine serum albumin used as the standard.

### *Organisms and Culture Conditions*

The isolation of filamentous fungi able to grow on hydrocarbons was performed using as source petroleum contaminated soil samples collected from the Salamanca refinery (Guanajuato, México). The primary and secondary selections were achieved using minimal media supplemented with 1% methanol or 1% hexadecane, respectively. The isolates were named YR, and the particular strain used was named YR-1; in all cases, colonial and microscopic morphologies were established as criteria for the assessment of the isolated strains as filamentous fungi. Yeast-peptone-glucose (YPG) complete media *(6)*, and salt minimal medium supplemented with 0.1% peptone (named sMMP), containing the specified amounts of glucose or hydrocarbons as carbon sources, were used to cultivate the fungus. The strain was maintained in agar slant tubes and spores were obtained after grown in YPG medium as described. Liquid cultures (600 mL) were propagated in 2-L Erlenmeyer flasks inoculated with spores at a final cell density of $5 \times 10^5$/mL and incubated in a water bath shaker at 28°C for different periods of time. To obtain aerobic mycelia, spores were inoculated in either YPG or sMMP media supplemented with glucose (0.1%), decane (1.0%), or hexadecane (1.0%) and the cultures were incubated aerobically *(6)*.

### *Preparation of Cell-Free Extracts*

Mycelium cells were processed and broken as described by Torres-Guzman et al. *(7)*, with some modifications. Briefly, mycelia cells were washed and suspended in buffer TP8.5 (20 m*M* Tris-HCl [pH 8.5] containing 1 m*M* PMSF). A volume of about 20 mL of cells was mixed with an equal volume of glass beads (0.45–0.50 mm in diameter) and disrupted in a Braun Model MSK cell homogenizer (Braun, Melsungen, Germany) for four 30-s periods under a stream of $CO_2$. The homogenate was centrifuged at 4360*g* for 10 min to remove cell walls and unbroken cells. The cell wall–free supernatant (crude extract) was centrifuged at 164,500*g* for 45 min; the resulting pellet, a mixed membrane fraction, was discarded and the 164,500*g* supernatant (cytosolic fraction) was saved for enzymatic determinations.

### *Enzyme Assays*

To evaluate the enzyme activity in cell extracts, it was necessary to implement a spectrophotometric method. Then, some variations to the lipoxygenase method were made to detect oxygenase in zymograms after a PAGE run *(8)*. Briefly, the basic principle was based on the property of oxygenases to use molecular oxygen to oxidize the hydrocarbon-substrate molecules by means of an electron transport system *(2,4)*. Similarly, the enzyme is capable of oxidizing *o*-dianisidine, producing a brown solution.

Enzyme activity was calculated by interpolating readings at 460 nm in a peroxidase activity calibration curve made with different concentrations of hydrogen peroxide as substrate of the enzyme in the presence of *o*-dianisidine *(9)*. In the case of zymograms, activity bands were detected by submerging the gel in the reaction mixture (*o*-dianisidine added to the substrate and buffer) and incubating the gel at room temperature with gentle agitation until the activity bands appeared.

The enzyme assays were performed at 25°C in reaction mixtures of 1.0 mL total volume containing 490 µL of 0.05 *M* of potassium phosphate buffer (pH 7.5), 400 µL of reactant A (20 mg of *o*-dianisidine dissolved in 15 mL of absolute ethanol and 0.025 *M* Tris-HCl buffer, pH 4.5, to 100 mL), 10 µL of the appropriate substrate (aliphatic or aromatic hydrocarbons), and 100 µL of cell-free extract (100–200 µg of protein). The reaction was started by adding substrate, and color development was determined by measuring the absorbance at 460 nm in a Beckman DU-650 spectrophotometer. In experiments in which the pH of the reaction was varied, phosphate (50 m*M*) and Tris-HCl (50 m*M*) buffers were employed. One unit of enzyme activity was defined as the amount of enzyme that leads to the production of 1 µmol $O_2$·min at 25°C. Oxygenase specific activity was expressed as units per milligram of protein. Detection of oxygenase activity in zymograms was performed by nondenaturing polyacrylamide gel electrophoresis (PAGE), following a variation of a spectrophotometric method. Briefly, after nondenaturing 8% (w/v) PAGE, the gel was submerged in the following solution: 10 mL of *o*-dianisidine reactive (reactive A); 9.0 mL of 0.05 *M* Tris-HCl, pH 8.5; and 1.0 mL of substrate (aliphatic or aromatic hydrocarbons). After incubating at 25°C for 60–120 min with gentle shaking, oxygenase electromorphs were observed as brown bands.

### *Substrate Specificity*

To test oxygenase specificity, enzyme activity was assayed in the presence of one of the following substrates, each at a final concentration of 100 m*M*:

Aliphatic: octane, decane, hexadecane, and tetracosane;

Aromatic: benzene, naphthalene, phenanthrene, anthracene, pyrene, toluene, and chlorobenzene.

### *Partial Enzyme Purification*

The 164,500*g* supernatant was used as the starting material for the purification steps, which were performed at 4°C. The supernatant was loaded onto a DEAE-Prep-Biogel (Bio-Rad) low-pressure chromatography column (2.5 4.5 cm) previously equilibrated with 0.5 *M* Tris-HCl (pH 8.5) buffer. Elution was started with Tris-HCl buffer, followed by elution with 0.1 *M* NaCl and subsequently 0.5 *M* NaCl (flow rate of 0.6 mL/min) in the same buffer. Fractions (3 mL) were collected, and those containing enzyme activity were pooled, concentrated by vacuum centrifugation

(Savant system), dialyzed against 20 m*M* Tris-HCl (pH 8.5), and stored at –70°C. The concentrated fractions were applied to a preparative 6% (w/v) polyacrylamide gel and electrophoresed under nondenaturing conditions; oxygenase activity was assayed, the active band was electroeluted from the gel, and the eluted protein was used in spectrophotometric assays of oxygenase activity.

### *Electrophoresis*

Sodium dodecyl sulfate (SDS) PAGE analysis of samples was carried out in slab gels using 10% (w/v) polyacrylamide with the buffer system of Laemmli *(10)*. Standard proteins of 14.4–106.0 kDa were used as markers; after electrophoresis, proteins were visualized in the gels using a Sigma silver-staining kit.

### *Immunoblotting and Immunodetection*

After SDS-PAGE, proteins were transferred to a nitrocellulose membrane in a Mighty small Transphor unit (Hoefer TE22; Pharmacia Biotech, San Francisco, CA). Detection was made with polyclonal antibodies raised against commercial soybean lipoxygenase and revealed with a second antibody coupled to peroxidase using 3,3'-diaminobenzidine (Sigma).

## Results

### *Oxygenase Activity in Cell Extracts from Mycelial Cells*

Oxygenase activity with hexadecane as substrate was analyzed in a 164,500*g* supernatant of aerobically grown mycelium of strain YR-1 obtained in sMMP containing 1.0% hexadecane as carbon source. The presence of oxygenase activity was clearly detected in the cytosolic fraction (164,500*g* supernatant) of these-mentioned cells. The appearance of oxygenase activity as a function of incubation time in growth medium with decane was estimated. Enzyme production reached its maximum after 22 h and then declined (Fig. 1); this decrease coincided with the onset of the stationary phase of growth.

Oxygenase activity with hexadecane as substrate was only detected when the fungus was grown in minimal media containing decane or hexadecane as carbon sources. This suggests that the fungus contains an oxygenase that recognizes hexadecane as substrate and is induced in the presence of the hydrocarbons decane or hexadecane (not shown).

Oxygenase activity from aerobically grown mycelial cells was measured over a range of pH using hexadecane as a substrate. Fig. 2 shows that the optimum pH for the oxidation of hexadecane to the respective alcohol was approx 8.5 in Tris-HCl buffer. No effect of incubation temperature was observed at 28 or 37°C (not shown).

To test the possibility of the presence of more than one oxygenase activity in cell extracts of the strain YR-1, the zymogram for oxygenase activity was obtained using different concentrations of the 164,500*g* super-

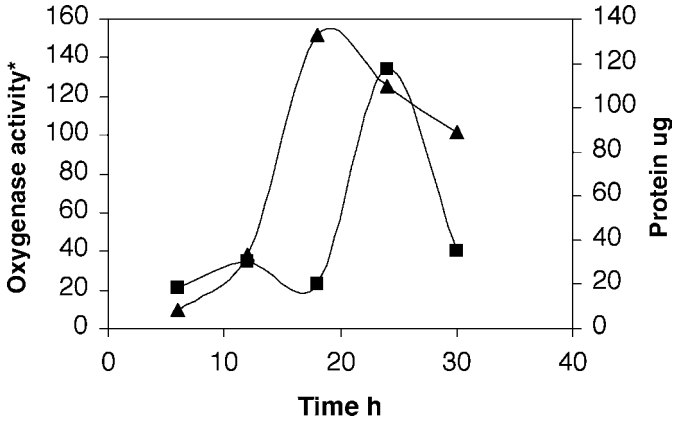


Fig. 1. Time course of oxygenase activity with respect to incubation time. Enzyme activity was determined in the 164,500*g* supernatant from aerobically grown mycelial cells in sMMP medium supplemented with decane. (▲) Protein; (■) oxygenase activity. *Oxygenase activity expressed as 460 nm/min absorbance.

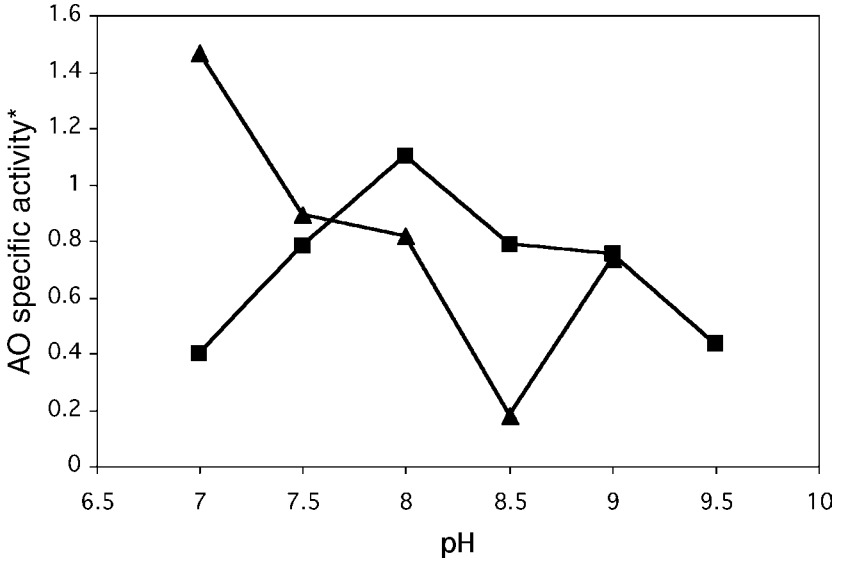


Fig. 2. Effect of pH on oxygenase activity. The enzyme activity was determined in the 164,500*g* supernatant from aerobically grown mycelial cells in sMMP supplemented with decane as the sole carbon source. The reaction mixture contained 25 m*M* potassium phosphate between pH 6.0 and 10.0, Tris-HCl between pH 6.0 and 10.0, (0.5 *M*), 0.025 *M* decane 3,3'-diaminobenzidine in 0.1 *M* HCl and 100 µg of protein of the 164,500*g* supernatant. (■) Tris-HCl buffer; (▲) potassium phosphate buffer. *Oxygenase specific activity expressed as units per milligram of protein.

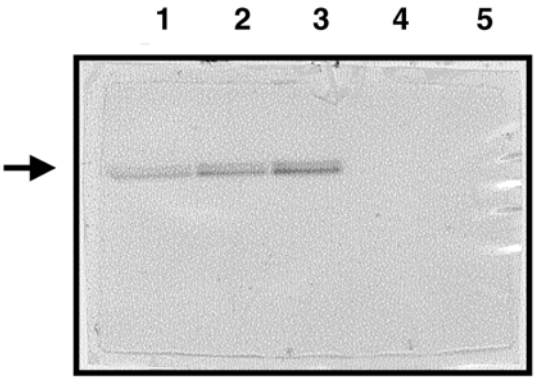


Fig. 3. Zymogram of oxygenase activity revealed with hexadecane as substrate. After native PAGE, in 6% acrylamide, activity bands were revealed using the proposal method. Lane 1, 100 µg of protein; lane 2, 200 µg of protein; lane 3, 300 µg of protein; lane 4, 300 µg of 5-min boiling protein; lane 5, 300 µg of protein extract from YR-1 strain grown in sMMP-glucose medium. Arrow shows the band of oxygenase activity.

natant from mycelial cells grown in hexadecane as sole carbon source. Figure 3 shows the oxygenase zymogram using hexadecane as the enzyme substrate; as can be observed, under these conditions there was a dose response in function of the increase in the amount of enzyme in the assay, and only one major band of oxygenase activity was detected in all cases. The main activity band appeared only when the microorganism was grown in the absence of glucose and in the presence of decane or hexadecane. In addition to the major oxygenase activity band, a minor activity band can be observed with hexadecane (Fig. 3, lanes 1–3). To determine whether the enzyme was a monooxygenase, a dioxygenase, or a dioxygenase with the activity of a monooxygenase as well *(12)*, a PAGE was run, and half of it was revealed with benzene and the other half with hexadecane. Figure 4A shows only a single dioxygenase activity band (benzene), and Fig. 4B shows monooxygenase activity (hexadecane), both cases with a similar relative electrophoretic mobility, indicating that the enzyme is a dioxygenase with monooxygenase capacity.

### *Partial Purification of Oxygenase*

Oxygenase from mycelium grown aerobically for 24 h was partially purified from the 164,500*g* supernatant by a combination of DEAE-Prep Biogel column and preparative native PAGE of the pool of samples with the highest activity level, followed by electroelution of the activity band. After DEAE-Prep Gel chromatography, the samples with highest oxygenase activity (fractions 22–26) were pooled and passed through a Superose column in a high-performance liquid chromatography system. Figure 5 shows that the bound proteins in the column were differentially eluted by means of the change in NaCl concentration when a discontinuous NaCl

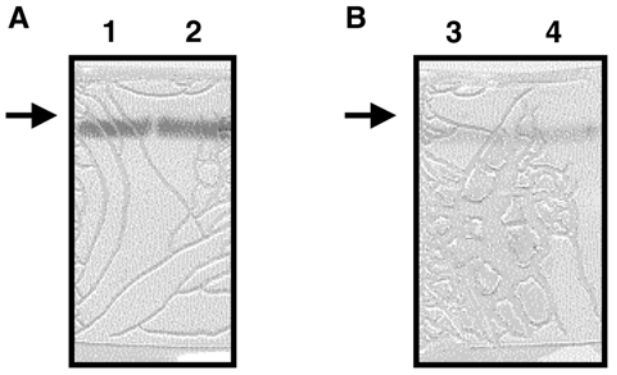


Fig. 4. Zymogram of oxygenase activity revealed with different substrates. Oxygenase activity was developed with **(A)** benzene or **(B)** hexadecane as substrates. Crude extracts (164,500*g* supernatant) of YR-1 strain grown in sMMP-hexadecane were used as sample. Lanes 1–4 were loaded with 300 µg of each protein. Arrows shows the bands of oxygenase activity.

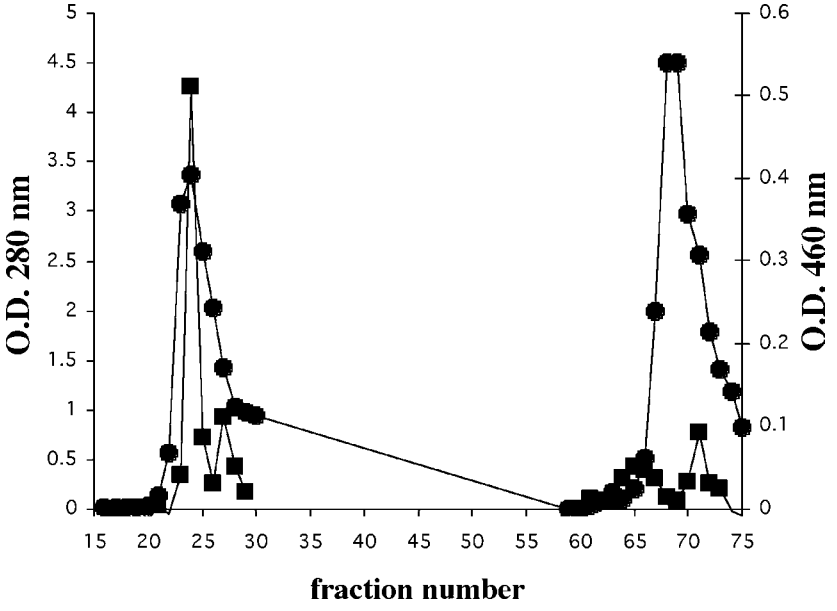


Fig. 5. Oxygenase activity elution profile after ion-exchange chromatography. The 164,500*g* supernatant of mycelium cells grown aerobically in sMMP medium supplemented with hexadecane as the sole carbon source was applied onto a DEAE-PREP Gel column. Elution was done with a discontinuous NaCl gradient (0–0.5 *M*). Oxygenase activity is expressed as optical density (OD) at 460 nm (■). Protein content of YR-1 is expressed as OD at 280 nm (●).

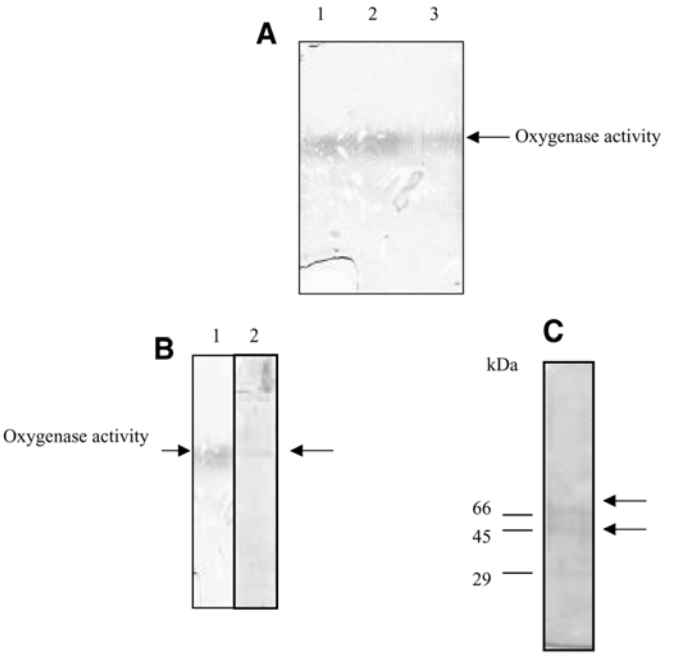


Fig. 6. Heterologous immunodetection of oxygenase subunits from partially purified fractions of oxygenase. The oxygenase activity band obtained after PAGE was electroeluted, submitted to SDS-PAGE, and the protein pattern was revealed with silver stain (not shown) or blotted to a nitrocellulose membrane and immunodetected using a heterologous antibody directed against lipoxygenase from soybean. **(A)** lanes 1–3 represent oxygenase activity revealed with hexadecane as substrate; **(B)** lane 1 represents oxygenase activity after PAGE, and lane 2 represents immunodetection of native oxygenase; **(C)** immunodetection of putative oxygenase subunit after SDS-PAGE. Arrows show oxygenase activity bands ([A] lanes 1 to 3; [B] lane 1); immunodetection of native form of oxygenase ([B]2); immunodetection of putative oxygenase subunits under denaturing conditions (C).

gradient was used. This procedure allowed the removal of most of the oxygenase activity from the column. As can be seen, there are two peaks of oxygenase activity; one (fractions 22–26) showed highest activity when benzene was used as the substrate. The second peak (fractions 64–71) showed minor activity. Additional steps in the purification protocol included the concentration of active fractions from the column, electrophoresis under nondenaturing conditions in a preparative PAGE gel, and then electroelution of the enzymatic activity band. In later experiments, only fractions 22–26 were used, because the other "peak" could be an artifact owing the fact that its localization is in almost the final volume of the column.

To gain information regarding the subunit composition of the native enzyme, after PAGE a zymogram was performed and the activity band (Fig. 6A) was cut and electroeluted from a preparative gel and submitted

to SDS-PAGE. As shown in Fig. 6C, partially purified samples of oxygenase were submitted to immunodetection experiments with polyclonal antibodies against soybean lipoxygenase. The results obtained indicated that two bands of 56 and 76 kDa were immunodetected, suggesting that these protein bands could be related to lipoxygenase enzyme from other organisms and may have some conserved epitopes. Additionally, the antibody was capable of recognizing only one protein band in native conditions that corresponded to the activity band in the zymogram (Fig. 6B).

### *General Properties: Substrate Specificity and Preliminary Kinetic Parameters*

The partially purified oxygenase was examined regarding substrate specificity using different aliphatic and aromatic substrates in the enzymatic assay; oxygenase activity with different substrates was expressed as specific activity (Table 1). The enzyme oxidized aliphatic and cyclic substrates, although much higher activity was observed with cyclic substrates. The complexity of the substrate molecule is important because when the number of rings increased the activity was reduced. The same phenomenon was observed with the aliphatic hydrocarbons; that is, the activity was lower with the increase in the number of carbon atoms in the molecule. $K_m$ and $V_{max}$ values were determined for benzene and decane (Table 2); the $K_m$ values for benzene (2.79 μ*M*) and decane (0.56 μ*M*) suggest that the higher affinity of the enzyme is for aliphatic substrates.

## Discussion

We consider that the method implemented in our laboratory to detect activity in zymograms and measure the oxygenase activity by spectrophotometric assay is easy and rapid. Furthermore when compared with the electrode measurements of consumed oxygen in this oxidation reaction, it is very inexpensive. This methodology allows us to make precise oxygenase measurements and detect with high qualityactivity in zymograms.

Oxygenase activity in aerobically grown mycelium cells of strain YR-1 obtained under different nutritional conditions was mainly found in the 164,500*g* supernatant. This observation indicated that this fungus oxygenase activity is cytosolic when using the ballistic homogenization of cells method (drastic rupture). However, it is important to use a gentle method of cellular homogenization, such as protoplast formation by lytic enzymes and osmotic shock homogenization, to be sure of the intracellular localization of this enzyme. The enzyme was detected only when the culture media contained a hydrocarbon as the sole carbon source, indicating a possible induction mechanism. However, the absence of activity when the microorganism was grown in glucose as the sole carbon source could indicate a possible regulatory effect of glucose.

The principal difference between the oxygenase from strain YR-1 and other oxygenases in different organisms is the capacity of the former to use

Table 1
Oxygenase Activity from Strain YR-1
Using Different Substrates[a]

| Substrate | Oxygenase specific activity (U/mg protein) |
|---|---|
| Aromatic | |
| Benzene | 18.58 |
| Naphthalene | 5.17 |
| Phenanthrene | 4.63 |
| Anthracene | 4.54 |
| Pyrene | 1.054 |
| Toluene | 14.35 |
| Chlorobenzene | 2.94 |
| Aliphatic | |
| Octane | 12.09 |
| Decane6.72 | |
| Hexadecane | 3.59 |
| Tetracosane | 0.106 |

[a]Partially purified oxygenase activity by ion-exchange DEAE-PREP chromatography was assayed as described in Materials and Methods, except that the substrates listed here were used in place of decane or hexadecane. All substrates were of 60 m*M* final concentration. Activities are expressed as activity units. The given values are the mean of two independent experiments with triplicate determinations in each instance.

Table 2
Kinetic Constants of Oxygenase from Strain YR-1[a]

| Substrate | $K_m$ (μ*M*) | $V_{max}$ (U/mg protein) |
|---|---|---|
| Benzene | 2.79 | 260 |
| Decane | 0.56 | 352 |

[a]Oxygenase activity from peak 1 (fractions 23–26) from the ion-exchange chromatography purification step was assayed with benzene and hexadecane at pH 8.5, as described in Materials and Methods. The given values are the mean of two independent experiments.

complex cyclic hydrocarbons as well as different aliphatic hydrocarbons as substrates. Kinetic parameters of oxygenase enzyme of strain YR-1 indicated differences regarding the affinity for substrates, mainly the use of decane over benzene (Table 2); however, maximum activity was obtained with benzene as the substrate (Table 1). This could be explained in terms of the $K_{cat}$ (catalytic constant) of the enzymatic system. The enzyme has a low $K_m$ value for decane over benzene but could perhaps have a higher $K_{cat}$ value

for benzene over decane. Nevertheless, it is necessary to make a more detailed determination of these constants, using samples with high purity level. The enzyme seems to have two subunits in its native conformation, made of two proteins of 76 and 56 kDa (Fig. 5A); the 56-kDa protein band was recognized by a heterologous antibody against lipoxygenase from soybean. These results suggested that the oxygenase present in the YR-1 strain is a dioxygenase with the capacity to use both cyclic and aliphatic substrates, i.e., a dioxygenase with both mono- and dioxygenase activities. In addition, our study will be of importance in establishing the role of these enzymes in hydrocarbon metabolism by the YR-1 strain.

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