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Presence and Physiologic Regulation of Alcohol Oxidase Activity in an Indigenous Fungus Isolated from Petroleum-Contaminated Soils

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Abstract

A soluble alcohol oxidase (AO) activity was detected in the mycelium of a filamentous fungus strain named YR-1, isolated from petroleum-contaminated soils. AO activity from aerobically grown mycelium was detected in growth media containing the hydrocarbons decane or hexadecane; the enzyme activity exhibited optimum pH for the oxidation of different alcohols (methanol, ethanol, and hexadecanol) similar to that of the corresponding aldehyde. Zymogram analysis conducted with purified fractions from aerobic mycelium of YR-1 strain extracts indicated the existence of two AO enzymes (AO-1 and AO-2). Purified samples of both enzymes analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis indicated the presence of three protein bands with molecular sizes 20, 38, and 46 kDa that could be part of the native enzyme. In samples of both enzymes, the 46-kDa protein gave a positive reaction in immunodetection experiments with antibodies directed against AO from *Hansenula polymorpha*. The purified AO-2 enzyme oxidized different alcohols, although higher activity was displayed with hexadecanol. K_m values obtained for methanol and hexa-decanol indicated a higher affinity for the latter. Analysis of the aminoter-minal sequence of the 46-kDa protein of AO-2 enzyme indicated significant similarity to enzymes involved in the metabolism of biphenyl polychloride compounds.

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Index Entries: Alcohol oxidase; filamentous fungi; hydrocarbon biodegradation; petroleum contamination.

Introduction

Hydrocarbon compounds are major environmental pollutants, as a result of improper disposal processes or spilling of petroleum or petroleum-derived products. This fact and the continuous increase in the number of toxic compounds generated by the oil industry have emphasized the importance of developing of new enzymatic or microbiologic processes to detoxify and degrade these waste products *(1)*. In nature, there exist many types of microorganisms useful in the biodegradation processes of these hazardous materials. Because natural biodegradation rates of these kinds of contaminants, principally in soils, it is very low and limited by environmental factors and by adaptation difficulties of microbial populations, is important to develop methods to eliminate these compounds from soils *(1)*.

Many microorganisms are capable of using hydrocarbons as the only carbon and energy source; however, when the number of carbon atoms in the hydrocarbon chain is increased to a certain amount, some bacteria and fungi are capable of metabolizing the hydrocarbonated chains *(2)*. The first step in hydrocarbon biodegradation is catalyzed by the protein complex Cytochrome P-450 followed by the action of alcohol oxidase (AO) *(3)*. Cytochrome P-450 is capable of using as substrates a wide range of xenobiotic compounds and by means of many types of chemical transformations, leading to the production of alcohols found in microorganisms as well as in plants and animals *(4)*. AO catalyzes the oxidation of alcohols to the corresponding aldehyde, which, in turn, is converted into the corresponding carboxylic acid *(5)*. The reactions catalyzed by Cyt-p450 and AO are a special point for bioremediation chemistry. So far most of the studies regarding the role of AO in hydrocarbon metabolism have been made on bacterial strains, and in several cases, AO enzymes from eukaryotic origin with physiologic roles related to hydrocarbon metabolism have been reported *(6)*.

In this article, we describe some physiologic characteristics of an indigenous fungus isolated from petroleum-contaminated soil, as well as the biophysical properties of AO, present in cell-free extracts of the microorganism.

Materials andMethods

Organisms and Culture Conditions.

The isolation of filamentous fungi able to grow on hydrocarbons was performed using as a source petroleum-contaminated soil samples collected from the Salamanca refinery (Guanajuato, Mexico). The primary and secondary selection were achieved using minimal media added to 1% methanol or 1% hexadecane, respectively. The isolates were named YR, and the particular strain used in this work 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. As a wild-type AO-proficient (AO^+) organism, we used strain R-25 of *Hansenula polymorpha (7)*. Yeast-peptone-glucose (YPG) complete media *(8)* and salts minimal media added to 0.1%, peptone (named sMMP) containing the specified amounts of glucose or hydrocarbons as carbon sources were used to cultivate the fungus. Strains were maintained in agar slant tubes, and spores were obtained after growth in YPG medium as described *(8)*. Liquid cultures (600 mL) were propagated in 2-L Erlenmeyer flasks inoculated with spores at a final cell density of 5×10^5/mL and incubated in a reciprocating water bath shaker at 28°C for the different time periods (see next section). To obtain aerobic mycelium, spores were inoculated in YPG medium and sMMP supplemented with glucose (0.1%), decane (1.0%), or hexadecane (1.0%), and the cultures were incubated aerobically *(8)*.

Preparation of Cell-Free Extracts

Mycelium cells were processed and broken as described by Torres-Guzman *(9)* with some modifications. Briefly, mycelial cells were washed and suspended in buffer TP8.5 (20 m*M* Tris-HCl [pH 8.5] containing 1m*M* phenylmethylsulfonyl fluoride [PMSF]). A volume of about 20 mL of cells was mixed with an equal volume of glass beads (0.45–0.50 mm 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

AO activity was measured according to Janssen, et al. *(10)*; the enzymatic assays were performed at 25°C in reaction mixtures of 1.0 mL total volume containing 780 µL of reactive A made of 1.2 mL of 0.2 *M* potassium-phosphate buffer (pH 7.5); 10 µL of 1.0% *o*-dianisidine dissolved in 0.025 *M* HCl, 5 µL of 3% peroxidase (0.01% final concentration), 150 µL of 0.2 *M* potassium-phosphate buffer, 15 µL of substrate (hexadecanol, decanol, or methanol) and 50 µL cell-free extract (100–200 µg protein). The reaction was started by the addition of substrate and development of color 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 of H_2O_2min at 25°C. AO specific activity was expressed as units per milligram of protein. AO activity in zymograms was detected by nondenaturing polyacrylamide gel electrophoresis (PAGE) following a variation of a spectrophotometric method *(10)* . Briefly, after nondenaturing 6% (w/v) PAGE,

the gel was submerged in the following solution: 4 mL of 0.2 *M* potassium-phosphate buffer (pH 7.5), 4 mL of 0.04% peroxidase, 0.4 mL of 0.01% *o*-dianisidine, 0.4 mL of substrate (methanol, ethanol, hexadecanol, and so on) and 31.8 mL of H_2O. After incubating at 25°C for 60 min with gentle shaking, AO electromorphs were observed as brown bands.

Substrate Specificity and Determination of Kinetic Constants

To test the specificity of AO, enzyme activity was assayed in the presence of one of the following substrates, each of them at a final concentration of 50 m*M*: methanol, ethanol, propan-2-ol, butan-1-ol, pentan-1-ol, 2-propen-1-ol, pentan-2-ol, octan-1-ol, cyclohexanol, benzyl alcohol, decanol, and hexadecanol. Apparent equilibrium constants were determined by Lineweaver-Burke plots *(11)*, using duplicate assays at five subsaturating concentrations for each substrate tested. Correlation coefficients from linear regression analyses for each kinetic constant determination were all >0.95.

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-Biogel (Bio-rad, Hercules, CA) 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 the latter buffer containing 0.1 *M* NaCl and, subsequently, 0.5 *M* NaCl (flow rate, 0.6 mL/min). Fractions (3 mL) were collected, and those containing enzyme activity were pooled. The pooled fractions were applied onto a high-pressure Mono Q HR 10/10 ionic exchange column in a high-performance liquid chromatography (HPLC) system. The elution was started with a 0.1–0.3 *M* NaCl continuous gradient in 50 m*M* Tris-HCl (pH 8.5). 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. AO activity was assayed, the active band electroeluted from the gel, and the eluted protein used to determine amino-terminal sequence and spectrophotometric assay of AO activity.

Electrophoresis

Sodium dodecylsulfate (SDS)-PAGE analysis of samples taken from the stages of the purification procedure was carried out in slab gels using 10% (w/v) polyacrylamide with the buffer system of Laemmli *(12)*. Standard proteins of 14.4–106.0 kDa were used as markers; after electrophoresis, proteins were visualized in the gels by a Sigma silver staining kit (St. Louis, MO).

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 done with polyclonal antibodies raised against commercial AO from *H. polymorpha* and revealed with a second antibody coupled to peroxidase using 3, 3' diaminobenzidine (Sigma).

Amino-Terminal Sequence

Sequencing of the 46-kDa protein of purified AO-1 and AO-2 was done by Bio-Synthesis (Lewisville, TX). Comparative analysis of protein sequence was performed with the FASTA3_t program using Swissprot and SWNEW database.

Miscellaneous

Molecular weight standards, PMSF, and yeast AO were purchased from Sigma. 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 *(13)* with bovine serum albumin used as the standard.

Results

AO Activity in Cell Extracts from Mycelial Cells

The presence of AO activity with methanol or hexadecanol as substrates was analyzed in different subcellular fractions (crude extracts, 164,500*g* supernatant and mixed membrane fractions) of aerobically grown mycelium of strain YR-1 obtained in sMMP containing 1.0% hexadecane as the carbon source; the enzyme activity was only detected in the cytosolic fraction (not shown). Table 1 shows AO activity levels when the strain was grown in different culture media, using methanol, ethanol, or hexadecanol to assay enzyme activity. AO activity was detected with methanol or ethanol, but not with hexadecanol, as enzyme substrates when the fungus was grown in the different media. On the other hand, AO activity with hexadecanol as substrate was only detected when the fungus was grown in minimal media containing decane or hexadecane as carbon sources. These results suggest that the fungus could contain different AO activities, and that the enzyme recognizing hexadecanol as substrate is induced by culture in the presence of the hydrocarbons decane or hexadecane.

AO activity from aerobically grown mycelial cells was measured over a range of pH using hexadecanol as a substrate. Figure 1 shows that the optimum pH for the oxidation of hexadecanol to the respective aldehyde was approx 7.0 in phosphate buffer. The effect of two different incubation temperatures, 25 and 37°C, on AO activity assay was investigated; no significant difference was found between these temperatures on AO activity (not shown). To test the possibility of the presence of more than one AO

Table 1
AO Activity of YR-1 Strain Grown in Different Culture Media

Growth medium	Specific activity of AO with the indicated substrate		
	Methanol	Ethanol	Hexadecanol
YPG	0.79 ± 0.02	0.13 ± 0.02	0.0
YP	0.17 ± 0.01	0.24 ± 0.04	0.0
YP-Methanol	1.36 ± 0.05	2.00 ± 0.02	0.0
YP-Ethanol	1.95 ± 0.04	0.85 ± 0.02	0.0
YP-Decane	0.78 ± 0.02	1.3 ± 0.05	0.0
YP-Hexadecane	0.57 ± 0.01	0.58 ± 0.02	0.0
MMS-Glucose	0.86 ± 0.05	0.18 ± 0.02	0.0
MMS-Decane	1.7 ± 0.03	4.50 ± 0.03	2.28 ± 0.1
MMS-Hexadecane	3.3 ± 0.08	3.80 ± 0.04	2.37 ± 0.08

[a]Enzyme activity was determined in the 164,500*g* supernatant from mycelial cells grown in the indicated culture media. Mycelial cells were broken and the cytosolic fraction was obtained by centrifugation. AO was measured with methanol, ethanol, or hexadecanol as substrates, as described in Materials and Methods. Enzyme activity is expressed as units per milligram of protein. The values are the means and their standard deviations in three independent experiments with triplicate determinations in each instance.

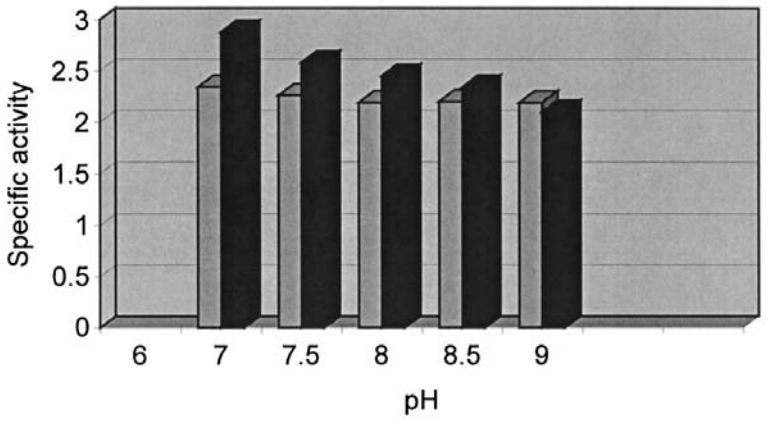

Fig. 1. Effect of pH on AO activity. The enzyme activity was determined in the 164,500*g* supernatant from aerobically mycelial cells grown in sMMP supplemented with decane. The reaction mixture contained 25 m*M* sodium phosphate between pH 6.0 and 10.0 (black bars) and Tris-HCl between pH 6.0 and 10.0 (gray bars), decanol (0.5 *M*), 0.025 *M* 3,3' diaminobenzidine in 0.1 *M* HCl and 100 µg of protein of the 164,500*g* supernatant.

activity in cell extracts of strain YR-1, the zymogram for AO activity was obtained using the 164,500*g* supernatant from mycelial cells grown under different conditions. Figure 2 shows AO zymograms using different alcohols as enzyme substrates. Under these conditions, only one major band of AO activity was detected in all cases, irrespective of the alcohol used as substrate. The main activity band appeared only when the micro-

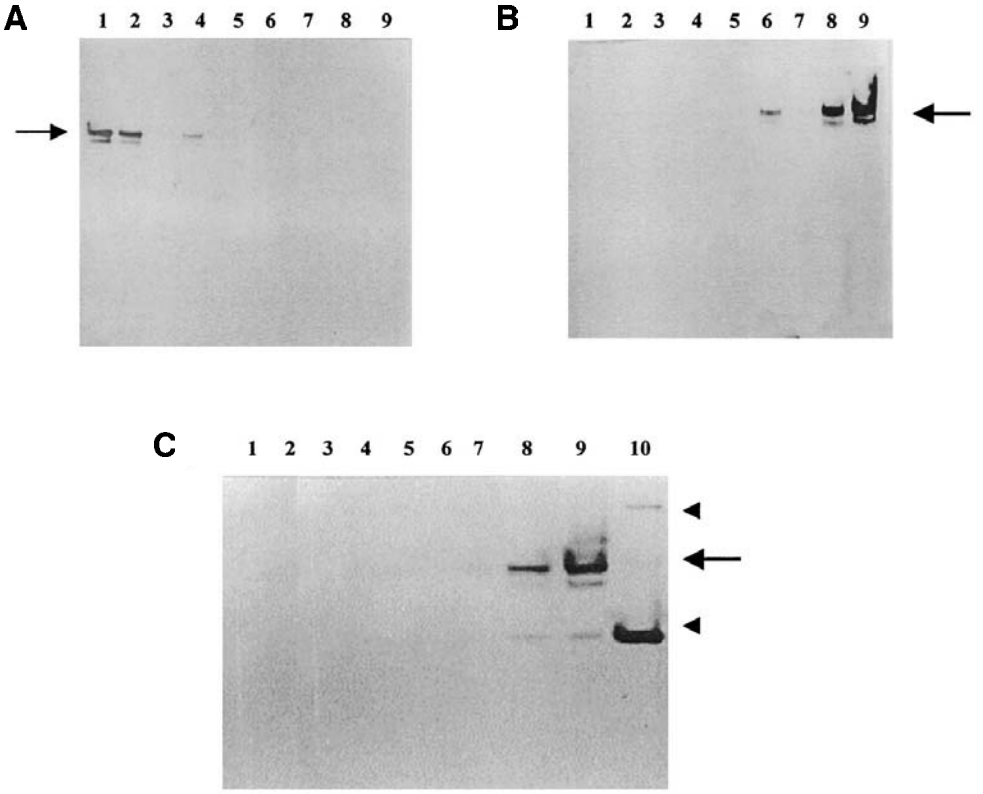

Fig. 2. Zymograms of AO activity from mycelium cells of YR-1. The 164,500*g* supernatant of mycelium cells grown aerobically for 22 h in different media was electrophoresed using 6% acrylamide. AO activity was developed in the gel with **(A)** methanol, **(B)** ethanol, or **(C)** hexadecanol as substrates. In all cases, 300 µg of protein was loaded in each lane. (A) Lane 1, MMS-decanol; lane 2, MMS-hexadecanol; lane 3, MMS-glucose; lane 4, YP-decanol; lane 5, YP-hexadecanol; lane 6, YP-methanol; lane 7, YP-ethanol; lane 8, YP; lane 9, YP-glucose. (B,C) Lane 1, YP-glucose; lane 2, YP; lane 3, YP-methanol; lane 4, YP-ethanol; lane 5, YP-hexadecanol; lane 6, YP-decanol; lane 7, MMS-glucose; lane 8, MMS hexadecanol; lane 9, MMS-decanol. (C) Lane 10, 164,500*g* supernatant of *H. polymorpha* cells grown in 0.5% YP-methanol (100 µg of protein loaded). Arrows indicate the bands of AO activity from YR-1; arrowheads indicate AO activity bands from *H. polymorpha*.

organism was grown in the absence of glucose and the presence of decanol or hexadecanol. In addition to the major AO activity band, minor activity bands are observed with hexadecanol as a substrate when the fungus was grown in minimal medium with hexadecane or decane (Fig. 2C, lanes 8 and 9).

The appearance of AO as a function of incubation time in growth medium with decane was estimated. Enzyme production reached its maximum after 22 h and then declined; this decrease in enzyme activity coincided with the onset of the stationary phase of growth (not shown).

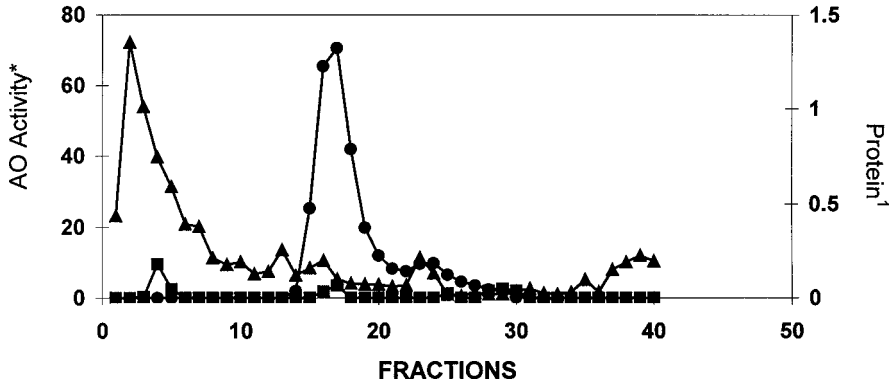

Fig. 3. Elution profile of AO activity from mycelium cells after separation by HPLC. The 164,500*g* supernatant of mycelium cells grown aerobically in sMMP supplemented with 1 % decanol, was applied onto a DEAE-Bio-gel column and eluted with a discontinuous gradient of 0–0.5 *M* NaCl. Fractions with highest activity were pooled and applied onto an Mono Q HR 10/10 ionic exchange column in an HPLC system. The elution pattern shown was made with a continuous gradient of NaCl from 0.15–0.25 *M*. Activity in methanol (●) or decanol (■)as substrates as well as protein (▲) is indicated. *AO activity = nmol of H_2O_2/min; [1]as 280 nm.

Purification of AO

AO from aerobically grown mycelium during 24 h was purified from the 164,500*g* supernatant by a combination of DEAE Biogel column, Superose column in an HPLC system, and preparative native PAGE of the pool of samples with the highest activity level followed by electroelution of the activity band. After DEAE chromatography, the samples with highest AO activity (fractions 5–12) were pooled and passed through a Superose column in an HPLC system. Figure 3 shows that the bound proteins in the column were differentially eluted by means of the change in the NaCl concentration when a discontinuous NaCl gradient was used. This procedure allowed the removal of most of the AO activity from the column. There are two peaks of AO activity; one of them (fractions 4 and 5) showed highest activity when decanol was used as substrate. The second peak (fractions 15–19) showed high activity with methanol as substrate and diminished activity with decanol. Additional steps in the purification protocol included the concentration of active fractions from the HPLC column, electrophoresis under nondenaturing conditions in a preparative PAGE gel, and then electroelution of the enzymatic activity band.

Zymogram analysis of the purified samples indicated the presence of two activity bands with different relative mobility in native PAGE (Fig. 4). The AO enzyme present in these bands differed in its preference for substrates. Fraction 1 (upper band, named AO-1) preferentially used methanol (Fig. 4A) and could also be used in lower-extension decanol and hexadecanol as substrates (Fig. 4B), whereas fraction 2 (lower band, named AO-2) only used decanol and hexadecanol as substrates. To gain information

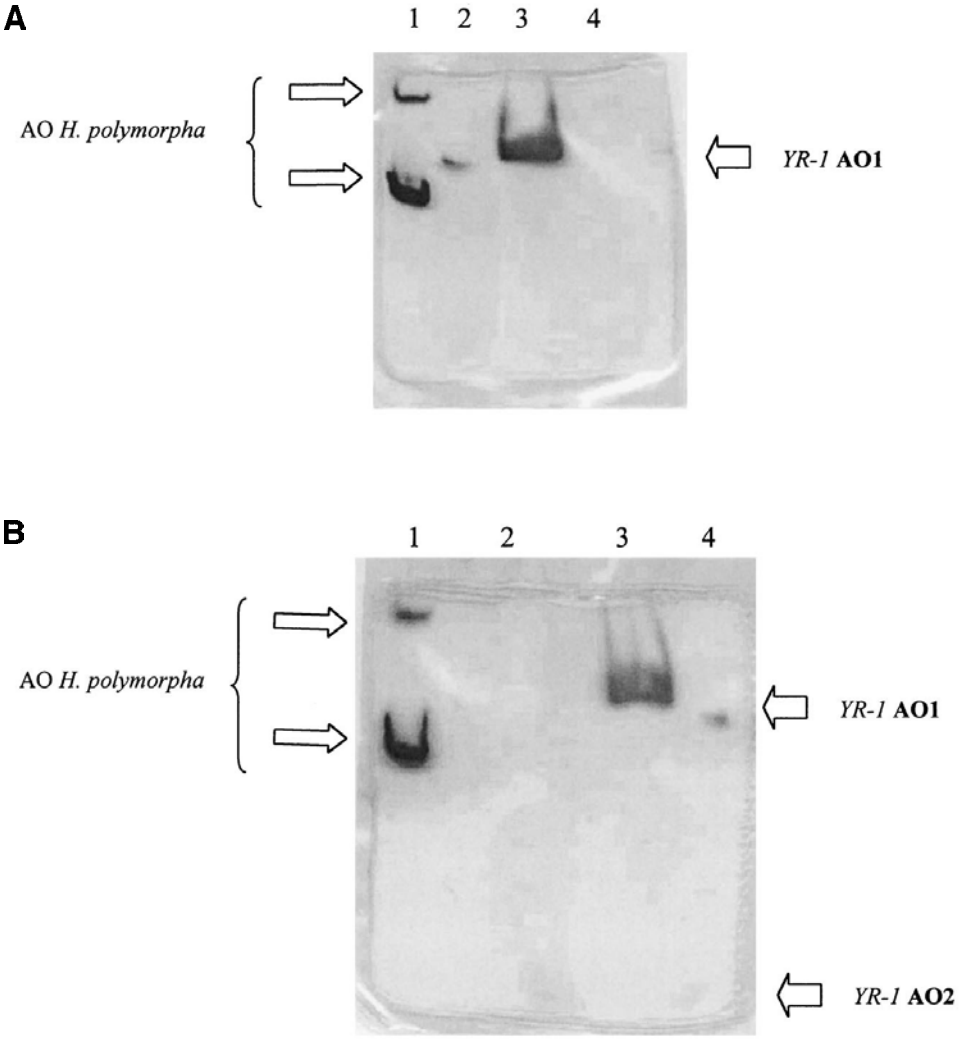

Fig. 4. Zymogram of AO activity of HPLC-purified fractions. AO activity was developed with **(A)** methanol or **(B)** decanol as substrates. (A) Lane 1, crude extracts (164,500*g* supernatant) of *H. polymorpha* grown in YP-methanol (control); lane 2, 164,500*g* supernatant of YR-1; lane 3, HPLC-purified fraction number 2; lane 4: purified fraction number 1. (B) Lane 1: crude extracts (164,500*g* supernatant) of *H. polymorpha* grown in YP-methanol (control); lane 2, HPLC-purified fraction number 1; lane 3, HPLC-purified fraction number 2; lane 4, 164,500*g* supernatant of YR-1.

regarding the subunit composition of the native enzyme in both fractions, in each case the activity band was electroeluted from a preparative gel and submitted to SDS-PAGE. As shown in Fig. 5A, the protein pattern of both fractions is similar, consisting of three protein bands with molecular weights of 46, 38, and 20 kDa, respectively. Partially purified samples of AO-1 and AO-2 were submitted to immunodetection experiments with

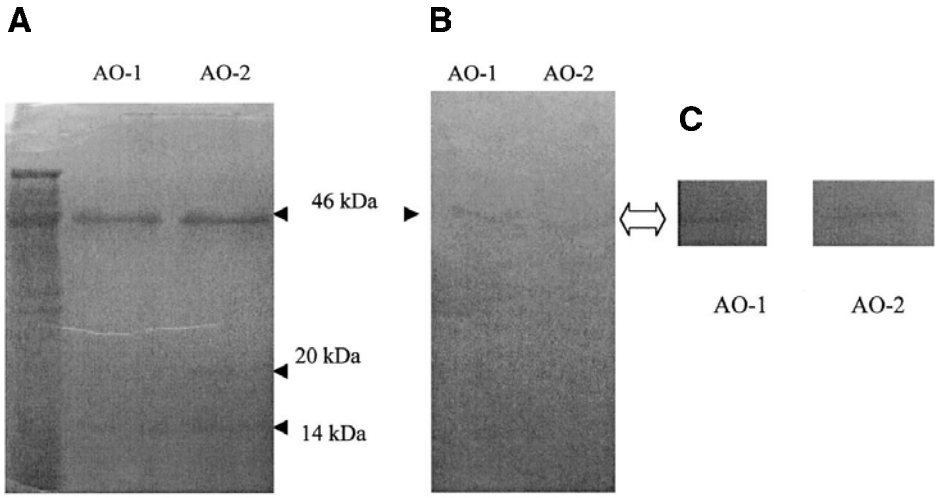

Fig. 5. Protein composition of purified fractions with AO activity. AO activity bands obtained in the experiment in Fig. 4 were electroeluted, submitted to SDS-PAGE, and the proteins were revealed **(A)** with silver stain, or **(B)** blotted to a nitrocellulose membrane and immunodetected with an antibody against-AO from *H. polymorpha*. The protein band recognized by the antibody (46 kDa) was transferred to a polyvinyl difluoride membrane **(C)** and submitted to amino terminal sequencing.

polyclonal antibodies against AO from *H. polymorpha*. The results obtained indicated that only the 46-kDa protein band was immunodetected in both samples (Fig. 5C), suggesting that this protein band is related to AO enzymes from methylotrophic yeasts.

Amino Terminal Sequence

Samples of purified 46-kDa protein of fractions 1 (AO-1) and 2 (AO-2) were submitted to amino-terminal sequencing; the amino acid sequence found for the AO-2 protein was as follows: **REYRFLAPSVXEAIDLAF**. Comparison of this sequence in databases indicated 30% similarity to a 3,3 biphenyl-diol-dioxygenase from *Rodhococcus globerulus*, an enzyme involved in the degradation of biphenyl-polychloride compounds *(14)*. The sequence results for the AO-1 46-kDa protein indicated that the amino terminal was blocked. These observations suggested that AO-1 and AO-2 could be two different entities.

General Properties: Substrate Specificity and Kinetic Parameters

Purified AO-2 was examined regarding substrate specificity using different alcohols in the enzymatic assay; OA with different substrates was expressed as percentage of the activity with hexadecanol (Table 2). The enzyme oxidized primary, secondary, and cyclic alcohols, although much higher activity was observed with primary alcohols no matter its chain size. The secondary highest AO activity was observed with isoamyl alcohol and,

Table 2
Relative Activities of AO from Strain YR-1 with Various Alcohols as Substrates

Substrate	Activity
Methanol	162.9
Ethanol	46.15
Propan-2-ol	45.0
2-Propen-1-ol	45.31
Butan-1-ol	72.6
Pentan-1-ol	78.16
Pentan-2-ol	90.06
Octan-1-ol	12.23
Hexadecan-1-ol	100.00
Cyclohexan-1-ol	80.11
Benzyl alcohol	37.86

[a] AO partially purified by ion exchange DEAE chromatography was assayed as described in Materials and Methods, except that the substrates listed below were used in place of methanol (127 m*M*) or hexadecanol (0.5 m*M*). All substrates were at 60 m*M* final concentration. Activities are expressed as percentage of the activity obtained with hexadecanol as the substrate, which was 0.98 units (mg protein)-1. The given values are the mean of two independent experiments with triplicate determinations in each instance.

Table 3
Kinetic Constants of AO-2 from Strain YR-1

Substrate	K_m (m*M*)	V_{max} (U/mg protein)
Methanol	89.58	1.86
Hexadecanol	5.0×10^{-4}	1.62

[a] AO activity from peak 1 (fractions 4 and 5) from the HPLC purification step was assayed with methanol and hexadecanol at pH 7.5, as described in Materials and Methods. The given values are the mean of two independent experiments with triplicate determinations in each instance.

in less extension, with the cyclic alcohol hexadecanol. Short chain alcohols, with the exception of methanol, were oxidized at a lower rate than butan-1-ol, pentan-1-ol or hexadecanol. K_m and V_{max} values were determined for methanol and hexadecanol (Table 3); the K_m values for methanol (89.5 m*M*) and hexadecanol (0.5 μM) suggest that the principal substrate for the enzyme is the latter compound.

Discussion

AO activity in aerobically grown mycelium cells of strain YR-1 obtained under different nutritional conditions was mainly found in the 164,500*g* supernatant. These observations indicated that in this fungus AO activity is cytosolic, independent of the carbon source in the growth medium.

The finding of two different AO activities in the 164,500*g* supernatant (Fig. 4A,B) is not unexpected; for instance, in the methylotrophic yeast *Candida boidinii*, the presence of two AO enzymes has been reported *(15,16)*. In filamentous fungi, the presence of AO enzymes has been reported in a limited number of cases, such as *Penicillium simplisimum*, which has a vanillyl oxidase *(17)*. The principal difference between the AOs of strain YR-1 and the AO of methylotrophic organisms is the capacity of the former to use complex alcohols as well as methanol as substrates. Kinetic parameters of AO-1 and AO-2 enzymes of strain YR-1 indicated differences regarding the affinity for substrates; AO-1 mainly uses methanol (Fig. 3A) whereas AO-2 shows preference for decanol (Fig. 3B). Both enzymes seem to have a similar subunit composition made of three proteins of 20, 34, and 46 kDa (Fig. 5A); the 46-kDa protein band of both AO-1 and AO-2 was recognized by heterologous antibody against AO from the methylotrophic yeast *H. polymorpha*. However, only the protein band from AO-2 had a free amino terminal and could be sequenced, whereas the 46-kDa protein band corresponding to AO-1 enzyme was blocked. These results suggested that AO-1 and AO-2 enzymes are different though related entities and that they have a component protein with a conserved epitope that is also present in the AO enzyme from methylotrophic yeasts. On the other hand, is interesting that the 46-kDA protein of AO-2 enzyme has significant similarity to one enzyme of the pathway of biphenyl-diol degradation in *Rhodococcus globerulus (14)*. Further studies will indicate the relationship of AO-1 and AO-2 to each other as well as to AO enzymes from other microorganisms. In addition, these studies will be of importance in establishing the role of these enzymes in hydrocarbon metabolism by the YR-1 strain.

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