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Detection of NAD$^+$-Dependent Alcohol Dehydrogenase Activities in YR-1 Strain of *Mucor circinelloides*, a Potential Bioremediator of Petroleum Contaminated Soils

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Abstract

Different soluble NAD$^+$-dependent alcohol dehydrogenase (ADH) isozymes were detected in cell-free homogenates from aerobically grown mycelia of YR-1 strain of *Mucor circinelloides* isolated from petroleum-contaminated soil samples. Depending on the carbon source present in the growth media, multiple NAD$^+$-dependent ADHs were detected when hexadecane or decane was used as the sole carbon source in the culture media. ADH activities from aerobically or anaerobically grown mycelium or yeast cells, respectively, were detected when growth medium with glucose added was the sole carbon source; the enzyme activity exhibited optimum pH for the oxidation of different alcohols (methanol, ethanol, and hexadecanol) similar to that of the corresponding aldehyde (~7.0). Zymogram analysis conducted with partially purified fractions of extracts from aerobic mycelium or anaerobic yeast cells of the YR-1 strain grown in glucose as the sole carbon source indicated the presence of a single NAD$^+$-dependent ADH enzyme in each case, and the activity level was higher in the yeast cells. ADH enzyme

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from mycelium grown in different carbon sources showed high activity using ethanol as substrate, although higher activity was displayed when the cells were grown in hexadecane as the sole carbon source. Zymogram analysis with these extracts showed that this particular strain of *M. circinelloides* has four different isozymes with ADH activity and, interestingly, one of them, ADH4, was identified also as phenanthrene-diol-dehydrogenase, an enzyme that possibly participates in the aromatic hydrocarbon biodegradation pathway.

Index Entries: Alcohol dehydrogenase; aromatic hydrocarbon biodegradation; contaminated soil; petroleum contamination; *Mucor Circinelloides*.

Introduction

Hydrocarbon compounds are major environmental pollutants, as a result of improper disposal processes or spills 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 the development of new enzymatic or microbiologic techniques to detoxify and degrade these waste products *(1).* There exist many types of microorganisms useful in the biodegradation processes of these contaminant compounds in nature. It is known that natural biodegradation rates of this typed of contaminant, principally in soils, are very low and limited by environmental factors as well as by adaptation difficulties of microbial populations *(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, only bacteria and fungi show a capacity to use these compounds as the only carbon and energy source *(2).* The hydrocarbon biodegradation pathway uses alcohol oxidase or alcohol dehydrogenase (ADH) to convert to the corresponding aldehyde the alcohol formed in the first step of the pathway by the complex protein oxygenase; the aldehyde formed will be converted into the corresponding carboxylic acid *(3)*. The combined reactions catalyzed by both oxygenases and alcohol oxidoreductases are central for bioremediation chemistry. So far, most studies concern the role of ADHs in alcohol metabolism; there are only a few reports about their role in aromatic-hydrocarbon metabolism.

In this article, we describe some characteristics of an indigenous fungus isolated from petroleum-contaminated soil, as well as several biophysical properties of ADH activity present in cell-free extracts of the microorganism. We also describe detection of different NAD^+-dependent ADH activities by zymograms as well as a phenanthrene-diol-dehydrogenase that may participate in the aromatic biodegradation pathway.

Materials and Methods

Reagents and Chemicals

Molecular weight standards, phenylmethylsulfonyl fluoride (PMSF), and yeast ADH were purchased from Sigma (St. Louis, MO), and 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 *(4)* with bovine albumin as the standard.

Organisms and Culture Conditions

The strain YR-1 of *Mucor circinelloides* was isolated using as source petroleum-contaminated soil samples collected from the Salamanca refinery, in the state of Guanajuato central México. As wild-type ADH-proficient (ADH^+) control we used a commercial preparation of *Saccharomyces cerevisiae* ADH (Sigma). Yeast-peptone-glucose (YPG) complete medium *(5)*, and salts minimal medium with 0.1% peptone added (named sMMP) containing the specified amounts of glucose or hydrocarbons as carbon sources, were used for cultivation of the fungus. Strains were maintained in agar slant tubes and spores were obtained after growth in YPG medium as described previously *(5)*. 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 indicated periods of time (see below). To obtain aerobic mycelium, spores were inoculated in YPG medium, sMMP-glucose (0.1%) or sMMP-decane, or hexadecane (1.0%), and the cultures were incubated aerobically *(6)*. The anaerobic yeast phase was obtained in YPG medium sparged with a sterile mixture of N_2/CO_2 (70:30 [v/v]) *(5)*.

Preparation of Cell-Free Extracts

Mycelial cells were processed and broken as described by Torres-Guzman et al. *(7)* with some modifications. Briefly, mycelial 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 the cells was mixed with an equal volume of glass beads (0.45 to 0.50-mm diameter) and disrupted in a Braun Model MSK cell homogenizer (Braun, Melsungen, Germany) for four 30-s periods while cooling with 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, and the 164,500 *g* supernatant (cytosolic fraction) were saved for enzymatic determinations or electrophoretic studies.

Enzyme Assays

All enzyme assays were carried out in a final volume of 1 mL and incubated for different times at 25°C. NAD^+-dependent ADH activity was assayed in the oxidative direction according to Bergmayer *(8)*. The enzymatic assays were performed in reaction mixtures of 1.0-mL total volume containing 25 m*M* Tris-HCl (pH 8.5), 2 m*M* NAD^+, cell-free extract (100200 µg of protein), and 0.8 *M* substrate (ethanol, decanol, or hexadecanol). The reaction was started by the addition of alcohol, and reduction of NAD^+ was monitored by the increase in absorbance at 340 nm in a Beckman DU-650 spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme required to reduce 1 µmol of NAD^+/min at 25°C. Specific ADH activity was expressed as units per milligram of protein. Detection of ADH activity in zymograms was performed by nondenaturing polyacrylamide gel electrophoresis (PAGE) following the method of Nikolova and Ward *(9)*, using a 6% running gel. After incubating at 25°C for 30 min (in the dark) with gentle shaking, ADH electromorphs were observed as brown bands.

Substrate Specificity

To test ADH specificity, enzyme activity was assayed in the presence of either ethanol or decanol, with each substratesat a final concentration of 50 m*M*.

Electrophoresis

PAGE analysis of samples taken from the stages of the purification procedure was carried out in slab gels using 6–8% (w/v) polyacrylamide without sodium dodecyl sulfate in the buffer system of Laemmli *(10)*.

Results

Influence of Oxygen on Morphology and ADH Activity in YR-1 Strain

Spore germination of the fungus *M. circinelloides* like other dimorphic species of *Mucor,* can lead to the production of either filamentous (mycelial) or spherical (yeast) cells, depending on environmental conditions. In the presence of oxygen, the fungus was grown as mycelium presenting large hyphae (Fig. 1A), whereas in its absence the fungus was grown in the yeast morphology (Fig.1B)

The biosynthesis of ADH in aerobically grown mycelial cells was estimated. The enzyme's appearance as a function of incubation time in growth medium with decane as the sole carbon source was estimated. Enzyme production reached its maximum level after 22 h and then declined (Fig. 2); this decrease coincided with the onset of the stationary phase of growth.

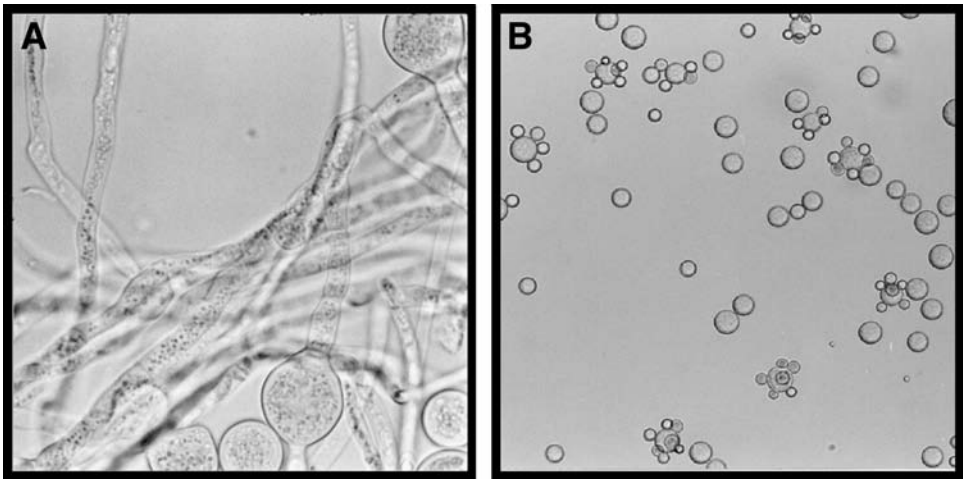

Fig. 1. Morphologic patterns of YR-1 strain grown in different environmental conditions. Mycelia or yeast morphologies were developed after **(A)** aerobic or **(B)** anaerobic growth of cells after incubation in glucose as sole carbon source. Magnification: 400×.

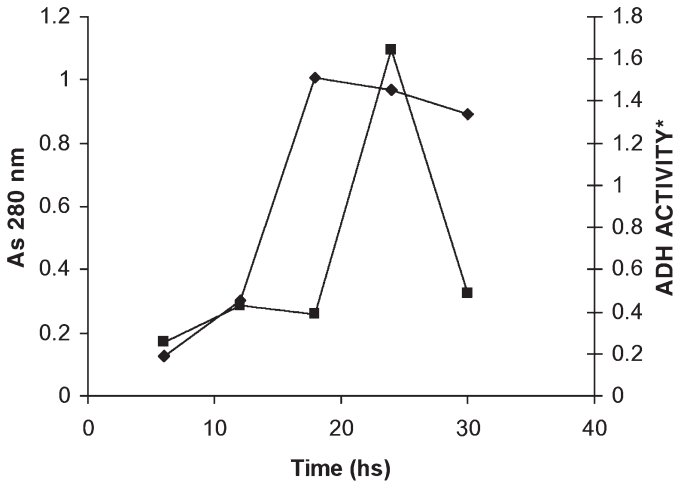

Fig. 2. Time course of ADH activity with respect to incubation time. Enzymatic activity was determined in the 164,500*g* supernatant from aerobically grown mycelial cells in sMMP supplemented with decane: (◆) protein; (■) ADH activity. *ADH activity is expressed as 340 nm/min absorbance.

Figure 3 shows that a single activity band was clearly observed in extracts from aerobically grown mycelium and anaerobically grown yeast cells (lanes 1 and 2, respectively). In both morphologic stages, the electrophoretic migration of the active band was similar, but differed from that shown by the commercial ADH from *S. cerevisiae* (lane 3). It is very

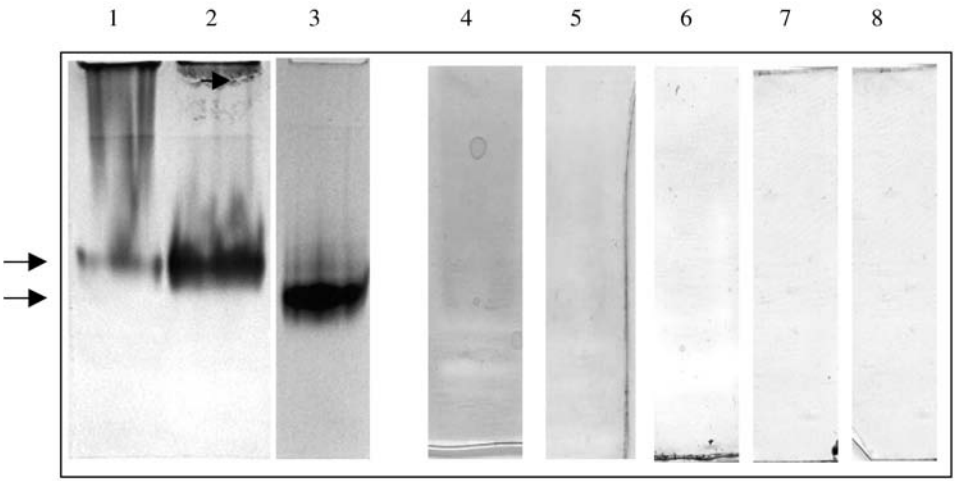

Fig. 3. NAD^+-dependent ADH zymogram band pattern from cell-free extracts of mycelium or yeast cells of strain YR-1 grown in glucose as sole carbon source. The 164,500*g* supernatant of mycelium or yeast grown aerobically (22 h) or anaerobically (16 h), respectively, was electrophoresed using a 6% acrylamide running gel and was stained for ADH activity with ethanol as substrate. Lane 1, aerobically grown mycelium cells; lane 2, anaerobically grown yeast cells; lane 3, commercial ADH of *S. cerevisiae;* lanes 4–8, Controls; lanes 4–6 cell extracts heated in boiling water for 10 min (mycelium and yeast from YR-1, respectively; ADH of *S. cerevisiae*); lanes 7 and 8, *r*eaction mixture without ethanol as enzyme substrate (mycelium and yeast from YR-1, respectively). All cases for YR-1 were loaded with 160 μg of protein in each lane. Arrows show ADH band activities.

interesting that ADH activity from yeast cells was higher than mycelial cells by as much as fivefold.

ADH Activity in Cell Extracts from Mycelial Cells

The presence of ADH activity with ethanol as substrate 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 detected only in the cytoplasm fraction (not shown). Table 1 gives ADH activity levels using ethanol as substrate in the assay, when the strain was grown in different culture media. ADH activity was higher when the fungus was grown in media with peptone added and containing hexadecane as the sole carbon source but also in alcohols because methanol and ethanol were high. These results suggest that it is possible that different ADH activities in the fungus could exist and that there might be a specific enzyme that could recognize hexadecanol or other complex alcohols (which may be aromatic) as substrate and be induced by the presence of hydrocarbons in the culture media.

ADH activity from aerobically grown mycelial cells was measured over a range of pH values in the forward (ethanol r acetaldehyde) reaction

Table 1
ADH Activity of YR-1 Strain Grown in Different Culture Media[a]

Growth medium	ADH activity (U/mg protein)
YPG	48.84 ± 0.02
YP	11.62 ± 0.01
YP- 1% methanol	239.00 ± 0.05
YP- 2% ethanol	255.00 ± 0.04
YP- 1% decane	8.15 ± 0.02
YP- 1% hexadecane	335.00 ± 0.01
MMS- 1% glucose	0.44 ± 0.05
MMS- 1% decane	1.58 ± 0.03
MMS- 1% hexadecane	7.94 ± 0.08

[a]ADH activity was determined in the 164,500*g* supernatant from mycelial cells grown in the indicated culture media. Mycelial cells were broken and the cytoplasmic fraction was obtained by centrifugation. ADH was measured with ethanol as substrate, as the described in Materials and Methods. The values are the means and their standard deviations in three independent experiments with triplicate determinations in each instance.

using NAD^+ and hexadecanol as enzyme cofactor and substrate, respectively. The optimum pH for the oxidation of hexadecanol to the respective aldehyde was approx 7.0 in phosphate buffer (not shown). In additions, the effect of two different incubation temperatures, 24 and 37°C, on ADH activity assay was investigated. It was found that there was significant difference between these temperatures on ADH activity (not shown).

To test the possibility of more than one ADH activity in cell extracts of strain YR-1, zymograms for ADH activity were obtained using the 164,500*g* supernatant from mycelial cells obtained under different growth conditions. Figure 4 shows that there are different activity bands in the cell extracts, depending of the carbon source used in the culture media. We used 8% acrylamide in gel for PAGE, and Fig. 4A shows three activity bands with different relative mobilities in each one. In lanes 2, 3, and 5 is not very clear whether there is only one activity band. To eliminate this doubt, we ran another PAGE experiment using 6% acrylamide; Fig. 4B shows the results. It is very interesting that in lanes 2 and 3 it is be possible to separate two different bands and a new activity band, named ADH4, is clearly seen. In lane 5, there is perhaps a double band, but with this result, it is difficult to measure the relative mobilities of these bands. Actually our results show that the YR-1 strain has four NAD^+-dependent ADH activities, but our principal aim in this work was to establish the presence of an ADH activity that could participate in the aromatic hydrocarbon biodegradation

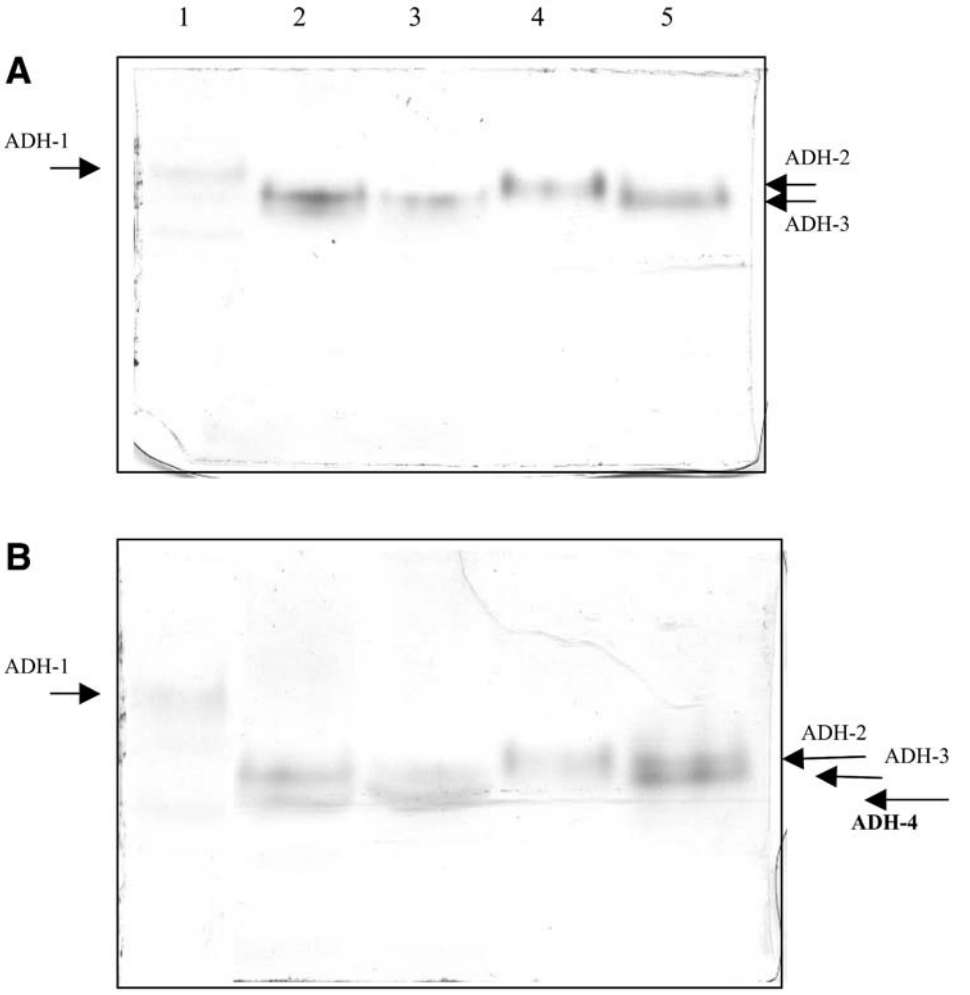

Fig 4. Zymogram of ADH activity in extracts from aerobically grown cells of YR-1 strain in different carbon sources. The 164,500*g* supernatant of mycelial cells grown for 22 h in different media was electrophoresed using **(A)** 8% or **(B)** 6% acrylamide running gel, and it was stained for ADH activity with ethanol as substrate. ADH activity was developed in the gel with ethanol as substrate. In all cases, 300 μg of protein was loaded in each lane. (A, B) Lane 1, sMMP-glucose; lane 2, sMMP-hexadecane; lane 3, sMMP-methanol; lane 4, sMMP-decane; lane 5, sMMP-decanol. Arrows show the bands of ADH activity.

pathway. To reveal this activity, it was necessary to synthesize the organic substrate for the putative di-hydro-diol-dehydrogenase enzyme. We chemically synthesized phenanthrene-diol (we proved that its structure was correct by nuclear magnetic resonance; not shown), and we used this product as the substrate in zymograms to reveal enzymatic activities of ADHs in the presence of NAD^+. The results are shown in Fig. 5, where it is clear that only one activity band is detected in lane 3, corresponding to

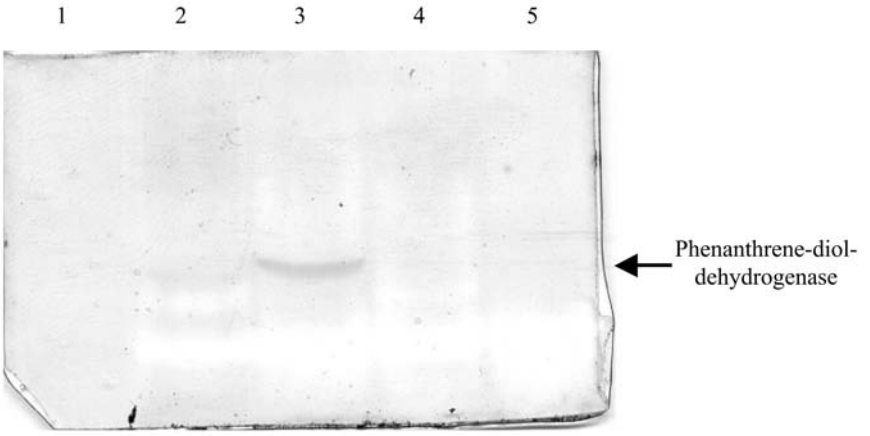

Fig. 5. Zymogram of ADH activity in extracts from aerobically grown cells of YR-1 strain in different carbon sources. The 164,500*g* supernatant of mycelium cells grown for 22 h in different media was electrophoresed using 6% acrylamide running gel. ADH activity was developed in the gel with Phenanthrene-diol as substrate. In all cases, 300 μg of protein was loaded in each lane. Lane 1, sMMP-glucose; lane 2, sMMP-hexadecane; lane 3, sMMP-methanol; lane 4, sMMP-decane; lane 5, sMMP-decanol. The arrow shows the band of ADH activity.

the extract obtained when YR-1 strain was grown in methanol as the sole carbon source. The activity band corresponds to ADH4.

Discussion

In Fig. 1, one can clearly see the differences between the morphologic pattern of growth of the fungus when it was growing in glucose as the sole carbon source and in the presence of O_2 or a flow of N_2/CO_2 (70–30% [v/v], respectively). This dimorphic pattern presented by YR-1 strain is consistent with different results with other Species of *Mucor (5)*. Figure 2 shows the time course of ADH synthesis. Interestingly, the higher activity was obtained in the stationary phase of growth, but it is possible to see a good activity level between 10 and 20 h of growth. In the zymogram show in Fig. 3, it is clear that there is only one ADH activity in both aerobically and anaerobically grown cells (lanes 1 and 2, respectively). In this particular case, that ADH activity was higher (maybe five times) in the 164,500*g* supernatant from cells grown in an N_2/CO_2 than in an O_2 atmosphere. This phenomenon has been observed in many other fungi and it has been proved that ADH activity is essential for the growth of some other fungi in the absence of oxygen *(5,7)*.

To investigate the presence of different ADH isozymes,ethanol was used as substrate, and ADH activity was revealed in zymograms. The results (Fig. 4A,4B) show that there are four different ADH activities, with each haring different relative mobility in the zymograms. ADH1 could be

a fermentative ADH with a physiologic role in the anaerobic development of the fungus. It is not subjected to a catabolite repression by glucose, and the presence of an inducer for its expression is not necessary. ADH2 and ADH3 possibly are important enzymes in the alcohol's biodegradation pathway. They were inducible by alcohols including methanol and also by aliphatic hydrocarbons. The most important contribution of this work is the detection of ADH4, because this enzyme was capable using as substrate a very complex compound chemically synthesized by us: phenanthrene-diol. This is a possible substrate for the putative ADH involved in the aromatic hydrocarbon biodegradation pathway, and in YR-1 cells it could be the product from the first enzymatic step in the aromatic biodegradation pathway when phenanthrene was the carbon source for growth of the YR-1 strain. The results shown in Fig. 5 strongly suggest that ADH4 could be a phenanthrene-diol-dehydrogenase, and it could be involved in the biodegradation of aromatic hydrocarbons. Interestingly, this enzyme was observed only in the high-speed supernatant corresponding to the growth of the YR-1 strain in methanol as the sole carbon source (Fig. 5, lane 3). In Fig. 4B, lane 2, there is an activity band with similar mobility to ADH in lane 3, but if one compares the same lanes in Fig. 5, only lane 3 shows the activity band using phenanthrene-diol as substrate, indicating that there could be two different ADH activities and that one participates in aromatic hydrocarbon biodegradation (lane 3) and the other in the biodegradation of aliphatic alcohols.

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