14

Polyacrylamide Gel Electrophoresis an Important Tool for the Detection and Analysis of Enzymatic Activities by Electrophoretic Zymograms

Reyna Lucero Camacho Morales, Vanesa Zazueta-Novoa,
Carlos A. Leal-Morales, Alberto Flores Martínez,
Patricia Ponce Noyola and Roberto Zazueta-Sandoval
University of Guanajuato
México

1. Introduction

Gel electrophoresis of enzymes is a very useful and powerful analytical method, which is at present widely used in many distinct fields of both biological and medical sciences and successfully applied in many different fields of human activity. The tremendous expansion of this methodology is mainly due to its simplicity and its high ability to separate both isoenzymes and alloenzymes, which have proven to be very useful genetic markers. The most important step of enzyme electrophoresis is the detection of native enzymes on electrophoretic gels; it means the procedure of obtaining electropherograms, or zymograms. Detection of enzymes on electrophoresis gels means the visualization of gel areas occupied by specific enzyme molecules after their electrophoretic separation. From this point of view, sometimes the testing of an enzyme-coding DNA sequence for expression of catalytically active enzyme is performed by zymograms, where the use of this technique for this purpose is very effective, cheap, and time saving. The number of applications of zymogram techniques for testing cloning enzyme-coding genes for their expression at the protein level is growing (Pfeiffeer-Guglielmi et al., 2000; Lim et al., 2001; Okwumabua et al., 2001). An absolute prerequisite for this is the specific and sensitive zymogram technique suitable for detection of the enzyme inside the gel and the use of the appropriate substrates.

The zymograms has been used to detect a variety of oxidoreductases (Bergmeyer, 1983), including isoenzymes (Jeng & Wayman, 1987) and to classify various genera of yeast based upon the relative mobility of the activity bands produced by selected enzymes (Goto & Takami, 1986; Yamasaki & Komagata, 1983). Electrophorezed gels are placed in a staining solution containing a reduced substrate such as an alcohol, oxidized cofactor such as NAD^+ or $NADP^+$, a dye such as nitroblue tetrazolium, and an electron acceptor-donor such as phenazine methosulphate. At the location of the appropriate enzyme catalyzing oxidation of substrate and reduction of cofactor, a dark-purple band appears as a result of the precipitate that forms upon reduction of the dye.

Previously we have investigated the use of zymogram staining of native electrophoretic gels as an initial approach to the identification of carbonyl reductase activities against both aliphatic (Silva et al., 2009; Zazueta et al., 2008) and aromatic hydrocarbons (Durón et al., 2005; Zazueta et al., 2003) in *Mucor circinelloides* YR1, an indigenous fungus isolated from petroleum contaminated soil.

Oil spills sometimes occur during routine operations associated with the exploration and production of crude oil. Crude oils vary widening in composition depending on factors such as source bed type and generation temperatures (Hunt, 1979). Biodegradation rates for crude oils will vary due to differences in composition, as reflected by hydrocarbon class distribution: saturates, aromatics, and polars, and the amount *n*-alkanes *versus* branched and cyclic alkanes within the saturated hydrocarbon class (Cook et al., 1974). In nature exist many types of microorganisms useful in the biodegradation processes of contaminant compounds (Atlas, 1995), such as the polycyclic aromatic hydrocarbons (PAH's) that are persistent soil contaminants and many of which have toxic and carcinogenic properties (Hyötyläinen and Oikari, 1999; Cerniglia, 1997).

In bacterial aerobic degradation of aromatic compounds, reactions of metabolic pathways generally lead to the formation of aromatic intermediates containing two hydroxyl constituents, which are subsequently ring-cleaved by excision dioxygenases (Neidle et al., 1992). In many catabolic pathways the formation of such intermediates is carried out by two successive enzymatic steps namely dihydroxylation of the polyaromatic substrate to produce *cis*-diols followed by dehydrogenation (Harayama & Timmis, 1989). The ring hydroxylation is catalyzed by multi-component dioxygenases, while the dehydrogenation is catalyzed by *cis*-diol-dehydrogenases. In mammalian tissues the enzyme dihydro-diol dehydrogenase (DD, EC 1.3.1.20) exists in multiple forms (Hara et al., 1990; Higaki et al., 2002) and catalyses the $NADP^+$-linked oxidation of *trans*-dihydro-diols of aromatic hydrocarbons to the corresponding catechols (Penning et al., 1999). Studies on the metabolism of aromatic hydrocarbons by fungi are limited, nevertheless have been shown to posses the ability to metabolize aromatic compounds (Auret et al., 1971; Ferris et al., 1976) and the aryl oxidative enzymes of fungi appear to be similar to monooxygenases of hepatic microsomes (Cerniglia & Gibson, 1977; Ferris et al., 1976). Smith & Rosazza (1974) have also presented evidence that naphthalene is metabolized to 1-naphtol by six different genera of fungi.

In this work we analyze the cytosolic fraction of YR-1 strain by electrophoretic zymograms, methodology that there is not described in the literature for the $NADP^+$-dependent dihydrodiol dehydrogenase (DD) activities. We analyze all the activity bands corresponding to proteins with DD activity present in an enzymatic extract in only one lane of the electrophoretic gel. Our results show eleven different DD activity bands, five of them are constitutive, DD1-5, since they appears when the strain is growth on glucose, and the others six are induced by different compound added to the culture media as a sole carbon source. Some biochemical-enzyme characteristics as pH, optimal temperature, cofactor dependence, substrate specificity and the effect of cations, EDTA and pyrazole were investigated for DD activities when YR-1 strain was grown in naphthalene as sole carbon source.

2. Materials and methods

2.1 Organisms used and culture conditions

Mucor circinelloides strain YR-1 originally isolated from petroleum-contaminated soil in Salamanca, Guanajuato, Mexico was used as enzymatic source. A defined media containing

yeast-peptone-glucose-agar (YPGA) (Bartnicki-García & Nickerson, 1962) was used for strain maintenance, spore collection and mycelium growth. Aerobic mycelium growth was also carried out on salt minimal medium (Alvarado et al. 2002) added with 0.1% (w/v) peptone (sMMP). As a carbon source we added D-glucose (1% w/v) or glycerol (1.0% v/v) or ethanol (2.0% v/v) or *n*-decanol (1.0% v/v) or *n*-pentane (1.0% v/v) or *n*-decane, (1.0% v/v) or *n*-hexadecane (1.0% w/v) or naphthalene (0.5% w/v) or anthracene (0.5% v/v) or phenanthrene (0.5% w/v) or pyrene (0.5% w/v). Liquid cultures (600 ml) inoculated with spores at a final cell density of 5 x 10^5/ml were propagated in 2-l Erlenmeyer flasks and incubated in a reciprocating water bath at 28°C for 22 h at 125 rpm for all substrates except glucose that was incubated for 12 h at same other conditions.

2.2 Preparation of purified fractions

Mycelium of 22 h of incubation was harvested by filtration and exhaustively washed with cold sterile-distilled water; mycelial mass was suspended in 15 ml of 20 mM Tris-HCl pH 8.5 buffer containing 1 mM phenylmethanesulphonyl fluoride (previously dissolved in ethanol). Approximately 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 periods of 30 sec each under a CO_2 stream. The homogenate (crude extract) was centrifuged at 4,300*g* for 10 min in a J2-21 Beckman rotor in a Beckman JA-20 centrifuge to remove cell walls and unbroken cells, a 1 ml sample of the supernatant was saved. The rest of the supernatant (low speed supernatant) was centrifuged at 31,000*g* for 20 min in a 70Ti Beckman rotor in a Beckman L8-80 ultracentrifuge and samples of 1 ml of the supernatant was saved; the resulting pellet (mitochondrion rich sample) was resuspended in 2 ml of buffer and saved. The rest of the supernatant was high-speed centrifuged at 164,500*g* for 45 min in a 70Ti Beckman rotor at 4°C in a Beckman L8-80 ultracentrifuge; the supernatant (cytosolic fraction) was put aside, and the pellet, the mixed membrane fraction (MMF), was resuspended in 2 ml and saved. In all cases samples of different fractions were kept at -70 °C for further studies.

2.3 Gel electrophoresis

The slab gels were 1.5 mm-thick contained 6% (w/v) acrylamide/4% (w/v) bisacrilamide, loaded with the cytosolic fraction of each culture and run in the mini-gel system manufactured by Bio-Rad. The continuous buffer system described by Laemmli (1970) without SDS (native conditions) was used to run for 2.5 h at 80 V. The *Rm* values were calculated as the ratio of the distance migrated by the stained band divided by the distance migrated by tracking dye; standard deviation was calculated with Excel from three independent experiments and each experiment was made by triplicate on each substrate.

2.4 Enzymatic 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 Bergmeyer (1983). The enzymatic assays contained 25 mM Tris-HCl (pH 8.5), 2 mM NAD^+ or $NADP^+$, cell-free extract (100-200 µg protein), and 100 mM of the substrate (1R, 2S)-*cis*-1,2-di-hydro-1,2-naphthalene-diol. The reaction was started by dihydrodiol addition, and reduction of NAD^+ or $NADP^+$ 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 required reducing 1 μmol of NAD^+ or $NADP^+$ per minute at 25ºC. Specific dihydrodiol dehydrogenase (DD) activity was expressed as units per milligram of protein.

For DD activity in gels we developed an appropriate methodology because there is not any report in the literature about the detection of these enzymes by means of electrophoretic zymograms, so for we modified the method described for Nikolova & Ward (1991) for alcohol dehydrogenase. Briefly, after non-denaturing 6% (w/v) PAGE, described above, the activity was revealed as follows. The gel was submerged for 120 min in 4 ml of 0.5 M Tris-HCl buffer pH 8.5 containing 0.5 mg phenazine methosulphate (PMS), 7.5 mg *p*-nitro-blue tetrazolium (PNBT), 14.34 mg $NADP^+$ or NAD^+, 1 mM EDTA, 1 mM DTT and 100 mM of (1R, 2S)-*cis*-1, 2-dihydro-1, 2-naphthalene-diol as substrate. After incubating at 25 °C for 30 min (in dark) with gentle shaking at 80 rpm, the dihydrodiol dehydrogenases or ADH electro-morphs were observed as blue-dark bands.

When substrate specificity of DD was tested, different single alcohols were added to the mixture reaction at a final concentration of 100 mM. The following substrates were tested: *N*-decanol, *n*-hexadecanol, *n*-octadecanol, hexane-1,2,3,4,5,6-hexaol, benzyl alcohol, cholesterol, *cis*-naphthalene-diol, ethylene-glycol, poly-ethylene-glycol 3350, and sorbitol, were previously dissolved in dioxan and others were prepared in water: methanol, ethanol, propane-1-ol, propane-2-ol, butane-1-ol, pentane-1-ol, propane-1,2,3-triol and methyl propane-1-ol.

The pH, optimal temperature, substrate specificity, and effect of cations, EDTA and pyrazole were performed after a non-denaturing gel, 6% acrylamide, loading 300 μg of protein. The pH determination was performed from 3 to 9 with citrate buffer for 3 to 5, phosphate buffer for 5 to 7 and Tris/HCl buffer for 7 to 9. The temperature effect was tested in a range of 4 to 45 °C, using a freezer or metabolic bath at the desired temperature. The cation effect was tested using 1 mM of $CaCl_2$, $MgSO_4$, $ZnSO_4$ and $FeSO_4$, and for the EDTA, 1mM was also used. The assays were performed in the presence of *cis*-naphthalene-diol as substrate and $NADP^+$ as electron acceptor; the enzymatic activity was measured over a range of pH values in the forward reaction dihydrodiol → diol.

2.5 Miscellaneous

Protein concentration was measured according Lowry et al. (1951), using bovine serum albumin as standard. Phenylmethanesulphonyl fluoride and *cis*-naphthalene-diol were purchased from Sigma (St. Louis, MO, USA), the alcohol used as substrates were from J.T. Baker (Phillipsburg, NJ, USA). All reagents were analytical grade.

Densitometric analysis was performed in a Gene Genius Bio-Imaging System V. 6.05.01, SYNGENE, Synoptics Systems. Software used was Gene Tools V. 3.06.02, Syn. Ltd.

3. Results

3.1 Sub-cellular distribution of dihydrodiol dehydrogenase activity

The first approach was to know the sub-cellular distribution of the dihydrodiol dehydrogenase (DD) activity by means of a differential-centrifugation procedure and the spectrophotometer detection of the DD activity from *M. circinelloides* YR-1 grown in

different carbon sources, using a variation of the method described by Bergmeyer (1983). For this purpose we use the commercial substrate *cis*-naphthalene-diol. If the low speed supernatant is compared, the enzymatic activity was almost 8 times higher when naphthalene rather than glucose was the carbon source and $NADP^+$ was used as electron acceptor (Table 1).

	DD activity (x 10^{-2})					
	$NADP^+$			NAD^+		
Sample	Carbon source					
	Glucose	Ethanol	Naphthalene	Glucose	Ethanol	Naphthalene
4,300 x *g*						
Supernatant	42	4.0	270	131	12	39
31,000 x *g*						
Pellet	1.7	ND[a]	ND	23	ND	12
Supernatant	4.5	1.3	91	57	23	61
164,500 x *g*						
Pellet (MMF)	0.7	0.1	0.1	5.2	1.2	ND
Supernatant (Cytosol)	21	0.5	178	59	2.8	1.4

Table 1. $NADP^+$ or NAD^+-dependent dihydrodiol dehydrogenase activities present in sub-cellular fractions of *Mucor circinelloides* YR-1 grown on different carbon sources. Mycelial cells, grown in the indicated carbon sources, were broken (Braun) and fractions obtained by differential-centrifugation. DD activity of the different fractions was measured with *cis*-naphthalene-diol as substrate and $NADP^+$ or NAD^+ as electron acceptor. The values are the means of three independent experiments with triplicate determinations.
[a] ND, no detected.

This suggests that at least some of the detected activity could be inducible, and as can be seen, the major enzymatic activity is present in the soluble fractions. When NAD+ was used as electron acceptor, the activity found in the low speed supernatant when the fungus was grown in glucose as a carbon source is more than 3 times higher than the one present when naphthalene was used, and more than ten times higher if compared with the activity obtained with ethanol as a carbon source.

These results enhance the interest to investigate how many different activities will be revealed by electrophoretic zymograms in the cytosolic fraction of the fungus when it grown on different carbon sources.

3.2 Use of zymograms to reveal the presence of several dihydrodiol dehydrogenase activities in cytosolic fraction of *M. circinelloides* YR-1 grown on different carbon sources

Aerobically mycelium grown in different carbon sources (see Materials and Methods) was used to obtain the corresponding cytosolic fraction and each one was run on no-denaturing polyacrylamide gels and stained for $NADP^+$-dependent dihydrodiol dehydrogenase activity with *cis*-naphtalen-diol as substrate.

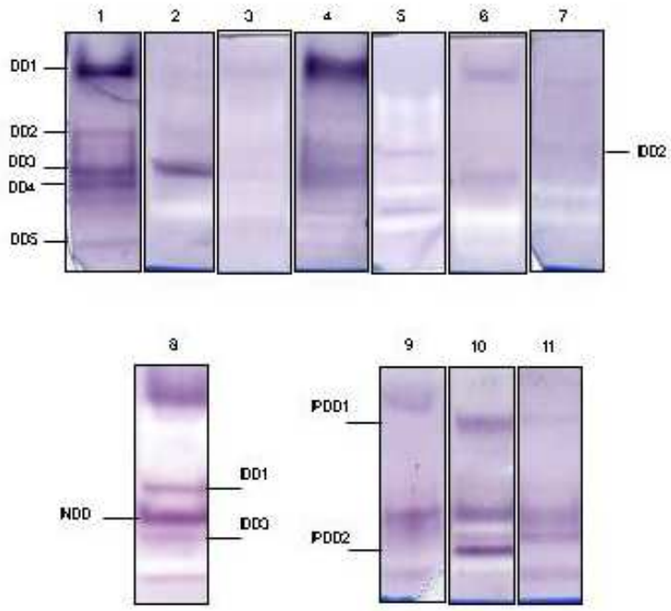


Fig. 1. Dihydrodiol dehydrogenase enzymatic activity present in cytosolic fraction of *M. circinelloides* YR-1 grown in different carbon sources. Mycelia in lane 1 was grown for 12 h and lanes 2-11 were grown for 22 h at 28ºC on sMMP medium added with the carbon source indicated. *Lanes*: *1,* D-glucose; *2*, glycerol; *3*, ethanol; *4*, *n*-decanol; *5*, *n*-pentane; *6, n*-decane; *7*, *n*-hexadecane; *8,* naphthalene; *9,* anthracene; *10,* phenanthrene; *11,* pyrene, at the concentrations described in Material and Methods. The extracts were electrophoresed and stained as described in Materials and Methods. In all cases 300 μg of protein were loaded in each lane. In this gel 100 mM *cis*-naphthalene-diol was the substrate and $NADP^+$ the electron acceptor. These results are representative gels and mycelia were grown up and run on the gels at least three times. The induction patterns were always reproducible.

Under the conditions tested, five bands were seen in glucose as carbon source (Fig. 1, lane 1) which were considered as constitutive dihydrodiol dehydrogenases and identified with a number (1-5) considering their decreasing *Rm* (DD1-5) (Table 2), and six inducible bands of activity were detected, depending of the carbon source in the culture media used for growth (Fig. 1; Table 3). One of the inducible bands (iDD1) was seen when *n*-decanol or *n*-pentane or *n*-hexadecane or naphthalene was the carbon source (Fig. 1, lanes 4, 5, 7 and 8 respectively, Table 3).

A second inducible $NADP^+$-dependent dihydrodiol dehydrogenase activity was seen when *n*-decanol or *n*-pentane was the carbon source (iDD2) (Fig. 1, lanes 4 and 5; Table 3). The third inducible enzymes (iNDD) was seen only when aromatic hydrocarbons were used as sole carbon source in the growth media (Fig. 1, lanes 8 to 11, Table 3). A fourth inducible naphthalene-diol dehydrogenase (iDD3) was induced by some of the alcohols, alkanes and aromatic polycyclic compounds tested (Fig. 1, lanes 3, 4, 6, 8, 10 and 11; Table 3). When phenanthrene was used as the carbon source, two new bands with different relative motilities were revealed, iPDD1 and iPDD2, (Fig. 1, lane 10; Table 3). The iPDD1 was also observed when pyrene was the carbon source (Fig. 1, lane 10 and 11; Table 3).

Carbon source	*Rm*[a] of DD constitutives				
	1	2	3	4	5
D-Glucose	0.22±0.04	0.4±0.03	0.62±0.01	0.69±0.02	0.90±0.01
Glycerol	-	-	-	-	-
Ethanol	-	-	-	-	-
n-Decanol	0.21±0.01	-	0.61±0.02	0.69±0.03	0.89±0.03
n-Pentane	-	-	-	0.7±0.03	-
n-Decane	0.21±0.01	-	-	-	-
n-Hexadecane	-	-	-	-	-
Naphthalene	0.21±0.01	-	-	-	0.89±0.006
Anthracene	0.22±0.006	-	-	-	0.89±0.02
Phenanthrene	-	-	-	-	0.89±0.01
Pyrene	-	-	-	-	0.89±0.01

Table 2. Constitutive $NADP^+$-dependent dihydrodiol dehydrogenase activities in cytosolic fraction of *Mucor circinelloides* YR-1 grown in different carbon sources.
[a]*Rm*; was calculated as described in Materials and Methods section as its standard deviation.

Carbon source	*Rm*[a] of DD inducibles					
	iDD1	iDD2	iDD3	iPDD1	iPDD2	iNDD
D-Glucose	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-
Ethanol	-	-	-	-	-	-
n-Decanol	0.55±0.002	0.66±0.005		-	-	-
n-Pentane	0.55±0.001	0.66±0.003	0.74±0.002	-	-	-
n-Decane	-	-	-	-	-	-
n-Hexadecane	-	-	0.73±0.010	-	-	0.66±0.005
Naphthalene	0.56±0.003	-	0.73±0.020	-	-	0.67±0.008
Anthracene	-	-	-	-	-	0.67±0.008
Phenanthrene	-	-	0.74±0.010	0.23±0.006	0.78±0.003	0.67±0.008
Pyrene	-	-	0.74±0.004	0.22±0.020	-	0.67±0.008

Table 3. Inducible $NADP^+$-dependent dihydrodiol dehydrogenase activities in cytosolic fraction of *Mucor circinelloides* YR-1 grown in different carbon sources.
[a]*Rm*; was calculated as described in Materials and Methods section as its standard deviation.

3.3 Bands intensity of dihydrodiol dehydrogenase activities in cytosolic fraction of *M. circinelloides* YR-1

With the comparing purpose the activity showed for the denominated iNDD enzyme that is induced when naphthalene was used as a carbon source was taking as a 100% and the others enzymes where referred to this value (Table 4).

In all cases *cis*-naphthalene-diol and $NADP^+$ were used in the enzymatic assay. When phenanthrene was used as a carbon source there are four different inducible enzymes, iDD3, iPDD1, iPDD2 and iNDD, being iPDD1 and iPDD2 best induced by this carbon source, and

the latest is the one that showed the highest induction value of all inducible enzymes (103.5%) (Table 4). The iPDD2 enzyme is the only one that it is induced by only one carbon source (Table 3). In the case of the iDD1, the highest induction value obtained was when naphthalene was used as a carbon source as iDD3 and iNDD enzymes (Table 4). The iDD2 showed its best induction value when n-pentane was used as a carbon source (Table 4). It is noticeable that glycerol, ethanol and *n*-decane do not induce any of the DD activities.

Carbon source	Band intensity[a] (relative units)					
	iDD1	iDD2	iDD3	iPDD1	iPDD2	iNDD
D-Glucose	0.0	0.0	0.0	0.0	0.0	0.0
Glycerol	0.0	0.0	0.0	0.0	0.0	0.0
Ethanol	0.0	0.0	0.0	0.0	0.0	0.0
n-Decanol	10.2	5.3	0.0	0.0	0.0	0.0
n-Pentane	8.7	18.2	14.6	0.0	0.0	0.0
n-Decane	0.0	0.0	0.0	0.0	0.0	0.0
n-Hexadecane	0.0	0.0	6.3	0.0	0.0	4.5
Naphthalene	56.8	0.0	16.3	0.0	0.0	100.0
Anthracene	0.0	0.0	0.0	0.0	0.0	90.5
Phenanthrene	0.0	0.0	8.1	82.9	103.5	84.5
Pyrene	0.0	0.0	10.2	8.0	0.0	38.4

Table 4. Relative inducibility of $NADP^+$-dependent dihydrodiol dehydrogenase activities in cytosolic fraction of *Mucor circinelloides* YR-1 grown in different carbon sources.
[a]Relative units were obtained by densitometry, using the value from iNDD as 100% when the fungus was growth on naphthalene.

3.4 Effect of ethanol as substrate and NAD^+ as electron acceptor on induced dihydrodiol dehydrogenase activities in cytosolic fraction of *M. circinelloides* YR-1 grown on different carbon sources

It is interesting to compare if the inducible DD enzymes are able to use NAD^+ as electron acceptor and/or ethanol as substrate because some dehydrogenases are able to use both of them. In the presence of NAD^+ as electron acceptor and *cis*-naphthalene-diol as substrate, there was not any staining in the region of either constitutive or inducible dihydrodiol dehydrogenase activities (Fig. 2A, lanes 1-5; Table 5). In the presence of ethanol as substrate and $NADP^+$ as electron acceptor, two ADH activity in cytosolic fraction from mycelium grown on glucose were revealed (Fig. 2B, lane 1) one with a *Rm* of 0.42±0.008 (denominated ADH1) and the other with a *Rm* of 0.84±0.003 (denominated ADH2). Under these conditions we also observed the inducible dihydrodiol dehydrogenase enzymes denominated DD3, suggesting that this enzyme also possesses an ADH activity $NADP^+$-dependent (Fig. 2B, lane 1). Also, under these assay conditions, two bands were observed when phenanthrene was used as a carbon source to growth the mycelia (Fig. 2B, lane 4). The bands correspond to the DD5 (*Rm* of 0.90±0.010) and a new ADH, denominated ADH3 with a *Rm* of 0.94±0.010 (Fig. 2B, lane 4).

When we used NAD^+ as electron acceptor and ethanol as substrate the denominated ADH1, ADH3 and DD5 activity bands were revealed when the cytosolic fraction from mycelium grown on glucose (Fig. 2C, lane 1).

As a control, a sample of the culture media lacking carbon source (Fig. 2A to C, lane 6) and an assay lacking substrate in the reaction mixture for the activity in zymograms (Fig. 2A to C, lane 7) did not showed any enzymatic activity.

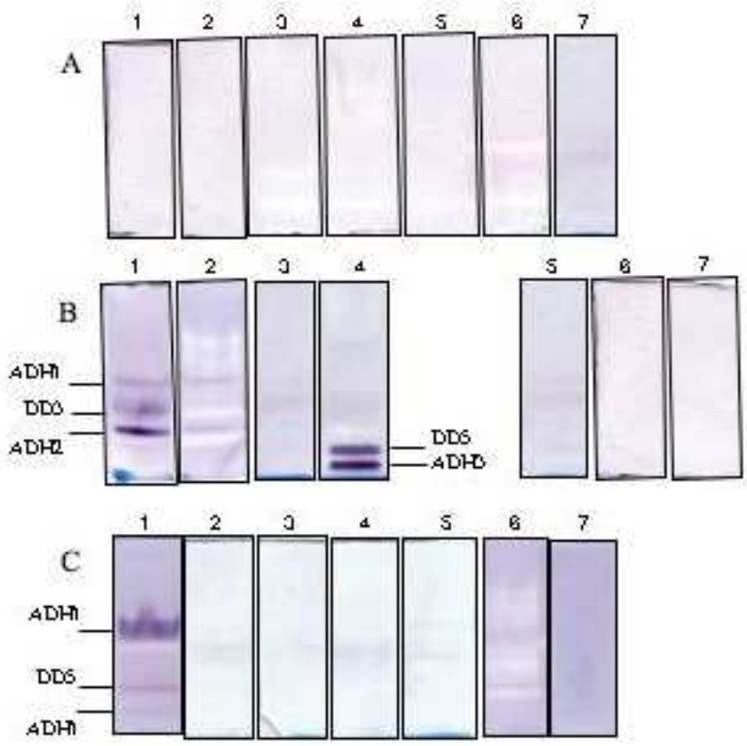


Fig. 2. Detection of dihydrodiol dehydrogenase activities in cytosolic fraction of *M. circinelloides* YR-1 by activity-stained gels. All mycelia were obtained after grown for 22 h at 28ºC on the carbon source indicated. A. *Lanes 1,* 1.0% glucose; *2,* 0.5% naphthalene; *3,* 0.5% anthracene; *4,* 0.5% phenanthrene; *5,* 0.5% pyrene; *6,* a sample of the culture media without carbon source; *7,* without substrate in the activity-reaction mixture. The reaction was performed with NAD^+ and 100 mM cis-naphthalene-diol as substrate, described in Materials and Methods section. B. identical samples as A, but the activity was developed with $NADP^+$ and 100 mM ethanol as substrate. C. identical samples as A, but the activity was developed with 100 mM ethanol as substrate. The amount of protein loaded per track was equalized to 300 μg. These results are representative gels and mycelia were grown up and run on the gels at least three times. The activity patterns were always reproducible.

In order to compare the observed activities we performed a densitometric analysis to the bands intensity and the Table 5 shows the obtained values under the condition where the activity have its highest value, taking the iNDD activity as a 100%. As show, the denominated DD1 enzymes, a constitute one, has the highest activity of all, in contrast the activity denominated iDD3 and DD5 measured with ethanol and NAD^+ are the lowest

(Table 5). It is interesting that only the denominated ADH 1 to 3 have activity with ethanol as a substrate, being the ADH3 the enzyme with the highest activity (Table 5). Surprisingly not a single one activity was revealed when *cis*-naphthalene-diol and NAD^+ were used as a substrate (Table 5).

3.5 General properties of NDD activities

DD activities were assayed only in crude cell-free extracts of aerobically-naphtahalene grown mycelia cells because at these moments, we were strongly interested in the NDD activity.

In all cases, the DD activities were tested in cell-free extracts of *M. circinelloides* YR-1.grown in 0.5% of naphthalene as sole carbon source for 22 h at 28 ºC. Cytosolic fraction was separated in a native electrophoresis and the amount of protein loaded per track equalized and was equivalent to 300 μg, $NADP^+$ as electron acceptor and 100 mM *cis*-naphthalene-diol as enzyme substrate were used to reveal the zymograms.

	Band intensity (relative units)[a]				
	cis-naphthalene-diol		Ethanol		
Enzyme	$NADP^+$	NAD^+	$NADP^+$	NAD^+	*Rm*[b]
DD 1	104.2	0.0	0.0	0.0	0.21±0.006
DD 2	1.2	0.0	0.0	0.0	0.40±0.010
DD 3	3.4	0.0	0.0	0.0	0.61±0.020
DD 4	3.2	0.0	0.0	0.0	0.69±0.030
DD 5	1.2	0.0	67.6	0.4	0.90±0.010
iDD1	56.8	0.0	0.0	0.0	0.56±0.010
iDD2	18.2	0.0	0.0	0.0	0.66±0.050
iDD3	16.3	0.0	3.6	0.4	0.73±0.010
iPDD1	82.9	0.0	0.0	0.0	0.23±0.006
iPDD2	103.5	0.0	0.0	0.0	0.78±0.003
iNDD	100.0	0.0	0.0	0.0	0.67±0.008
ADH1	0.0	0.0	1.2	3.1	0.42±0.008
ADH2	0.0	0.0	45.2	0.0	0.84±0.003
ADH3	0.0	0.0	74.8	0.0	0.94±0.010

Table 5. Activities of *cis*-naphthalene-diol and alcohol dehydrogenase of cytosolic fraction of *M. circinelloides* YR-1 grown in the best inducer for each one. Densitometric analysis was carried out as described in Materials and Methods. The enzymatic determination was on the gel with *cis*-naphthalene-diol or ethanol as the substrate and $NADP^+$ or NAD^+ as the electron acceptor.

[a] Relative units were obtained by densitometry, using the value from iNDD as 100% when naphthalene was the carbon source.

3.5.1 pH dependence

The Fig. 3 shows that the optima pH value for all five activities expressed with n-naphthalene as carbon source and $NADP^+$ and naphthalene-diol in the enzymatic reaction was 8.5. It is noticeable that only the iNDD show activity at pH 3 and little DD activities

were showed at pH values below 8.5. It is important to say that the background in the lane for activity revealed at pH 9, was darken because of pH.

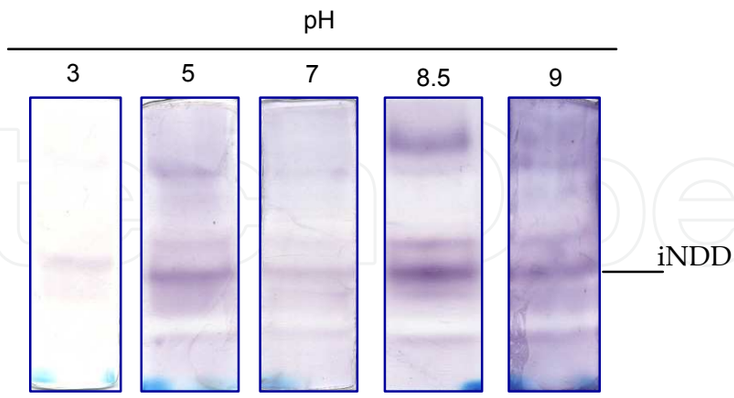


Fig. 3. Effect of pH on dihydrodiol dehydrogenase activities present in cytosolic fraction of *M. circinelloides* YR-1grown in naphthalene. Each track was cut and stained at the indicated pH value.

3.5.2 Temperature

The effect of the temperature on DD activities was tested on cytosolic fraction in a range of temperatures oscillating between 4 and 45 ºC. The optimum temperature was 37 ºC, notice that even at 45 ºC the activity band corresponding to the iNDD can be seen in the zymogram (Fig. 4). It is important to specify that the background in the lanes for activity revealed at 37 or 45 ºC were darker because of the incubation temperature.

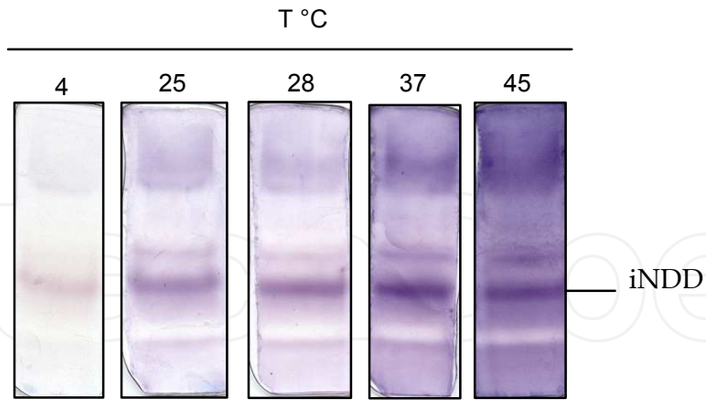


Fig. 4. Temperature effect on dihydrodiol dehydrogenase activity present in cytosolic fraction of *M. circinelloides* YR-1. Each track was cut and activity developed at the indicated temperature.

3.5.3 Requirement of different divalent ions

Different divalent ions were used to prove if some of them were required for DD activities. The Fig. 5 shows that only Ca^{2+} had an enhancing effect on DD activities meanwhile the other divalent metals tested and also EDTA were inhibitory Fe^{2+} > Zn^{2+} > EDTA> Mg^{2+}.

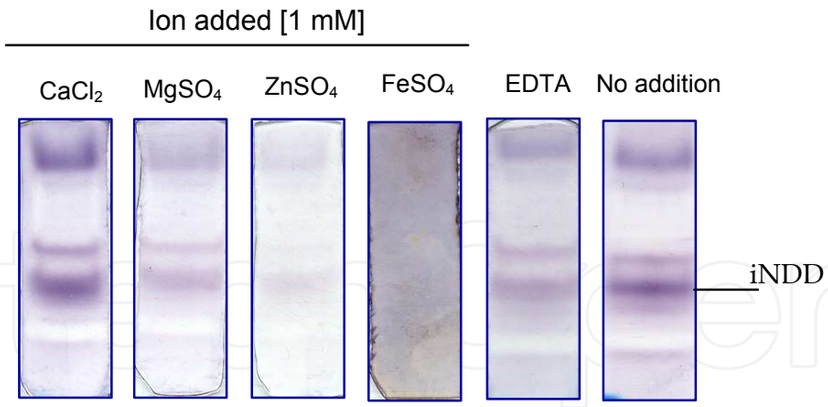


Fig. 5. Divalent ions effect on dihydrodiol dehydrogenase activity present in cytosolic fraction of *M. circinelloides* YR-1. Each track was cut and stained adding to the reaction mixture the divalent ion indicated at the concentrations described in Materials and Methods.

3.5.4 Pyrazole effect

Pyrazole is a well known ADH competitive inhibitor (Pereira et al., 1992) and this is the principal reason we decide to prove its effect on the different DD activities present in crude cell-free extracts obtained from *M. circinelloides* YR-1 mycelia grown in naphthalene as the sole carbon source. As can be seen in Fig. 6, pyrazole has a little inhibitory effect on the different DD's. In addition, iNDD showed a mild decrease in the level of its activity when measured by staining for activity in gels in presence of pyrazole (Fig. 6)

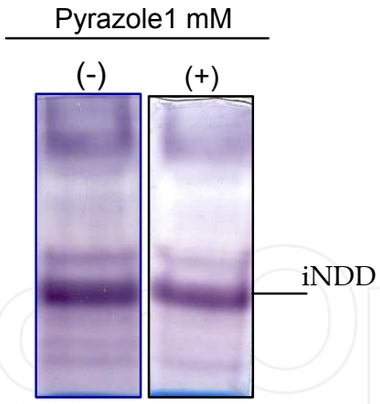


Fig. 6. Pyrazole effect on dihydrodiol dehydrogenase activity present in the cytosolic fraction of *M. circinelloides* YR-1 grown in naphthalene. Each track was cut and stained adding or not to the reaction mixture 1.0 mM pyrazole.

3.5.5 Substrate specificity of the different inducible and constitutive dihydrodiol dehydrogenase activities

To test the substrate specificity we chose naphthalene as the carbon source in the culture media since we can observe two constitutive band of activity (DD1 and DD5) and three inducible bands (iDD1, iDD3 and iNDD) (Fig. 1, Table 2 and 3). A variety of substrates were tested in the gel making the assay with $NADP^+$ as electron acceptor. The constitutive DD1

enzyme it is the one that shows in a majority of substrates, 14 out of 18, being ethanol, propane-1-ol, benzyl alcohol and sorbitol the substrates where the activity did not show, but when show its intensity is really low (Fig. 7). In contrast, the band with the highest intensity is the DD2 enzyme when propane-1,2,3-triol was the substrate (Fig. 7, lane 10), even when this enzyme cannot be seeing when cis-naphtalene-diol is used as a substrate (Fig. 7, lane 18). As this DD2 enzyme there are others enzymes that did not show with cis-naphthalene-diol and can be seen with others substrates, as it is DD3 and iPDD2, that shows with three and four different substrates respectively (as an example see Fig. 7, lane 10). It is surprising that the iPDD2 enzyme that only showed when phenanthrene was the carbon source to growth the fungus, it is present here depending on the substrate used, propane-1,2,3-triol, 2-methyl propane-1-ol, ethylene-glycol and benzyl alcohol (Fig. 7, lanes 10, 11, 13 and 15).

In the particular case of NDD1, it was present only when naphthalene was the carbon source (Fig. 7) but it was absent in all other aromatic hydrocarbons used as carbon source (Fig. 1) and this enzyme practically only uses *cis*-naphthalene-diol as substrate (Fig. 7, lane 18). In the case of iDD3 and DD5, both present broad substrate specificity, showing a special preference for short-chain alcohols, including 1-decanol (Fig. 7). There are five bands that show with different substrates, but its intensity is really low and they were not taken in account (Fig. 7).

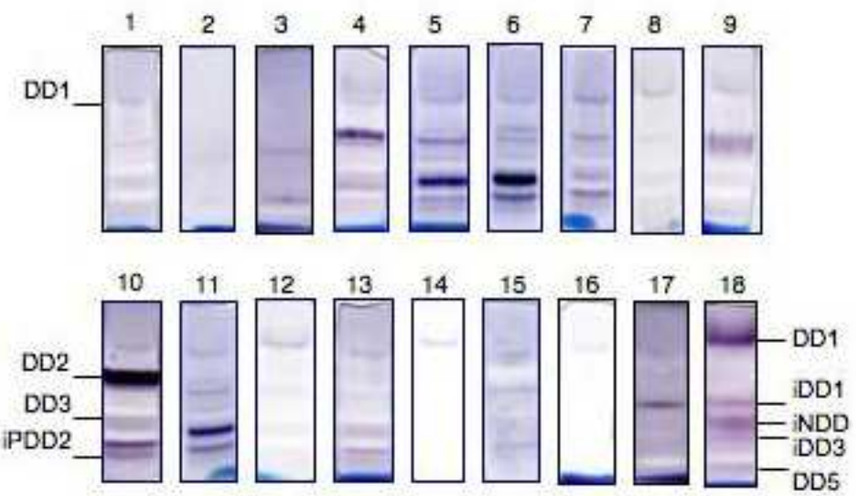


Fig. 7. Substrate specificity of constitutive and inducible DD activities of cytosolic fraction of *M. circinelloides* YR-1 grown on naphthalene, revealed by activity-stained gels. A variety of substrates were used with $NADP^+$ as electron acceptor. All substrates were tested at 100 mM of final concentration. Lane *1* methanol*; 2* ethanol*; 3* propane-1-ol*; 4* propane-2-ol*; 5* butane-1-ol*; 6* pentane-1-ol*; 7* 1-decanol*; 8* 1-hexadecanol*; 9* hexane-1,2,3,4,5,6- hexaol*; 10* propane-1,2,3-triol*; 11* 2-methyl propane-1-ol*; 12* 1-octadecanol*; 13* ethylene-glycol*; 14* poly-ethylene-glycol 3350*; 15* benzyl alcohol*; 16* cholesterol*; 17* sorbitol*; 18 cis*-naphthalen-diol.

4. Discussion and conclusion

Many studies have been done on NAD^+-dependent *cis*-dihydrodiol dehydrogenases (DD) in bacteria (Jouanneau & Meyer, 2006; Van Herwijnen et al., 2003). In the case of $NADP^+$-

dependent *trans*-dihydrodiol dehydrogenases, almost all investigations have been done in mammalian tissues (Carbone et al., 2008; Chang et al, 2009; Chen et al., 2008) but only a few reports about these important enzymes have been done in fungi (Bezalel et al., 1997; Hammel, 1995; Sutherland et al., 1993) particularly in *Phanerochaete chrisosporium* (Bogan & Lamar, 1996; Muheim et al., 1991). At date, there is no any report about the detection of dihydrodiol dehydrogenase activities by means of electrophoretic zymograms in any organism. This methodology represents an interesting approach because in this way it is possible to detect, study and compare the different isoenzymes present in the cell-free extracts of the organism used as enzymatic source. In our own work, YR-1 strain possesses extraordinary metabolic machinery that premises it to survive in a very dangerous place how is a petroleum-contaminated soil.

The results about the localization of DD activities in a differential centrifugation procedure from YR-1 grown in different carbon sources (Table 1), revealed that the activity measured with *cis*-naphthalene-diol as substrate and $NADP^+$ as electron acceptor was only present in the supernatant fractions of each centrifugation speed, suggesting that all DD activity observed must be a soluble enzyme. At date, we cannot discard the possibility that the DD activity could be located in the lumen of some kind of microsomal bodies, because of the drastic ballistic treatment used to homogenize the cells. Actually, we are conducting different experiments employing density gradients and electron transmission microscopy to resolve this question.

Complementary analysis of DD activities by electrophoretic zymograms led us to detect eleven different activities and all of them were $NADP^+$-dependent (Fig. 2) this represents the first report about the detection of DD activities by electrophoretic zymograms, a non-denaturing gel electrophoresis stained with a colored product of the enzymatic reaction.

Of the eleven bands detected, we described five different constitutive DD activities, DD 1-5, since them were observed when D-glucose was the carbon source and only DD-2 was solely induced by this sugar, since the others are induced at least for another carbon source. When *n*-decanol was used as a carbon source, we observed four out of five of the constitutive bands, lacking only the DD-2 band. Its noticeable that only when glycerol, ethanol, n-pentane and n-hexadecane were the carbon source to grow the fungus, not a single constitutive band was observed, may be due to the fact that these compounds only can be metabolized specifically by the induced enzymes. In glucose grown mycelium, all inducible dihydrodiol dehydrogenase activities were absent suggesting that they could be subject to carbon-catabolite repression (Fig. 2).

Surprisingly all the activities described here as DD are able to use *cis*-naphthalene-diol, since this substrate has been describes as bacterial specific (Cerniglia & Gibson 1977). The substrate reported for eukaryotic cells is the *trans*-naphthalene-diol (Cerniglia 1977).

Phenanthrene was the best inducer since when used as a carbon source four out of six inducible bands were observed, *n*-decanol and naphthalene were the second best inducers since each one led the induction of three different enzymes, sharing the bands denominated iDD1 and iDD2. Also in the case of the inducible enzymes glycerol, n-pentane and n-hexadecane were unable to induce any activity. The specific induction of an activity must be due to substrate specificity.

We have shown that on naphthalene, anthracene, phenanthrene or pyrene used as sole carbon source, there exist three different inducible $NADP^+$-dependent dihydrodiol dehydrogenase activities. One of them iNDD, was the only isoenzyme inducible by all aromatic hydrocarbons which presumably is involved in the aromatic hydrocarbon biodegradation pathway in YR-1 strain (Fig. 1). In particular iNDD was capable to use only *cis*-naphthalene-diol as substrate (Fig. 7) suggesting that this enzyme is specific part of the metabolic pathway of the naphthalene; all other activity bands are ADH substrate unspecific

It is interesting that iPDD2 has broad substrate specificity when $NADP^+$ was the electron acceptor, suggesting that it could be one of the different dehydrogenases belonging to the microsomal system of alcohol (ethanol) oxidation [MEOS (Krauzova et al., 1985)]. No one of all inducible DD's activities showed to be able to use NAD^+ as electron acceptor.

For iNDD, naphthalene was the best inducer and pyrene the worst. In the case of both iPDD1 and 2, phenanthrene was the only inducer of these enzymes, however pyrene shows a very low inducer effect on iPDD1 (Table 3). The finding of two inducible (iNDD and iPDD1) and one constitutive (DD1) enzymes that uses specifically a dihydrodiol as substrate is in agreement with the number of three possible DD's of predicted function, reported in database of *Mucor circinelloides* (Torres-Martínez et al., 2009).

With regard to the constitutive dihydrodiol dehydrogenase activities present in YR-1 strain, four of them use only *cis*-naphthalene-diol as substrate: DD1, 2, 3 and 7; DD2, use both *cis*-naphthalene-diol and with high efficiency propane-1,2,3-triol, indicating that it can be the glycerol dehydrogenase-1 (iGlcDH1 inducible by 1-decanol) described previously by ourselves in YR-1 (Camacho et al., 2010).

Our above-mentioned findings with *M. circinelloides* YR-1 dihydrodiol dehydrogenase activities are indicative of developmental regulation of the different DD's enzymes; this interpretation is supported by following observations: in zymograms for DD's activities when YR-1 was grown in different carbon sources is showed a differential pattern of the activity bands depending of the carbon source used in the culture media. The present results suggest the existence of eleven enzymes with dihydrodiol dehydrogenase activity. Particularly important the DD1 that could be the constitutive DD, and iNDD iPDD1, that could be part of the aromatic hydrocarbon biodegradation pathway in YR-1 strain for naphthalene or the others aromatic hydrocarbon, respectively. Future genomic analysis after isolation of the respective genes should prove the existence of one gene for each constitutive or inducible activity in agreement with the *M. circinelloides* data base prediction. The details of the possible interaction between alcohols or hydrocarbons metabolism remain to be determined.

5. Acknowledgments

Support for this research by Universidad de Guanajuato (México), is gratefully acknowledged.

6. References

Alvarado, C. Y., Gutiérrez-Corona, F. & Zazueta-Sandoval, R. (2002). Presence and physiologic regulation of alcohol oxydase activity in an indigenous fungus isolated from petroleum-contaminated soils. *Applied Biochemistry and Biotechnology*. Vol. 98-100, No. 1-9 (March 2002), pp. 243-255. ISSN: 0273-2289.

Atlas, R. M. (1995). Bioremediation. *Chemical and Engineering News*. Vol. 73, No. 14 (April 1995), pp. 32-42. ISSN 0009-2347. DOI: 10.1021/cen-v073n014.p032

Auret, B. J., Boyd, D. R., Robinson, P. M. & Watson, C.G. (1971). The NIH Shift During the Hydroxylation of Aromatic Substrates by Fungi. *Journal of the Chemical Society D: Chemical Communications* No. 24, (December 1971), pp. 1585-1587. ISSN 1364-548X.

Bartnicki-García, S. & Nickerson, W. J. (1962). Nutrition, Growth and Morphogenesis of *Mucor rouxii*. *Journal of Bacteriology*. Vol. 84, No. 4 (October, 1962), pp. 841-858. ISSN 0021-9193.

Bergmeyer, H. U. (1983). Alkoholdehydrogenase. *In*: Bergmeyer, H.U. (ed) *Methods of enzymatic analysis*, Verlag Chemie, Weinheim, Vol. 11, 3rd edn. pp-139-145.

Bezalel, L., Hadar, Y. & Cerniglia, C.E. (1997). Enzymatic Mechanisms Involved in Phenanthrene Degradation by the White-Rot Fungus *Pleurotus ostreatus*. *Applied and Environmental Microbiology*. Vol. 63, No. 7, (July 1997) pp. 2495-2501. ISSN 0099-2240.

Bogan, B. W. & Lamar, R. T. (1996). Polycyclic Aromatic Hydrocarbon-Degrading Capabilities of *Phanerochaete laevis* HHB-1625 and Its Extracellular Ligninolytic enzymes. *Applied and Environmental Microbiol*ogy. Vol. 62, No. 5, (May 1996), pp. 1597-1603. ISSN 0099-2240.

Camacho, M. R. L., Durón, C. A. & Zazueta-Sandoval, R. (2010). Analysis of Glycerol Dehydrogenase Activities Present in *Mucor circinelloides* YR-1. *Antonie Van Leeuwenhoek*. Vol. 98, No. 4, (November 2010), pp. 437-445, ISSN 0003-6072.

Carbone, V., Hara, A. & El-Kabbani, O. (2008). Structural and Functional Features of Dimeric Dihydrodiol Dehydrogenases. *Cellular and Molecular Life Science*. Vol. 65, No. 10, (May 2008), pp. 1464-1474, ISSN 1420-682X.

Cerniglia, C. E. (1997). Fungal Metabolism of Polycyclic Aromatic Hydrocarbons: Past, Present and Future Applications in Bioremediation. *Journal of Industrial Microbiology and Biotechnology*. Vol. 19, No. 5-6 (November 1997), pp. 324-333. ISSN 1367-5435.

Cerniglia, C. E. & Gibson, D. T. (1977). Metabolism of Naphthalene by *Cunninghamella elegans*. *Applied and Environmental Microbiology*. Vol. 34, No. 4, (October 1977), pp. 363-370. ISSN 0099-2240.

Chang, C. H., Chen, L. Y., Chan, P. C., Yeh, T. K., Kuo, J. S., Ko, J. C. & Fang, Y. H. (2009). Overexpression of Dihydrodiol Dehydrogenase as a Prognostic Marker in Resected Gastric Cancer Patients. *Digestive Diseases and Sciences*. Vol. 54, No. 2 (February 2009), pp. 342-347. ISSN 0163-2116.

Chen, J., Adikari, M., Pallai, R., Parckh, K. H. & Simpkins, H. (2008). Dihydrodiol Dehydrogenases Regulate the Generation of Reactive Oxygen Species and the Development of Cisplatin Resistance in Human Ovarian Carcinoma Cells. *Cancer Chemotherapy and Pharmacology*. Vol. 61, No. 6 (May 2008), pp. 979-987. ISSN 0344-5704.

Cook, F. D., Jobson, A., Phillippe, R. & Westlake, D. W. S. (1974). Biodegradability and crude oil composition. *Canadian Journal of Microbiology*. Vol. 20, No. 7 (July 1974), pp. 915-928. ISSN 0008-4166.

Durón-Castellanos, A., Zazueta-Novoa, V., Silva-Jiménez, H., Alvarado-Caudillo, Y., Peña Cabrera, E. & Zazueta-Sandoval, R. (2005). Detection of NAD^+-Dependent Alcohol Dehydrogenase Activities in YR-1 Strain of *Mucor circinelloides,* a Potential Bioremediator of Petroleum-Contaminated Soils. *Applied Biochemistry and Biotechnology*. Vol. 121-124, No. 1-3 (March 2005), pp. 279-288. ISSN 0273-2289.

Ferris, J. P., MacDonald, L. H., Patrie, M. A. & Martin, M. A. (1976). Aryl Hydrocarbon Hydroxylase Activity in the Fungus *Cunninghamella bainieri*: Evidence for the Presence of Cytochrome P-450. *Archives of Biochemistry and Biophysics*. Vol. 175, No. 2 (August 1976), pp. 443-452. ISSN 0003-9861.

Goto, S. & Takami, H. (1986). Classification of Ascoideaceous Yeasts Based on the Electrophoretic Comparison of Enzymes and Coenzymes Q Systems. *The Journal of General and Applied Microbiology*, Vol. 32, No. 4 (June 1986), pp. 271-282. ISSN 1349-8037.

Hammel, K. E. (1995). Mechanisms for Polycyclic Aromatic Hydrocarbon Degradation by Ligninolytic Fungi. *Environmental Health Perspectives*, Vol. 103. Suppl. 5 (June 1995), pp. 41-43.

Hara, A., Taniguchi, H., Nakayama, T. & Sawada, H. (1990). Purification and Properties of Multiple Forms of Dihydrodiol Dehydrogenases from Human Liver. *Journal of Biochemistry*. Vol. 108, No. 2 (August 1990), pp. 250-254. ISSN 0021-924X.

Harayama, S. & Timmis, K. N. (1989). *In: Genetics of bacterial diversity*. Hopwood, D.A., and Chater K.E.. eds. Academic Press, pp. 151-174. New York, USA.

Higaki, Y., Kamiya, T., Usami, N., Shintani, S., Shiraishi, H., Ishikura, S., Yamamoto, I. & Hara, A. (2002). Molecular Characterization of Two Monkey Dihydrodiol Dehydrogenases. *Drug Metabolism and Pharmacokinetics*. Vol. 17, No. 4 (September 2002), pp. 348-356, ISSN 1347-4367.

Hunt, J. M. (1979). *Petroleum geochemistryand geology.* W. M. Freeman, San Francisco, CA. pp. 221. ISBN 0 7167 1005 6.

Hyötyläinen, T. & Olkari, A. (1999). The Toxicity and Concentrations of PAHs in Creosote-Contaminated Lake Sediment. *Chemosphere.* Vol. 38, No. 5 (February 1999), pp. 1135-1144. ISSN 0045-6535.

Jeng, R. S., Shiyuan, Y. & Wayman M. (1987). Isoenzyme and Protein Patterns of Pentose-Fermenting Yeasts. *Canadian Journal of Microbiology*. Vol. 33, No. 11 (November 1987), pp. 1017-1023. ISSN 0008-4166.

Jouanneau, Y. & Meyer, C. (2006). Purification and Characterization of an Arene *Cis*-Dihydrodiol Dehydrogenase Endowed with Broad Substrate Specificity Toward Polycyclic Hydrocarbon Hydrodiols. *Applied and Environmental Microbiology.* Vol. 72, No. 7 (July 2006), pp. 426-434. ISSN 0099-2240.

Krauzova, V. I., Il'chenko, A. P., Sharyshev, A. A. & Lozinov, A. B. (1985). Possible Pathways of the Oxidation of Higher Alcohols by Membrane Fractions of Yeasts Cultured on Hexadecane and Hexadecanol. *Biochemistry.* Vol. 50, No. 5 (May 1985), pp. 609-615. ISSN 0006-2960.

Laemmli, U. K. (1970). Cleavage of Structural Proteins During the Assembly of Head of Bacteriophage T6. *Nature*. Vol. 227, No. 5259 (August 1970), pp. 680-685. ISSN 0028-0836.

Lim, W. J., Park, S. R., Cho, S. J. Kim, M. K., Ryu, S. K., Hong, S. Y., Seo, W. T., Kim, H. & Yun, H. D. (2001). Cloning and characterization of an intracellular isoamylase gene from *Pectobcterium chrysantemi* PY35. *Biochemical and Biophysical Chemical Communications.* Vol. 287, No. 2 (September 2001), pp.348-354. ISSN 0006-921X.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein Measurement with the Folin Phenol Reagent. *Journal of Biological Chemistry*, Vol. 193, No. 1 (November 1951), pp. 265-275. ISSN 0021-9258.

Muheim, A., Waldner, R., Sanglard, D., Reiser, J., Schoemaker, H. E. & Leisola M. S. (1991). Purification and Properties of an Aryl-Alcohol Dehydrogenase from the White-Rot Fungus *Phanerochaete chrysosporium*. *European Journal of Biochemistry*. Vol. 195, No. 2 (January 1991), pp. 369-375. ISSN 0014-2956.

Neidle, E., Hartnett, C., Ornston, L. N., Bairoch, A., Rekik, M. & Harayama, S. (1992). *Cis*-Diol Dehydrogenases Encoded by the TOL pWWO Plasmid *xylL* Gene and the *Acinetobacter calcoaceticus* Chromosomal *benD* Gene Are Members of the Short –

Chain Alcohol Dehydrogenase Superfamily. *European Journal of Biochemistry*. Vol. 204, No. 1, (February 1992), pp. 113-120. ISSN 0014-2956.

Nikolova, P. & Ward, O. P. (1991). Production of L-phenylacetyl Carbinol by Biotransformation: Product and By-Product Formation and Activities of the Key Enzymes in Wild Type and ADH Isoenzyme Mutants of *Saccharomyces cerevisiae. Biotechnology and Bioengineering*. Vol. 38, No. 5 (August 1991), pp. 493-498. ISSN 1097-0290.

Okwumabua, O., Persaud, J. S., & Reddy, P. G. (2001). Cloning and characterization of the gene encoding the glutamato dehydrogenase of *Streptococcus suis* serotype 2. *Clinical and Diagnostic Laboratory Immunology.* Vol. 8, No. 2 (March 2001), pp. 251-257. ISSN 556-6811.

Penning, T. M., Burczynski, M. E., Hung, C. F., McCoull, K. D. Palackal, N. T. & Tsuruda, L. S. (1999). Dihydrodiol dehydrogenases and polycyclic aromatic hydricarbon activation: Generation of reactive and redox *o*-quinones. *Chemical Research in Toxicology.* Vol. 12, No. 1 (December 1998), pp. 1-18. ISSN 0893-228X.

Pereira, E. F., Aracava, Y., Aronstam, R. S., Barreiro, E. J. & Alburquerque, E. S. (1992). Pyrazole, an Alcohol Dehydrogenase Inhibitor, Has a Dual Effects on N-methyl-D-asp. *Journal of Pharmacology and Experimental Theory*. Vol. 261, No.1 (April 1991), pp. 331-340. ISSN 0022-365.

Pfeiffeer-Guglielmi, B., Broer, S., Broer, A. & Hamprecht, B. (2000). Isoenzyme pattern of glycogen phosphorylase in the rat nervous system and rat astroglia-rich primary cultures: electrophoretic and polymerase chain reaction studies. *Neurochemical Research*, Vol. 25, No. 11 (November 2000), pp. 1485-1491. ISSN 0364-3190.

Silva, J. H., Zazueta-Novoa, V., Durón, C. A., Rodríguez, R. C., Leal-Morales, C. A. & Zazueta-Sandoval, R. (2009). Intracellular Distribution of Fatty Alcohol Oxidase Activity in *Mucor circinelloides* YR-1 Isolated from Petroleum Contaminated Soils. *Antonie Van Leeuwenhoewk*. Vol. 96, No. 4 (November 2009), pp. .527-535. ISSN 0003-6072.

Smith, R. V. & Rosazza, J. P. (1974). Microbial Models of Mammalian Metabolism. Aromatic Hydroxylation. *Archives of Biochemistry and Biophysics*. Vol. 161, No. 2 (April 1974), pp. 551-558. ISSN 003-9861

Sutherland, J. B., Fu, P. P., Yang, S. K., Von Tungeln, L. S., Casillas, R. P., Crow, S. A. & Cerniglia, C. E. (1993). Enantiomeric Composition of the *Trans*-Dihydrodiols Produced by Phenanthrene by Fungi. *Applied and Environmental Microbiology.* Vol. 59, No. 7 (July 1993), pp. 2145-2149. ISSN 0099-2240.

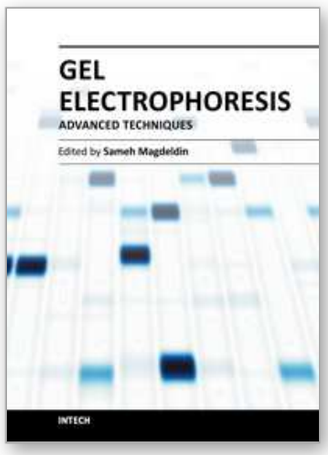
Torres-Martínez, S., Garre, V., Corrochano, L., Eslava, A. P., Baker, S.E. & others. (2009). Project CBS277.49, v1.0 http//genome.jgi-PSF.org.

Van Herwijnen, R., Springael, D., Slot, P., Govers A. J. H. & Parsons, R. J. (2003). Degradation of Anthracene by *Mycobacterium* sp. Strain LB501T Proceeds Via a Novel Pathway Throught o-Phtalic Acid. *Applied and Environmental Microbiology*. Vol. 69, No. 1 (January 2003), pp. 186-190. ISSN 0099-2240.

Yamazaki, M. & Komagata, K. (1983). An Electrophoretic Comparison of Enzymes of Ballistosporogenous Yeasts. *The Journal of General Applied Microbiolgy*. Vol. 29, No. 2 (February 1983), pp. 115-143. ISSN 0022-1260.

Zazueta-Sandoval, R., Durón, C. A. & Silva, J. H. (2008). Peroxidases in YR-1 strain of *Mucor circinelloides* a Potential Bioremediator of Petroleum-Contaminated Soils. *Annals of Microbiology*. Vol. 58, No. 3 (September 2008), pp. 421-426. ISSN 1590-4261.

Zazueta-Sandoval, R., Zazueta-Novoa, V., Silva-Jiménez, H. & Ortíz, R. C. (2003). A Different Method of Measuring and Detecting Mono and Di-Oxygenase Activities: Key Enzymes in Hidrocarbon Biodegradation. *Applied Biochememistry and Biotechnology*. Vol. 108, No. 1-3 (March 2003), pp. 725-736. ISSN 0273-2289.



Gel Electrophoresis - Advanced Techniques

Edited by Dr. Sameh Magdeldin

ISBN 978-953-51-0457-5
Hard cover, 500 pages
Publisher InTech
Published online 04, April, 2012
Published in print edition April, 2012

As a basic concept, gel electrophoresis is a biotechnology technique in which macromolecules such as DNA, RNA or protein are fractionated according to their physical properties such as molecular weight or charge. These molecules are forced through a porous gel matrix under electric field enabling uncounted applications and uses. Delivered between your hands, a second book of this Gel electrophoresis series (Gel Electrophoresis- Advanced Techniques) covers a part, but not all, applications of this versatile technique in both medical and life science fields. We try to keep the contents of the book crisp and comprehensive, and hope that it will receive overwhelming interest and deliver benefits and valuable information to the readers.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Reyna Lucero Camacho Morales, Vanesa Zazueta-Novoa, Carlos A. Leal-Morales, Alberto Flores Martínez, Patricia Ponce Noyola and Roberto Zazueta-Sandoval (2012). Polyacrylamide Gel Electrophoresis an Important Tool for the Detection and Analysis of Enzymatic Activities by Electrophoretic Zymograms, Gel Electrophoresis - Advanced Techniques, Dr. Sameh Magdeldin (Ed.), ISBN: 978-953-51-0457-5, InTech, Available from: http://www.intechopen.com/books/gel-electrophoresis-advanced-techniques/polyacrylamide-gel-electrophoresis-an-important-tool-for-the-detection-and-analysis-of-enzymatic-act

INTECH
open science | open minds

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

IntechOpen