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Effects of Carbon Source on Expression of Alcohol Oxidase Activity and on Morphologic Pattern of YR-1 Strain, a Filamentous Fungus Isolated from Petroleum-Contaminated Soils

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Abstract

Soluble alcohol oxidase (AO) activity was detected in the supernatant fraction of a high-speed centrifugation procedure after ballistic cellular homogenization to break the mycelium from a filamentous fungus strain named YR-1, isolated from petroleum-contaminated soils. AO activity from aerobically grown mycelium was detected in growth media containing different carbon sources, including alcohols and hydrocarbons but not in glucose. In previous work, zymogram analysis conducted with crude extracts from aerobic mycelium of YR-1 strain indicated the existence of two AO enzymes originally named AO-1 and AO-2. In the present study, we were able to separate the AO-1 band into two bands depending on culture conditions, carbon source, and polyacrylamide gel electrophoresis (PAGE) separation conditions; the enzyme activity pattern in zymograms from cell-free extracts exhibited three different bands after native PAGE. New nomenclature was used for upper bands AO-1 and AO-2 and lower band AO-3, respectively. The expression of AO activity was studied in the absence of glucose in the culture media and in the presence of hydrocarbons or petroleum as sole carbon source, suggesting that AO expression could be subjected to two regulatory possibilities: carbon catabolite regulation by glucose and induction by hydrocarbons. The possibility of catabolic inhibition of AO by glucose in the active enzyme was also tested, and the results confirm that this kind of regulatory mechanism is not present in AO activity.

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Index Entries: Alcohol oxidase; filamentous fungi; hydrocarbon biodegradation; petroleum contamination; YR-1 strain.

Introduction

Hydrocarbon represents an enormous energy resource without which our actual modern lifestyle would be impossible. Principally exploited as fossilized fuels, hydrocarbons must be considered a finite resource although methane and lignin are certainly renewable. As well as being the predominant energy source in most countries, hydrocarbons are also an important feedstock for the chemical industry. Their potential impact in biotechnology processes is enormous. Unfortunately, hydrocarbon compounds are major environmental pollutants, as a result of improper disposal processes or spills of petroleum or petroleum-derived products. These facts and the continuous increase in the number of toxic compounds generated by the oil industry have emphasized the importance of the development of effective processes to eliminate these waste products *(1)*. In nature, there exist many types of microorganisms that are useful in the biodegradation processes of these hazardous materials. Because natural biodegradation rates of this kind of contaminant, principally in soils, are very low and limited by environmental factors and by adaptation difficulties of microbial populations, it is important to develop methods to increase the bioavailability of contaminants and, finally, 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, only several microorganisms are capable of metabolizing the hydrocarbonated chains *(2)*. The first step in aliphatic-hydrocarbon biodegradation is catalyzed by an oxygenase named cytochrome P-450, followed by the action of an alcohol oxidase (AO) *(3)*. This enzyme is capable of using as substrates a wide range of xenobiotic compounds, and by means of many types of chemical transformations, this leads to the production of alcohols; P-450 can be found in microorganisms as well as in plants and animals *(4)*. The oxidation of alcohols to the corresponding aldehyde is catalyzed by AO; aldehyde is in turn converted to the corresponding carboxylic acid *(5)*. The reactions catalyzed by Cyt-p450 and AO are special points 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 the present work, we continue the studies of AO activity in the YR-1 strain. We describe some differences in morphologic development as a result of the presence of glucose or hydrocarbons present in culture media. Regarding AO activity, the results suggest the possibility that AO expression could be regulated by two mechanisms—catabolite repression by glucose and induction by hydrocarbons—and discard the inhibition of AO activity by glucose as a regulatory mechanism.

Materials and Methods

Chemical and Reagents

Phenylmethylsulfonyl fluoride (PMSF) and yeast AO 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 *(7)* using bovine serum albumin as standard.

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, México). The primary and secondary selections were achieved using minimal medium with the addition of 1% methanol or 1% hexadecane, respectively. The isolates were named YR, and the particular strain used was named YR-1; in all cases colonial and microscopic morphologies were established as criteria for the assessment of the isolated strains as filamentous fungi. As a wild-type AO-proficient (AO^+) organism, we used strain R-25 of *Hansenula polymorpha (8)*. Yeast-peptone-glucose (YPG) complete medium *(9)*, and salts minimal medium with the addition of 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 previously *(9)*. 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 different periods of time (*see* Fig. 3). To obtain aerobic mycelium, spores were inoculated in YPG medium, and in sMMP supplemented with glucose (0.1%), decane (1.0%), or hexadecane (1.0%), and the cultures were incubated aerobically *(9)*.

Preparation of Cell-Free Extracts

Mycelial cells were processed and broken as described by Torres-Guzmán et al. *(10)* with some modifications. Briefly, mycelial cells were washed and suspended in buffer (20 m*M* Tris-HCl [pH 8.5] containing 1 m*M* PMSF). A volume of about 20 mL of cells was mixed with an equal volume of glass beads (0.45–0.50 mm in diameter) and disrupted in a Braun Model MSK cell homogenizer (Braun, Melsungen, Germany) through four periods of 30 s each one 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 enzyme determinations.

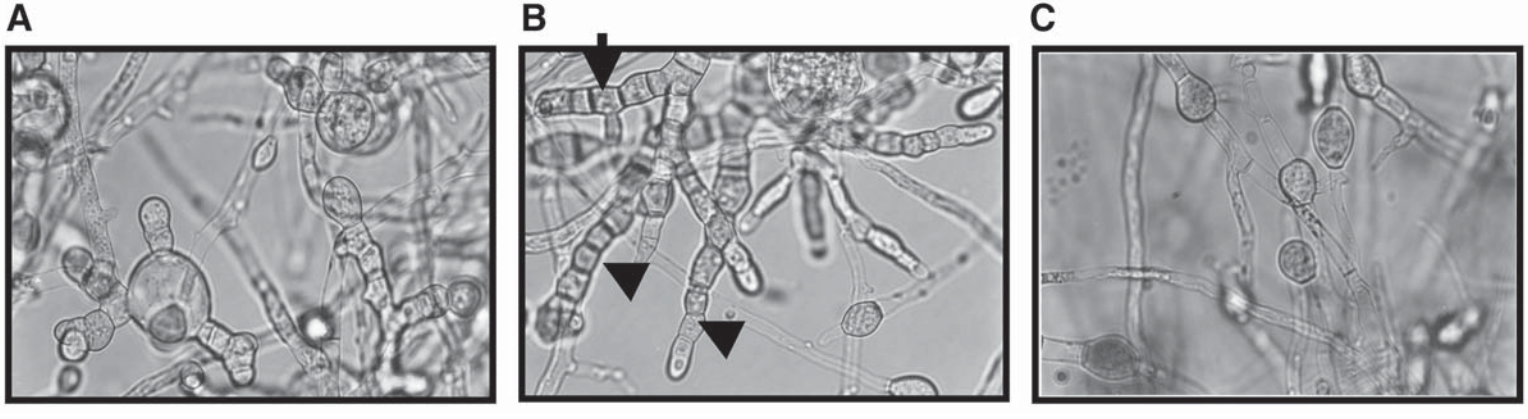

Fig. 1. Growth of YR-1 strain in different carbon sources. The microorganism was grown in sMMP with added glucose **(A)**, hexadecane **(B)**, or petroleum **(C)**. Magnification: ×400. Arrows shows specific structures formed only in medium with hexa-decane as the sole carbon source.

Enzyme Assays

AO activity was measured according to Janssen et al. *(11)*; the enzyme assays were performed at 25ºC 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 of cell-free extract (100–200 µg of protein). The reaction was started after adding 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_2 . min at 25°C. AO specific activity was expressed as units per milligram of protein. AO activity in zymograms was detection by nondenaturing polyacrylamide gel electrophoresis (PAGE), following a variation of a spectrophotometric method *(11)*. Briefly, aftera 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.

Electrophoresis

PAGE analysis of protein from different samples was carried out in slab gels using 6% (w/v) polyacrylamide with the buffer system of Laemmli *(12)*. After electrophoresis, proteins were visualized in the gels by Coomassie Blue R-250 staining (Sigma).

Results

Morphologic Patterns of YR-1 Strain in Different Carbon Sources

Strain YR-1 exhibited different morphologic patterns depending on the carbon source added to the sMMP culture medium. As can be seen in Fig. 1, the growth of YR-1 in glucose was typically the hyphae. However, in both hexadecane and petroleum the hyphae exhibited structures quite different from normal growth. In hexadecane, some segmented structures (Fig. 1B) were present only in this carbon source.

AO Activity in Cell-Free Extracts from Mycelial Cells

The presence of AO activity was analyzed in different cell extracts (crude extracts, 164,500*g* supernatant fractions) of aerobically grown mycelium of strain YR-1 obtained in sMMP or sMM containing 1.0% glycerol, decane, hexadecane, or glucose at different concentrations as carbon

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

	AO activity (U)[b]		
Sample	Methanol	Ethanol	Hexadecanol
SMMP	6.87×10^{-7}	1.97×10^{-5}	1.67×10^{-5}
sMMP–1% glycerol	4.25×10^{-7}	1.64×10^{-5}	1.14×10^{-6}
sMMP–1% hexadecane	0.050	0.27	0.20
sMMP–1% decane	0.053	0.28	0.25
sMM–1% hexadecane	No growth	No growth	No growth
sMM–0.2% glucose sMM–1.0% glucose sMMP–0.2% glucose sMMP–0.4% glucose	Growth and no AO detected	Growth and no AO detected	Growth and no AO detected

[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. The values are the means of three independent experiments with triplicate determinations in each use.

[b]One unit of activity, 1 µmol of H_2O_2 /[mg of protein (min)].

source. 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, ethanol, or hexadecanol as enzyme substrates only when the fungus was grown in media with the addition of peptone and without glucose. On the other hand, AO activity was only detected when the fungus was grown in minimal media containing decane or hexadecane as carbon sources. Figure 2A shows AO zymograms using hexadecanol as substrate. As can be observed in lane 1 of Fig. 2A, under these conditions it was possible to detect the presence of three bands of AO activity. This is an important point for us because in previous work *(13)*, we detected only two bands in our zymograms. We ran several experiments, but it was not possible separate the upper activity named AO-1. In the present work, it was possible to detect three activity bands (Fig. 2A, lanes 1 and 2) by means of different changes in PAGE concentration and run conditions. For the lanes in Fig. 2A corresponding to the cell-free extracts obtained when the fungus was grown in different conditions including no carbon source, glucose, or glycerol (lane 3, 4, and 5, respectively), in these cases the activity bands are absent. Figure 2B shows the protein pattern stained by Coomassie blue, which is similar in all cases. In all lanes, 100 µg of protein was loaded.

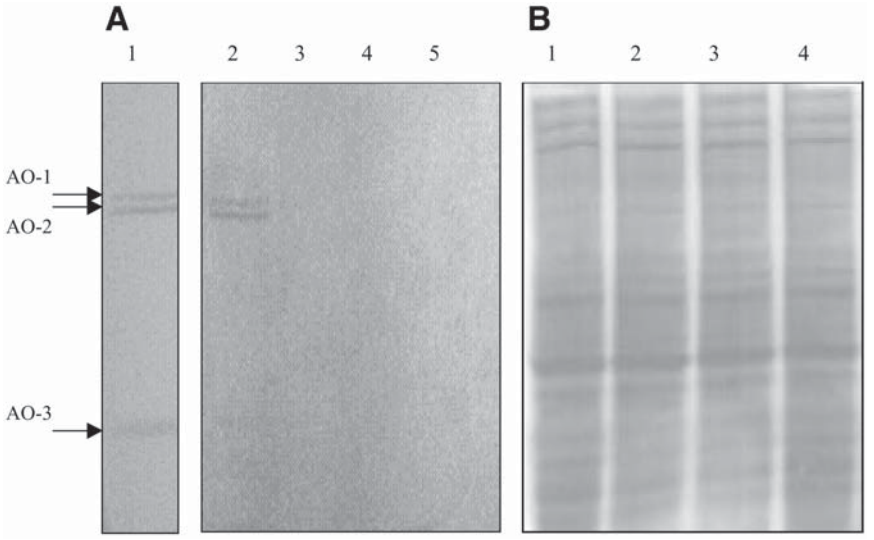

Fig. 2. AO activity zymograms from mycelium cells of YR-1. The 164,500*g* supernatant of mycelium cells grown aerobically for 22 h in sMMP media with added different carbon sources was electrophoresed using 6% acrylamide. AO activity was developed in the gel with hexadecanol as substrate. **(A)** Lane 1, sMMP-hexadecane; lane 2, sMMP-hexadecane; lane 3, sMMP without carbon source; lane 4, sMMP-glucose; lane 5, sMMP-glycerol. In all cases 300 (g of protein was loaded in each lane. **(B)** Protein patterns of samples stained by Coomassie blue R-250. Lane 1, sMMP-hexadecane; lane 2, sMMP without carbon source; lane 3, sMMP-Glucose; lane 4, sMMP-glycerol. One hundred micrograms of protein was loaded in each lane. Arrows show the bands of AO activity.

Specific Growth Rate and Generation Time

The appearance of AO activity as a function of incubation time in growth medium with hexadecane or glucose was estimated. Figure 3 shows that in the case of hexadecane as sole carbon source, the 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. Meanwhile, in glucose there was no activity during the incubation period. The kinetic growth parameters specific growth rate (μ) and generation time (T_g) were calculated. Table 2 shows that the microorganism presents a value of T_g for growth in hexadecane twice that obtained for glucose and a value of μ for hexadecane half that of the glucose value, indicating that glucose is a much better substrate for growth than hexadecane, but hexadecane is a possible inducer of AO activity and glucose could be a repressor or possibly an activity inhibitor; the latter observation can not be disregarded.

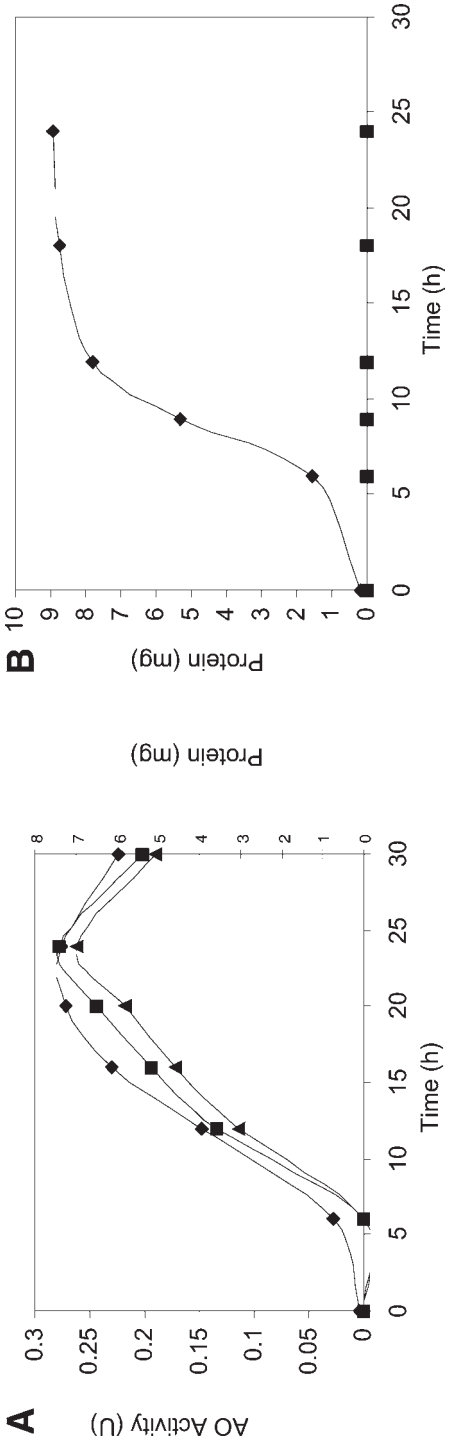

Fig. 3. Growth kinetics of YR-1 strain and appearance of AO activity as function of incubation time in different carbon sources. The 164,500*g* supernatant of samples from different incubation times from mycelium cells grown aerobically in sMMP supplemented with 1% hexadecane **(A)** or glucose **(B)**, was used to measure the AO activity using decanol (■)or hexadecanol (▲) as substrates. Protein (◆) indicates fungus growth measure. One unit of activity, µmol of H_2O_2 /(mg of protein (min); protein, $A_{280\,nm}$.

Table 2
Specific Growth Rate (μ) and Generation Time (T_g) of YR-1 Strain Grown in Different Carbon Sources[a]

	μ (h^{-1})	T_g (h)
SMMP–1% Glucose	0.536	1.19
SMMP–1% Hexadecane	0.330	2.10

[a]The values given are the mean of two independent experiments with triplicate determinations in each case.

Table 3
AO Activity in Strain YR-1 with Various Alcohols as Substrates in Experiments of Mycelium Transfer[a]

	AO activity (U)[b]		
Transfer from glucose to	Methanol	Ethanol	Decanol
sMMP–0.5% Decane	0.0870	0.0570	0.58
sMMP–0.5% Hexadecane	0.0690	0.0480	0.67
sMMP–1% Methanol	0.0027	0.0023	0.35
sMMP	7×10^{-5}	4.5×10^{-5}	8×10^{-6}
sMMP–1% Glycerol	2.2×10^{-5}	2.6×10^{-5}	3.6×10^{-5}
sMMP–1% Glucose	ND	ND	ND

[a]The microorganism was grown for 8 h in YPG medium. The mycelium was then exhaustively washed and transferred to minimal salts media (sMMP) supplemented with different carbon sources. AO was assayed as described in Materials and Methods, the substrates listed were used at different concentrations: 127 m*M* methanol; ethanol and decanol were at 60 m*M* final concentration. Activities are expressed as units of activity. The values given are the mean of two independent experiments with triplicate determinations in each case. ND, not detected.

[b]One unit of activity, 1 μmol of H_2O_2 /[mg of protein (min)].

Induction of AO Activity

All previous results of the present study strongly suggest that AO activity is negatively affected by the presence of glucose and also that the presence of a hydrocarbon as a possible inducer is necessary. To obtain more information about the inducible mechanism of AO synthesis, we conducted some experiments transferring mycelia grown in minimal medium with the addition of 1.0% glucose for 8 h. After this incubation period, the mycelium was exhaustively washed in sterile conditions and transferred into fresh minimal medium with the addition of different carbon sources—glucose, glycerol, methanol, and hydrocarbons—and, finally, a flask with the minimal medium but without carbon source. It is clear from the data in Table 3 that the transference of mycelium grown in the presence of glucose (not induced) to an induction medium (hydrocarbon present) was able to permit AO expression. By contrast, in cases when the fresh medium contained glucose, glycerol (a nonfermentable carbon source), or methanol, no AO activity was detected, suggesting that the induction mechanism is present in the expression of AO enzyme.

Table 4
Effect of Presence of Glucose on AO Activity[a]

Sample	AO activity (U) in decanol
Control (no glucose added)	0.0368
0.1% Glucose	0.0239
0.5% Glucose	0.0339
1.0% Glucose	0.0323
2.0% Glucose	0.0280

[a]Glucose at different concentrations was added to the reaction mixture to determine AO activity. The activity of AO from strain YR-1 using 0.5 m*M* decanol (final concentration) as substrate was assayed as described in Materials and Methods. The values given are the mean of two independent experiments with triplicate determinations in each case.

Inhibition of AO Activity by Glucose

To explore whether the presence of glucose in the reaction mixture of the AO activity measure could negatively affect the enzymatic activity of AO by means of an inhibitory effect, we conducted several experiments in which we added different amounts of glucose to the reaction mixture in order to determine enzyme activity. The results, shown in Table 4, clearly demonstrate that the presence of glucose at any concentration had no effect on AO activity.

Discussion

Figure 1 clearly shows the differences in the morphologic pattern of growth of the fungi when it was growing in glucose or hexadecane. Interesting, in hexadecane there was a formation of "septa-like" structures that could be similar to the structures formed when the fungi were in the late stationary phase of growth and the nutrients in the culture medium are exhausted.

AO activity in aerobically grown mycelial cells of strain YR-1 obtained under different nutritional conditions was measured in the 164,500*g* supernatant of each growth condition. Activity was present only when glucose in the culture medium was absent. This result could suggest that AO synthesis is subjected to catabolic repression, but more experiments investigating the amount of mRNA synthesized by cells growing in glucose or hydrocarbon are necessary to establish whether there is an effect of catabolite repression by glucose in the synthesis of the mRNA. A complementary experiment was conducted to prove the inhibitory effect of glucose over AO activity. In this case, the enzyme activity was not under the mechanism of glucose inhibition.

The previous finding of two different AO activities in the 164,500*g* supernatant *(13)* and the actual result of three activity bands in zymo-

grams are not unexpected; for instance, in the methylotrophic yeast *Candida boidinii*, the presence of two AO enzymes has been reported *(14)*. It is very important to be extremely sure about the exact number of AO activities present in the YR-1 strain and which one is the most important for hydrocarbon metabolism. To date, we are studing the purification of the three activity bands, and by means of double dimension PAGE, we will be able to determine whether these bands correspond to different enzymes, and whether they are isozymes or could be the same enzyme with different aggregation states. 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 alcohol oxidase *(15)*. The principal difference between 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.

When the microorganism was transferred from growth in glucose to different carbon sources, the results in the presence of glucose indicate the possible effect of catabolite repression, but more important, was the transference from glucose to glycerol in which no activity was detected, because this result strongly suggests that the presence of a hydrocarbon is necessary to induce the synthesis of AO activity. 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 important in establishing the role of these enzymes in hydrocarbon metabolism in the YR-1 strain. Finally, we are conducting molecular experiments to identify taxonomically the YR-1 strain.

References

1 Atlas, R. M. (1995), *Chem. Eng. News* **73(14),** 32–42.
2 Alper, J.(1993), *Biotechnology* **11,** 973–975.
3 Guenguerich, F. P. and Macdonald, T. L. (1990), *FASEB J.* **4,** 2453–2459.
4 Kellner, D. G., Maves, S. A., and Sligar, S. P. (1997), *Curr. Opin. Biotechnol.* **8,** 274–278.
5 Jones, J. G. and Bellion, E. (1991), *Biochem. J.* **280,** 475–481.
6 Evers, M. E., Titorenko, V., Harder, W., ven der Hlei, Y., and Veehhuis, M. (1996), *Yeast* **12,** 917–923.
7 Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem.* **193,** 265–275.
8 Gleason, M. A., Ortori, S., and Sudberry, P. E. (1986), *J. Gen. Microbiol.* **132,** 3459–3465.
9 Bartnicki-Garcia, S. and Nickerson, W. J. (1962), *J. Bacteriol.* **84,** 841–858.
10 Torres-Guzman, J. C., Arreola-Garcia, G. A., Zazueta-Sandoval, R., Carrillo-Rayas, T., Martínez-Cadena, G., and Gutiérrez-Corona F. (1994), *Curr. Genet.* **26,** 166–171.
11. Janssen, F. W., Kerwin, R. M., and Ruelius, H. W. (1975), in *Methods in Enzymology*, vol. XLI, Academic, London, UK, pp. 364–369.
12 Laemmli, U. K. (1971), *Nature* **227,** 680–685.
13 Alvarado-Caudillo, Y., Bravo-Torres, J. C., Zazueta-Novoa, V., Silva-Jiménez, H., Torres-Guzmán, J. C., Gutiérrez-Corona, J. F., and Zazueta-Sandoval, R. (2002), *Appl. Biochem. Biotechnol.* **98–100,** 243–255.
14 Sakai, Y. and Tani, Y. (1992), *Gene* **114,** 67–73.
15. Benen, J. A. E., Sánchez-Torres, P., Wagemaker, M. J. M., Fraaije, M. W., van Berkel, W. J. H., and Visser, J. (1998), *J. Biol. Chem.* **273,** 7865–7872.