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Production of Alcohol Oxidase by Several Basidiomycetes

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An Amber MPH-Cerelose medium was devised on which the unidentified basidiomycete B 191039 produced high titers of alcohol oxidase in shake cultures. Another, fully synthetic medium was developed on which the production was nearly as good, but very slow. No cofactors were required to produce this flavine adenine dinucleotide-containing enzyme. The B 191039 organism underwent frequent mutations. The mutant forms were morphologically distinct from the parent strain and elaborated little or no alcohol oxidase. Several other basidiomycetes were shown to produce alcohol oxidase in submerged culture.

The occurrence of a novel enzyme, alcohol oxidase, in mycelial extracts of a basidiomycete grown in submerged culture was described by Janssen, Kerwin, and Ruelius (3). This enzyme catalyzes the oxidation, by molecular oxygen, of lower primary alcohols to the corresponding aldehydes and hydrogen peroxide. Methanol is the preferred substrate. The rate of oxidation of primary alcohols decreases rapidly with increasing chain length. The enzyme has been crystallized and characterized as a flavoprotein that contains flavine adenine dinucleotide (FAD) as the prosthetic group (4).

The organism that produces this enzyme in submerged culture was collected by M. A. Espenshade in the field during a program to screen basidiomycetes for the production of antitumor principles (1, 2). The organism belongs to the genus *Polyporus*, but unfortunately it was not fully identified at the time it was collected. A tissue culture was prepared from the wild sporophore, and the number B 191039 was assigned to that culture by the Cancer Chemotherapy National Service Center.

The present study was undertaken to determine whether alcohol oxidase is produced by other basidiomycetes and to investigate the conditions which promote enzyme production by the B 191039 organism in submerged culture.

MATERIALS AND METHODS

Organisms and maintenance. Basidiomycete B 191039 was supplied by M. A. Espenshade of these laboratories. The other organisms that were investigated and the sources from which they were obtained are as follows: *P. obtusus* Berk, (no. 136) Forest Research Institute, India; *Irpex flavus*, Klotzsch Centraalbureau voor Schimmelcultures, Baarn, Netherlands; *P. versicolor*, (L)Fr. (no. 22794), Department of Agriculture, Ottawa, Canada; *Lenzites trabea*, Persoon ex Fries (no. 8715), American Type Culture Collection; *Radulum casearium* (Morgan) Hoyd, Department of Agriculture, Ottawa, Canada (no. 17532); *Pellicularia filamentosa* var. *microsclerotia*, (Matz) Exner (no. 6298), Institute for Fermentation, Osaka, Japan; and *Armillaria mellea*, Vahl ex. Fr. (no. 5070), Department of Agriculture, Ottawa, Canada. These organisms were maintained on slants of Malt or Mycophil Agar (BBL) or on Medium A of Stevens into which 1.5% agar had been incorporated (J. A. Stevens, Ph.D. Thesis, Michigan State Univ., East Lansing, 1957). The inoculated slants were incubated and stored at 23 ± 2 C.

Substrains of basidiomycete B 191039 were obtained by plating out the organism on petri dishes containing Malt Agar (BBL). The plates were incubated at 25 C until the colony reached a diameter of about 5 to 6 cm. The mycelium from the various sectors was carefully removed and transferred to a series of Malt Agar slants (BBL).

Fermentation equipment. Enzyme production was investigated in 500-ml Erlenmeyer flasks containing 150 ml of medium and 2,000-ml Erlenmeyer flasks containing 1,000 ml of medium. The 500-ml flasks were incubated on a reciprocating shaker (model 94; New Brunswick Scientific Co., New Brunswick, N.J.) set for 90 four-inch strokes per minute, and the 2,000 ml flasks were incubated on a gyratory incubator shaker (model G-25; New Brunswick Scientific Co.) set at 160 rev/min. Both shakers were maintained at a temperature of 25 ± 1 C.

Inoculum. Inoculum for shake flasks was prepared as follows. A 10-ml amount of sterile water was added to a slant, and the growth was suspended by scraping it off the slant with a sterile needle. The suspension was added aseptically to a heavy-walled, 250-ml Erlenmeyer flask containing 100 ml of a medium composed of 1.0% Phytone (BBL) and 1.0% dextrose (*p*H unadjusted). The flasks were closed with cotton plugs

TABLE 1. *Media used in the production of alcohol oxidase by various basidiomycetes*

Medium constituents[a]	Organisms						
	Polyporus obtusus	*Radulum casearium*	*Lenzites trabea*	*Irpex flavus*	*Polyporus versicolor*	*Pellicularia filamentosa*	*Armillaria mellea*
Cerelose	10	10		10		10	10
Glucose					10		
Brown sugar[b]			20				
Maltose				10			
Amber MPH	30	10					10
Phytone				10	10		
NZ-Amine				10			
Vico P-200[c]				33			
Difco malt extract			10			5	
NZ-Amine YTT			20				
Sheffield soy peptone						20	
KH_2PO_4		1	1			1	1
Mineral solution[d]		10	10				10

[a] Amounts are given in grams per liter of water, except for mineral solution which is given in milliliters. The *p*H of the media before autoclaving was 6.5 for all of the organisms except for *P. versicolor*, for which it was 7.1.

[b] Franklin Sugar Refinery, Division of American Sugar Co., Philadelphia, Pa.

[c] A. E. Staley Manufacturing Co., Decatur, Ill.

[d] Composition: $MgSO_4 \cdot 7H_2O$, 100 g; NaCl, 20 g; $CaCl_2 \cdot 6H_2O$, 2 g; $MnSO_4 \cdot H_2O$, 5 g; $ZnCl_2$, 0.15 g; $FeCl_3 \cdot 6H_2O$, 0.5 g; $CuSO_4 \cdot 5H_2O$, 0.005 g; water, 1,000 ml. See J. H. Litchfield, R. C. Overbeck, and R. S. Davidson, Agr. Food Chem. *11*:158–162, 1963.

TABLE 2. *Influence of carbon source on alcohol oxidase production by basidiomycete B 191039*[a]

Carbon source	Maximum enzyme yield (Klett units/ml of mycelial extract)	Time required for maximum enzyme yield
		days
Cerelose	3,100	9
Lactose	3,000	10
Glycerol	2,800	11
Sucrose	2,050	14

[a] Each flask contained 1.5 g of Amber MPH and 1.5 g of one of the carbon sources listed above in 150 ml of water (*p*H adjusted to 6.5 prior to autoclaving). Flasks were sampled daily and assayed for enzyme titer.

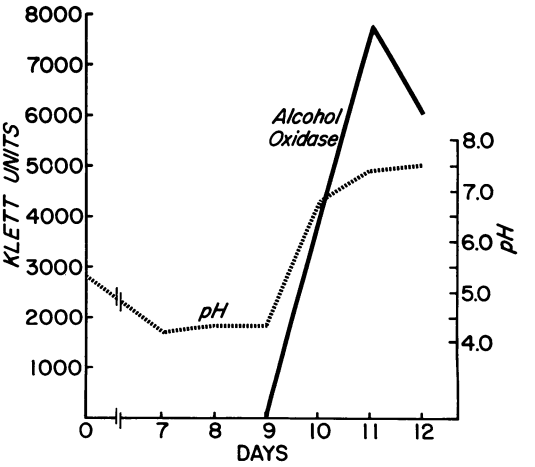

FIG. 1. *Production of alcohol oxidase by the B 191039 organism on a Cerelose-Amber MPH-ferrous sulfate medium. Solid line, alcohol oxidase content in Klett units per milliliter of mycelial extract. Broken line, pH.*

and were incubated at 25 ± 1 C for 7 days on a gyratory shaker (model G-25; New Brunswick Scientific Co.) set at 280 rev/min. Each 500-ml shake flask was inoculated with 5 ml of this inoculum and each 2,000-ml flask was inoculated with 10 ml of this inoculum.

Media. Crude glucose (Cerelose; Corn Products Co., Argo, Ill.), sucrose, lactose, glycerol, and corn dextrin (Fisher Scientific Co., Pittsburgh, Pa.) were investigated as carbon sources for the B 191039 organism. In attempts to increase enzyme production, we used Amber MPH and Amber OMP-60 (Amber Laboratories, Milwaukee, Wis.), N-Z-Amines E and and ET (Sheffield Chemical Co., Norwich, N.Y.), malt extract (Difco), corn steep liquor (A. E. Staley, Decatur, Ill.), L-leucine, L-asparagine, and glycine as nitrogen sources.

We also examined a fully synthetic medium having the following composition: D-glucose, 25 g; L-asparagine, 2.28 g; KH_2PO_4, 2.0 g; $Fe_2(SO_4)_3$, 1 mg; $ZnSO_4 \cdot 7H_2O$, 0.88 mg; $MnSO_4 \cdot H_2O$, 0.3 mg; thiamine, 100 μg; biotin, 5 μg; and water, 1,000 ml. The *p*H of this medium was adjusted to 5.0.

TABLE 3. *Morphological characteristics of B 191039 substrains and alcohol oxidase production*

Substrain no.	Alcohol oxidase production			Morphological characteristics		
	Maximum titer (Klett units/ml)	Time required for maximum enzyme titer	*p*H of medium at maximum enzyme titer	Color	Appearance	Clamp connections
		days				
0	7,750	11	7.4	Buff yellow	Cottony	Yes
9	50	6	4.8	Tan	Cottony	Yes
10	1,850	6	7.6	Tan	Dry, fragmentary	No
11	1,000	6	5.3	White	Cottony	Yes
12	200	5	5.7	White	Fragmentary	No

The media used in the investigation of alcohol oxidase production by other basidiomycetes are given in Table 1.

Enzyme assay. Production of the enzyme was monitored as follows. The contents of one 500-ml flask were filtered through coarse paper under reduced pressure. The mycelium was added to 150 ml of 0.05 M sodium phosphate buffer (*p*H 7.5) and was homogenized for 5 min in a water-cooled Micro Waring Blendor (Central Scientific Co., Chicago, Ill.). From 2,000-ml flasks, a sample of the contents (e.g., 100 ml) was withdrawn aseptically and filtered as above. The mycelium was suspended and homogenized in a volume of phosphate buffer equal to the volume of the sample withdrawn. The homogenates were filtered as above, and the filtrates were assayed for enzyme content as described by Janssen and Ruelius (4).

Alcohol oxidase activity was expressed in Klett units per milliliter; to accomplish this the colorimeter reading was multiplied by the dilution factor of an appropriately diluted sample of the filtrate.

RESULTS

Production of alcohol oxidase by basidiomycete B 191039. We obtained good enzyme production with a medium composed of Amber MPH and crude glucose (Cerelose) (Table 2). The carbon source did not appear critical; the enzyme titers produced with lactose or glycerol were almost as high as those obtained with the glucose medium. When glucose was replaced by sucrose, enzyme production was delayed and not quite as high. Other nitrogen sources gave results that were similar to or not as good as those obtained with Amber MPH.

The addition of 1 g of ferrous sulfate to 1 liter of the Amber MPH-Cerelose medium increased the production of alcohol oxidase 1.5- to 2.5-fold. Maximum titers of 4,650 to 7,750 Klett units/ml were obtained in 9 to 12 days. The beginning of enzyme production always coincided with a sharp rise of the *p*H value in the medium on the 7th to 9th day of the fermentation. The maximum concentrations were reached 2 to 3 days later as the *p*H leveled off. Thereafter, the titers declined rapidly. These observations are presented graphically in Fig. 1.

When greater or lesser amounts of ferrous sulfate were added to the Amber MPH-Cerelose medium, inferior enzyme levels were observed. Magnesium sulfate increased alcohol oxidase production somewhat, but not as much as ferrous sulfate. Manganese sulfate, zinc sulfate, copper sulfate, sodium molybdate, and boric acid either had no effect or decreased enzyme formation. Methanol, the preferred substrate of alcohol oxidase (4), reduced enzyme titers considerably when added to the Amber MPH-Cerelose medium in concentrations of 0.05 to 1%.

The B 191039 organism also produced large amounts of alcohol oxidase on the fully synthetic medium; the maximum yields (2,600 to 3,000 Klett units) were nearly as high as with the Amber MPH-Cerelose medium, but much longer fermentation times were required (22 to 28 days). Neither biotin nor thiamine proved essential for enzyme production; in fact, omission of the vitamins gave higher yields, although the fermentation time was extended considerably (30 to 36 days).

Since alcohol oxidase contains FAD as the prosthetic group (4), the influence of riboflavine, flavine mononucleotide, and FAD on enzyme production was investigated. No increased enzyme levels were detected when these flavines were added to the fully synthetic medium. With added FAD, the time required to produce maximum enzyme titers was shortened to 18 days, but the other two flavines had no effect.

Morphological instability of the B 191039 organism. The work with basidiomycete B 191039 was hampered by frequently occurring mutations. We observed morphological as well as metabolic changes in subcultures derived from the slants intended for the preparation of inoculum (Table 3). The original culture had a buff yellow color and an abundance of aerial hyphae that gave it a cottony appearance. This strain (substrain 0) had

TABLE 4. *Production of alcohol oxidase by various basidiomycetes*

Organism[a]	Maximum titer of mycelial extract (Klett units/ml)	Fermentation time	Final *p*H value
		days	
Polyporus obtusus	4,700	9	7.4
Radulum casearium	3,200	11	7.7
Lenzites trabea	700	22	7.0
Irpex flavus	550	4	6.9
Polyporus versicolor	400	7	6.8
Pellicularia filamentosa	50	5	7.0
Armillaria mellea	0	8	6.6

[a] Grown on the media indicated in Table 1.

many clamp connections. The most mutated form (substrain 12) was white, had a dry, fragmentary appearance, and had no clamp connections. Various intermediates were observed between these two extreme forms (e.g., substrains 10 and 11).

The ability of mutant forms to elaborate alcohol oxidase was strongly reduced (substrains 10 to 12). However, low enzyme production was also observed with cultures that had a normal appearance (e.g., substrain 9) but were incapable of bringing the *p*H value of the medium up to neutrality. Substrains 11 and 12 showed this defect in addition to morphological changes. Alcohol oxidase is unstable at *p*H values below 6, as was reported earlier (3, 4), and during fermentation appreciable enzyme titers began to appear only after the *p*H value had started to rise (*see* Fig. 1).

Production of alcohol oxidase by other organisms. Table 4 lists the organisms that were investigated for alcohol oxidase production. The amounts obtained with *P. obtusus* and *R. casearium* are impressive, and the fermentation times required are comparable to those of the B 191039 organism. Fair titers were produced by *I. flavus*, *P. versicolor*, and *L. trabea*.

DISCUSSION

The titers of alcohol oxidase produced by the B 191039 organism on the Amber MPH-Cerelose-ferrous sulfate medium were quite adequate for the purification studies that culminated in the crystallization of the enzyme (4). Care had to be taken to harvest the mycelium at the moment of maximum enzyme content. Attempts to maintain maximum enzyme levels over several days were unsuccessful. The reason for the fast decline after the peak had been reached (Fig. 1) is not known. It is conceivable that the enzyme is released from the cells at this time. This possibility could not be verified, since the fermentation liquor contained a substance that interfered with the determination of alcohol oxidase. However, similar situations were encountered in related studies with other basidiomycetes. The carbohydrate oxidase of *P. obtusus* is located in the mycelium of that organism. Several days after maximum enzyme production was reached, lysis of the mycelium occurred and the enzyme levels dropped sharply (6). Poricin, the antitumor factor produced by *Poria corticola* in submerged culture, was found to be released into the medium after the substance had reached its peak concentration in the mycelium (5).

The reason for the increased enzyme titer in the presence of ferrous sulfate is not known. Alcohol oxidase is probably not a metalloprotein (4).

That the ability to produce alcohol oxidase is not limited to the B 191039 organism is fortunate, for this organism is morphologically and metabolically unstable, changing quite easily to what appears to be a mutant form which produces little or no enzyme. The B 191039 organism also produces an antitumor factor of high molecular weight (H. W. Ruelius, R. T. Schillings, and R. M. Kerwin, *unpublished data*), but loses this ability whenever it changes to the "white" form.

Our attempts to prevent mutation by changing the maintenance conditions were unsuccessful. During the course of this investigation, the culture had to be plated out several times so that sections with the "right" morphological characteristics could be selected and transferred to slants. In this way, the potential for alcohol oxidase production was temporarily restored. In view of the unstable nature of the B 191039 organism and attendant problems in the production of alcohol oxidase, *P. obtusus* or *R. casearium* may prove to be more reliable sources of the enzyme.

Six of the seven other basidiomycetes that we examined were found to elaborate alcohol oxidase (Table 4). The six belong to several orders of *Basidiomycetes*, which suggests that related organisms may produce the enzyme.

The role played by alcohol oxidase in the metabolism of these organisms is not known. The crystalline enzyme is so specific for lower primary alcohols (4) that it is difficult to imagine a metabolic role involving the oxidation of other substrates.

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