Oxidation of Methanol by the Yeast, Pichia pastoris. Purification and Properties of the Alcohol Oxidase  

R. COUDERC and J. BARATTI*  
Centre de Biochimie et de Biologie Moléculaire du C.N.R.S., 31 chemin Joseph Aiguier, 13274 Marseille Cedex 2, France  
Received January 25, 1980

The enzymes of methanol oxidation were investigated in a new yeast strain, Pichia pastoris IFP 206, with high yield (0.42 g cell per g of methanol). The following enzymes were detected in cell free extracts of P. pastoris: alcohol oxidase, catalase, formaldehyde and formate dehydrogenases. The alcohol oxidase was purified from cell free extracts of P. pastoris containing high amount of the enzyme (33%) with a good yield (55%). The preparation was homogenous by immunochemical methods. The enzyme had a molecular weight of 675,000 and was composed of eight identical subunits of M.W. 80,000. Each subunit contained one FAD. The N-terminal sequence was found to be: Ala-Ile-Pro-Glu-Glu-Phe-Asp-Ile-Leu-Val-Leu-Gly. The protein had 65 free –SH groups per molecule. The optimum temperature for the enzyme activity was 37°C and the activation energy was 11.1 kcal/mol. Optimum pH was 7.5 and the enzyme activity was unstable at acidic pH. The apparent Km for methanol were 1.4 and 3.1 mM at oxygen concentrations of 0.19 and 0.93 mM. Similarly, the apparent Kms for oxygen were 0.40 and 1.0 mM at methanol concentrations of 1, 10 and 100 mM. The enzyme oxidized primary alcohols with short carbon chains like ethanol and propanol. Inhibition of enzyme activity by hydrogen peroxide was a consequence of the oxidation of essential –SH groups. The inhibition was reversed by reducing agents.

A new yeast growing on methanol as sole carbon and energy source has been isolated at the Institut Français du Pétrole (IFP 206). The usefulness of this yeast for Single Cell Protein production from methanol has been also demonstrated and a process developed. The yeast was identified as a strain of Pichia pastoris by the Centraal Bureau Voor Schimmelcultures (Holland).

In all the yeasts so far studied the oxidation of methanol to formaldehyde is catalyzed by an oxidase (E.C. 1.1.3.13). The electron acceptor is oxygen and hydrogen peroxide is formed during the reaction. Consequently no energy is available for the cell from this oxidation step. In bacteria the oxidation of methanol is catalyzed by a dehydrogenase and the electrons are transferred to the electron transport chain. In this case the energy of methanol oxidation is available to the cell. The higher yields observed with bacteria as compared to yeasts were attributed to the first step of methanol oxidation. Since the yield on methanol of the yeast, Pichia pastoris IFP 206, was high compared to other yeasts (0.42 g of cell per g of methanol) the question was raised whether the oxidation of methanol is catalyzed by an oxidase or a dehydrogenase.

Formaldehyde formed by the oxidation of methanol is further oxidized to carbon dioxide by a two steps reaction involving formaldehyde (E.C. 1.2.1.1) and formate dehydrogenases (E.C. 1.2.1.2). Both enzymes are NAD dependent and were found in all the yeasts so far studied. Carbon assimilation occurs probably from formaldehyde but the exact mechanism in yeast is still unknown. The enzymes of methanol metabolism have been studied in yeasts especially the alcohol oxidases from Kloeeckera, Candida.  

* To whom all correspondence should be addressed.
and \textit{Hansenula polymorpha}\textsuperscript{12,13,14,17} but also formaldehyde and formate dehydrogenases.\textsuperscript{11,14,17} The present work is a study of methanol oxidation by the yeast, \textit{P. pastoris} IFP 206. Alcohol oxidase, formaldehyde and formate dehydrogenases activities were detected in cell free extracts of cells grown on methanol. The structural and enzymatic properties of the alcohol oxidase are reported and compared to the enzymes of other species.

**MATERIALS AND METHODS**

**Microorganisms.** \textit{P. pastoris} IFP 206 was grown by continuous culture on a mineral medium containing methanol as sole carbon and energy sources at 34°C, pH 3.5 at a dilution rate of 0.12 h\textsuperscript{-1}. Cells were harvested by filtration, lyophilized and stored at -15°C until used. These operations were done by Dr Ballerini at the Institut Français du Pétrole.\textsuperscript{1} \textit{H. polymorpha} ATCC 26012 was grown in continuous culture as described by Levine and Cooney.\textsuperscript{18}

**Enzyme purification.** The alcohol oxidases from the two yeasts, \textit{P. pastoris} and \textit{H. polymorpha}, were purified according to published procedures\textsuperscript{8-13} with some modifications. In a typical experiment, 7 g of \textit{P. pastoris} cells were suspended in 420 ml of a 50 mM potassium phosphate buffer pH 7.5 (KPB) containing 0.1 mM \(\beta\)-mercaptoethanol. Cell disruption was obtained by a two minutes treatment with glass beads in a Braun homogenizer (KOD 648) as previously described.\textsuperscript{9} Cell debris was eliminated by a 15 min centrifugation at 30,000 g and the resulting supernatant was purified by an ammonium sulfate fractionation between 40 and 60\% of saturation at 0°C.

The ammonium sulfate was then eliminated by dialysis and the crude preparation was chromatographed on a DEAE-Sephadex (A-50) column (3.5 × 40 cm) equilibrated with a 50 mM KPB, pH 7.5. The absorbed proteins were eluted with a linear gradient of sodium chloride (2 × 1 liter) from 0 to 0.5 M in the equilibration buffer. The fractions with alcohol oxidase activity emerged at a sodium chloride concentration of 0.28 M. The active fractions were pooled, concentrated by precipitation with 60\% ammonium sulfate and dialyzed. The resulting enzyme preparation was then filtrated through an Ultrogel AcA 34 (Industrie Biologique Française) column (2 × 200 cm) equilibrated with 50 mM KPB pH 7.5. The active fractions were pooled and stored at 4°C until used. Lyophilization resulted in a powdered protein mainly insoluble in buffers.

The alcohol oxidase from the yeast, \textit{H. polymorpha}, was also prepared for comparison. The same method was used except that the ammonium sulfate fractionation was done between 50 and 70\% saturation. Both enzyme preparations were found homogeneous by different methods given in the results section.

**Assays of enzyme activities.** The alcohol oxidase activity was assayed by measuring the amount of formaldehyde formed at 37°C in a 15 min incubation with 10 mM methanol in a 100 mM KPB, pH 7.5.\textsuperscript{12} The total volume of the reaction mixture was 1.4 ml. The reaction was stopped by addition of 0.1 ml of 4 N HCl and the formaldehyde formed was estimated by addition of 1.5 ml of Nash reagent\textsuperscript{20} by incubation for 10 min at 60°C. A reference curve was obtained in the same conditions with a standard solution of formaldehyde. Standardization of the formaldehyde solution was done by the dimedone reaction.\textsuperscript{19} Three assays with different enzyme concentrations were run in parallel and the activity was calculated from the slope of the straight lines obtained by plotting formaldehyde formed versus enzyme concentration. One enzyme unit was defined as the amount of enzyme which liberated one micromole of formaldehyde per minute in our conditions.

In some experiments, the alcohol oxidase activity was determined by the oxygen consumption of the reaction with a Gilson K-ICC Oxigraph. Incubations were done at 37°C in a 10 mM methanol, and 100 mM KPB, pH 7.5. Enzyme activities were determined from the slope of the lines oxygen concentration versus time. In this case one enzyme unit was the amount of enzyme which consumed one micromole of oxygen per minute in our conditions. Since all the activities determined by this method were done with the purified enzyme, catalase was not present during incubation and one mole of oxygen consumed corresponds to the formation of one mole of formaldehyde.\textsuperscript{21} Then the two enzyme units are equivalent. Good agreement was observed between the two methods.

Formaldehyde and formate dehydrogenases were assayed spectrophotometrically with a Gilford 2400 spectrophotometer as described by van Dijken.\textsuperscript{17} The incubation mixture for formaldehyde dehydrogenase contained: 100 mM KPB, pH 7.5, NAD 0.4 mM, reduced glutathione 60 mM and HCHO 1 mM. Incubation was done at 37°C and the appearance of NADH was followed at 340 nm as a function of time. A blank was run simultaneously without formaldehyde. When formate dehydrogenase was assayed glutathione was omitted and 125 mM formate was added instead of formaldehyde. A molar extinction coefficient of 6.22 × 10\(^{4}\) cm\(^2\)/mol was taken for NADH.\textsuperscript{17} One enzyme unit was the amount of enzyme which reduced one micromole of NAD per min in our conditions. Catalase was assayed spectrophotometrically according to Bergmeyer.\textsuperscript{22} The incubation mixture contained a 100 mM KPB, pH 7.0 and 12.5 mM \(\text{H}_2\text{O}_2\). The decrease of the absorbance at 240 nm was monitored at 25°C. A molar extinction coefficient of 36,000 cm\(^2\)/mol was used for \(\text{H}_2\text{O}_2\).\textsuperscript{12}
Oxidation of Methanol by the Yeast *Pichia pastoris* 2281

Analytical methods. Proteins were determined by the method of Lowry.\(^{22}\) Using bovine serum albumin as standard. The concentration of pure enzyme preparations were determined spectrophotometrically by their adsorption at 280 nm. Values of 20.9 and 16.5 were used respectively for the \(A_{1%}^{1cm}\) of the methanol oxidases from *P. pastoris* and *H. polymorpha*. The prosthetic group, FAD, was determined according to Crestfield and Moore.\(^{26}\) FAD was identified by chromatography on a Whatman Paper No. 1. The elution solvent was *n*-butanol–acetic acid–water (4:1:5, v/v/v). The quantitative determination of the liberated FAD was done spectrophotometrically at 450 nm using an extinction coefficient of \(1.3 \times 10^6\) cm\(^2\)/mol.\(^{12}\)

The absorption and fluorescence spectra of the purified enzymes were made respectively with a Cary 14 double beam spectrophotometer and a Fica 55 differential spectrofluorometer. Carboxymethylation of the apoenzyme was done according to Crestfeld and Moore\(^{26}\) and free sulfhydryl groups were determined by titration with the Ellman reagent.\(^{27}\)

Amino acid composition and NH\(_2\) terminal sequence. The amino acid composition of the protein was determined after hydrolysis with 6 N hydrochloric acid for 24, 48 and 72 hr at 110°C. The hydrolyzate was chromatographed on a Beckman 120 C autoanalyzer for the quantitative determination of the amino acids. Cystine and cysteine were determined as carboxymethyl cysteine. Tryptophan was assayed spectrophotometrically according to Spies and Chambers.\(^{28}\) The NH\(_2\) terminal amino acid was identified by the dansyl method of Hartley\(^{29}\) modified by Charles.\(^{30}\) The NH\(_2\) terminal sequence was determined using a Beckman 990 C automatic sequencer. Phenylthiohydantoines were identified by thin layer, gas liquid and high pressure liquid chromatographies.

Ultracentrifugation analysis. All the experiments were performed with a Spinco Model E analytical ultracentrifuge. The sedimentation coefficient was determined in a 50 mm KPB, pH 7.5, at enzyme concentrations of 2.9 to 5.1 mg/ml. The molecular weight determination were performed by the equilibrium method \(^{31}\) in the same buffer as above. The protein concentration was \(1 \sim 1.5\) mg/ml. The density of enzyme solutions were measured at different concentrations with a Parr densitometer model DNA 02. A partial specific volume of \(\bar{\varepsilon} = 0.753\) ml/g was calculated from the density measurements. The relation between the protein concentration and the fringe shift was used to calculate the extinction coefficient of the enzyme.\(^{32}\) The values were in good agreement with dry weight determination and an average value of \(A_{1%}^{1cm} = 20.9\) was determined.

Acrylamide gel electrophoresis and electrofocalization.

The standard polyacrylamide gel electrophoresis was performed with a Canalco apparatus in a Tris-glycine buffer pH 8.6 with an acrylamide concentration of 7.5%. Electrofokalization was carried out according to Fairbanks.\(^{33}\) The following reduced and carboxymethylated proteins were used as standard: bovine trypsinogen (24,000), ovalbumin (43,000), catalase (60,000) and \(\beta\)-galactosidase (130,000). The isoelectric point was determined by electrofocalization on a polyacrylamide gel using a pH gradient of ampholines from 3 to 11.

Immunological studies. Antibodies against a cell free extract of *P. pastoris* were raised in rabbit by the following procedure: each animal received a total of \(3\) mg of proteins in four injections, \(2 \times 1\) mg subcutaneously and 6 weeks later \(2 \times 0.5\) mg intravenously. Antisera were collected 1 week after the last injection, filtered and stored at 4°C. These antisera were used to check the homogeneity of our enzyme preparation. Two different methods were employed: the classical immunodiffusion technique of Ouchterlony and the more sensitive "rocket" method of Bjerrum.\(^{34}\)

RESULTS

The enzymes of methanol oxidation in the yeast, *P. pastoris*

The level of alcohol oxidase, catalase, formaldehyde and formate dehydrogenases and proteins in the two yeasts, *P. pastoris* and *H. polymorpha*, are given in Table I. The results are expressed for the proteins in mg and for the enzymes in units per g of cells (dry cell weight). The specific activities in cell free extracts are also given. All these enzymes of methanol metabolism are found in the cell-free extract of *P. pastoris* at levels very similar to the one observed for *H. polymorpha*. Then it seems likely that in *P. pastoris* the methanol oxidation proceeds via formaldehyde and formate to CO\(_2\). Although the presence of a methanol dehydrogenase was not checked, it seems likely that such an enzyme does not exist in *P. pastoris* since the amount of alcohol oxidase was very high: 33% of the proteins in cell free extracts (35% for *H. polymorpha*). Such high values were also reported by van Dijken\(^{13}\) who examined the alcohol oxidase content of the cell (in continuous culture), at low dilution rate. It is well known that the cells maintain a high rate of methanol oxidation by increasing the amount of the rate limiting
TABLE I. ENZYMES OF THE METHANOL OXIDATION IN CELL FREE EXTRACTS

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>Amount of proteins and enzymes per g of cell</th>
<th>Specific activity units/mg protein</th>
<th>Amount of proteins and enzymes per g of cell</th>
<th>Specific activity units/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteins (P. pastoris IFP 206)</td>
<td>366 (mg)</td>
<td>3.9</td>
<td>354 (mg)</td>
<td>294</td>
</tr>
<tr>
<td>Alcohol oxidase</td>
<td>1,430 (units)</td>
<td>965</td>
<td>1,485 (units)</td>
<td>320</td>
</tr>
<tr>
<td>Catalase</td>
<td>671 (units)</td>
<td>1.8</td>
<td>63 (units)</td>
<td>0.9</td>
</tr>
<tr>
<td>Formaldehyde dehydrogenase</td>
<td>118 (units)</td>
<td>0.32</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Enzyme activities were determined as described in MATERIALS AND METHODS. P. pastoris IFP 206 was obtained in continuous culture at a dilution rate of 0.105 hr⁻¹ with a medium containing 50 g/liter of methanol. H. polymorpha ATCC 26012 was cultivated at a dilution rate of 0.05 hr⁻¹ with a medium containing 4 g/liter of methanol. In both cases the cells were harvested and immediately used for enzyme determinations.

TABLE II. PURIFICATION OF THE ALCOHOL OXIDASE FROM P. pastoris IFP 206

<table>
<thead>
<tr>
<th>Step</th>
<th>Total* protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell free extract</td>
<td>3071</td>
<td>6650</td>
<td>2.2</td>
<td>100</td>
</tr>
<tr>
<td>Supernatant ammonium sulfate 40%</td>
<td>1638</td>
<td>5680</td>
<td>3.5</td>
<td>85</td>
</tr>
<tr>
<td>Pellet ammonium sulfate 60%</td>
<td>1448</td>
<td>5521</td>
<td>3.8</td>
<td>83</td>
</tr>
<tr>
<td>DEAE Sephadex chromatography</td>
<td>355</td>
<td>3754</td>
<td>10.6</td>
<td>56</td>
</tr>
<tr>
<td>Ultrogel AcA 34 filtration</td>
<td>310</td>
<td>3700</td>
<td>11.9</td>
<td>55</td>
</tr>
</tbody>
</table>

* Protein was assayed by spectrophotometry.

Enzyme purification

A typical result of an alcohol oxidase purification is given in Table II. From 7 g of lyophilized cells, 310 mg of purified alcohol oxidase was obtained. The yield in enzyme units was high (55%). The specific activity of our enzyme preparation was nearly identical to other enzymes. The homogeneity was checked by polyacrylamide gel electrophoresis. One single band was observed even at high protein loading on the gel. However it should be noted that a very intense staining of alcohol oxidase with Coomassie brilliant blue was observed resulting in a large band for the protein.

Consequently, we decided to check the

FIG. 1. Immunochemical Precipitation of the P. pastoris Alcohol Oxidase.
A, cell free extract (3 μg of protein); B, purified alcohol oxidase (0.5 μg of protein).
purity of our preparation by several other methods. The protein showed a single peak by ultracentrifugation, one band by electrofocalization and one NH₂ terminal amino acid (see later). Finally, we found one line of precipitation by the immunochemical method of Bjerrum34) (Fig. 1). A cross reaction was also observed between the enzymes from P. pastoris and H. polymorpha.

**Molecular weight and subunit structure**

The sedimentation coefficient of our protein was $S_{20,w} = 20.0S$. It was measured in a 50 mm KPB pH 7.5 at enzyme concentrations from 2.9 to 5.1 mg/ml. No dependance with the concentration was detected.

The molecular weight of the protein was estimated by the equilibrium technique of Yphantis.31) From the slope of the curve log $c$ vs. $r^2$ and a partial specific volume of 0.753 cm³/g, a molecular weight of 675,000 was calculated. This value was in good agreement with the elution pattern of the enzyme in a gel filtration on Ultrogel AcA 34.

With the high value of molecular weight the protein might contain subunits. Indeed they were detected by polyacrylamide gel electrophoresis in presence of SDS 1%. Both denatured enzyme (treated 10 min at 60°C with SDS 1%) and denatured and reduced enzyme (same conditions but with 1% β-mercaptoethanol) gave a single band of molecular weight 80,000.

These results suggest that the alcohol oxidase molecule is formed of eight subunits, probably identical, and not linked by disulfide bridges.

**Flavin content**

The probable presence of a flavinic coenzyme was first detected by the absorption spectrum of the protein in the visible region. As shown in Fig. 2 two absorption maxima were found at 370 nm and 447 nm. Values of 375 nm and 450 nm are reported for pure FAD. The involvement of FAD in methanol oxidation was shown by addition of methanol under nitrogen. A decrease in the intensity of absorption at both wavelengths was observed. The initial spectrum was recovered after oxygen bubbling.

The fluorescence spectrum is given in Fig. 3. Two excitation wavelengths were found at 375
and 440 nm and maximum intensity of emission was observed at 545 nm for the methanol oxidase and 535 nm for pure FAD. It should be noted that the intensity of fluorescence was much less (5%) for the FAD in the protein than free at the same concentration. The intensity of fluorescence was depressed (39%) by addition of methanol under nitrogen and the initial value was recovered after oxygen bubbling.

Finally the presence of FAD was confirmed by direct identification by paper chromatography of the extract obtained after treatment with ammonium sulfate under acidic conditions. A single spot with the same Rf value than pure FAD was obtained. A quantitative determination by spectrophotometry gave 7.3 mol of FAD per mol of enzyme (MW 675,000). Then each subunit contains one mol of FAD.

Table III. Amino Acid Composition of Alcohol Oxidases

<table>
<thead>
<tr>
<th></th>
<th>P. pastoris IFP 206</th>
<th>H. polymorpha</th>
<th>Kloeckera</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys</td>
<td>41</td>
<td>39</td>
<td>44</td>
</tr>
<tr>
<td>His</td>
<td>23</td>
<td>21</td>
<td>19</td>
</tr>
<tr>
<td>Arg</td>
<td>35</td>
<td>39</td>
<td>38</td>
</tr>
<tr>
<td>Asp</td>
<td>88</td>
<td>106</td>
<td>94</td>
</tr>
<tr>
<td>Thr</td>
<td>43</td>
<td>50</td>
<td>43</td>
</tr>
<tr>
<td>Ser</td>
<td>41</td>
<td>56</td>
<td>53</td>
</tr>
<tr>
<td>Glu</td>
<td>63</td>
<td>64</td>
<td>73</td>
</tr>
<tr>
<td>Pro</td>
<td>46</td>
<td>50</td>
<td>47</td>
</tr>
<tr>
<td>Gly</td>
<td>69</td>
<td>64</td>
<td>66</td>
</tr>
<tr>
<td>Ala</td>
<td>48</td>
<td>41</td>
<td>48</td>
</tr>
<tr>
<td>Val</td>
<td>40</td>
<td>35</td>
<td>29</td>
</tr>
<tr>
<td>Met</td>
<td>17</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>Ile</td>
<td>37</td>
<td>29</td>
<td>24</td>
</tr>
<tr>
<td>Leu</td>
<td>60</td>
<td>52</td>
<td>48</td>
</tr>
<tr>
<td>Tyr</td>
<td>33</td>
<td>31</td>
<td>38</td>
</tr>
<tr>
<td>Phe</td>
<td>30</td>
<td>36</td>
<td>32</td>
</tr>
<tr>
<td>1/2 Cys</td>
<td>11</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td>Trp</td>
<td>8</td>
<td>11</td>
<td>17</td>
</tr>
<tr>
<td>Total</td>
<td>733</td>
<td>739</td>
<td>751</td>
</tr>
</tbody>
</table>

Structural properties

The amino acid composition of the P. pastoris alcohol oxidase is given in Table III and compared with that of H. polymorpha and Kloeckera. These profiles are not very different except for the higher content in methionine of the P. pastoris enzyme. A calculation of the minimum molecular weight of the subunits by the method of Delaage gave a value of 81,000 in good agreement with the other determination.

The NH₂ terminal amino acid was found to be an alanine residue by dansylation. This result was confirmed by the sequence given by the automatic sequencer using Edman degradation:

Ala-Ile-Pro-Glu-Glu-Phe-Asp-Ile-Leu-Val-Leu-Gly-

The high yield in alanine for the first degradation step (84%) strongly suggest that all the subunits have the same NH₂ terminal. Then all the subunits must be identical.

Isoelectric point of the enzymes from P. pastoris and H. polymorpha were respectively 6.3 and 6.2 as determined by electrophoresis on polyacrylamide gel. Free —SH groups were detected and titrated with the Ellman reagent. In non denaturing conditions, 12 —SH groups per mole were titrated. Higher values of 65 and 64 —SH groups per mol were
Oxidation of Methanol by the Yeast Pichia pastoris

FIG. 4. Effect of Temperature on Alcohol Oxidase Activity.
A: Effect of temperature on activity of the alcohol oxidases from P. pastoris (●—●) and H. polymorpha (○—○).
B: Arrhenius plot of data presented in A.
C: Stability against temperature. The enzyme solutions were incubated for 10 min at different temperatures and the remaining activity was assayed at 37°C under standard conditions.

detected in the presence of 0.7% SDS in the enzymes from P. pastoris and H. polymorpha respectively. Occurrence of free −SH groups in alcohol oxidases must be paralleled to the high sensitivity of these enzymes to hydrogen peroxide. It is well known that enzyme inactivation occurred by treatment with PCMB.13) In our case the number of free −SH groups fall to 36 per mol after a 30 min treatment at 0°C with a 10 mM solution of hydrogen peroxide. Under these conditions the remaining enzyme activity was less than 1%. This is the first evidence that the enzyme inactivation by hydrogen peroxide proceeds via the oxidation of essential −SH groups. Reactivation of the alcohol oxidase by reducing agents was observed after treatment of the enzyme with hydrogen peroxide solutions.19)

Effect of temperature on alcohol oxidase activity
The optimum temperature for the P. pastoris enzyme was 37°C (Fig. 4A) which is significantly lower than the 45°C reported for the H. polymorpha enzyme by Kato12) and also found in our laboratory. A similar shift was also observed in the stability curves of both enzymes against temperature. Half inactivating temperatures of 38 and 48°C were found respectively (Fig. 4C). Activation energies of 11.1 and 10.8 kcal/mol were calculated from the Arrhenius plots (Fig. 4B) of the preceding data for P. pastoris and H. polymorpha respectively. This later value is higher than the 5.98 kcal/mol reported by Kato.12)

Effect of pH on the alcohol oxidase activity
From the pH activity curves given in Fig. 5 an optimum pH of 8.5 was observed for the H. polymorpha enzyme which is in good agreement with the reported value.12) In sharp contrast the P. pastoris enzyme showed maximum activity near pH 7.5 and very low activity above pH 9. This is mainly due to the relative instability of the later enzyme above pH 9 as shown in Fig. 6. Both alcohol oxidases were unstable at acidic pH.

Substrate kinetics
The kinetic of methanol oxidation by the P. pastoris enzyme followed Michaelis Menten kinetic as shown in Fig. 7. However the
apparent Michaelis constant and catalytic constants were function of substrate concentrations. For instance the $K_m$ (app) for methanol was varied from 1.4 to 3.1 mM for oxygen concentrations of 0.19 and 0.93 mM (Table IV). Very similar values were reported\textsuperscript{13} for the $H. polymorpha$ enzyme: 1.2 and 2.8 mM at the same oxygen concentrations. Similarly (Fig. 8) the $K_m$ (app) for oxygen varied from 0.4 to 1 mM for methanol concentrations of 1 to 100 mM. Values of 0.24 and 0.4 mM were reported for the $H. polymorpha$ enzyme.\textsuperscript{13}

**Alcohol specificity**

The alcohol oxidase was not specific for methanol since other primary alcohols like ethanol and propanol were also oxidized (Table V). However the activity rapidly decreased with chain length of primary alcohols and no activity was detected with $n$-octanol. Secondary alcohols were oxidized at much lower rate than the relative primary ones.

---

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
Oxygen Concentration* (mM) & $K_m$ (app) for methanol (mM) & Methanol Concentration** (mM) & $K_m$ (app) for oxygen (mM) \\
\hline
0.19 & 1.4 & 1 & 0.4 \\
0.93 & 3.1 & 10 & 0.7 \\
 & & 100 & 1.0 \\
\hline
\end{tabular}
\caption{Apparent Affinities of Methanol and Oxygen}
\end{table}

---

\textsuperscript{*} Activity was assayed by the standard procedure.
\textsuperscript{**} Activity was assayed by oxygen consumption with an oxygraph.
FIG. 8. Effect of Oxygen Concentration on Alcohol Oxidase Activity.

Activity was assayed with a Gilford oxygraph by monitoring oxygen consumption. Concentration of methanol 1 mM (■—■), 10 mM (○—○) and 100 mM (●—●).

TABLE V. SUBSTRATE SPECIFICITY OF ALCOHOL OXIDASE

| Substrate    | Relative activity (%)
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>100</td>
</tr>
<tr>
<td>Ethanol</td>
<td>82</td>
</tr>
<tr>
<td>Propanol-1</td>
<td>43</td>
</tr>
<tr>
<td>Propanol-2</td>
<td>2</td>
</tr>
<tr>
<td>Butanol-1</td>
<td>20</td>
</tr>
<tr>
<td>Isobutanol</td>
<td>1.2</td>
</tr>
<tr>
<td>Butanol-2</td>
<td>0.2</td>
</tr>
<tr>
<td>tert-Butanol</td>
<td>0</td>
</tr>
<tr>
<td>n-Octanol</td>
<td>0</td>
</tr>
</tbody>
</table>

Activities were assayed by the oxygen consumption rate with an oxygraph. The oxygen and alcohol concentrations were 0.93 mM, respectively.

These results agreed well with the specificity of alcohol oxidases from *H. polymorpha*, *Kloeckera* and *Candida*. Inhibition by hydrogen peroxide

Without added catalase, the purified alcohol oxidase is rapidly inhibited by one reaction product: hydrogen peroxide. In our case a 50% inhibition was observed at 30°C after a 20 min treatment with 0.8 mM solution of hydrogen peroxide. Values of 7 and 0.92 mM were reported for the alcohol oxidase from *H. polymorpha* and *Kloeckera*. Protection against inactivation was observed in the presence of mercaptoethanol or dithiothreitol at a concentration of 1 mM. This protection was not due to a direct reaction of hydrogen peroxide with the reducing agent since reactivation of the enzyme could be observed. For instance when a solution containing 3.5 units/ml of alcohol oxidase was incubated at 0°C with 1 mM hydrogen peroxide for 20 min, the remaining activity was 2.3 units/ml. Total recovery of activity was obtained after a 10 min treatment at 0°C with 1 mM β-mercaptoethanol. These results strongly suggest that some essential -SH groups were reversibly oxidized by hydrogen peroxide.

DISCUSSION

The oxidation of methanol by the yeast, *P. pastoris*, proceeds very likely via alcohol oxidase, formaldehyde and formate dehydrogenases. The cell levels of these enzymes are very similar to the ones observed in other yeasts like *H. polymorpha*. Then the high yield observed with *P. pastoris* (0.42 g of cell per g of methanol) was not due to a better utilization of the energy of methanol oxidation to formaldehyde by an alcohol dehydrogenase. The exact reason for the observed high yield may be in the mechanism of methanol assimilation rather than the methanol oxidation. Some structural and enzymatic properties of the alcohol oxidase from *P. pastoris* are summarized in Table VI and compared with those of the enzymes from the yeasts *H. polymorpha*, *Kloeckera* and *Candida*. Like the other enzymes the alcohol oxidase from *P. pastoris* is a flavoprotein of high molecular weight (675,000) composed of eight identical subunits containing one mol of FAD per mol of enzyme. The absorption spectrum showed maxima at 370 and 447 nm. The *P. pastoris* and *H. polymorpha* enzymes showed a close resemblance in isoelectric point and free -SH groups, and had the same NH₂ terminal
The main difference between these enzymes was observed in optimum temperature. The value of 37°C for *P. pastoris* is in the same range as that found for the other yeasts except for *H. polymorpha*. The fact that optimum temperatures for enzyme activities are in the same range as optimum growth temperatures of the microorganisms confirms the conclusion that limitation of growth at high temperature was due to alcohol oxidase inactivation.\(^{12}\)

It may be also pointed out that at optimum growth temperature the rate limiting step in methanol metabolism might be the oxidation of methanol. Then one may expect that a small variation in the rate of methanol oxidation affects the growth rate of the microorganism. This variation will be facilitated by the fact that the enzyme maximal velocity is not reached because of the low affinity of methanol for the oxidase. Then the rate of methanol oxidation will be dependent on the methanol concentration and lower growth rate are expected at low methanol concentrations.

In continuous culture at low dilution rates, the methanol concentrations are low but the growth rate remains high.\(^{13}\) The rate of methanol oxidation is maintained constant by increasing the alcohol oxidase concentration in the cell to the high values observed in this paper or by others.\(^{13,19}\)

The apparent Michaelis constant of methanol and oxygen for alcohol oxidase are higher than the concentrations of these substrate during cell growth. When a rapid increase for instance in methanol concentration occurred it was observed that cell growth stopped (P. Jara and J. Baratti, unpublished results). For instance, the wash-out technique for the determination of maximum growth rate could not be applied to *P. pastoris* in our hands. The growth of the yeast was completely inhibited by a fast increase in the methanol concentration. After such an inhibition the specific activity of the alcohol oxidase falls to less than 1% of the original value indicating that growth probably ceased by lack of methanol oxidation.

---

**Table VI. Comparison of Structural and Enzymatic Properties of Alcohol Oxidases**

<table>
<thead>
<tr>
<th></th>
<th><em>P. pastoris</em></th>
<th><em>H. polymorpha</em></th>
<th><em>Kloeckera</em>(^{12})</th>
<th><em>Candida boidini</em>(^{12})</th>
<th><em>Candida N-16</em>(^{11,16})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>675,000</td>
<td>669,000(^{12})</td>
<td>673,000</td>
<td>600,000</td>
<td>600,000</td>
</tr>
<tr>
<td>Molecular weight of subunits</td>
<td>80,000</td>
<td>83,000(^{12})</td>
<td>83,000</td>
<td>74,000</td>
<td>—</td>
</tr>
<tr>
<td>(S_{20,w})</td>
<td>20.0</td>
<td>19.8(^{12})</td>
<td>19.8</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>(\delta) (cm(^3)/g)</td>
<td>0.753</td>
<td>0.743(^{12})</td>
<td>0.743</td>
<td>0.733</td>
<td>—</td>
</tr>
<tr>
<td>(E_{1%})</td>
<td>20.9</td>
<td>16.5(^{12})</td>
<td>23.6</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Absorption maxima</td>
<td>370, 440 nm</td>
<td>373, 398, 470 nm</td>
<td>372, 395, 460 nm(^{12})</td>
<td>383, 458 nm</td>
<td></td>
</tr>
<tr>
<td>Fluorescence maxima</td>
<td>545 nm</td>
<td>545 nm</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Isoelectric point</td>
<td>6.3</td>
<td>6.2</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>N-Terminal residue</td>
<td>Ala</td>
<td>Ala</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>SH group per molecule</td>
<td>65</td>
<td>64</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Optimum temperature (°C)</td>
<td>37</td>
<td>45</td>
<td>35</td>
<td>30</td>
<td>35</td>
</tr>
<tr>
<td>Activation energy (kcal/mol)</td>
<td>11.1</td>
<td>5.98(^{12})</td>
<td>5.98</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Heat stability*</td>
<td>38</td>
<td>50(^{12})</td>
<td>43</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Inhibition by (H_2O_2)**</td>
<td>0.8 mm</td>
<td>0.98 mm</td>
<td>0.92 mm</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* Temperature resulting in 50% inactivation for a 10 min treatment.
** Concentration giving 50% inhibition for a 20 min treatment at 30°C.
Oxidation of essential -SH groups of the alcohol oxidase might be the reason of the observed inhibition since hydrogen peroxide might be produced in high amount as a result of an increase rate of methanol oxidation.

In vitro, protection against the hydrogen peroxide inactivation was obtained in the presence of reducing agents. In vivo, it is well known that the substrate of formaldehyde dehydrogenase is not formaldehyde itself but the hemimercaptal formed spontaneously by reaction with reduced glutathione. Then one may expect that a reducing environment is maintained around the alcohol oxidase and constitutes a second barrier to hydrogen peroxide inactivation, the first barrier being the presence of the catalase which is able to degrade hydrogen peroxide by catalasic or peroxidative reactions.

Acknowledgments. The authors wish to thank M. Ballerini and the Institut Francais du Pétrole for given us cells of P. pastoris and interest during this work. The technical assistance of J. Bonicel for sequence determination, P. Sauve for ultracentrifugation analysis and A. Guidoni for amino acid analysis are gratefully acknowledged.

REFERENCES