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Purification and Characterization of Alcohol Oxidase from a Genetically Constructed Over-producing Strain of the Methylotrophic Yeast *Hansenula polymorpha*

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Abstract—Alcohol oxidase (AOX) has been purified 8-fold from a genetically constructed over-producing strain of the methylotrophic yeast *Hansenula polymorpha* C-105 (*gcr1 catX*) with impaired glucose-induced catabolite repression and completely devoid of catalase. The final enzyme preparation was homogeneous as judged by polyacrylamide gel electrophoresis and HPLC. Some physicochemical and biochemical properties of AOX were studied in detail: molecular weight (~620 kD), isoelectric point (p*I* 6.1), and UV-VIS, circular dichroism (CD), and fluorescence spectra. The content of different secondary structure motifs of the enzyme has been calculated from the CD spectra using a computer program. It was found that the native protein contains about 50% α-helix, 25% β-sheet, and about 20% random structures. The kinetic parameters for different substrates, such as methanol, ethanol, and formaldehyde, were measured using a Clark oxygen electrode. The rate of enzymatic oxidation of formaldehyde by alcohol oxidase from *H. polymorpha* is only twice lower compared to the best substrate of the enzyme, methanol.

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Key words: alcohol oxidase, over-producing strain, secondary structure, spectral and kinetic properties

Alcohol oxidase (alcohol:oxygen oxidoreductase, EC 1.1.3.13) is an oligomeric enzyme with molecular weight approximately 600 kD consisting of eight identical subunits arranged in a quasi-cubic orientation, each containing a strongly bound flavin adenine dinucleotide (FAD) cofactor [1]. The binding of the cofactor to the protein depends rather on the adenine than on the flavin moiety. The adenine moiety is believed to bind to the N-terminal domain of each monomer, which contains a typical nucleotide-binding fold [2]. It has been found that the FAD molecules are bound to the surface of the monomers rather than to the interface between subunits [3].

Alcohol oxidase (AOX) catalyzes the oxidation of primary low molecular weight alcohols in the presence of dissolved oxygen to produce the corresponding aldehydes and hydrogen peroxide. The enzyme has been found in a number of methanol-utilizing yeasts [4-6]. Therefore, AOX is available for the determination of lower alcohols and formaldehyde and it has been widely used for the construction of alcohol sensors, in conjunction with oxygen and hydrogen peroxide sensors [7, 8]. During the catalytic reaction, the AOX cofactor (FAD) is first reduced to its hydrogenated form ($FADH_2$) and then re-oxidized to its native form by molecular oxygen, with a concomitant formation of hydrogen peroxide. The kinetics of AOX has been studied by two different strategies: by monitoring the consumption of oxygen, e.g., using a Clark oxygen electrode, or by monitoring the production of hydrogen peroxide [9].

The basic biochemical and kinetic properties including thermal and operational stabilities of AOX from the methylotrophic yeast *Hansenula polymorpha* have been studied previously [5, 10, 11]. Electron microscopy and

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image analysis of two-dimensional crystals and single molecules of AOX from *H. polymorpha* were also performed [1]. However, to our knowledge no paper describing three-dimensional structure of any AOX has been published so far. Thus the investigation of the structure of AOX by spectroscopic analysis can result in important and significant information about the enzyme functioning.

In this paper we report on the purification and characterization of AOX produced by a genetically constructed over-producing mutant strain of the methylotrophic yeast *H. polymorpha* C-105 (*gcr1 catX*) including molecular weight, subunits structure, p*I*, UV-VIS, circular dichroism (CD), and fluorescence spectra, and kinetic parameters. Additionally, we have investigated the secondary structure motifs of the enzyme based on the CD spectra. The physicochemical and kinetic properties are compared with those of the enzymes obtained from other sources as well as with data reported previously for AOX from *H. polymorpha*.

MATERIALS AND METHODS

Chemicals. DEAE-cellulose Toyopearl 650M was from Toyo Soda (Japan); ampholine and standard set of markers for gel filtration were from Pharmacia LKB Biotechnology (Sweden); Coomassie G-250 and R-250, EDTA, and markers for electrophoresis and isoelectrofocusing were from Serva (Germany); 2-mercaptoethanol was from Ferak (Germany); paraformaldehyde and phenylmethylsulfonyl fluoride (PMSF) were from Sigma (USA). Tris and glycine were from ICN (USA); acrylamide, N,N′-methylenebisacrylamide, and ammonium persulfate were from Reanal (Hungary); Na_2HPO_4, KH_2PO_4, $MgSO_4$, $CaCl_2$, and NaCl were from Merck (Germany); methanol, ethanol, formaldehyde, KCl, H_3PO_4, $(NH_4)_2SO_4$, and KOH were of the highest purity available from domestic sources. Buffers were prepared using water (18 MΩ) purified with a Milli-Q system (Millipore, USA).

Formaldehyde solution (1 M) was prepared by hydrolysis of paraformaldehyde (300 mg in 10 ml water) by heating in a sealed ampoule at 105°C for 6 h.

Strains. As a producer of alcohol oxidase, the mutant strain of the thermotolerant methylotrophic yeast *H. polymorpha* C-105 (*gcr1 catX*) constructed in the Institute of Cell Biology (Lviv, Ukraine) [12, 13] was used. It has an impairment in glucose catabolite repression of AOX synthesis, is catalase-defective, and has the ability to over-produce AOX in glucose medium.

Cultivation and preparation of cell-free extracts. Cells of the mutant *H. polymorpha* C-105 were cultivated in flasks on a shaker (200 rpm) at 30°C to the middle of the exponential growth phase (~24 h) in medium containing (g/liter): glucose, 10; $(NH_4)_2SO_4$, 3.5; KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2$, 0.1; yeast extract, 3.0. The pH of the medium was adjusted to 5.5 with KOH.

After washing, freshly grown cells (about 15 g wet weight) were resuspended in two volumes of 0.05 M phosphate buffer, pH 7.5. The cells were disrupted in the presence of glass beads (d = 0.45-0.50 mm) in a planetary disintegrator at 1000 rpm (r_{av} 10 cm) and 4°C for 6 min. Whole cells and cell debris were removed by centrifugation at 15,000 rpm (r_{av} 8 cm) for 40 min. The supernatant was used as the cell-free extract for isolation of AOX. Activity of AOX was determined by the rate of hydrogen peroxide formation in reaction with methanol as monitored by the peroxidative oxidation of *o*-dianisidine in the presence of horseradish peroxidase [14]. The millimolar extinction coefficient of the colored product in acidic solution (2.5 M HCl) at 525 nm was shown to be 13.38 $mM^{-1} \cdot cm^{-1}$. Protein concentration was determined by the Lowry method or spectrophotometrically in accordance with methodology presented in [15].

Isolation and purification of AOX. AOX was isolated from cell-free extract of the mutant C-105 by a two-step precipitation with ammonium sulfate (at 40 and 60% of saturation) in the presence of 1 mM EDTA and 0.4 mM PMSF to inhibit proteases. At 40% saturation, the protein precipitate was discarded, and the AOX precipitate obtained at 60% saturation was collected by centrifugation.

Low performance ion-exchange liquid chromatography was carried out for purification of the crude preparation of AOX using a DEAE-cellulose Toyopearl 650 M column. The enzyme was eluted from the column using a linear gradient of phosphate buffer, pH 7.0, with the concentration range 50-500 mM. The analytical variant of HPLC for AOX was carried out in a TSK G 4000 SW TOSOHAAS column (Japan) using a series 1100 chromatographic system (Hewlett Packard, USA).

Analysis of AOX preparations by electrophoresis. Electrophoretic patterns of proteins from methylotrophic yeasts were obtained by native and sodium dodecyl sulfate electrophoreses in 6 and 10% polyacrylamide gels (SDS-PAGE), respectively, in accordance with the methods of Davis [16] and Laemmli [17]. The gels were stained with Coomassie Blue G-250 and R-250 to reveal protein bands (for native and SDS-PAGE, respectively) and by a mixture for assay of AOX [14] to visualize enzymatically active bands (for native electrophoresis).

Physicochemical characterization of AOX. The molecular mass of native AOX was determined by high performance size-exclusion liquid chromatography on a TSK G 4000 SW column calibrated with a standard set of markers (thyroglobulin, 669 kD; ferritin, 440 kD; catalase, 232 kD; aldolase, 158 kD) from Pharmacia LKB Biotechnology. The column was eluted with 50 mM phosphate buffer, pH 7.5, containing 0.5 M KCl with a flow rate of 0.5 ml/min, and the proteins were detected spectrophotometrically at 280 nm.

The molecular mass of the AOX subunit was calculated from the electrophoretic mobility values of AOX and a set of the standard proteins after SDS-PAGE (Fig. 1).

Analytic isoelectric focusing was performed in a 5% horizontal polyacrylamide gel with ampholines (gradient pH 3-10) using the following p*I*-markers: basic lentil lectin (p*I* 8.65), middle lentil lectin (8.45), acidic lentil lectin (8.15), basic band myoglobin (7.35), human carbonic anhydrase B (6.55), bovine carbonic anhydrase B (5.85), β-lactoglobulin A (5.20), trypsin inhibitor (4.55), methyl red dye (3.75), amyloglucosidase (3.50). The gels were stained with Coomassie Blue R-250 [18].

The p*I* value for AOX was calculated by measuring the running paths for AOX and markers as well as by direct measuring of pH in gel slices.

Spectral investigation. Absorbance spectra for AOX were recorded with a Beckman Coulter DU 650 spectrophotometer (Germany) with different scan rates and slits widths. Fluorescent spectra were recorded with a Shimadzu spectrofluorometer RF-5301 (Japan) with a scan rate of 120 nm/min and a bandwidth of 5 nm. Circular dichroism (CD) spectra were recorded using a Jasco J-715 spectropolarimeter (Japan) with a scan rate of 120 nm/min and a bandwidth of 1 nm. The parameters of the secondary structures were calculated using an in house computer program Protein-CD (Russia) [19].

The FAD content in the enzyme was calculated by the method described in [20].

Kinetic study. When substrate specificity was investigated, AOX activity was determined by estimation of the initial rates of oxygen consumption using a Clark type oxygen electrode (final volume 1 ml) at 20°C in 0.1 M phosphate buffer, pH 7.5, with constant stirring. Protein concentration was determined by the Lowry method. The concentrations of substrates were chosen to ensure a measurable linear rate for the first 40 sec of the reaction, which was started by the addition of AOX. The potential at the platinum electrode of the Clark electrode was controlled by a three-electrode potentiostat (BAS CV-50W Electrochemical Analyzer with BAS CV-50W software v. 2.1; Bioanalytical Systems, USA). The Clark electrode was of the two-electrode type. The kinetic parameters (K_m and k_{cat}) of the enzymatic reaction catalyzed by AOX were calculated using the Michaelis–Menten equation with the Microcal Origin program to take into account the initial concentration of oxygen in the buffer to be 0.26 mM.

RESULTS AND DISCUSSION

As mentioned in the introduction, the goal of the present work was to purify and characterize AOX from the genetically constructed mutant strain of the thermotolerant methylotrophic yeast *H. polymorpha* C-105 (*gcr1 catX*) [12, 13] and to compare the properties of the isolated AOX with the enzyme from other sources. The used AOX-overproducing strain, isolated at the Institute of Cell Biology, is impaired in glucose catabolite repression of AOX synthesis, is catalase-defective, and has the ability to over-produce AOX in glucose medium. During growth of the mutant cells in a medium without methanol, formaldehyde is not produced and formation of the modified AOX with covalently bound formaldehyde on lysine residues is avoided. As suggested for *in vivo* conditions, such reversible non-enzymatic modification of AOX is important for formaldehyde-buffering activity of the protein and plays a detoxifying role during methylotrophic growth [21]. Probably, due to this reaction some AOX preparations isolated from methylotrophically grown cells can produce hydrogen peroxide under conditions without adding any substrate. This process can negatively influence analytical procedures resulting in increased output of the blank samples. Another advantage of the used mutant strain is its defect in the catalase-coding gene that avoids some chromatographic procedures necessary to separate catalase from cell-free extracts during purification of AOX. Due to AOX-overproducing ability, isolation of AOX from the constructed mutant strain is much more economical when compared to the procedure with wild type strains.

The AOX was purified from a culture of the methylotrophic yeast *H. polymorpha* C-105, which was grown in medium containing 1% glucose as a carbon source. The

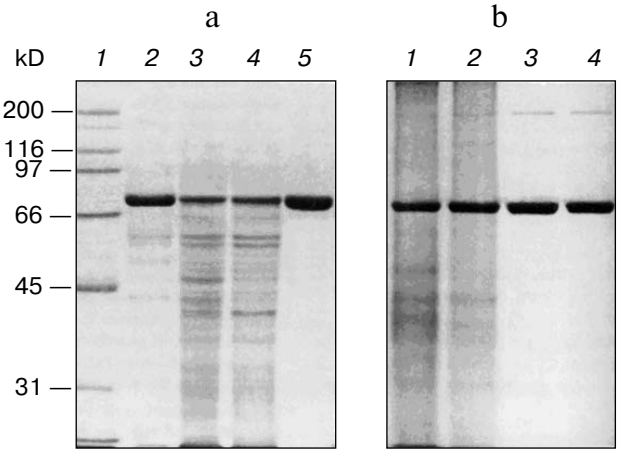

Fig. 1. Electrophoretic patterns of different preparations of alcohol oxidase (AOX) from methylotrophic yeast *H. polymorpha* C-105 (*gcr1 catX*) after SDS (a) and native (b) electrophoreses in 10 and 6% polyacrylamide gel, respectively. a) Lanes: *1*) protein standards; *2*) commercial preparation of AOX from *P. pastoris*, 5 μg protein; *3*) cell-free extract, 30 μg protein; *4*) crude preparation of AOX after second step of precipitation by ammonium sulfate (at 40-60% of saturation), 20 μg protein; *5*) AOX preparation after chromatography on DEAE-cellulose, 5 μg protein. b) Lanes: *1*) cell-free extract, 30 μg protein; *2*) crude preparation of AOX after second step of precipitation by ammonium sulfate (at 40-60% of saturation), 20 μg protein; *3*) AOX preparation after chromatography on DEAE-cellulose, 5 μg protein; *4*) commercial preparation of AOX from *P. pastoris*, 5 μg protein.

Table 1. Isolation and purification of AOX from the mutant strain *H. polymorpha* C-105 (*gcr1 catX*)

Purification stage	Total protein, mg	Total activity, U	Specific activity, U/mg*	Yield, %**	Purification, fold
Crude extract	825	2475	3.0	100	1.0
Precipitation by $(NH_4)_2SO_4$ (40-60% of saturation)	350	1748	5.0	71	1.7
DEAE-cellulose chromatography	36	825	23.0	33	7.7

* One unit of AOX activity (U) is defined as 1 μmol of H_2O_2 produced per minute at 30°C under standard assay conditions.
** Calculations of yields were carried out per liter of batch culture that corresponds to 15 g wet weight cells.

enzyme was purified by precipitation with ammonium sulfate (at 40-60% of saturation) followed by ion-exchange column chromatography. The purification procedures are summarized in Table 1, indicating eightfold purification with a yield of 33%. The specific activity of the final preparation was 23.0 units per mg protein. The homogeneity of the final enzyme preparation was proved by SDS-PAGE (Fig. 1) and HPLC chromatography (data not shown).

The molecular mass of native AOX was estimated to be approximately 620 kD by gel filtration chromatography on a TSK G 4000 SW column (data not shown). SDS-PAGE showed one protein band at about 80 kD (Fig. 1). From these results, alcohol oxidase is suggested to consist of eight identical subunits. The molecular weights of AOX from various methanol-utilizing yeasts were reported to be from 600 to 670 kD, and they consisted of eight subunits [4-6, 22]. From these results, it is clear that the molecular structure of the enzyme is similar to other alcohol oxidases. The isoelectric point of the alcohol oxidase determined by isoelectric focusing was found to be approximately 6.1. This value is very close to the value of 6.3 that is predicted on the basis of the AOX gene sequence, and this is consistent with our suggestion about the absence of post-translational modifications of this protein in mutant cells grown on glucose medium.

The absorption spectrum of native AOX has one maximum at 280 nm (data not shown) and two absorption

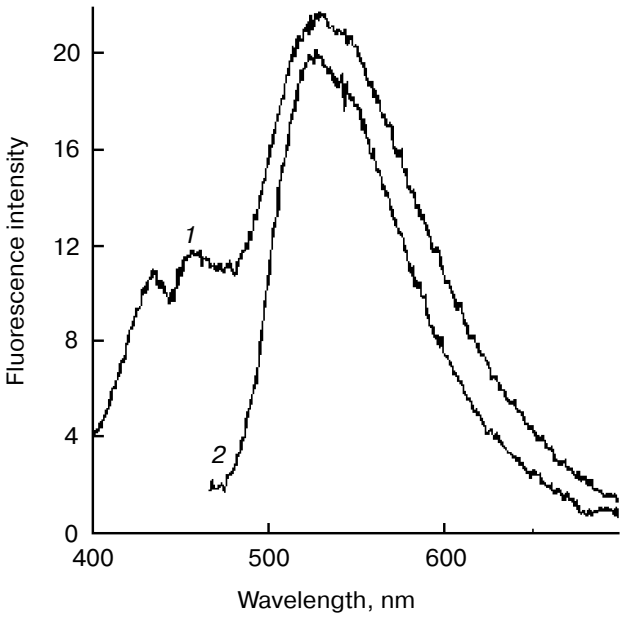

Fig. 2. Fluorescence emission spectra of AOX in 0.01 M potassium phosphate buffer, pH 7.5: *1*, *2*) excitation wavelength is 385 and 455 nm, respectively. The concentration of alcohol oxidase was 1.4 mg/ml.

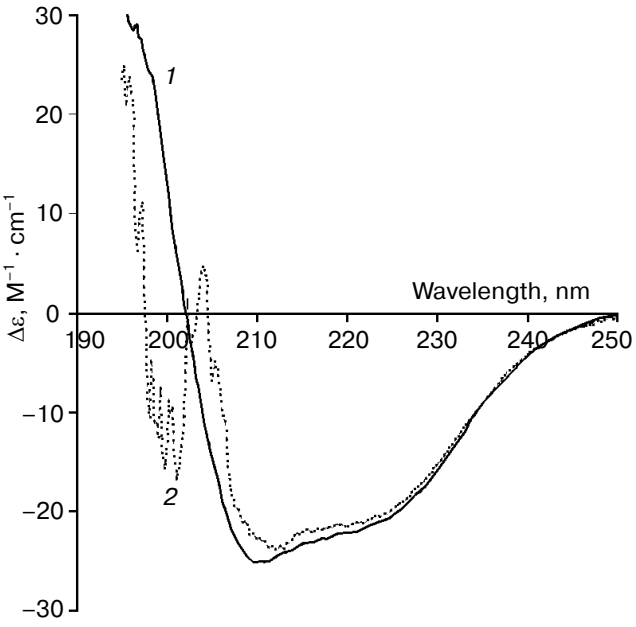

Fig. 3. Circular dichroism spectra of *H. polymorpha* alcohol oxidase (AOX) in the absence (*1*) and presence (*2*) of NaN_3. The concentration of AOX was 0.14 mg/ml in 0.01 M potassium phosphate buffer, pH 7.5.

maxima at 385 and 455 nm. The ratio of the light absorptions at 385 and 455 nm was found to be 1.30. Previously, peak values of 375 and 450 nm were reported for pure FAD [23]. In the presence of competitive inhibitor of the enzyme (NaN_3) the absorption spectrum of AOX changed and exhibited one absorption maxima at 370 nm and broad absorption in the region 400-600 nm with two peaks at 450 and 485 nm, as well as shoulder around 540 nm. Two characteristic absorption maxima with typical ratio of the light absorptions in the spectrum of the native AOX as well as significant spectral changes of the enzyme in the presence of NaN_3 suggest the contribution of a flavin derivative to the enzyme prosthetic group [5, 24, 25].

Fluorescence analysis of the enzyme has been performed in the presence and absence of NaN_3, a reversible inhibitor of AOX. Typical emission fluorescence spectra of native AOX are presented in Fig. 2. The maximum intensity of emission was observed at 530 nm for the native enzyme, and a value of 535 nm was reported previously for pure FAD [23]. It was found that fluorescence intensity of bound FAD significantly increased with protein denaturation, e.g., with time storage. The same results were obtained previously for *Pichia pastoris* AOX. It was even concluded that fluorescence intensity of the enzyme could serve as a criterion of its nativity [26]. It was also shown that the interaction of the enzyme with NaN_3 significantly decreased intensity of emission at 530 nm and stabilized the protein structure. These results are in good agreement with previously published data. It was found that the enzyme from *P. pastoris* has two azide binding sites, namely an allosteric site and another one in the region of the active center, and the latter site is responsible for the enzyme stabilization in the presence of NaN_3 [27].

Table 2. Characteristics of the secondary structure of AOX

Structure	AOX	AOX + NaN_3
α-Helix	53.0	34.0
γ-Helix	2.0	5.0
β-Antiparallel	4.0	27.0
β-Parallel	8.0	11.0
β-Turn	6.0	10.0
β-Turn type T1	7.0	0.0
Coil	6.0	2.0
S-Bend (PP)	14.0	11.0

Note: The secondary structures are presented in accordance with a classification given in [30] with some modifications.

Since all the spectral data for AOX from *H. polymorpha* are rather similar to those reported for AOXs from different sources (*Candida boidinii*, *Pichia* sp., *Poria* sp.) [20, 24, 28, 29], it can be concluded that the enzyme contains one FAD as the prosthetic group per subunit of the enzyme. Thus, the studied AOX is an oligomeric enzyme with molecular weight 620 kD consisting of eight identical subunits, each containing a tightly bound FAD cofactor.

CD spectra of the enzyme with and without NaN_3 were recorded in order to investigate the secondary structure of AOX. As shown in Fig. 3, the spectrum of the enzyme has a maximum at 210 nm, as well as shoulder at 250 nm. In the presence of NaN_3 the spectrum changed drastically, e.g., modification of the secondary structure of AOX could be observed. The content of different secondary structure motifs in the enzyme has been calculated on the basis of CD spectra using the computer program

Table 3. Kinetic parameters for AOXs from different sources

Source	Methanol		Ethanol		Formaldehyde	
	k_{cat}, sec^{-1}	K_m, mM	k_{cat}, sec^{-1}	K_m, mM	k_{cat}, sec^{-1}	K_m, mM
H. polymorpha C-105 (this study)	60	0.4	58	6.6	32	10.5
H. polymorpha (wild type) [5]	—	0.71	—	2.7	—	2.6
Candida boidinii [31]	—	0.42	—	1.78	—	—
Pichia pastoris [31]	—	0.85	—	3.68	—	—
Pichia sp. [28]	67	0.5	61	—	10	3.5
Poria contigua [24]	210	0.2	200	1.0	31	6.1

Notes: S.E.M. was calculated from five independent experiments and did not exceed 10%; "–", not determined; k_{cat} were calculated for octameric enzyme for all species and expressed in μmol O_2 per second per μmol of the enzyme.

Protein-CD v. 1.5 (Russia) [19]. The program compares the CD spectrum of interest (AOX in this paper) with the average of CD spectra associated with a cluster of related proteins with known secondary structures. Ideally the cluster would consist of proteins very similar to the protein of interest. But for AOX, to our best knowledge, no such examples of similar proteins are available. Therefore, clusters of proteins with known secondary structures and CD spectra from our database were constructed *ad hoc*. Average CD spectra for the clusters were correlated with the observed one. The best result was obtained for a cluster that contained 32 proteins with 99% correlation with the CD spectrum of AOX. On the basis of this cluster, the native protein was calculated to contain about 50% α-helix, 25% β-sheet, and about 20% random structures. The parameters of the secondary structure of the enzyme with and without NaN_3 are summarized in Table 2. Addition of NaN_3 resulted in significant changes in the secondary structure of AOX. Based on the spectral data (fluorescence and CD), it can be suggested that azide "ordered" and thereby stabilized the structure of AOX.

The kinetic parameters for different substrates, such as formaldehyde, methanol, and ethanol, were measured using a Clark oxygen electrode. The properties of the enzyme are summarized in Table 3. AOX oxidizes not only methanol but also lower primary alcohols (C_2-C_4) and formaldehyde. These data are in good agreement with previously published results about substrate specificity of AOX from different sources [20, 24, 28]. However, as can be seen from Table 3, the rate of enzymatic oxidation of formaldehyde by AOX from *H. polymorpha* is only twice lower compared to best substrate of the enzyme—methanol. This makes this enzyme attractive for application to different areas of biotechnology, including formaldehyde assay in food products, wastewater, and pharmaceuticals.

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REFERENCES

1. Vonck, J., and van Bruggen, E. F. J. (1990) *Biochim. Biophys. Acta*, **1038**, 74-79.
2. Wierenga, R. K., Terpstra, P., and Hol, W. G. J. (1986) *J. Mol. Biol.*, **187**, 101-107.
3. Averbakh, A. Z., Pekel, N. D., Seredenko, V. I., Kulikov, A. V., Gvozdev, R. I., and Rudakova, I. P. (1995) *Biochem. J.*, **310**, 601-614.
4. Fujii, T., and Tonomura, K. (1975) *Agric. Biol. Chem.*, **39**, 2325-2330.
5. Kato, N., Omori, Y., Tani, Y., and Ogata, K. (1976) *Eur. J. Biochem.*, **64**, 341-350.
6. Couderc, R., and Baratti, J. (1980) *Agric. Biol. Chem.*, **44**, 2279-2289.
7. Verduyn, C., van Dijken, J. P., and Scheffers, W. A. (1983) *Biotechnol. Bioeng.*, **25**, 1049-1055.
8. Guilbault, G. G., Palleschi, G., and Lubrano, G. (1995) *Biosens. Bioelectron.*, **10**, 379-392.
9. Nanjo, M., and Guilbault, G. G. (1975) *Anal. Chim. Acta*, **75**, 169-180.
10. Chen, H. F., Chen, T. J., and Fang, H. Y. (1988) *Zhongguo Nongye Huaxue Huizhi*, **26**, 287-301.
11. Azevedo, A. M., Cabral, J. M. S., Prazeres, D. M. F., Gibson, T. D., and Fonseca, L. P. (2004) *J. Mol. Cat. B: Enz.*, **27**, 37-45.
12. Gonchar, M. V., Ksheminska, G. P., Hladarevska, N. M., and Sibirny, A. A. (1980) in *Genetics of Respiratory Enzymes in Yeasts* (Lachowicz, T. M., ed.) *Proc. Int. Conf.*, Wroclaw University Press, Wroclaw, pp. 222-228.
13. Gonchar, M. V., Kostryk, L. B., and Sibirny, A. A. (1997) *Appl. Microbiol. Biotechnol.*, **48**, 454-458.
14. Sibirnyi, A. A., Titorenko, V. I., Efremov, B. D., and Tolstorukov, I. I. (1987) *Yeast*, **3**, 233-241.
15. Ehresmann, B., Imbault, P., and Weil, J. H. (1973) *Analyt. Biochem.*, **54**, 454-463.
16. Davis, B. J. (1964) *Ann. NY Acad. Sci.*, **121**, 404-427.
17. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
18. Osterman, L. A. (1983) *Study of Biological Macromolecules by Electrofocusing, Immunoelectrophoresis, and Radioisotopic Methods* [in Russian], Nauka, Moscow.
19. Shleev, S. V., Morozova, O. V., Nikitina, O. V., Gorshina, E. S., Rusinova, T. V., Serezhenkov, V. A., Burbaev, D. S., Gazaryan, I. G., and Yaropolov, A. (2004) *Biochimie*, **86**, 693-703.
20. Suye, S.-I. (1997) *Curr. Microbiol.*, **34**, 374-377.
21. Hopkins, T. R., and Muller, F. (1987) in *Microbial Growth on C1 Components* (van Verseveld, H. W., and Duine, J. A., eds.) Martinus Vijholf Publishers, Dordrecht, pp. 150-157.
22. Sahm, H., and Wagner, F. (1973) *Eur. J. Biochem.*, **36**, 250-256.
23. Whitby, L. G. (1953) *Biochem. J.*, **54**, 437-442.
24. Bringer, S., Sprey, B., and Sahm, H. (1979) *Eur. J. Biochem.*, **101**, 563-570.
25. Tani, Y., Miya, T., and Ogata, K. (1972) *Agr. Biol. Chem.*, **36**, 76-83.
26. Maskevich, A. A., Artsukevich, I. M., and Stepuro, V. I. (1997) *J. Mol. Struct.*, **408/409**, 261-264.
27. Maskevich, A. A., Artsukevich, I. M., Maskevich, S. A., and Chernikevich, I. P. (1997) *Bioorg. Khim.*, **23**, 479-485.
28. Patel, R. N., Hou, C. T., Laskin, A. I., and Derelanko, P. (1981) *Arch. Biochem. Biophys.*, **210**, 481-488.
29. Dickinson, F. M., and Wadforth, C. (1992) *Biochem. J.*, **282**, 325-331.
30. Kabsch, W., and Sander, C. (1983) *Biopolymers*, **22**, 2577-2637.
31. Woodward, J. R. (1990) *Adv. Autotroph. Microbiol. One-Carbon Metab.*, **1**, 193-225.