Chapter 15

Alcohol Oxidase from the Methylotrophic Yeast *Ogataea polymorpha*: Isolation, Purification, and Bioanalytical Application

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Abstract

Alcohol oxidase (EC 1.1.3.13; AOX) is a flavoprotein that catalyzes the oxidation of primary short-chain alcohols to corresponding carbonyl compounds with a concomitant release of hydrogen peroxide. It is a key enzyme of methanol metabolism in methylotrophic yeasts, catalyzing the first step of methanol oxidation to formaldehyde.

Here we describe the isolation and purification of AOX from the thermotolerant methylotrophic yeast *Ogataea* (*Hansenula*) *polymorpha*, and using this enzyme in enzymatic assay of ethanol, simultaneous analysis of methanol and formaldehyde, and in construction of amperometric biosensors selective to primary alcohols and formaldehyde.

Key words Alcohol oxidase, Isolation and purification, Methylotrophic yeast, Primary alcohols and formaldehyde, Enzymatic assay, Amperometric biosensor

1 Introduction

Alcohol oxidase (AOX, EC 1.1.3.13) is a peroxisomal enzyme detected in several genera of methylotrophic yeasts, such as *Candida*, *Pichia*, and *Ogataea* (*Hansenula*), that utilize methanol as a sole carbon and energy source [1]. The enzyme catalyzes the oxidation of methanol with oxygen to formaldehyde and hydrogen peroxide. AOX is a flavoprotein that contains FAD as a prosthetic group linked to the apoenzyme by noncovalent bonds. The native protein is an octamer consisting of eight identical FAD-containing subunits (664 amino acid residues) with a total molecular weight of about 600 kDa. The protein has a rather low isoelectric point: for example, the pI AOX from *Candida pastoris* is 5.7. Possibly, due to the high content of AOX in peroxisomes and the lower pH in these organelles (5.8–6.0), the enzyme easily transforms in vivo into an

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crystalloid state, fixed by electron microscopy. The structure of the corresponding genes and proteins are known for AOX of different origin, but a full X-ray analysis of the enzyme has been performed only for AOX from *Pichia pastoris* [2].

AOX is isolated in a highly purified state from several types of methylotrophic yeast. As a rule, a multistage procedure is used for purification, which includes mechanical destruction of cells, fractional precipitation with ammonium sulfate, ion exchange chromatography on DEAE-cellulose and gel filtration. The specific activity of the purified enzyme varies widely—from 5 to 30 and even 50 μmol/(min·mg), depending on the type of the yeast and purification scheme.

AOX is not a highly specific enzyme and, in addition to methanol, is able to oxidize in vitro other short aliphatic primary alcohols (from ethanol to hexanol), with virtually no effect on their secondary and tertiary isomers. The catalytic activity of the enzyme decreases with increasing length of the carbon chain of the substrate. Formaldehyde, which in its hydrated form is methylene glycol, is also oxidized by AOX with an efficiency of 13% when compared to methanol (Table 1).

AOX is able to oxidize allyl alcohol (relative efficiency compared to methanol—from 30 to 89%) with the formation of a very toxic compound, acrolein. This reaction is used for the positive selection of strains with reduced AOX activity (in particular, with impaired peroxisome biogenesis) by their resistance to allyl alcohol [5].

The concentration of dissolved oxygen as the second substrate of AOX significantly affects the catalytic activity of the enzyme. K_M for oxygen is 0.8 mM [6], which is significantly higher than the value of 0.23 mM, the saturated oxygen concentration at 30 °C in aqueous solutions at an air pressure of 1 atm. Moreover, oxygen concentration affects the kinetic parameters of AOX relative to alcohols. For example, the K_M for methanol, determined in oxygen saturated solutions, is three fold higher than the value obtained under air saturation.

Table 1
K_M (mM) values for AOX from various sources in reactions with alcohols [3] and formaldehyde [4]

	Substrate				
Yeast	**Methanol**	**Ethanol**	**Propanol-1**	***n*-Butanol-1**	**Formaldehyde**
O. polymorpha	0.712	2.7	27.3	54.6	2.6
P. pastoris	0.845	3.68	20.26	18.06	
C. boidinii	0.417	1.78	6.06	10.56	

Besides of to molecular oxygen, other electronic acceptors do not support the alcohol oxidase reaction, in particular, NAD^+, potassium ferricyanide, 2,6-dichlorophenolindophenol, methyl violet, methylene blue, and cytochrome *c*.

The temperature optimum of AOX of different origin differs slightly and is most shifted to higher temperatures for the enzyme from *O. polymorpha* (up to 45 °C), reflecting, perhaps, the relative thermophilicity of the strain and strongly contrasting with the behavior of the enzyme from *P. pastoris* (25 °C [3], although data with a value of 40 °C were presented [7]). AOX from *O. polymorpha* differs significantly from similar enzymes of other methylotrophic yeasts also in pH-optimum value: this enzyme has a wide range of optimum action—from pH 7 to 11, while for other AOXs there is a peak of maximum activity at pH of about 8. In addition, AOX from *O. polymorpha* is usually resistant to the inhibitory effect of chloride, whereas an enzyme from *P. pastoris* is very sensitive to the action of this anion.

The product of the alcohol oxidase reaction—hydrogen peroxide—adversely affects the enzymatic properties of the AOX, causing its inactivation. AOXs of most yeast species lose half their activity at a H_2O_2 concentration of 1–10 mM, while the enzyme from *P. pastoris* is markedly more resistant to peroxide inactivation, maintaining activity at 1 M H_2O_2 for 20 min at 30 °C [7]. The second oxidation product of methanol, formaldehyde, although being a substrate of AOX, is an inhibitor of the enzyme, especially at high concentrations. This is probably due to the ability of CH_2O to react with the functional groups of the protein, in particular, lysine residues. The functional role of covalent reversible adducts of AOX with formaldehyde in vivo is unknown, although it is suggested a specific buffer role of AOX in peroxisomes in maintaining a lower level of free formaldehyde in these organelles [7]. In addition to the binding of excess formaldehyde, this process can lead to a decrease in AOX activity and inhibition of further accumulation of this toxic metabolite in the cells.

AOX is completely inactivated by reagents for the sulfhydryl group (1 mM *p*-chloromercuribenzoate and 10 mM iodoacetamide), heavy metal ions (1 mM Cd^{2+}, Cu^{2+}, Hg^{2+}, and Ag^+), and sodium azide NaN_3. No metal activates AOX, which may explain the lack of enzyme inhibition by chelating agents, in particular, ethylenediaminetetraacetic acid (EDTA).

AOX is synthesized in cells of methylotrophically grown yeasts in large quantities—up to 37–40% of the total cellular protein, and with complete localization of the enzyme in peroxisomes, although the synthesis of the monomer chains occurs in the cytoplasm on membrane-bound endoplasmic reticulum. The need for protein synthesis is associated with a low AOX affinity for oxygen and methanol. The mechanism by which AOX enters the peroxisome and assembles into the crystal lattice has been extensively studied in

recent years. AOX monomer (Mr = 74 kDa) is known to be synthesized in the cytosol in the mature form, but how it binds to the FAD, translocates into the peroxisome, assembles into the octamer and is incorporated into the crystalloid matrix has not been studied in detail. AOX oligomerization has been shown to occur only after protein transfer to peroxisomes, after the prebinding of the monomers to the FAD [8].

A mutant strain C-105 (*gcr1 catX*) of the thermotolerant methylotrophic yeast *O. polymorpha* capable to overproduce AOX in glucose medium and avoiding catalase was selected [9, 10]. The simple scheme for the isolation and chromatographic purification of AOX was proposed and highly purified enzyme preparations were obtained [11, 12]. Partually purified enzyme was successfully used as a cheap catalyst of the bioreactor for removing formaldehyde from environmental media [13–15] and in enzymatic assay of primary alcohols and formaldehyde. Highly purified AOX was used as the biorecognition element of biosensors sensitive to primary alcohols and formaldehyde [16–22]. The fabrication and properties of a reagentless bienzyme amperometric biosensor based on alcohol oxidase/peroxidase in combination with an Os-complex modified electrodeposition paint was described [17].

There are published several reviews on AOXs from different yeast sources, including old papers [3, 7, 8, 23, 24] and more recent ones [25–27]. Undying scientific interest in AOXs is caused by complicity of the enzyme, being a model for structural and functional theoretical studies of flavoproteins. Due to ability of AOXs for irreversible and relatively selective oxidation of alcohols, easy availability, and no requirement in external cofactors, these enzymes are regarding as promising catalytic tools for modern technologies [25]: analytical biotechnology (enzymatic kits and biosensors for alcohol assay), biotransformation (synthesis of flavor and fragrance compounds, organic synthesis of optically pure compounds), and bioremediation (removing toxic methanol and formaldehyde contaminations).

The current review is devoted mainly to methodological aspects of the work with AOX from the yeast *O. polymorpha*: its isolation and purification and protocols of analytical procedures (enzymatic assay of alcohols, simultaneous analysis of methanol and formaldehyde, biosensor construction and their evaluation on real samples of food technology), for fields where authors of this review have many years' experience.

2 Materials and Methods

2.1 Strain, Medium and Cultivation Conditions

1. As a producer of AOX, the mutant strain of the thermotolerant methylotrophic yeast *O. polymorpha* C-105 (*gcr1 catX*), selected in the Institute of Cell Biology, National Academy of Sciences of Ukraine, is used. It has impairment in glucose catabolite repression of AOX synthesis, is catalase-defective, and has the ability to over-produce AOX in glucose medium [10, 16].
2. Cells of the mutant *O. polymorpha* C-105 are cultivated in flasks on a shaker (200 rpm) up to the late-logarithmic growth phase (~36 h) at 30 °C in medium containing (per 1 L of deionized water) 10 g glucose, 3.5 g $(NH_4)_2SO_4$, 1.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2$, and 2.0 g yeast extract. The pH of the medium is adjusted to 5.5 with KOH.
3. The cells are harvested by centrifugation (5000 × *g*, 10 min) and washed twice with water and once with 0.02 M phosphate buffer (PB), pH 7.5. The cells can be stored at −60 °C until use. The freezing process did not reduce the AOX activity of the cells.

2.2 Determination of Cells Concentration

The biomass of *O. polymorpha* cells (mg dry weight cells per 1 mL suspension) is determined by measuring optical density (D) of the suspension at 600 nm using a spectrophotometer (1 cm cuvette). The calculation is performed according to the equation:

$$C = D_{600} \cdot N/1.66,$$

where C—cells concentration; mg/mL; D_{600}—optical density at 600 nm; N—dilution of the initial suspension; 1.66—calibration coefficient.

2.3 Permeabilization of Yeast Cells

Freshly grown cells of the yeast *O. polymorpha* are washed from the medium and resuspended in 0.05 M PB, pH 7.5, containing 2 mM EDTA, to the cell concentration 20–50 mg dry weight cells in 1 mL buffer. The equal volume of aqueous solution of cetyltrimethylammonium bromide (2 mg/mL) is added to the suspension and mixture is incubated at 30 °C with careful shaking during 15 min. After permeabilization, the cells are washed three times with 0.02 M PB, pH 7.5, containing 2 mM EDTA.

2.4 Assay of AOX Activity

1. The AOX activity is measured in a reaction mixture with a final volume of 3.0 mL, containing 50 mM PB, pH 7.5, 0.25 mM *o*-dianisidine, horseradish peroxidase (0.07 mg/mL), 10 mM methanol, and appropriate amount of enzyme or permeabilized cells. Incubation is carried out for 5–10 min at 30 °C. The enzymatic reaction is stopped by the addition of 0.8 mL 12 M HCl.

2. The generated colored product is determined spectrophotometrically at 525 nm [28]. The millimolar extinction coefficient of the formed dye in acidic solution (2.5 M HCl) is shown to be 13.38 mM^{-1} cm^{-1}. One unit (U) of AOX activity is defined as the amount of enzyme releasing 1 μmol H_2O_2 per 1 min at 30 °C under standard assay conditions.
3. Protein concentration is determined by the Lowry method.

2.5 Preparation of Cell-Free Extracts

1. After washing, freshly grown cells (about 15 g wet weight cells from 1 L of culture) are resuspended in 0.1 M PB, pH 7.5, containing 2 mM EDTA and 0.4 mM phenylmethylsulfonyl fluoride (PMSF) to the final cell concentration 100–130 mg dry weight cell in 1 mL of buffer (approx. 1.0 g wet weight cells per 1 mL).
2. The cells are disrupted with glass beads ($d = 0.45$–0.50 mm) (2/3 of suspension volume) in Bead-Beater on ice for 6 min. After removal of cell debris by a low speed centrifugation (5000 × *g*, 10 min, 4 °C), the supernatant is centrifuged at 40,000 × *g* for 45 min at 4 °C. The supernatant is used as the cell-free extract for isolation of AOX.

2.6 Ammonium Sulfate Fractionation

AOX is isolated from cell-free extract of the mutant *O. polymorpha* C-105 by a two-step fractionation with ammonium sulfate (30–70% of saturation) in the presence of 2 mM EDTA and 0.4 mM PMSF to inhibit proteases.

1. To remove ballast proteins, crystalline ammonium sulfate fine powder is added to cell-free extract to 30% saturation under constant stirring and keeping the mixture on ice. The pH of the mixture is maintained at 7.5 with 2 M KOH. After complete dissolving of the salt, the mixture is left for 3–5 h at 4 °C. The pellet, formed at 30% saturation of ammonium sulfate, is discarded by centrifugation at 10,000 × *g* for 30 min at 4 °C.
2. To the supernatant, ammonium sulfate is added to 70% saturation and the mixture is left for 5 h at 4 °C. The obtained AOX precipitate is collected by centrifugation at 10,000 × *g* for 30 min at 4 °C, and washed three times with 70% saturated ammonium sulfate in 0.05 M PB, pH 7.5, containing 2 mM EDTA.

2.7 Dialysis

The AOX preparation obtained at 70% saturation ammonium sulfate is dialyzed against three changes (3 L each) of 0.01 M PB, pH 7.0, containing 1 mM EDTA, at 4 °C for 12 h under constant stirring. The insoluble pellet is removed by centrifugation at 10,000 × *g* for 10 min at 4 °C and discarded.

Table 2
Isolation and purification of AOX from the cells of mutant strain *O. polymorpha* C-105 (*gcr1 catX*)

Purification step	Total protein, mg	Total activity, U	Specific activity, U/mg	Yield[a], %	Purification, fold
Crude extract	340.0	1020.0	3.0	100	1.0
Protein precipitation with $(NH_4)_2SO_4$ (30–70% of saturation)	146.0	730.0	5.0	71.6	1.6
DEAE-Toyopearl 650 M chromatography	14.6	336.6	23.0	33.0	7.7

[a]Calculations of yields were carried out per 1 L batch culture that corresponds to 15 g wet weight cells

2.8 Ion-Exchange Liquid Chromatography

After dialysis, the protein solution is applied to a DEAE-Toyopearl 650 M column (1.6 × 23 cm), which is previously equilibrated with 0.05 M PB, pH 7.0. After removal of unspecific bound proteins by washing with the same buffer, enzyme is eluted from the column using a linear gradient of PB, pH 7.0, with the concentration range 0.05–0.5 M. The AOX appeared as a peak at 0.3 M concentration of the buffer. Obtained purified enzyme preparation exhibited specific activity at least of 23.0 U/mg. For AOX long-term storage, the fractions possessing the highest AOX activity are pooled together and enzyme is precipitated at 70% saturation of ammonium sulfate using 100% saturated solution of ammonium sulfate in 0.05 M PB, pH 7.5, containing 2 mM EDTA and 0.1 mM PMSF. A summary of a typical purification procedure is presented in Table 2 [11] and purity of the AOX preparation is illustrated in Fig. 1. AOX preparations can be stored at −10 °C without significant loss of activity for at least 6 months as a suspension in 0.05 M PB, pH 7.5 at 70% ammonium sulfate saturation.

2.9 Lyophilization of AOX

To obtain lyophilized powder of AOX, enzyme preparation after ammonium sulfate fractionation is mixed in a ratio 2:1 (v/v) with 24% trehalose solution or 1.5% gelatin and is subjected to freeze-drying at −55 °C. Trehalose is found to be the most effective stabilizing agent for AOX during lyophilization. It ensured maintenance of the enzyme's activity up to 85% during 12 months of storage of the dried powder at 4 °C, while the lyophilization of AOX without the addition of stabilizer leads to the loss of more than 60% of the original activity.

2.10 Analysis of AOX Preparations by Electrophoresis

1. Electrophoresis of proteins is performed under native (according to Ornstein and Davis) or denaturing (according to Laemmli) conditions, using 7.5% PAG or 10% PAG plus 0.1% SDS, respectively, and Tris–glycine buffer, pH 8.3.

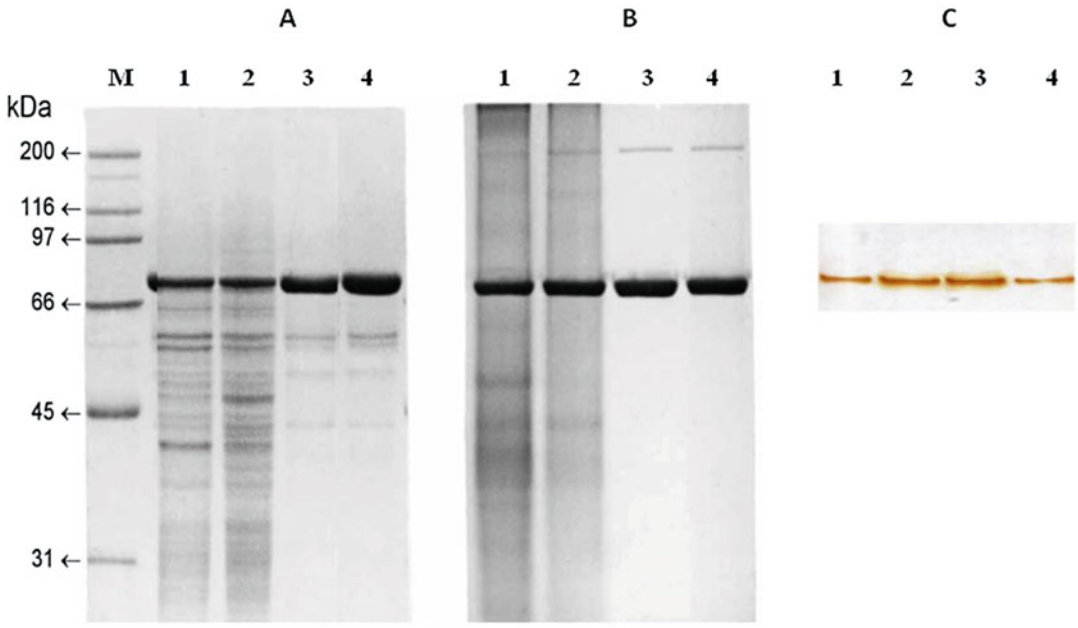

Fig. 1 Electrophoretic patterns of AOX preparations after SDS (**a**) and native (**b**, **c**) electrophoreses in 10% and 7.5% PAG, respectively; M—protein standards; lane 1—cell-free extract, 30 μg protein; lane 2—crude enzyme after second step of precipitation with ammonium sulfate (at 30–70% of saturation), 20 μg protein; lane 3—eluate from DEAE-Toyopearl (23 U/mg protein), 5 μg protein; lane 4—commercial preparation of AOX from *Pichia pastoris*, 5 μg protein. The visualization of AOX activity (**c**)

2. The protein zones are stained with Coomassie brilliant blue: R-250 for SDS-PAG (Fig. 1a) and G-250 for native PAG (Fig. 1b). Visualization of AOX for native PAGE in fractions of chromatographic eluates is presented on Fig. 1c. The enzyme is detected as a brown precipitate in a mixture of 0.01 M methanol, 0.05 mg/mL horseradish peroxidase, and 0.6 mM benzidine in 0.05 M PB, pH 7.0. After colored zones appearance, PAG are washed with water and with 10% acetic acid.

2.11 AOX-Based Enzymatic Assay of Ethanol, Methanol, and Formaldehyde

2.11.1 Assay of Ethanol Using Enzymatic Kit ALCOTEST

Alcohol oxidase of the yeast *O. polymorpha* together with commercial horseradish peroxidase are the enzymes exploited for quantitative determination of ethanol. As a chromogen, tetramethylbenzidine hydrochloride (TMB) can be used. Using these key components, the enzymatic kit *ALCOTEST* is developed for determination of ethanol in alcoholic beverages [29], fermented musts and wine products [30], human serum and blood [31].

The enzymatic kit *ALCOTEST* consists of three components:

1. "Chromogen," a mixture of chromogen and buffer components.
2. "Enzymes," a suspension of alcohol oxidase and peroxidase in ammonium sulfate solution.
3. "Standard," a calibrated ethanol solution, 10 g/L (stabilized).

1. Preparation of reagent and standard solutions: The content of bottle 1 ("Chromogen") is dissolved in 350 mL distilled water at 100 °C and then cooled to room temperature. The final concentration of TMB is 0.067 mM in 0.05 M PB, at pH 7.0. The solution is stable for 2 weeks at 20 °C, kept in the dark.
   (a) The content of bottle 2 ("Enzymes") containing 27 units of AOX and 2 mg horseradish peroxidase with RZ (Reinheitzahl) 3.0 in 0.5 mL 60% saturated ammonium sulfate was dissolved in cooled solution of chromogen. Fresh solution is prepared before analysis. If the number of samples to be analyzed are less than 100 (4 mL reaction mixture) corresponding aliquots of the well-stirred enzymes suspension and of the chromogen solution are mixed.
   (b) Ethanol solution from the bottle 3 ("Standard") is diluted 100-fold to the concentration of 0.1 g/L and used the same day.
2. Sample preparation: Samples of the tested alcoholic beverages are diluted with water to ethanol concentration of about 0.05–0.5 g/L. Usual dilution factor is 500–2000.
3. Analysis procedure:
   (a) In the separate test tubes, add 0.1 mL aliquots of the sample, standard or water (for blank).
   (b) To the test tubes, portions of 3.5 mL reagent solution are added in fixed time (sequentially in 30 s intervals).
   (c) The solutions are well mixed and left to stand at room temperature for 15–20 min.
   (d) After incubation during 10–15 min, the reaction is stopped by addition of 0.5 mL 0.8 M HCl.
   (e) Absorbance at 450 nm of the reaction mixtures is read against the blank. If the assay is performed using the "fixed time" regime, timing of the start and termination of the assay is very important. Therefore, the reaction is started (and also ended) by adding respective reagent sequentially in 30 s intervals.
4. Calculations: The calculation of ethanol content in alcoholic beverages is performed using the following formula:

$$C = A_{\text{sample}} \cdot 0.1 \cdot n / A_{\text{standard}}$$

$$\text{or } C_{\text{vol\%}} = 0.13 \cdot C \text{ (with respect to 96.5\% ethanol)},$$

where C—ethanol mass concentration, expressed in g/L; A_{sample}—optical density of the sample solutions; A_{standard}—optical density of the standard solution; n—dilution factor for initial sample; $C_{\text{vol\%}}$—ethanol volumetric content in %.

2.11.2 Assay of Formaldehyde in Fish Food Products

AOX-peroxidase method using enzymatic kit ALCOTEST can be applied for determination of formaldehyde [32]. Formaldehyde (FA) is classified as a mutagen and a possible carcinogen [33]. High level of FA toxicity necessitates the control over the content of this substance in environment, industrial products, medical preparations, and even in some food products, in particular, fish food products [15, 34, 35]. The AOX-based method compared with routine chemical approaches has several advantages: high sensitivity, good linearity, and insensitivity to the interference effect of the sample's components and usage of nonaggressive reagents for the sample pretreatment and assay procedure [32]. The linearity of calibration curve for this method is kept even at high optical densities—up to 0.9 which corresponds to 15 μM FA in final reaction mixture (15 nmol/mL), and the threshold sensitivity of the method is about 0.8 nmol/mL.

It is demonstrated that some fish products (hake and cod) contain high levels of toxic FA, up to 100 mg/kg wet weight, while the content of FA in carp is negligible [32].

1. Preparation of reagent and standard solutions:
   (a) Sample of solution of AOX from *O. polymorpha* with specific activity 3–5 μmol/(min·mg) of protein (3–5 U/mg).
   (b) Sample of horseradish peroxidase solution with specific activity 900 U/mg and RZ = 3.0.
   (c) Mixture "Enzymes" a suspension of AOX oxidase (42 U/mL) and peroxidase (4 mg/mL) in 60% saturated ammonium sulfate solution.
   (d) Mixture "Chromogen" a mixture of the chromogen TMB and buffer components.
   (e) The final reagent is prepared by dissolution of 490 mg mixture "Chromogen" in 60 mL water. The mixture is heated until it is dissolved, cooled to room temperature and supplemented with 0.5 mL of the enzyme mixture. The final composition of the prepared reagent is as follows: 75 mM PB, pH 7.5; 0.08 mM TMB; 0.05 mg/mL horseradish peroxidase with RZ = 3.0; 0.35 U/mL AOX.
   (f) Formaldehyde calibration solution is prepared by hydrolysis of paraformaldehyde in water (1 M concentration) in a sealed ampoule at 105 °C for 12 h and by dilution of hydrolysate to the necessary concentration.
   (g) FA solution ("Standard") is freshly prepared by 5000-fold dilution of the initial 1 M FA to the final concentration of 0.2 mM (or 6 mg/L) before analysis.
2. Sample preparation: For assay of FA in fish meat (muscle tissues of frozen hake and cod, as well as of freshly killed carp), the

following deproteinization procedure is used: 2.5 g cut muscle tissue is ground in a ceramic pot. After adding 10 mL water, the mixture is ground again, mixed with 2 mL of Carrez reagent I (15%, m/v, aqueous solution of $K_3Fe(CN)_6 \cdot 3H_2O$), 2 mL Carrez reagent II (30%, m/v, aqueous solution of $ZnSO_4 \cdot 7H_2O$), and 34 mL water (to obtain 50 mL volume) and stirred. Precipitated proteins are removed by filtering through folded filter. The filtrate is stored at 4 °C before the analysis.

3. Analysis procedure:
   (a) In separate test tubes, add 0.2 mL aliquots of the analyzed extract (5–50 nmol CH_2O), standards, and dilute with water to the volume of 0.5; or to the blank sample, add 0.5 mL water.
   (b) To the test tubes, add portions of 3 mL of freshly prepared reagent in fixed time (sequentially in 30 s intervals).
   (c) The samples are incubated at room temperature for 25 min.
   (d) The reaction mixture is stopped by adding 0.5 mL 0.8 M HCl to each sample.
   (e) The optical density of solutions is measured at 450 nm against blank sample which contains water instead of the analyzed extract.
4. Calculations: The calculation of FA content in fish products (in mg per 1 kg of wet weight of the sample) is performed using the following formula:

$$C_{mg/kg} = 600 \cdot 0.2 \cdot A_e/A_c = 120 \cdot A_e/A_c,$$

where A_e and A_c—values of optical density of the experimental and calibration samples, respectively; 0.2—millimolar (mM) concentration of FA in the calibration solution; 600—coefficient which arises from extraction ratio (2.5 g of fish tissue per 50 mL of the extract); the volume of extract taken for analysis (1 mL) and molecular weight of FA (30), that is, $600 = (50/2.5) \cdot 30$.

2.11.3 Simultaneous Assay of Methanol and Formaldehyde in Wastewater

An enzymochemical method using AOX and 3-methyl-2-benzothiazolinone hydrazone (MBTH) is assigned for the simultaneous analysis of methanol and formaldehyde in mixtures, including industrial wastewaters. AOX from the yeast *O. polymorpha* is used as a methanol oxidizing agent and MBTH as a reagent for colorimetric determination of generated formaldehyde [36].

1. Preparation of reagent and standard solutions:

(a) 25 mM MOPS/KOH buffer, pH 7.0. 5.23 g 3-(*N*-morpholino)propanesulfonic acid (MOPS) is dissolved in 1 L water. Mix 250 mL MOPS solution (5.23 g/L) and 87 mL 25 mM KOH (14.03 g/L). Dilute to 0.5 L with water. Store at 4 °C.

(b) Crude preparation of *O. polymorpha* AOX with specific activity 4 μmol/(min·mg) of protein (4 U/mg).

(c) Reagent **1** (0.05% MBTH-hydrochloride solution in 25 mM MOPS/KOH buffer, pH 7.0): 0.05 g MBTH hydrochloride is dissolved in 100 mL 25 mM MOPS/KOH buffer, pH 7.0.

(d) Reagent **2** (reaction mixture containing AOX): 0.1 mL crude preparation of *O. polymorpha* AOX (with activity of 50 U/mL) is dissolved in 10 mL Reagent **1** (final activity 0.5 U/mL).

(e) Reagent **3** (0.1% $FeCl_3$ solution in 30 mM HCl): 122 mg $FeCl_3 \cdot 6H_2O$ is dissolved in 100 mL 30 mM HCl. It is stable for at least 10 days of storage at 20 °C.

(f) FA calibration solution is prepared by hydrolysis of paraformaldehyde in water (1 M concentration) in a sealed ampoule at 105 °C for 12 h and by dilution of hydrolysate to the necessary concentration.

(g) FA solution (Standard **1**) is freshly prepared by 5000-fold dilution of the initial 1 M FA to the final concentration of 0.2 mM (or 6 mg/L) before analysis.

(h) Methanol solution (Standard **2**) is freshly prepared by dilution of the initial methanol to the final concentration of 0.156 mM (or 5 mg/L) before analysis.

2. Sample preparation: The wastewater samples are frozen and stored at −25 °C before analysis.

   (a) Assay of FA and methanol are carried out in distillates.

   (b) The sample's distillation is carried out in accordance with recommendations of the Polish Standard PN-71C/04568 [37].

   (c) After distillation, samples are diluted (2–100-fold), immediately analyzed or frozen and stored at −25 °C before analysis.

3. Assay of Formaldehyde :

   (a) In separate test tubes, add 0.2 mL aliquots of the diluted samples or Standard **1,** or water (for blank).

   (b) To the test tubes, add 1.8 mL Reagent **1**.

   (c) Samples are incubated for 15 min at 30 °C and Reagent **3** is added.

(d) Mixtures are incubated for 20 min at 30 °C and their optical densities are measured at 670 nm against a blank containing all components except a tested sample.

(e) Calculations: The formaldehyde content in the test samples is calculated using a calibration curve [36]. Calibration curve is constructed using formaldehyde calibration solutions. For calibration, formaldehyde solution (12 mg/L) is used. The linearity of calibration curve for MBTH-method is kept even at high optical densities—up to 0.8 which corresponds to 18.30 μM FA in final reaction mixture (18.3 nmol/mL), and the threshold sensitivity of the method is about 0.25 nmol/mL.

The calculation of formaldehyde content in the tested samples is performed according to the following formula:

$$C = A_{\text{sample}} \cdot 0.2 \cdot n / A_{\text{standard}}$$

or $C_{\text{mg/L}} = 30 \cdot C$, where C—millimolar concentration of formaldehyde in mM; A_{sample}—optical density of the tested sample solutions; A_{standard}—optical density of the standard solution; n—dilution factor for initial sample; $C_{\text{mg/L}}$—mass concentration of formaldehyde in mg/L.

2.11.4 Assay of Methanol

(a) In the separate test tubes, add 0.2 mL aliquots of the diluted samples or Standard **2,** or water (for blank).

(b) To the test tubes, add 1.8 mL Reagent **2**.

(c) Samples are incubated for 15 min at 30 °C and Reagent **3** is added.

(d) The samples are incubated for 20 min at 30 °C.

(e) The optical density of solutions is measured at 670 nm against blank sample which contains all components except a tested sample.

(f) Calculations: Calculations of methanol content in tested samples are carried out using calibration curve [36]. Calibration curve is constructed using methanol calibration solutions. For calibration, methanol solution (5 mg/L) is used. The linearity of calibration curve for AOX-MBTH-method is kept even at high optical densities—up to 0.8 which corresponds to 17.8 μM methanol in final reaction mixture (17.80 nmol/mL), and the threshold sensitivity of the method is about 0.475 nmol/mL.

The calculation of methanol content in the tested samples is performed according to the following formulas:

$$C = \Delta D_{\text{sample}} \cdot 0.156 \cdot n / D_{\text{standard}}$$

or $C_{\text{mg/L}} = 32 \cdot C$, where C—millimolar concentration of methanol in mM; ΔD_{sample}—difference in optical densities of

the samples tested for the content FA + methanol and samples tested for only FA; $D_{standard}$—optical density of the solution with methanol standard; n—dilution factor for initial sample; and $C_{mg/L}$—mass concentration of methanol in mg/L.

2.12 Application of AOX in Construction of Biosensors

The use of AOX in biosensorics is illustrated here by the fabrication of a reagentless bienzyme amperometric biosensor based on AOX (specific activity of 30 U/mg protein), commercial peroxidase (HRP, 30 U/mg protein) in combination with an Os-complex modified electrodeposition paint [17].

1. Electrodes and measurement:

   Constant-potential amperometry in a three-electrode configuration with Ag/AgCl/KCl (3 M) reference electrode and a Pt-wire counter electrode is used. As a working electrode, graphite rod (type RW001, 3.05 mm diameter) sealed in a glass tube using epoxy glue is exploited. Before sensor preparation, the graphite electrodes are polished on emery paper (P1200). Amperometric measurements are carried out using a bipotentiostat EP30 Biometra connected to a personal computer via a RS232 port for data acquisition. Measurements are performed in steady-state mode using a standard cell in volume 25 mL at 25 °C at continuous stirring. Between experiments, the enzyme electrodes are stored in 0.1 M PB, pH 7.6, at 4 °C.

2. Selection of electrodeposition paints: The paints are synthesized in the laboratory of Analytical Chemistry—Elektroanalytik & Sensorik, Ruhr-Universität Bochum, Germany, according to strategy of the radical copolymerization of acrylic acid (anodic paints) or (dimethylamino)ethylmethacrylate (cathodic paints) with suitable comonomers.

   A lot of osmium complex-integrated anodic paints are screened by their electron transferring activity and enzyme-inactivating/stabilizing property on the model of HRP-containing electrode. Cyclic voltammetry is used for these investigations. The most effective composition *AP59-Os* (Os-bis-*N*,*N*′-(2,2′-bipyridyl)-dichloro complex—an anodic paint 59 supplemented by the osmium complex) demonstrated the ability for direct electrochemical communication with immobilized HRP in the presence of hydrogen peroxide under the lowest working potential (−50 mV vs. SCE). *AP59-Os* composition is chosen for construction of bienzyme AOX-based biosensor. The scheme of electron transfer for this biosensor is shown in Fig. 2.

3. Entrapment of HRP in a *AP59-Os* layer: One microliter of a freshly prepared mixture of horseradish peroxidase (400 U/mL) solution in 0.02 M PB, pH 7.6 and of *AP59-Os*, ratio 1:1, is dropped onto the surface of the working electrode. In a

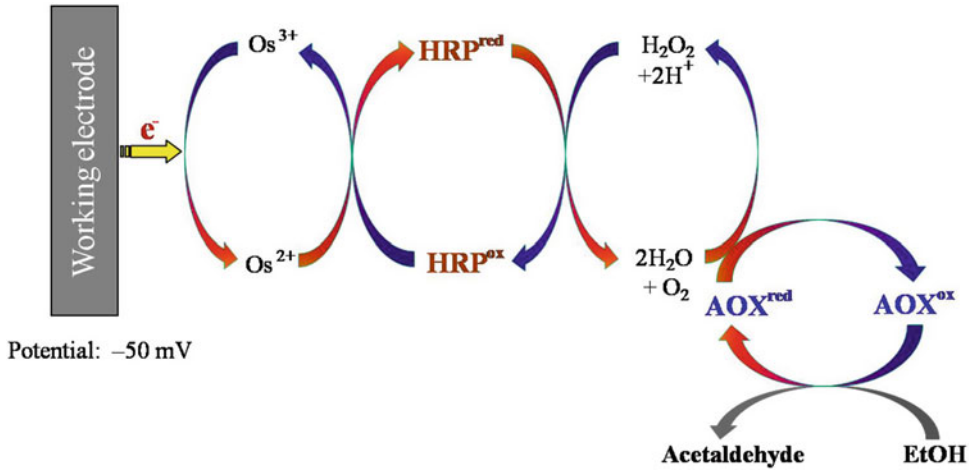

Fig. 2 Reaction scheme and electron-transfer pathway from ethanol *via* AOX and enzymatically generated H_2O_2 *via* HRP and polymer-bound Os-relays to the AOX-HRP-based electrode [17]

miniaturized electrochemical cell, the anodic paint is precipitated on the electrode surface using a potentiostatic pulse sequence of +2200 mV for 0.2 s and 0 mV for 5 s. During the electrochemical initiated water oxidation, the anodic paint is protonated concomitantly modulating the solubility of the carboxylate-containing polymer chains. The coprecipitated enzyme is thus entrapped in front of the electrode surface. The electrodes are rinsed with 0.02 M PB, pH 7.6.

4. Entrapment of AOX in a *CP9* layer: One microliter of AOX suspension (200 U/mL; pH 7.6) is dropped on the surface of the working electrode and allowed to dry. Forty microliters of the *CP9* suspension is added to the miniaturized electrochemical cell and the *CP9* is precipitated by means of a potentiostatic pulse sequence of −1200 mV for 0.2 s and 0 mV for 5 s. AOX is entrapped within the electrodeposited cathodic paint layer on the surface of the working electrode. The electrode is rinsed with 0.1 M PB, pH 7.6, before use. Finally, an optimal architecture of the developed alcohol-selective biosensor is proposed [17]: HRP- and *AP59-Os*-containing first layer followed by a second one consisting of AOX (or a mixture of AOX and HRP) entrapped in cathodic paint *CP9*.

The main bioanalytical properties of the constructed bienzyme sensor are presented in Fig. 3.

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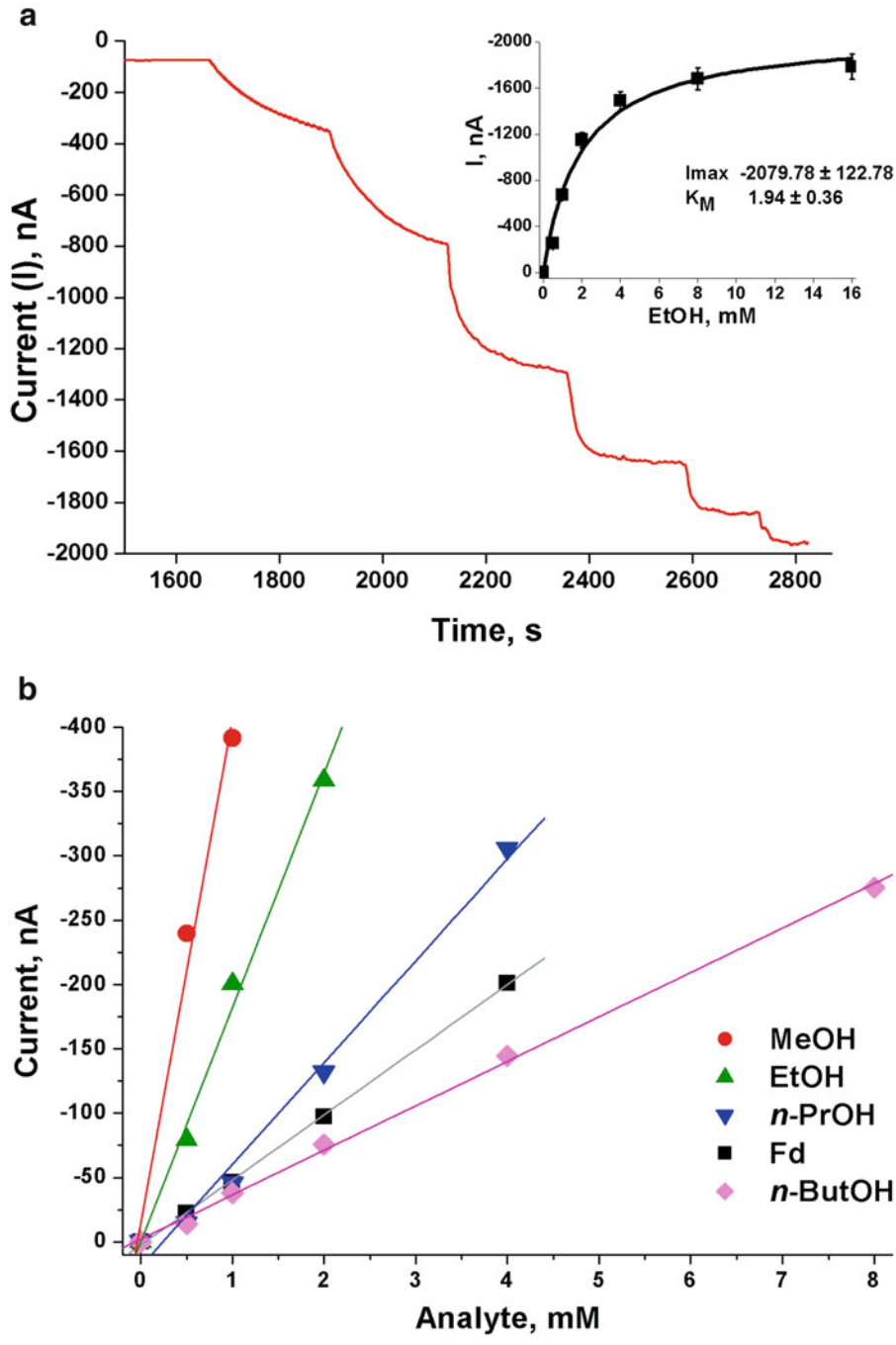

Fig. 3 The main bioanalytical properties of the constructed bienzyme sensor. (**a**) The typical dynamic response range of the sensor toward ethanol and corresponding calibration curve as dependence on added ethanol (inserted); (**b**) the linear dynamic range of sensor's response toward different analytes: MeOH, EtOH, n-PrOH, Fd, n-ButOH. 3.05 mm graphite electrode; −50 mV *vs.* Ag/AgCl

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