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Immobilized formaldehyde-metabolizing enzymes from *Hansenula polymorpha* for removal and control of airborne formaldehyde

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ABSTRACT

Formaldehyde (FA)-containing indoor air has a negative effect on human health and should be removed by intensive ventilation or by catalytic conversion to non-toxic products. FA can be oxidized by alcohol oxidase (AOX) taking part in methanol metabolism of methylotrophic yeasts. In the present work, AOX isolated from a *Hansenula polymorpha* C-105 mutant (*gcr1 catX*) overproducing this enzyme in glucose medium, was tested for its ability to oxidize airborne FA. A continuous fluidized bed bioreactor (FBBR) was designed to enable an effective bioconversion of airborne FA by AOX or by permeabilized mutant *H. polymorpha* C-105 cells immobilized in calcium alginate beads. The immobilized AOX having a specific activity of 6–8 U mg^{-1} protein was shown to preserve 85–90% of the initial activity. The catalytic parameters of the immobilized enzyme were practically the same as for the free enzyme (k_{cat}/K_m was 2.35×10^3 M^{-1} s^{-1} *vs* 2.89×10^3 M^{-1} s^{-1}, respectively). The results showed that upon bubbling of air containing from 0.3 up to 18.5 ppm FA through immobilized AOX in the range of 1.3–26.6 U g^{-1} of the gel resulted in essential decrease of FA concentration in the outlet gas phase (less than 0.02–0.03 ppm, i.e. 10-fold less than the threshold limit value). It was also demonstrated that a FBBR with immobilized permeabilized C-105 cells provided more than 90% elimination of airborne FA. The process was monitored by a specially constructed enzymatic amperometric biosensor based on FA oxidation by NAD^+ and glutathione-dependent formaldehyde dehydrogenase from the recombinant *H. polymorpha* Tf 11-6 strain.

1. Introduction

Formaldehyde is a very important commercial chemical, and is among the most toxic pollutants used in many industries. It is used as an adhesive material in pressed wood products, as a preservative in paints and coatings, in the production of fertilizer, paper, plywood and urea–formaldehyde resins and in numerous other applications (Yocom and McCarthy, 1991; Khoder et al., 2000; Otson and Fellin, 1992). FA was introduced into medical practice as a disinfectant and tissue hardener and is used extensively for preserving tissue specimens in hospitals and laboratories (Walker, 1964; Cox, 1994). It is also used as a sterilizer and a preservative in vaccine production (Geier and Geier, 2004; Offit and Jew, 2007).

In vivo FA is detoxified principally via the action of FdDH (EC 1.2.1.1), a specific enzyme that catalyzes the conversion of FA into S-formylglutathione (finally, into formic acid) and NADH in the presence of GSH and NAD^+ (Pourmotabbed and Creighton, 1986; Uotila and Mannervik, 1979). FdDH is widely used for bioanalytical purposes (Ali et al., 2006; Gonchar et al., 2002; Hämmerle et al., 2010; Herschkovitz et al., 2000; Kawamura et al., 2005; Korpan et al., 1993, 2000; Vastarella and Nicastri, 2005; Winter and Cammann, 1989).

Hydrated form of FA can be easily oxidized into formic acid without any exogenous cofactor by AOX (EC 1.1.3.13), an enzyme which is responsible *in vivo* for the first reaction of methanol metabolism in methylotrophic yeast (Kato et al., 1976; Klei van der et al., 1990).

It is regarded that exposure to 20 ppm FA in air is dangerous to life and health. Several methods have been proposed for the

Abbreviations: FA, formaldehyde; AOX, alcohol oxidase; FdDH, formaldehyde dehydrogenase; GSH, reduced glutathione; MB, meldola blue; MBTH, 3-methyl-2-benzothiazolinone hydrazone hydrochloride; TLV, threshold limit value; MSDS, material safety data sheets; PMSF, phenylmethylsulfonyl fluoride; CTMAB, cetyltrimethylammonium bromide; FBBR, fluidized bed bioreactor.

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removal of FA from indoor air. Physical adsorption of FA with activated carbon (Boonamnuayvitaya et al., 2005; Tseng et al., 2005), various fractions of karamatsu bark (Takano et al., 2008) and zeolites (Cazorla and Grutzeck, 2006) were demonstrated to yield good to high results. However, simple adsorption cannot provide a radical solution to the problem, since FA is not decomposed, but is only transferred from one phase (air) to another (solid). Attempts at physical FA decomposition with the help of photocatalytic, negative ion and ozone air cleaners resulted in only up to 50% elimination of FA and failed to supply an FA level that meets WHO guidelines (0.08 ppm) (Tseng et al., 2005). Chemical decomposition of FA by composite silica particles functionalized with amine groups and platinum nanoparticles demonstrated a very high capacity for removing FA (Lee et al., 2008). However, this method is expensive. Another approach to chemical elimination of airborne FA was developed by Sekine, where manganese dioxide was shown to be effective for FA oxidation (Sekine, 2002; Tian and He, 2009). Combustion of a formaldehyde–methanol mixture in an airstream on Mn/Al_2O_3 and $Pd–Mn/Al_2O_3$ catalysts was shown to afford total conversion of organic compounds (Álvarez-Galván et al., 2004). The chemical approach to FA decomposition exhibits high efficiency, but the solid wastes that remain after these processes usually contain harmful toxic components which cause subsequent utilization problems.

FA removal from air using biological decomposition is not well developed. Several biofilters and biotrickling filters containing natural microorganisms were tested for the treatment of a mixture of formaldehyde and methanol (Prado et al., 2004, 2006). A maximum FA elimination capacity of 180 g m^{-3} h^{-1} was reached.

To the best of our knowledge, enzyme-based bioreactors have not been tested as a potential tool for FA bioremediation of indoor air. The goal of the present work was to study a possibility to use the enzyme AOX, as well as mutant yeast *H. polymorpha* cells overproducing this enzyme, to design continuous bioreactor for catalytic oxidation of airborne FA. For quantitative monitoring of this process, FA-selective amperometric biosensor based on recombinant NAD^+- and GSH-dependent FdDH was to be constructed.

2. Materials and methods

2.1. Chemicals

DEAE-Toyopearl 650M was purchased from Toyo Soda (Tokyo, Japan); para-formaldehyde, Triton X-100, PMSF, chromotropic acid, MBTH, MB, and sodium alginate were obtained from Sigma–Aldrich (USA); CTMAB and GSH were purchased from Fluka (Buchs, Switzerland). NAD^+ and NADH were obtained from Gerbu Biotechnik (Gailberg, Germany), 1% Nafion solution in ethanol (Aldrich, Germany) and the cathodic electrodeposition paint "GY 83-0270 0005" was purchased from BASF Farben und Lacke (Munster, Germany).

All chemicals were of analytical reagent grade and all solutions were prepared using HPLC-grade water. FA solution (1 M) was prepared by hydrolysis of the corresponding amount of paraformaldehyde (Sigma–Aldrich, USA) in water (300 mg; 10 ml water) by heating the suspension in a sealed ampoule at 105 °C for 6 h.

2.2. Mutant and recombinant yeast strains

The permeabilized cells of two *H. polymorpha* mutants were used as a bioactive material for construction of cell bioreactors: (1) CSA-33 (*gcr1*) ("constitutive synthesis of alcohol oxidase") with impaired glucose catabolite repression of AOX synthesis (Sibirny et al., 1993) and (2) C-105 (*gcr1 catX*) with additional mutation inactivating catalase (Gonchar et al., 2001, 2002). The latter strain was also used as a source for AOX isolation.

The recombinant *H. polymorpha* Tf 11-6 strain constructed by us previously was used for isolation of thermostable NAD^+- and glutathione-dependent FdDH (Demkiv et al., 2005).

2.3. Assay of enzyme activities

AOX activity was measured as described previously (Shleev et al., 2006).

FdDH activity was determined by the rate of NADH formation monitored spectrophotometrically at 340 nm (Schutte et al., 1976) under the following conditions: 25 °C, 1 mM FA, 1 mM NAD^+, and 2 mM GSH in PB (50 mM phosphate buffer, pH 8.0). Instant activity of FdDH (A) was calculated as the difference between A^{+FA} (in the presence of FA) and A^{-FA} (without addition of FA).

One unit (1 U) of activity for both enzymes was defined as the amount of enzyme which forms 1 μmol of the product per 1 min under standard conditions of the assay.

Protein concentration was estimated by the Lowry method.

2.4. Isolation and purification of the enzymes

2.4.1. AOX from mutant overproducing H. polymorpha yeast cells

AOX was isolated from cell-free extracts of the yeast *H. polymorpha* C-105 (*gcr1 catX*) strain cultivated in glucose medium (Gonchar et al., 2001; Shleev et al., 2006). Purification of the enzyme was carried out using a two-step precipitation with ammonium sulfate (at 40 and 60% 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. The final activity of the enzyme preparations used for the bioreactor was 6–8 U mg^{-1} protein.

2.4.2. Recombinant FdDH

For enzyme isolation, cells of the recombinant *H. polymorpha* Tf 11-6 strain were cultivated in 1% methanol medium in the presence of 5 mM FA (Demkiv et al., 2007).

The purification procedure included preparation of a cell-free extract and a two-step column chromatography on anion-exchange sorbent DEAE-Toyopearl 650M (Demkiv et al., 2007). The final specific activity of the enzyme used for the biosensor construction was 18 U mg^{-1} protein.

2.5. AOX-based bioreactor

2.5.1. Immobilization of AOX in calcium alginate gel

Aliquots of 1 ml AOX solutions in 0.05 M PBS, pH 7.5 (buffer A) with activity of 2, 10, 20 or 40 U ml^{-1} were mixed with 2 ml of 3% (w/v) sodium alginate. The mixtures were dropped into a 2.5 mM $CaCl_2$ aqueous solution using a syringe with a 21G needle under stirring at room temperature and kept for 45 min for bead formation. The obtained gel beads were washed twice with 20 ml of buffer A.

2.5.2. Immobilization of the yeast cells in calcium alginate gel

Suspensions of 30 mg permeabilized mutant *H. polymorpha* C-105 cells (specific AOX activity 0.2 U mg^{-1}) and CSA-33 (activity 0.08 U mg^{-1}) were diluted in 1 ml of buffer A, mixed with 2 ml of 3% (w/v) alginate and treated as described above for immobilization of AOX.

Permeabilization of the yeast cells was carried out by their treatment with 0.1% CTMAB at 30 °C for 30 min. The permeabilizing agent was removed by centrifugation and washing the cells with