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Formaldehyde Oxidizing Enzymes and Genetically Modified Yeast *Hansenula polymorpha* Cells in Monitoring and Removal of Formaldehyde

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1. Introduction

Formaldehyde (FA), a very important commercial chemical, is one of the most toxic pollutants used in many industries. It is exploited as an adhesive material in pressed wood products, as a preservative in paints and coatings, in the production of fertilizers, paper and plywood, urea-formaldehyde resins and numerous other applications (Yocom Y.E., 1991; Otson, 1992; Khoder, 2000). It is also applied in the production of cosmetics and sugar, in well-drilling fluids, in agriculture as a preservative for grains and seed dressings, in the rubber industry in the production of latex, in leather tanning and in photographic film production. FA has been a popular constituent of embalming solutions since about 1900 (Kitchens et al., 1976; Plunkett and Barbella, 1977). Approximately 30 years following its discovery, FA was introduced into medical practice as a disinfectant and tissue hardener, used in many hospitals and laboratories to preserve tissue specimens (Walker, 1964; Cox, 1984). It has medical applications as a sterilizer and is employed as an anti-viral agent and preservative in the production of vaccines, instead of the harmful merthiolate, which can cause neurodevelopmental disorders including autism and autism spectrum disorders (Offit, 2007; Geier, 2004).

FA has a negative influence on human health, especially on the central nervous, blood and immune systems. Anatomists, technicians, medical or veterinary students and embalmers are among the people who have a great risk for FA toxicity. FA can also be found in the air

that we breathe at home and at work, in the food we eat, and in some products that we put on our skin. A major source of FA that we breathe everyday is found in smog in the lower atmosphere. Automobile exhaust from cars without catalytic converters or those using oxygenated gasoline also contain FA (Kitchens et al., 1976; National Research Council, 1982). At home, FA is produced by cigarettes and other tobacco products, gas cookers, and open fireplaces. It is found in many products used every day around the house, such as antiseptics, cosmetics, dish-washing liquids, fabric softeners, shoe-care agents, carpet cleaners, glues, lacquers, paper, plastics, and some types of wood products (Gerberich and Seaman, 1994). Inhaled FA primarily affects the airways; the severity and extent of the physiological response depends on its concentration in the air. Acute inhalation exposure to FA causes histopathologic damage (Chang et al.,1983) and DNA-protein cross-linking in the nasal mucosa of rats and rhesus monkeys (Auerbach et al.,1977; Martin et al.,1978; Griesemer et al.,1982; Casanova et al.,1989). Recently, a new health risk factor associated with FA has been revealed. Some advanced technologies of potable water pre-treatment include the ozonation process, during which FA is generated as a result of the reaction of ozone with humus traces (Schechter and Singer, 1995). FA has been in widespread use for over a century as a preservative agent in some foods, such as some types of Italian cheeses and dried foods. It has been found as a natural chemical in fruits and vegetables, and in human flesh and biological fluids (Gerberich and Seaman, 1994). In extreme cases, some frozen fish, especially of the *Gadoid* species, can accumulate up to 200 mg of FA per kg of wet weight due to the enzymatic degradation of a natural fish component - trimethylamine oxide (Rehbein, 1995; Pavlishko et al., 2003).

FA is classified as a mutagen and possible human carcinogen (Feron et al., 1991), one of the chemical mediators of apoptosis. FA is clearly genotoxic *in vitro*. It induces mutations and DNA damage in bacteria. DNA-protein cross-links, DNA single-strand breaks, chromosomal aberrations, sister chromatid exchanges and gene mutations are induced in human and rodent cells. Animal studies indicate that FA is a rat carcinogen at high levels ($\geq$ 10 ppm) of exposure, producing nasal tumours that are both exposure duration and concentration-dependent (Shaham J. et al., 1996.

At the same time, FA is a naturally occurring metabolite produced in very small amounts in our bodies as part of our normal, everyday metabolism of serine, glycine, methionine and choline and also by the demethylation of *N*-, *S*- and *O*-methyl compounds (Heck, 1984). It is estimated that endogenous FA concentration in blood is close to 0.1 mM. FA may be detoxified principally via action of formaldehyde dehydrogenase (FdDH, EC 1.2.1.1), a specific enzyme that catalyzes the conversion of FA in the presence of reduced glutathione (GSH) and NAD^+ to S-formylglutathione (finally, to formic acid) and NADH (Uotila and Mannervik, 1979; Pourmotabbed and Creighton,1986). S-formylglutathione (GSCH=O) is finally hydrolyzed to free formic acid:

$$CH_2O + GSH \leftrightarrow GS\text{-}CH_2OH \qquad (1)$$

$$GS\text{-}CH_2OH + NAD^+ \xleftrightarrow{FdDH} GS\text{-}CH{=}O + NADH + H^+ \qquad (2)$$

$$H_2O + GS\text{-}CH{=}O \longrightarrow GSH + HCOOH \qquad (3)$$

Since FdDH is a glutathione dependent enzyme, the pool of glutathione available for FA binding is important in regulating FdDH activity. Then FA can be metabolised to formate

and enter the one carbon pool for incorporation into the cells constituents (Casanova-Schmitz, 1984). At the moment, three different FdDHes, bacterial NAD^+-dependent, yeast NAD^+- and GSH-dependent and bacterial dye-linked NAD^+ and GSH-independent, are widely used for bioanalytical purposes (Ben Ali et al., 2006, 2007; Winter and Cammann, 1989; Vastarella and Nicastri, 2005; Herschkovitz et al., 2000; Korpan et al., 1993; Gonchar et al., 2002; Korpan et al., 2010; Achmann et al., 2008; Kawamura et al., 2005).
Besides FdDH, FA can be easily oxidized by alcohol oxidase (AOX) (EC 1.1.3.13), an enzyme which is responsible *in vivo* for the first reaction of methanol metabolism in methylotrophic yeast (Klei van der et al, 1990). AOX is not an absolutely selective enzyme and oxidizes the hydrated form of FA to formic acid without any exogenous cofactor (Kato et a*l*., 1976). The theoretical possibility of AOX using for FA assay is based on a known fact that FA exists in aqueous solutions in the hydrated form (95–99% of total concentration) which has a structural resemblance to methanol and can be oxidized by AOX` with the subsequent formation of formic acid and hydrogen peroxide according to the following reactions:

$$CH_2O + H_2O \leftrightarrow HOCH_2OH \quad (4)$$

$$HOCH_2OH + O_2 \xrightarrow{AOX} HCOOH + H_2O_2 \quad (5)$$

2. Methods of formaldehyde monitoring

2.1 Chemical and enzymatic methods

There are many chemical methods for the determination of FA (Sibirnyi et al., 2005; Bakar et al., 2009). The traditional Nash's method (Nash, 1953) is based on the reaction of FA with acetylacetone in the presence of ammonium ions. Another widely used photometric and sufficiently sensitive analytical method exploits the reaction of FA with chromotropic acid (Sawicki et al., 1962). This approach enables the determination of the analyte in the concentration range 0.05 - 1.0 mg dm^{-3} (Polish Standard, 1988). Unfortunately, determination of FA involves heating the sample with chromotropic acid under strongly acidic conditions.
4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (AHMT) was also proposed for FA assay (Avigad, 1983, Jung et al., 2001). FA and other aldehydes form products of different colors, which can be selectively tested spectrophotometrically. The sensitivity limit of the method is 1.5 nmol of FA in 1 ml sample. However, the main drawback of the AHMT method is the requirement of a very strong base.
High Performance Liquid Chromatography coupled to steam distillation and 2,4-dinitrophenylhydrazine derivatization (2,4-DNPH) displayed good selectivity, precision and accuracy (Li et al., 2007).
A polarographic method has been developed for the determination of FA traces by direct *in situ* analyte derivatization with (carboxymethyl)trimethyl ammonium chloride hydrazide (Girard T-reagent) (Chan & Xie, 1997). The drawback of this method is the expensive apparatus required, as well as the necessity to remove oxygen traces by sparging with pure nitrogen.
A flow injection analysis (FIA) system with an incorporated gel-filtration chromatography column has been applied to determine FA using FdDH (Benchman, 1996).

2.2 Biosensor methods

The degree of selectivity or specificity of a given biosensor is determined by the type of biocomponent incorporated into the biosensor. Biological recognizers are divided into 3

groups: biocatalytic, bioaffinity and hybrid receptors (Mello and Kubota, 2002). The selection of an appropriate immobilization method depends on the nature of the biological element, type of transducer used, physico-chemical properties of the analyte and operating conditions of the biosensor system (Luong et al., 1988). Biosensors can be categorized according to their transducer: potentiometric (Ion-Selective Electrodes (ISEs), Ion-Sensitive Field Effect Transistors (ISFETs)), amperometric, conductometric, impediometric, calorimetric, optical and piezoelectric.

FA selective biosensors are based on cells (Korpan et al., 1993) or enzymes used as biorecognition elements: either alcohol oxidase (AOX) (Korpan et al., 1997, 2000; Dzyadevych et al., 2001) or formaldehyde dehydrogenase (FdDH) (Herschkovitz et al., 2000; Kataky et al., 2002, Achmann et al., 2008). A number of sensor approaches for the detection of FA concentration have been published including systems operating in gas (Dennison et al., 1996; Hämmerle et al., 1996; Vianello et al., 1996) and organic phases. An optical biosensor has also been proposed for FA assay (Rindt & Scholtissek, 1989).

Potentiometric biosensors, consisting of a pH sensitive field effect transistor as a transducer and either the enzyme AOX, or permeabilised yeast cells (containing AOX) as the biorecognition element, have been described by Korpan et al. (2000). These biosensors have demonstrated a high selectivity to FA with no interference response to methanol, ethanol, glucose or glycerol.

Amperometric biosensors have been suggested for the determination of FA level using FdDH (Winter & Cammann, 1989; Hall et al., 1998). Conductometric enzymatic biosensors based on FdDH (Vianello et al., 2007) and AOX (Dzyadevych et al., 2001) have been developed for FA assay.

3. Microbial methanol and formaldehyde biodegradation in wastewater

The study of microbial methanol and FA biodegradation in wastewater is an important problem of environmental biotechnology. Different microorganisms are capable of FA degradation: bacteria *Pseudomonas* spp. (Kato et al., 1983), *Halomonas spp*. (Azachi et al., 1995) and various strains of *Methylotropha* (Attwood & Quayle, 1984); the yeasts of genera *Debariomyces* and *Trichosporon* (Kato et al., 1982), *Hansenula* (van Dijken et al., 1975), *Candida* (Pilat & Prokop, 1976) and the fungi *Gliocladium* (Sakagushi et al., 1975). Selected strains of *Pseudomonas putida*, *Pseudomonas cepacia*, *Trichosporon penicillatum* and the mixed culture of these three species were used for aerobic degradation of FA and formic acid in synthetic medium and wastewater generated by melamine resin production (Glancer-Šoljan et al., 2001). The selected mixed culture containing two bacterial strains of *Pseudomonas* (*P. putida and P. cepacia*) and *Trichosporon* yeast genera (*T. peicillatum*) exhibited highly efficient degradation of FA and formic acid in the synthetic medium. The mixed culture also degraded formaldehyde, methanol and butanol contained in the wastewater of the melamine resin production facility.

Nineteen bacterial strains able to degrade and metabolize FA as a sole carbon source were isolated from soil and wastewater of a FA production factory. The samples were cultured in complex and mineral salts media containing 370 mg FA/L. Some strains were identified to be *Pseudomonas pseudoalcaligenes*, *P. aeruginosa*, *P. testosteroni*, *P. putida*, and *Methylobacterium extorquens*. After adaptation to high concentrations of FA, microorganisms completely consumed 3.7 g FA/L after 24 h, and degraded 70% of 5.92 g FA/L after 72 h (Mirdamadi et al., 2005). The development of appropriate technologies for the treatment of FA discharged

into the environment is important to minimize its negative impact. Studies have shown that in a special reactor for treating FA, both *Methanosaeta* and *Methanosarcina* were found to thrive with influent FA concentrations higher than 394.0 mg HCHO/L. Microorganisms like *Methanosaeta* probably survived due to its preferential use of acetate while *Methanosarcina* preferentially used the methanol (Oliveira et al., 2004). Biodegradation of FA was also tested in the marine microalga *Nannochloropsis oculata* (Yoshida et al., 2009). Transformation of [^{13}C]-FA in the medium was monitored by nuclear magnetic resonance (NMR) spectrometry. FA was transformed into formate, and these two substances degraded in the medium as was clearly shown by the NMR spectrometry.

Environmental FA can be detected and remediated in a biological system that incorporates a bacterium *Rhodobacter sphaeroides* containing suitable genetic sequences encoding a FA-inducible regulatory system. The system includes a transcriptional promoter from *Rhodobacter sphaeroides* that can be specifically induced in the presence of FA to transcribe an operable linked gene (US Patent 6242244).

The application of the methylotrophic yeast *Hansenula polymorpha* to the treatment of methanol and FA containing wastewater was experimentally verified. A variety of wastewater samples originating from chemical industry effluent were examined (Kaszycki & Kołoczek, 2000; Kaszycki et al., 2001). The methylotrophic yeast *H. polymorpha* was shown to cooperate with activated sludge from biological wastewater treatment stations, enhancing substantially its potential to biodegrade FA in industrial wastewater. After integration with yeast cells, the modified sludge retained its original structure and activity whereas its resistance to elevated FA concentrations was significantly improved (Kaszycki & Koloczek, 2002). An yeast isolate revealing unique enzymatic activities and substrate-dependent polymorphism was obtained from the autochthonous microflora of soil heavily polluted with oily slurries. By means of standard yeast identification procedures, the strain was identified as *Trichosporon cutaneum.* Further molecular PCR product analysis of ribosomal DNA confirmed the identity of the isolate with the genus *Trichosporon*. As it grew on methanol as a sole carbon source, the strain appeared to be methylotrophic, able to utilize formaldehyde (Kaszycki et al., 2006).

Mitsui et al. (2005) isolated a bacterial strain that efficiently degraded FA and used it as a sole carbon source. The isolated strain was identified as *Methylobacterium* sp. MF1, which could grow on FA and methanol. The resistance to the toxic effects of FA exhibited by *Methylobacterium* sp. MF1 is related to factors other than C1 metabolism.

Microorganisms utilizing methanol have adopted several metabolic strategies to cope with the toxicity of FA. Mechanisms of FA detoxification in yeast, bacteria and archaea were studied (Yurimoto et al., 2005). The toxicity of FA in batch assays, using volatile fatty acids as co-substrates and the continuous anaerobic treatment of wastewaters containing FA in upflow anaerobic sludge blanket reactors was investigated (Vidal et al., 1999). The kinetic process of FA biodegradation in a biofilter packed with a mixture of compost, vermiculite powder and ceramic particles was studied by Xu et al. (2010).

4. FA-oxidizing yeast enzymes for FA monitoring

4.1 NAD^+- and glutathione-dependent formaldehyde dehydrogenase (FdDH)

4.1.1Yeast engineered for overproduction of FdDH

To construct strains of *H. polymorpha* that over-produce thermostable NAD^+- and glutathione-dependent FdDH, the *H. polymorpha FLD*1 gene with its own promoter

(Baerends et al., 2002) was inserted into the integrative plasmid pYT1 (Demkiv et al., 2005) containing the *LEU2* gene of *Saccharomyces cerevisiae* (as a selective marker). The constructed vector was used for multi-copy integration of the target gene into the genome of *H. polymorpha* by transformation of *leu 1-1* (Demkiv et al., 2005) and *leu 2-2* recipient cells (both *leu* alleles are complemented by *S. cerevisiae* gene *LEU2*). The transformation was performed using three different methods (Table 1): electroporation (Delorme, 1989), the lithium chloride method (Ito et al., 1983), and the protoplasting procedure (Hinnen et al., 1978).

Selection of FdDH-overproducing strains was carried out simultaneously by leucine prototrophy and by resistance to elevated FA concentrations in the medium. Of more than 150 integrative Leu$^+$- transformants with higher resistance to FA – up to 10-12 mM on solid plates, 14 stable clones, resistant up to 15-20 mM FA on plates, were selected and studied in more detail. The growth characterstics of selected clones in the liquid medium were shown in Fig.1: all transfomants grew better and were more resistant to elevated FA content in liquid medium with 1% methanol, compared to the recipient strains (Demkiv et al., 2005, Gayda et al, 2008). Finally, FdDH specific activities were tested in cell-free extracts (CE) of the best selected FA-resistant Leu-prototrophic transformants (Fig. 2).

Parental strains	Transformation method	Plasmid	Number of experiments	Average transformation efficacy, Leu$^+$-clones/μg DNA	Number of the tested clones with a higher resistance to FA
Leu1-1	Electroporation	pHpFLD1	3	$2x10^3$	12
Leu2-2		pHpFLD1	3	30	10
Leu1-1		$pHp(FLD1)_2$	3	$1.5x10^3$	50
Leu2-2		$pHp(FLD1)_2$	3	15	10
Leu1-1	LiCl	pHpFLD1	3	2	12
Leu2-2		pHpFLD1	3	20	80
Leu1-1	Protoplastes	$pHp(FLD1)_2$	1	0.5	1
Leu2-2		$pHp(FLD1)_2$	1	0.4	2

Table 1. Efficacy of different transformation methods for two strains of the yeast *H. polymorpha* by plasmids *pHpFLD1* and $pHp(FLD1)_2$

Activity of FdDH was determined by the rate of NADH formation monitored spectrophotometrically at 340 nm (Schutte et al., 1976). One unit (1 U) of the enzyme activity was defined as the amount of the enzyme which forms 1 μmole NADH per min under standard conditions of the assay: 25^{o}C, 1 mM FA, 1 mM NAD$^+$, 2 mM GSH in 50 mM Phosphate buffer (PB, pH 8.0).

Tf 11-6 and Tf-142 were the most effective recombinant strains, with the highest FdDH activity, up to 4.0 U/mg, which is a 4-5 fold increased as compared to the parental strains, *leu* 1-1 and *leu* 2-2, respectively. These transformants were characterized and chosen as a source for FdDH production. It was estimated by Southern dot-blot analysis, that genomes of the stable recombinant yeast clones contain 6-8 copies of the target *FLD1* gene, which confirmed the results obtained by the Southern-hybridization method (data not shown). Therefore, the recombinant yeast strain Tf 11-6 contains more than 8 copies of the integrated plasmid, as compared to 1 copy of the parental strain, probably due to the usage of the double-gene plasmid $pHp(FLD1)_2$ and its tandem integration into the genome of the recipient strain.

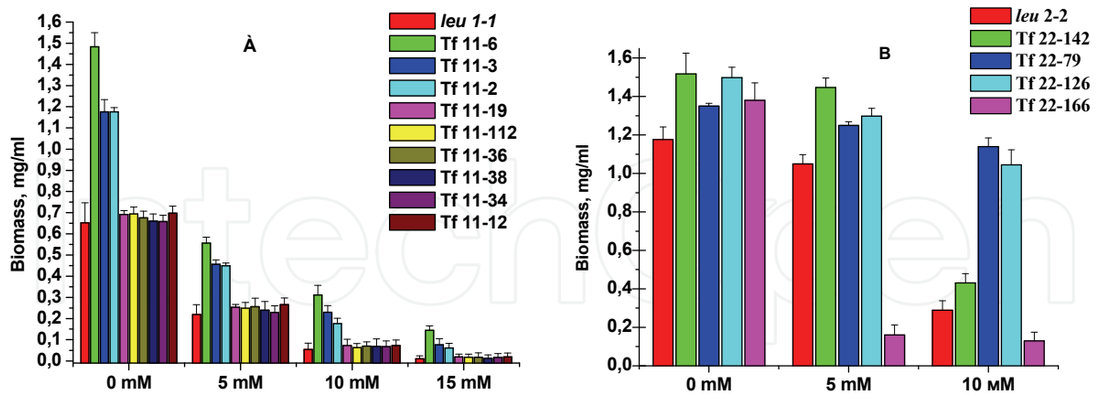


Fig. 1. Resistance to FA of the recipient yeast strains *leu1-1*(A) and *leu2-2* (B), *of H. polymorpha* and their transformants, grown in 1% methanol medium

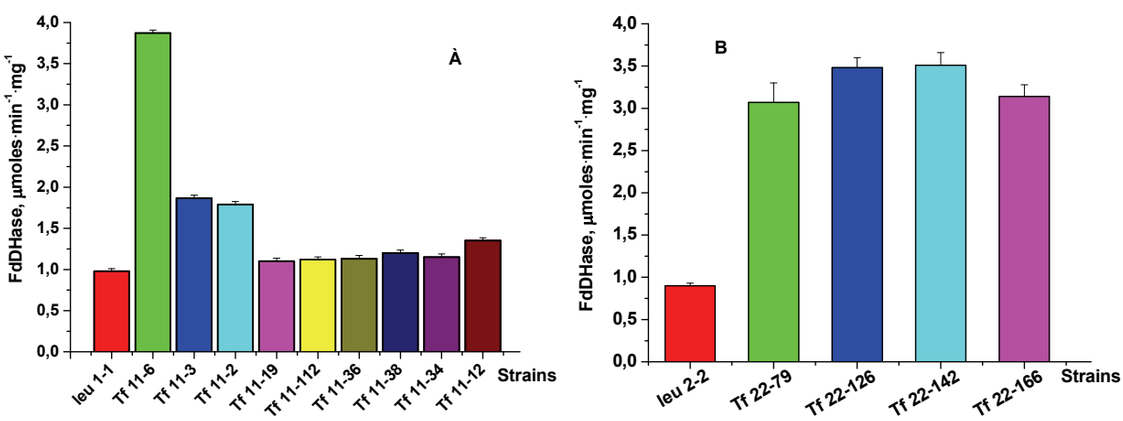


Fig. 2. Specific activity of FdDH in cell-free extracts of parental yeast strains *leu1-1*(A) and *leu 2-2* (B) *of H. polymorpha* and their transformants grown in 1% methanol medium

4.1.2 Optimization of cultivation conditions for FdDH-overproduction

In order to optimize cultivation conditions to obtain the highest enzyme yield, the influence of growth medium composition on FdDH concentration using the two best strains, Tf 11-6 and Tf 22-142, was studied. FdDH activity in cell-free extract was dependent on a carbon source. Cultivation in 1% methanol as a sole carbon source resulted in the highest levels of the enzyme synthesis for both of the tested strains (Fig. 3), which is in accordance with the literature concerning the wild type strains (Hartner et al., 2006; Harder et al.,1989; Egli et al.,1982).

The addition of FA to the methanol medium stimulated synthesis of FdDH. Under experimentally determined optimal conditions, *i.e.* methanol as carbon source, methylamine as nitrogen source and 5 mM FA as an additional inductor of FdDH synthesis, target

Fig. 3. FdDH activity in CE of the recombinant strains Tf 11-6 and Tf 142, cultivated on the media with methylamine, 5 mM FA and different carbon sources: 1% ethanol (EtOH), 1% methanol (MeOH), 1% glucose (Glc) or glycerol (Glyc).

enzyme activity achieved was 6.2 U/mg, 1.6-fold higher than under normal growth conditions, as described in Fig. 2. The addition of up to 10 mM FA to the optimal culture medium resulted in FdDH activity of 8.3 U mg^{-1}, a 2-fold increase as compared to medium without FA (Fig.2). The strong correlation between FA concentration in the medium and FdDH activity in cultivated cells of recombinant yeast strain Tf-11-6, demonstrates the important role of FA as a FdDH-synthesis inducer (Fig. 4).

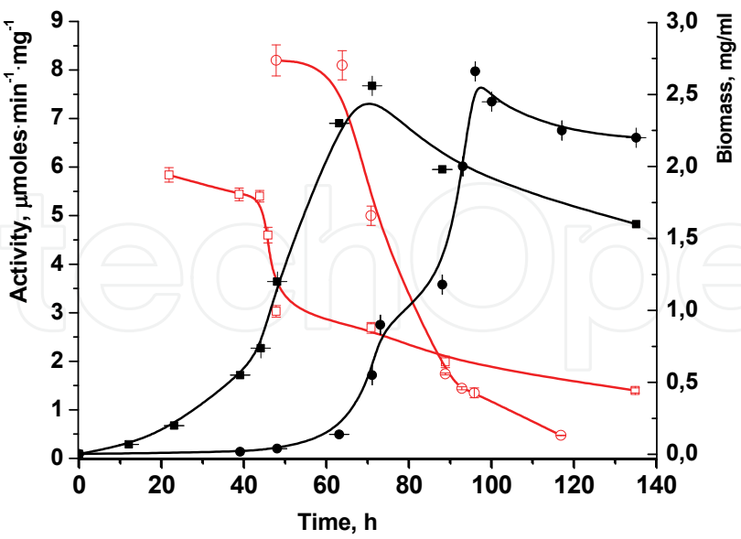


Fig. 4. FdDH activity (red), and biomass (black) of the enzyme-overproducer Tf-11-6 during cultivation in a medium with 1% methanol supplemented with 5 mM (■, □) and 10 mM (●, ○) formaldehyde.

4.1.3 FdDH purification and characterization

For enzyme isolation from cell-free extracts, cells of the recombinant over-producer strain Tf 11-6, cultivated in 1 % methanol medium supplemented with 5 mM FA during 20 h, were used. A simple scheme for FdDH isolation and purification on anion-exchange sorbent was proposed, resulting in a FdDH preparation with specific activity about 27 U units per mg of protein. For comparison, specific activities of commercially available FdDH preparations from *Ps. putida* and from the yeast *C. boidinii* are 3-5 U mg^{-1} and 17-20 Umg^{-1}, respectively (Demkiv, et. al. 2007). The purity of the isolated enzyme preparation was controlled by PAAG electrophoresis in denaturizing conditions (Laemmly, 1970).
Some physico-chemical characteristics of the purified FdDH are shown in Table 2.

Strains/ property		*Candida boidinii*	*Pichia pastoris*	*H. polymorpha* strains	
				wild type	recombinant Tf11-6
M, kDa	Enzyme	80/82	84	82	-
	Subunit	40 /42	39/41	40.6	40
K_M, mM	FA	0.25/0.29	0.43/0.31	0.21	0.18
	GSH	0.13/-	0.48/0.16	0.18	-
	NAD^+	0.09/0.025	0.24/0.12	0.15	0.21
	Methylglyoxal	1.2/2.8	-	-	-
	Formylglutation	-/0.12	-/0.6	-	-
	NADH	-/0.025	-/0.25	-	-
Temperature optimum, ^{0}C		35/-	47/-	-	50
Thermostability, ^{0}C*		52/-	52/-	-	57
pH optimum		8.5/-	7.9/-	8.2	7.5-8.5
Reference		Schutte et al., 1976 /Kato et al., 1990	Allais et al.,1983; /Patel et al., 1983	Uotila et al., 1979	Demkiv et al., 2007

Table 2. Comparison of structural and enzymatic properties of FdDH.

The molecular mass of the FdDH subunit, estimated by SDS-electrophoresis, was shown to be approximately 40 kDa, similar to the 41 kDa found for *C. boidinii* (Melissis et al., 2001). It was reported that the predicted *FLD1* gene product (Fld1p) is a protein of 380 amino acids (Baerends et al., 2002). Taking into account, that the M of the native enzyme from various methanol-utilizing yeasts were estimated to be from 80 to 85 kDa, isolated thermostable, NAD^+- and GSH-dependent FdDH can be assumed to be dimeric. As shown in Table 2, values of the Michaelis-Menten constant (K_M) for FA and NAD^+ calculated for this enzyme are close to K_M for the wild-type enzyme.
Optimal pH-value and pH-stability (during incubation in the appropriate buffer at room temperature for 60 min) of the enzyme were evaluated. Optimal pH was found to be in the range of 7.5-8.5, and the highest stability of FdDH was observed at pH 7.0-8.5.

The optimal temperature for enzyme activity was 50^0C. At 65^oC the enzyme retained about 60% of its highest activity (assay time 5 min), *i.e.* equal to the level of FdDH activity at 30^0C. The enzymatic activity at 37^0C was 1.6-fold higher than under the standard conditions of the FdDH activity assay (at 25^0C). Study of the thermal stability of the enzyme demonstrated that its activity was completely preserved after 10 min of incubation at 40^0C, and was partially preserved at 55^0C (up to 70%) and 60^0C (25%). Complete inactivation occurred after heating of the enzyme solution at 70^0C for 5 min. These results indicate that the thermostability of the enzyme is apparently high, enabling its usage for bioanalytical purposes, namely, for FA assay in food products, wastewater, and pharmaceuticals, as well as for biotransformation of FA to formic acid.

The effect of a number of inhibitors on the enzymatic properties was studied. Table 3 shows an influence of some compounds on enzymatic activity in purified FdDH preparation tested before and after its incubation with additives, for 30 min at 4°C. Bivalent cations (Zn^{2+}, Cu^{2+} and Mn^{2+}), as well as an ionic detergent SDS were shown to inhibit FdDH activity. According to the literature, enzymes from two other yeasts, *P. pastoris* and *C. boidini* (Allais et al., 1983; Kato et al., 1990, Patel, 1983) were also inhibited in a similar fashion.

4.2 Enzymatic methods for FA monitoring

4.2.1 The development of FdDH- and AOX based enzymatic kits

FdDH preparation isolated from the recombinant strain of the yeast *H. polymorpha* with the specific activity 17.0 units per mg of protein at 25°C (that is about 27 U·mg^{-1} at 37°C) was proposed for the enzymatic assay of FA. In methylotrophic yeasts, NAD^+- and glutathione-dependent FdDH catalyzes the oxidation of FA to formic acid with the simultaneous reduction of NAD^+ to NADH.

Additive	FdDH activity (%) under different additives levels	
	1 mM	10 mM
$ZnSO_4$	23.3	0
$CuSO_4$	0	38.3
$FeCl_3$	78.3	0
$MnCl_2$	27.8	60.0
$MgCl_2$	84.8	85.0
EDTA	96.5	85.0
PMSF	91.7	56.3
2-mercaptoethanol	72.7	66.7
SDS	0	0
Dithiotreitol	96.33	85.2

Table 3. The influence of different additives, in concentrations 1 and 10 mM, on enzymatic activity of purified FdDH preparation (100 % activity has initial enzyme preparation)

The proposed enzymatic method is based on the photometric detection of colored product, formazan, formed from nitrotetrazolium blue (NTB) in reaction coupled to FdDH-catalyzed oxidation of FA in the presence of an artificial mediator, phenasine methosulfate (PMS) and detergent Triton X-100 (Demkiv et al., 2007, Demkiv et al., 2009):

$$NADH + NTB^{+} \longrightarrow NAD^{+} + \text{Formazan} \quad (6)$$

The enzymatic kit "Formatest" was developed on the base of these reagents. The assay was performed in conditions of incomplete conversion of the analyte (approximately, 10 %), using a limited concentration of the enzyme (23 mU/ml) in the reaction mixture. These conditions are economic and reasonable, because of the high FA content in the tested samples. Under conditions of complete oxidation of FA (excess of the enzyme), assay sensitivity was determined to be 2.5 µM (in final reaction mixture) or 20 µM in the tested samples.

Alcohol oxidase (AOX) from the thermotolerant methylotrophic yeast cells *H. polymorpha* can be an alternative to FdDH, used for analytical purposes. This enzyme is quite stable, contains tightly bound FAD and does not need any exogenous co-enzyme for catalytic activity (Woodward J., 1990). Theoretically, AOX can be used to assay FA because in aqueous solutions FA exists in hydrated form (95–99% of total concentration) which structurally resembles methanol, and can be oxidized by AOX with the subsequent formation of formic acid and hydrogen peroxide (see reactions 4 and 5).

AOX preparations were isolated from the strain *H. polymorpha* C-105 - catalase-defective mutant (Gonchar et al., 1990) with impaired glucose catabolite repression of AOX synthesis (*gcr1, catX*). The mutant cells, grown in glucose medium, were disrupted and cell-free extract was used for partial purification of AOX by two-step ammonium sulfate precipitation (Gonchar et al., 1998). Using this simple procedure, enzyme preparation in a form of suspension in 60 % saturated $(NH_4)_2\ SO_4$, with specific activity of 7.5 U/mg, was obtained. This is close to activity of some commercial AOX preparations. As shown by PAAG electrophoresis, the isolated crude AOX preparation is not homogenous, but still suitable for analytical application. AOX preparation can be stored at 4 °C in 60 % saturated ammonium sulfate in the presence of protease inhibitors for at least 1 year without loss of activity.

The oxidase–peroxidase-based method (AOP) and enzymatic kit "Alcotest" were developed on the base of two enzymes - alcohol oxidase (AOX) and peroxidase (PO) (Gonchar et al., 2001). As a chromogen, 3,3',5,5'-tetramethyl-benzidine dihydrochloride (TMB) was used. The principle of FA determination by AOP-method is based on the measurement of the dye-product accumulation in peroxidative oxidation of chromogen by H_2O_2 (Sibirny et al., 2008) generated from FA in AOX reaction (see reactions 5, 7):

$$\underset{\text{chromogen}}{H_2O_2 + S\text{-}[2H]_{reduced}} \xrightarrow{PO} \underset{\text{dye}}{2H_2O + S_{ox}} \quad (7)$$

The analytical parameters of the FdDH-based method have been determined (Fig. 5) in comparison with enzymatic AOP-method and several chemical methods based on the use of Nash's reagent, 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald), chromotropic acid and 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH). It was clearly shown, that AOP-method has the highest sensitivity. The slope of the corresponding calibration curve is equal 59.3, corresponding to an apparent millimolar extinction of the formed coloured product (in $mM^{-1}\ cm^{-1}$). Actually, the observed slope value equals the millimolar extinction coefficient (ε_{mM}) multiplied by conversion factor for the enzymatic reaction (k):

$$\text{Slope} = \varepsilon_{mM} \times k \quad (8)$$

The value of ε_{mM} for the oxidized TMB is equal 81.7 mM^{-1} cm^{-1} (Gonchar et al., 2001), so the conversion coefficient of the analyte for AOP-method at the used experimental conditions is 72.6 %. For the FdDH-based method, conversion factor of the analyte is 32.9 %, assuming a milimolar extinction for NTB-formazane as 10.2 mM^{-1} cm^{-1} at 570 nm in acidic medium.
The linearity of calibration curve for AOP-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^{-1}), and the threshold sensitivity of the method is about 0.8 nmol ml^{-1}. These analytical parameters are the best as compared to four chemical methods, even with the use of MBTH or Purpald.
FdDH-based method reveals linearity (at enzymatic conversion above 33 %) at least to 100 μM FA, and its sensitivity is close to Nash's method (the corresponding slopes are 3.36 and 4.46, respectively). Compared to AOP-method, sensitivity of FdDH-based-method is 18-fold less.

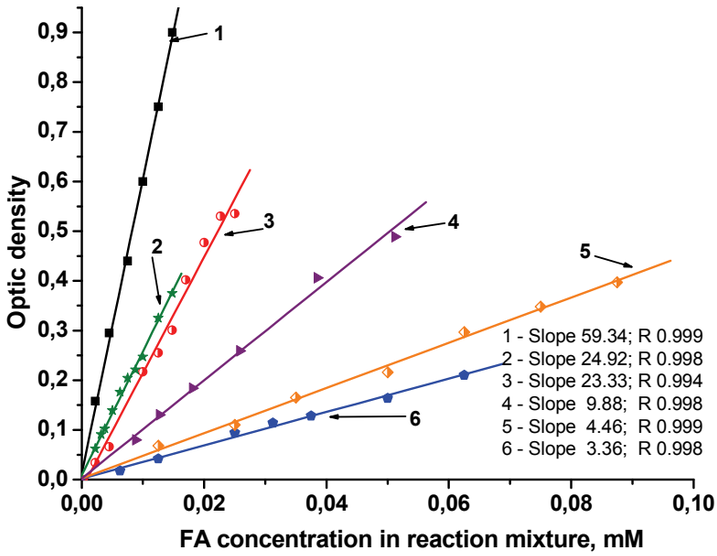


Fig. 5. Comparative analysis of FA-assay methods, using: 1- AOX and PO (AOP); 2 - MBTH; 3 - "Purpald"; 4 -chromotropic acid; 5 - Nash's reagent and 6 - FdDH. The slopes of calibration curves characterize the sensitivity of the methods. Slope values and coefficients of linear regression are shown for each calibration curve.

4.2.2 The reliability of the enzymatic methods for assay of FA in real samples

The FdDH- and AOX-based methods were tested on the real wastewater samples containing FA. We have tested FA in real samples of wastes by the developed enzymatic methods in comparison with standard chemical approaches. It has been demonstrated that in order to evaluate the possible interfering effect of real samples' components on FA assay, it was necessary to perform a standard addition test in both approaches (chemical and enzymatic) and that analytical data obtained by enzymatic method are more reliable than chemical ones.
As shown in Table 4, the comparison of FA concentrations for the FdDH- and AOX-based methods and two routinely used chemical ones (chromotropic acid and MBTH), showed a good correlation between the four approaches. Only in some cases (samples of wastewater DK5 and DK7), with a lower FA content, the difference between the compared methods is higher, than 15 % - 41 % and 26 %, respectively. A relatively high difference is also observed between two chemical methods for the mentioned above samples – 37 % and 21 %. This can

be explained by a higher error in measurement of low optical density values obtained for samples with a low FA content. On the other hand, it is worth emphasizing that the chemical approaches used are not free from possible mistakes due to interference from co-impurities, usually present in wastewater samples, for example, phenol, which is an attendant pollutant of FA-containing wastes (Polish standard, 1988).
To evaluate the possible interfering effect of the components of wasterwater samples on FA assay by the FdDH-based and chromotropic acid methods, we used a standard addition test (SAT) for sample WW-A (Table 4, Fig. 6A and B).

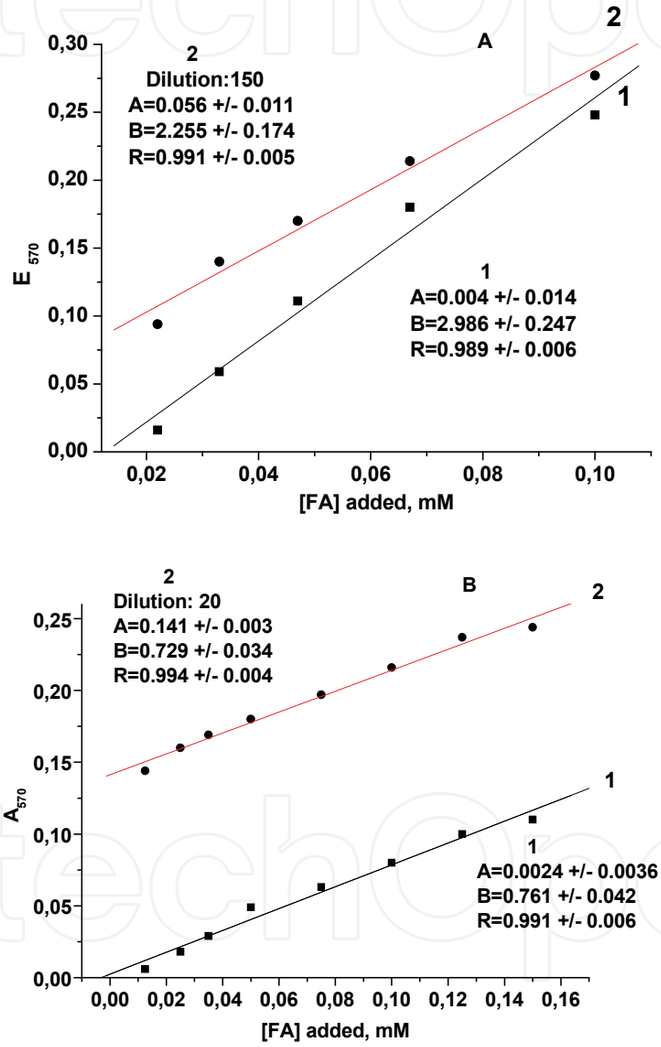


Fig. 6. Standard addition test for the FA assay by the chromotropic acid method (A) and the FdDH-based method (B). Curve 1 corresponds to the calibration experiment performed for FA solutions (traditional calibration), and curve 2 corresponds to the standard addition calibration (FA was added at different concentrations to the diluted wastewater sample; WW-A). Some statistical data are presented on the graphs: parameters of linear regression (coefficients of the equation Y =A+BX, where Y = OD, X = FA concentration (mM), A = OD of the variant without addition of exogenous FA, and B = slope value); R = linear regression coefficient.

Sample/ Method	Enzymatic methods		Chemical methods	
	FdDH-based	AOX-based	Chromotropic acid	MBTH
DK 1	7.89±0.59	9.60±0.45	9.30±0.61	9.56±0.51
DK 2	6.66±0.26	8.12±0.20	8.70±0.50	8.06±0.32
DK 3	6.88±0.41	8.01±0.44	7.20±0.33	7.84±0.36
DK 4	7.58±0.32	6.86±0.9	7.10±0.36	6.30±0.46
DK 5	2.32±0.08	1.97±0.12	1.65±0.35	1.20±0.15
DK 6	5.73±0.32	5.60±0.28	4.64±0.24	4.99±0.06
DK 7	2.47±0.15	2.19±0.2	1.62±0.17	1.96±0.20
WW-A	112±4.5 (SAT) 84.4±6.5 (routine test)	-	116±5.1(SAT) 111±6.1 (routine test)	-

Table 4. Comparison of different methods for FA assay (mg/L) in wastewater samples

As can be seen from Fig. 6, the chromotropic method is more sensitive to the interfering effect of the wasterwater sample components than the enzymatic method: the slope values of the calibration curves obtained for FA in water and in the background of wasterwater sample (WW-A) differed by 24% (2.986 and 2.255, respectively). The respective values obtained for the enzymatic method were 0.761 and 0.729, a difference of only 4.2 %, which is within the limit of statistical deviation. Thus, we can conclude that analytical data obtained by the FdDH-based method are more reliable than the chemical ones. Due to this very important analytical feature of the enzymatic method, it can be recommended for practical application in lieu of chemical methods, which are labour-intensive and time consuming, thereby eliminating the need to distil the samples or perform standard addition test (as in the case of phenol contamination).

The FdDH-based method was tested on different FA-containing vaccines (Paryzhak et al., 2007). As shown in Table 5, the comparison of FA concentration obtained by the FdDH-based method and by two routinely used chemical ones, showed a good correlation between both approaches. Lower levels of FA in anti-diphtheria vaccines, obtained using the enzymatic method as compared to the chemical methods may be due to the inhibitory effect on the enzyme by the Hg-containing compound, 0.01% merthiolate, a vaccine preservative.

Sample/ Method	Enzymatic methods	Chemical methods	
	FdDH-based	Chromotropic acid	MBTH
Anti-diphtheria vaccine	15±2.5	36±1.4	31±2.0
Anti-diphtheria and tetanus vaccine	17±1.9	27±2.0	29±2.7
Polio-vaccine "Imovax"	30±3.0	27±2.6	–
Tetanus vaccine	10.2±0.6	9.0±0.2	12.0±0.2

Table 5. Comparison of FA assay methods (mg/L) in the different vaccines

4.2.3 AOX based method for simultaneous assay of methanol and FA in industrial wastewater

We describe a new enzymo-chemical method for the simultaneous assay of methanol and FA in mixtures, which exploits AOX and aldehyde-selective reagent - 3-methyl-2-benzothiazolinone hydrazone, MBTH (Sibirny et al., 2008). Pre-existing FA content is detected without treating samples by AOX (CD_0 in reaction 9); and methanol content is determined by an increase in colored product concentration due to the methanol-oxidising reaction (CD_Δ in reaction 10).

$$[CH_2O]_0 + MBTH \rightarrow [MBTH\text{-}CH_2O]_0 \xrightarrow[Azine]{FeCl_3} \text{Cyanine dye } (CD_0) \quad (\lambda max=670 \text{ нм}) \quad (9)$$

$$CH_3OH + O_2 \xrightarrow{AOX} [CH_2O]_\Delta \xrightarrow{MBTH} [MBTH\text{-}CH_2O]_\Delta \xrightarrow[-H_2O_2]{FeCl_3} \text{Cyanine dye } (CD_\Delta) \quad (10)$$

Methanol is oxidized to FA by AOX, and FA is oxidized further by AOX. In the presence of MBTH, FA reacts with MBTH, to form an azine adduct that prevents the further enzymatic oxidation of FA by AOX. In this reaction MBTH plays a double role. During the first step of reaction, it forms a colorless azine adduct with pre-existing and enzymatically formed FA, and masks it from further oxidation by AOX, and during the second step of reaction, MBTH facilitates the non-enzymatic oxidation of the azine product to cyanine dye in the presence of ferric ions in acid medium. Pre-existing FA content is assayed by colorimetric reaction with MBTH, without treating samples by AOX, and methanol content is determined by a gain in a colored product due to methanol-oxidising reaction. This enzymo-chemical method of differential detection of FA and methanol in mixtures was used to analyze samples of a commercial product, formalin, which is a concentrated FA solution containing methanol as a stabilizer that inhibits FA polymerization. The results of this analysis, shown in Table 6, are in a good agreement with the data obtained by traditional chemical methods and gas-chromatography.

Sample	Methanol (MeOH) and formaldehyde (FA) content, % (M±m, n=4)				
	AOX-chemical method		Gas-chromatography	Chemical method (chromotropic acid, permanganate)	
	MeOH	FA	MeOH	MeOH	FA
I	2.59 ± 0.19	4.36 ± 0.23	3.3 ±0.5	2.7 ±0.13	4.62 ±0.11
II	4.61± 0.34	7.15 ± 0.37	5.39 ± 0.5	4.72 ± 0.27	7.27 ± 0.2
III	3.29 ±0.38	6.95 ± 0.23	3.4 ± 0.5	3.01 ± 0.08	6.49 ± 0.28
IV	2.8 ± 0.32	6.23 ± 0.25	3.53 ± 0.5	2.70 ± 0.05	6.58 ± 0.33
V	0	1.72 ± 0.2	0	0	1.85 ± 0.1
VI	0	1.48 ± 0.13	0	0	1.73 ± 0.08
VII	3.77 ± 0.30	2.66 ± 0.16	3.13 ± 0.2	3.79 ± 0.12	3.82 ± 0.15
VIII	4.15 ± 0.32	2.14 ± 0.27	3.06 ± 0.5	2.93 ± 0.31	4.11 ± 0.13

Table 6. Results of enzymo-chemical assay of methanol and FA in distillate of wastewaters (compared with the reference methods)

The threshold sensitivity of the assay method for both analytes is near 1 μM which corresponds to 30-32 ng analyte in 1 ml of reaction mixture and is 3.2-fold higher when compared to the chemical method using permanganate and chromotropic acid. The linearity of the calibration curve is reliable ($p < 0.0001$) and standard deviation for parallel measurements of test samples does not exceed 7%. The proposed method, in contrast to the standard chemical approach, does not need the use of aggressive chemicals (concentrated sulfuric, phosphoric, chromotropic acids, permanganate), it is easier to perform, and can be used for industrial waste verification and certification of formaline-containing materials**.**

4.2.4 AOX- and FdDH-based methods for FA assay in fish food products

Fish products are an important source of food protein. The fish species *Gadidae* are second only to *Clupeidae* in the size of industrial catch, but are preferred as food products whereas *Clupeidae* are more frequently used in agriculture and industry. The tissues of the *Gadidae* fish under inappropriate storage, that is, at non-deep freezing conditions (t°>–30 °C), accumulate highly toxic concentrations of FA due to endogenous metabolic reactions, involving namely the natural osmoprotectant trimethylamine-N-oxide, which acts as antifreeze (Reihbein, 1995). Generated FA can cause the fish to spoil, and even make it dangerous for human health if consumed. These two important reasons highlight the necessity for selective, sensitive and reproducible method to control the content of this dangerous metabolite in some fish products.

The applicability of both enzymes simultaneously used, AOX with peroxidase (AOP-method) and FdDH for FA assay in fish products was demonstrated. Test samples of frozen fish of the *Gadidae* family (hake and cod), most frequently sold in European markets, as well as freshly-killed carp were used. The optimal protocols for obtaining of protein-free extracts and for testing procedures have been elaborated (Pavlishko et al., 2003). The analytical parameters of both enzymatic methods have been determined in comparison with several chemical methods based on the use of Nash's reagent, Purpald, chromotropic acid and MBTH. Fig. 5 presents calibration curves for the two enzymatic methods and compares them with the best of the chemical methods. It is clearly shown, the AOP-method has the highest sensitivity.

The FdDH-based method is nearly 18-fold less sensitive, compared to AOP-method, because of a lower molar extinction of the corresponding formazane: the analyte conversion factor is 32.9 %, assuming a milimolar extinction for NTB-formazane as 10.2 mM^{-1} cm^{-1} at 570 nm in acidic medium (own data). FdDH-based method sensitivity is close to Nash's method (the corresponding slopes are 3.36 and 4.46, respectively). Linearity of FdDH-based method is at least to 100 μM FA.

There was a good correlation between the analytical results of both enzymatic methods as compared with chemical approaches, though AOX-based assay is preferred due to its higher sensitivity, good linearity, insensitivity to the interference by test sample contaminants and the usage of non-aggressive reagents for the sample pre-treatment and assay procedure (Table 7 and Table 8).

Table 8 shows FA concentrations as measured by all of the tested methods. To compare the validity of both enzymatic methods, and to evaluate possible interference by the chemical background of the test samples on analytical results, FA content was analyzed using in fish protein-free extracts using a routine method (with an external calibration) as well as a multiple standard addition test (MSAT). Simultaneously, FA concentration was also analyzed by two chemical methods, using chromotropic acid and MBTH.

Approach \ Fish	Hake	Cod	Carp
AOX- based, M±m	90.0±2.6	74.1±1.1	0
Nash's, M±m	121.6±0.9	96.8±3.1	0
Purpald, M±m	100.6±1.6	59.7±3.1	0

Table 7. Results of FA assay (in mg per 1 kg of wet weight of muscle tissue) in protein-free extracts of fish using three independent approaches: AOX- based method, Nash's and Purpald methods

Method	Multiple standard addition test	Routine test
FdDH- based, M±m	101.8±3.2 (p<0.05)*	64.3±8.6
AOX- based, M±m	95.3±3.7 (p>0.05)**	98.0±3.5
MBTH, M±m	104.3±5.6 (p>0.05)**	106.4±7.9
Chromotropic acid, M±m	100.5±1.2 (p>0.05)**	102.8±7.3

**Difference between routine test and MSAT is statistically significant; **Difference between routine test and MSAT is statistically insignificant.*

Table 8. Results of FA assay (in mg FA per kg of wet weight of muscle tissue) in protein-free extract of the fish hake, using three independent approaches: FdDH-metod, MBTH and chromotropic acid.

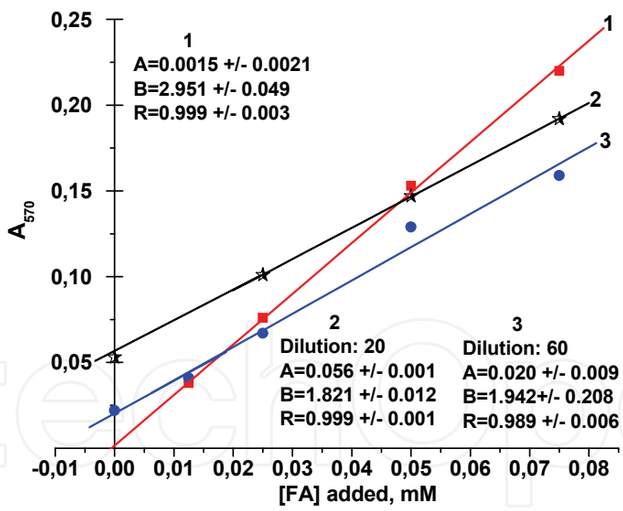


Fig. 7. Multiple standard addition test for FA assay in hake, using the FdDH-based method. Curve 1 corresponds to the calibration experiment performed for aqueous solutions of FA (external traditional calibration), and curves 2 and 3 correspond to the multiple standard addition test (FA was added at different concentrations to the diluted real sample). Some statistical data are presented on the graphs: parameters of linear regression (coefficients of the equation Y =A+BX, where Y - optical density, X - FA concentration (mM), A – optical density for the sample without addition of exogenous FA, and B - slope value); R - linear regression coefficient

It was demonstrated that some fish products (hake and cod) contain high FA concentrations, up to 100 mg/kg wet weight, while FA content in carp was negligible.
The slopes of the calibration curves prepared on fish extracts are dependent upon the dilution factor, a bigger dilution results in a higher the slope (meaning there is less of an interfering effect). For the external calibration (that has no test sample background, and corresponds to an infinite dilution), the slope is the highest, 2.95 as compared to 1.94 (a 60-fold dilution of the test sample) and 1.82 (20-fold dilution of the sample). For the AOP and chemical methods, there is no significant difference between routine and MSAT-variants of the assay.
As shown in Table 8, there is a good correlation between all analytical data obtained in the MSAT-variant of analysis, which was not the case for the results obtained by the routine variant of analysis with external calibration. This may be due to the interference of some components which are co-extracted by TCA from the fish tissue. This suggestion is clearly supported by the data obtained by the FdDH-based method (Fig. 7).

5. Construction and investigation of FA-selective biosensors

5.1 AOX- based enzymatic and microbial sensors

For the quantitative analysis of FA there have been developed potentiometric biosensors using whole cells of mutant strains of methylotrophic yeasts and AOX as the biorecognition elements and pH-Sensitive Field Effect Transistors (pH-SFETs) as a transducer. As an analytical signal in the pH-SFET-based sensor, the production of protons due to FA conversion into formic acid was exploited.
To develop cell-based FA - sensitive potentiometric sensor (Korpan et al., 1993), the mutant strain *H. polymorpha* A3-11 with repressed activities of AOX and formate dehydrogenase and blocked activity of formaldehyde reductase was obtained. The biosensor demonstrated high specificity/selectivity to FA with no response to several organic acids, methanol and other alcohols, except for the very low sensitivity to ethanol. The linear dynamic range of the sensor's response corresponds to FA concentration of 2 to 200 mM.
Partially purified AOX preparations have also been used as recognition elements of pH-SFET-based potentiometric sensor selective to FA (Korpan et al., 1997; Korpan et al., 2000). The response time in steady-state measurement mode is in the range of 10–60 s, but if measured in kinetic mode the response time of the created biosensors was less than 5 s. The linear dynamic range of the sensor output signals corresponds to 5–200 mM of FA concentration. It was quite suprisingly that AOX-based sensors gave no signal to methanol and was highly selective to FA. These results seem rather unusual because methanol is the preferred substrate for most AOX's, being directly oxidised to FA. The absence of a measurable response to methanol may be explained as follows: a) the rate of methanol oxidation in AOX reaction is about 10-fold higher than that of FA; b) effective oxidation of methanol is likely to result in the local oxygen depletion in the bioactive zone limiting the oxygen available for subsequent FA oxidation; c) FA produced from methanol can diffuse from the bioactive zone back into the bulk solution without oxidation; d) FA, being very reactive, is likely to bind covalently with NH_2-groups of AOX.
All these factors may result in a decrease of the concentration of formic acid produced from methanol in bioactive membranes to a level lower than the sensitivity of the potentiometric biosensor described and therefore no response to methanol is apparent. It is noteworthy that most of the described factors do not work for intact yeast cells where FA and methanol are oxidised in different reactions.

It should be noted that contrary to other pH-SFET-sensors, the signal of AOX-based sensors (Korpan et al., 2000) to FA is not repressed, but even enhanced in the presence of Tris-HCl buffer. The chemical nature of this effect seems to be the reaction of FA with aminogroup of tris(hydroxymethyl)aminomethane with production of a hydroxymethylamine derivative, which is a weaker base compared to the parent compound and this reaction results in releasing free protons. This unexpected effect is the first reported example of specific "chemical enhancement" of the pH-SFET biosensor response.

A highly stable and sensitive amperometric bi-enzyme biosensor (Smutok et al., 2006) was developed for assay of ethanol, as well as of FA, using the highly-purified AOX preparation (Shleev et al., 2006), isolated from the yeast cells of *H. polymorpha* C-105. The sensor's layer was created with a non-manual electrochemically-induced immobilization procedure using a new type of Os-complex modified electrodeposition paints (EDP) for horseradish peroxidase placing in a first layer and a cathodic EDP for AOX immobilization and stabilization in a second layer. The used redox EDP assures fast electron transfer between the integrated peroxidase and the electrode surface at a low working potential.

Bioanalytical properties of an optimized biosensor such as response time, dynamic range for different analytes (FA and alcohols), operational and storage stability were investigated. The obtained sensors showed significantly improved stability as compared to previously reported sensors based on AOX. But such biosensor can be used for FA assay in wastes water only in the absence of aliphatic alcohols in tested probes.

For amperometric assay of FA, permeabilized and intact cells of the mutant strain *H. polymorpha* C-105 with a high activity of AOX as the biorecognition elements, were tested. Different approaches were used for monitoring FA-dependent cell response including analysis of their oxygen consumption rate by the use of a Clark electrode, as well as of oxidation of redox mediator at a screen-printed platinum electrode covered by cells entrapped in Ca-alginate gel. It was shown that oxygen consumption rate of permeabilized cells reached its saturation at 4 mM of FA (23 ^{o}C). The detection limit is 0.27 mM. In the presence of redox mediator 2,6-dichlorophenolindophenol (DCIP), the screen-printed platinum band electrode covered by permeabilized cells did not show any current output to FA. In contrast, well-pronounced amperometric response to FA was observed in the case of intact yeast cells in the presence of DCIP. However, intact cells did not show a strict substrate selectivity, because of functioning of the whole electron transport chain. In contrast, essentially improved substrate selectivity was observed in the case of permeabilized cells where only AOX is responsible for the oxygen consumption. Obviously, it is necessary to perform a directed metabolic engineering of the yeast cells to improve their bioanalytical characteristics in the corresponding biosensors (Khlupova et al., 2007).

5.2 FdDH-based capacitance, impedance and conductometric biosensors

Recombinant yeast FdDH (Demkiv et al., 2007) was used as a FA-recognising element coupled with semiconductor-based structure $Si/SiO_2/Si_3N_4$ as a transducer (Ben Ali et al., 2007). The bio-recognition element had a bi-layer architecture and consisted of FdDH, cross-linked with albumin, and two cofactors (NAD^+ and GSH) in the high concentrations (first layer); the second layer was a negatively charged Nafion membrane which prevented a leakage of negatively charged cofactors from the bio-membrane. Changes in capacitance properties of the bio-recognition membrane were used for monitoring FA concentration in a bulk solution. It has been shown that FA can be detected within a concentration range from 10 μM to 25 mM with a detection limit of 10 μM (Fig. 8 and Table 9).

A similar FA sensitive biosensor based on commercial preparation of bacterial FdDH as recognizing element has been also described (Ben Ali et al., 2006). As transducers, gold electrodes SiO_2/Si/SiO_2/Ti/Au and electrolyte- insulator-semiconductor Si/SiO_2 structures (EIS) have been used. Changes in capacitance and impedance properties of the bio-recognition membrane have been used for FA monitoring, It has been shown that FA can be detected within a concentration range from 1 μM to 20 mM depending on the used type of transduction, with a detection limit 1 or 100 μM for gold- and EIS-based transducers, respectively.

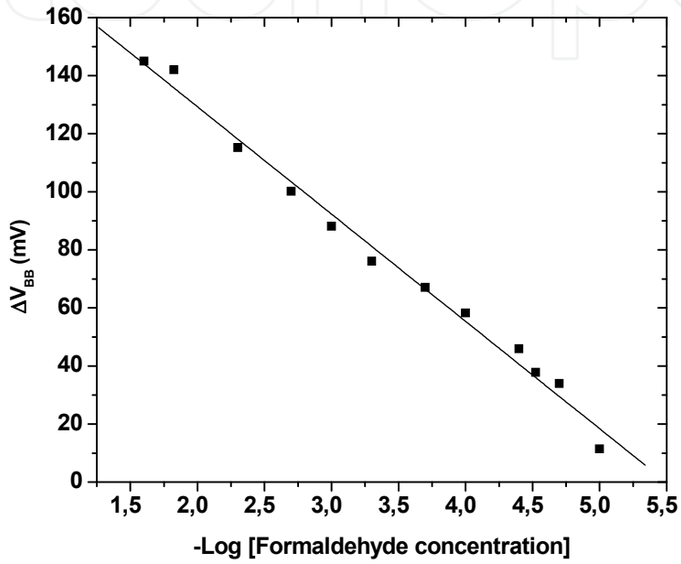


Fig. 8. Response of bio-functionalized Si/SiO_2/Si_3N_4 structure for FA (in logarithms of the molar concentration) in the tested solution

To construct FA-selective conductometric biosensor, the novel method was proposed using thin-film interdigitated gold planar electrodes and FdDH (commercial bacterial or recombinant yeast rFdDH) co-immobilized with cofactor(s) in the presence of DEAE-Dextran (Korpan et al., 2010).

DEAE-Dextran prevents active leakage of cofactors out of the bioselective layer and allows for the reuse of the biosensor without the regeneration of the cofactor(s), since its(their) concentration(s) in the layer is (are) high, constituting 100 mM and 20 mM for NAD^+ and GSH, respectively.

The working signals obtained for the conductometric biosensors based on rFdDH and FdDH, allow to assume that the apparent value of pH shift in the bioselective membrane does not exceed 0.4 units of pH. Due to the pH of the working solution (exactly, 8.7) used for measurements and some processes of protons masking, the steady-state value of protons inside the selective membrane can be estimated as 10^{-7} M. This means that during one assay the concentration of cofactor (NAD^+) consumed during FA transformation is about 6 orders lower in comparison with the concentration of NAD^+ (10^{-1} M) immobilized on the transducer surface. Even taking into account that 90% of NAD^+ could leak out of the membrane due to the washing procedures, and assuming that transformation efficiency of

the analyte is approx. 1%, the remaining effective level of NAD^+ has to be quite enough to perform up to 1.000 measurements using the same transducer. These calculations correlate well with the data obtained experimentally by tests performed on the developed biosensors over a 3 month period: more than 200 measurements on the same transducer have been made without any loss of the sensor signal and sensitivity. At the same time, most of the previously reported FdDH-based sensors (Vianello et al., 1996; Kataky et al., 2002) are based on the usage of covalently bounded NAD^+, which essentially decreases its availability and effectiveness for enzyme-substrate interactions.

It was studied the dependence of the developed rFdDH-based conductometric biosensor response on FA concentrations (Table 9). It has been shown (Fig. 9), that the conductometric biosensor is highly selective and even specific to FA, the basic substrate of FdDH. Other compounds, in particularly ethanol and ethanol, caused the significant changes in responces of the proposed conductometric biosensor. However, the usage of methanol and ethanol mixtures with FA leads to slight alteration of rFdDH-based conductometric sensor response values toward FA.

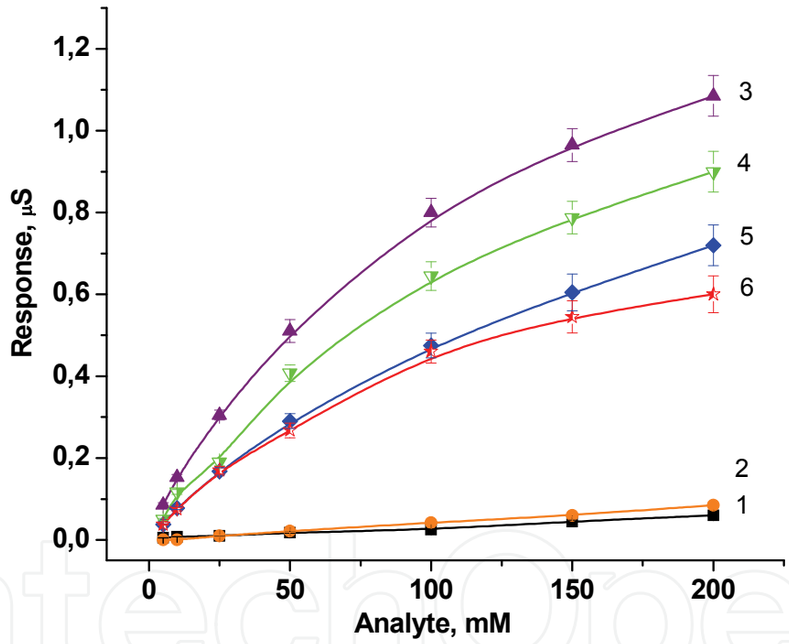


Fig. 9. Selectivity of the developed rFdDH-based biosensor to different substrates: 1 – methanol, 2 – ethanol, 3 – formaldehyde, 4 - equimolar mixture of FA and methanol, 5 - equimolar mixture of FA and ethanol, 6 – methanol, ethanol and FA. The measurements were performed in 10 mM borate buffer, pH 8.7

5.3 FdDH- and cells-based amperometric biosensors

The recombinant yeast cells Tf 11-6 *H. polymorpha* and FdDH isolated from these cells were used as biorecognition elements of amperometric biosensors. In the physiological electron-transfer pathway, electrons are transferred from FA via intermediate G-SH to the active centre of FdDH with the simultaneous reduction of NAD^+ to NADH. To design an electron-transfer pathway for the immobilised FdDH as a bioselective element of the sensor, the

enzymatically generated NADH has to be re-oxidised additionally at the electrode surface using a suitable redox mediator (Fig. 10).

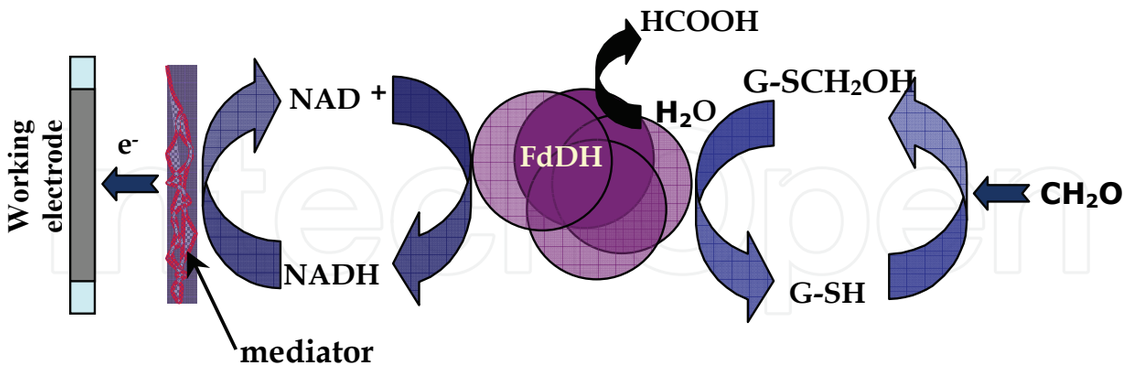


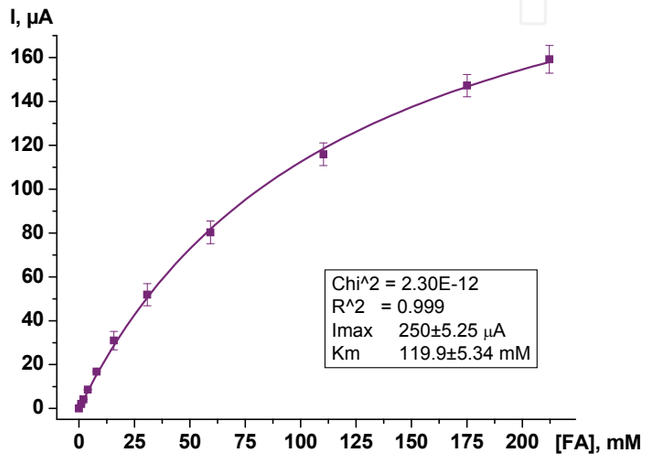
Fig. 10. Schematic representation of the electron pathway for FA detection by FdDH-based amperometric biosensor

The best mediators for enzyme biosensors were shown to be positively charged cathodic electrodeposition paints modified with Os-bis-N,N-(2,2′-bipyridil)-dichloride ([$Os(bpy)_2Cl_2$]) complexes (Ngounou et al., 2004). Among five tested Os-containing redox polymers of different chemical structures and properties, complex *1CPOs* of osmium-modified poly(4-vinylpyridine) with molecular mass of 60 kDa and containing diaminopropyl groups was selected as the most suitable redox polymer. At the same time, the polymer layer served as a matrix for keeping the negatively charged low-molecular cofactors, GSH and NAD^+, within the bioactive layer. In cells-based biosensors phenazine methosulfate (free-diffusing redox mediator, PMS) exhibited the best electron transfer characteristics (Demkiv et al., 2008; Gayda et al., 2008).

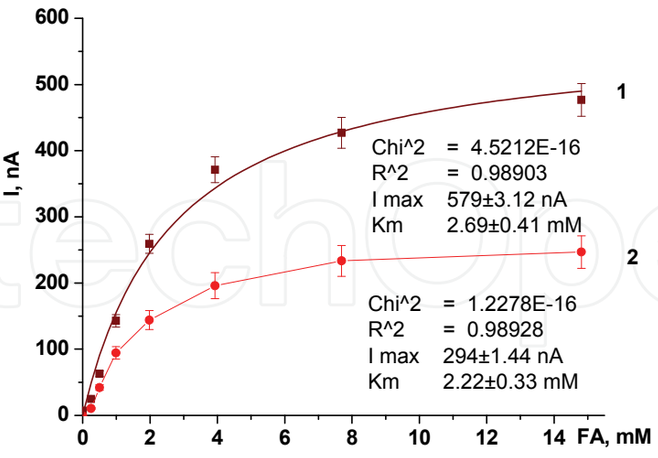
For construction of the envisaged FA biosensor, it was proposed a sophisticated sensor architecture with the aim of securely fixing all sensor components in a bioactive layer onto the transducer surface. Especially, the sensor architecture was designed to prevent any leakage of the low-molecular and free-diffusing cofactors of the enzyme, thus enabling FA determination without the addition of the cofactors to the analyte solution [(Demkiv et al., 2008; Gayda et al., 2008). In the optimized biosensor's construction, platinised graphite electrode, with 3.05 mm diameter, was used as a transducer and [$O_s(Me2bpy)_2Cl_2$]-modified positively charged cathodic paint *1CPOs* was found to be the best redox mediator, as well as a good matrix for enzyme or cells electrodeposition and for holding enzyme's co-factors, GSH and NAD, in a bioactive layer. Covering of the bio-layer by a negatively charged Nafion membrane additionally prevented the leakage of cofactors and enhanced sensor's stability.

Bioanalytical characteristics of the constructed biosensors were studied in detail: kinetics, dynamic and linear range, selectivity, and effect of temperature on sensor output (Table 9). For the enzyme-based biosensor, the maximum current value was 250 ± 5.25 μA and the apparent Michaelis-Menten constant (K_M^{app}), derived from the FA calibration curves, was 120 ± 5.3 mM with a linear detection range for FA up to 20 mM. For the cell-based biosensor, the maximum current value was 1.07 ± 0.04 μA, and K_M^{app} was 20.1 ± 1 mM with a linear detection range for FA up to 8 mM. The optimal pH-value for the developed biosensors was in the range of 7.6 to 8.3 with an optimal temperature between 45-50 °C (Gayda et al., 2008), due

to a higher thermostability of the enzyme used, and thermotolerance of recombinant yeast cells. The bioanalytical properties of the developed biosensors were evaluated with the specific aim of improving the long-term operational stability of the sensor. The novel biosensors demonstrated a good sensitivity, high selectivity to FA and a good stability (Fig. 11-13).
A typical response of the developed *1CPOs-NAD*$^+$*-FdDH*-modified electrodes towards FA is shown in Fig. 11 A. As can be seen from Fig. 11 B, K_M values for FA, obtained by using intact- and permeabilized recombinant cells-based biosensors, are similar, but I_{max} value for the intact-cell-based sensor is 2-fold higher than for the sensor with permeabilized cells.



(A)



(B)

Fig. 11. Chronoamperometric determination of FA, using A - *1CPOs-NAD*$^+$*-FdDH-GSH*-Nafion -based biosensor; B - *Cells*-Tf *11-6*-NAD+-GSH-Nafion-modified graphite electrode (1) and perm*Cells*-Tf *11-6*-NAD$^+$-GSH-Nafion-modified graphite electrode (2). Potential 0 mV against Ag/AgCl/3M KCl in phosphate buffer, pH 8.0, with 0.2 mM PMS

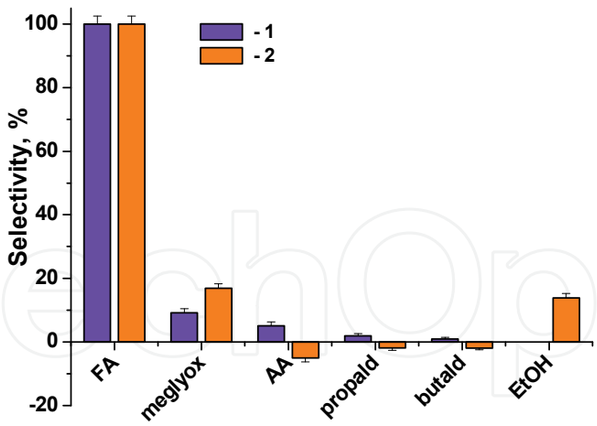


Fig. 12. Selectivity of the developed amperometric enzyme-(1) and cell-based (2) biosensors to different substrates: meglyox – methylglyoxal; AA – acetaldehyde; propald – propionaldehyde; butald – butyraldehyde; EtOH – ethanol

Another amperometric biosensor, developed (Nikitina et al., 2007) in a co-operation with the scientific group headed by Prof. E. Czoregi (University of Lund, Sweden), was a bilayer bi-enzyme sensor based on diaphorase and FdDH, together with [Os(4,4'-dimethylbipyridine)$_2$Cl]/(PVP-Os). The sensor's architecture was comprised of a first layer containing diaphorase from *Bacillus stearothermophilus,* cross-linked with the PVP-Os redox polymer. On the top of this layer, a second layer was formed by additional cross-linking of FdDH with poly(ethylene glycol)(400)diglycidyl ether. The sensor architecture was optimised with respect to efficient electron transfer and stability of the enzyme(s). The developed bi-enzyme FdDH-DPH-PVP-Os-modified electrode displayed the best characteristics for FA detection in flow injection mode of a sample.

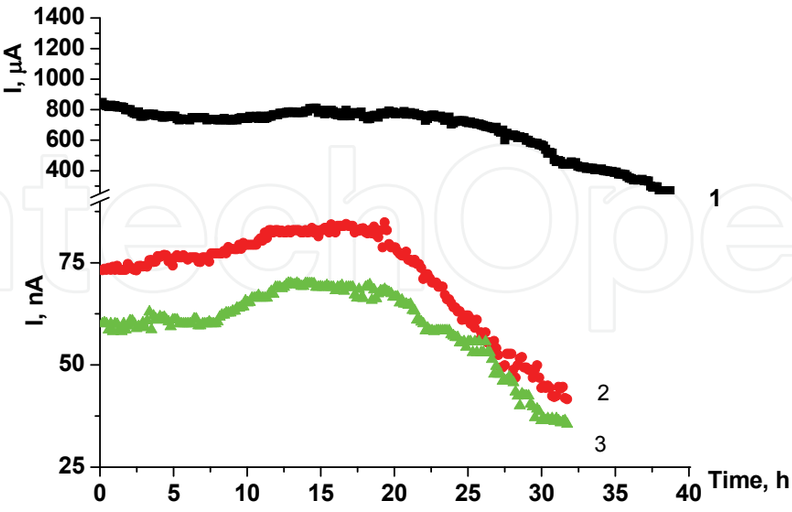


Fig. 13. Operational stability of the optimised FA biosensors: enzymatic-based (1), cell-based (2) and permeabilized cell-based (3). Bioelectrodes were tested in an automatic sequential injection analyser. Flow-rate 5 ml min^{-1}; sample injection every 4 min. Potential 0 mV against Ag/AgCl/3M KCl in phosphate buffer, pH 8.0, with 0.2 mM PMS

The basic bioanalytical characteristics of the bi-enzyme biosensor, polarized at +180 mV vs. NHE, are presented in Table 9 and Fig. 14. The biosensor-FA reaction obeys typical Michaelis-Menten kinetics. The detection limit was found to be 32 μM, while the dynamic range was shown to be linear between 0.05 and 0.5 mM FA. The slope of the calibration curve (sensitivity) and the linear correlation coefficient were 22 $Am^{-2}M^{-1}$ and 0.998, respectively. The stability of the FdDH immobilized on the electrode was also evaluated. When the biosensors were stored at 4 ^{0}C in phosphate buffer, pH 7.5, the response was linear with a loss of 50% of the activity after 24 h. Dry storage of the immobilized electrode at the same temperature resulted in the complete inactivation of the immobilized enzyme.

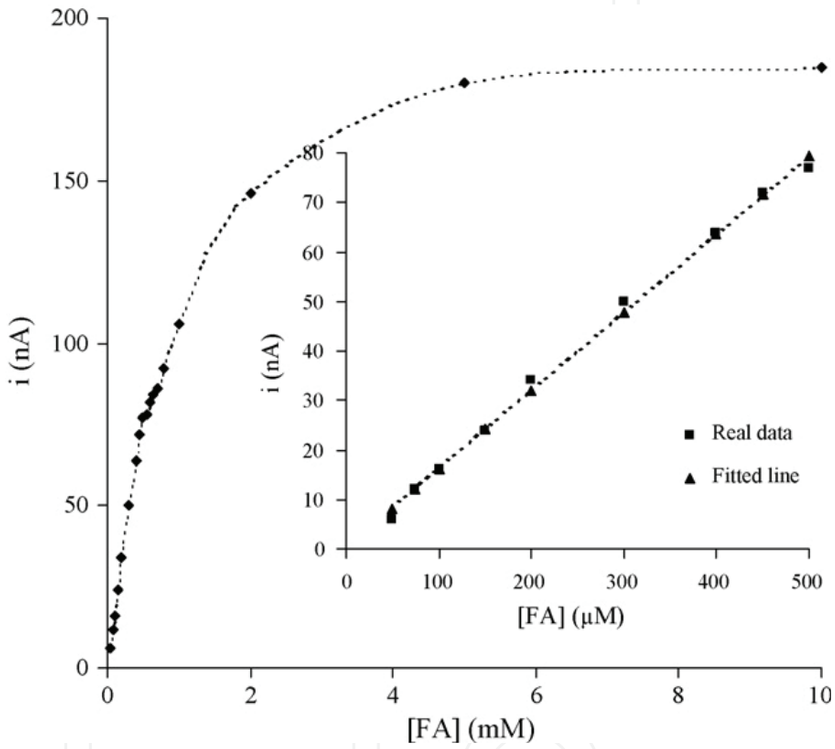


Fig. 14. Calibration curve of the FdDH-DPH-PVP-Os-modified electrode (0.5 mM NAD^+; 0.25 mM GSH; 0.1 M phosphate buffer, pH 7.5; *E* appl = 160 mV; 0.4 ml/min flow rate)

5.4 The comparison of the developed FA-selective biosensors

Tables 9 and 10 represent a brief summary of the published results on the developed microbial and enzyme-based FA biosensors with differend types of signal detection. The amperometric biosensors, enzyme- and cell-based, work at a very low applied potential, compared with other known biosensors (zero or 160 vs. 340, 610 or 560 mV), thus the possible interferences (*e.g.*, methanol, ethanol, acetic acid) should be considerably reduced. Different approaches were used for biosensor monitoring FA-dependent cell response: 1) analysis of their oxygen consumption rate by using a Clark electrode; 2) assay of oxidation of redox mediator at a screen-printed platinum electrode covered by cells entrapped in Ca-alginate gel (Khlupova et al., 2007).

The dynamic ranges of all described biosensors were of micromolar values. As can be seen from Tables 9 and 10, AOX- and FdDH-based biosensors, constructed for potentiometric and conductometric signals registration, have high storage stability.

Characteristics	FdDH-based				AOX-based	
	Bi-enzyme	Mono-enzyme			Mono-enzyme	Bi-enzyme
Type of signal detection	Amperometric		Capaci-tance	Conducto-metric	Potentio-metric	Ampero-metric
Detection limit, mM	0.032	0.003	0.01	10	-	0.024
Linear range, mM	0.05-0.5	up to 20.0	0.01-25	10-200	5-200	4
$I_{max,}$ μA	0.18	250	-	-	-	0.8
Sensitivity, $*A \cdot m^{-2} \cdot M^{-1}$	22*	358*	31 mV/ decade	-	50 mV/ decade	114*
Storage stability, days	1	3	-	140	120	14
Reference	Nikitina et al., 2007	Demkiv et al., 2008	Ben Ali et al., 2007	Korpan et al., 2010	Korpan et al., 2000	Smutok et al., 2006

Table 9. Bioanalytical characteristics of enzyme-based biosensors

Parameter	Cells *H. polymorpha* C-105				Cells *H. polymorpha* Tf 11-6		
	Intact		Permeabilized		Intact		Permea-bilized
Applied potential (mV)	-600	+200	-600	-	+200	0	0
Mediator	-	DCIP	-	-	*CP58-Os*	PMS	PMS
Registration type	Clark electrode	Ampe-rometric	Clark electrode	Potenti-ometric	Amperometric		
Linear dynamic range, mM	up to 3.0	1.0-7.0	0.3-4.0	5-50	0.5-6.0	0.25-8.0	1.0-2.5
Detection limit, mM	0.6	0.74	0.27	3.5	0.003	0.11	0.5
Sensitivity	*1.15	8.62 nA·mM^{-1}	*0.44	-	2.65 μA mM^{-1}	37.5 nA·mM^{-1}	-
Storage stability	-	-	-	30	16	20	7
Reference	Khlupova *et al.*, 2007			Korpan *et al.*, 2000	Demkiv *et al.*, 2008, Paryzhak *et al.*, 2008		

* Oxygen consumption rate per 1 mM of FA ($\mu M\ O_2\ s^{-1} \cdot mM^{-1}$)

Table 10. Comparison of microbial (yeast cells-based) FA-sensitive biosensors. DCIP - 2,6-dichlorophenolindophenol; PMS - phenazine methosulfate

Such excellent stability is intrinsic for cell-based sensors, too. Both amperometric and capacitance biosensors, AOX-, FdDH- and cells Tf 11-6 based, are very sensitive to low FA concentrations (Demkiv et al., 2008, Smutok et al., 2006, Ben Ali et al., 2007). FdDH-based biosensors have very important property for FA analysis in real samples – high selectivity to FA, compared with AOX-and cells-based sensors (Gayda et al., 2008).

5.5 Application of biosensors for FA-monitoring in real samples

The purified FdDH, as well as recombinant *H. polymorpha* cells overproducing this enzyme were used for construction of enzyme-based and microbial electrochemical biosensors selective to FA. The reliability of the developed analytical approaches was tested on real samples of wastewaters, pharmaceuticals, and FA-containing industrial products. As we can see from table 11, the proposed methods, approved on the real FA-containing samples, are well correlated with the results of the known chemical methods and novel FdDH-based analytical kit "Formatest" (Demkiv et al., 2009).

The constructed amperometric biosensors revealed a high selectivity to FA (100 %) and a very low cross-sensitivity to other structurally similar substances: butyraldehyde (0,93%), propionaldehyde (1,89%), acetaldehyde (5,1%), methylglyoxal (9,12%) (Paryzhak et al., 2007). These sensors were applied for FA testing in some industrial goods: Formidron, Descoton forte, formalin and rabbit vaccine against viral hemorrhage. A good correlation was observed between the data of FA testing (Table 11) by the amperometric biosenor's approaches (FdDH and cells-based), proposed enzymatic method "Formatest" and standard chemical methods.

Sample/ Method	Chemical methods			FdDH-based methods				
					Biosensors			
	MBTH	Chromo-tropic acid	Purpald	Forma-test	Amperometric			Conducto-metric
					FdDH	FdDH*	Cells	FdDH
Formidron	1.64± 0.61	1.48±0.26	1.20 ± 0.20	1.53± 0.31	1.57± 0.13	1.50 ± 0.60	1.48± 0.06	1.69±0.13
Descoton forte	3.57± 0.30	3.59±0.44	3.30 ± 0.30	3.25± 0.80	3.61± 0.13	3.50 ± 0.30	3.29± 0.12	14.10±0.80
Formalin	12.6± 0.73	14.0±0.81	12.9± 0.70	13.5± 0.54	13.6± 0.6	13.6± 0.6	13.8± 0.54	12.99±0.18
Rabbit vaccine against viral hemorrhage	0.038± 0.003	0.029±0.005	0.043± 0.005	0.042± 0.004	0.041± 0.005	-	0.042± 0.002	-
Reference	Demkiv, et al., 2008, Demkiv, et al., 2009				Demkiv, et al., 2008			Korpan et al., 2010

Table 11. FA content in molar concentration in real samples, M±m, determined by different methods: chemical (MBTH, Chromotropic acid); enzymatic method "Formatest", FdDH-based, and biosensor approaches (FdDH- and recombinant cells Tf 11-6 -based). *FdDH - enzyme was Integrated in analyzer "OLGA" with Flow Injection mode.

The conductometric sensors, FdDH- and rFdDH-based (Korpan et al., 2010), were evaluated in determining the FA content in real samples of the industrial product Formalin and two pharmaceuticals, the antimicrobial agent Descoton forte and antiperspirant Formidron, and the results of these tests are summarized in Table 11. As for the amperometric rFdDH-based sensor, the maximal interfering effect for the proposed conductometric biosensors was observed for Descoton, less for Formidron, and the smallest for Formalin. The results obtained for Descoton are due to the presence in this preparation of high quantities of glutaric aldehyde, which consequently changing substantially the mechanical and catalytic properties of the bioselective layer, since it can cause cross-linking reactions. For all investigated samples, a good correlation was observed between the conductometric sensor values and enzymatic or chemical methods. These analytical data confirm the possibility to exploit the developed biosensors for FA assay at least in real samples of non-complicated compositions such as pharmaceuticals, potable water and wastewater.

6. FA removal from indoor air

For removal of FA from indoor air a number of methods have been proposed. Physical adsorption of FA with activated carbon (Boonamnuayvitaya et al., 2005; Tseng et al., 2003), by various fractions of karamatsu bark (Takano et al., 2008) and by zeolites (Cazorla & Grutzeck, 2006) was shown to demonstrate good to high results, but simple adsorption cannot provide a radical solution to the problem, since FA does not decompose, but is only transferred from one phase (air) to another (solid). Efforts, attempting to carry out the physical decomposition of FA, with the help of photo-catalytic, negative ions and ozone air cleaners resulted in the elimination of only up to 50% FA, and failed to reach acceptable FA concentrations as specified by WHO guidelines (0.08 ppm) (Tseng et al, 2003). 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), but this process is expensive. Another approach to the chemical elimination of FA from air was developed in the work of Sekine, where manganese dioxide was shown to be effective in the oxidation of FA (Sekine, 2002; Tian & He, 2009). Combustion of a formaldehyde-methanol mixture in an air stream on Mn/Al_2O_3 and $Pd\text{-}Mn/Al_2O_3$ catalysts was shown to result in a total conversion of organic compounds (Álvarez-Galván, et al., 2004). Some chemical approachs to FA decomposition are highly effective, but solid wastes still remain as a by-product of these processes, in most cases containing harmful toxic components that cause subsequent utilization problems.

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

Recently, enzyme-based approaches have been proposed for FA bioremediation of indoor air. To this aim, continuous flow bioreactors based on the immobilized FA-oxidizing enzyme AOX or mutant yeast cells overproducing this enzyme were constructed (Sigawi et al., 2010).

AOX isolated from mutant *H. polymorpha* C-105 cells was immobilized in calcium alginate beads and applied for the bioconversion of airborne FA. The AOX preparation had a specific activity in the range of 6-8 U·mg^{-1} protein and was shown to preserve 85-90% of the initial

activity after incorporation into the calcium alginate gel. This activity was proven to remain unchanged for up to seven months upon storage of the immobilized enzyme at 4°C.
A fluidized bed bioreactor (FBBR) based on glass columns was filled with gel beads containing immobilized AOX and suspended in phosphate buffer-saline. Columns filled with gel alone were used as control. FA-containing air was bubbled through the columns from the bottom to the top (Fig. 15) as described previously in Sigawi et al, 2010. The results showed that in the case of the 20 ml reactors, the outlet FA concentration was less than 0.03 ppm, i.e. ten-fold less than the threshold limit value (TVL), and the 750 ml reactor outlet air contained no FA at all. The FA concentration in the gas phase at the outlet from the control columns without the enzyme was essentially higher (0.09-0.1 ppm) than the test columns, but also relatively low compared to the input level, evidently due to FA dissolution in the liquid phase of the column and possibly also due to adsorption by the gel. The FA concentration in the bioreactor liquid phase of the test column was ca. 1-2 mM (Fig. 16), and in the control experiment ranged from 6 mM (750 ml reactor, Fig. 16) to 20 mM (20 ml reactor).

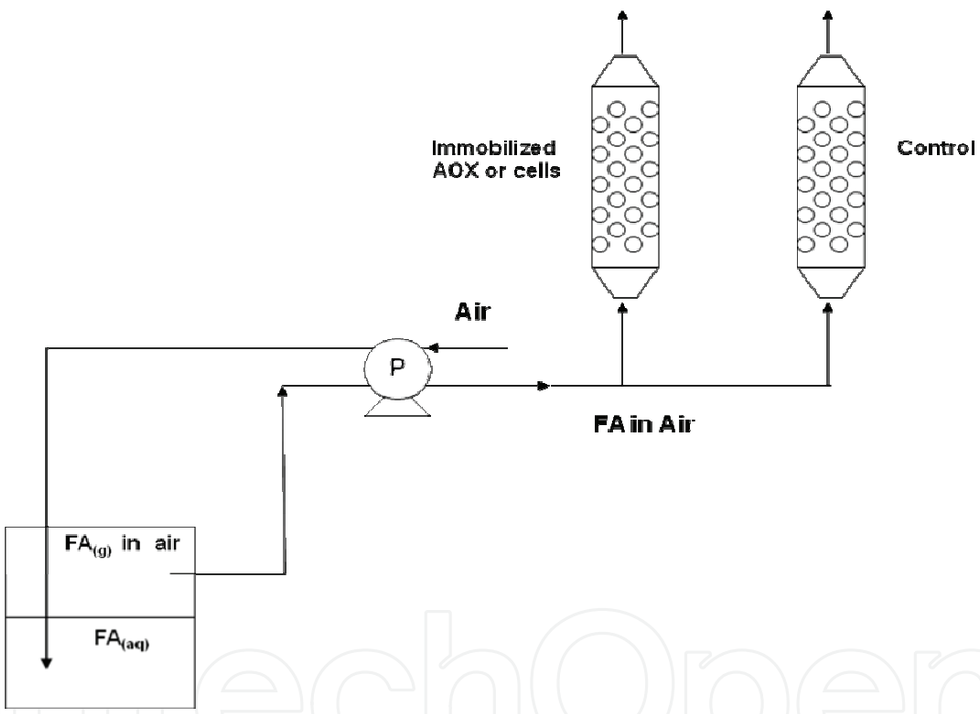


Fig. 15. Scheme for oxidation of airborne FA by AOX immobilized in calcium alginate or cells in a continuous FBBR. 1.5 or 38 g gel beads containing AOX with 6.6 $U \cdot g^{-1}$ of the gel in 20 or 750 ml 0.05 M PBS, pH 7.5, were applied onto a 1x30 cm or 10x10 cm column, which was connected at the bottom to the source of FA in air at 25°C. The 0.3-18.5 ppm FA concentrations in air were generated by bubbling 7-152 $ml \cdot min^{-1}$ airflow through an aqueous FA solution at concentrations of 2.7-100 mM. The control columns contained gel beads without immobilized material. The FA concentrations were tested for about three weeks in the outlet gas phase with a Formaldehyde Gas Detector (Model FP-40 Riken Keiki, Japan) and also in the aqueous column phase by a standard photometric method using a reaction with 1% chromotropic acid (Sawicki et al., 1961), as well as by the amperometric FdDH-based biosensor (Sigawi, 2010).

The proposed method for FA removal from indoor air by the enzyme AOX entrapped in alginate gel provides not only an effective bioconversion of FA in the gas phase, but also a safe FA level in the liquid phase of the continuous FBBR. After termination of the process the contents of the bioreactor can be used as organic fertilizer, since the gel beads together with the liquid phase are free of hazardous components. The entire process can therefore be considered as entirely environmentally friendly. It can be concluded that the proposed bioreactor is suitable for treating air containing various FA concentrations.

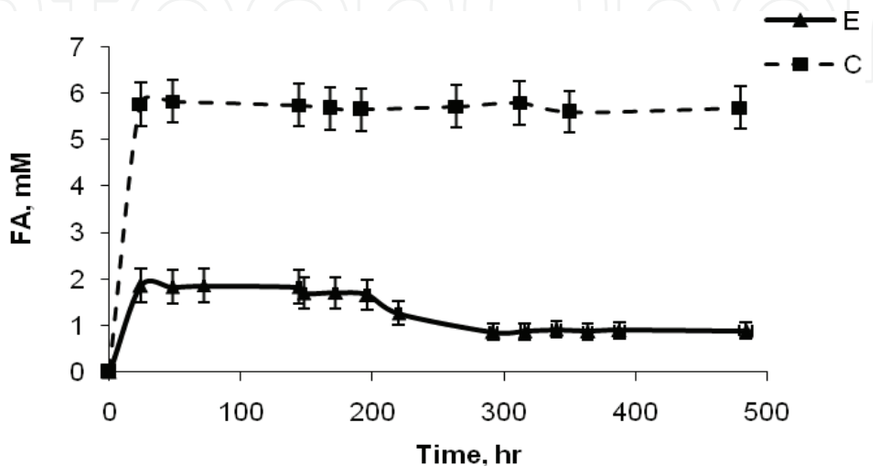


Fig. 16. FA concentration in the aqueous phase of the continuous FBBR upon oxidation of FA in the air by AOX immobilized in 1.5% calcium alginate gel (E). Air flow was 152 ml·min^{-1}, initial FA concentration in air was 18 ppm. The air was bubbled through a 10x10 cm column with 38 g gel beads, containing AOX with 6.6 U·g^{-1} of the gel. FA concentration in the aqueous phase was monitored by a standard photometric method using a reaction with chromotropic acid, as well as by the amperometric FdDH-based biosensor. In the control experiment (C), calcium alginate gel alone was used.

7. Conclusion

Bioremediation of wastes polluted by formaldehyde (FA) and monitoring of this toxic compound in environment, commercial goods, potable water and food products is an important challenge for science and practiclal technology.

In this review, there are described enzymes- and cells-based approaches to monitor FA content in different sources (wastes, indoor air, industrial products, vaccines, and fish food). As the main analytical instrument selective to FA, it has been used recombinant formaldehyde dehydrogenase (FdDH) isolated from the gene-engineered strains of the thermotolerant methylotrophic yeast *Hansenula polymorpha.* The stable recombinant clones, containing 6-8 copies of the target *FLD1* gene, were resistant to 15-20 mM FA in a medium due to over-synthesis of a homologous NAD^+- and glutathione-dependent FdDH. A simple scheme for FdDH isolation and purification from the recombinant overproducers was developed, physico-chemical and catalytic properties of the purified enzyme were studied.

The enzymatic method for FA assay, based on recombinant FdDH (with linear detection range from 0.01 to 0.05 mM and detection limit 0.007 mM) and analytical kit "Formatest"

were developed. In comparison with the known methods, the described procedure is rather simple: a method does not require transformation of FA into chemical adduct for the extraction of the target analyte from the tested sample. As compared to chemical methods, the analysis time is shorter and some dangerous operations (*e.g.* heating in strong acid) are not required. The developed method is approved on the FA-containing real samples, and data are well correlated with the results of the known chemical methods.

Another FA-oxidizing enzyme, alcohol oxidase (AOX) isolated from the mutant *H. polymorpha* (*gcr1 catX*), defective in glucose repression of AOX synthesis and avoid of catalase, was shown to be useful for enzymatic FA determination in wastes and industrial products. AOX *in vivo* oxidizes methanol, but *in vitro* has ability to catalyze the oxidation of other primary alcohols and hydrated form of FA ($HO\text{-}CH_2\text{-}OH$). For simultaneous assay of both FA and methanol in wastes, the specific chemico-enzymatic method was elaborated. AOX was also successfully used for FA assay in *Gadoid* fish products.

The purified preparations of FdDH and AOX, as well as *H. polymorpha* cells overproducing these enzymes were used for construction of enzyme-based and microbial electrochemical biosensors selective to FA. The reliability of the developed analytical approaches was tested on real samples of waste waters, pharmaceuticals, and FA-containing industrial products.

AOX and permeabilized mutant yeast cells of *H. polymorpha* (*gcr1 catX*) were shown to be used as the catalytic unit in cartridges for removing of formaldehyde from the indoor air. Experimental data confirm the possibility to exploit the developed bioreactors based on crude preparations of AOX or methylotrophic yeast cells for effective formaldehyde oxidation coupled with FdDH-based biosensor for accurate control of this process.

8. Acknowledgements

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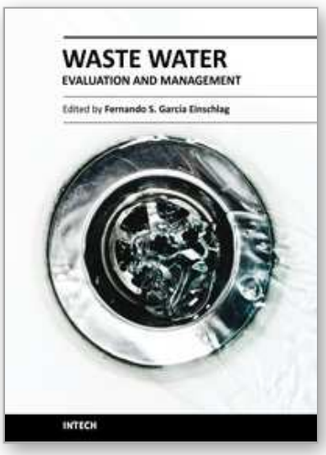

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Fresh water resources are under serious stress throughout the globe. Water supply and water quality degradation are global concerns. Many natural water bodies receive a varied range of waste water from point and/or non point sources. Hence, there is an increasing need for better tools to asses the effects of pollution sources and prevent the contamination of aquatic ecosystems. The book covers a wide spectrum of issues related to waste water monitoring, the evaluation of waste water effect on different natural environments and the management of water resources.

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