Chapter 5
Effective Technologies for Isolating Yeast Oxido-Reductases of Analytical Importance

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Abstract Microbial enzymes have gained interest for their widespread use in industries and medicine due to their stability, catalytic activity, and low-cost production, compared to plant and animal analogues. Microbial enzymes are capable of degrading toxic chemical compounds of industrial and domestic wastes by degradation or via conversion to nontoxic products. Enzyme technology broadly involves production, isolation, purification, and use of enzymes in various industries (e.g., food, medicine, agriculture, chemicals, pharmacology). The development of simple technologies for obtaining highly purified novel enzymes is an actual task for biotechnology and enzymology. This chapter presents a review of the main achievements in the elaboration of modern techniques for obtaining recombinant and novel enzymes. The results of a series of the authors' investigations into the development of novel enzymatic approaches, including biosensors, for determination of practically important analytes are summarized. The described methods are related to isolation of highly purified yeast oxido-reductases: alcohol oxidase, flavocytochrome b_2, glycerol dehydrogenase, and formaldehyde dehydrogenase. The enzymes were isolated from selected or recombinant yeast cells using the simple and effective technologies developed by the authors.

Keywords Yeasts · Oxido-reductases · Isolation and purification · Analytical application

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5.1 Introduction

Enzymes are used in many different spheres of human activity, and this use is increasing rapidly due to reduced processing time, low energy input, cost effectiveness, nontoxic, and eco-friendly characteristics (Singh et al. 2016). Enzymatic reactions are the basis of many production processes, and application of microbial enzymes has been widely used since the early twentieth century. Enzymes are necessary in genetic engineering and biotechnology, and in particular for developing the ethanol fuel technology, in various industries (e.g., food, agriculture, chemicals, pharmaceuticals), and in medicine. Enzymes also play an important role in analytical applications (Reyes-De-Corcuera and Powers 2017; Chapman et al. 2018). Analytical systems which contain microbial enzymes possess high selectivity and sensitivity and are widely used in analytical laboratories of the food and microbiological industries, as well as for clinical diagnostics.

The driving force for the growth of the enzymatic test-systems and biosensors market is the need for security control of the environment and food, as well as health status due to the aging population and its related disorders, growth of chronic and infectious diseases, and environmental disasters. Furthermore, people care about their health and want to live comfortably and have fresh air, clean water, and high-quality food. These factors resulted in the development of various services for environmental monitoring of toxic compounds, as well as the emergence of new areas of medicine, including personalized medicine that requires high-performance noninvasive portable test-systems for application in the clinic and at home.

Production of recombinant proteins is a rapidly growing area of research and development. This area emerged in the early 1980s with the development of genetic engineering tools, which represented a compelling alternative to protein extraction from natural sources. Over the years, a high level of heterologous protein was made possible in a variety of cell factories (hosts) ranging from the bacteria *Escherichia coli* to mammalian cells (Vieira Gomes et al. 2018; Owczarek et al. 2019). It is worth mentioning that the production of recombinant biopharmaceutical proteins is a multibillion-dollar industry. The global market for industrial enzymes was valued at USD 4.61 billion in 2016 and is projected to grow at a compound annual growth rate of 5.8% from 2017 and reach USD 6.30 billion by 2022 (Singh et al. 2016; Pharmaion 2019; Feed enzymes market 2019; Industrial Enzymes Market 2019). This branch of science and industry requires rationally chosen cell factories (hosts) and cost-efficient protein isolation protocols.

Of the established hosts, yeasts combine the advantages of unicellular organisms such as fast biomass growth and relatively easy genetic manipulations, with eukaryotic features such as correct post-translational modifications of recombinant proteins and efficient secretory pathways (Rueda et al. 2016; Sibirny 2017; Baghban et al. 2018; Huang et al. 2018a, b; Ekas et al. 2019). This is the reason why yeasts have, in recent decades, become attractive hosts for the production of heterologous proteins, enzymes, organic compounds, biopharmaceuticals, etc. (Daly and Hearn 2005; Idiris et al. 2010; Mattanovich et al. 2014; Singh et al. 2016; Stasyk 2017; Vogl et al. 2018; Yang and Zhang 2018a, b; Ekas et al. 2019; Owczarek et al. 2019).

Analysis of the literature data and our own research experience indicates that the following general approaches are employed for obtaining highly purified yeast enzymes:

1. Screening or gene engineering of the effective yeast strain as a producer of the target enzyme(s)
2. Optimization of cell cultivation conditions for achieving the highest specific activity of a target enzyme in the cells
3. Optimization of disruption conditions for producing intracellular enzymes
4. Development of effective methods for enzyme isolation and purification
5. Selection of methods for enzyme concentration and stabilization during storage

In the current chapter, we focused on the main achievements in the elaboration of modern techniques for isolation and purification of recombinant enzymes. Our investigations into effective technologies for obtaining yeast oxido-reductases of analytical importance are related to two aspects. One is the development of a cost-effective scheme for obtaining several enzymes from the same yeast source. The other is to summarize our previous results on isolation and purifications methods as well as the analytical application of some oxido-reductases for use in bioanalyses.

5.2 Non-conventional Yeasts as Hosts for Production of Heterological Proteins/Enzymes

Yeasts combine the ease of genetic manipulation and fermentation of cells with the capability of secreting and modifying foreign proteins according to a general scheme. Their rapid growth, microbiological safety, and high-density fermentation in simplified medium have a high impact, particularly in the large-scale industrial production of recombinant proteins (Singh et al. 2016; Sibirny 2017; Ekas et al. 2019).

Historically, *Saccharomyces cerevisiae* was the dominant yeast host for heterologous protein production (Muller et al. 1998; Kim et al. 2016; Chen et al. 2018). Lately, other yeasts, including non-conventional ones have emerged as advantageous cell factories. Non-conventional yeasts are considered as convenient expression platforms and promising industrial producers of recombinant proteins of academic and industrial interest (Reiser et al. 1990; Sudbery 1996; Stasyk 2017; Rebello et al. 2018). The yeasts *Kluyveromyces lactis*, *K. marxianus*, *Scheffersomyces stipitis*, *Yarrowia lipolytica*, and *Schizosaccharomyces pombe*, as well as the methylotrophic yeasts *Ogataea* (*Hansenula*) *polymorpha* and *Komagataella phaffii* (*Pichia pastoris*), have been developed as eukaryotic hosts because of their desirable phenotypes, including thermotolerance, assimilation of diverse carbon sources, and secretion of high quantities of proteins (Gellissen et al. 2005; Wagner and Alper 2016; Weninger et al. 2016; Juturu and Wu 2018; Vandermies and Fickers 2019).

Several value-added products – vaccines (Smith et al. 2012; Liu et al. 2014; Bredell et al. 2016; Xiao et al. 2018), mammalian proteins of pharmaceutical

interest (Thömmes et al. 2001; Mack et al. 2009; Bawa et al. 2014; Arias et al. 2017; Walker and Pretorius 2018), therapeutic proteins (Griesemer et al. 2014; Kim et al. 2015; Love et al. 2018; Pobre et al. 2018; Zepeda et al. 2018a, b; Baghban et al. 2018; Owczarek et al. 2019), enzymes (Curvers et al. 2001; Hemmerich et al. 2014; Engleder et al. 2018; Vogl et al. 2018; Liu and Zhu 2018; Juturu and Wu 2018), industrial proteins (Singh et al. 2016; Shang et al. 2017; Barrero et al. 2018; Baghban et al. 2018), food additives, bio-renewable value-added chemicals, and biofuels (Jullessen et al. 2015; Kim et al. 2016; Białkowska 2016; Dmytruk et al. 2017; Semkiv et al. 2017; Porro and Branduardy 2017; Avalos et al. 2017; Passoth 2017; Rahman et al. 2017; Xu 2018; Ekas et al. 2019) were generated by the above-mentioned yeasts.

Yeast expression systems are economical and do not contain pyrogenic or viral inclusions. Unlike prokaryotic systems, the eukaryotic subcellular organization of yeasts enables them to carry out many of the post-translational folding, processing, and modification events required to produce "authentic" and bioactive mammalian proteins (Buckholz and Gleeson 1991, Domínguez et al. 1998; Talebkhan et al. 2016; Stasyk 2017). However, secretory expression of heterologous proteins in yeasts is often subject to several bottlenecks that limit yield.

Yeast engineering by genetic modification has been the most useful and effective method for overcoming the drawbacks in yeast secretion pathways (Muller et al. 1998; Daly and Hearn 2005; Pobre et al. 2018; Vogl et al. 2018; Thomas et al. 2018; Bao et al. 2018; Chang et al. 2018; Nora et al. 2019). Metabolic engineering and synthetic biology methods are promising for production of native and transgenic enzymes and proteins on an industrial scale (Krivoruchko et al. 2011; Fletcher et al. 2016; Fernandes et al. 2016; Yang and Zhang 2018a; Theron et al. 2018; Ekas et al. 2019). Methanol-free mutant strains were constructed as alternatives to the traditional system, as a result of synthetic biology tools for reprogramming the cellular behavior of methylotrophic yeasts. The creation of a methanol-free induction system for eliminating the potential risks of methanol and for achieving enhanced recombinant protein production efficiency has been reviewed (Shen et al. 2016).

The methylotrophic yeasts *K. phaffii* (*Pichia pastoris*) and *O. polymorpha* are the most effective sources of recombinant proteins (Sudbery 1996; Berg et al. 2013; Rajamanickam et al. 2017; Peña et al. 2018; Sibirny 2017). The methylotrophic yeast *O. polymorpha* has remarkable thermotolerance. This yeast is therefore successfully used as a cell factory for the production of thermostable enzymes and proteins (Gellissen et al. 2005; Krasovska et al. 2007). Many of the expression platforms, including circular plasmids with the *P. pastoris*-specific autonomously replicating sequence (*PARS1*), were developed in order to facilitate genetic manipulation for plasmid replication and distribution (Song et al. 2003; Sturmberger et al. 2016; Nakamura et al. 2018; Portela et al. 2018).

Yeasts with beneficial native phenotypes and genetically modified cells were therefore selected and used for the heterologous production of recombinant enzymes, proteins, and other products. A detailed and comprehensive description of methylotrophic yeasts as producers of recombinant proteins was published by Stasyk (2017).

5.3 Scheme for Obtaining Several Yeast Enzymes from the Same Source

We carried out a research in order to develop a cost-effective approach for simultaneous isolation and purification of several enzymes with practical importance from the same yeast source. A mutant strain of the thermotolerant methylotrophic yeast *O. polymorpha* C-105 (*gcr1 catX*), overproducer of yeast alcohol oxidase (AO), was selected as the producer of enzymes (Gonchar et al. 1990). This strain has impairment in glucose catabolite repression of AO synthesis, is catalase-defective, and is able to overproduce AO in glucose medium. Contrary to *P. pinus*, the yeast *O. polymorpha* C-105 can generate a single AO isoform, due to the presence of the AO-coding gene only in its genome (Cregg et al. 1985, 1989; Gunkel et al. 2004). However, cultivation of *O. polymorpha* C-105 cells in glucose medium prevents the formation of multiple forms of AO which can be generated in methanol-containing medium as a result of chemical modification of the AO protein by formaldehyde (FA).

Yeast cells were cultivated in mineral medium with 1% glucose and 0.2% yeast extract up to the middle of the exponential growth phase (Gonchar et al. 2002). The cells were washed, resuspended in 30 mM phosphate buffer, pH 7.5 (PB); supplemented with protease inhibitors; and disrupted with glass beads in a planetary disintegrator. The pellet of disrupted cells was removed by centrifugation, and the supernatant (cell-free extract) was used for a two-step ammonium sulfate fractionation (at 40 and 60% saturation).

Pellet 60% (see scheme in Fig. 5.1) was dissolved in 30 mM PB and placed on a column with ion-exchange sorbent DEAE-Toyopearl 650 M (Shleev et al. 2006; Sigawi et al. 2011). The sorbent was washed step by step with 30–200 mM PB; the target enzyme was eluted with 0.25 M PB and collected by fractions. Each fraction was tested for AO activity and protein concentration. AO activity was determined at 30 °C by the rate of hydrogen peroxide formation in reaction with methanol as monitored by the peroxidative oxidation of *o*-dianisidine in the presence of horseradish peroxidase (Gonchar et al. 2001). Chromatographic fractions with the highest specific activity of AO were combined and analyzed electrophoretically in PAG under denaturation conditions.

The resultant AO preparations with a specific activity up to 20 U per mg protein were fourfold purer than in pellet 60% and sevenfold purer than in the cell-free extract. The yield of purified AO from 1 L of yeast culture was about 800 U, which is 30% of the initial activity in the cell-free extract. The purified enzyme was stable, but still not homogeneous in SDS-PAG. AO preparations were stored as a suspension in 70% saturated ammonium sulfate (with an activity of 200 U mL^{-1} suspension) at −10 °C, without any remarkable decrease in activity over a period of 2 years. Before use, the enzyme suspension was centrifuged, and the enzyme precipitate was dissolved in 0.05 M phosphate buffer, pH 7.6.

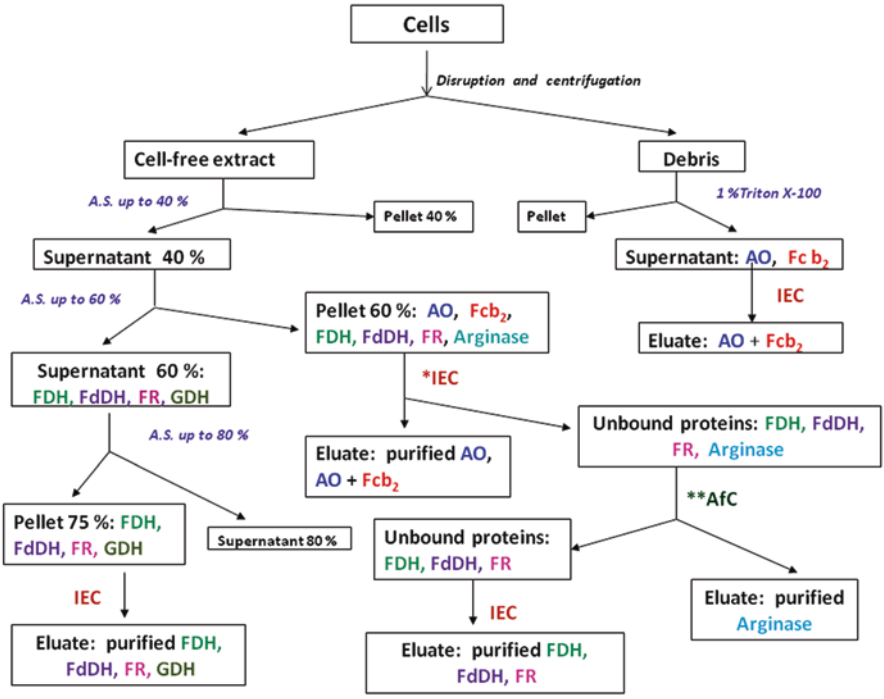


Fig. 5.1 Scheme of obtaining several enzymes from the *O. polymorpha* C-105 cells. Abbreviations: ∗ IEC, ion-exchange chromatography on DEAE-Toyopearl M-650; ∗∗AfC, affinity chromatography on Arg-containing sorbent

We tested additionally the activity of flavocytochrome b_2 (Gaida et al. 2003) during AO purification in order to obtain the highly purified AO preparations (up to 40 U per mg protein) that were homogeneous during electrophoresis in SDS-PAG (see Fig. 5.1). The proposed approach for optimizing the technology for obtaining the target enzyme with the highest purity is characterized by the following points:

1. Simplification of express qualitative or semiquantitative enzyme activity assays for target enzyme identification in a mixture of different proteins
2. Visualization of enzymatic activity in native PAG for testing the presence of target and waste enzymes in each stage of the purification procedure (from the initial cell-free extract to the final chromatographic fractions)

The examples of express qualitative or semiquantitative assays for AO and Fcb_2 are presented in Fig. 5.2.

A cost-effective technology for obtaining several enzymes from the same yeast producer was thus proposed. This technology demonstrated (Fig. 5.1) the possibility of isolating and chromatographically purifying several yeast enzymes from *O. polymorpha* C-105 cells: alcohol oxidase (AO), flavocytochrome b_2 (Fc b_2), glycerol

dehydrogenase (GDH), methyl aminooxidase (AMO), arginase, formaldehyde dehydrogenase (FdDH), formate dehydrogenase (FDH), and formaldehyde reductase (FR). The best yields were observed for AO and Fc b_2. Activities of other enzymes (Fig. 5.1) were tested as described earlier: for GDH by Synenka et al. (2015), for AMO by Krasovska et al. (2006), for arginase by Stasyuk et al. (2013), and for FdDH, FDH, alcohol dehydrogenase (ADH), methyl formiate synthase (MFS) and FR by Demkiv et al. (2011).

The proposed scheme for enzymes, presented in Fig. 5.1, was also applied to other yeast producers (see Table 5.1).

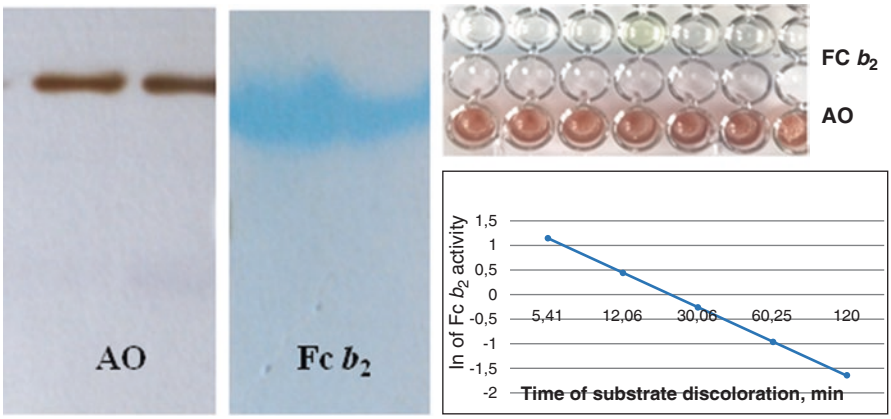


Fig. 5.2 Visualization of enzymatic activities in native PAG and screening tests for AO and Fc b_2 assays

Table 5.1 Enzymes of analytical importance and their producers

Enzyme	Yeast strain – the source of enzyme	Reference
Yeast alcohol oxidase (AO)	Mutant *O. polymorpha* C-105 (*gcr1 catX*)	Gonchar et al. (1990), Shleev et al. (2006)
Yeast alcohol oxidase (mAO)	Mutant *O. polymorpha* CA4 and CA2	Dmytruk et al. (2007)
Yeast formaldehyde dehydrogenase (FdDH)	Recombinant *O. polymorpha* NCYC 495 (*leu*1-1)	Demkiv et al. (2005, 2007, 2011)
Yeast glycerol dehydrogenase (GDH)	Recombinant *Saccharomyces cerevisiae W303* (*HpGDH*)	Nguyen and Nevoigt (2009), Synenka et al. (2015)
Yeast flavocytochrome (Fc b_2)	*O. polymorpha 356*	Gaida et al. (2003), Smutok et al. (2011)
Yeast amine oxidase (AMO)	Recombinant *O. polymorpha* CBS4732	Krasovska et al. (2006)
Human liver arginase I (arginase)	Recombinant *O. polymorpha* NCYC 495-*pGAP1-HsARG1-(leu2car1 Sc:LEU2)*	Stasyuk et al. (2013)

5.4 Oxido-reductases of Analytical Importance

5.4.1 Alcohol Oxidase

Alcohol oxidases (alcohol: O_2 oxidoreductase; EC 1.1.3.x) are flavoenzymes that catalyze the oxidation of alcohols to the corresponding carbonyl compounds with a concomitant release of hydrogen peroxide. Based on substrate specificity, alcohol oxidases may be categorized broadly into four different groups, namely, (a) short-chain alcohol oxidase, (b) long-chain alcohol oxidase, (c) aromatic alcohol oxidase, and (d) secondary alcohol oxidase (Ozimek et al. 2005; Leferink et al. 2008; Goswami et al. 2013; Romero and Gadda 2014; Pickl et al. 2015; van Berkel 2018; Sützl et al. 2018). The sources reported for these enzymes are mostly limited to bacteria, yeasts (Sahm and Wagner 1973; Sagiroglu and Altay 2006; Shleev et al. 2006; Koch et al. 2016; Vonck et al. 2016; Mangkorn et al. 2019), fungi (Janssen and Ruelius 1968; Bringer et al. 1979; Kondo et al. 2008, Isobe et al. 2009; Hernández-Ortega et al. 2012), plants (Panadare and Rathod 2018), insects (Sperry and Sen 2001), and mollusks (Grewal et al. 2000).

Alcohol oxidase (EC 1.1.3.13) also known as methanol oxidase (AO) is a key enzyme of methanol methabolism in methylotrophic yeasts; it catalyzes the first step of methanol oxidation to formic acid (Mincey et al. 1980; Eggeling and Sahm 1980; Sibirny et al. 1988; Lusta et al. 2000; Gadda 2008; Wongnate and Chaiyen 2013; Dijkman et al. 2013; Liu et al. 2018). In addition to the physiological substrate methanol, AO can typically oxidize also short aliphatic primary alcohols consisting of up to four carbons.

AO is a flavoprotein with flavin adenine dinucleotide (FAD) as a prosthetic group, non-covalently, but very tightly bound with apoenzyme. Native protein is an octamer of approximately 600 kDa composed of eight identical FAD-containing subunits (Bringer et al. 1979; Mincey et al. 1980; Boteva et al. 1999; Gunkel et al. 2004; Ozimek et al. 2005; Isobe et al. 2009; van der Klei et al. 1991). Only octameric enzyme has catalytic activity. The mechanism of oligomerization into catalytical active octamers, as well as the role of AO octameric structure in catalysis, is not yet elucidated.

Although AO was discovered 50 years ago, its tertiary structure (for *Pichia pastoris* or *Komagataella phaffii*) was elucidated only in 2016 using crystallography and cryoelectron microscopy (Koch et al. 2016; Vonck et al. 2016).

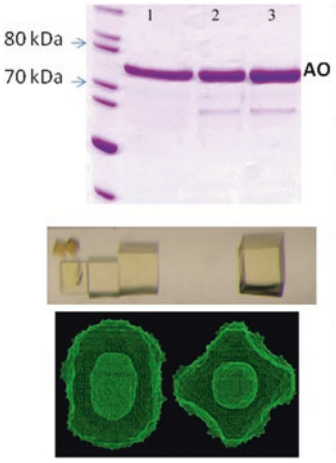
AO shows a high structural homology to other members of the GMC ("glucose-methanol-choline") family of oxidoreductases, which share a conserved FAD binding domain but have different substrate specificities (Gvozdev et al. 2012; Dijkman et al. 2013; Romero and Gadda 2014; Pickl et al. 2015; Sützl et al. 2018; Liu et al. 2018). The preference of AO for small alcohols is explained by the presence of conserved bulky aromatic residues near the active site. Compared to the other GMC enzymes, AO contains a large number of amino acid inserts, the longest

being 75 residues. These segments are found at the periphery of the monomer and make extensive inter-subunit contacts which are responsible for the very stable octamer. A short surface helix forms contacts between two octamers, explaining the tendency of AO to form crystals in the peroxisomes (Vonck et al. 2016).

The crystal structure analysis of the methanol oxidase from *P. pastoris* was described (Koch et al. 2016). The crystallographic phase problem was solved by means of molecular replacement in combination with initial structure rebuilding using Rosetta model completion and relaxation against an averaged electron density map. The subunit arrangement of the homo-octameric AO differs from that of octameric vanillyl alcohol oxidase and other dimeric or tetrameric AOs, due to the insertion of two large protruding loop regions and an additional C-terminal extension in AO. In comparison to other AOs, the active site cavity of AO is significantly reduced in size, which could explain the observed preference for methanol as substrate. All AO subunits of the structure reported here harbor a modified FAD, which contains an arabityl chain instead of a ribityl chain attached to the isoalloxazine ring.

The recently described AO from the white-rot basidiomycete *Phanerochaete chrysosporium* (PcAOX) was reported to feature very mild activity on glycerol. PcAOX was expressed in *Escherichia coli* in high yields and displayed high thermostability. Steady-state kinetics revealed that PcAOX is highly active toward methanol, ethanol, and propanol-1 (k_{cat} = 18; 19 and 11 s^{-1}, respectively), but showed a very limited activity toward glycerol (k_{obs} = 0.2 s^{-1}at 2 M substrate). The crystal structure of the homo-octameric PcAOX was determined at a resolution of 2.6 Å. The catalytic center is a remarkable solvent-inaccessible cavity located at the re-side of the flavin cofactor. Its small size explains the observed preference for methanol and ethanol as best substrates (Nguyen et al. 2018). The catalytic center is a remarkable solvent-inaccessible cavity located at the re-side of the flavin cofactor. Its small size explains the observed preference for methanol and ethanol as best substrates (Nguyen et al. 2018).

In our research, we have obtained a stable highly purified AO (up to a specific activity of 40 U mg^{-1}) from the overproducing strain of the yeast *O. polymorpha* C-105 *(gcr1 catX)* with impaired glucose-induced catabolite repression of AO synthesis and completely devoid of catalase (see Sect. 5.3). Such purity permitted to obtain the enzyme in crystalline form. The crystals of highly purified AO were obtained by different methods, including crystallization in space weightless conditions. X-ray study followed by the calculations for AO complexes with competitive inhibitors structures was performed. Comparative analysis of X-ray database for AO structure with the known protein structures of some oxidases was done, and the model of AO subunit tertiary structure was proposed (Fig. 5.3). Some recommendations for site-specific mutagenesis in AO gene for obtaining enzyme with significantly decreased affinity to ethanol compared to the wild-type AO have been done (Gayda et al. unpublished data).



Stages of procedure	AO activity		Yield, %	Factor of purification
	Total, U	Specific, U mg^{-1} protein		
Cell-free extract	1500	3.5	100	1
Two-step fractionation with AS	1200	5.0	80	1.4
Ion-exchange chromatography, pH 7.0, unbound proteins	300	15–20	20	4-6
Ion-exchange chromatography, pH 8.8, eluates	150	25–30	10	7-8
	75	35 - 40	5	10-11

Fig. 5.3 Isolation and purification of AO from *O. polymorpha* C-105 cells

5.4.2 *NAD$^+$- and Glutathione-Dependent Formaldehyde Dehydrogenase*

Formaldehyde (FA) is a natural metabolite found in tissues, cells, and body fluids. It is present in fruits, vegetables, meat, and fish. FA is also a large-scale product, used extensively in industry. FA is very toxic and is an extremely active chemical compound which causes modifications of bioorganic molecules in living organisms. That is why this dangerous compound is monitored in environmental, industrial, and medical laboratories. Enzymatic methods for valid selective determination of FA are based on using FA-selective enzymes, including formaldehyde dehydrogenase (FdDH). The aim of our research was to obtain highly purified FdDH and develop analytical approaches for FA assay. For this aim, yeast engineering for construction of FdDH-overproducing strains was carried out.

The *O. polymorpha FLD*1 gene with its own promoter was inserted into the integrative plasmid pYT1 (Demkiv et al. 2005) containing the *LEU2* gene of *Saccharomyces cerevisiae* (as a selective marker) in order to construct strains of *O. polymorpha* that overproduce thermostable NAD+- and glutathione-dependent FdDH. The constructed vector was used for multicopy integration of the target gene into the *O. polymorpha* genome by transformation of *leu 1-1* (Demkiv et al. 2005, 2011; Sibirny et al. 2011b) and *leu 2-2* recipient cells (both *leu* alleles were complemented by *S. cerevisiae* gene *LEU2*).

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 which were resistant to 15–20 mM FA on plates were selected and studied in greater detail. The growth characteristics of selected clones in the liquid medium are shown in Fig. 5.4. All transformants grew

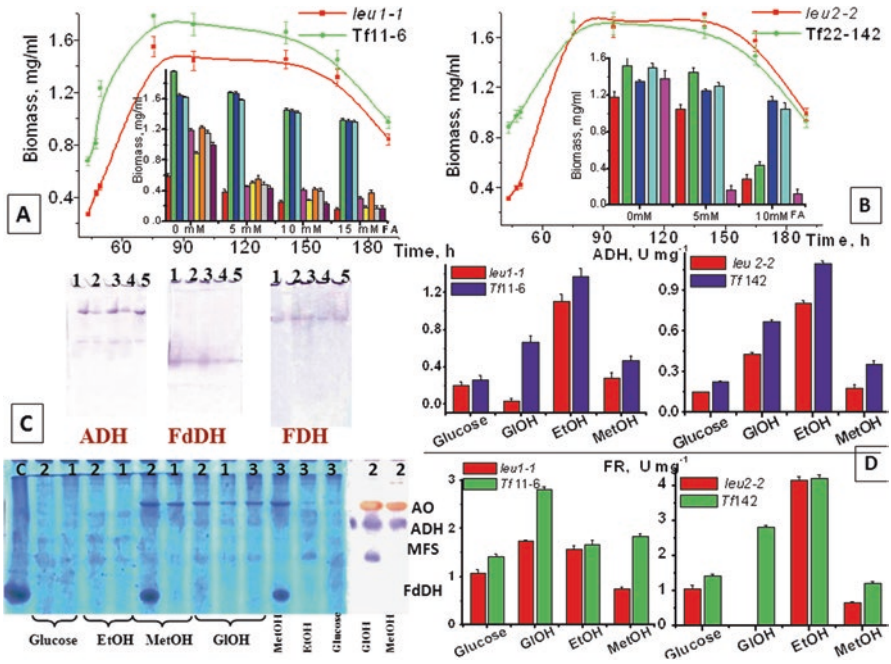


Fig. 5.4 Effect of cultivation conditions on growth and specific activity of FA-utilizing enzymes in cell free extracts. A, B, inductive effect of FA on growth of parental and recombinant cells in a medium with 1% methanol; C, visualization of enzymes activities in native PAG. At a top: *Tf* 11-6 cells, cultivated in medium with MetOH, $(NH_4)_2SO_4$, FA (1); MetOH, $(NH_4)_2SO_4$ (2); MetOH, CH_3NH_3HCl (3); MetOH, NH_4Cl (4); GlOH, NH_4Cl, FA (5). At a bottom: activities of AO, ADH, MFS, and FdDH in *leu 1-1* (1), *Tf* 11-6 (2), and *Tf* 22-142 (3) cells in comparison with purified FdDH (c); D, activities of ADH and FR in parental and recombinant cells.

better and were more resistant to elevated FA content in liquid medium with 1% methanol, compared to the recipient strains. A detailed description of this research has been done by Demkiv et al. (2005, 2011). Finally, FdDH specific activities were tested in cell-free extracts (CE) of the best selected FA-resistant Leu-prototrophic transformants (Fig. 5.4).

Tf 11-6 and Tf 22-142 were the most effective recombinant strains with the highest FdDH activity (up to 4.0 U mg^{-1}), which was four- to fivefold higher compared to the parental strains, *leu* 1-1 and *leu* 2-2, respectively. These transformants were characterized and chosen as sources for FdDH production.

Southern dot-blot analysis showed that genomes of the stable recombinant yeast clones contain 6–8 copies of the target *FLD1* gene (Demkiv et al. 2011). The recombinant yeast strain Tf 11-6 contains more than eight copies of the integrated plasmid, as opposed to one copy in the parental strain, probably due to use of the double-gene-containing plasmid $pHp(FLD1)_2$ and its tandem aggregation into the genome of the recipient strain.

The influence of growth medium composition on FdDH concentration was studied for the best two strains, Tf 11-6 and Tf 22-142, in order to optimize cultivation conditions for obtaining the highest enzyme yield. FdDH activity in cell-free extract was shown to be dependent on a carbon source. Cultivation in medium with 1% methanol resulted in significant levels of the enzyme synthesis for both tested strains (Fig. 5.4). This is in accordance with the literature data (Hartner and Glieder 2006; van der Klei et al. 1991; Eggeling and Sahm 1980).

We demonstrated that the addition of FA to the methanol medium stimulated synthesis of FdDH. The target enzyme activity was 6.2 U mg^{-1} under experimentally determined optimal conditions (with methanol as a carbon source, methylamine as a nitrogen source, and 5 mM FA as an additional inducer of FdDH synthesis). This is 1.6-fold higher than under normal growth conditions. The addition of 10 mM FA to the optimal culture medium resulted in a FdDH activity of 8.3 U mg^{-1}, which is twofold higher than in the medium without FA. 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 an inducer of FdDH synthesis (Fig. 5.4).

The enzyme was isolated from a cell-free extract of the recombinant overproducing Tf 11-6 strain. Cells were cultivated in 1% methanol medium supplemented with 5 mM FA for 20 h (Demkiv et al. 2007). A simple scheme for FdDH isolation and purification from the recombinant strain by two-step column chromatography on an anion-exchange sorbent was proposed, resulting in a FdDH preparation with specific activity of 27 U mg^{-1} protein.

In the first step, cell-free extract (CE) was applied to the sorbent, equilibrated by PB (pH 8.0). The fraction of unabsorbed proteins, which contained FdDH, was diluted with water (1:3). Tris base solution was added to adjust the pH to 8.8, and the final solution was applied to the same column (the second step), previously washed with 1 M NaCl and equilibrated with 40 mM Tris buffer, pH 8.8 (TB). The enzyme was eluted with 0.1 M NaCl in the initial TB buffer, and the specific activity of FdDH was assayed in each fraction. The fractions of eluate with enzyme activity higher than 10 U mg^{-1} and devoid of AO activity were combined, and dithiothreitol (DTT) up to 2 mM and ammonium sulfate (up to 80% saturation, pH 8.0, at 0 °C) were added. After incubation at 0 °C for 1 h, the enzyme was collected by centrifugation, and the pellet was resuspended in a minimal volume of ammonium sulfate solution (80% saturation) in 40 mM TB with 2 mM DTT.

The specific activity of resulted FdDH was 27 U mg^{-1}. For comparison, the specific activities of commercially available FdDH preparations from *P. putida* and from the yeast *C. boidinii* are 3–5 U mg^{-1} and 17–20 U mg^{-1}, respectively. The purity of the isolated enzyme preparation was controlled by PAG electrophoresis under denaturation conditions according to Laemmly (Demkiv et al. 2007; Sibirny et al. 2011b).

It was reported that the predicted *FLD1* gene product (Fld1p) is a protein of 380 amino acids (Baerends et al. 2002). Since the molecular mass of native FdDH from various methanol-utilizing yeasts was estimated to be between 80 and 85 kDa, the

isolated thermostable NAD^+- and GSH-dependent FdDH can be assumed to be dimeric. The molecular mass of the FdDH subunit, estimated by SDS-electrophoresis, was shown to be approximately 40 kDa, which is similar to the 41 kDa found for *C. boidinii* (Yurimoto 2009).

Optimal pH and pH stability of the enzyme were evaluated by incubation in the appropriate buffer at room temperature for 60 min. The 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.

Values of the Michaelis-Menten constant (K_M) for FA and NAD^+ calculated for this enzyme are close to the K_M for the wild-type enzyme. The effect of several inhibitors on the enzymatic properties was studied. The bivalent cations Zn^{2+}, Cu^{2+}, and Mn^{2+} were shown to inhibit FdDH activity, as did the ionic detergent SDS. According to the literature, enzymes from two other yeasts, *P. pastoris* and *C. boidini* (Allais et al. 1983; Kato 1990; Patel et al. 1983), were also inhibited in a similar manner.

A limited number of publications on the isolation and characterization of formaldehyde reductase (FdR) and the absence of a corresponding commercial preparation of the enzyme led us to screen potential microbial FdR producers among wild-type and recombinant strains of the yeast *O. polymorpha.* The gene-engineered integrative transformants with the highest FA resistance originating from *leu* 2-2 were shown to overproduce NADH-dependent FdR upon cultivation on 1% ethanol or glycerol. The best integrative clone, T22-126, was chosen as a source for FdR isolation, and optimal cultivation conditions for the highest yield of FR were established (Demkiv et al. 2011). The simple scheme for isolation of FR (Gayda et al. 2008b) from *O. polymorpha* T22-126 yeast cells and chromatographic purification of the enzyme on anion-exchange sorbent was proposed, resulting in *electrophoretically homogeneous* enzyme preparations.

The enzymatic methods and analytical kits for FA assay were developed based on FdDH (Demkiv et al. 2007; Gayda et al. 2008a, 2015; Sibirny et al. 2011a, b). In methylotrophic yeasts, FdDH catalyses the oxidation of FA to formic acid under simultaneous reduction of NAD^+ to NADH. The proposed enzymatic method is based on the photometric detection of a colored product, formazan, which is formed from nitrotetrazolium blue in a reaction coupled with FdDH-catalyzed oxidation of FA in the presence of an artificial mediator, PMS (Demkiv et al. 2007).

Purified preparations of FdDH were also used for construction of FA-selective electrochemical biosensors. Several FA-selective FdDH-based biosensors with different types of signal detection were developed and described in detail (Nikitina et al. 2007; Ali et al. 2007; Demkiv et al. 2008; Gayda et al. 2008a; Sibirny et al. 2011a, b). All constructed biosensors were characterized by high storage and good operational stability, high sensitivity, broad dynamic range, and low applied potential compared to known biosensors. A comparative analysis of different FA-sensitive biosensors was presented in reviews (Sibirny et al. 2011a, b; Gayda et al. 2015).

5.4.3 NAD⁺-Dependent Glycerol Dehydrogenase

Analysis of glycerol (GlOH) is important in clinical diagnostics for assessing the level of triglycerides in obesity and metabolic disorders, in particular lipid metabolism, that cause the development of type II diabetes and cardiovascular disease and in the wine industry for controlling wine quality during the production process. Simple methods for monitoring GlOH content (as a by-product of this technology) are currently in high demand, due to the growth in biodiesel production (Gerpen 2005; Talebian-Kiakalaieh et al. 2018). Additionally, GlOH assay is necessary for new technologies of GlOH conversion to valuable chemical products, including dihydroxyacetone (DHA) (Li et al. 2010; Cho et al. 2015; Kumar and Park 2018; Oh et al. 2018).

A key component of enzymatic kits for glycerol assay are glycerol-selective enzymes, including glycerol dehydrogenase (GDH). GDH (EC 1.1.1.6) is synthesized by mammalian tissues and microorganisms, including bacteria and yeasts (Yamada et al. 1982; Gartner and Kopperschlager 1984; Ruzheinikov et al. 2001; Yamada-Onodera et al. 2002). Three types of enzymes have been described: NAD^+-dependent GDH that converts GlOH to DHA and vice versa, $NADP^+$-dependent GDH that catalyzes the conversion between GlOH and DHA (glycerol 2-dehydrogenase, EC 1.1.1.156), and $NADP^+$-dependent GDH that catalyzes the conversion of GlOH on glyceraldehyde (EC 1.1.1.72). GDH is a promising enzyme for the development of analytical methods for assaying GlOH and other alcohols as well as for GlOH conversion. Commercial preparations of GDH from *Cellulomonas speciaes*, *Enterobacter aerogenes*, and *Bacillus megaterium* are present on the enzymes market (Sigma products).

The aim of our work was to obtain a thermotolerant NAD^+-dependent GDH of *O. polymorpha* (previously *H. polymorpha*) and to investigate its properties in order to develop a reliable and sensitive glycerol assay. We used the recombinant *Saccharomyces cerevisiae* strain that harbors the gene *HpGDH* (Mallinder et al. 1992). This strain was created for the biotransformation of glycerol to DHA (Nguyen and Nevoigt 2009). It contains an extrachromosomal multicopy plasmid p424GDH with an integrated *O. polymorpha GDH* gene under a strong constitutive glyceraldehyde-3-phosphate dehydrogenase (*GPH*) promoter. During cultivation of the cells in a medium with glucose, GDH of *O. polymorpha* was constitutively expressed, and this enzyme oxidized glycerol to DHA. GDH overexpression resulted in DHA extrusion into the extracellular liquid up to 100 mg L^{-1}, which is 60-fold higher than in the wild-type strain.

Optimal conditions for cell cultivation were studied (Fig. 5.5).

The influence of the growth medium composition on biomass and enzyme yield for the chosen producer was studied in order to obtain the highest GDH yield (Fig. 5.5A-E). GDH activity in cell-free extract was dependent on a carbon source. Cultivation in a medium containing 1% GlOH and 0.1% glucose resulted in considerable levels of enzyme activity (Fig. 5.5C). It is noteworthy that two GDH

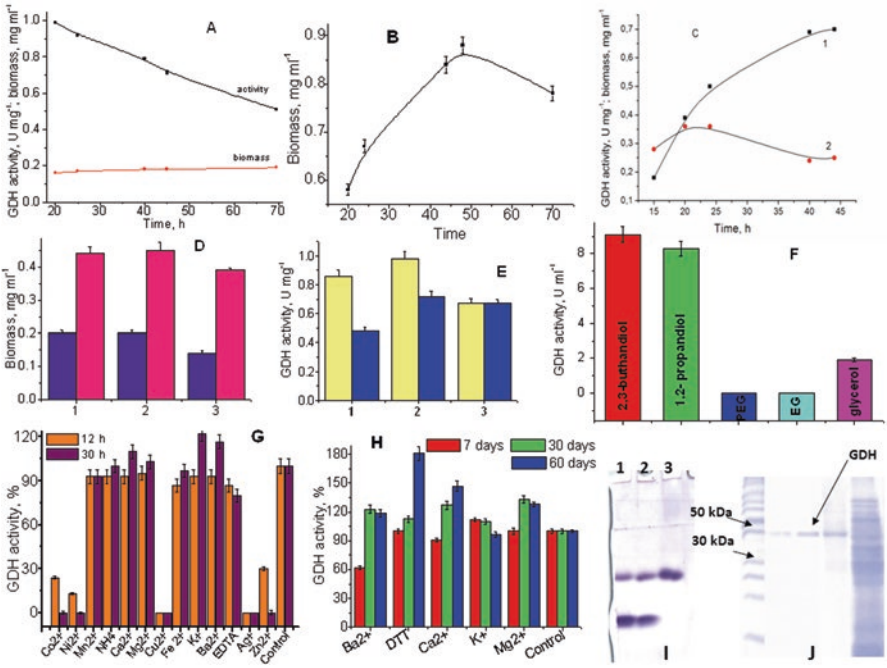


Fig. 5.5 Isolation and characterization of GDH. Effect of cultivation conditions on cells growth (A–D) and specific activity of GDH in cell-free extracts (A, C, E) of producing cells. Properties of the purified GDH: substrate specificity (F), effect of metallic ions (G), storage stability (H), visualization in native PAG after incubation at 40 °C, 50 °C, and 60 °C during 10 min (I), control of purity and estimation of molecular mass of enzyme's subunit in SDS-PAG (J).

isoforms were visualized in native PAG when cells were cultivated in a medium with GlOH and glucose. However, only one thermostable form of GDH was found in cells cultivated in GlOH without glucose (Fig. 5.5I).

A simple scheme for *O. polymorpha* GDH isolation from a cell-free extract of the recombinant strain *S. cerevisiae* was developed. It includes desintegration of the cells by vigorous vortexing followed by two-stage ion-exchange chromatography of cell-free extract on DEAE-Toyopearl M-650 (at pH 8.0 and pH 8.8). This approach was proposed earlier for purification of recombinant FdDH (Demkiv et al. 2007). As a result, a highly purified (tenfold) enzyme preparation with specific activity of 34 U mg^{-1} of protein and 10 % yield was obtained (Fig. 5.5J). For comparison, specific activities of commercially available GDH preparations are 50 U mg^{-1} for enzyme from *Cellulomonas* sp., 15 U mg^{-1} of solid for *B. megaterium*, and 20–80 U mg^{-1} of protein – for *E. aerogenes.* The purity of the isolated enzyme preparation was controlled by PAG electrophoresis under denaturation conditions according to Laemmly. Molecular weight of enzyme's subunit was shown to be 40 kDa (Fig. 5.5J).

The enzymatic method for GlOH assay based on GDH was developed (Gayda et al. 2013a, b). In yeasts, GDH catalyzes the oxidation of GlOH to DHA under simultaneous reduction of NAD^+ to NADH. The proposed enzymatic method includes photometric detection of a colored product, formazan, which is formed from nitrotetrazolium blue in a reaction coupled with GDH-catalyzed oxidation of GlOH in the presence of an artificial mediator, PMS. The same approach was used for the development of an enzymatic method for FA assay (Demkiv et al. 2007). The optimal conditions for an effective reaction were determined. Calibration graphs for GlOH estimation, using a GDH-based method, are presented in Fig. 5.5. This method was used for analysis of GlOH in real samples of commercial wines (see Sect. 5.5.2).

5.4.4 Flavocytochrome b_2

L-Lactate is an important metabolite in glucose metabolism. Monitoring lactate levels is a useful indicator of the balance between tissue oxygen demand and utilization and is useful when studying cellular and animal physiology. Lactate detection plays a significant role in healthcare, and food industries and is specially necessitated in conditions like hemorrhage, respiratory failure, hepatic disease, sepsis, and tissue hypoxia.

For L-lactate analysis a lot of physicochemical and chemical methods have been proposed: spectrophotometry, fluorimetry, pH potentiometric measurements, and amperometric biosensors based on O_2 and H_2O_2 electrodes. The available methods include enzymatic approaches which generally use NAD^+-dependent lactate dehydrogenase (LDH) from animal muscle or heart and bacterial lactate oxidase. These classic approaches as well as modern methods were described in detail in the last years (Rassaei et al. 2014; Sharma et al. 2017; Rosati et al. 2018; Rathee et al. 2016; Dagar and Pundir 2018; Bollella et al. 2019). Most of these methods need a lot of time and previous labour-consuming procedures such as filtration, chromatography, deproteinization, etc. On the other hand, most of them require an expensive equipment or are non-selective.

We proposed using the yeast L-lactate-cytochrome *c*-oxidoreductase (EC 1.1.2.3; flavocytochrome b_2, Fc b_2) of the thermotolerant methylotrophic yeast *O. polymorpha* as a promising biocatalyst for enzymatic-chemical analytical methods and amperometric biosensors. Fc b_2 is a tetramer of identical subunits, where each subunit contains FMN- and haem-binding domains. The enzyme exhibits absolute specificity for L-lactate, but application of Fc b_2 from baker's yeast in bioanalytics is hampered by its instability and difficulties in purification of the enzyme. We used the following stages for obtaining Fc b_2:

1. Screening potential yeast producers in order to choose the best source of thermostable Fc b_2

2. Optimizing cultivation conditions in order to achieve the maximal yield of the enzyme
3. Testing different cell disruption methods in order to obtain a stable enzyme with the highest yield
4. Developing a simple scheme for Fc b_2 isolation, chromatographic purification, and stabilization

The highly purified target enzyme was used for developing enzymatic-chemical and biosensor methods for L-lactate assay. Screening of 16 yeast species was carried out in order to choose the most effective producer of the stable form of FC b_2. For this aim, a method of visualization of the activity of Fc b_2 in electrophoretograms was used. This method was based on the interaction between ferrocyanide (generated during the enzymatic reaction) and Fe^{3+}, resulting in the formation of intensely colored precipitates of Berlin blue (Gaida et al. 2003) The main advantages of this method were its high sensitivity (less than 0.005 U Fc b_2 was detected within a suitable time period) and the stability of the dye formed. The method developed can be used for determining Fcb_2 activity in cell-free extracts (e.g., in the selection of Fc b_2 producers) and monitoring chromatographic purification of proteins, as well as in other cases associated with Fc b_2 assessment. *O. polymorpha*, *Kluyveromyces lactis*, and *Rhodotorula pilimanae*, which exhibited the highest specific activities in cell-free extracts, were chosen as the best producers of Fc b_2 (Fig. 5.6). A study of the enzyme's thermostability in the cell-free-extracts revealed that only Fc b_2 from *O. polymorpha* remains active after heating for 10 min at 60 °C or 3 min at 70 °C (Smutok et al. 2006c).

To obtain the target enzyme, *O. polymorpha* cells were cultivated in flasks to the beginning of the stationary growth phase at 30 °C on a shaker with strong aeration (240 rpm) in a mineral medium containing 1% glucose, 0.2% L-lactate sodium, and 0.05% yeast extract. Freshly grown cells were collected by centrifugation, washed, lyophilized, and kept at –20 °C until used (Smutok et al. 2006a).

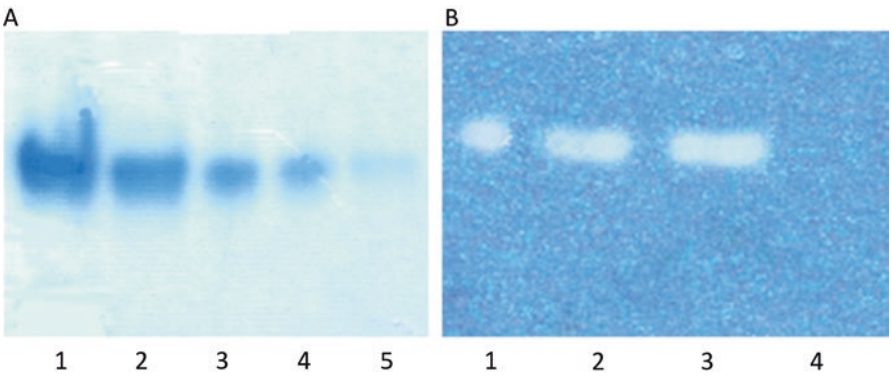


Fig. 5.6 Visualization of Fc b_2 activity in cell-free extracts in native PAG. A, by Berlin Blue method, 0.03 (1), 0.015 (2), 0.010 (3), 0.005 (4), and 0.003 (5) units of enzyme are in PEG's cell; B, methylene Blue method, 0.08 (1), 0.15 (2), 0.2 (3), and 0.02 (4) units of enzyme are in PEG's cell

Cell-free extract (CE) was obtained by incubating lyophilized cells in a lysing mixture with 10% *n*-butanol (Gaida et a. 2003). CE was separated from the cell debris by centrifugation, cell fragments were washed twice with a lysing mixture, and the supernatants were combined (C1). It is worth mentioning that cell fragments in the C1 contained a significant amount of Fc b_2. The pellets were therefore also extracted with 1% Triton X-100 in 50 mM PB, pH 7.5, and the supernatants were combined (C2).

It was demonstrated that the specific activity and stability of Fc b_2 was higher in the C1 and C2 extracts than in the CE. Furthermore, use of Triton X-100 allows an extraction of up to 95% of the total Fc b_2 activity from the cell debris (C1+C2). The enzyme was purified from the (C1+C2) extracts with a total activity of 60 U by column chromatography on the anion-exchange sorbent DEAE-Toyopearl 650 M (TSK-Gel, Japan). The enzyme was eluted by 15% (of saturation at 0 °C) ammonium sulfate in 50 mM PB, pH 7.5, containing L-lactate. Monitoring of enzyme activity and purity was carried out by estimation of the Fc b_2 specific activity in each fraction and by PAG-electrophoresis under native (Gaida et al. 2003) and denaturation conditions according Lemmly.

The most active fractions were combined and treated with ammonium sulfate up to 70% saturation. The highest specific activity of FC b_2 in some fractions was 20 U·mg^{-1} protein; the yield was 10%. Precipitation with ammonium sulfate allows to purify the target enzyme additionally (1.5-fold) up to 30 U mg^{-1} (Smutok et al. 2006a).

Fc b_2 preparation isolated from the methylotrophic yeast *O. polymorpha* 356 has been chosen as a biorecognition element in biosensor's construction (Smutok et al. 2005, 2006a, 2011, 2017; Goriushkina et al. 2009) as well as for the development of enzymatic-chemical methods for L-lactate assay (Gonchar et al. 2009; Smutok et al. 2013).

5.5 Bioanalytical Application of the Isolated Oxido-reductases

Enzymes AO, FdDH, GDH, and FC b_2, isolated from the cells *O. polymorpha* C-105 and other yeast producers, including recombinant cells (see Table 5.1), were used as biocatalysts for the development of analytical approaches to determine correspondent analytes (Fig. 5.7).

5.5.1 AO, FdDH, and FC $\mathbf{b}_2$

The functional characteristics of the constructed amperometric biosensors for analysis of practically important analytes, based on the purified yeast oxido-reductases, are summaried in Table 5.2. The main advantage of the developed enzyme-based

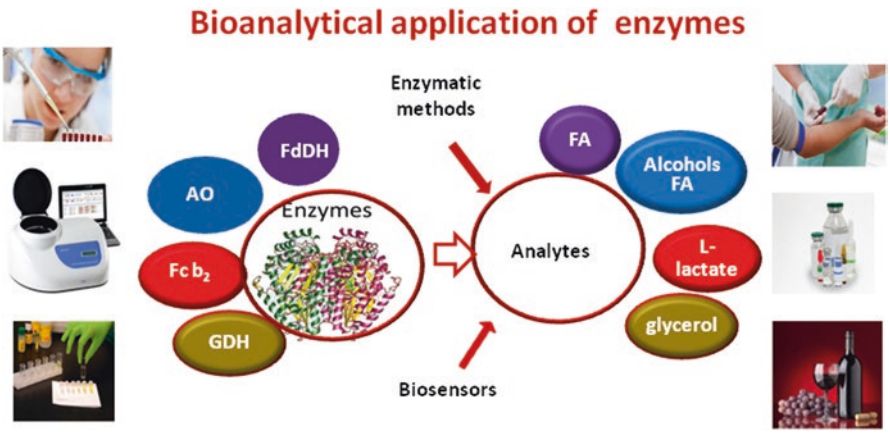


Fig. 5.7 Bioanalytical application of the isolated enzymes

Table 5.2 Analytical characteristics of the developed amperometric biosensors

Analyte	Biomembrane	Linearity range, mM	Response time, s (95%)	Stability, days (50%)	K_M^{app}, mM	Reference
Ethanol	AO/PO	Up to 1.8	45	16	1.94 ± 0.37	Smutok et al. (2006b, 2011)
Ethanol	Mutated AO/[a]PO	Up to 4.0	[b]ND	14	5.4 ± 0.8	Dmytruk et al. (2007)
Ethanol	AO-[c]nAu-enriched/[d]p-cells/PO	Up to 2.0	20	30	1.93 ± 0.08	Karkovska et al. (2017)
Ethanol	AO/PO-like nanozyme	0.01–0.25	15	12	1.99 ± 0.08	Stasyuk et al. (2019)
Methanol	AO/PO-[e]gnPd	Up to 0.8	55	3	0.64 ± 0.02	Gayda et al. (2019)
FA	FdDH/ diaphorase	0.05–0.5	ND	1	ND	Nikitina et al. (2007)
FA	NAD/FdDH/ glutathione	Up to 20.0	165	3	119 ± 5.34	Demkiv et al. (2008)
FA	AO/PO	Up to 4.0	ND	16	3.1	Sibirny et al. (2011b)
L-Lactate	Fc b_2	Up to 0.5	6	16	0.52 ± 0.02	Smutok et al. (2005)
L-Lactate	nAu-FC b_2 on Au-electrode	0.3–2.0	20	34	0.79 ± 0.03	Smutok et al. (2017)
L-Lactate	Fc b_2 -nAu-enriched/p-cells/ PO	0.3–2.7	5	ND	ND	Karkovska et al. (2015, 2017)

Remarks: [a]*PO* horseradish peroxidase, [b]*ND* not determined, [c]*nAu* gold nanoparticles, [d]*p-cells* permeabilized yeast cells, [e]*gnPd* Pd nanoparticles obtained via "green" synthesis

biosensors is a simple procedure of sample preparation: neither pretreatment of the samples nor their derivatization is required.

Highly stable and sensitive amperometric biosensors on primary alcohols and FA were developed using AO isolated from thermotolerant methylotrophic yeast *O. polymorpha* as biorecognition elements (Smutok et al. 2006b, Shkotova et al. 2006; Smutok et al. 2011; Sigawi et al. 2011, 2014). To construct bi-enzyme sensor, immobilization of AO was performed by means of electrodeposition paints; horseradish peroxidase (PO) and Os-complex modified polymer were used to decrease the working potential (Smutok et al. 2006b). Mono-enzyme AO-based biosensor was also developed by electrochemical deposition of the Resydrol polymer, conjugated with AO (Shkotova et al. 2006). To facilitate electron transfer between the enzyme and the electrode surface, electroactive polymers were used in biosensor's construction. Both biosensors demonstarted a good reproducibility and operational and storage stability, so they were used for ethanol assay in real alcoholic beverages. For optimization of the electrochemical communication between the immobilized enzymes and the electrode surface, a variety of sensor architectures were tested. Bioanalytical properties of the most effective AO-/HRP-based biosensor were investigated (Table 5.2). The best biosensor with architecture *HRP/Os-Ap59//AOX/CP9* was applied for the determination of ethanol in wine samples (Smutok et al. 2006b).

For construction of highly selective biosensors on FA, FdDH being highly selective to FA was used (see Table 5.2). The developed biosensors on FA show high sensitivity and selectivity to FA and good operational and storage stability. The reagentless biosensor on FA with fixation of all sensor components in a bioactive layer on the transducer surface was proposed (Demkiv et al. 2008). This biosensor was designed to prevent any leakage of the low-molecular and free-diffusing cofactors of FdDH, thus enabling FA determination without addition of the cofactors to the analyte solution. A validity of this biosensor for FA analysis in real samples was approved by testing formalin-containing commercial goods.

A number of amperometric L-lactate-selective biosensors were developed using Fcb_2 and the enzyme-producing yeast cells (Table 5.2). Different immobilization methods and low-molecular free-diffusing redox mediators were tested for optimizing the electrochemical communication between the immobilized enzyme and the electrode surface. The possibility of direct electron transfer from the reduced form of Fc b_2 to carbon electrodes was evaluated. The bioanalytical properties of Fc b_2-based biosensors, such as signal rise time, dynamic range, dependence of the sensor output on the pH value, temperature, and storage stability, were investigated, and the proposed biosensor demonstrated a very fast response and a high selectivity for L-lactate determination (Smutok et al. 2005, 2006a; Goriushkina et al. 2009). The proposed biosensor was successfully tested for L-lactate analysis on the samples of commercial wines.

Combining nanobiotechnology with electrochemical enzyme-based biosensors has become a crucially novel strategy for the development of simple and reliable monitoring systems for food quality and safety. Nanomaterials also endow electrochemical biosensors with device miniaturization and high sensitivity and

specificity. They, therefore, have a great potential for on-site food safety assessment (Nikolelis and Nikoleli 2016, Gonchar et al. 2017; Lv et al. 2018).

The improved biosensors were created using a combination of genetic technology and nanotechnology approaches, namely, by overexpression of the corresponding gene in the recombinant yeast cells and by the transfer of enzyme-bound gold nanoparticles (Fcb_2-nAu and AO-nAu) into the cells (Smutok et al. 2005; Karkovska et al. 2017). The resulted biosensors were shown to possess the high sensitivity and the fast response. A novel mono-enzyme AO-based nanobiosensor on ethanol was constructed with the usage of peroxidase-like PtRu-nanoparticles. This biosensor, being rather stable and very sensitive, was successfully tested on the several real samples of wines (Stasyuk et al. 2019). Recently, we demonstrated the possibility of developing reagentless AO-based amperometric biosensors using nanoparticles of noble metals, synthesized via "green synthesis" in the presence of extracellular metabolites of the yeast *O. polymorpha.* It was shown that AO-based electrode, modified with green-synthesized Pd nanoparticles, although having a lower sensitivity to methanol, reveals a broader linear range of detection and a higher storage stability, compared with unmodified control electrode. Such bioelectrode characteristics are desirable for enzymes, possessing a very high sensitivity for their substrates, because in such cases the tested samples must be very diluted, which is problematic for online analysis of real samples (Gayda et al. 2019).

To achieve the excellent characteristics of enzyme-based sensor, the usage of gold electrode, modified by Fcb_2-nAu claster, was proposed recently (Smutok et al. 2017). This biosensor was shown to demonstrate a ninefold higher sensitivity to L-lactate and a wider linear range in comparison with the characteristics for free enzyme, immobilized on the same electrode.

Enzymatic-chemical methods for ethanol (Gonchar et al. 2001; Pavlishko et al. 2005), FA (Demkiv et al. 2007; Gayda et al. 2008a; Sibirny et al. 2011a, b; Sigawi et al. 2011), and L-lactate (Gonchar et al. 2009; Smutok et al. 2013) determination were described also earlier. All these methods were successfully tested on the real samples of food products, beverages, as well as biological liquids (Smutok et al. 2011; Pavlishko et al. 2005; Sibirny et al. 2011a, b).

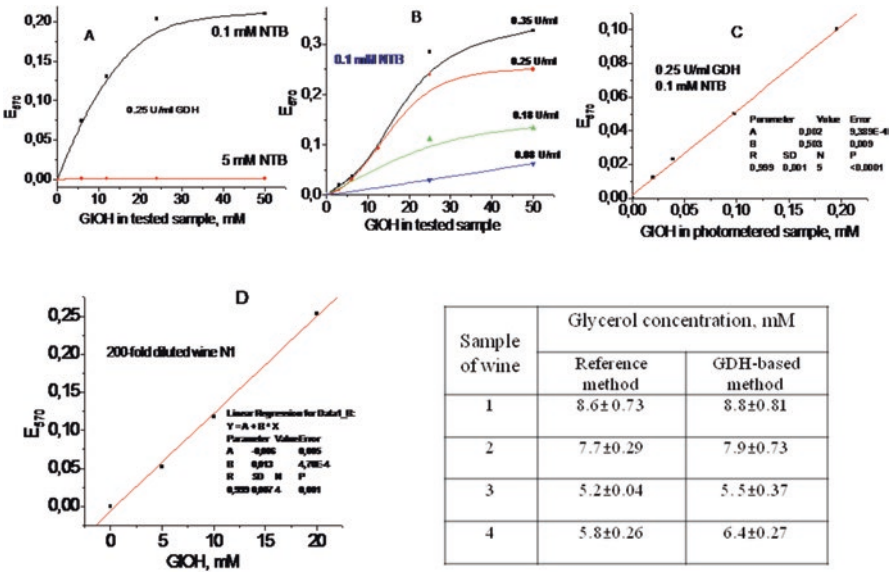
5.5.2 GDH-Based Methods for Glycerol Assay in Wines

The general principles of amperometric detection of glycerol (GlOH) using enzyme-based biosensors were previously reviewed in detail (Goriushkina et al. 2010; Smutok et al. 2011; Synenka et al. 2015; Mahadevan and Fernando 2016). The described methods are based on NADH and mediators-aid registration, on the use of oxygen and hydrogen peroxide electrodes, conductive organic salts, and wiring electrodes. GlOH assay using recombinant yeast GDH was not investigated as thoroughly. We therefore focus on demonstrating the applicability of the proposed enzymatic-chemical method to GlOH assay in real samples: wines.

GlOH is an important by-product of glycolysis and is quantitatively one of the major components of wine (Nieuwoudt et al. 2002). GlOH positively influences the taste of table wines, giving them viscosity, sweetness, and softness. The production of GlOH is closely linked to the availability of the fermentable sugars presented in musts.

We developed an enzymatic-chemical method for GlOH assay based on spectrophotometric detection of solubilized formazan (see Sect. 5.4.2) which is generated in the reaction of nitrotetrazolium blue with NADH, a product of GDH-catalyzed oxidation of GlOH. The validity of the proposed method was tested on commercial wines and compared with a referent method. A standard addition test was used in order to evaluate the negative influence of wine components on enzyme-catalyzed reactions. The results of graphical estimation of the GlOH content in the samples of some commercial wines are presented in Fig. 5.8. It was shown that the estimated values are in good correlation with the data of reference methods, as well as with literature data.

The GlOH content, usually formed by *Saccharomyces cerevisiae* in wine, varies between 1.36 and 11 g/L (15–120 mM) (*http://www.lallemandwine.com/wp-content/uploads/2014/12/Wine-Expert-120321-WE-Glycerol-and-WInemaking.pdf*). Higher GlOH levels are generally considered as improving wine quality. The mean GlOH concentrations in dry red (10.49 g L^{-1}), dry white (6.82 g L^{-1}), and noble late harvest wines (15.55 g L^{-1}) were found to be associated with considerable variation within each respective style (Nieuwoudt et al. 2002). GlOH content is greater in wines from must that was processed with sulfite (Goold et al. 2017; Belda et al. 2017; Rankine and Bridson 1974; Remize et al. 2003) and also in wines made from grapes



Sample of wine	Glycerol concentration, mM	
	Reference method	GDH-based method
1	8.6±0.73	8.8±0.81
2	7.7±0.29	7.9±0.73
3	5.2±0.04	5.5±0.37
4	5.8±0.26	6.4±0.27

Fig. 5.8 Glycerol dehydrogenase as biocatalyst enzymatic-chemical method for glycerol assay

affected by *Botrytis cinerea*, the "noble mold" – up to 30 g L^{-1} or 330 mM (Nieuwoudt et al. 2002). Thus, comparison of the measured and expected contents of GlOH enables confirmation or challenging the originality of tested wines.

5.6 Conclusions

Enzymes possess high selectivity and sensitivity and are thus widely used in analytical test-systems for control of the environment and food, as well as for clinical diagnostics. Obtaining a wide range of target enzymes on an industrial scale from different sources, including recombinant microorganisms, is an urgent problem of biotechnology and enzymology. This chapter presents the main achievements in the elaboration of modern techniques for recombinant enzymes isolation from selected or recombinant yeasts. The results of a series of the authors' investigations of these problems are summarized.

Some steps are necessary for isolating a highly purified, stable, and active yeast enzyme: selection or construction of the effective yeast producer, optimization of its cultivation conditions for achievement of the highest specific activity of enzyme in a cell-free extract, and development of an effective technology for target enzyme purification. The scheme for simultaneous isolation of several thermostable yeast enzymes from cells of the thermotolerant methylotrophic yeast *O. polymorpha* followed by their chromatographic purification using ion-exchange sorbent was proposed. The possibility of obtaining alcohol oxidase (AO), flavocytochrome b_2 (Fc b_2), glycerol dehydrogenase (GDH), methylamine oxidase (AMO), arginase, formaldehyde dehydrogenase (FdDH), formate dehydrogenase (FDH), and formaldehyde reductase (FR) from the cell-free extract of the same yeast source was demonstrated.

The highly purified yeast oxido-reductases (AO, FdDH, GDH and Fc b_2) were isolated from *O. polymorpha* cells overproducing alcohol oxidase, as well as from other special yeast producers, including recombinant cells. The target enzymes were characterized and used as biocatalysts for the development of analytical methods for assaying primary alcohols, formaldehyde, glycerol, and L-lactate, respectively.

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