Serbian Biochemical Society
Fourth Conference

" Biochemistry - molecular life science "

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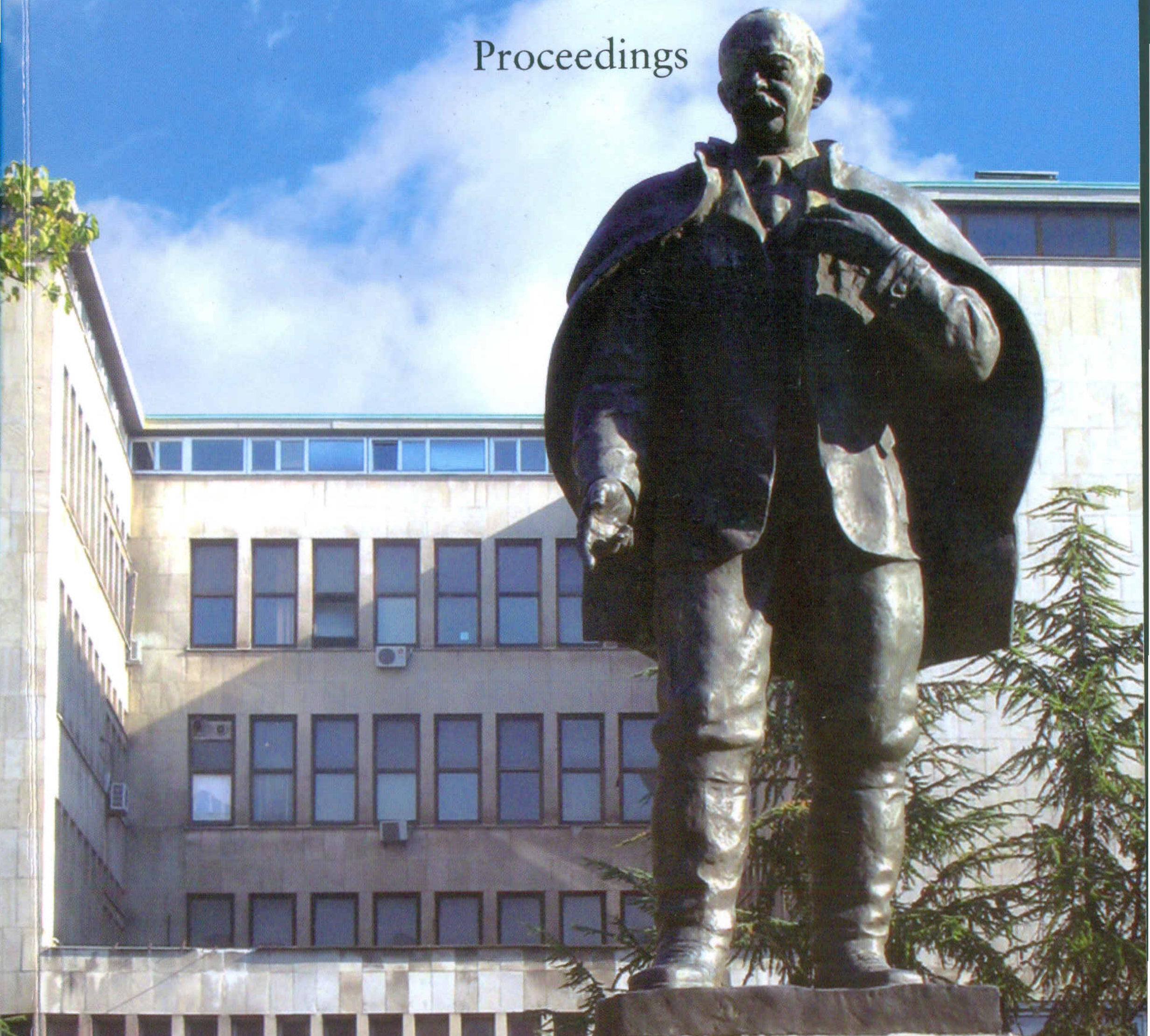

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Enzymes in unusual environments

Lidija Izrael Zivkovic*

** Department of Chemistry, School of Medicine, University of Belgrade, Visegradska 26, Belgrade, Serbia*
e-mail: lidija.izrael-zivkovic@mfub.bg.ac.rs, lidijajob@yahoo.com

The idea of carrying out enzyme reaction in organic solvents was initially considered with skepticism. Now biocatalysis in organic solvents is investigated and used in numerous academic and industrial laboratories. Alkaline lipase from Pseudomonas aeruginosa NCAIM(P) B 001380, capable of growing in a water-restricted medium has excellent properties and good potential for biotechnological applications in the metal industry. Its marked stability and activity in organic solvents suggest that this lipase is highly suitable as a biotechnological tool in a water-restricted medium with a variety of applications including organosynthetic reactions and the control and prevention of Metal Working Fluid (MWF) putrification in the metal industry.

Introduction

Potential of enzymes as catalysts in water solutions is well known, but their remarkable stability and capacity to function in water-restricted environments, including organic solvents, has potentiated numerous advantages. Using organic solvent system for enzymatic reaction has importance for the bioconversion of substrates that are moderately soluble, or even insoluble, in water. The introduction of organic solvents in enzymology has provided possibility of broad variation of enzymatic reactions. The substrate, stereo-, regio- and chemoselectivities of the enzymes markedly differ in nonaqueous solutions[1]. Likewise, thermodynamic equilibrium favors synthesis over hydrolysis which has been extensively used in organosynthetic reactions. Discovery that enzymatic selectivity can be markedly affected and even inverted by the solvent is of particular importance[1]. Interactions between an enzyme molecule and the surrounding water are of the crucial importance for enzyme catalysis. The effect of organic solvents on an enzyme is primarily due to interactions with the enzyme-bound essential layer of water rather than with the enzyme itself. The water required by enzymes in nonaqueous solvents provides them sufficient conformational flexibility needed for catalysis[2]. When placed in anhydrous solvent, enzyme acquire remarkable new properties: enhanced stability, altered substrate specificity, ability to catalyze new reactions. In the absence of water, which acts as a molecular lubricant, enzymes are very rigid, so in dry solvents enzyme drive to unfold but it does not possess conformational flexibility to do so. Protein becomes kinetically "trapped"[1].

Significant advantages of using enzymes in organic solvent systems are numerous: increased solubility of nonpolar substrates, suppression of water-dependent side reactions, thermodymnamic equilibria favors synthesis over hydrolysis, alteration of specificity, recovery and reusability of enzyme, often enhanced thermostability, and elimination of microbial contamination. Some of the disadvantages are: inactivation of enzymes, mass-transfer limitations, expensive preparation of biocatalysts, water activity control[3].

Organic solvent systems

There are three main types of organic solvent systems, depending on the miscibility with water and relative ratio of the solvent and water in the system: 1-water-water miscible organic solvent system; 2- water-water immiscible organic solvent system; 3-nearly anhydrous solvent system.

Water-water miscible organic solvent system is produced when water miscible cosolvents are added to the medium to improve the solubility of compounds. These systems are monophasic which lead to more rapid reaction rates for hydrophobic compounds by reducing the mass-transfer limitations. Moreover, this system has advantages because thermodynamic equilibrium favor synthesis over hydrolysis. The concentration of substrates and products around enzymes can be easily controlled in order to prevent an excessively high concentration of substrates or products around enzymes. However, direct contact of the organic solvent with the enzyme can result in rapid denaturation and inactivation[4].

Water-water immiscible organic solvent system consist of two phases, the aqueous containing a dissolved enzyme and the phase of an immiscible organic solvent, and between them the interfacial area forms. Inactivation rates of enzymes in two-phase systems are lower than those in cosolvents systems because the direct contact of an enzyme with organic solvent is prevented. Although minimized, denaturation of the enzyme occurs at the interface. The reaction rates are relatively low because of a low rate of mass-transfer across the interface. This obstacle can be overcome by intensive stirring. However, the increased interface can results in denaturation and inactivation of the enzyme[5]. When enzymes and substrates are soluble in the aqueous phase and products are soluble in the organic phase a water-water immiscible organic solvent system is desirable. The enzymatic reaction proceeds in the aqueous phase and product are partitioned in the organic phase, so the product yield is high and separation is easy.

Native enzymes are insoluble in nearly anhydrous solvent systems. The stability of solid enzymes is sometimes high but their activities are lower than those of solubilized enzymes. Conformation mobility of enzyme at such low water content is restricted and therefore the proteins are more rigid. The increased rigidity results in lower activity from one side but higher stability from another. Enzymes can be solubilized by modification with amphipatic compounds. In these systems conformational mobility of enzymes is restricted. The rigidity

of the enzyme structure results in higher thermal stability and provides the possibility of use of the techniques such as molecular imprinting [6]. The water participates in all noncovalent interactions maintaining the native, catalytically active enzyme conformation, so the water is indeed required for enzyme action[7]. As long as water is localized in few monolayers around the enzyme, it can be catalytically active, and this is situation depicting an enzyme functioning in nearly anhydrous organic medium[8].

Effect of organic solvents on the stability and activity of enzymes

In organic solvent containing media the protein unfolding occurs due to disturbance of the balance between hydrophobic interactions, electrostatic interactions, Van der Waals forces, and hydrogen bonds. Water plays an important role in enzyme structure and function in aqueous media. That role becomes even more important when one focuses on enzymes in low water media.

Different approaches (experiments as well as molecular dynamic simulations of enzymes) have contributed the understanding of the factors affecting stability and activity of enzymes in organic solvents. Enzyme activity in organic solvents depends on parameters such as: water activity, partition coefficient log*P*, nature of solvent, pH control, and enzyme form[3]. These parameters are often helpful in describing the behavior of enzymes in organic solvents but so far none of them have provided serious predictive analysis[9].

Analysis focused on enzyme structure, flexibility, and the details of enzyme hydration in organic solvents have been performed[10]. The enzyme surface and the active site region are well hydrated in aqueous medium, whereas with increasing polarity of the organic solvent the hydration water is stripped from the enzyme surface. Water stripping is accompanied by the penetration of organic solvent molecules into crevices on the enzyme surface and especially into the active site. More polar organic solvents replace mobile and weakly bound water molecules in the active site. In contrast, the lack of water stripping in octane allows efficient hydration of the active site. These differences in the active site hydration are consistent with the inverse dependence of enzymatic activity on organic solvent polarity and indicate that the behavior of hydration water on the enzyme surface and in the active site is an important determinant of biological function especially in low water media. Wet lab and in silico experiments show the inverse dependence of enzyme activity on the polarity of the organic medium employed[10,11].

The protonation state of the various groups of enzyme is undoubtedly important for enzyme activity. Protonation in water is controlled by pH adjustment, but this is not the same in organic solvent. A way to control enzyme protonation was developed by Zaks and Klibanov. They have showed that enzyme activity in organic solvent was dependent on pH of the solution from which the enzyme was lyophilized or precipitated. The enzyme lyophilized at its pH optimum exhibit high activity (phenomenon of pH memory)[2].

Organic solvent-tolerant lipases

Many enzymes are easily denatured and inactivated in the presence of organic solvents. Therefore protein engineering and several methods such as immobilization, modification and entrapment for stabilizing enzymes have been developed [12, 13]. But, if enzymes are naturally stable and active in the presence of organic solvents, such treatment is not necessary.

Lipases are among the most promising and important biocatalysts for carrying out reactions in both aqueous and nonaqueous media. This is primarily due to their ability to utilize a broad spectrum of substrates[14] having high chemo-, regio- and enantioselectivities in the synthesis or hydrolysis of lipid compounds, as well as their stability under a wide range of conditions. Most of organic solvent tolerant lipases have been isolated from microorganisms including organic solvent tolerant bacteria such as *Pseudomonas spp*. Their stability in organic solvents has been reported for lipases from *Pseudomonas pseudomalei* 12sm (*P. pseudomalei)*[15], *P. aeruginosa* YS-7[16], *P. Mendoncina*[17], *P. aeruginosa* LST-03[4], *P. aeruginosa* ATCC 27853[18] and *P. Fluorescens*[19]. In addition, lipases from *P. aeruginosa*, *P. cepacia* and *P. fluorescens* have been manufactured and extensively used in organosynthetic reactions, particularly in chiral production of racemic compounds[20], which includes manipulation in nonaqueous solutions. One selected organic solvent tolerant lipase is summarized below.

A lipase from *P.aeruginosa* NCAIM (P) B 001380[21]

P. aeruginosa NCAIM(P) B 001380 (previously named *P. aeruginosa* san-ai) has been isolated from putrid mineral cutting oil used as metalworking fluid (MWF) in the metal industry[22]. It was assumed that *P. aeruginosa* san-ai grown in a water-restricted medium, could secrete enzymes stable and active in organic solvents. It has been shown that the bacteria beside lipase[21] produces protase stable in organic solvents [22], biosurfactant rhamnolipid and polysaccharide[23,24]. Both enzymes, lipase and protease have shown stability and activity in organic solvents and their use as detergent additives has been evaluated[25].

Characterization of lipase protein

Basic characteristics of the lipase are presented in Table 1. The relative molecular mass of the san-ai lipase was estimated to be 54 kDa which is higher than that of other lipases from *P. aeruginosa*. The optimum pH and temperature were 11 and 70 °C, respectively. The enzyme is stabile over a broad pH range (pH 4-11.5). Remaining activities of the enzyme at 70 °C and 60 °C after 15 min were 30% and 75% of the initial activity, respectively. The lipase was inhibited strongly by Zn^{2+}, Hg^{2+}, Cu^{2+} and slightly by Ca^{2+} and Mg^{2+}. Non-ionic detergents and sodiumdeoxycholate enhanced lipase activity.The divalent metal-chelating agents EDTA and *o*-phenathroline as well as the S-S reducing agent DTT and SH-carboxymethylation reagent IAA caused no inhibition of lipase activity. The lipase preferably acted on triacylglycerols with medium-chain fatty acids.

Table 1. Characteristics of the lipase

Characteristic	
Mw	54 kDa
pH optimum	11
Temperature optimum	70 °C
Temperature stability	60 °C 15 min (75%)
Inhibition (%)	Hg^{2+}
Substrate specificity	The highest activity on middle higher faty acids

Organic solvent stability

The effects of various organic solvents: methanol, ethanol, acetone, butanol, *iso*-propanol, chloroform, *n*-hexane and DMFA, on the lipase from *P. aeruginosa* san-ai were examined at 30 °C for 2 days. The san-ai lipase was stable for 24 h in selected organic solvents, with the exceptions of *n*-butanol and *iso*-propanol. Nonmiscible organic solvents with a much higher log P, such as chloroform (2.0) and *n*-hexane (3.5) (compared with the following alcohols butanol, 0.80; *iso*-propanol, 0.28) had a stabilizing effect on the enzymatic activity in aqueous solution. The lipase retained complete activity in chloroform and *n*-hexane, even after a long exposure of 2 days. Lipase exhibit high stability in organic solvents as expected since obtained from microorganisms growing in specific medium.

Thermal stability in organic solvents

The thermal instability of enzymes is a consequence of protein unfolding on exposure to high temperature; however an improved thermal stability of enzymes in non-aqueous media has been documented[1]. The thermal stability of the lipase from *P. aeruginosa* san-ai was measured as remaining activity of the crude enzyme solution supplemented with n-hexane, DMFA, ethyl-methyl ketone, methanol and ethanol at a final concentration of 30% at 50 °C and 60 °C (Table 2.). It was found that the enzyme retained 100% activity in *n*-hexane for 15 min at 60 °C and 50% activity at the same temperature for 30 min. The enzyme retained 100% activity in methanol for 30 min at 50 °C and 75% activity in ethyl-methyl ketone for 10 min at 50 °C. However, the enzyme was not stable at 60 °C in the presence of DMFA, ethyl-methyl ketone, methanol and ethanol. The enzyme is clearly stable in organic solvents mixtures at 50 °C. Furthermore, the thermal stability in *n*-hexane was slightly improved in comparison with that in aqueous solutions.

Table 2. Thermal stability in organic solvents

Organic solvent	Remaining activity (%)	
	50°C	60°C
Methanol	100 (for 30 min)	-
Ethanol	55 (for 10 min)	-
Ethyl-methyl ketone	75 (for 10 min)	-
DMFA	100 (for 30 min)	-
n-Hexane	85 (for 30 min)	100 (for 15 min) 50 (for 30 min)

Activity in organic solvents

Although some enzymes are stable in organic solvents, their activity is reduced. To assess the potential of water to abate organic solvent-related activity loss, the san-ai lipase was tested for its activity against *p*-NPP in organic solvents supplemented with 5% water. Results have indicated that the enzyme is active against *p*-NPP in ethanol, methanol, ethyl-methyl ketone, acetone and *n*-hexane supplemented with 5% water (Table 3.). The activity in ethanol and methanol was significantly higher than that in other solvents. Alcohols are solvents capable of forming multiple hydrogen bonds, mimicking the effect of water and, thereby, enhancing enzyme action[1]. This activation effect is particularly obvious when methanol is the solvent.

Table 3. Lipase activity on *p*-nitrophenyl palmitate in 95% organic solvents

Organic solvent	Activity (mU/mg of protein)
Ethanol	5.22
Methanol	231.02
Ethyl-methyl ketone	0.12
Acetone	0.06
n-Hexane	0.13

Potential application in metal industry

During growth in MWF, *P. aeruginosa* san-ai causes the degradation of the mineral oil, a serious problem in the metal industry. In addition, the strains of *P. aeruginosa* found in MWFs have shown to possess natural resistance to many commonly used antimicrobial agents and biocides[26].

The present study describes the production, purification, and characterization of the lipase from *P. aeruginosa* san-ai grown in MWF. The high enzymatic stability of the lipase in an alkaline water-restricted medium opens the possibility for the potential application of this lipase in the construction of an intelligent Drug Delivery System (DDS) designed for the prevention of putrefication of MWF as depicted in Fig.1. In short, if the bacterium produces an extracellular lipase, then antibacterial reagents (combinations of a bacteriolytic enzyme and a sterilizer) immobilized in a water/oil/water (W/O/W) emulsion, can be released through lipase activity. The released bacteriolytic enzyme and sterilizer will then act against the bacteria- cause of the MWF putrefication, triggering its death, that way any further secretion of the lipase will be discontinued. On the other hand, this action will also stop the release of now unnecessary antibacterial agents. Thus, the construction of such a DDS could be considered as a specific enzymatically controlled on-off mechanism of antibacterial reagent delivery in the control of MWF putrefication.

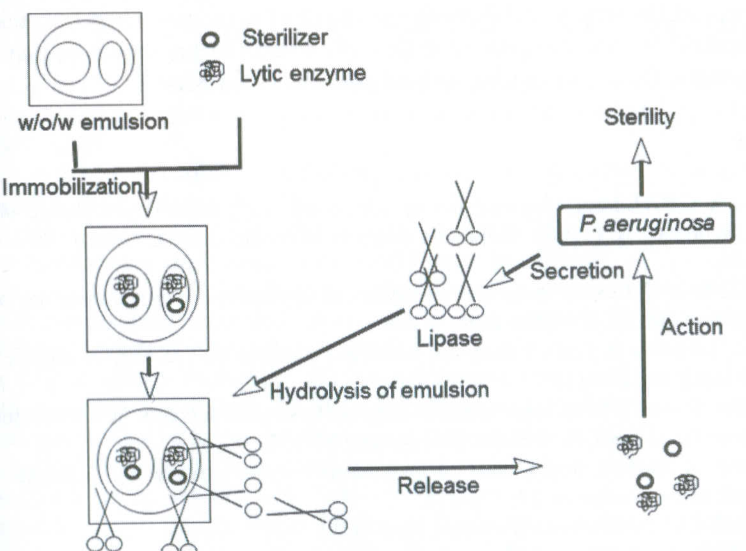

Figure 1. Model of intelligent DDS. Antibacterial agents (combinations of a bacteriolytic enzyme and a sterilizer) immobilized in a w/o/w emulsion are released by an extracellular lipase.

Enzyme immobilization

Current demand of the world's biotechnological industries is further immprovment of enzyme characteristics. Potential of different immobilisation techniques has been studied in order to overcome instability, enhance enzyme reuse, offer easier separation, making production economically profitable. Immobilization of *Candida rugosa* lipase, one representative lipase was successfully performed onto nano and meso porous materials with improvement of thermal stability and excellent reuse potential[27]. In light of the obtained encouraging results immobilisation of *P. aeruginosa* lipase and protease is underway.

Conclusion

Enzyme activity in organic solvents described as an unusual phenomenon is important instrument used im diferent aplication. Besides, it has stimulated fundamental research in order to answer questions related to the performances of enzyme in unusual environments.

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