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review

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Non-Aqueous Biocatalysis in Homogeneous Solvent Systems

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Summary

Enzymes are highly specific catalysts that typically function in aqueous solvents. However, many enzymes retain their catalytic activities at high concentrations in non-aqueous environments, including neat hydrophilic organic solvents. In fact, enzymes can be used to carry out reactions in organic solvents that are not possible in aqueous systems. Therefore, biocatalysis in homogenous non-aqueous solvents offers possibilities for producing useful chemicals and several synthetic reactions have already been developed using this type of system. The current review discusses factors that influence enzyme catalysis in non-aqueous solvents such as water content, solvent concentration, interaction of solvent with protein structure, stability and activity. Also, new strategies for non-conventional biocatalysis using extremophiles and ionic solvents are mentioned.

Key words: homogeneous biocatalysis, homogeneous solvent mixtures, enzyme stability, enzyme solvent interactions

Introduction

Enzymes are highly specific and extremely enantio- and regio-selective catalysts. Due to these attributes, they are widely used in biotransformations for the production of fine chemicals and optically active compounds of industrial interest, in processes that represent an effective and environmentally-friendly alternative to chemical synthesis (1,2). In particular, non-aqueous biocatalysis is suitable for synthesis of commercially important pharmaceutical precursors and drugs, such as pure enantiomers, chiral molecules, single isomers and biopolymers.

Over the last twenty years, biocatalysis in organic solvents has emerged as an area of systematic research and industrial development, fueled mainly by chemical and pharmaceutical interest. Attention has been especially focused on enzymes as catalysts for asymmetric

synthetic transformations (3,4). The main advantages that biocatalysis in organic solvents offers compared to traditional aqueous enzymology can be summarized as: (i) increased solubility of non-polar substrates and products, which markedly speeds up overall reaction rates; (ii) reversal of thermodynamic equilibrium in favor of synthesis over hydrolysis, allowing reactions usually not favored in aqueous solutions to occur (e.g. transesterification, thioesterification, aminolysis); (iii) drastic changes in the enantioselectivity of the reaction when one organic solvent is changed to another; (iv) suppression of unwanted water-dependent side reactions, which often degrade common organic reagents; and (v) elimination of microbial contamination in the reaction mixture (1,5–14). Also, it has been reported that low-water environments can be used to stabilize enzyme conformations that exhibit unpredictable catalytic properties (8).

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However, it is very well documented that enzymes are denatured or highly inactivated in the presence of organic solvents, and specific catalytic activities of enzymes that are stable in non-aqueous environments are generally lower than those in aqueous systems (12,13). This observation has led to numerous attempts to improve enzyme activity and stability in non-aqueous media, using strategies including: protein engineering (site-directed mutagenesis and directed evolution) (15–19); covalent attachment of amphipathic compounds (PEG, aldehydes and imidoesters) (12,20,21); non-covalent interaction with lipids or surfactants (12,22); entrapment in water-in-oil microemulsions or reverse micelles (23); immobilization on appropriate insoluble supports (synthetic polyhydroxylic matrices, porous inorganic carriers, polymers and molecular sieves) (24,25); and utilization of »solid« enzymes (lyophilized enzyme powders and cross-linked crystals suspended in organic solvents) (14,26).

In addition, most of the biocatalysts that have been used in non-aqueous solvent systems were employed simply because they were commercially available, and generally were not selected specifically for catalyzing reactions in non-aqueous environments. Screening of extremophiles is a novel alternative in this field that aims specifically to obtain enzymes with high stability and high reaction rates (see paragraph Homogeneous Aqueous-Organic Mixtures).

Currently, the main industrial applications of non-aqueous enzymology are based on the use of suspended (»solid«) and immobilized enzymes, both representing heterogeneous biocatalytic systems. However, heterogeneous biocatalysis in organic solvents has the important drawbacks of low reaction rates that are difficult to control and poor reproducibility at large scale, such drawbacks being detrimental to any industrial process. In homogeneous systems these problems do not occur: due to the absence of a water-organic solvent interface, diffusional limitations for substrates and products are eliminated. Also, substrate and product concentrations at the enzyme surface can be easily controlled in homogenous systems in order to manipulate the product conversion rate and to prevent enzyme inhibition.

In this review we conduct a comprehensive survey of homogeneous biocatalysis in non-aqueous systems, emphasizing recent developments in the field.

Solubility of Enzymes in Organic Solvents

Non-aqueous homogeneous biocatalysis not only has an absolute prerequisite of enzyme solubility in the organic solvent, but also requires the enzymes to be stable and to have good catalytic activity within the solvent.

Protein solubility in organic solvents is variable and depends on many parameters including the nature of the solvent, the properties of protein and the physicochemical conditions at the protein-solvent boundary. Until some years ago, the vast majority of organic solvents were considered unsuitable for proteins, due to the proteins being insoluble in them, with the exception of classical protein-dissolving organic solvents like DMSO, ethylene glycol and formamide (27). However,

solvents such as DMSO and formamide tend to render enzymes inactive. More recently it has been shown that it is possible to obtain high soluble enzyme concentrations in various neat non-aqueous solvents, with the enzymes being stable and maintaining their catalytic activity (28–35). Attempts have been made to correlate protein solubility with physicochemical solvent properties, based on studies carried out with hen egg-white lysozyme, subtilisin, and other enzymes dissolved in a wide range of organic solvents (28,29). Protein solubility showed reasonable correlation with solvent hydrophobicity ($\log P$) and in some cases also with empirical solvent polarity (E_T^N). Lysozyme was highly soluble (>10.0 mg/mL) in fourteen polar water-miscible non-aqueous solvents, including formamide, DMSO, phenol, methanol and some diols and polyols (28). Subtilisin showed high solubility (>2.0 mg/mL) in glycerol, phenol and 1,2-diaminoethanol (29). All strongly protein-dissolving solvents are very hydrophilic, polar and protic; which suggests that electrostatic and hydrogen interactions between protein and solvent are important.

Chin *et al.* demonstrated a marked dependence of the solubility of lysozyme on the pH of the buffer used prior to lyophilisation: the solubility was greater when the pH value of the buffer was far from the isoelectric point of the protein (28). This behavior was also observed in other proteins such as insulin, growth hormone, myoglobin and glucagon, which were dissolved in methanol and ethanol at high concentrations (30). The same results were described for BSA previously lyophilized at pH=9.0, which is far from the isoelectric point of pH=4.8. It had a solubility of at least 10.0 mg/mL in glycerol, ethylene glycol and other polyols (29). This fact was initially interpreted as a »pH memory« effect of the protein in organic solvent, meaning that the protein »remembered« the pH of the original protein solution before freeze-drying. Despite the fact that the pH concept can be applied only to aqueous environments, the »pH memory« phenomenon was later demonstrated to be the result of the net charge of buffer ions present in the lyophilized powder, these ions allowing the ionogenic groups of the protein to preserve their ionization states. By use of volatile buffers, which are removed during the drying process, the »pH memory« effect can be overridden (36).

Effect of Organic Solvents on the Stability and Activity of Enzymes

The three dimensional structure of folded enzymes is the result of the exquisite balance between hydrogen bonds, the hydrophobic effect, van der Waals forces and dipole interactions within the protein and between the protein and the solvent. These interactions are also influenced by boundary conditions such as the temperature and the ionic strength of the solvent. In homogeneous systems, the intimate contact of dissolved enzymes with mixtures of organic solvents can result in severe distortion of enzyme structure, rapid denaturation and inactivation. A prevailing assumption is that the hydrophobic domains that are normally buried inside the protein are exposed and dispersed when it is placed in organic solvents. In other words, the protein

unfolds, losing its native tertiary structure. However, this conjecture was challenged by the fact that 63 % of protein polar side chains and 54 % of charged side chains are hidden inside the protein molecule, and are also inaccessible to the solvent (37). Further, 53 % of the hydrophobic groups of average globular proteins are exposed at the surface of the protein (38). Also, from the energetic point of view, the contribution of peptide bonds and buried polar groups to protein stability is about 25 % higher than that of non-polar groups (39–41). Considering that protein interiors are extremely compact (42), ionic and polar interactions between inside atoms of the protein will be more favorable, and a key element for enzyme stability. In fact, it has been proposed that the catalytic sites of enzymes provide specific polar environments for the substrates, these sites being structured in such a manner as to stabilize the enzyme-substrate transition state (43).

In non-polar solvents, the main protein-solvent interactions are of a hydrophobic nature, consequently the interaction of polar groups located at the protein surface is reinforced. As a result of both driving forces, enzymes tend to aggregate, which allows destabilizing polar interactions inside the protein structure, producing enzyme inactivation. Another unfavorable effect of organic solvents is the dehydration of polar groups, which also reduces the ionic and charged form of these groups.

In anhydrous organic solvents, high enzyme stability has been observed in many cases, but with reduced activity. This phenomenon was primarily attributed to the absence of water, which supposedly places enzymes in a restrained conformation. This leads to a more rigid protein molecule; this rigidity has the double effect of increasing resistance to thermal vibrations and reducing the enzyme-substrate interaction which is associated with conformational changes during the catalytic process. Therefore the substrate conversion rate is reduced (13).

Addition of small quantities of water to anhydrous solvents can increase enzyme activity significantly (29). This »water effect« is only partially explained by the increased flexibility conferred by the presence of water at the enzyme surface (44). Electrostatic interactions between the enzyme and the substrate provide the major driving force for the enzymatic reaction. Water is initially bound at the active site and the replacement of this water by the substrate provides the free energy change necessary to promote conformational changes of the enzyme-substrate complex as it passes through the transition state (45).

Due to the stripping effect of hydrophilic organic solvents, which sequester bound water molecules from the enzyme, anhydrous hydrophobic solvents result in better systems than hydrophilic ones for enzymatic reactions under similar experimental conditions (46). However, proteins are insoluble in all hydrophobic solvents tested and form aggregates, which reduces the reproducibility of the biocatalytic system, and makes the control of the reaction rates difficult. In general, use of media with hydrophilic solvents requires more water than hydrophobic ones in order to obtain high catalytic activity. However, very high enzyme activities can be obtained in certain neat hydrophilic solvents, such as glycerol

and ethylene glycol, which are capable of forming multiple hydrogen bonds with enzyme molecules, thereby stabilizing the transition state of the reaction, and partially mimicking the effect of water (13).

The structure and activity of enzymes dissolved in organic solvents has received some attention. There is a good correlation between enzyme activity and retention of the secondary structure of the protein subtilisin Carlsberg dissolved in different organic solvents: when the α -helix and β -sheet contents in non-aqueous solvents are the same as in water, then the activity is also similar (32). However, sometimes changes in enzyme structure improve biocatalytic activity. For example, proteinase K activity was considerably enhanced when the enzyme was exposed to acetonitrile at 70 °C for 3 h. X-ray diffraction analysis revealed that the overall structure of the enzyme in acetonitrile was similar to the native structure in water. Nevertheless, the organic solvent displaced water molecules in the recognition site of the active center, where acetonitrile interacted with atoms of the protein, introducing a conformational change in the amino acid Ile 107 (47).

A correlation between solvent hydrophobicity (log P) and structure and activity of enzymes has been reported (28,29). A study carried out with lysozyme dissolved in DMSO, DMF, formamide, methanol and ethylene glycol suggested that the retention of native protein structure is favored in very hydrophilic solvents with strong hydrogen bonding propensities (35). In another study, subtilisin was dissolved in several organic solvents and solvent hydrophobicity (log P) was the only solvent parameter that exhibited some correlation with the enzymatic activity, which was only detected in hydrophilic solvents such as glycerol, ethylene glycol and 1,3-propanediol (29). These results contradict the rule proposed by Laane *et al.*, which states that hydrophilic solvents (log P < 2) are not suitable for enzymatic catalysis; however, the ionization state of the protein is not taken into account in the log P model (48). As already mentioned, there are hydrophilic solvents, such as glycerol, capable of mimicking the effect of water and thereby preserving enzyme structure (13). Owing to its properties, glycerol is one of the most studied organic solvents in homogeneous solvent mixtures. The dielectric constant of glycerol is approximately a half of that of water and is therefore not so low, compared to the natural organic solvents, as to increase the rigidity of the molecules (due to strong intra-protein interactions), nor so high as to destabilize the tertiary structure of the proteins as other organic solvents do (14,49). Motions in proteins are governed mostly by electrostatic interactions (46,47) and are particularly influenced by the dielectric constant of the solvent: with an increasing solvent dielectric constant the motions in proteins become faster (49). Therefore, glycerol can improve both the catalytic activity and the structural stability of the enzymes dissolved in it. Some of the advantageous properties observed in enzymes dissolved in glycerol are summarized in Table 1. In comparative studies, which included structurally related glycols, glycerol was the only solvent in which the secondary and tertiary structures of lysozyme were similar to those in water and also in which unfolded lysozyme could be correctly refolded

Table 1. Advantages of biocatalysis in glycerol

Catalytic and Structural Properties of Enzymes Dissolved in Glycerol	
Activity	(1) High catalytic activity of subtilisin in neat glycerol compared to other organic solvents (29,30) (2) Improvement of enzymatic activity compared to other organic solvents due to: (a) glycerol mimicking the effect of water (b) higher dielectric constant of glycerol (14,51) (3) Great increase of enzymatic activity with the increase of water content from 0 to 1 % in the reaction medium, observed in subtilisin, α -chymotrypsin, thermolysin and trypsin (29,30)
Structure and Stability	(1) Higher structural stability than in water and other organic solvents (a) correct folding of reduced and unfolded lysozyme in glycerol containing 1 % water; retention of catalytic activity in the refolded enzyme (30,31) (b) secondary and tertiary structures of lysozyme remain similar to those in water (34,35) (c) correct folding of subtilisin; reverses the lyophilization-induced perturbation of secondary structure (32) (2) Higher thermostability than in water (a) higher thermostability of subtilisin and α -chymotrypsin than in water (29,31) (b) higher thermostability of esterases in a water-glycerol mixture than in pure water (Fig. 1) (c) thermostability of tertiary structure of lysozyme identical to that in water (34) (d) higher stability of secondary structure of lysozyme than in water (33) (e) positive variation of standard free energy (ΔG) for the conversion of substrate to the product with increase in temperature for α -chymotrypsin in glycerol compared with water (33) (3) Higher stability related to pH changes than in water (a) higher stability of α -chymotrypsin at high pH than in water (33) (b) α -chymotrypsin conformation remains approximately constant in different pH (conformation changes were observed in water for different pH) (33)

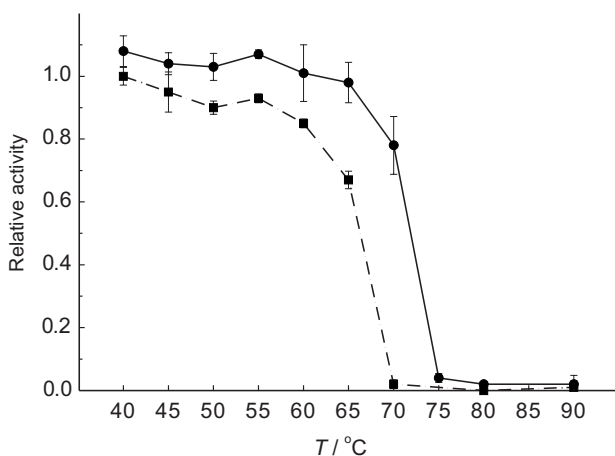


Fig. 1. Effect of temperature on thermal stability of *B. licheniformis* S-86 esterase in water (■), and water-25 % glycerol mixtures (●). The thermal stability of the enzyme was studied by incubating the reaction mixtures at pH=7 (30 mM Tris-HCl) at various temperatures for 15 min. The remaining activities are shown as values relative to those before the incubation (non-incubated sample)

(30,34,35). Also, in glycerol-water mixtures, an esterase produced by a thermoresistant and solvent-tolerant *Bacillus licheniformis* S-86 isolated in our laboratory displayed higher enzymatic activity in 25 to 50 % glycerol mixtures than those observed in aqueous buffer solutions (Table 2).

On the other hand, the main disadvantage of aqueous glycerol solutions is high viscosity, which represents a serious problem for mass transfer process, especially at room temperature. In addition, according to Kramer's

Table 2. Influence of glycerol concentration on *B. licheniformis* S-86 esterase activity

Glycerol/%	Relative activity*
0	1.00
25	1.54
50	1.36
65	0.77
80	0.73

*enzymatic reactions were carried out using 0.92 mM *p*-nitrophenyl acetate as substrate in 30 mM Tris-HCl (pH=7.0) and 37 °C for 30 min

theory, high viscosity can slow down or inhibit conformational changes during catalysis (50–52). This problem can be overcome by diluting the aqueous-organic mixture to reach 70 to 80 % of water and increasing the temperature of reaction (14,31).

The main problem of enzymatic catalysis at high temperatures is enzyme stability. In aqueous media, water promotes conformational mobility of protein molecules, deamidation of Asn and Gln residues and hydrolysis of peptide bonds, causing the unfolding of enzyme molecules and loss of enzymatic activity (49). These water-dependent deleterious reactions cannot proceed in anhydrous organic solvents and therefore, in general, enzymes are extremely thermostable in such systems. For example, the thermostability of lysozyme dissolved in neat glycerol at temperatures above 80 °C was higher than that in water (35). Trypsin and α -chymotrypsin dissolved in 99 % glycerol retained 80 % of their enzymatic activities at 100 °C after 4 and 10 h respectively,

whereas in water these enzymes were inactivated in less than 1 min (29). Note that thermal stability of enzymes in aqueous systems depends on the properties of the buffers used, suggesting that ionic interactions are vital for enzyme stability (41–43).

Interestingly, due to the presence of water, it would be expected that enzyme thermostability in aqueous-organic mixtures should not be improved. Nevertheless, improved thermal stability was observed in various water-organic mixtures, particularly in water-polyol mixtures. Thermal stabilization effects of polyols are discussed in the next section.

Homogeneous Aqueous-Organic Mixtures

In homogeneous aqueous-organic mixtures, the presence of the organic solvent changes the properties of the medium, which may cause distortion of the protein molecule core. Also, the presence of water confers on protein molecules sufficient conformational flexibility to unfold (13,53–57). Thus, enzymes that are stable in a certain neat organic solvent could denature in a mixture of the same solvent with water, or they could show reduced stability and activity than in an aqueous medium. Despite this, numerous enzymes that are stable and active in aqueous-organic mixtures have been reported and in some cases enhancement of enzyme stability and activity, compared to the values in water, was observed. Further, microbial enzymes, especially those produced by extremophiles, are often particularly stable and active in non-aqueous solvents. As an example, *Bacillus licheniformis* S-86 displays high esterase activities in 50 % homogeneous solvent mixtures of DMSO and glycols. Principally in the case of glycerol and ethylene glycol solutions, enzymatic activity was higher compared to that in water (Table 3). Furthermore, esterase activity showed a similar relationship with temperature in water and in a 25 % glycerol-water mixture and showed comparable maximum activities in the 65 to 70 °C range in both systems (58). However, there were drastic differences in the kinetics of thermal inactivation of *B. licheniformis* S-86 esterase: after 15 min at 70 °C less than 5 % activity remained in pure water, whereas in the 25 % glycerol-water mixture residual activities were 98 and >75 %, for 15 min incubation at 65 and 70 °C, respectively (Fig. 1). These results clearly indicate that the presence of

glycerol confers thermostability on the enzymes, in spite of the presence of water. A similar stabilization effect was observed for the lipase from *Bacillus* GK8 in the presence of 30 % glycerol and also in ethylene glycol-water mixtures (59), and for the xylanase produced by *Bacillus amyloliquefaciens* MIR-32, which was thermo-stabilized by the addition of polyols at concentrations lower than 40 % (60). Another esterase, produced by the *B. subtilis* MIR-16 isolate in our laboratory, exhibited a more than 2-fold higher activity in 50 % acetone than in water; while in the same concentration of methanol, ethanol, propan-2-ol and dioxan the enzyme displayed activities similar to those in aqueous medium (61).

Hydrolases have been selected in many studies because they are very stable in non-aqueous environments, this being particularly true for esterases, lipases and proteases produced by strains of *Pseudomonas* and *Bacillus*. These hydrolases are often used for resolution of racemates either by hydrolysis, esterification or transesterification of suitable precursors (61–74). In fact, some microbial hydrolases are activated in aqueous mixtures of 10 to 50 % water-miscible organic solvents. In addition, some enzymes produced by extremophiles have remarkable properties: a protease from *Thermus* strain Rt4A2 is extremely stable in 90 % of acetone, methanol, ethanol and propan-2-ol (65), while a lipase from the hyperthermophilic archaeon *Pyrobaculum calidifontis* VA1 is stable for 1 h in 80 % of DMSO, DMF, acetonitrile, methanol, ethanol and propan-2-ol (73).

In general, an increase of organic solvent content in the reaction medium results in reduction of enzyme activity. However, this was not the case for the *Pseudomonas* sp. AG-8 lipase, which was activated in 20 % of ethanol, methanol and acetone. Note that further increase in organic solvent concentration reversed the effect, leading to a sharp decline in enzyme activity. In contrast, increasing the concentration of DMSO to 90 % enhanced the activity 5.7-fold (72). As in neat organic solvents, stability and activity of enzymes in water-organic mixtures depend not only on the properties and concentration of the organic solvent, but also on the nature of the enzyme, since enzymes from different sources display different behaviors in the same organic solvent (70,75).

Systems involving mixtures of water and organic solvents are mostly used to enable the bioconversion of substrates that are moderately soluble in water, to modify the enantioselectivity of enzymes or to decrease the water content in the reaction medium in order to favor synthesis over hydrolysis (14). Moreover, intimate interactions between the organic solvent and the enzyme can lead to changes in the enzymatic reaction that is catalyzed. For example, when DMF was added to a reaction medium containing subtilisin BPN, the aminolysis reaction increased and the hydrolysis reaction was suppressed. This was associated with changes in the active site His residue caused by the presence of 50 % DMF (76). Also, the enantioselectivity of subtilisin for ethyl-2-(4-substituted phenoxy) propionates was enhanced, owing to the deformation of the enzyme that occurred in water-DMSO mixtures (77).

In addition, a germinal area of non-aqueous homogeneous biocatalysis was recently opened with the

Table 3. *B. licheniformis* S-86 esterase activity in 50 % organic-aqueous homogeneous mixtures at 37 °C

$\varphi(\text{solvent})/\%$	Relative activity*
Ethylene glycol	1.09
DMSO	0.57
Glycerol	1.36
Methanol	0.77
Propan-2-ol	0.23
Propylene glycol	0.73
Water	1.00

* enzymatic reactions were carried out using 0.92 mM *p*-nitrophenyl acetate as substrate in 30 mM Tris-HCl (pH=7.0) and 37 °C for 30 min

discovery of ionic liquids soluble in water, these ionic liquids mostly being derivatives of 1-alkyl-3-methylimidazolium tetrafluoroborate. However, some controversial results have been reported. The controversy probably arises as a result of the way that the ionic liquid is synthesized and purified, rather than being a result of the enzyme properties (78,79). More recently, Malhotra and co-workers reported the successful use of *alcalase* from *B. licheniformis* for the resolution of homophenylalanine esters using 1-ethyl-3-methylimidazolium tetrafluoroborate and *N*-ethyl pyridinium tetrafluoroborate instead of acetonitrile (80).

Conclusions

Homogeneous non-aqueous enzymology emerges as an attractive alternative to other non-aqueous systems in order to overcome some inherent drawbacks associated with heterogeneous non-aqueous biocatalysis. However, homogeneous organic systems require enzymes that are both soluble and stable in organic solvents. Several techniques have been developed to improve enzymes for non-aqueous biocatalysis, from chemical and physical approaches to site-directed mutagenesis and directed evolution. At the same time, some natural enzymes produced by extremophiles have been re-discovered recently and shown to have good solvent stability; however this area has not been exploited extensively. In addition, biocatalysis in ionic liquids is a very promising area that is still under development and probably will offer a myriad of new possibilities and challenges in non-conventional biocatalysis.

Finally, elucidation of protein structure-function relationships will allow us to determine the major mechanisms that diminish enzyme stability and activity in organic solvents, while the discovery of enzymes that are naturally stable and active in organic solvents will be fundamental for the progress of homogeneous non-aqueous enzymology.

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References

- G. Carrea, S. Riva, *Angew. Chem.-Int. Edit.* 39 (2000) 2226–2254.
- A. Schmid, J. S. Dordick, B. Hauer, A. Kiener, M. Wubolts, B. Witholt, *Nature*, 409 (2001) 258–268.
- K.-E. Jaeger, B. W. Dijkstra, M. T. Reetz, *Ann. Rev. Microbiol.* 53 (1999) 315–351.
- E. Garcia-Junceda, J. F. Garcia-Garcia, A. Bastida, A. Fernandez-Mayoralas, *Bioorg. Med. Chem.* 12 (2004) 1817–1834.
- J. S. Dordick: Principles and Applications of Non-Aqueous Enzymology. In: *Applied Biocatalysis*, J. S. Dordick (Ed.), Marcel Dekker Inc., New York (1991) pp. 1–53.
- J. S. Dordick, *Biotechnol. Progr.* 8 (1992) 259–267.
- J. S. Dordick, Y. L. Khmelnsky, M. V. Sergeeva, *Curr. Opin. Microbiol.* 1 (1998) 311–318.
- M. Tuena de Gomez-Puyou, A. Gomez-Puyou, *Crit. Rev. Biochem. Mol. Biol.* 33 (1998) 53–89.
- J. A. Akkara, M. S. R. Ayyagari, F. F. Bruno, *Tibtech*, 17 (1999) 67–73.
- Y. L. Khmelnsky, J. O. Rich, *Curr. Opin. Chem. Biol.* 3 (1999) 47–53.
- P. J. Halling, *Curr. Opin. Chem. Biol.* 4 (2000) 74–80.
- H. Ogino, H. Ishikawa, *J. Biosci. Bioeng.* 91 (2001) 109–116.
- M. T. Ru, J. S. Dordick, J. A. Reimer, D. S. Clark, *Biotechnol. Bioeng.* 63 (1999) 233–241.
- G. R. Castro, T. Knubovets, *Crit. Rev. Biotechnol.* 23 (2003) 195–231.
- F. H. Arnold, *Chem. Eng. Sci.* 51 (1996) 5091–5102.
- F. H. Arnold, *Adv. Biochem. Eng. Biotechnol.* 58 (1997) 1–14.
- F. H. Arnold, *Nature*, 409 (2001) 253–257.
- S. H. Krishna, *Biotechnol. Adv.* 20 (2002) 239–267.
- L. M. Pera, M. D. Baigori, G. R. Castro: Hybrid Enzymes. In: *Enzyme Technology*, A. Pandey, C. Webb, C. R. Soccol, C. Larroche (Eds.), Asiatech Publisher Inc. (2004) pp. 652–671.
- G. DeSantis, J. B. Jones, *Curr. Opin. Biotechnol.* 10 (1999) 324–330.
- A. B. Salleh, M. Basri, M. Taib, H. Jasmani, R. N. Rahman, M. B. Rahman, C. N. Razak, *Appl. Biochem. Biotechnol.* 102 (2002) 349–357.
- N. Kamiya, M. Inoue, M. Goto, F. Nakashio, *Biotechnol. Progr.* 16 (2000) 52–58.
- L. M. Pera, M. D. Baigori, G. R. Castro, *Indian J. Biotechnol.* 2 (2003) 356–361.
- M. Persson, E. Wehtje, P. Adlercreutz, *Chembiochem*, 3 (2002) 566–571.
- A. X. Yan, X. W. Li, Y. H. Ye, *Appl. Biochem. Biotech.* 101 (2002) 113–129.
- V. P. Torchilin, K. Martinek, *Enzyme Microb. Technol.* 1 (1979) 74–82.
- S. J. Singer, *Adv. Protein Chem.* 17 (1962) 1–68.
- J. T. Chin, S. L. Wheeler, A. M. Klivanov, *Biotechnol. Bioeng.* 44 (1994) 140–145.
- G. R. Castro, *Enzyme Microb. Technol.* 25 (1999) 689–694.
- L. E. Bromberg, A. M. Klivanov, *Proc. Natl. Acad. Sci.* 92 (1995) 1262–1266.
- R. V. Rariy, A. M. Klivanov, *Biotechnol. Bioeng.* 62 (1999) 704–710.
- K. Xu, K. Griebenow, A. M. Klivanov, *Biotechnol. Bioeng.* 56 (1997) 485–491.
- G. R. Castro, *Enzyme Microb. Technol.* 27 (2000) 143–150.
- T. Knubovets, J. J. Osterhout, P. J. Connolly, A. M. Klivanov, *Proc. Natl. Acad. Sci. USA*, 96 (1999) 1262–1267.
- T. Knubovets, J. J. Osterhout, A. M. Klivanov, *Biotechnol. Bioeng.* 63 (1999) 242–248.
- E. Zacharis, P. J. Halling, D. G. Rees, *Proc. Natl. Acad. Sci. USA*, 96 (1999) 1201–1205.
- G. J. Lesser, G. D. Rose, *Proteins*, 8 (1990) 6–13.
- T. V. Chalikian, M. Trovov, R. Abaygan, K. J. Breslauer, *J. Mol. Biol.* 260 (1996) 588–603.
- B. A. Shirley, P. Stanssens, U. Hahn, C. N. Pace, *Biochemistry*, 31 (1992) 725–732.
- Y. Harpaz, M. Gerstein, C. Clothia, *Structure*, 2 (1994) 641–649.
- C. N. Pace, *Biochemistry*, 40 (2001) 310–313.
- M. H. Klapper, *Biochim. Biophys. Acta*, 229 (1971) 557–566.
- A. Washel, *J. Biol. Chem.* 273 (1998) 27035–27038.
- A. Shurki, M. Strajbl, J. Villa, A. Warshel, *J. Am. Chem. Soc.* 124 (2002) 4097–4107.
- T. Anthonsen, B. J. Sjursnes: Importance of Water Activity for Enzyme Catalysis in Non-Aqueous Organic Systems.

- In: *Methods in Non-Aqueous Enzymology*, M. N. Gupta (Ed.), Birkhäuser Verlag, Basel (2000) pp. 14–35.
46. L. Gorman, J. Dordick, *Biotechnol. Bioeng.* 39 (1992) 392–397.
47. S. Sharma, R. Tyagi, M. N. Gupta, T. P. Singh, *Indian J. Biochem. Biophys.* 38 (2001) 34–41.
48. C. Laane, S. Boeren, K. Vos, C. Veeger, *Biotechnol. Bioeng.* 30 (1987) 81–87.
49. R. Affleck, C. A. Haynes, D. S. Clark, *Proc. Natl. Acad. Sci. USA*, 89 (1992) 5167–5170.
50. H. A. Kramer, *Physica*, 7 (1940) 284–304.
51. M. Jacob, F. X. Schmid, *Biochemistry*, 38 (1999) 13773–13779.
52. S. Uribe, J. G. Sampedro, *Biol. Proced. Online*, 5 (2003) 108–115.
53. V. V. Mozhaev, K. G. Poltevisky, V. I. Slepnev, G. A. Badun, A. V. Levashov, *FEBS Lett.* 292 (1991) 159–161.
54. C. A. Schiffer, V. Dötsh, *Curr. Opin. Biotechnol.* 7 (1996) 428–432.
55. G. L. Makhatadze, P. L. Privalov, *Protein Sci.* 5 (1996) 507–510.
56. M. N. Gupta, *Eur. J. Biochem.* 203 (1992) 25–32.
57. K. Gekko, S. N. Timasheff, *Biochemistry*, 20 (1981) 4667–4676.
58. S. Torres, G. R. Castro: Organic Solvent Resistant Lipase Produced by Thermoresistant Bacteria. In: *New Horizons in Biotechnology*, S. Roussos, C. R. Soccol, A. Pandey, C. Augur (Eds.), Kluwer Academic Publishers (2003) pp. 113–122.
59. N. S. Dosanjh, J. Kaur, *Biotechnol. Appl. Biochem.* 36 (2002) 7–12.
60. J. D. Breccia, A. C. Moran, G. R. Castro, F. Siñeriz, *J. Chem. Technol. Biotechnol.* 71 (1998) 241–245.
61. M. D. Baigori, G. R. Castro, F. Siñeriz, *Biotechnol. Appl. Biochem.* 24 (1996) 7–11.
62. A. Sugihara, M. Ueshima, Y. Shimada, S. Tsunasawa, Y. Tominaga, *J. Biochem.* 112 (1992) 598–603.
63. Y. Shabtal, N. Daya-Mishne, *Appl. Environ. Microbiol.* 58 (1992) 174–180.
64. Y. Shimada, C. Koga, A. Sugihara, T. Nagao, N. Takada, S. Tsunasawa, Y. Tominaga, *J. Ferment. Bioeng.* 75 (1993) 349–352.
65. S. A. Freeman, K. Reek, M. Prescott, R. Daniel, *Biochem. J.* 295 (1993) 463–469.
66. H. Ogino, K. Miyamoto, H. Ishikawa, *Appl. Environ. Microbiol.* 60 (1994) 3884–3886.
67. H. Ogino, K. Yasui, T. Shiotani, T. Ishihara, H. Ishikawa, *Appl. Environ. Microbiol.* 61 (1995) 4258–4262.
68. D. W. Choo, T. Kurihara, T. Suzuki, K. Soda, N. Esaki, *Appl. Environ. Microbiol.* 64 (1998) 486–491.
69. H. Ogino, K. Miyamoto, M. Yasuda, K. Ishimi, H. Ishikawa, *Biochem. Eng. J.* 4 (1999) 1–6.
70. H. Ogino, F. Watanabe, M. Yamada, S. Nakagawa, T. Hirose, A. Noguchi, M. Yasuda, H. Ishikawa, *J. Biosci. Bioeng.* 87 (1999) 61–68.
71. H. Ogino, S. Nakagawa, K. Shinya, T. Muto, N. Fujimura, M. Yasuda, H. Ishikawa, *J. Biosci. Bioeng.* 89 (2000) 451–457.
72. A. K. Sharma, R. P. Tiwari, G. S. Hoondal, *J. Basic Microbiol.* 41 (2001) 363–366.
73. Y. Hotta, S. Ezaki, H. Atomi, T. Imanaka, *Appl. Environ. Microbiol.* 68 (2002) 3925–393.
74. N. S. Dosanjh, J. Kaur, *Prot. Expr. Purif.* 24 (2002) 71–75.
75. M. N. Gupta, R. Batra, R. Tyagi, A. Sharma, *Biotechnol. Progr.* 13 (1997) 284–288.
76. R. D. Kidd, P. Sears, D. H. Huang, K. Witte, C. H. Wong, G. K. Farber, *Protein Sci.* 8 (1999) 410–417.
77. K. Watanabe, S. Ueji, *Biotechnol. Lett.* 22 (2000) 599–603.
78. T. L. Husum, C. T. Jorgensen, M. W. Christensen, O. Kirk, *Biocatal. Biotransform.* 19 (2001) 331–338.
79. N. Kaftzik, P. Wasserscheid, U. Kragl, *Org. Process Res. Dev.* 6 (2002) 553–557.
80. H. Zhao, R. G. Luo, S. V. Malhotra, *Biotechnol. Progr.* 19 (2003) 1016–1018.

Nevodena biokataliza u homogenim sustavima otapala

Sažetak

Enzimi su visokospecifični katalizatori koji uobičajeno djeluju u vodenim otapalima. Međutim, mnogi enzimi zadržavaju svoju katalitičku aktivnost pri velikim koncentracijama u nevodenom okolišu, obuhvaćajući čisto hidrofilna organska otapala. Zapravo se mogu koristiti za provođenje reakcija u organskim otapalima koje nisu moguće u vodenim sustavima. Primjenjujući biokatalizu u homogenim nevodenim otapalima omogućena je proizvodnja korisnih kemikalija i provedba nekih sintetskih reakcija. U radu su razmatrani faktori koji utječu na enzimsku katalizu u nevodenim otapalima, kao što su udjel vode, koncentracija otapala i njegova interakcija s proteinskom strukturom, te utjecaj otapala na stabilnost i aktivnost proteina. Ujedno su spomenuti novi pristupi za nekonvencionalnu biokatalizu koristeći ekstremofile i ionska otapala.