

## Enzyme Behaviour in Non-conventional Systems

Licia M Pera<sup>1</sup>, Mario D Baigori<sup>1</sup> and Guillermo R Castro<sup>2\*</sup>

<sup>1</sup> PROIMI, Av. Belgrano y Pasaje Caseros. 4000 MTV Tucumán, Argentina

<sup>2</sup> Department of Biomedical Engineering, Tufts University. 4 Colby Street, Medford, MA 02155, U S A

*Received 16 November 2002; accepted 21 February 2003*

**Most important recent advances in biocatalytic biphasic systems such as aqueous two-phase reactor systems using environmental sensitive polymers, two-phase aqueous-organic systems, reverse micelles, microemulsion-based organogels, and trapped aqueous-organic solvent continuous biphasic reactors are reviewed.**

**Keywords: heterogeneous biphasic systems; non-conventional media; aqueous two-phase systems; partitioning; aqueous-organic two-phase systems; trapped Aqueous-organic biphasic system; reverse micelles; W/O microemulsions; microemulsion-based gels; extraction systems**

### Introduction

Since last decade, biotransformations are well-know alternative avenues to conventional chemistry for the production of fine chemicals and active intermediate compounds of industrial interest. In particular, synthesis of chiral molecules, enantiomers, and single isomers in pharmaceutical industries produced by enzymes is becoming increasingly important (McCoy, 2001). In addition, biocatalysis techniques are environmentally friendly, and allow the development of sustainable production processes on large scale. However, from the synthesis point of view, important disadvantages of enzymatic processes at industrial scale are: poor operational stability, poor volumetric productivity, and also side reactions produced by impurities (Thayer, 2002).

Most of commercial biotransformations are performed in monophasic aqueous media using hydrophobic reagents, e.g. low-water solubility, which can lead to low volumetric productivities and poor enzyme-stability in mono-phase aqueous systems. New techniques to improve biocatalytic performance under unfavourable experimental conditions, which involves solvent engineering and multiphase systems, have been recently reported. Alternatively, non-aqueous homogenous biocatalysis received in the last five years considerable attention, but it is out of the scope of this review.

In the present review, some of the most relevant research works done in biphasic biocatalytic systems have been summarized. The system models include recent advances on aqueous two-phase reactor systems, two-phase aqueous-organic systems, water-in-oil microemulsions or reverse micelles, microemulsion-based organogels, and trapped aqueous-organic solvent continuous biphasic reactor.

### Aqueous Two-phase Systems (ATPS)

ATPS have been showing many advantages in biotechnology field (Hatti-Kaul, 2001). In the case of aqueous systems, the second immiscible phase posses reduced water activity and also ionic interactions between the molecules and solvent are reduced.

Novel ATPS have been developed base on a wide variety of stimuli-responsive polymers, i.e. polymers capable of reversible phase transition in response to small changes in the environment, such as changes in pH or temperature (Johansson *et al*, 1999; Pietruszka *et al*, 2000). Examples of these systems are ethylene oxide-propylene oxide random copolymers (PEO-PPO), which are linear and non-ionic compounds. The water solubility of PEO-

**Short running title:** PERA *et al*: ENZYME BEHAVIOUR IN NON-CONVENTIONAL SYSTEMS

-----  
\*Author for correspondence:

Tel: 617-627.4320

Fax: 617-627.3231

PPO shows temperature dependence. When the copolymer is heated above the lower critical solution temperature, it can be separated from the aqueous solution. The temperature at which this phenomenon occurs is known as the cloud point (CP) of the polymer. The CP can be lowered either by increasing the PO/EO ratio or by increasing the amount of sodium sulphate concentration in the aqueous phase. After ATPS protein isolation, a protein-free PEO-PPO copolymer is produced, which can be recycled to the primary phase system for a new extraction (Persson *et al*, 2000). One of the major advantages of these systems is that it can be lowered by the salt concentration in the media, avoiding the risks of protein aggregation, precipitation, and also salting out. In addition, the target protein can be recovered in an aqueous solution after PEO-PPO copolymer thermo-separation. Another important advantage at industrial level is due to the polymer recycling process of the system, with a substantial reduction of economic costs. A very good example of an extractive bioconversion of starch to glucose is to use  $\alpha$ -amylase and amyloglucosidase (Li *et al*, 2002, a, b). It is very well known that the enzymatic process is usually inhibited by glucose. The presence of PEO-PPO-2500 (molecular weight of 2,500)/  $\text{MgSO}_4$  enhance the enzymatic starch hydrolysis reaction by lowering the glucose concentration in the aqueous phase. Both  $\alpha$ -amylase and amyloglucosidase are strongly maintained in the bottom salt phase, starch is completely partitioned into the bottom phase meanwhile glucose is partitioned to the top phase. This system reduces the hydrolysis time to almost half of that for single-phase processing and shows promise for enzymatic reactions process where the product severely inhibits the enzyme (Li *et al*, 2002b). It is also interesting to use a stimuli-responsive polymer as a modifier of the top phase of an ATPS in order to increase the protein-refolding yield. Recently, it was reported that the addition of PPO-Ph-PEG (Polyethylene glycol) in PEG/dextran at 52°C, improved the bovine carbonic anhydrase refolding yield (1.7 times) and suppressed aggregate formation during refolding process (Kuboi *et al*, 2000).

In addition, the concept of two-phase partitioning bioreactor can also be applied to separation, purification and concentration of biocatalysts to improve process. The partitioning of endo-polygalacturonase (endo-PG) and total protein from a clarified fermentation broth in polyethylene glycol (PEG)-polyvinyl alcohol (PVA 10000) and PEG hydroxypropyl starch (Reppal PES 100) aqueous two-phase systems are clear examples of the high versatility of the biphasic systems (Wu *et al*, 2001). Endo-PG can be concentrated in the top phase of PEG 8,000-PVA10,000 system with a partition coefficient up to 6, while the total protein still dominates in the bottom phase. The separation scheme consisting of a first extraction in PEG 8,000-PVA 10,000 system, followed by a second extraction in PEG 8,000- $(\text{NH}_4)_2\text{SO}_4$  system. This allowed the separation of endo-PG from polymer and the recycling of PEG polymer, since endo-PG was very strongly partitioning into the bottom phase of the PEG 8,000- $(\text{NH}_4)_2\text{SO}_4$  system. The authors reported an enzyme recovery higher than 91% with a total purification factor of about 1.9 and a concentration factor of more than 5 (Wu *et al*, 2001). These results show that aqueous two-phase extraction methods can be a powerful tool for downstream processing of endo-PG starting from its crude fermentation broth.

Another interesting application of two-phase aqueous system is the detection of enzyme complexes and intermediates formation. A high rate cellular pathway requires very close interactions between sequential enzymes involved in the metabolic route. In this case, active sites of two sequential enzymes must be close to each other to produce and channelling intermediates molecules. An enzyme-enzyme complex formation would have several biological advantages for the living cell such as regulation of metabolism, direct transfer of metabolites between enzymes and shielding metabolites from the aqueous cytosol. Recently, a complex formation between pure phosphofructokinase and aldolase with different partitioning in dextran-polyethylene glycol two-phase systems compared to that of either enzyme has been reported. Complex formation took place in the presence and absence of AMP, but presence of AMP was obligatory to get enhanced activities of individual enzyme (Matic *et al*, 2001).

Collén *et al* (2002) recently demonstrated how partitioning of a hydrophilic enzyme can be directed to hydrophobic detergent-enriched phase of an aqueous two-phase system by addition of short stretches of amino acid residues to the protein molecule. The target enzyme was the industrially important endoglucanase I (EGI) of *Trichoderma reesei*. The authors investigated the partitioning of three EGI variants containing various C-terminal peptide extensions including Trp-Pro motifs of different lengths and localizations. Additionally, the thermo-separating copolymer, HM-EOPO was utilized to study the effect of fusion tags. Fusion of the peptide  $(\text{WP})_4$  to EGI resulted in an improvement of partitioning to the micelle-enriched phase in both aqueous two-phase system studied, HM-EOPO (random copolymer of ethylene oxide and propylene oxide chains, which flank an isophoronediisocyanate group in the center of the molecule)/water and Agrimul NRE 1205 (consists mainly of  $\text{C}_{12}\text{E}_5$ )/water. The strongest effect was obtained in the Agrimul NRE 1205/water system, in which 90% of the fusion protein  $\text{EGI}_{\text{core-P5}}(\text{WP})_4$  partitioned to the detergent-enriched phase (Collén *et al*, 2002).

## Two-phase Aqueous-Organic Systems

Two-phase aqueous-organic system increases the productivity of the conversion of sparingly aqueous soluble substrates. Baldascini *et al* (2001) showed that the kinetic resolution of racemic epoxides by an enantioselective epoxide hydrolase can be carried out in an n-octane/aqueous two-phase system to overcome the problem of low epoxide aqueous solubility. Epoxides are important intermediates for the production of a wide range of fine-chemical and pharmaceutical products. Biological activity of these products often lies with only one enantiomer and the potential toxic side effect of the other enantiomer have made their production in enantiomerically pure form increasingly important (Crosby, 1992). The resolution of a 39 g/l solution of racemic styrene oxide in octane was successfully carried out in an emulsion batch reactor to obtain (S)-styrene oxide in high enantiomeric excess (>95% e.e.) with a yield of 30% (Baldascini *et al*, 2001).

Another very interesting system to stabilize and enhance biocatalytic activities is by enzyme coating. A lipid-coated lipase was reported as an efficient catalyst for hydrolysis of lipophilic esters in the two-phase aqueous-organic systems (Mori *et al*, 2001). The coating lipid produces a lipophilic environment surrounded the enzyme, but did not change the substrate selectivity of the enzyme. Both the lipid-coated lipase and substrates are solubilized in the organic phase and the hydrolysis occurs with water molecules from the aqueous phase. Therefore, the reaction was 40-100 times faster compared with the traditional “native lipase system”, in which the enzyme and substrate exist separately in the aqueous and organic phase, respectively, and the reaction proceeded at the interphase. Also, another advantage of the system is that hydrolysis rates for the lipid-coated lipase were not affected by the aqueous pH and agitation speed of the two-phases. Considering the extensive use of lipases at industrial level, the coated-lipase system could be a very important tool for hydrolysis of lipophilic esters in the two-phase system, including the practical hydrolysis of viscous triglyceride (olive oil) emulsion.

Recently, the aqueous-organic biphasic system concept was applied to peptide synthesis catalysed by a new cysteine-type protease (Barberis *et al*, 2002). Morrenain b II, isolated from the latex of a South American climbing plant, *Morrenia brachystephana* Griseb (*Asclepiadaceae*) was found to be stable in aqueous-organic biphasic systems. In the report, the authors investigated the ability of morrenain b II to perform peptide synthesis using Phe.Ome and Asp as substrates, Cys as an activator in 100 mM Tris-HCl buffer (pH 8.5) and chloroform as reaction media. Morrenain b II showed high specificity towards bonds between Cys and Phe.Ome amino acids. A significant amount of the cysteine-Phe.Ome peptide was produced within 1 hour (Barberis *et al*. 2002).

## Water-in-oil Microemulsion or Reverse Micelles and Microemulsion-based Organogels

It is well established that many enzymes can be entrapped in water-in-oil (W/O) microemulsions or reverse micelles, retaining their catalytic activity. The main advantages of this system are the possibility to provide an adequate environment to the enzyme, and therefore protect it against denaturation by the organic solvent. Additionally, microemulsions can provide an extremely large interfacial area between the oil-continuous phase and the dispersed aqueous phase (approximately  $100\text{m}^2\text{ ml}^{-1}$ ). The water-insoluble compound is solubilized by the surface layer of the microemulsions and therefore can come into contact with the entrapped enzyme. Interestingly, many W/O microemulsions can be “gelled” by the addition of aqueous gelatin, yielding a matrix suitable for enzyme immobilization. Gelatin-containing microemulsion-based organogels (MBGs) were first described in 1986 (Haering & Luisi), and their physical-structural characterization has since been the subject of a number of studies (Luisi *et al*, 1990; Quellet *et al*, 1991). It is proposed that the MBGs comprise an extensive, rigid, interconnected network of gelatin/water rods stabilized by a monolayer of surfactant, in coexistence with a population of “conventional” W/O microemulsion droplets. The MBGs are stable in contact with apolar solvents, and fully retained the surfactant, gelatin, and water components in most conditions. They could be used as a solid-phase biocatalyst in the presence of organic solvent or liquid substrate. The MBGs offer considerable advantages over W/O microemulsion, such as higher enzyme stabilities, product isolation and facilitates reuse.

This type of system was used to study the kinetic behaviour for the esterification of octanoic acid with 1-octanol, catalysed by *Candida lipolytica* (CL) lipase in water/ bis-(2-ethylhexyl) sulfosuccinate sodium (AOT)/isooctane (Zhou *et al*, 2001). The reaction follows a Ping-Pong BI BI mechanism with inhibition by excess of 1-octanol. The values of apparent kinetic parameters were:  $V_{\text{max}} = 4.7 \times 10^{-3}\text{ mmol l}^{-1}\text{ min}^{-1}\text{ mg}^{-1}$ ,  $K_{\text{m acid}} = 49.3\text{ mmol l}^{-1}$ , and  $K_{\text{m alcohol}} = 47.6\text{ mmol l}^{-1}$ , respectively. CL lipase was also immobilized in gelatin-containing AOT microemulsion-based organogels with retention of catalytic activity. These lipase-containing MBGs proved to be novel solid-phase catalysts for use in apolar organic solvent.

However, denaturation or deactivation of protein and enzymes due to the hydrophobic and electrostatic interactions with AOT is a severe problem in the practical application of reverse micelle. In addition to the use of bile salt described recently (Yang *et al*, 2001), one promising approach to overcome this problem is to reduce the

surface charge density by modifying the reverse micellar interface via the addition of non-ionic surfactant (Adachi *et al*, 1999). In general, it was found that the stabilities of enzyme in reverse micelles could be significantly improved by this approach (Yang *et al*, 2001). Alcohol dehydrogenase, which catalyses the oxidation of alcohols and the reduction of carbonyl compounds, such as aldehydes and ketones has received considerable attention because of its potential applications in the production of various starting materials and intermediates in chemical industry, the synthesis of chiral compounds, biosensors, and the regeneration of coenzymes NAD(P) and NAD(P)H. Some studies of circular dichroism (CD) and electron paramagnetic resonance (EPR) showed that the conformation of horse liver alcohol dehydrogenase (HLADH) in AOT reverse micelles was altered and suggested that AOT molecules might interact with HLADH via adsorption and active-site contamination (Creagh *et al*, 1993). Chen *et al* (2002), studied the effects of polyoxyethylene lauryl ether (Brij 30) concentration on the stabilities and activities of yeast alcohol dehydrogenase (YADH) at various  $\omega_o$  values. The stability of YADH in AOT/Brij 30 mixed reverse micelles were tested. In general, the residual activity of YADH decreased rapidly first, and then approached to a steady state value. At  $\omega_o = 10$ , the stability of YADH was enhanced with the increase of Brij30 concentration as theoretically expected. However, at  $\omega_o = 20$ , almost no significant improvement was observed and the stabilities under some conditions were even lower than that in the absence of Brij 30. This phenomenon was quite different from those reported previously for other enzymes such as lipase and  $\alpha$ -chymotrypsin (Yang *et al*, 2001), and probably it can be interpreted on the basis of different enzyme structures. By the investigation on the hydrodynamic diameter (HD) of mixed reverse micelles and its distribution, four important factors affecting the structure of mixed reversed micelles and the stability of YADH could be clarified. The properties are surface charge density, bound water, reverse micellar size, and entrapment of water by hydrophilic-hydrophilic interaction of AOT and Brij 30. A value decrease of these factors would favour the stability of YADH, although they were opposite under some conditions. Furthermore, it was found that the activity of YADH in AOT/Brij 30 mixed reverse micelles was affected by the hydrophobic and electrostatic interactions between enzyme and surfactants, the reverse micellar size, and the bound degree of water molecules. Except an optimal reverse micellar size was needed, the activity of YADH might be enhanced by the reduction of the other two factors.

### **Trapped Aqueous-organic Solvent Biphasic Continuous Reactor**

According to the pioneering work of Peter Halling in this field, when the aqueous-organic biphasic system has such low proportions of aqueous phase that they are essentially restricted to the pores or interior of solid particles, then the term *trapped aqueous-organic biphasic* system was used to distinguish the emulsion aqueous-organic biphasic system (Halling, 1987). Xin *et al* (2000) presented a process that consists of a stereoselective hydrolysis of the racemic Naproxen methyl ester by *Candida rugosa* lipase in a trapped aqueous-organic biphasic system. (S)-(+)-2-(6-Methoxy-2-naphthyl) propionic acid (Naproxen) is a nonsteroidal anti-inflammatory drug that belongs to the family of 2-aryl propionic acid derivatives, it is widely used as a drug for human connective tissue diseases. The physiological activity of the *S* form of Naproxen is 28-folds that of the *R* form (Margolin, 1993). Hence, only the *S* form is used as a drug for humans. The reaction has been carried out in a laboratory-scale continuous-flow stirred tank reactor (CSTR). The starting material was added, remaining substrate recovered from the organic phase. To increase the activity and stability of lipase and to prevent the formation of an emulsion in aqueous-organic biphasic system, a YWG-C6H5 support was used to immobilize the lipase and to restrict the aqueous phase to its pore or in interior. YWG-C6H5 is poorly polar, high specific surface (100 m<sup>2</sup>/g), amorphous multi-porous silica on which the -CH<sub>2</sub>CH<sub>2</sub>C<sub>6</sub>H<sub>5</sub> groups have been bounded. In order to overcome product inhibition and to facilitate product recovery, a dialysis membrane tube containing a continuous-flow closed loop phosphate buffer solution was applied in the CSTR. Such a system overcomes the problems that agitation conditions may affect the interfacial area between the two phases and emulsion formation in an aqueous-organic biphasic system. Because the aqueous part consumed by the hydrolysis may be recruited from the dialysis membrane tube, the thermodynamic reaction equilibrium is favourable. The reaction system offers several advantages for industrial application. First, because the high specific surface support at which the lipase was immobilized and the aqueous phase was trapped offers a high lipid-aqueous interface, under mild agitation, the support may be suspended and the highest reaction rate may be obtained. Secondly, because the aqueous phase was restricted to the pores or in interior of support, the problem of emulsion formation may be overcome. Thirdly, because the products were extracted by the continuous-flow closed-loop phosphate buffer solution contained in dialysis membrane tube, the product inhibition effecting the activity and stability may be overcome and the product may be easily separated. At steady-state operating conditions, an initial conversion of 35% has been obtained. The CSTR was allowed to operate continuously for 60 days at 30°C with a 30% loss of activity. The hydrolysis reaction yielded (S)-(+)-Naproxen with >90% enantiomeric excess and overall conversion of 30%.

In conclusion, traditional two-phase systems combined with recent discoveries of environmental sensitive polymers, reverse micelles and diverse organic solvents open a myriad of new possibilities in basic and applied biocatalysis.

### Acknowledgement

The authors gratefully acknowledge the financial support from CABBIO (2000, # 0012), CONICET (Argentina), and Prof. David L. Kaplan (Tufts University, USA).

### References

- Adachi M *et al*, 1999. Selective separation of trypsin from pancreatin using bioaffinity in reverse micellar system composed of a nonionic surfactant. *Biotechnol Bioeng*, **58**, 649-653.
- Baberis S *et al*, 2002. Peptide synthesis in aqueous-organic biphasic systems catalyzed by a protease isolated from *Morrenia brachystephana* (Asclepiadaceae) *J Mol Catal B: Enzymatic*, **17**, 39-47.
- Baldascini H *et al*, 2001. Effect of mass transfer limitations on the enzymatic kinetic resolution of epoxides in a two-liquid-phase system. *Biotechnol Bioeng*, **73**, 45-54.
- Chen D & Liao M, 2002. Effects of mixed reverse micellar structure on stability and activity of yeast alcohol dehydrogenase. *J Mol Catal B: Enzymatic*, **18**, 155-162.
- Collèn A *et al*, 2002. Primary recovery of a genetically engineered *Trichoderma reesei* endoglucanase I (Cel 7B) fusion protein in cloud point extraction systems. *Biotechnol Bioeng*, **78**, 385-394.
- Creagh M *et al*, 1993. Structural and catalytic properties of enzymes in reverse micelles. *Enzyme Microb Technol*, **15**, 383-92.
- Crosby J, 1992. Chirality in industry-An overview. *in* Chirality in industry, edited by A N Colling, G N Shelldrake & J Crosby. Chichester. John Wiley & Sons, U K. Pp 1-66.
- Haering G & Luisi P, 1986. Hydrocarbon gels from Water-in-Oil microemulsions. *J Phys Chem*, **90**, 5892-5898.
- Halling P, 1987. Biocatalysis in multi-phase reaction mixtures containing organic liquids. *Biotechnol Adv*, **5**, 47-84.
- Hatti-Kaul R, 2001. Aqueous two-phase systems. A general overview. *Mol Biotechnol*, **19**, 269-277.
- Johansson H *et al*, 1999. Thermo-separating water/polymer system: A novel one-polymer aqueous two-phase system for protein purification. *Biotechnol Bioeng*, **66**, 247-257.
- Kuboi R *et al*, 2000. Protein refolding using stimuli-responsive polymer-modified aqueous two-phase systems. *J Chromatogr B*, **743**, 215-223.
- Li M *et al*, 2002a. Amylase partitioning and extractive bioconversion of starch using thermoseparating aqueous two-phase systems. *J Biotechnol*, **93**, 15-26.
- Li M *et al*, 2002b. Kinetic enhancement of starch bioconversion in thermoseparating aqueous two-phase reactor systems. *Biochem Eng J*, **11**, 25-32.
- Luisi P *et al*, 1990. Organogels from water-in-oil microemulsions. *Colloids & Polym Sci*, **268**, 356-374.
- Margolin A, 1993. Enzymes in the synthesis of chiral drugs. *Enzyme Microb Technol*, **15**, 266-279.
- Matic S *et al*, 2001. Interaction between phosphofructokinase and aldolase from *Saccharomyces cerevisiae* studied by aqueous two-phase partitioning. *J Chromatogr B*, **751**, 341-348.
- McCoy M, 2001. Making drugs with little bugs. *Chem Eng News*, **79**, 37-41.

- Mori T *et al*, 2001. A lipid-coated lipase as an efficient hydrolytic catalyst in the Two-phase aqueous-organic system. *Biotechnol Bioeng*, **76**, 157-163.
- Persson J *et al*, 2000. Polymer recycling in aqueous two-phase extractions using thermoseparating ethylene oxide-polypropylene oxide copolymers. *J Chromatogr B*, **743**, 115-126.
- Pietruszka N *et al*, 2000. New polymers forming aqueous two-phase polymer systems. *Biotechnol Prog*, **16**, 408-415.
- Quellet W *et al*, 1991. Formation of microemulsion-based gelatin gels. *J Phys Chem*, **95**, 5642-5655.
- Thayer A M, 2002. Biocatalysis. *Chem Eng News*, **79**, 27-34.
- Wu Y *et al*, 2001. Separation of endo-polygalacturonase using aqueous two-phase partitioning. *J Chromatogr A*, **929**, 23-29.
- Xin J *et al*, 2000. Enzymatic resolution of (S)-(+)- Naproxen in a trapped aqueous-organic solvent biphasic continuous reactor. *Biotechnol Bioeng*, **68**, 78-83.
- Yang H *et al*, 2001. Effects of bile salts on the solubility, activity of yeast alcohol dehydrogenase in AOT reversed micelles. *J Mol Catal B: Enzymatic*, **14**, 7-14.
- Zhou G *et al*, 2001. Kinetic studies of lipase-catalyzed esterification in water-in-oil microemulsions and the catalytic behaviour of immobilized lipase in MBGs. *Colloids & Surf A: Phys-chem Eng*, **194**, 41-47.