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Review

Oxidation Catalysis by Enzymes in Microemulsions

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Abstract: Microemulsions are regarded as "the ultimate enzyme microreactors" for liquid oxidations. Their structure, composed of water nanodroplets dispersed in a non-polar medium, provides several benefits for their use as media for enzymatic transformations. They have the ability to overcome the solubility limitations of hydrophobic substrates, enhance the enzymatic activity (superactivity phenomenon) and stability, while providing an interface for surface-active enzymes. Of particular interest is the use of such systems to study biotransformations catalyzed by oxidative enzymes. Nanodispersed biocatalytic media are perfect hosts for liquid oxidation reactions catalyzed by many enzymes such as heme peroxidases, phenoloxidases, cholesterol oxidase, and dehydrogenases. The system's composition and structural properties are important for better understanding of nanodispersion-biocatalyst interactions.

Keywords: biocatalysis; detergentless microemulsions; liquid oxidations; heme peroxidases; phenoloxidases; cholesterol oxidase; dehydrogenases

1. Introduction

Enzymes are macromolecular biological catalysts evolved in nature to catalyze and accelerate chemical reactions necessary to develop and maintain life. Biocatalysis can be defined as the use of enzymes as biocatalysts for industrial synthetic chemistry, under controlled conditions in a bioreactor. Enzyme catalysis has several advantages over chemical catalysis. Enzymes are active under mild reaction conditions (low temperature, pressure, and pH values), more efficient (lower concentration of enzyme needed) and have high specificity which indicates minimum or no production of by-products. As a result, the use of enzymes reduces the energy requirements for an industrial scale process [1]. The use of enzymes has been applied in oxidation reactions, among others. Biocatalysts have traditionally been used in aqueous media with detailed studies on kinetics and stereoselectivity of bioorganic reactions [2,3]. However, some groups have studied the use of enzymes in anhydrous organic solvents for the synthesis of products insoluble in water [4,5], as aqueous environments frequently give rise to unwanted side reactions making the product recovery difficult. Alcohol dehydrogenases [6] and horseradish peroxidase [7] are some of the oxidoreductases that have been used in such solvents. However, the interest during past decades has turned from aqueous or organic to low water media (non-conventional systems) because most of the enzymes retain their catalytic activity in low water content systems in contrast to organic solvents. Additionally, these systems have the ability to host substrates of different polarities. One of the most intensively studied approaches has been the use of water-in-oil (W/O) microemulsions as "microreactors".

The most widely accepted definition for microemulsions is that proposed by Danielson and Lindman: a microemulsion is a system of oil, water, and an amphiphile, which is a single optically isotropic and thermodynamically stable liquid solution [8]. The presence of surfactant and co-surfactant

molecules (amphiphiles) decreases the surface tension between oil and water to very low values, thus formulating a microemulsion with an extensive interface area.

Enzymatic reactions in microemulsions and microemulsion related systems gained interest on the grounds that through those systems the correlation between the in vitro and the in vivo behavior of enzymes can be studied, as the micro-domain structure of these systems simulates cell compartmentalization. In addition, they provide a larger surface area due to their conformation, protecting the molecule—because of the presence of water molecules in the micelle—from the denaturation effect of the organic solvent. In addition, the biocatalysts are able to react with substrates of different polarities. Microemulsions provide an aqueous phase for hydrophilic enzymes, an interface for surface-active enzymes, and an organic phase for hydrophobic substrates or products [9]. The determination of the properties of enzymes in a microemulsion system (catalytic activity, kinetic parameters, and mechanism) can be achieved by the techniques applied in aqueous solutions. This is due to the microemulsion's transparency allowing the application of photometric methods, which gives to these formulations a big advantage over other non-conventional systems. Since the late 1980s microemulsions have been extensively studied, on account of their microstructure properties, using different methods such as NMR (Nuclear Magnetic Resonance) [10], TEM (Transmission Electron Microscopy) [11], SAXS (Small-angle X-ray scattering) [12], SANS (Small-angle neutron scattering) [13], DLS (Dynamic Light Scattering) [14], and EPR (Electron Paramagnetic Resonance) [15].

Nevertheless, microemulsions present a disadvantage as the isolation of the products from the reaction medium may be hindered by the presence of surfactants. This problem leads to the formulation of stable systems, which are able to incorporate enzymes in the absence of surfactants [16].

W/O microemulsions are capable of hosting proteins in their dispersed water-pools such as lipases [17,18], oxidases [19], phosphatases [20] etc. The studies of enzyme behavior in microemulsions have been focused in altering several parameters including water content [21–25], pH [26], ionic strength [27,28], size of the reverse micelles [29], and nature of the surfactant [21,28,30]. From the parameters above the most widely studied is the water amount in the microemulsions which can alter the equilibrium of the reactions. Early studies indicated that in W/O microemulsions the properties of water are different from those of bulk water [31,32]. More recent studies revealed that water in reverse micelles exists in four hydration states [33]. Free water is defined as out-core water, because it is isolated from the in-core water by the oriented monolayers formed by surfactant molecules. The other three types are defined as in-core water, namely, anion-bound, cation-bound, and bulk-like water. This diversity of the water state can influence the behavior of the encapsulated enzyme. The water/surfactant molar ratio is abbreviated as w_o, and refers to the number of water molecules per surfactant molecule.

A particular case of enzymatic biocatalysis in microemulsions is that involving oxidative enzymes, which will be presented in this review. Extensive studies have been performed on peroxidases (with main representative horseradish peroxidase (HRP)), polyphenol oxidases (tyrosinase, laccase), and hydrogenases.

2. Oxidative Enzymes in Microemulsions

2.1. Heme Peroxidases

Peroxidases are hydrogen peroxide oxidoreductases that catalyze the oxidation of various substrates (such as phenols, aromatic amines, non-aromatic compounds etc.) [34,35] using hydrogen peroxide or organic peroxides as oxidants (reaction 1). Products of such reactions are insoluble polymers of free radicals, which can be removed from the solution by sedimentation and filtration.

$$2AH + H_2O_2 \rightarrow 2A + 2H_2O \quad (1)$$

2.1.1. Horseradish Peroxidase

Horseradish peroxidase (HRP, EC 1.11.1.7) is a heme containing enzyme of paramount importance obtained from plant sources. It is a metalloenzyme of many isoforms, which is active over a great range of pH and has the ability to catalyze various substrates such as phenols, biphenols, benzidines etc. [36]. HRP in biocatalysis has already been used in a variety of systems, ranging from biphasic [37] and sol-gel [38] supports to microemulsions and ionic liquids [39]. It is generally known that one of the main factors necessary for HRP to retain its activity in a reverse micellar system is the appropriate hydration of the enzyme molecule. Additionally, an outstanding "superactivity" has been observed and many research groups have tried to modulate different factors in order to achieve this state [40,41]. The main substrates used for the investigation of HRP activity in microemulsions and microemulsion-related systems are ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)), pyrogallol (1,2,3-trihydroxybenzene) and guaiacol (2-methoxyphenol). Oxidation of ABTS by HRP generates the ABTS radical cation, whereas oxidation of pyrogallol leads to the formation of purpurogallin and that of guaiacol gives 3,3′–dimethoxy-4,4′-biphenoquinone.

Effect of Surfactant Nature and Concentration

The properties of W/O microemulsions can affect the structure of an enzyme and as a consequence its catalytic activity. The first reports obtained were with systems based on anionic surfactants, such as AOT (Bis(2-ethylhexyl) sulfosuccinate sodium salt) and SDS (Sodium dodecyl sulfate). Chen et al. [42] investigated by FTIR (Fourier Transform Infrared Spectroscopy) the alterations in the secondary structure of HRP in isooctane/surfactant/water microemulsions and concluded that AOT has the least influence on the structure of the molecule and as a result on its activity, followed by SDS. It is generally observed, that in microemulsions with SDS as surfactant, increased concentration of this component leads to enhanced activity of HRP. The above observation can be explained by the fact that SDS leads to a decrease in the buffer pH that is favorable for the enzyme [43]. Parida et al. [44] enhanced the catalytic activity of the enzyme at higher temperatures and pH values with the addition of cholesterol molecules which interacted with the head group of AOT in an isooctane/AOT/cholesterol/water system and hardened the membrane (substrates: *o*-phenylenediamine, pyrogallol). HRP activity has been studied, also, in microemulsions formulated with mixed surfactants, AOT and a nonionic one such as lauryl alcohol ethoxylate ($C_{12}E_3$). An enhanced activity of the enzyme was observed by reducing the charge on the surface where the enzyme is located with a concurrent increase in the droplet diameter of the dispersed phase [44].

Since microemulsions are used to provide a biomimetic system for hosting enzymes, it is also of great importance to study cationic surfactants because living cells also contain positively charged molecules. DTAB (dodecyltrimethylammonium bromide) and CTAB (cetyltrimethyl ammonium bromide) are the main representatives of this group of surfactants. Mahiuddin et al. [41] studied the oxidation of ABTS by hydrogen peroxide catalyzed by HRP in *n*-dodecane/SDS/DTAB/*n*-hexanol/buffer system where the low DTAB weight fraction in combination with high buffer content presented higher enzymatic activity than in the *n*-dodecane/SDS/*n*-hexanol/buffer microemulsion, even though DTAB acts as an enzyme inhibitor. Increase of CTAB concentration in microemulsions reduced dramatically the HRP activity, a fact that confirms that for surface-active enzymes, enzyme efficiency increases with a decrease in surfactant concentration. Roy et al. [45] investigated the improvement of HRP activity towards pyrogallol in cationic microemulsions by varying the CTAB concentration in an isooctane/CTAB/*n*-hexanol/water system, with constant values of pH = 7 and w_o = 24. The activity of the enzyme was found to increase with an increase in surfactant concentration (higher activity at 20–25 mM) but declined for higher surfactant concentrations (30–50 mM). Debnah et al. [46] studied the activity of HRP in isooctane/cationic surfactant/1-hexanol/water systems where the cationic surfactants vary depending on the degree of saturation of their head groups. The use of surfactants with unsaturated head groups leads to a decreased HRP activity towards pyrogallol oxidation, in contrast to the use of

surfactants with acyclic saturated polar heads. Many studies have investigated the effect of the mixture of anionic and cationic surfactants on HRP activity, revealing that anionic surfactants suppress the inhibition effect caused by cationic surfactants. Moreover, Biswas et al. [40] measured the activity of HRP towards the amount of SDS in the system dodecane/DTAB/SDS/*n*-hexanol/citrate buffer. The highest activity of HRP was observed in the case of the highest SDS concentration. These observations can be attributed to the changes to the pH values induced by the nature and amount of surfactant ions (Debye–Hückel effect), strongly affecting the affinity of the substrate to the biomolecule, as was demonstrated by Bauduin et al. [47]. As a result, the presence of charged surfactants (anionic or cationic) in a microemulsion affects the catalytic activity of a biomolecule.

Although nonionic surfactants are not charged and they are generally characterized as "mild", the studies regarding this particular type of microemulsion lag behind the amount of work done in other categories. In such a report, Gébicka and Jurkas [48] studied the effect of polyethylene lauryl ethers (Brij35 and Brij30) towards the oxidation of ABTS and guaiacol by H_2O_2. In isooctane/Brij/phosphate buffer microemulsions, the activity of HRP was comparable with that in aqueous solution but after replacing isooctane with cyclohexane and dodecane the enzymatic activity was lost, a fact that indicates the dependence of the activity upon the oily phase in such systems. The small amount of water in the last two systems was not able to protect the enzyme from denaturation. Motlekar and Bhagwat [49] observed that the activity of HRP towards the oxidation of guaiacol by H_2O_2 was slightly enhanced in the presence of Tween 40 (polyoxyethylenesorbitan monopalmitate) and suppressed by Tween 80 (polyoxyethylenesorbitan monooleate) at concentrations above their CMC (Critical Micellar Concentration) values. Kumar [50] observed that the spectral characteristics of HRP in cyclohexane/TritonX-100 (4-(1,1,3,3-Tetramethylbutyl) phenyl-polyethylene glycol)/1-hexanol/water system within a pH range from 5 to 11.8 are similar to those in aqueous solution, indicating no enzyme conformational changes. Nonionic surfactants combined with cationic ones enhance the activity of HRP by modification of the anisotropic interface of the microemulsion (Figure 1). Brij30 or 90 in combination with appropriate amounts of CTAB reduce the positive charge at the interface of the micro-domain, inducing thus, enhanced HRP activity [51].

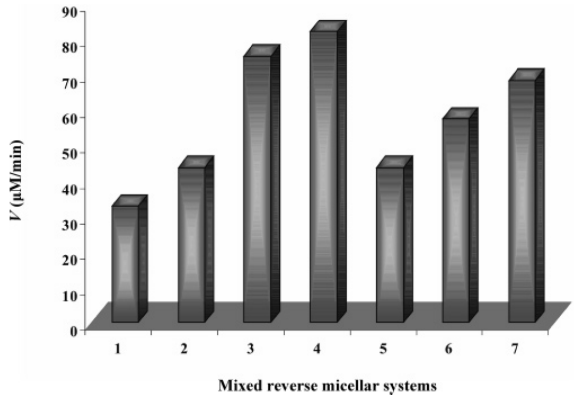

Figure 1. Dependence of horseradish peroxidase (HRP) activity on the composition of mixed reverse micelles at z = 8, 25 °C, pH = 7 (25 mM phosphate buffer). [HRP] = 1 μg/mL, [Pyrogallol] = 0.3 mM. Numbers 1–7 referred to different surfactants in surfactant/*n*-hexanol/isooctane/water system. (1: cetyltrimethyl ammonium bromide (CTAB), 2–4: CTAB + Brij30, 5–7: CTAB + Brij92); z = [co-surfactant]/[surfactant] [51]. Reprinted with permission from Shome, A.; Roy, S.; Das, P.K. Nonionic surfactants: A key to enhance the enzyme activity at cationic reverse micellar interface. *Langmuir*, **2007**, *23*, 4130–4136. Copyright 2007, American Chemical Society.

Effect of Co-Surfactant

In microemulsion systems, usually, small molecules such as alcohols and short chain polyols are used in order to "assist" the amphiphilic surfactants to reduce the surface tension of the interface

between the immiscible components of the system. These molecules are called co-surfactants and affect both microemulsion size and structure as their short alkyl chains strongly influence the interfacial composition [52–55]. In some cases the partition of the co-surfactant between the oil and water phases may disturb the encapsulated enzyme functionality [56]. In the case of microemulsions loaded with oxidative enzymes, increase of alcohol chain length leads to a bell shaped curve versus the catalytic activity, with the lowest reaction rates in dodecane/SDS/*n*-alcohol/buffer system recorded for 1-octanol and 1-butanol, respectively (Figure 2). This was attributed to the solubility of each alcohol in the buffer. The study underlies the importance of a quick thermodynamic equilibrium for the microemulsion because in the opposite case (as happens with the addition of long-chain alcohols) the enzyme is exposed to denaturation factors [43]. The above conclusion supports the fact that enzymatic activity in microemulsions based on SDS is lower than in systems based on the more hydrophobic AOT, where the equilibrium is immediately reached. It should be mentioned that AOT does not need a co-surfactant to formulate a microemulsion (feature of the surfactants with two hydrocarbon chains) while SDS needs the presence of a co-surfactant [57]. In the majority of the W/O microemulsions used for HRP encapsulation, *n*-hexanol is used as co-surfactant. Variations in its concentration can influence HRP activity. Increase of *n*-hexanol in microemulsions [40,41] generally leads to a decrease in the catalytic activity due to the fact that more alcohol is in contact with the surface-active enzyme. Surprisingly, Roy et al. [45] demonstrated that increase of *n*-hexanol in isooctane/CTAB/*n*-hexanol/water system enhances the activity of HRP towards the oxidation of pyrogallol at pH = 7, even though alcohols are inhibitors for this enzyme.

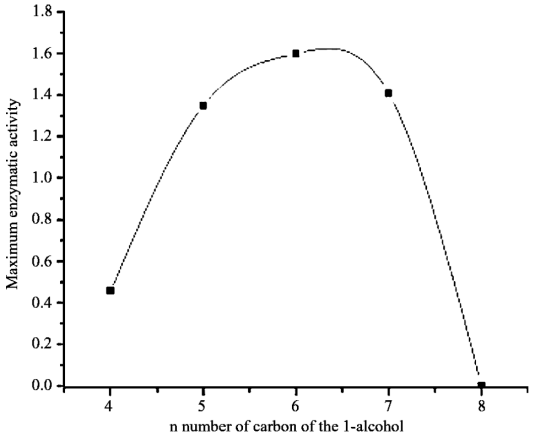

Figure 2. Maximum enzymatic activity, defined as $A = V/V_0$ (with V initial velocity of the enzymatic reaction in the microemulsion and V_0 initial velocity of the same reaction in the standard buffer solution), observed in microemulsion vs. n, the number of carbon atom of the chain of 1-alcohol used as co-surfactant in dodecane/sodium dodecyl sulfate (SDS)/*n*-alcohol/buffer microemulsion [43]. Reprinted from *J. Colloid Interface Sci.*, *292*, Bauduin, P.; Touraud, D.; Kunz, W.; Savelli, M.P.; Pulvin, S.; Ninham, B.W., The influence of structure and composition of a reverse SDS microemulsion on enzymatic activities and electrical conductivities, 244–254. Copyright 2005, with permission from Elsevier.

Effect of the System's Water Content

The activity of enzymes in microemulsions has been extensively studied towards w_o. This parameter, being an indicator of the biomolecule's hydration rate and the protection that it has towards the organic solvent, is crucial for the enzymatic activity in W/O microemulsions. At low water content, denaturation of the enzyme occurs because part of the water is spent on solvation of the surfactant molecules. The consequent dehydration can affect the conformation of the protein and decrease its catalytic activity. As water content increases the conformation of the enzyme reverts, but with a further water content increase, decrease of the enzymatic activity takes again place due to the

conformational rearrangements in the micelles. This leads to the characteristic bell-shaped curve of enzymatic activity towards w_o which also applies in the case of HRP.

Bauduin et al. [43] demonstrated that for w_o values of approximately 20, the system dodecane/SDS/*n*-alcohol/water (with different alcohols as co-surfactants) offers a properly hydrated environment for the enzyme and as a result its maximum activity occurs when tested towards the oxidation of ABTS. In the same study, it was shown that when using 1-butanol and 1-heptanol, enzymatic activity presents two different linear parts when plotted versus w_o, with a sharp increase in the first part and a slight decrease in the second part, whereas, when 1-hexanol and 1-pentanol were used, the plot exhibits three distinctive regions. In another study by Biswas et al. [40] on *n*-dodecane/SDS/DTAB/*n*-hexanol/water system, as the water content increased an increased activity was observed, although the droplet size of the microemulsion decreased. This was attributed to the reduction of *n*-hexanol concentration on the surface of the droplets, which further reduces the denaturation effect of the alcohol on the biocatalyst. Mahiuddin et al. [41] calculated an optimum percentage of buffer concentration of approximately 15%, for the same system used for the oxidation of ABTS. In addition, Pietikainen and Adlercreutz [58] changed the buffer strength of the aqueous phase of the microemulsion and observed a shift in the optimum w_o. According to their study, Tris-HCl buffer with five times bigger concentration shifts the optimum w_o from 23 to 29. Roy et al. [45] also asserted that buffer solutions with lower strength cause the enzyme to show a higher enzymatic activity, as with higher buffer strengths CTAB microemulsions are not sufficiently stable to carry out the enzymatic reaction. An exception in the above pattern was reported for microemulsions based on synthesized surfactants with unsaturation at the head group. In isooctane/surfactant/hexanol/water system [46], increase in the water content did not affect the activity of HRP towards pyrogallol oxidation. The polarity of the surfactant's polar head seems to play a key role in the enzymatic activity towards enzymes located on the interface.

Effect of pH

pH alterations in the microemulsion can change dramatically HRP activity. Decrease in pH values (lower than 4) has been shown to induce enhanced activity of HRP towards different substrates. Even changes of 0.5 in pH values, e.g., pH change from 5 to 4.5, can lead to 100% increase in the activity of HRP in *n*-dodecane/SDS/DTAB/*n*-hexanol/buffer microemulsion [59]. It is crucial to underline that the properties of water in a microemulsion are different to those of the aqueous solution. However, in microemulsions based on cationic surfactants, especially CTAB-based systems, alterations in pH do not affect the activity of the enzyme, with the only reported exception of a system was that based on monohydroxylated surfactants [46].

In recent years, interest has shifted to biocatalysis in biocompatible and edible W/O microemulsions, which are appropriate for future biotechnological applications. Microemulsions with olive oil as organic phase have successfully incorporated HRP. Even with a low amount of water (0.8% *w*/*w*) these systems can serve as microreactors, but the absence of free water in the enzyme microenvironment can induce changes to its activity and substrate specificity [60,61].

HRP has also been used in water-in-ionic liquid microemulsions (W/IL) in a study by Moniruzzaman et al. [62]. These systems feature some unique properties such as negligible vapor pressure, high thermal stability etc., therefore, they are used in synthetic reactions as "green" solvents. In the above study, HRP was solubilized in the aqueous droplets of [C8mim][Tf2N] (1-octyl-3-methylimidazolium bis(trifluromethylsulfonyl)amide)/AOT/1-hexanol system and used for the oxidation of pyrogallol by H_2O_2. An enhanced activity was observed. The insolubility of the oxidation product in the aqueous phase with the subsequent reduction of product inhibition and the change in enzyme microenvironment were some of the possible explanations for the observed enhanced activity. Moreover, in this study [62], the plot of enzymatic activity versus w_o reported for pyrogallol oxidation does not follow the bell shaped pattern expected for enzymes in microemulsions, but shows a saturation curve (Figure 3).

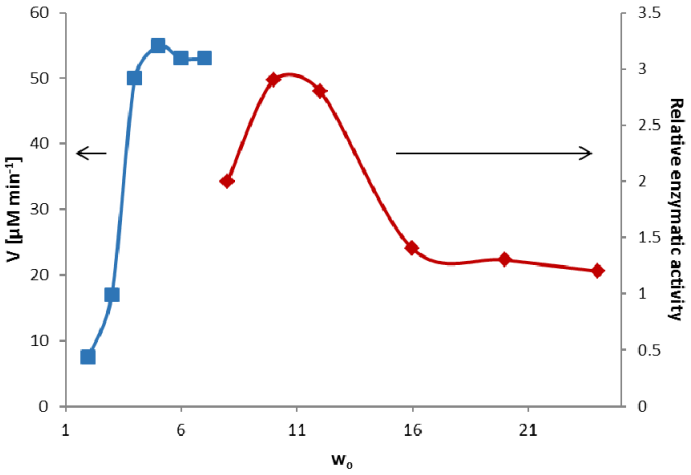

Figure 3. Variation in the enzymatic activity for the HRP-catalysed oxidation of pyrogallol in (♦) W/O microemulsion consisted of isooctane/AOT/*n*-hexanol/buffer (25 mM phosphate buffer, pH = 7.0, z = 9.6) [46] and (■) a W/IL microemulsion consisted of [C8mim][Tf2N] (1-octyl-3-methylimidazolium bis(trifluromethylsulfonyl) amide)/AOT/1-hexanol/buffer (50 mM Tris buffer, pH = 8.0) [62]. AOT: Bis(2-ethylhexyl) sulfosuccinate sodium salt; IL: ionic liquid; z = [*n*-hexanol]/[surfactant].

2.1.2. Chloroperoxidases

Chloroperoxidases (CPO, EC 1.11.1.10) are the most versatile enzymes in the heme peroxidase family. Morris and Hager discovered CPOs in the marine fungus *Calduriomyces fumago* [63]. Although CPO is a member of the heme peroxidase family, the proximal ligand is cysteine, which is identical to that of cytochromes P450. Chloroperoxidase catalyzes a variety of reactions, such as halogenation, peroxidation, oxygen insertion reactions, and decomposition of hydrogen peroxide to oxygen and water, and has been considered as a potential candidate for industrial applications [64]. CPO from *C. fumago* has not been so extensively studied in microemulsions or microemulsion-based systems, as HRP. The biggest problems when using this enzyme are the formation of side products, the limited stability, and the low aqueous solubility of the substrates. The use of microemulsions as a solution to these problems started back in 1988 when Franssen et al. [65] used the peroxidase in octane/CTAB or CTAC/pentanol/phosphate buffer system. The use of these surfactants leads to stabilized reversed micelles but also provides counter ions that are used as a substrate. The compounds 2-monochlorodimedon and 1,3-dihydrobenzene were halogenated within the system, giving the 2-halo and 4-halo derivatives, respectively, in the presence of hydrogen peroxide. Both reactions were characterized by about twice higher rates compared to aqueous media. Of exceptional importance was the enzyme's enhanced stability due to the labile behavior of the enzyme in aqueous media. The CTAC system provides a more efficient environment for enzyme stabilization in comparison with CTAB, a fact that has been explained by the oxidation of the bromide ions, which results in enzyme inactivation. For the above CTAC system, the study by Chen et al. [66] revealed that the specific activity of CPO increased significantly with the addition of CO_2 in the microemulsion and was 10 times higher than in the "conventional" microemulsion. This study was the first indicator that the enzyme catalytic reaction can be effectively controlled by CO_2. This was attributed to the reduction of viscosity (due to the addition of CO_2) which enhanced the diffusion of the substrates. The same enzyme was used for the peroxidation of ABTS and oxidation of indole in isooctane/CTAB or DTAB/pentanol/water system [67]. Because the anionic surfactant AOT inhibits the enzyme activity [68], the above system was used as an alternative and proved an excellent host for the enzyme as it causes a favorable strengthening of a-helix structure that results in enhanced activity for both reactions. Due to the extremely enhanced activity and thermostability exhibited by the enzyme, the group studied the influence of surfactant, water, and organic solution concentration towards the activity of the enzyme

in the peroxidation reaction, obtaining a bell-shaped pattern in each case. The decrease in the activity of CPO as a result of the high concentration of surfactants was explained by the increase of the micro-interface net charges, resulting in weakly electrostatic interactions of the surfactant with CPO, by interaction of the enzyme with the micellar matrix and by the thicker interfacial film, which becomes a barrier between the enzyme and its substrates. High concentrations of water can lead to increased activity due to the position of the enzyme in the structured water, near the polar head, that affects its activity. Finally, kinetic studies showed that k_{cat} increased and K_m decreased in comparison to that obtained in aqueous solution, which confirms that encapsulation of the enzyme into reverse micelles may induce a favorable conformational change on the enzyme molecule improving both the catalytic activity and stability of CPO [67].

VCPO (vanadium chloroperoxidase) from *Curvularia inaequalis* was active towards chlorination and single oxygenation in microemulsions of octane/$C_{10}E_4$/water, where $C_{10}E_4$ is a nonionic surfactant [69]. The use of ethoxylated fatty alcohols for the formulation of microemulsions leads to systems that can host the oxygenation of 9,10-dimethylanthracene (DMA) giving similar rates as in aqueous buffer, a result that is very interesting as very few studies have dealt with biocatalysis in microemulsions based on nonionic surfactants although the results were not encouraging.

2.1.3. Lignin Peroxidases (LiP)

LiP belongs to the family of oxidoreductases, specifically those acting on peroxide as acceptor and can be included in the broad category of ligninases [70]. LiP (EC 1.11.1.14) is a heme peroxidase that catalyzes the one-electron oxidation of non-phenolic aromatic compounds with high redox potentials via the formation of a substrate cation-radical [71]. It has the potential to oxidize a wide range of environmentally persistent compounds, such as polyaromatic hydrocarbons etc. Due to the fact that these substrates are hydrophobic, in 2004 Kimura et al. [72] used an isooctane/AOT/water microemulsion in order to formulate an appropriate environment for the oxidation of aromatic pollutants such as *p*-nonylphenol, Bisphenol A, and 2,4-dichlorophenol. It has to be mentioned here that LiP did not show any activity in organic solvents, so the use of microemulsion was one-way for a solution. Alterations in the organic phase of the microemulsion indicated that decane as external phase creates an environment more appropriate for the enzyme as the initial rate was increased four times compared to the one observed in isooctane, but it has the disadvantage that at higher temperatures phase separation occurs. The general opinion that the enzymatic activity is the highest when the size of the micelle is equal to the diameter of the enzyme is verified in this study, with the w_o effect towards the reaction's initial rate displaying a bell-shaped curve. Zhang et al. [73] compared the above system with a novel reverse micellar system in order to enhance the catalytic activity of LiP. They synthesized a two-tail nonionic surfactant, namely GGDE (*N*-glutamic acid didecyl ester) which they used to prepare a cyclohexane/GGDE/Triton X-100/water system. They observed, towards the oxidation of veratryl alcohol, a 40 times higher catalytic efficiency than the one observed in AOT microemulsions. LiP was studied also in W/IL microemulsions and more specifically in a [BMIM][PF6]/Triton X-100/water microemulsion [74]. The correlation of w_o with the initial rate of the enzyme catalyzed reaction showed a bell-shaped curve, and compared to the pure or water saturated [BMIM][PF6] the catalytic activity of the enzyme increased, probably due to the protective effect of Triton X-100.

2.2. Cholesterol Oxidase

The use of cholesterol oxidase (EC 1.1.3.6) in W/O microemulsions has been studied with a great deal of interest since 1988 because in these systems the water insoluble substrates can be dissolved. Cholesterol oxidase is an enzyme produced by several microorganisms such as: *Arthrobacter*, *Nocardia erythropolis*, *Rhodococcus erythropolis*, *Mycobacterium* etc. [75].

Cholesterol oxidase catalyzes the oxidation by dioxygen of the 3β-hydroxyl group of cholesterol and related steroids to a keto group and the isomerization of the Δ5 double bond, which leads to the formulation of a Δ4 cholestone [76]. The above reaction is of great importance as the cholestone is

a precursor of androst-1,4-diene-3,17-dione which can be chemically modified to manufacture oral contraceptives. In 1988 Lee and Biellmann [76] studied this reaction in the presence of cholesterol oxidase from *Streptomyces* and *Nocordia* in W/O microemulsions using cyclohexane and butanol as organic media with different surfactants. The kinetic studies showed K_m values of 10–14 mM whereas Laane et al. [77] for the same reaction in heptane/CTAB/octanol microemulsion found the K_m value decreased about 10 times. The above difference supports the opinion that the nature of the microemulsion's ingredients plays a crucial role to the catalytic efficiency of the entrapped enzyme. Laane et al. [77] observed that as the a_o values increase (with a_o being the molar ratio of hexanol to CTAB in the interface) the activity of the enzyme increases too, as the substrate has a higher solubility in those solvents and more substrate is available for the interface-active enzyme. Hedström et al. [78] studied the same reaction in an isooctane/AOT/water system, where the reaction rate in the presence of cholesterol oxidase from *Brevibacterium* sp., increased linearly versus w_o in discordance to the "classic" bell-shaped profile. Cholesterol oxidase from *N. erythropolis* showed a similar pattern [79]. In this study, Bru and co-workers studied the dependence of the enzymatic reaction on the micelle size concluding that the activity in the reverse micellar system approaches that in the aqueous medium as the micelle size increases [79]. Backlund et al. [19] used cholesterol oxidase from *Brevibacterium* sp. in a bicontinuous microemulsion composed of hexadecane/soybean lecithin/ethanol/water, in which they observed higher enzymatic activity with increasing ethanol. They assumed, due to the nature of the system, the majority of the enzyme and substrate molecules are distributed in different phases, which explains the low reaction yields. Lee & Biellmann [80] studied the oxidation of cholesterol in the presence of cholesterol oxidase from *N. erythropolis* in three different types of microemulsions composed of nonionic (cyclohexane/Triton X-100/*n*-butanol/water), anionic (cyclohexane/SDS/*n*-butanol/water) or cationic (cyclohexane/CTAB/*n*-butanol/water) surfactants. In the microemulsions based on SDS and CTAB the activity of the enzyme decreased rapidly to 25% and 75% with respect to the initial ones, whereas in the presence of Triton X-100 the enzymatic activity remained stable for a long period of time. The enzyme, remarkably, retained its activity in an extremely low water content microemulsion with nonionic surfactants. A more enlightened study by Gupte et al. [81] focused on different microemulsions based on the same surfactants (AOT, CTAB, Triton X-100) in isooctane. The study revealed that the surfactant of the microemulsion plays a crucial role in the optimum pH conditions for the enzymatic reaction. More specifically, in an isooctane/AOT microemulsion the optimum pH moved to lower values than in aqueous solution, a result that is in contrast to the study by Bru et al. [79]. In isooctane/CTAB/buffer microemulsion, the optimum pH is more basic whereas in microemulsions with nonionic surfactants, such as Triton X-100, the optimum pH is the same as in aqueous buffer. In the first two cases, the profile of the enzymatic activity versus w_o gives a bell-shaped curve, in contrast to the third case where the activity increases linearly with the molar ratio. The enzymatic reaction in those systems follows the classical Michaelis–Menten kinetics, with 100–1000 fold higher kinetic constant $k_{cat,app}$ and $K_{m,app}$ values, in comparison to those obtained in aqueous solution.

2.3. Phenoloxidases

Laccase (EC 1.10.3.2) and tyrosinase (EC 1.14.18.1) are two groups of phenoloxidases that catalyze the transformation of a large number of phenolic and non-phenolic aromatic compounds.

2.3.1. Laccases

The laccase molecule is a dimeric or tetrameric glycoprotein, which usually contains four copper atoms per monomer distributed in three redox sites [82]. This enzyme catalyzes the oxidation of *ortho*- and *para*-diphenols, aminophenols, polyphenols, polyamines, lignins and aryl diamines, as well as some inorganic ions coupled to the reduction of molecular dioxygen to water [83]. In a typical laccase reaction, a phenolic substrate is subjected to a one-electron oxidation giving rise to an aryloxyradical. This active species can be converted to a quinone in the second stage of the oxidation.

Laccases, which use molecular oxygen as electron acceptor, are attractive catalysts especially for waste treatment. There is increasing interest in their use to treat textile wastewaters which also contain auxiliary chemicals such as surfactants and salts [84,85]. Environmental pollutants (dioxins, polycyclic aromatic hydrocarbons, chlorophenols) which are some of the substrates of laccases, are not water soluble so the use of microemulsions was unavoidable [86].

Yellow laccases catalyze the oxidation of non-phenolic aromatic compounds but they lose their activity in apolar organic solvents. As a result, the use of microemulsions for the extension of their applications is essential. Rodakiewicz-Nowak et al. [87] demonstrated that yellow lacasse from *Pleurotus ostreatus* in isooctane/AOT/water microemulsions retained its catalytic activity, however, it was markedly reduced in comparison to that obtained in aqueous solution. This observation was extensively investigated with respect to the microemulsion's w_o and the conclusion was that w_o versus the oxidation rates for both yellow laccases and blue laccases have a linear dependence [88]. Moreover, laccase from *Coriolus versicolor* was active when immobilized in isooctane/AOT/water microemulsions towards Bisphenol A (BPA) oxidation and the addition of mediator 1-hydroxybenzotriazole (HBT) was not necessary, unlike in aqueous solutions. The above system can be used not only for the oxidation of BPA but also for *p*-nonylphenol and chlorophenols [89]. The main advantage of these systems is their ability to solubilize higher concentrations of substrates with low solubility in water. Nevertheless, the stability of the enzyme in the micellar system is decreased, showing a lower initial reaction rate and a significant loss of activity.

Laccase is a phenoloxidase that was also solubilized in W/IL microemulsions showing a higher catalytic activity when compared to aqueous solution [87]. Xue et al. [90] constructed a W/IL microemulsion consisting of [BMIM][PF6]/AOT/Triton X-100/water and studied the activity of laccase. In their previous work [74] they observed that in a system with the same ingredients but without AOT the enzyme was active, so they studied the effect of a different surfactant on the activity of the enzyme. In fact the addition of AOT (the solubility of which in the ionic liquid is increased by the presence of Triton X-100) creates a microemulsion in which the activity of laccase is higher, in contrast to the one without AOT. In addition, they observed that the catalytic activity versus w_o in both cases gives a linear pattern, which indicates that the specific interface has an inhibitory effect on the enzyme and specifically, the increase of Triton X-100 decreases the enzymatic activity.

2.3.2. Tyrosinase

Tyrosinase (EC 1.14.18.1, monophenol monoxygenase) is widely distributed throughout the phylogenetic scale from bacteria to mammals. Tyrosinase is an enzyme that has drawn attention due to its application in enzyme biosensors for the detection of phenolic pollutants. This enzyme is one of the first that has been proven to function in reversed micelles, such as those formed by AOT surfactant in isooctane [91], cyclohexane [92], and hexane [93] and others composed of nonionic Brij96 (poly-(10)-oxyethylene oleyl ether) in cyclohexane [94]. Yang and Robb [95] studied two different microemulsions composed of AOT and isooctane and CTAB, hexane and chloroform, where mushroom tyrosinase was immobilized and effectively used to catalyze the formation of *o*-quinones by oxidation of *o*-diphenols and presented superactivity in AOT microemulsion. These results are in disagreement with the findings by Rojo et al. [92], where the same enzyme in cyclohexane/AOT reverse micelles demonstrates equal activity towards the oxidation of 4-methylcatechol to 4-methylquinone in comparison with the one obtained in aqueous solution. In addition, Yang and Robb [95] observed that the kinetic constant, K_m, showed a small increase in reverse micelles based on AOT due to substrate partitioning, an observation that was also made by Bru et al. [91] for AOT microemulsions towards the oxidation of 4-methylcatechol. Moreover, K_m was observed to be higher in microemulsions than in aqueous solutions, while the higher V_{max} value was calculated for the AOT system. The previous conclusion that the activity is preserved in chloroform but is rapidly lost in hexane was confirmed by the above study [96].

Studies on the dependence of enzymatic activity towards temperature showed that surfactant affects the enzymatic activity. In AOT microemulsions enzymatic activity shows a peak on the increase of temperature, whereas in CTAB microemulsions there is no optimum found [95]. Rodakiewicz-Nowak et al. [87] investigated the oxidation of 4-*t*-butylcatechol by tyrosinase in the same system and showed that w_o affected the enzyme activity, with the enzyme being active in highly concentrated AOT microemulsions even at a low water content. Nevertheless, the effect of water content of the microemulsion towards the initial rate of the reaction did not demonstrate a bell-shaped dependence, but showed an increase followed by a plateau. Shipovsov et al. [97], in contrast to the previously reported data, found that when using isooctane and cyclohexane the profile of specific enzymatic activity ($\log(V_{max}/[E])$) versus w_o displayed two peaks, at w_o = 12 and w_o = 25. Estimation of the volume of reverse micelles and correlation with the molecular volume of tyrosinase monomers and multimers provided evidence that tyrosinase is likely to exist in two different catalytically active forms when entrapped in reverse micelles, specifically, a monomeric form at w_o = 12 and a tetrameric form at w_o = 25.

Furthermore, Papadimitriou et al. [98] constructed biocompatible W/O microemulsions with lecithin as surfactant and extra virgin olive oil, in which the oxidation of oleuropein by tyrosinase successfully took place. The same systems based on different oils (refined olive oil and isooctane) disabled the activity of tyrosinase. This result leads to the conclusion that the minor components of extra virgin olive oil influence the accessibility of enzyme by oleuropein. In contrast to other studies, in such systems substrate inhibition was very intense, probably due to the eventual accumulation of oleuropein and the corresponding *o*-quinone products around the protein molecule and within the amphiphile monolayer [99].

2.4. Dehydrogenases

Dehydrogenases are another group of oxidoreductases. They oxidize a substrate by transferring hydrogen to an electron acceptor, with NAD^+ (nicotinamide adenine dinucleotide) or FAD (flavin adenine dinucleotide) being common electron acceptors. Alcohol dehydrogenases (ADHs, EC 1.1.1.1) are oxidoreductases that catalyze the reversible oxidation of alcohols to aldehydes or ketones, with the concomitant reduction of NAD^+ or $NADP^+$ (nicotinamide adenine dinucleotide phosphate) [100]. These enzymes all have the same catalytic center but the size and the number of the active groups is different depending on the enzyme's source.

Meier and Luisi studied horse liver alcohol dehydrogenase (HLADH) successfully incorporated in a simple isooctane/AOT/water microemulsion following acetaldehyde reduction [101]. Martinek et al. [102] studied the oxidation of aliphatic alcohol in reverse micelles. The specificity (expressed in terms of k_{cat}/K_m) was higher for butanol when HLADH was encapsulated in the microemulsion (saturated with NAD^+) in contrast to the aqueous buffer solution where the enzyme had higher specificity towards octanol. The above finding is an example of alteration in substrate specificity of the enzyme in the environment of a reverse micelle. Samama et al. [103] saw the same alteration in substrate specificity in AOT micelles towards the oxidation of cinnamyl alcohol. Berezin et al. [104] studied the oxidation of isobutanol to isobutyraldehyde and particularly the equilibrium constants of the reaction in an isooctane/AOT/water system. In 1987 Larsson et al. [105] studied the activity and the stability of the above enzyme in a cyclohexane/AOT/water system for the oxidation of ethanol and the reduction of cyclohexanone. The two reactions are part of a reaction system in which the co-factor (NAD^+) was regenerated by the enzyme itself. The apparent K_m for ethanol in reverse micelles was about eight times lower as compared to the one in buffer, in contrast to the K_m of cyclohexanone that was almost unaltered. For the storage stability, the enzymatic activity decreased quickly in the first 24 h, but it further remained stable for two weeks.

Vos and coworkers [106] studied the catalytic properties of the enzyme in two different microemulsions in order to investigate the effect of the surfactant on the enzymatic behavior. AOT and CTAB were used in isooctane/surfactant/water microemulsions. For both systems the enzymatic

activity in the highest values of w_o (w_o = 40) was lower than in aqueous solution with the lowest activity value for CTAB microemulsion. They claimed that the incorporation of the enzyme in the reverse micellar system barely affects the secondary/tertiary structure, as revealed by CD (Circular Dichroism) studies. These results disagree with the results of phosphorescence lifetimes of LADH that revealed significant alterations in the conformational state of the macromolecule, as Strambini and Gonnelli [107] claimed in the most studied isooctane/AOT/water system. Samama et al. [103] studied different microemulsions composed of anionic surfactant-SDS and cationic-CTAB in cyclohexane with or without octane as oil phase and 1-butanol, 1-pentanol, 1-hexanol, *t*-butanol as co-surfactants. In SDS microemulsions, the enzyme quickly lost its activity, which was explained by the subunit dissociation of the enzyme by the SDS in aqueous solutions. On the contrary, in CTAB microemulsions the enzyme activity seemed to be more stable especially in the presence of higher alcohols. The difference between the two systems was the dependence of the velocity towards the water content. In the case of SDS microemulsion, the velocity increased as the water content increased but in CTAB microemulsion, the velocity did not show a clear dependence on the water content. The same group studied the behavior of HLADH in a cyclohexane/Triton X-100/1-butanol/water system where 1-butanol worked as both co-surfactant and a substrate [108]. They concluded that the enzyme in the above microemulsion has a higher stability over time in comparison to other microemulsions with anionic surfactants.

Another enzyme from the family of ADHs is the yeast alcohol dehydrogenase (YADH) which is a tetramer whereas HLADH is a dimer. Sarcar et al. [109] focused on the activity and stability of the enzyme in an isooctane/AOT/water system, when used for the oxidation of ethanol. The enzyme showed a maximum activity at w_o = 28 and pH = 8.1. The plot of k_{cat} of the reaction versus w_o and pH showed a bell shaped curve. In the micellar system, the activity of the enzyme sharply increased at 30 °C (double as in aqueous buffer) and then decreased rapidly. The enzyme's stability in the microemulsions decreased rapidly in comparison to that in aqueous buffer solution, probably due to the inactivation of the enzyme caused by the ionic interaction between the surfactant head group and the enzyme. The same group studied the same reaction and system by CD and concluded that the inactivation probably can be explained by the transformation of α-helix into either β-sheet structures or random coil conformations after the entrapment of the enzyme in the micelles [110]. Chen and Liao [111] examined the effect of nonionic surfactants in the activity of YADH. They studied a reduction reaction in isooctane/AOT/Brij30/water microemulsion and they concluded that the presence of Brij30 in concentrations lower than 0.1 M would be helpful for the enhancement of the steady residual activity of YADH in AOT reverse micelles.

YADH was also used in W/IL microemulsions. As alcohol dehydrogenase was inactive in [BMIN][PF6] due to the presence of immidiazole group which was a competitor of NAD^+ for the binding site, [BMIN][PF6] was used in microemulsion in the presence of Tritox X-100. The nonionic surfactant constructed a protective interfacial membrane for the enzyme. This is a very interesting approach which can lead to the replacement of pure ionic liquids by the related microemulsions [112].

3. Superactivity

Some enzymes are more active in nanodispersions than in reference buffered solutions. This behavior is called superactivity and is defined as a significant increase in the activity of a specific enzyme [113]. The three explanations that can be found in the literature for this phenomenon are: (i) conformational changes in the enzymes after the encapsulation in the microemulsions; (ii) state of the micellar water and (iii) ionic effect of the surfactants head group.

Mahiuddin et al. [41] studied HRP in *n*-dodecane/SDS/*n*-hexanol/buffer system, where the presence of a low DTAB weight fraction led to higher enzymatic activity, even though DTAB acts as an inhibitor of the enzyme. Gébicka et al. [48] studied the effect of different w_o values in a *n*-heptane/AOT/water system where HRP was entrapped and used for the oxidation of the hydrophilic substrate ABTS. The activity of HRP indicated a case of superactivity from which they assumed that the incorporation in the reverse micelles changed the secondary structure of HRP and as a

result, the mechanism of the catalyzed reaction was influenced. On the other hand, the rate of oxidation of guaiacol (2-methoxyphenol), which is better solubilized in organic phase than in water, by HRP encapsulated in AOT reverse micelles, appeared lower than in homogeneous aqueous solution. Biswas et al. [40] investigated the effect of the addition of SDS on the HRP structure. Due to the fact that very small changes in the pH can have a drastic effect on the initial velocity of the enzyme catalyzed reaction, the addition of the above surfactant to the citric buffer led to a decrease of the pH, as does the addition of conventional inorganic electrolytes.

The strong dependence of the enzymatic activity on pH in systems with ionic surfactants can be attributed to the Debye–Hückel effect [47], which also affects the partition of the substrates in the different microemulsion domains [114]. Therefore, the addition of different alcohols, such as pentanol, hexanol, and heptanol cause superactivity due to the high ionic strength in the nanodroplets and the pH decrease. Biswas et al. also reported that the superactivity of HRP encapsulated in cationic microemulsions was correlated with the slowest solvent dynamical modes of the confined water pool [40]. The application of W/IL microemulsions (composed of [C8mim][Tf2N] (1-octyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide)/AOT/1-hexanol/ water) as the reaction medium for the enzymatic oxidation of pyrogallol catalyzed by HRP was also investigated. The results demonstrated that the rate of HRP-catalyzed reactions in these microemulsions in the presence of imidazolium-based IL was significantly increased when compared with the results obtained in the classic microemulsions (without IL) [62].

Another peroxidase, the Iraqui Turnip peroxidase, was studied in a W/O microemulsion composed of chloroform, buffer, SDS, and the alcohols 1-propanol, 1-butanol, 1-pentanol, and 1-hexanol as co-surfactants. The enzymatic activity of the enzyme entrapped in the microemulsions was increased in the presence of alcohols with longer chain lengths. The above result was combined with the alterations in the size of the water pool [115].

Chloroperoxidase from *C. fumago* showed two-fold reaction rates in octane/CTAC/pentanol/water microemulsion in comparison to aqueous media towards the conversion of apolar compounds. Chen et al. [66] revealed that the specific activity of CPO increased significantly by 10 times with the addition of CO_2 in the CTAC-based microemulsion.

Laccase from *C. versicolor* also presented superactivity in AOT/isooctane microemulsion in BPA oxidation. This was probably due to an interaction of the surfactant with the enzyme inducing some renaturation or refolding of the partly inactivated enzyme when incorporated in the microemulsion systems. The correlation of w_o with the initial rate showed two optimal values for w_o, 15 (higher activity) and 30, a fact that can be explained by the size of the protein which corresponds with the one of the reverse micelle, as Martinek et al. [59] also observed for other enzymes. In water-in [BMIM][PF6] microemulsions LiP and laccase showed an increased catalytic activity, as well [74]. This was due to the carefully chosen amounts of Triton X-100 added to create a membrane to separate the enzyme from the ionic liquid that inhibits the enzyme.

Tyrosinase from *Agaricus bisporus* [96] also presented superactivity toward the conversion of *o*-disphenols to *o*-quinones entrapped in AOT-based microemulsion.

4. Surfactantless Microemulsions

The high cost of biocatalysis procedures based on microemulsions, especially at large scale, due to the high concentration of surfactants and the difficulty of product isolation led the researchers to a new approach. The use of detergentless (also called surfactantless or surfactant-free) microemulsions is a challenging perspective. To begin with, detergentless W/O microemulsions, as well as the conventional ones, are capable of simultaneously solubilizing water-soluble and water-insoluble reactants. They consist of a hydrocarbon, a short chain alcohol (hydrotrope) and water. The hydrotrope is not a typical surfactant but an amphiphilic substance, which is partially miscible with the aqueous and the oil phases. The combination of these three categories of components results in the formulation of two distinct domains (water-rich and oil-rich) where the hydrotrope is partitioned between the

coexisting pseudo-phases [116]. This was recently demonstrated by Diat et al. [117] using the powerful techniques of SANS and SWAXS (small- and wide-angle X-ray scattering). Water pools are stabilized by alcohol molecules absorbed at their surface [118]. The result is a stable and of low viscosity micro-heterogeneous dispersion of water in an organic solvent, optically transparent and thermodynamically stable.

The presence of the hydrotrope in a surfactantless microemulsion does not induce the formation of a similar monolayer at the interface as in the case of conventional microemulsions. This results in the interaction between the solubilized biomolecule and the organic solvent, a fact that does not take place in surfactant-based microemulsions where the surfactant monolayer provides a "barrier" between the aqueous and the oil phase [119]. A typical example is given in Figure 4.

Since this environment provides a low-cost "reactor" which permits easy product isolation, several surfactant-free systems were investigated in respect to their use as biocatalysis media. The first report in the literature appears 10 years after the first studied surfactant-free microemulsion by Smith et al. [120], when Khmelnitsky et al. [121] investigated the use of trypsin in ternary systems composed of hexane, isopropanol, and water. Since then, a wide range of enzymes has been successfully solubilized in that type of microemulsion, such as different types of hydrolases with emphasis on lipases [122] and oxidoreductases such as laccases [123] and peroxidases [124].

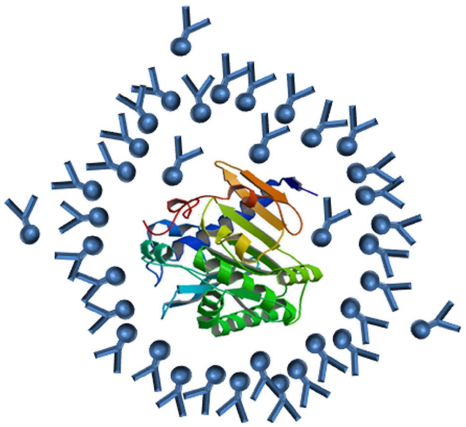

Figure 4. Model arrangement of the protein molecule within a water pool, with propanol molecules forming the interface between hexane and water [124]. Reprinted from *Curr. Opin. Colloid Interface Sci.*, 22, Xenakis, A.; Zoumpanioti, M.; Stamatis, H., Enzymatic reactions in structured surfactant-free microemulsions, 41–45. Copyright 2016, with permission from Elsevier.

These surfactantless systems have been mainly composed of non-polar solvents (as the "oil phase") with hexane [16,121–123,125–127] and toluene [126,128,129] being the most frequently used. Tziala et al. demonstrated for the first time the use of cyclic terpenes as natural organic solvents, such as α-pinene and D-limonene [130]. Such oils were efficiently used to formulate a stable surfactantless microemulsion where fungal chloroperoxidases and laccases retained their catalytic activity [131]. Furthermore, these systems provided higher bioconversion yields of hydrophobic and hydrophilic compounds in comparison to the yields obtained in aqueous-based media. The above study was of great importance because the use of surfactantless systems based on essential oils provides a new approach in bioconversions while offering an environment where the catalytic activity of oxidative enzymes can be enhanced. The stabilization of the dispersed aqueous phase in such systems occurs with the addition of short-chain alcohols such as isopropanol, 1-propanol, and *t*-butanol. As the microstructure of a surfactant-based microemulsion, in detergentless microemulsions a transparent dispersion is obtained by two immiscible liquids with the simultaneous construction of an interfacial film that separates the two liquids but also provides the appropriate compartmentation for the activation of surface active enzymes [132]. The most crucial and as a result the most frequently

studied factor is the effect of the components' ratio on the activity of the encapsulated enzymes in detergentless microemulsions.

Khmelnitsky et al. [123] studied the activity patterns for laccase and cholesterol oxidase in isopropanol/*n*-hexane/water system. In the first case lacasse revealed higher activity in the monophasic region of the ternary phase diagram that corresponds to water-in-oil microemulsions, in contrast to cholesterol oxidase and polyphenoloxidase [126], which in the same region presented the lowest activity. The results were inversed regarding the inactivation rate constant [118]. The difference is that in the case of cholesterol oxidase, hydrophobic substrates are involved whereas in the case of laccase the substrates are hydrophilic. Khmelnitsky et al. [123] revealed also that the catalytic activity of the enzyme towards the oxidation of pyrocatechol was similar to that obtained in aqueous solution, whereas the Michaelis constant was invariably much higher in media with high concentrations in organic solvents. This is due to the extremely high solubility of this substrate in the organic solvents. Water content is one of the most decisive factors that affects the catalytic activity of the entrapped enzyme. By increasing the water content of the systems a higher enzymatic activity for laccases, chloroperoxidases, and other enzymes can be observed. This is because there are sufficient water molecules for adequately hydrating the enzyme. This behavior could also be attributed to the partition and therefore the availability of the lipophilic substrates in the enzyme aqueous microenvironment [130–132]. Nevertheless, in the case of cholesterol oxidase at the optimum water content, the entrapped enzyme showed lower stability in a temperature range of 25–40 °C in comparison to aqueous solutions [118,120].

Surfactantless microemulsions were proven to be appropriate systems to host oxidative enzymes, providing an environment where the enzymes can retain their catalytic activity, even an enhanced one. In some cases, enzymes immobilized in surfactant-free microemulsions exhibit a greater stability in comparison to the one they present in surfactant-based microemulsions, a fact that makes the surfactantless microemulsions attractive for biotechnological applications.

5. Conclusion

The use of low water content media has attracted the interest of researchers for various enzymatic reactions. The use of microemulsions provides a medium for the solubilization of hydrophobic compounds and a region for activation of surface active enzymes. They can shift reaction equilibrium, may improve the thermal stability of the enzymes thereby enabling reactions to be carried out at higher temperatures, and can enhance enzymatic activities. The problem of product isolation or the reuse of the enzymes that arises due to the high concentration of surfactants can be solved by the formulation of surfactant-free microemulsions, which maintain the properties of conventional microemulsions. The use of microemulsions both conventional and non-conventional in different aspects of life is undeniable. The understanding of the enzymes' behavior within the micelles, as well as the interactions of different surfactants in the protein's molecule structure has to be further clarified. Of great importance is the application of the acquired knowledge on oxidation catalysis in microemulsions to the large scale in order to optimize the appropriate procedures. The replacement of "classic" organic solvents with more environmentally friendly ones, such as essential and edible oils, can be a very promising aspect in the use of microemulsions. Additionally, future enzymatic studies on water-in-ionic liquid microemulsions need to focus on the reuse of the enzyme, crucial for large-scale reactions.

Conflicts of Interest: The authors declare no conflict of interest.

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