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ENZYMATIC CATALYSIS OF HYDROCARBONS OXIDATION "*IN VITRO*" (REVIEW)

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Abstract. Reactions proceeding at ambient temperature very promptly and selectively, as it occurs in live organisms, imitation of the wildlife processes, and, eventually, study of mechanism of enzymes action are important challenges of contemporary chemistry. This short review gives information on recent achievements in the field of enzymatic catalysis of oxidation of hydrocarbons. The literature material was gathered using the Thomson Reuters electronic database (Web of Science) and mostly covers the last decades.

Keywords*: enzyme, oxidereductase, hydrocarbon, aerobic oxidation, biocatalysis, organic pollutant, biodegradation.

1. Introduction

Oxidation of hydrocarbons is value-enhancing chemical transformation with the goal that has long been pursued – to make the process manageable, high-yielded and selective. An additional challenge is to make such processes environmentally friendly, for example by using nontoxic reagents and energy-efficient catalytic methods. Excellent examples are naturally occurring enzymes, and extensive studies have revealed the key chemical principles that underlie their efficacy as catalysts for aerobic oxidations. Important inroads have been made in applying this knowledge to the development of synthetic catalysts modeling enzymes [1].

Actually, the current industrial catalysts often operate under harsh conditions and produce large amounts of heavy-metal waste. Enzymatic oxidations operate under relatively mild conditions and produce little if any waste. The enzymes catalyze the chemo-, regio-, and stereoselective oxygenation of hydrocarbons producing alcohols, aldehydes, epoxides, and carboxylic acids [2, 3].

The matter is interesting from the scientific point of view, *e.g.* to contribute more important details to geochemical aspects of genesis of oxygenated petroleum hydrocarbon or to the mechanism of biomimetic catalysis, to make enable new routes for difficult-to-synthesize complex substances of pharmaceutical and agricultural industries as well. Such biologically inspired hydrocarbon oxidation catalysts hold great promise for wide-ranging synthetic applications [4-8].

2. Different Enzymes to Catalyze the Oxidation of Different Hydrocarbons

The extension of enzymatic catalysis, classically carried out in aqueous media, to organic media can be first ascribed to the possibility of using substrates that are poorly soluble or insoluble in water. In biphasic media consisting of an aqueous phase containing the enzyme in solution and of a non-water-miscible organic solvent, the enzyme is kept in a suitable aqueous environment. A variant biphasic system consists in creating reverse micelles by the addition of a surfactant in order to increase the interfacial area and thus to improve the transfers between the aqueous phase where the enzyme is located and the organic phase. In these two cases, the partition coefficient of the different reactants plays a crucial role by governing the rates and yields of reaction. Micro-aqueous media constitute a new system for biocatalysis in organic media. In this case, a solid enzyme is incorporated into an organic solvent, preferably a hydrophobic one, wherein it exhibits new properties, especially a higher stability and a modified selectivity. The enzymes most studied in this type of medium are hydrolytic ones, such as lipases or proteases, which can then catalyze synthesis reactions. Yet, other classes of enzymes have also been used in this way [9].

The first step in the aerobic degradation of alkanes by bacteria, yeasts, and fungi is catalyzed by oxygenases.

These enzymes, which introduce oxygen atoms derived from molecular oxygen into the alkane substrate, play an important role in oil bioremediation and in the co-metabolic degradation of compounds such as trichloro-ethylene and fuel oxygenates [10]. In addition, they are useful biocatalysts and important models for a difficult chemical reaction – regio- and stereospecific activation of C–H bonds. Several unrelated enzyme classes catalyze the oxidation of alkanes. Alkane-degrading yeast strains contain multiple alkane hydroxylases belonging to the P450 superfamily, while many bacteria contain enzymes related to the *Pseudomonas putida* GPol membrane-bound alkane hydroxylase system. Short-chain alkanes are probably oxidized by alkane hydroxylases related to the soluble and particulate methane monooxygenases. Only the membrane-bound enzymes have been studied with respect to their prevalence in environments such as soils or aquifers [10].

"*In vitro*" enzymes applications are usually restricted by cofactor requirements and sensitive nature of oxygenases, which are often multicomponent systems with oxidizing and electron-shuttling subunits. Many of these issues have been explored in the works [11-14], where an extensive discussion of the application of oxygenases with many examples of the associated problems and possible solutions are given.

2.1. Oxidases

It is well-known that lignin peroxidase (LiP) is able to catalyze oxidation of aromatic compounds with redox potentials higher than 1.4 V (NHE (Normal hydrogen electrode)) by single electron abstraction, but the exact redox mechanism is still poorly understood [15]. Lignin peroxidase has been purified to homogeneity using a process of concentration by ultrafiltration and anion exchange chromatography on diethylaminoethyl (DEAE) cellulose from the liquid culture filtrate of the brown rot fungi Gleophyllum striatum MTCC-1117. The molecular mass of the purified enzyme is 43 kDa as determined by sodium dodecyl sulfate polyacrylamide gel electropho-resis (SDS-PAGE) analysis. It has been established that the lignin peroxidase stimulates the aerobic oxidation of such polycyclic aromatic hydrocarbons as pyrene, acenaphthene, anthracene, dibenzothiophene, and 9-methyl anthracene [16]. The reaction products were mainly quinones, suggesting the oxidation mechanism for the biocatalyst [17].

Lignin peroxidase from *Phanerochaete chrysosporium* was used to study the oxidation of aromatic compounds, including polycyclic aromatic hydrocarbons and heterocyclic compounds that are models of moieties of asphaltene molecules. The oxidations were done in systems containing water-miscible organic solvents, including methanol, isopropanol, N,N-dimethylforma-mide, acetonitrile, and tetrahydrofuran. Nine of the 20 aromatic compounds tested were oxidized by lignin peroxidase in the presence of hydrogen peroxide. Among the compounds studied, lignin peroxidase was able to oxidize those with ionization potentials of < 8 eV (measured by electron impact). The oxidizing substances were anthracene, 1-, 2-, and 9-methylanthracenes, acenaphthene, fluoranthene, pyrene, carbazole, and dibenzothiophen [18]. The reaction products contain hydroxyl and keto groups. In one case, carbon-carbon bond cleavage, yielding anthraquinone from 9-methyl-anthracene, was detected. Kinetic constants and stability characteristics of lignin peroxidase were determined by using pyrene as the substrate in systems containing different amounts of organic solvent [18].

The new approach was applied to maximize the catalytic efficiency of chloroperoxidase (CPO, from *Caldariomyces fumago*) toward the oxidation of hydrocarbons [19]. The reaction system consisted of an organic/aqueous emulsion comprising pure substrate and aqueous buffer supplemented with the surfactant dioctyl sulfosuccinate. The emulsion system attenuated not only the destabilizing effects of the substrate and product on the enzyme by emulsifying the compounds, but also oxidant toxicity (oxidative stress) by increasing substrate availability. As a result, CPO exhibited total turnover numbers (TTNs, defined as the amount of product produced over the catalytic lifetime of the enzyme) of ca. 20,000 mol product/mol enzyme for the oxidation of styrene, toluene, and *o*-, *m*-, *p*-xylenes. The TTNs are over 10-fold higher than those previously reported for the oxidation of benzylic hydrocarbons by CPO. This study represents a significant step toward the development of CPO as a practical catalyst for large-scale organic syntheses [19].

Based on the previous results CPO was analyzed for its ability to oxidize ten different monoterpenes with hydrogen peroxide as oxidant. In the absence of halide ions the terpenoids like geraniol and, to a lesser extent, citronellol and nerol were converted into the corresponding aldehydes, whereas terpene hydrocarbons were recalcitrant under these conditions. In the presence of chloride, bromide and iodide ions, every terpene tested was converted into one or more products. (1S)-(+)-3-carene was chosen as a model substrate for the CPO-catalysed conversion of terpenes in the presence of sodium halides. With chloride, bromide and iodide, the reaction products were the respective (1S, 3R, 4R, 6R)-4-halo-3,7,7-trimethyl-bicyclo[4.1.0]-heptane-3-ols, as iden-tified by H-1 and C-13 nuclear magnetic resonance [20]. These product formations turned out to be strictly regio- and stereoselective and proceeded very rapidly and almost quantitatively. These results represent the first examples of the application of CPO as a highly efficient biocatalyst

for monoterpene functionalization. The authors indicate the promising strategy for "green" terpene chemistry overcoming drawbacks usually associated with cofactor-dependent oxygenases and conventional chemical methods used for terpene conversions [20].

The authors described several steps in the oxidative pathway leading from *p*-xylene to terephthalic acid that are catalyzed by enzymes. CPO from *Caldariomyces fumago* was used to oxidize *p*-xylene [21]. However, only one of the two aromatic methyl groups was oxidized. To examine the route from 1,4-benzenedimethanol (1,4-BDM) to terephthalic acid numerous peroxidase and oxidase enzyme systems were investigated. A combination of two enzymes, CPO and xanthine oxidase (XO), was found to produce the highest yield of terephthalic acid from 1,4-BDM. Oxidation of 1,4-BDM to a mixture of predominantly terepthaldicarboxaldehyde, 4-carboxybenzaldehyde, and 4-hydroxymethylbenzaldehyde was carried out by CPO with the continuous addition of hydrogen peroxide as an oxidant. Subsequent addition of XO resulted in a 65 % yield of terephthalic acid. A tandem system in which both CPO and XO were present enabled the initial H_2O_2 to be enzymatically regenerated an average of 1.6 times. However, much lower final yields (ca. 2 %) of terephthalic acid were obtained [21].

The catalytic potential of chloroperoxidase (CPO) immobilized on mesoporous materials was evaluated for the oxidation of 4,6-dimethyldibenzothiophene in water/acetonitrile mixtures [22]. Two different types of materials were used for the immobilization: a metal containing Al-MCM-41 material with a pore size of 26 angstrom and SBA-16 materials with three different pore sizes: 40, 90 and 117 angstrom. The SBA-16 40 angstrom did not retain any CPO. The nature and the pore size of the material affected the catalytic activity of the enzyme as well as its stability. Compared to the free enzyme, the thermal stability of CPO at 45 angstrom was two and three times higher than when immobilized on Al-MCM-41 and SBA-16 90 angstrom, respectively [22].

As oil products are typical hydrophobic compounds, it was of interest in view of possible applications in the petroleum and petrochemical industries to report the present knowledge about the functioning of enzymes in organic media.

Results have been obtained at the enzymatic oxidation of sulfur-containing straight-run diesel fuel [23]. The diesel fuel contained 1.6 % of sulfur and was enzymatically oxidized with the CPO. The oxidative treatment was applied to remove or at least to diminish the sulfur content. Most organosulfides and thiophenes were transformed to form sulfoxides and sulfones. The oxidized organosulfur compounds can be effectively removed by distillation. The resulting fraction after distillation

contained only 0.27 % of sulfur, while the untreated straight-run diesel fuel after the same distillation process still showed 1.27 % of sulfur [23].

The line comprising bio-oxidative desulfurization of liquid hydrocarbon fuels was utilized in the patent of Indian authors [24]. The patent holders announced that methods for producing ultra-low-sulfur content hydrocarbon fuel and desulfurized product are disclosed. The offered method involves selective biocatalytic oxidation of sulfur containing compounds present in the fuels employing enzyme lipase (class of esterases) as biocatalyst in the presence of controlled and incremental amount of hydrogen peroxide and carboxylic acid without using water or any co-factor for the reactivity of the enzymes. The biocatalyst is recoverable and reusable in the reaction (claimed). The process is industrially feasible, less time consuming, more selective, and cost effective. The process provides for the removal of a significant amount of sulfur from the fossil fuel employing a lipase enzyme as a biocatalyst. The process involves minimum steps and demonstrates greater selectivity towards the conversion of alkylated aromatic sulfur-bearing compounds, which are relatively unattacked under normal hydrodesulfurization, with no apparent change in the non-sulfur-bearing components of the diesel fuel. The process is carried out without using water or any co-factor for the reactivity of the enzyme [24].

Other enzyme representative, associated with the oxidases – horseradish peroxidase (HRP) has been utilized for the synthesis polyaromatic quinones in organic media by means of the biochemical oxidation of antracene and pyrene. The resulting products were 9,10-anthraquinone and mainly 1,2 and 4,5-pyrenequinones [25].

A combination of ultrasound and oxidative enzyme, HRP, was explored for the oxidative degradation of substituted phenols (phenol, *p*-chlorophenol, *p*-bromophenol, *p*-iodophenol, *p*-methoxyphenol, *p*-cresol and *p*-nitrophenol) in aqueous environment [26]. These phenols, which are commonly considered as organic pollutants, were conventionally divided into three groups according to their degradation behavior. In group (I), HRP in the presence of hydrogen peroxide had a negligible effect on decomposition of *p*-nitrophenol. The combined method showed the same effect as sonolysis solely. Therefore, it has been concluded that the degradation of *p*-nitrophenol by means of ultrasonic waves alone is convenient and sufficient. In group (II), consisting of *p*-methoxyphenol and *p*-cresol, the combined method gave approximately the same effect as enzyme treatment. It means that the ultrasound had a negligible effect and the enzyme treatment alone was more favorable. Phenol and its halogenated compounds (chloro-, bromo and iodo) were placed in group (III) and showed different behaviour than the two mentioned cases. The combined method was

more efficient than the sonolysis and enzyme treatment individually. It should be noted that the effect of enzyme on this group (III) was in the middle level as compared to the two other groups (I and II), where the enzyme had a lower and higher effects, respectively [26].

Continuing the HRP line, the catalytic capability of HRP and hydrogen peroxide (H_2O_2) coupled oxidation of aromatic hydrocarbons (*o*-xylene-*d*(10) and naphthalene-*d*(8)) was investigated by J. Fung *et al.* [29]. Batch experiments were conducted using horseradish peroxidase prepared in potassium phosphate buffer in the presence of H_2O_2. The oxidation of aromatic hydrocarbon was tested as a function of HRP at a fixed concentration of H_2O_2, and as a function of the concentration of H_2O_2 at a constant HRP activity (4000 units/ml). The mass removal of the aromatic compounds increased with increasing HRP enzymatic activity to be up to 54 % and 51 % for *o*-xylene-*d*(10) and naphthalene-*d*(8), respectively. The same pattern for hydrogen peroxide – increasing the concentration of H_2O_2 resulted in increased mass removal of aromatic hydrocarbons [27].

Degradation of nitroaromatics, which are significant environmental pollutants, is difficult to achieve. Zero-valent iron reduction of nitroaromatics coupled with peroxidase-catalyzed capture of the resulting anilines as a two-step strategy for removing nitroaromatics from wastewater and process water has been investigated by R. Mantha *et al.* [28]. The concentration range of nitroaromatics studied was adjusted to that which would be present in industrial wastewater streams. The studies were done in continuous-flow columns. The enzymatic treatment following zero-valent iron reduction was carried out in a plug-flow reactor using a crude preparation of the enzyme soybean peroxidase extracted from soybean hulls. The complete reaction time for the two steps was 5–5.5 h. Operating parameters including pH, peroxide/substrate ratio, enzyme concentration, and alum concentration were optimized. Optimum conditions obtained were approximately neutral pH with a hydrogen peroxide/substrate molar ratio of 1.5 for all of the nitroaromatics tested. Alum concentrations of 50–100 mg/l were useful in removing the apparent color from the treated water.

Biotransformation of organic pollutants is a promising area of effective ecological application of enzymatic oxidation. Actually, the biotransformation is often most effective and complete under oxidant-abundant conditions. At the same time dissolved oxygen availability is frequently limiting the biotransformation of these organic compounds in the subsurface due to the limited aqueous solubility of oxygen, the relatively slow rate of re-aeration of groundwater in the saturated zone, and therefore significant biological oxygen demand is exerted during aerobic metabolism [29]. Addition of hydrogen peroxide (H_2O_2) can augment the oxidant capacity of the aquifer. The paper reviews several reactions pertinent to remediating contaminated ground waters *via* H_2O_2 addition. H_2O_2 is disproportionated by the action of microbial catalase and several inorganic catalysts such as iron oxide species to give 0.5 mol O_2 per mole of H_2O_2 consumed. If disproportionation occurs too quickly evolution of oxygen gas can form bubbles that lower aquifer permeability. Another type of reaction is direct oxidation of organic compounds by H_2O_2 in the presence of enzymes (peroxidases) or metal oxide catalysts. Molecular oxygen is not evolved as a result of this type of H_2O_2-consuming reaction [29].

The ability of peroxidases and laccases enzymes (the latter is Cu-containing oxidase enzymes) to treat organic pollutants is reviewed [30]. Authors declared that enzymatic methods generally have low energy requirements, are easy to control, can operate over a wide range of conditions and have a minimal environmental impact. Peroxidases and laccases have broad substrate specificities and can catalyze the oxidation of a wide range of toxic organic compounds. The results show that an enzymatic oxidation can diminish the toxicity of some polycyclic aromatic hydrocarbons (PAHs), phenols, organophosphorus pesticides, and azo dyes in laboratory and some field conditions. Due to the hydrophobicity and low aqueous solubility of these substrates, reactions are usually performed in the presence of organic solvents. However, it was detected that organic solvents can provoke enzyme denaturation, unfavorable substrate partition, inhibition or stabilization of enzyme-substrate complexes, depending on the enzyme, substrate and organic solvent used. Strategies to overcome these problems are proposed [30]. Additionally, low stability of heme-containing peroxidases to hydrogen peroxide, low reaction rates of laccases, mediators toxicity, limited availability and high costs of these enzymes are other limitations detected for commercial applications. Since field reaction conditions are more complex than laboratory conditions efforts have to be made to achieve cheap overproduction of these biocatalysts in heterologous hosts and also their modification by chemical means or protein engineering to obtain more robust and active enzymes.

Up to now little is known about the biotransformation potential of single-walled carbon nanotubes (SWNTs). In the paper the enzymatic transformation of SWNTs and oxidized (carboxylated) SWNTs (O-SWNTs) using three ligninolytic enzymes was compared: lignin peroxidase, manganese peroxidase (MnP), and laccase. Only MnP was capable of transforming SWNTs, as determined by Raman spectroscopy, near-infrared spectroscopy, and transmission electron microscopy. Interestingly, MnP degraded SWNTs but not O-SWNTs. The recalcitrance of O-SWNTs to enzymatic transformation is likely attributable to the binding of Mn^{2+} by

their surface carboxyl groups of O-SWNTs at the enzyme binding site. This inhibits critical steps in the MnP catalytic cycle (*i.e.*, Mn^{2+} oxidation and Mn^{3+} dissociation from the enzyme). The results suggest that oxygen-containing surface functionalities do not necessarily facilitate the biodegradation of carbonaceous nanomaterials, as is commonly assumed [31].

The transformation profiles of PAHs by pure laccases from *Trametes versicolor* and *Pycnoporus sanguineus*, and the optimal reaction conditions (acetonitrile concentration, pH, temperature and incubation time) were determined in the investigation [32]. Anthracene was the most transformable PAH by both laccases, followed by benzo[α]pyrene, and benzo[α]anthracene. Laccase-mediator system (LMS) could not only improve the PAHs oxidation but also extend the substrate types compared to laccase alone. 5 or 10 % (v/v) of acetonitrile concentration, pH 4, temperature of 313 K, and incubation time of 24 h were most favorable for anthracene oxidation by laccase from *T. versicolor* or *P. sanguineus*. The gas chromatography-mass spectrometry analysis indicated that 9,10-anthraquinone was the main product of anthracene transformed by laccase from *T. versicolor*. Microtox test results showed that both anthracene and its laccase-transformation products were not acute toxic compounds, suggesting that laccase-treatment of anthracene will not increase the acute toxicity of contaminated site [32].

The chemically modified laccase from *Trametes versicolor* has been used in the "*in vitro*" oxidation of two PAHs: acenaphthylene and anthracene, in combination with 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as a redox mediator. The results indicate that the maleic anhydride modified laccase (MA-Lac) improved the stability of laccase to temperature, pH and storage time compared with the free enzyme. After incubation for 72 h, the MA-Lac-ABTS system oxidized acenaphthylene and anthracene to more than 70 % from the reaction mixture [33].

Enzymatic complexes constructed by linear-dendritic copolymers and laccase are used for the unprecedented one-pot biotransformation of fullerene (C(60)) into epoxide-and hydroxyl-derivatives under mild and environmentally friendly reaction conditions (318 K and aqueous medium) [34]. The reaction is catalyzed by mediator pairs – N-hydroxy-5-norbornene-2,3-dicarboxylic acid imide/1-hydroxybenzotriazole or 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)/1-hydroxybenzotriazole used in equimolar amounts. After 24 and 48 h, the biotransformation products – C(60)O(n), C(60)(OH)(n), C(60)(H)(n)(OH)(n), and/or C(60)O(n)(H)(m)(OH)(m) range between 50 and 78 %, respectively. Their structure is revealed by FTIR, NMR,

and mass-spectrometry. The mechanism of the process is discussed and elucidated. The reaction procedure allows the repeated usage of the enzyme/linear-dendritic complex, which retains its catalytic activity after several cycles [34].

Laccase partially purified from residual compost of *Agaricus bisporus* by an aqueous two-phase system (Lac ATPS) was used in degrading PAHs: fluorene (Flu), phenanthrene (Phe), anthracene (Ant), benzo[α]pyrene (BaP), and benzo[α]anthracene (BaA). The capacity of the enzyme to oxidize polyaromatic compounds was compared to that of the crude laccase extract (CE). After treatment for 72 h, Lac ATPS and CE were not capable of oxidizing Flu and Phe, while Ant, BaP, and BaA were oxidized, resulting in percentages of oxidation of (11.2 ± 1), (26 ± 2) and (11.7 ± 4) % with CE, respectively. When Lac ATPS was used, the following percentages of oxidation were obtained: (11.4 ± 3) % for Ant, (34 ± 0.1) % for BaP and (13.6 ± 2) % for BaA. The results reported here demonstrate the potential application of Lac ATPS for the oxidation of PAHs [35].

Laccase possesses the ability to oxidize a broad range of persistent organic pollutants, including benzo[α]pyrene, the most carcinogenic, mutagenic and teratogenic PAHs. In the study [36] the reaction conditions for benzo[α]pyrene oxidation by laccase from Trametes versicolor were optimized in a liquid medium by a series of single-factor experiments. The maximal benzo[α]pyrene oxidation rate was observed at 313 K, pH 4, 10% of acetonitrile and an incubation time of more than 24 hr, and the benzo[α]pyrene oxidation was enhanced significantly by the addition of a mediator, 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonicacid) (ABTS). Laccase was also applied to aged PAHs polluted soil to examine the efficiency of enzymatic bioremediation. The results showed that the enzyme was still effective in the degradation of anthracene, benzo[α]pyrene and benzo[α]anthracene in soil. Moreover, the degradation rate of most PAHs increased by the addition of ABTS. These results indicated that the bioremediation of PAHs contaminated soil using laccase is feasible but suboptimal pH might be a limiting factor in the enzymatic treatment of soil [36].

Manganese peroxidase (MnP) produced by *Anthracophyllum discolor*, a Chilean white rot fungus, was immobilized on nanoclay obtained from volcanic soil and its ability to degrade PAHs compared with the free enzyme was evaluated [37]. The immobilized enzyme was able to degrade pyrene (> 86 %), anthracene (> 65 %), alone or in mixture, and to a less extent fluoranthene (< 15.2 %) and phenanthrene (< 8.6 %). Compared to free MnP from *A. discolor*, the enzyme immobilized on nanoclay enhanced the enzymatic transformation of anthracene in soil. Overall results indicate that nanoclay, a

carrier of natural origin, is a suitable support material for MnP immobilization. In addition, immobilized MnP shows an increased stability to high temperature, pH and time storage, as well as an enhanced PAHs degradation efficiency in soil. All these characteristics may suggest the possible use of nanoclay-immobilized MnP from *A. discolor* as a valuable option for in situ bioremediation purposes [37].

It has been indicated that phenols are present in petroleum refining wastewater. An enzymatic method for removing phenols from industrial aqueous effluent has been developed in the past several years. In this method, a peroxidase enzyme catalyzes the oxidation of phenol by hydrogen peroxide generation of phenoxyl radicals. These radicals diffuse from the active center of the enzyme into solution and react nonenzymatically to eventually form higher oligomers and polymers, which can be removed from wastewater by conventional coagulation and sedimentation or filtration. In the Ref. [38], Arthromyces ramosus peroxidase (ARP) was applied to treat a petroleum refining wastewater containing 2 mM (188 mg/l) phenol in a batch and continuous-flow system. The latter consisted of a plug-flow reactor (PFR) where the reaction took place between phenol and hydrogen peroxide catalyzed by the enzyme in the presence of polyethylene glycol (PEG). A flocculation tank followed the PFR where alum and sodium hydroxide were added and then the polymers formed were settled in a sedimentation tank and removed from the system. Most (95–99 %) of the phenol was removed by the same dose of ARP required for the treatment of synthetic wastewater containing an equal amount of phenol. Polyethylene glycol, as an additive, reduced enzyme inactivation and consequently reduced the enzyme dose and the cost of the treatment process. Step feeding of hydrogen peroxide was not effective in reducing the enzyme requirement. A significant removal of chemical oxygen demand was achieved when using PEG to reduce the enzyme dose [38].

2.2. Monooxygenases

Among the enzymes which provoke the oxidation cycle, the systems-termed-monooxygenases is one of the most important. It can now be accepted as evidenced that hydroxylation is accomplished namely by means of this class of oxidoreductases. Enzymes of this type have a distinguishing feature – during the reactions which they catalyze one atom of an oxygen molecule is consumed in oxidizing the substrate (S), while the other atom is reduced to water, so that the overall hydroxylation is represented by the equation

$$S + O_2 + DH_2 \rightarrow \bullet SO + H_2O + D$$
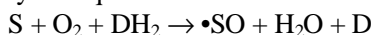
where DH_2 is the co-substrate, which is dehydrogenated by the reaction. Mono-oxygenases were classified according to types of reactions and structure, to assess their role in biological oxidation, and to discuss their composition and properties [39]. A special emphasis was made on the role of models in the study of the mechanism of reactions catalyzed by mono-oxygenases.

The control over the regio- and/or stereo-selective aliphatic C–H oxidation by metalloenzymes is of great interest to scientists. Typically, these enzymes invoke host-guest chemistry to sequester the substrates within the protein pockets, exploiting sizes, shapes and specific interactions such as hydrogen-bonding, electrostatic forces and/or van der Waals interactions to control the substrate specificity, regio-specificity and stereo-selectivity. Aromatic C–H bond hydroxylation of 1-methoxynaphthalene was efficiently catalyzed by the substrate misrecognition system of the hydrogen peroxide dependent cytochrome P450 (BS beta) (CYP152A1), which usually catalyzes hydroxylation of long-alkyl-chain fatty acids [40].

Cytochrome P450 monooxygenases (the enzymes containing a heme cofactor) are the catalysts of choice in the biological world for mediating the oxidation of $sp(3)$ and $sp(2)$ C–H bonds with a high degree of chemo-, regio-, and stereoselectivity and in a wide array of compounds of varying complexity. The efficiency of these enzymes, compared with chemical methods, to catalyze the insertion of oxygen into unactivated C–H bonds under mild reaction conditions has sparked interest among researchers toward investigating and exploiting P450s for a variety of synthetic applications. Realizing the synthetic potential of these enzymes, however, depends upon the availability of effective strategies to tune the reactivity of natural P450s to obtain viable oxidation catalysts for the desired transformation. The review describes recent efforts in this area involving the use of protein engineering, substrate engineering, guest/host activation, and functional screening strategies [41]. The development of engineered P450s for drug metabolite production and emerging methodologies involving the integration of P450-catalyzed transformations in preparative-scale chemoenzymatic syntheses are also presented. Key challenges that need to be addressed to capitalize on P450 oxidation catalysis for chemical synthesis are discussed [41].

Cytochrome P450 monooxygenase (CYP63A2) from the model ligninolytic white rot fungus *Phanerochaete chrysosporium*, that was found to possess a broad oxidizing capability toward structurally diverse hydrocarbons belonging to mutagenic/carcinogenic fused-ring higher-molecular-weight polycyclic aromatic hydrocarbons (HMW-PAHs), endocrine-disrupting long-chain alkylphenols (APs), and crude oil aliphatic hydrocarbon *n*-alkanes was reported [42]. A homology-based three-dimensional (3D) model revealed the presence of an extraordinarily large active-site cavity in CYP63A2 compared to the mammalian PAH-oxidizing

(CYP3A4, CYP1A2, and CYP1B1) and bacterial aliphatic-hydrocarbon-oxidizing (CYP101D and CYP102A1) P450s. This structural feature in conjunction with ligand docking simulations suggested potential versatility of the enzyme. Experimental characterization using recombinantly expressed CYP63A2 revealed its ability to oxidize HMW-PAHs of various ring sizes, including 4 rings (pyrene and fluoranthene), 5 rings [benzo(α)pyrene], and 6 rings [benzo(ghi) perylene], with the highest enzymatic activity being toward the 5-ring PAH followed by the 4-ring and 6-ring PAHs, in that order [42]. Recombinant CYP63A2 activity yielded monohydroxylated PAHs metabolites. The enzyme was found to also act as an alkane omega-hydroxylase that oxidized *n*-alkanes with various chain lengths (C9 to C12 and C15 to C19), as well as alkyl side chains (C3 to C9) in alkylphenols (APs). CYP63A2 showed preferential oxidation of long-chain APs and alkanes. The authors postulated that this is the first P450 identified from any of the biological kingdoms that possesses such broad substrate specificity toward structurally diverse xenobiotics (PAHs, APs, and alkanes), making it a potent enzyme biocatalyst candidate to handle mixed pollution, *e.g.* crude oil spills [42].

Over the years, the researchers have developed a series of deuterated and fluorinated variants of the hydrocarbon substrates as probes to gain insights into the controlled C–H oxidations of hydrocarbons facilitated by the enzymes [43]. They have published the review to illustrate the application of these designed probes in the study of three monooxygenases: (i) the particulate methane monooxygenase (pMMO) from *Methylococcus capsulatus* (Bath), which oxidizes straight-chain C1–C5 alkanes and alkenes to form their corresponding 2-alcohols and epoxides, respectively; (ii) the recombinant alkane hydroxylase (AlkB) from *Pseudomonas putida* GPol, which oxidizes the primary C–H bonds of C5–C12 linear alkanes; and (iii) the recombinant cytochrome P450 from *Bacillus megaterium*, which oxidizes C12–C20 fatty acids at the omega-1, omega-2 or omega-3 – CH positions [43].

According to the periodic literature both enzymatic and abiotic factors (such as UV light) can mediate the initial oxidation of polyethylene chains, and given the chemical similarity between polyethylene and olefins it has been suggested that the metabolic pathways for degradation of hydrocarbons can be used once the size of polyethylene molecules decrease to an acceptable range for enzyme action (typically from 10 to 50 carbons) [44]. The long-range structure and morphology of polyethylene have shown important roles, with amorphous regions being more prone to microbial attack than the crystalline ones. The work indicated focuses on the recent hypotheses and experimental findings regarding to the oxidative biodegradation of polyethylene [44].

Summary

As demonstrated in the above mentioned review the enzymes engaged as catalysts for oxidation of C–H bond in the presence of dioxygen and hydrogen peroxide were: 1) oxidases: peroxidase, lighin peroxidases, chloro-peroxidases, xanthine oxidase, horseradish peroxidase, laccase, manganese peroxidase; 2) monooxygenases: different cytochromes P450, methane monooxygenase, alkane hydroxylase. It should be noted that one enzyme was specified [42], Cytochrome CYP63A2, which was estimated as the first P450 identified from any of the biological kingdoms that possesses broad substrate specificity toward structurally diverse xenobiotics – higher-molecular-weight polycyclic aromatic hydrocarbons, long-chain alkylphenols, and crude oil aliphatic hydrocarbon n-alkanes. It makes this enzyme an effective handler of mixed hydrocarbon pollutions, *e.g.* crude oil spills or contaminants.

The substrates that have been oxidized or degraded or hydroxylated by the enzymes were: 1) polycyclic aromatic hydrocarbons and their functional derivatives: a) monocyclic – toluene, *p*-alkyl (C3–C9) phenols, 1-metoxy phenol, *p*-cresol, styrene, *o*-, *m*-, *p*-xylenes, *p*-xylene glycol, aniline; b) bicyclic – naphthalene, 1-metoxy-naphthalene; c) tricyclic – anthracene, 1,2,9-methyl anthracenes, phenanthrene, acenaphthene, acenaphthylene, dibenzothiophene, carbazole; d) tetracyclic – pyrene, fluoranthene, benzo([α]anthracene; e) pentacyclic – benzo[α]pyrene; f) hexacyclic – benzo(ghi)perylene; 2) monoterpenes; 3) alkanes C1–C5, C5–C17 and C9–C12; 4) alkenes C1-C5; 5) C12-C20 fatty acids; 6) different organosulfides and thiophenes presented in the hydrocarbon fuels; 7) organophosphorus compounds (pesticides); 8) azo-dyes; 9) polyethylene 10) buckminsterfullerene (C_{60}); and 11) single-walled carbon nanotubes (SWCNTs).

The products resulting from the oxidation were: quinones, anthraquinones, pyrenequinones, phenols, different enantiomers of terpineols (monoterpene alcohols), terephtalic acid, sulfoxides, sulfones, oxygen-containing surface functionalities (in the case of fullerene C_{60} and SWCNTs), higher oligomer and polymer products, low-molecular alcohols and epoxides, oxyacids, and desulfurized products (in fuels).

3. Conclusions

Aromatic hydrocarbons – mono- and polycyclic compounds are most amenable to the enzymatic catalytic oxidation by dioxygen and hydrogen peroxide. This oxidation affords compounds containing keto- and

hydroxyl groups, *i.e.* the enzymes are able to catalyze selectively generation of only initial stable products of a long and complex oxidation transformation cycle. The next compounds of the oxidation cycle: aldehydes, oxy-acids, ethers and polymeric products may be obtained by the enzymatic oxidation of the respective starting alcohols, acids and recombining active intermediates.

The enzymes considered, oxidases and monooxy-genases, are able to catalyze oxidation and hydroxylation of linear alkanes, alkenes, terpenes as well as nanocarbons with obtaining oxygen containing primary (–C=O, –OH) functionalities.

The enzymes are extremely useful as effective remedy for desulfurization of hydrocarbon fuels, *i.e.* universal degrader of organic pollutants.

Acknowledgements

References

[1] Que L. and Tolman W.: Nature, 2008, **455,** 333.
[2] Hayaishi O. (Ed.): Molecular Mechanisms of Oxygen Activation. Elsevier, Amsterdam 2012.
[3] Ayala M. and Torres E.: Appl. Catal. A, 2004, **272**, 1.
[4] Straathof A., Panke S. and Schmid A.: Curr. Opin. Biotechnol., 2002, **13**, 548.
[5] Schmid A., Hollmann F., Park J. and Buhler B.: Curr. Opin. Biotechnol., 2002, **13**, 359.
[6] van Beilen J., Duetz W., Schmid A. and Witholt B.: Trends Biotechnol., 2003, **21**, 170.
[7] Seifert W.: Fortschritte der Chemie Organischer Naturstoffe/Progr. in the Chem. of Org. Nat. Products, 1975, **32**, 1.
[8] Seifert W., Gallegos E. and Teeter R.: J. Am. Chem. Soc., 1972, **94**, 5880.
[9] Zaks A. and Klibanov A.: Science, 1984, **224**, 1249.
[10] van Beilen J., Li Z., Duetz W. *et al.*: Oil & Gas Sci. and Techn., 2003, **58**, 427.
[11] Buhler B. and Schmid A.: J. Biotechnol., 2004, **113**, 183.
[12] Duetz W., van Beilen J. and Witholt B.: Curr. Opin. Biotechnol., 2001, **12**, 419.
[13] van Beilen J. and Funhoff E.: Curr. Opin. Biotechnol., 2005, **16**, 308.
[14] van Beilen J. and Funhoff E.: Appl. Microbiol. Biotechn., 2007, **74**, 13.
[15] Piontek K., Smith A. and Blodig W.: Biochem. Soc. Transact., 2001, **29**, 111.
[16] Yadav M., Singh S., Sharma J. and Yadav K.: Environ. Techn., 2011, **32**, 1287.
[17] Torres E., Tinoco R. and Vazquez-Duhalt R.: Water Sci. Techn., 1997, **36**, 37.
[18] Vazquez-Duhalt R., Westlake D. and Fedorak P.: Appl. Environ. Microbiol., 1994, **60**, 459.
[19] Park J. and Clark D.: Biotechn. & Bioeng., 2006, **94**, 189.

[20] Kaup B., Piantini U., Wust M. and Schrader J.: App. Microbiol. Biotechn., 2007, **73**, 1087.
[21] Morgan J., Lu Z. and Clark D.: J. Mol. Catalysis B, 2002, **18**, 147.
[22] Terres E., Montiel M., Le Borgne S. and Torres E.: Biotechn. Lett., 2008, **30**, 173.
[23] Ayala M., Robledo N., Lopez-Munguia A. and Vazquez-Duhalt R.: Environ.l Sci. & Techn., 2000, **34**, 2804.
[24] Singh M., Kumar M., Kalsi W. *et al.*: US Pat. 20090217571, Publ. Sept. 3, 2009.
[25] Hernandez C., Diogo S. and Eliane D'Elia: Electrochem. Commun., 2008, **10**, 108.
[26] Entezari M. and Petrier C.: Ultrasonics Sonochem., 2003, **10**, 241.
[27] Fang J. and Barcelona M.: Chemosphere, 2003, **50**, 105.
[28] Mantha R., Biswas N., Taylor K. and Bewtra J.: Water Environ. Res., 2002, **74**, 280.
[29] Pardieck D., Bouwer E. and Stone A.: J. Contaminant Hydrology, 1992, **9**, 221.
[30] Torres E., Bustos-Jaimes I. and le Borgne S.: Appl. Catalysis B, 2003, **46**, 1.
[31] Zhang C., Chen W. and Alvarez P.: Environ. Sci. & Techn., 2014, **48**, 7918.
[32] Li X., Cheng Q., Wu Y. *et al.*: Pedosphere, 2014, **24**, 359.
[33] Liu Y. and Hua X.: RSC Adv., 2014, **59**, 31120.
[34] Gitsov I., Simonyan A., Wang L. *et al.*: J. Polym. Sci. A, 2012, 50, 119.
[35] Mayolo-Deloisa K., Machin-Ramirez C., Rito-Palomares M. and Trejo-Hernandez M.: Chem. Eng. & Techn., 2011, 34, 1368.
[36] Li X., Lin X., Yin R. *et al.*: J. Health Sci., 2010, **56**, 534.
[37] Acevedo F., Pizzul L., Gonzalez M. *et al.*: Chemosphere, 2010, **80**, 271.
[38] Ibrahim M., Ali H., Taylor K. *et al.*: Water Environ. Res., 2001, **73**, 165.
[39] Akhrem A., Metelitsa D. and Skurko M.: Rus. Chem. Rev., 1975, **44**, 398.
[40] Shoji O., Wiese C., Fujishiro T. *et al.*: JBIC J. Biolog. Inorg. Chem., 2010, **15**, 1109.
[41] Fasan R.: ACS Catalysis, 2012, **2**, 647.
[42] Syed K., Porollo A., Lam Y. *et al.*: Appl. & Environ. Microbiol., 2013, **79**, 2692.
[43] Chen Y., Luo W., Yang C. *et al.*: J. Inorg. Biochem., 2014, **134**, 118.
[44] Restrepo-Florez J., Bassi A. and Thompson M.: Int. Biodeter. & Biodegrad., 2014, **88**, 83.

ФЕРМЕНТАТИВНИЙ КАТАЛІЗ ОКИСНЕННЯ ВУГЛЕВОДНІВ «IN VITRO» (ОГЛЯД)

Анотація. Розглянуто важливі проблеми сучасної хі-мії – швидкий і селективний перебіг реакції за звичайних умов, як це відбувається в живих організмах, імітація процесів з життя живої природи, вивчення механізму дії ферментів. Представлено інформацію про останні досягнення в області ферментативного каталізу окиснення вуглеводнів. Приведено огляд джерел літератури за останні десятиліття з викорис-танням електронної бази Thomson Reuters (Web of Science).

***Ключові слова:** ензим, оксиредуктаза, вуглеводень, аеробне окиснення, біокаталіз, органічний забруднювач, біодеструкція.*