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Application of mushroom cultures and isolated enzymes for biodegradation of organic environmental pollutants

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Summary:

White-rot fungi can degrade a wide spectrum of recalcitrant organopollutants, often carcinogenic, mutagenic or toxic, that arise from industrial operations, petroleum released into environment and plant protection. These include polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls (PCB), polychlorinated phenols, organophosphorus compounds, even neuroparalytic VX gases. The oxidoreductases, that are part of the ligninolytic complex of mushroom belonging to the class of *Basidiomycetes*, play an increasingly important role in this process. The stability of these extracellular enzymes, their good solubility, and a multitude of catalyzed reactions enable their use in biodegradation of organic environmental pollutants. This review focuses on use of white-rot fungi in bioremediation, to highlight the numerous possibilities for the application of this organisms as well as isolated fungal oxidoreductases in degradation of organic pollutants

Key words: biodegradation, white-rot fungi, laccase, *Basidiomycetes*, organopollutants.

Introduction

The detrimental consequence of intensive industrial growth during the twentieth century was pervasive pollution of natural environment with man-made toxic compounds, called xenobiotics. Despite developing environmental awareness and introduction of international regulations concerning release of the xenobiotics, their concentration in soil, sludge, surface water and living organisms constantly increase. The most dangerous pollutants ubiquitous in most ecosystems are aromatic hydrocarbons and their halogenated compounds. Polychlorinated biphenyls (PCBs) were extensively applied in chemical industry, primarily as transformer oil components and pesticides (DDT). Ability of PCBs to bioacumulate in food chain accompanied by their wide distribution in ocean waters pose health risk to both wildlife and humans [1]. Products of PCBs hydroxylation (HO-PCBs) interact with hormone receptors leading to serious endocrine disorders in mammals [2]. Similar alterations in hormone metabolism can be caused by dibenzofurans, mostly butyl benzyl phthalate (BBP) used as plasticizer in polyvinyl chloride (PCV) and leaching from wasted plastic products into environment. BBP also can easily accumulate in tissues of aquatic organisms and together with

polychlorinated dibenzo-p-dioxins (PCDDs), introduced to ecosystem mostly in herbicides, can cause teratogenesis, neoplastic transformation and thymic atrophy [3, 4]. The physicochemical properties of those compounds include good stability, liphophilicity and high biological activity, preserved in a large proportion of their metabolites. Due to their extreme resistance to environmental degradation, PCBs, PCDDs and dibenzofurans were classified as persistent organic pollutants (POPs) and severely restricted by the United Nation Environment Programme. Neutralization of the other group of xenobiotics, polycyclic aromatic hydrocarbons (PAHs), is also hindered by their lipophilicity. They are strictly bound to soil particles and therefore could not be presented for bacterial degradation [5]. Human exposure on PAHs toxic effects including carcinogenesis and mutagenesis is wide, since they are extensively distributed into natural environment as the major components of petroleum. Synthetic azo dyes are other additives in petroleum products. In a quantity as large as 10-15% they are released in the waste water from dyeing industries [6]. Synthetic dyes are usually recalcitrant to bacterial degradation and their prospective metabolites are carcinogenetic [7]. Alkylphenols (AP) and their derivatives are detergents and emulsifiers prevalently used in various industrial processes and has been proved to persist in water, atmosphere, soil and food [8]. Aromatic structure incorporating fluorine is responsible for limited biotransformation of fluoroquinolone antibiotic, enrofloxacin (EFL). EFL is used in veterinary treatment of livestock and pets and can be spread in soils and pastures by fertilization with animal waste [9].

Considerable research attention has focused on the effective and environmental friendly methods for elimination or neutralization of resistant toxic pollutants. Bioremediation, involving naturally occurring microorganisms, is one of the currently investigated methods. In studies on biodegradation of severely resistant aromatic and halogenated hydrocarbons, the white-rot *Basidiomycetes* and their oxidoreductases, laccases, has shown promising results [2-9].

Fungal oxidoreductases

White-rot fungi belonging to the class of *Basidiomycetes* and Ascomycetes are unique in their ability to completely degrade lignin. This process enable fungal redox enzymes: lignin peroxidases (LiP), Mn-dependent peroxidases (MnP), versatile peroxidases (VP), other peroxidases, laccases, and tyrosinases [10]. The redox potentials of the mentioned above enzymes, which are the determinants of their oxidizing properties, are high and equal for LiP, VP, MnP, peroxidase, laccase and tyrosinase: 1.2–1.5 V , ~1.1 V, ~1.0 V, 0.7–0.9 V, 0.26–0.35 V, respectively [11]. These enzymes are non-specific, non-stereoselective, therefore able to transform a broad spectrum of organic pollutants, including polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) [12-14]. It has been even reported to successfully degrade the nerve gases by use of fungal oxidases [15]. The lignolytic enzymes of white rot fungi are active extracellulary, therefore these organisms are better candidates for the biotransformation of apolar pollutants than non-lignolytic microorganisms [16]. Among the fungal oxidoreductases, the laccases are probably the most studied group of enzymes, that catalyze transformation of a large number of the phenolic and nonphenolic aromatic compounds.

Mushroom laccases, structure and mechanism of action

Fungal laccases are glycoproteins with molecular weight of 50-130 kD and about of 10-20% the carbohydrate contents. Probably the carbohydrate moiety of the laccase molecule is responsible for the stability of the protein globule [17]. The carbohydrate moiety has also been supposed to protect the enzyme molecule against proteolysis and inactivation by free radicals [18-19]. Laccase belong to a small group of enzymes termed as blue oxidases, cuproproteins containing in their active site four copper atoms [20]. According to their electron paramagnetic resonance (EPR) features they are clasiffied as: Type I or blue, Type 2 or normal and Type 3 or coupled binuclear copper site (Fig.1). The oxidation of various xenobiotics, catalysed by laccase, is combined with the simultaneous reduction of oxygen to water. The active site of laccase in the binding and multielectron reduction of dioxygen, consists of the Type 2 and Type 3 copper atoms [21] (Fig.1).

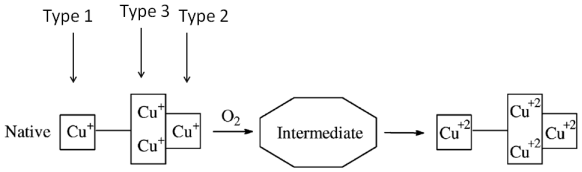

Figure 1: Reactivity of laccase with oxygen (from [86]).

During the oxidation of the substrate, reactive radicals may be formed, which are involved in further non-enzymatic reactions. In summary, the catalytic action of laccases can be divided into two stages: (1) an enzymatic oxidation of the substrate with formation of reactive intermediates – aryl or phenoxy radicals either quinines, (2) non-enzymatic spontaneous reactions of free radicals giving the product as a result [14] (Fig.2).

Figure 2: The catalytic action of laccases in two stages process.

The above-described processes can result in: substrate polymerization via radical cross-coupling or substrate degradation via bond cleavage, aromatic ring opening, demethoxylation, demethylation, substituents release, etc. The oxidation of xenobiotics catalysed by laccase may occur as a direct, or as a mediated reaction. The direct, mediator-free oxidation is based on the possibility of immediate exchange of the electron between the active site of the enzyme and the substrate in the absence of mediator [22, 23]. This type of reaction can take place only in the case of oxidation of simple organic compounds, e. g. mono- di- and polyphenols [24] (Fig 3a).

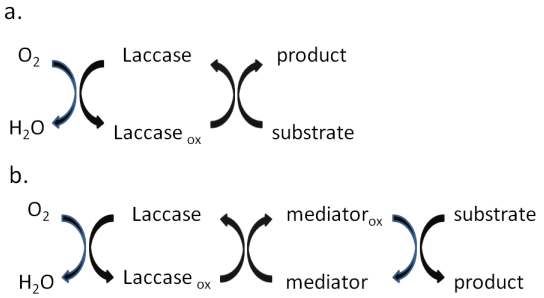

Figure 3: The oxidation of xenobiotics catalysed by laccase as a direct (a), and as a mediated (b) reaction.

The range of compounds which can be oxidized with involvement of laccases may be enlarged by use of so called redox mediators. These are laccase substrates that produce high-potential intermediates as a result of enzymatic oxidation. Those intermediates can chemically react with the substrates, which cannot be oxidized with involvement only of laccases. Then, the oxidized mediator is reduced to the initial state by the compound subjected to oxidation, and thus a closed cycle is created [25,26]. This mechanism is essential in the case of oxidation of the molecules that exhibit high redox potential, e.g. non-phenolic compounds, aromatic amines, polynuclear aromatic compounds (Fig 3b).

For bioremediation, understood as series of processes directed for biotransformation of a pollution-changed environment in order to recover its initial state, laccases are very promising owing to their wide substrate specificity and ability to degrade many phenolic compounds [27-30]. Oxidation of toxic substrates by laccase with production of insoluble products and the subsequent separation of the precipitate is a promising approach to purification of industrial sewage. Organophosphorus compounds (in particular, various insecticides and neuroparalytic poisons) are very toxic and cannot be sufficiently hydrolyzed nonenzymatically. Purified laccase from the fungus *P. ostreatus* in the presence of ABTS completely and at high rate oxidatively destroyed neuroparalytic VX gases and the insecticide diisopropylamiton [15].

Mycoremediation

According to the Environmental Protection Agency definition, bioremediation is the treatment that uses naturally occurring organisms to break down hazardous substances into less toxic or non toxic substances. It should be interpreted as a waste management technique, that involves the use of organisms to remove or neutralize pollutants from a contaminated site. Mycoremediation in turn, is a form of bioremediation that uses conditioned native fungi or fungal mycelium to remove and degrade contaminants [10]. The unique enzymatic apparatus of *Basidiomycetes* fungi allows their use in bioremediation, to degrade extremely toxic contaminants, that are not transformed by other organisms.

The technologies used in mycoremediation may be generally classified as in situ or *ex situ*. In situ bioremediation involves treating the contaminated material at the site. This technique requires the introduction of wood-degrading fungi in the soil, where are confronted with an environment different from wood in many aspects. *Ex situ* methods, however, involve the removal of the contaminated material, to be treated in the apparatus appropriately adapted for the cultivation of mushrooms (bioreactors). Most often for the biodegradation of contaminants a fungal mycelium or isolated mushroom enzymes are used.

Ex situ methods in mycoremediation

White rot fungi do not assimilate organic pollutants as a sole carbon source, therefore they require cometabolites to degrade them [10]. The degradation process is performed either in solid substrates mixed with contaminated soils, such as wood chips, wheat straw, peat, sawdust, a nutrient-fortified mixture of grain and sawdust, bark, rice, annual plant stems and wood, spent mushroom compost, or in different liquid-phase in bioreactors. Fungal bioreactors are also termed *mycoreactors*. Different *mycoreactors* are operated under aerobic or anaerobic conditions [10]. The most common is submerged cultivation of mushroom mycelia in stirred tanks.

Ex situ bioremediation may also be performed using isolated oxidoreductase enzymes of fungal origin, in particular laccases, native or immobilized [31-39].These processes are also carried out in the liquid phase, in the bioreactors of appropriate to the process construction.

Degradation of polychlorinated biphenyls (PCB)

White-rot fungi have shown their ability to degrade many complex mixtures of PCBs and single congeners. Degradation of these xenobiotics is also mediated by a lignin-degrading enzyme system. Besides the most widely examined species *Phanerochaete chrysosporium* [40], several other white-rot fungi ,e.g., *Pleurotus ostreatus* [41, 42], *Coriolopsis polysona* [43, 44], *Coriolus (Trametes) versicolor* [41, 45, 46]. *Bjerkandera adusta* [41], *Lentinula edodes* [47, 48] are also known to metabolize PCBs.

Degradation of polycyclic aromatic hydrocarbons (PAH)

Bacterial bioremediation of the PAH polluted soil is hampered by a very low solubility in water and, therefore, low availability for bacteria. The observation, that white rot fungi can oxidize PAH with their lignolytic enzyme systems, has raised interest in their use for bioremediation of PAH polluted soils [49]. The promising strains for PAH detoxification are able to degrade even 90% of the organic pollutants added to the fungal cultures. The white rot fungus *Phanerochaete chrysosporium* is able to degrade simultaneously all polycyclic aromatic hydrocarbons termed as BTEX compounds (benzene, toluene, ethylbenzene, xylenes) [50, 51]. The other mushroom genus, Trametes trogii was found to be able to decompose nitrobenzene and antracene, added to the cultivation medium in concentration of 500 ppm [52].

Fungal degradation of dioxins

Dioxins, some of the most toxic compounds known to science, are released in the environment as the by-products of several industrial processes involving chlorine, and are found as impurities in pentachlorophenol, which is used as a wood preservative. These compounds are highly stable and lipophilic in nature. Fungi are the most effective degraders of dioxins. A white-rot fungus, *Phlebia lindtneri*, has been shown to be able to transform polychlorinated dibenzo-p-dioxins (PCDDs), such as 2,7-dichloro-, 2,3,7-trichloro-, and 1,2,6,7 – and 1,2,8,9-tetrachlorodibenzo-p-dioxins to hydroxylated and methoxylated compounds [10, 53]. The degradation of the PCDDs is related to the chlorination patterns of these substrates; the degradation rate of 1,2,6,7-tetrachlorodibenzo-p-dioxin is higher than that of 2,3,7-trichlorodibenzo-p-dioxin. *P. lindtneri* also metabolized 2,8-dichlorodibenzofuran (2,8-diCDF) to hydroxy-diCDF [10,54].

Three transformants of Coriolus hirsutus efficiently converted 2,7/2,8-dichlorodibenzo-p-dioxins n [55]. Phanerochaete sordida degrades nearly 60% of 2,3,7,8 – tetrachlorodibenzo-p-dioxin [56]. Valli *et al*. [57] suggested a multistep pathway for the degradation of 2,7-dichlorodibenzo-p-dioxin by LiP and MnP of *Phanerochaete chrysosporium*. These findings

suggest the possibility of use of fungal cultures and isolated enzymes in the bioremediation of dioxin-contaminated soils.

Degradation of pesticides

Due to the extreme toxicity and persistence in the environment, they have been the target of research throughout the world for the past four decades. Selected white-rot fungi can be used in bioremediation systems, but mainly in situ. However, several instances of liquid and solid state fermentation may be cited. For instance: Pleurotus pulmonarius degrades more than 75% of atrazine in liquid culture and produces mainly N-dealkylated metabolites [58], *Hypholoma fasciculare* and *Stereum hirsutum* are the best degraders of terbuthylazine in liquid culture [59], *Trametes versicolor* exhibited the highest ability to degrade atrazine in liquid media. *H. fasciculare* attained a high level of degradation of atrazine in the biobed matrix. Solid-state fermentation is also suggested as a means to detoxify carbofuran and atrazine [60].

Degradation of organophosphorus compounds

The organophosphorus insecticides extensively used in agriculture mostly are easily degraded by bacteria. However, number of them appear to be moderately persistent and have half-lives of several months – in these cases bioremediation using fungal cultures appears to be useful. There are several examples of the efficient process of decomposition pollutants by fungal cultures e.g.: transformation of malathion to β-monoacid and dicarboxylic acid occurs due to carboxyesterase activity by *Aspergillus oryzae* [61], *Penicillium waksmanii* isolated from flooded acid sulfate soil is able to degrade parathion to aminoparathion [62], the chlorinated pyridinyl ring of chlorpyrifos and the phenyl ring of fonofos undergo cleavage during biodegradation by *P. chrysosporium* [63]. A mixed population of several fungi species, such as *Alternaria alternata*, *Cephalosporium* sp., *Cladosporium cladosporioides*, *Cladorrhinum brunnescens*, *Fusarium* sp., *Rhizoctonia solani*, and *Trichoderma viride*, reveal the degradation of chlorpyrifos in liquid culture [10, 64]. The white-rot fungus, *Trametes versicolor* degrade chlorpyrifos in a biobed matrix [65].

Decomposition of azo dyes

Synthetic dyes, employed in the textile, paper, cosmetic, pharmaceutical, and food industries, are highly stable. Dye color in industrial wastewaters is one of the most important environmental concerns in the textile industry. 10 to 15% of dyestuffs are released into the water unutilized [66]. Therefore the search for the eco-friendly biodegradation process of these compounds is gained maximal attention. The white rot fungus *Phanerochaete chrysosporium* has been shown to completely degrade synthetic dyes due to its extracellular enzymatic system. [67, 68, 69]. Of 26 white-rot fungi, 10 strains decolorize and decompose all structurally different dyes and produce laccase, lignin peroxidase (LiP), and manganese peroxidase (MnP) on solid or liquid medium [70].

Ex situ methods in mycoremediation

The potential of *Basidiomycetes* to neutralize aromatic hydrocarbons has been confirmed in the research studies on isolated mycelial cultures [2, 4,5,9]. Application of white rot fungi in bioreactor systems mostly affected decolourization of reactive azo dyes and has been performed mostly for experimental purposes [7,71,72]. However, in order to perform a large-scale decontamination of polluted environment implementation of in situ bioremediation is required. Results of the recent studies on field use of white-rot fungi in decontamination are ambiguous ([73,74]. Despite physiological differences between wood-inhabiting and soil-inhabiting *Basidiomycetes*, several wood-degrading species can survive in the soil and preserve their ability to produce extracellular ligninolytic enzymes. Those species are characterized by strong competitive abilities, since interaction with indigenous organisms is the main factor impairing colonization of soil by wood-inhabiting *Basidiomycetes* [75]. The most successful competitors affect both the count and composition of the soil bacteria. It has been shown that *Pleurotus ostreatus* and *Phanerochaete chrysosporium* representing the competitive ligninolytic fungi caused 50-70% decrease in soil total bacterial biomass [76,77].

Concurrently, metabolic activity of wood-degrading *Basidiomycetes* is a source of simple organic substrates utilized by mycoparasitic and opportunistic bacteria. The level of inhibition of native soil bacteria appears therefore to depend on their relation with introduced wood-inhabiting fungi and availability of lignocelluloses-based nutrients in soil [78].

Organic nutrient content influences also the growth of ligninolytic *Basidiomycetes*. Comparing with wood, soil contains low amounts of available carbon and nitrogen varying with different soil types. Insufficient nutrition is the important factor impairing colonization of soil by white-rot fungi [79]. Addition of external substrate in form of lignocelluloses is required in order to successfully introduce wood-degrading fungi into soil and stimulate production of extracellular ligninolytic enzymes [78, 80]. Nevertheless, fungi are able to transport nutrients through mycelial cords supporting growth of mycelium also in locations with low substrate content.

The production of fungal oxidoreductases, including laccases, is essential in the proccess of bioremeditation. Laccase activity is mainly induced by the presence of phenolic substrates [81]. Among other factors affecting laccases production in soils, temperature is of great significance. Low temperatures decrease growth of fungal colonies and their enzymatic activity [82]. Concerning application of laccases in bioremediation in situ, the impact of seasonal temperature changes in temperate climatic zone is non-negligible. Interesting aspect of laccase regulation is increase in activity in response to the presence of xenobiotic compounds and concurrent soil microorganisms [83, 84]. This phenomenon has been considered the 'stress reaction' responsible for defense from harmful factors and appears to be advantageous in the context of effective neutralization of organic contaminants [75].

Some toxic compounds, mainly heavy metals, may however reduce ligninolytic activity of extracellular enzymes [85]. This problem, together with temperature demands, requirement of cooperation with native soil bacteria (different nutritional preferences), preparation of contaminated site to treatment (homogenization, substrate addition) and selection of fungal species appropriate for the type of encountered toxic agent are the major limitations for use of wood-inhabiting *Basidiomycetes* in large-scale soil bioremediation in situ.

Conclusions

Idea to use fungi to degrade pollutants dates back more than 30 years and bases on the discovery of the extracellular oxidative ligninolytic fungal enzymes. The aim of this review was to show the multitude of applications of white rot fungi, the intact organisms or isolated enzymes thereof, in a variety of biotechnological applications. The application recommended in this review is bioremediation or waste treatment. The search for low-cost methods for biodegradation of pollutants justifies favoring the use of the whole microorganism or unpurified culture broth. As demonstrated by several studies, mushroom ligninolytic enzymes can degrade the pollutants that otherwise would be difficult to eliminate from the environment. The application of the pure, isolated enzymes or of mixtures with defined composition ensures specific reactions of substrates without side-products being formed. The successful implementation of the mycoremediation to the technical scale involves, according to Singh [10] the development of three phases of the process:

- the inoculum preparation techniques and their improvements,
- the clear technical protocols for the final design and associated engineering processes,
- the remediation protocols for the monitoring, adjustment, continuity, and maintenance of the engineering system.

The significant part of biotechnological processes described in this work is still is at the stage of preliminary experiments. However, the processes using white-rot fungi for degradation of the environmental pollutants have been patented. Recently a few companies, including Gebruder Huber Bodenrecycling in Germany and Earth-Fax Development Corporation inUnited States employ white rot fungal cultures for soil bioremediation and a broader use probably will take place in the future.

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