Review article

Nitroaromatic enzymatic biodegradation system in *Phanerochaete chrysosporium*

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Abstract**:** *Phanerochaete chrysosporium is an ubiquitous fungus having huge potential for application in biodegradation processes. Its enzymatic system, consisting of ligninases, membrane-associated oxidases and hydrogen peroxide generating enzymes is capable of degrading a wide range of pollutants like 2,4,6-trinitrotoluene, 2,5-dinitrophenol, 3,5-dinitrosalicylic acid or azodyes produced by military or civilian industry. Synergetic action between enzymes, based on providing substrates essential for their activity and their extreme low-specificity guarantees successful degradation of recalcitrant pollutants. Nevertheless, a development of a technique, taking into the account the type of pollutant, its concentration in the environment, its metabolic pathway and maintenance of the system is required. This paper presents a literature survey related to enzymatic system of a white rot fungus Phanerochaete chrysosporium and its potential application in biodegradation processes.*

Keywords: *applied biochemistry, biochemical engineering, biotechnology, biotransformation, denitrification, detoxification.*

Introduction

Nitrocompounds belong to the group of organic substances containing one or more nitro (-NO_2) groups within their aliphatic or aromatic structures. Vast majority of them are known for toxicity [1], cancerogenic properties [2] and strong recalcitrance to biodegradation. They also tend to accumulate in environment [3, 4, 5], what associated with their massive release into the atmosphere from military production and fossil fuel combustion pose a serious threat to the environment [5, 6, 7, 8]. Several methods were developed to deal with contamination of environment with nitrocompounds. Depending on the type of compounds, they were mostly based on physicochemical degradation including incineration and sonolysis [9],UV radiation, oxidation with H_2O_2, O_3 or Fentons's reagent [10, 11]. Such processes are usually supported or combined with

coagulation, adsorption and membrane processes [12, 13, 14]. Worth mentioning are high costs and limited efficiency of methods described above.

Therefore, biological methods are proposed as an alternative to physicochemical degradation, as under proper conditions they generate much lower costs with concomitant good biodegradation efficiency. An extensive research focused especially on microorganisms known for secretion of enzymes with low substrate-specificity, providing possibility to degrade a wide range of substrates. Their example are basidiomycetes belonging to white-rot fungi. They are capable of decomposing lignin [15, 16, 17], being the extremely recalcitrant natural polymer. Their effectiveness derives from the cooperation of three enzymes: manganese peroxidase, lignin peroxidase and laccase, secreted when the environment is short in nutrients [9].

This article is a literature survey of *Phanerochaete chrysosporium's* enzyme system enabling it to decompose very recalcitrant and toxic substances like nitroexplosives, azodyes or chloropesticides. Potential application of microbiological methods is also proposed.

White-rot fungus *Phanerochaete chrysosporium*

P. chrysosporium is a well-known basidiomycete, which genus can be find worldwide. It is a wood-inhabiting, saprobic microorganism forming an uniform white rot on the wood surface [18] and also forms a flat, reproductive fruiting bodies. It has branched hyphae network of 3-9 μm in diameter, ended with thick-walled chlamydospores 50-60 μm thick. Conidiophore has around 6-9 μm in diameter [19, 20]. *P. chrysosporium* is known to grow at various temperatures, with the optimal one for growth of 37°C [19].

Due to the synthesis of extracellular, low-specific enzymes it is known for its ability to degrade chemicals like 2,4,6-trinitrotoluene, 2,4-dinitrophenol, lindane, pentachlorobenzene, polychlorinated biphenyls, and azodyes [21, 22, 23, 24, 25, 26]. Moreover, the fungus is known to bioaccumulate heavy metals like lead, mercury, cadmium or selenium [27, 28, 29, 30].

Enzymatic system of selected white rot fungi

Synergetic work of enzymes secreted by *P. chrysosporium* is a key to efficient biodegradation of toxic compounds. It is based on the following enzymes: Manganese peroxidase (MnP; E.C.1.11.1.13), lignin peroxidase (LiP; E.C.1.11.1.14) and laccase (E.C. 1.10.3.2). Putatively, when the fungus encounters nitro-based compounds like TNT, an essential role in biodegradation is played by nitroreductases, reducing nitro groups to amino substituents through hydroxyamino- and nitroso- derivatives [31, 32, 33]. All work done by LiP and MnP is supported by hydrogen peroxide generating enzymes, providing the essential substrate for their activity. Selected properties of the aforementioned enzymes are described below.

Lignin peroxidase

This oxidoreductase possesses the high redox potential for the non-phenolic structure oxidation and is also able to oxidize various aromatics. Several isozymes of LiP from *P. chrysosporium* were described, differing in their physical properties, stability, specificity and activities[34, 35]. Their molecular mass ranges from 38 to 43 kDa [36, 37,38], detected activity within pH scale is set between 2.5 to 6, with optimum between 3 to 5, depending on the substrate used. Lignin peroxidase is also known for its thermal stability. While optimum temperature for its synthesis and catalysis usually is about 37°C, its activity was reported within the range 25 to 75°C [39, 40, 41, 42].

Lignin peroxidase is a hemeprotein consisting of about 350 amino acid residues. Its folding motif is made of sixteen helices – eight major α-helices, eight smaller ones and three antiparallel β-sheets [43]. Eight Cys residues of LiP are involved in four disulfide bonds, and the enzyme does not contain tyrosine residues.

Catalytic reactions of LiP include three steps. The first is oxidation of the resting ferric enzyme by H_2O_2, forming the oxo-ferryl intermediate (becoming deficient of $2e^-$), which is reduced by a substrate molecule in the second step, forming the second intermediate, deficient of $1e^-$. The second intermediate undergoes subsequent reduction by the reduced substrate hence completing the cycle [44, 45].

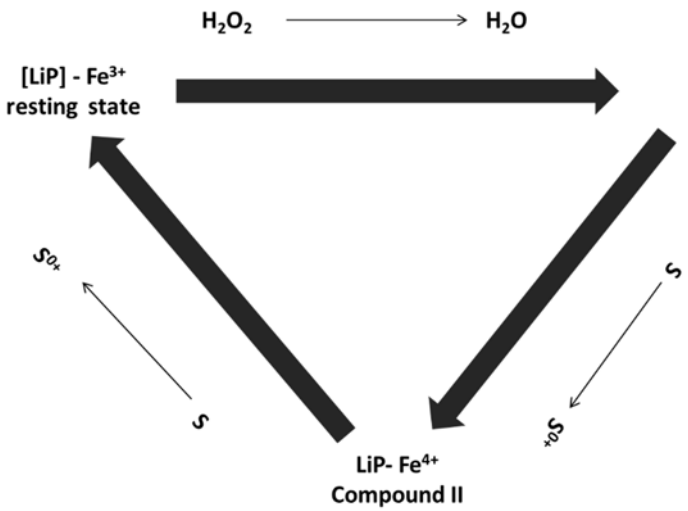

Figure 1. Catalytic cycle of lignin peroxidase [44]

Laccase

It is a copper-containing glycoprotein catalyzing oxidation of aromatic and aliphatic compounds using molecular oxygen[46, 47]. Usually, several isozymes of laccase are synthesized, depending on species. Their average molecular weights are around 66 kDa, including around 7-10% N-linked saccharides. Laccase has a very wide pH activity range, from 2 to 11, with the optimum at pH around 3.5-5.0 [48, 49]. Some of the isozymes are very thermostable. While optimum temperatures for the majority of enzymes from basidiomycetes is around 35-40°C, some laccases were active even at 90-100°C [50, 51]. There are some contradictive opinions about laccase synthesis by *P. chrysosorium*. Some authors suggest that many false positive reactions for laccase activity were caused by the high concentrations of Mn(II) ions, leading to oxidation of most commonly used reagent, 2.2'-azino-bis(3-etilbenz-tiazolin-6-sulfonate) (ABTS) [52]. Other authors report that laccase is synthesized in several isoforms [47, 53].

Fungal laccases usually form multimeric complexes consisting of isozymes. The enzyme is stabilized by carbohydrate residues. For the catalytic activity of an enzyme, four atoms of Cu per protein molecule are required [54]. Polypeptide chains of laccases consist of about 500 amino acid residues, with the highly conserved cluster of three copper ions (Type 3, the coupled copper-copper pair and Type 2, the single paramagnetic copper), coordinated by eight histidine residues and four other amino acid residues (two histidines, methionine and cysteine) coating the remaining copper atom (Type 1) [54, 55].

Laccases are capable of natural or synthetic polymers degrading, as well as aromatic ring cleavage using molecular oxygen as the second substrate. It derives from four monoelectronic oxidations at the copper type 1 and then transfer of $4e^-$ to the Type 2 and 3 cluster.

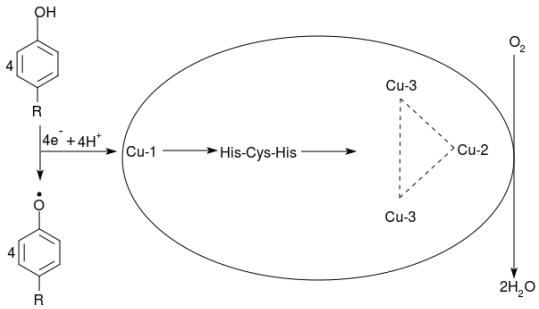

Figure 2. Catalytic cycle of laccase [56]

Manganese peroxidase

This heme enzyme, like LiP, catalyzes oxidation of various phenolic and non-phenolic compounds. The difference lies in substrate oxidized - manganese (II) ions, essential for any reaction catalyzed by MnP.

P. chrysosporium produces three isozymes of manganese peroxidase. Their molecular weight is between 40 and 46 kDa [57, 58]. Similarly to LiP, its active form's pH range is set between 3 to 8, with optimum, depending on the substrate investigated, between 3 and 5 [59, 60, 61]. MnP is less thermostable than LiP and laccase, it is active in the temperature range between 25 and 45°C, with optimum below 37°C [62, 63, 64]. MnP, like LiP, is a glycoprotein that forms monomeric structures, made of about 360 amino acid residues. The manganese binding site consists of two Glu residues, enabling the enzyme to oxidize Mn(II) to Mn(III), which becomes an oxidant of lignin and other substrates [65].

The catalytic cycle of manganese peroxidase resembles that of lignin peroxidase. The difference lies in electron donor for catalysis – Mn(II) ions. The first step is identical like in case of LiP – oxidation of the native ferric enzyme, providing intermediate I - Fe(IV) radical complex. The second step is an oxidation of Mn(II) to Mn(III) with subsequent formation of intermediate II – Fe(IV) , that can be reduced only in the presence of other Mn(II) ions to complete the cycle. As mentioned before, the Mn(III) ions obtained in the cycle and stabilized by organic acids attack organic molecules, leading to their reduction [66].The unique property of MnP is that its action on some compounds like glutathione or NADPH yields hydrogen peroxide as product, being by chance essential for its activity [67].

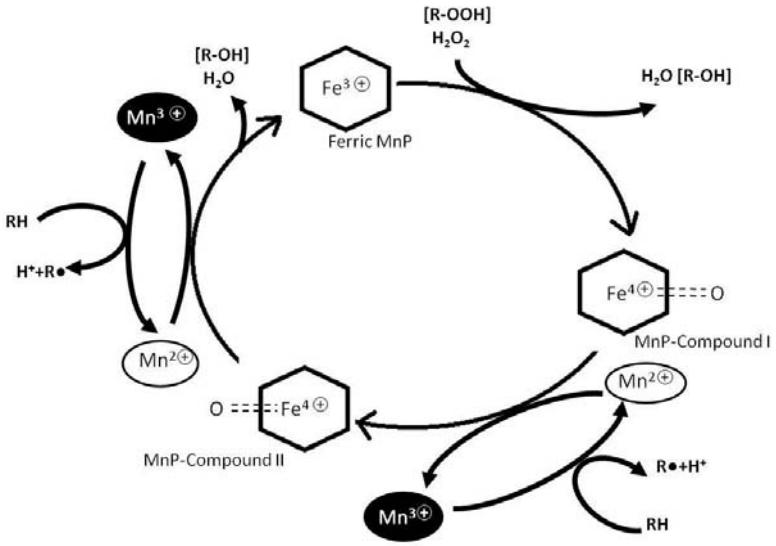

Figure 3. Catalytic cycle of manganese peroxidase [66]

Enzyme activity assay

In order to construct mentioned catalytic cycle, all LiP, MnP and laccase, it is required required to concentrate extracellular fluid and then use substrate specific for each enzyme. However, due to certain substrates used for activity assays, it is not required to perform purification or isolation, as each enzyme has well-examined and determined substrate, ensuring lack of interference. In case of LiP, one may choose either veratryl alcohol or methylene blue. In case of veratryl alcohol as a substrate, assay mixture contain 25mM tartrate buffer (pH = 2.5), 2 mM of substrate, 0.4 mM H_2O_2 and concentrated extracellular fluid, where H_2O_2 initiates the reaction. Reaction is monitored spectrophotometrically at A_{310}. For methylene blue method, the substrate (1.2 mM) is mixed with sodium tartrate buffer (0.5 M, pH = 4.5), extracellular fluid and 2.7 mM H_2O_2 for activation. The decrease in absorbance is measured at A_{664}[68, 69].

Manganese peroxidase assay is based on phenol red oxidation, monitored spetrophotometrically at A_{610}. Reaction mixture consists from the 0.1M substrate, 50mM sodium succinate and sodium lactate buffers (pH = 4.5), 0.1mM $MnSO_4$, 3mg/$mL_{mixture}$ of gelatin. Oxidation is initiated by addition of H_2O_2 [68].

Laccase protocol assay is based on oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)(ABTS). Reaction mixture consists of extracellular culture fluid and 14 μmol ABTS dissolved in glycine-HCl buffer (pH = 3.0). Reaction is monitored spectrophotometrically at A_{436}. However, it is essential to prevent H_2O_2 generation by other enzymes present in the solution [70].

Hydrogen peroxide generating enzymes

These enzymes - glyoxal oxidase (GLOX, E.C. 1.2.3.15), aryl-alcohol oxidase (AAO, E.C. 1.1.3.7) and pyranose oxidase (POX, E.C. 1.1.3.10) are not directly involved in fungal metabolism of nitrocompounds and lignin, but provide hydrogen peroxide, essential for manganese and lignin peroxidases activity [44,71].

They are either monomeric proteins with molecular masses of about 60 kDa (GLOX), 70 kDa (AAO) or tetramers with molecular mass of around 250 kDa (POX). Like peroxidases, they are active in the pH range between 5 and 9, with optimum close to 7 [72, 73, 74]. Also the dependence of the activity on temperature is similar – the activity was detected between 20 and 60°C, with optimum between 30 and 45 [69, 75].

Like ligninases, the hydrogen peroxide generating enzymes of *P. chrysosporium* have quite broad substrate specificity. Depending on enzyme, they catalyze conversion of several derivatives of aromatic alcohols(AAO), carbohydrates (POX) and aldehyde acids (GLOX), yielding the oxidized substrate and hydrogen peroxide [68, 70, 76].

Plasma membrane redox system

Reduction of some pollutants is a key to their successful degradation. In case of nitroaromatics like TNT, lack of any hydroxyl or amino group may significantly impede the decomposition process conducted by LiP/MnP/Laccase system It was identified that an enzyme catalyzing the reduction of nitro groups attached to the aromatic ring that enabled further decomposition by laccase or other peroxidases.

This enzyme, or a group of enzymes, are NAD(P)H- dependent oxidases bound to cell membrane [77]. Unfortunately, this plasma-membrane redox system, in terms of nitroreduction, has not been well identified and described yet. Putatively, not only does this system facilitate biodegradation of nitropollutants, but also protects the fungus against the damage caused by free radicals generated by lignin degradation system [78].

Table 1. Properties of *P. chrysosporium* ligninolytic enzymes

Enzyme	MW (kDa)	Number of isoforms	pH range (stability)	pH range (optimum)	Temp. range (°C) (stability)	Temp. range (°C) (optimum)
LiP	38-43	7	2.5-6	3-5	25-75	Around 37
MnP	40-46	3	3-8	3-5	25-45	≤37
Laccase	60	Unknown	2-11	3.5-5	20-100	35-40

Cooperation between selected enzymes

Synergistic action of described enzymes is a key to efficient decomposition of recalcitrant polymers like lignin or pollutants like nitroaromatics, chloropesticides or azo-dyes. But unless the environment or growth medium is limited in nitrogen or carbon, the enzymes are not synthesized [79, 80].

In case of lignin, the first step is its oxidation catalyzed by laccase, providing the substrate for hydrogen peroxide generation by glyoxal oxidase through different reactions like ring cleavage, ether linkage breakdowns or demethoxylation [81]. Glyoxal oxidase and other peroxide-generating enzymes, while not directly involved in lignin degradation, provide H_2O_2, essential for MnP and LiP activity. At this step, generation of oxidized lignin compounds occurs, yielding products like phenoxy radicals, which through carbon-carbon linkage breakdown turn into quinones. Now, through cooperation of plasma-membrane redox system and further reduction by laccase and peroxidase the superoxide cation radicals are generated, which contribute to further lignin oxidation and degradation.

Such system, efficient in case of lignin, enables also degradation of various xenobiotics. One of them is 2,4,6-trinitrotoluene (TNT), which is resistant to enzymatic attack of oxidoreductases but its nitro groups is reduced by the plasma-membrane redox system, yielding a mixture of aminodinitro, hydroxyamino and azoxy derivatives [82, 83]. This provides an opportunity for peroxidases to perform conversion of some of these products to quinones that results in further mineralization of TNT metabolites [84, 85]. The efficiency of total biodegradation

of the xenobiotic varied, but even up to 85% of investigated TNT was mineralized within 90 days in liquid cultures [86].

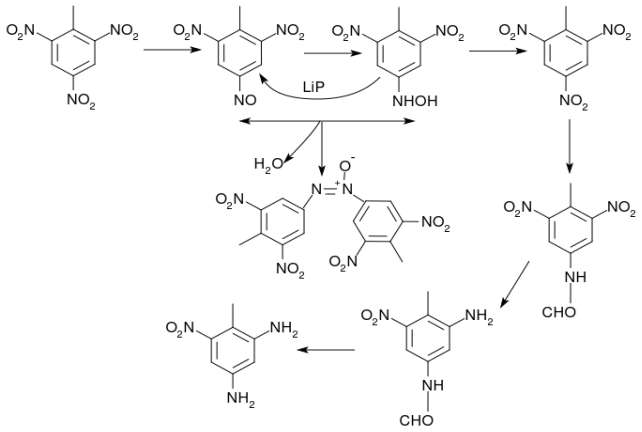

Figure 4. Reduction pathway of TNT through nitroso, hydroxylamino and azoderivatives [3]

However, some nitrocompounds do not require pretreatment with plasma-membrane redox system to be efficiently degraded. While textile azodyes undergone the similar pathway of reactions, including breaking N-N linkage, oxidation into quinones and further mineralization [87], most of them possess polar groups like hydroxyl (-OH) or carboxyl (-COOH), significantly facilitating enzymatic degradation, and making the plasma-membrane redox system almost redundant. The similar situation is encountered in case of another common nitropollutant – 2,4-dinitrophenol (DNP), decomposed with only laccase immobilized in the fungal mycelium, degrading more than 90% of substratein 12 h treatment [88]. The efficiency of the process was satisfyingas *P. chrysosporium* was capable of mineralizing up to 48% of initial concentration of selected azodyes within 12 days.

Worth mentioning is fact that despite good mineralization efficiency of pollutants like TNT in laboratory conditions, not only is biodegradation *in situ* much less efficient, but also part of metabolites, due to altered physicochemical properties, instead of being degraded may be e.g. entrapped in soil, accumulated in plants or may leak into ground water. For example – 2,4-dinitrotoluene – one of potential metabolites – is almost two times more soluble in water than TNT [89]. It is also essential to underline, that sometimes certain metabolites may be more toxic that initial compounds, like hydroxy- and aminoderivatives of TNT [90].

Discussion

Enzymatic system secreted by *P. chrysosporium*, along with its membrane-associated enzymes can be a powerful tool to be applied in bioremediation of polluted areas or disposal of toxic reaction byproducts that lack any usability. The

activity under various environmental conditions (wide pH and temperature range) significantly facilitates application of immobilized mycelium and/or enzymatic system in flow or batch processes. This provides an opportunity to deploy cheap, easy-to-handle system in poor and highly-polluted regions. Depending on the substrate, one would choose either whole *P. chrysosporium* mycelium (must be alive and intact) or just enzymatic preparations. Their selection depends on physicochemical properties of substrates. The most important feature is the presence and type of substituents attached to the aromatic ring. In case of 2,4,6-trinitrotoluene, the three highly electrophilic groups are accompanied by the nucleophilic methyl group that provides the strong resistance to oxidation, thus the nitro groups reduction is essential for successful biodegradation. Therefore, the whole fungal mycelium would be required, due to the presence of membrane-associated oxidation system. But in case of several azodyes or aromatic nitrocompounds that contain the non-recalcitrant to reduction or conversion substituents (hydroxyl, nitroso, hydroxyamino, carboxyl), just the enzymatic system can be applied. However, the strict control of pH and H_2O_2 concentration must be maintained, what would require either utilization of additional enzyme (GLOX, AAO, POX) or constant monitoring and correction of H_2O_2 concentration. Such system could be applied for remediation of areas polluted with dinitrophenol derivatives, picric acid, 3,5-dinitrosalcylic acid, 4-aminobenzene etc. The second important property is the solubility in water. Some nitroaromatic compounds have very low dipole moment, resulting in poor solubility, e.g. TNT (13 mg/L), or are completely insoluble (hexogen, octagen). In case of such materials, the plasma-membrane system owing to its reduction capabilities, not only enables enzymatic degradation, but also enhances their solubility in water, therefore providing more substrate for reduction. However, for other soluble pollutants like 3,5-DNS or picric acid, such system does not seem to be necessary. Aforementioned solutions, using either immobilized enzymes or whole mycelia, could be applied in continuous-flow systems. Such technology could use immobilized preparations of fungal mycelia based on lignocellulosic waste materials - sugar beet pulp, sugar cane pulp, wood chips enriched with biomass hydrolysates etc. or porous synthetic carriers, like polyurethane foams. As enzymatic system of *P. chrysosporium* has very low specificity, along with available carbon sources from immobilization media it would utilize nitrocontaminants with good efficiency. The system employing intact mycelium immobilized on waste materials would consist of: vessels containing waste water contaminated with soluble nitrocompounds, peristaltic pump providing continuous flow through reactors filled with mycelia immobilized on selected matrices, and temperature and aeration control systems. The exact composition of solution containing nitrocompounds would depend on immobilization media used. Agricultural wastes are usually rich in carbon and nitrogen sources as well as contain necessary microelements like Mn^{2+} or Fe^{2+}, therefore enrichment in additional substances would be redundant. However, in case of artificial matrices like polyurethane foams it is essential to supplement

carbon and nitrogen sources as well as microelements. One could apply Czapek medium, rich in all necessary nutrients, however agar exclusion and decrease in glucose and nitrate concentration would be necessary. Another problem is pH control in such system. Buffering components are required to be non-toxic for fungus (therefore Ca^{2+}, Mg^{2+}-based buffers are excluded) and need to buffer pH in range 5-8, optimal for fungal activity. Therefore phosphate or organic buffers could be applied. The drawback of buffering organic compounds is an addition of another carbon source to the system, so decrease in pollutants' metabolism is suspected.

On the other hand, organic acids and salts are natural for fungi while phosphate buffer could enhance contamination risk and it is unknown whether it can impede enzymes activity. Aeration system is much less complex. The most important factor is biological oxygen demand (BOD) of the system. It does not require continuous aeration, just maintenance of dissolved oxygen concentration at minimal value is required. However, if air is delivered, it should be sterilized in order to avoid any contamination. In laboratory scale, small aeration pump programmed to deliver air periodically with sufficient volume would be enough. Sterilization of air could be solved with flushing the air through, e.g. solutions of NaOH or $KMnO_4$; at laboratory scale it would be enough. An example of such system is presented in Fig. 5.

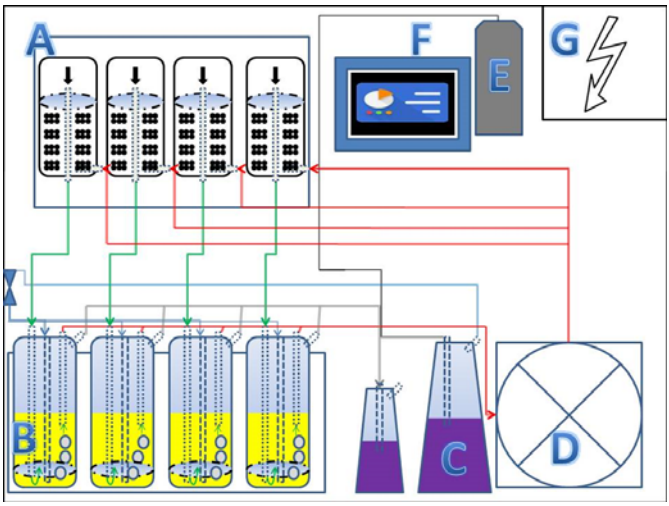

Figure 5. A scheme of a system for continuous-flow biodegradation process in laboratory scale. **A** depicts reactors filled with immobilized fungus, **B's** are tanks with contaminated water, placed in thermostated (**F)** water bath. The reaction liquid is pumped by peristaltic pump (**D;** red arrows) to the reactors and returns to tanks (green arrows). The air is delivered by air pump **E**; it is flushed through $KMnO_4$ solution (**C)** in order to ensure sterility. **G** is an electric current source

Such system, containing all mentioned elements, could be sufficient for conducting a research regarding biotransformation of water soluble nitrocompounds.

However, such application has certain drawbacks. Although the fungi is resistant to toxic environment, it is not clear whether it could withstand significant concentrations of pollutant or its mixtures. For example, 2,4,6-trinitrotoluene maximum concentrations safe for fungal activity in case of liquid culture was below 0.02%(w/v) [91], albeit it is unknown whether mixture with additional RDX or 2,4-dinitrophenol would not impede degradation process. Another problem is source of compounds essential for catalytic activity. As mentioned before, manganese(II) and iron(II) cations are essential for enzymes to perform substrates' oxidation, as well as hydrogen peroxide. As long as cations' concentration just must be monitored and corrected during the process, hydrogen peroxide concentration can be maintained not only by its generation by hydrogen-peroxide generating enzymes like GLOX or POX, but also by choosing proper immobilization matrix composed of polysaccharides. On the one hand, they do not provide easily accessible carbon source, thus do not impede biodegradation process, while on the other, they encourage activity of hydrolases and lytic polysaccharide monooxygenase (E.C. 1.14.99.54) and cellobiose dehydrogenase (E.C. 1.1.99.18), responsible for oxidation of recalcitrant polysaccharides, providing H_2O_2 and hydroxyl radicals as one of the products [92, 93]. Another problem, especially in case of *in situ* remediation, is potential contamination with antagonistic bacteria, e.g. *Pseudomonas fluorescens*. As long as in the laboratory or industrial process it is relatively easy to prevent contamination, biodegradation process *in situ* would require careful utilization of additional antibacterial agents [94].

Conclusions

The growing abundance of nitrocompounds in the biosphere pose a serious threat to the environment. Due to their high toxicity and recalcitrance to biodegradation, the immediate actions to prevent further contamination and the development of utilization methods are necessary. *P. chrysosporium* is a versatile organism capable of mineralizing nitrocompounds, what provides the huge opportunity for application in bioremediation of areas where the ecosystems were exposed to these pollutants. Depending on contaminating substances, either the whole organism or only its extracellular enzymes could be used. As neither the fungus nor its enzymes are challenging in maintenance, and the costs of their usage are low, they may be applied for pollutants biodegradation in many regions. The system proposed in this work seems to be suitable for research at laboratory scale. The maintenance costs would not be high owing to mild temperature conditions (around 10°C higher than room temperature). Besides, neither the air pump nor peristaltic pump work at high pressure, thus they are not highly energy-consuming. Also the costs of operation of the relevant large-scale system would not be high.

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