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Degradation of pharmaceuticals and personal care products by white-rot fungi-a critical review

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Abstract
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Keywords
pharmaceuticals, personal, care, products, white-rot, fungi-a, critical, degradation, review

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Abstract:

White-rot fungi (WRF) mediated treatment can offer an environmentally friendly platform for the removal of pharmaceuticals and personal care products (PPCPs) from wastewater. These PPCPs may have adverse impacts on aquatic organisms and even human and thus their removal during wastewater treatment is of significant interest to the water industry. Whole-cell WRF or their extracellular lignin modifying enzymes (LMEs) have been reported to efficiently degrade PPCPs that are persistent to conventional activated sludge process. WRF mediated treatment of PPCPs depends on a number of factors including physicochemical properties of PPCPs (e.g., hydrophobicity and chemical structure) and wastewater matrix (e.g., pH, temperature and dissolved constituents), type of WRF species and their specific extracellular enzymes. This review critically analyzes the performance of whole-cell WRF and their LMEs for the removal of PPCPs: particularly it offers insights into PPCP removal mechanisms (*e.g.*, biosorption *vs.* biodegradation) and degradation pathways as well as the formation of intermediate byproducts.

Keywords: White-rot fungi; lignin modifying enzymes (LMEs); Pharmaceuticals and personal care products (PPCPs); Biodegradation; Redox-mediators; metabolites.

1. Introduction

Trace organic contaminants (TrOCs) include diverse groups of chemicals such as pharmaceuticals and personal care products (PPCPs), pesticides, surfactants and industrial chemicals. Due to their ineffective removal by the conventional wastewater treatment processes, these TrOCs are commonly detected in the aquatic environment including surface water and groundwater, and even seawater [1, 2, 3]. Most TrOCs are biologically active even at trace concentrations (in the range of few ng/L) and can impose detrimental impacts on aquatic environment as well as on human health [4, 5]. For example, PPCPs such as synthetic hormones can induce endocrine disrupting effects in aquatic lives [6, 7]. Some of these TrOCs can also accumulate in aquatic biota. Exposure to a non- lethal dose of antibiotics may result in the development of antibiotic resistant genes in bacteria which is an emerging concern for human health according to the World Health Organization [8, 9, 10, 11]. Biological treatment processes are environmentally friendly and cost effective [12, 13, 14]. The conventional activated sludge and membrane bioreactor processes can efficiently remove bulk organics, nutrients and pathogens. However, certain groups of PPCPs such as significantly hydrophilic compounds and/or compounds with strong electron withdrawing functional groups are poorly removed by the conventional biological treatment processes [2, 15, 16, 17]. Thus, effective treatment processes to remove PPCPs from wastewater is urgently needed.

Extracellular enzymes of white-rot fungi (WRF) are characterized by their capacity to degrade the complex structure of lignin, which also enables them to degrade a wide range of resistant pollutants including synthetic dyes, chlorophenols, polychlorinated biphenyls and polycyclic aromatic hydrocarbons [18, 19, 20, 21, 22, 23]. WRF and their lignin modifying enzymes (LMEs) have been investigated recently for the degradation of a broad spectrum of PPCPs [24, 25, 26, 27]. The potential of WRF for the removal of PPCPs has been investigated mostly in batch mode. There are only a few studies on continuous flow reactor configurations [24, 26, 28, 29, 30]. Despite a significant research effort, efficient removal of PPCPs by WRF mediated treatment, their removal mechanisms and degradation pathways remain largely to be elucidated. There have been a few excellent reviews on bioremediation of

recalcitrant compounds by WRF and their LMEs [31, 32, 33, 34, 35]. However, aspects such as comparative performance of different whole-cell WRF species, the impacts of different reactor configurations as well as the performance under non-sterile environment deserve further attention. Moreover, factors controlling the performance of the crude and/or purified LMEs for the degradation of PPCPs, particularly the redox-mediator enhanced laccase systems remain largely unaddressed. This critical review fills these gaps. Degradation mechanisms and pathways for the removal of PPCPs are also critically discussed and the major metabolites or intermediate products following WRF treatment are identified. In addition, the technological challenges associated with the continuous fungal bioreactors are discussed and possible solutions are outlined. Directions for future research are also provided.

2. Enzymatic Systems of WRF

Several features of WRF make their application for PPCP removal attractive: (i) the non-specificity of their enzyme systems, facilitating degradation of a broad spectrum of pollutants [36, 37]; (ii) the hyphal growth, a defining property of filamentous fungi including WRF, enabling them to access pollutants by colonizing fast; (iii) the secretion of extracellular enzymes enabling them to degrade pollutants with low water solubility; and (iv) the ability of some WRF species to degrade pollutants in nutrient deficient environment over a wide range of pH. Notable, however, is that resistant pollutant degradation by WRF is a co-metabolic process, *i.e.*, occurs in presence of an easily degradable substrate. Since, PPCP degradation by WRF depends mainly on their enzyme systems, these are described in this section.

Three different types of extracellular LME, namely, lignin peroxidases (LiPs), laccase and manganese-dependent peroxidases (MnPs), are secreted by WRF. The main difference between laccase and peroxidases is the electron acceptor where oxygen and hydrogen peroxide are the respective electron acceptors. [38, 39]. Not every WRF species produces all three enzymes, and combination of major LME varies from one WRF species to another. Even the secretion pattern of enzymes varies within a WRF species. For instance, different strains of *T. versicolor* has been reported to secrete all three enzymes but laccase is the main enzyme secreted by the strain ATCC 7731 [40, 41, 42]. In addition, composition of

growth medium and culture conditions can influence the secretion of a specific enzyme. Degradation of some pollutants such as phenolic compounds, peptides, and organic acids by WRF may result in the formation of low molecular weight mediators which can enhance the spectrum of compounds degraded by WRF [37, 39, 43]. Based on the secretion patterns of enzymes, WRF can be categorized as [44]: (i) MnP-laccase group such as *T. versicolor*, *P. ostreatus*, *D. squalens* and *P. tigrinus*; (ii) LiP-laccase group such as *P. ochraceofulva*; and (iii) LiP-MnP group such as *P. chrysosporium*.

In addition to the extracellular enzymes, intercellular enzymes may play an important role in the degradation of xenobiotics. Intracellular cytochrome P450 enzyme system has been observed to play a vital role in the degradation of some PPCPs such as and chlorinated hydrocarbons and polycyclic aromatic hydrocarbons [45, 46, 47]. Cytochrome P450 is a group of monooxygenases which can degrade PPCPs by catalyzing a number of reactions such as heteroatom oxygenation, dehalogenation and hydroxylation [48].

3. Modes of PPCP Degradation by WRF

Different WRF and their LME have been investigated under different experimental conditions for the removal of PPCPs - either by whole-cell WRF culture or by using the crude and/or purified enzyme extracts. In this section, different modes of PPCP degradation via WRF and their LMEs are critically reviewed.

3.1. Removal by Whole-cell WRF

3.1.1. Degradation capacity of different WRF

Although whole-cell WRF cultures, either in submerged or solid media, have been used for the removal of PPCPs, submerged whole-cell WRF cultures have been more commonly reported. Since WRF species harbor different enzyme systems, the extent of PPCP removal achieved by different WRF species also varies. PPCP removal performance by different WRF species is presented in Table 1. Notably, temperature and pH for these studies were in the range of 25-30^{o}C and 4.5-5, respectively, with glucose used as the common electron donor.

T. versicolor, also known as *Coriolus versicolor* [49, 50], has been investigated in several studies for the removal of PPCPs both in batch and continuous reactors [24, 26, 27, 43, 51, 52, 53]. Depending on the strain, *T. versicolor* may contain laccase, LiP and MnP, with laccase being the predominant enzyme in some strains. It can be observed from Table 1 that *T. versicolor* achieved significant removal (>70%) for most of the tested PPCPs. PPCPs such as steroid hormones, nonylphenol and octocrylene can induce endocrine disrupting effects [54, 55]. Their removal by *T. versicolor* in literature ranges from 80-99% regardless of the operating conditions. Conversely, poor and unstable removal was reported for particular pharmaceuticals namely ciprofloxacine (35%), salicylic acid (0-5%), azithromycin (26%), tetracyclin (0-5%) and carbamazepine (negligible to 90%). Low and/or unstable removal of these compounds can be attributed to the presence of strong electron withdrawing functional groups in their chemical structure [31]. Compared to the pharmaceuticals, the removal of personal care products has been communicated in only a limited number of studies [27, 52]. However, *T. versicolor* has been shown to achieve high removal of ingredients of personal care products such as triclosan, oxybenzone, octocrylene and nonylphenol (Table 1).

[TABLE 1]

Other WRF species such as *P. chrysosporium* [56, 57], *B. adusta* , *D. squalens* and *P. ostreatus* containing different combinations of LMEs have also been investigated for the removal of PPCPs (Table 1). However, these studies reported the removal of only a few PPCPs such as ibuprofen, diclofenac and triclosan. Despite the difference in enzyme secretion pattern of these WRF, efficient removal (in the range of 70-99%) was achieved for most tested PPCPs, with uncertain/unstable removal reported for carbamazepine, which is a known persistent pharmaceutical. It is important to note that a direct comparison of PPCP removal data from different studies may not be valid due to the differences in operating conditions and reactor configurations as well as difference in their enzymatic systems. However, Table 1 serves the purpose of providing a general overview.

3.1.2. WRF Reactor configurations

Different reactor configurations have been explored for the continuous treatment of PPCPs in WRF-based systems (Table 1). Since whole-cell WRF based treatment systems are still in their development phase, the reactor design and configurations are of significant importance. Hence, the salient features of the reactor configurations studied to date for the removal of PPCPs, namely, stirred tank bioreactors [56, 57], bubble column bioreactors, fluidized or packed bed bioreactors [59, 60, 61] and membrane bioreactors [62, 63, 64, 65] are briefly discussed here to provide a general overview.

Among all the reactor configurations, stirred tank bioreactor has been the most common type of reactor used for the treatment of PPCPs in WRF based treatment systems. This reactor type has been explored mostly in batch and sterile modes [56, 57]. In this reactor aeration is provided usually at the bottom of the reactor which is dispersed via mechanical agitation. Mechanical mixing also ensures the uniform mixing of the growth medium and wastewater in the reactor [59]. Enhanced production of extracellular enzymes could be achieved in stirred tank bioreactors compared to other reactor configurations. For instance, Babič and Pavko [66] investigated the production of laccase and MnP from *D. squalens* in stirred tank bioreactor and bubble column reactor under different operating conditions such as incubation time and agitation speed: they observed that laccase production was as much as 70% higher in the former, although the production of MnP was comparable [66]. Agitation speed and high shear rate may influence the morphology of the fungal biomass. In a study by Cao et al. [67], impact of two reactor configurations, namely stirred tank bioreactor and airlift bioreactor, on the morphology of *P. sanguineus* was investigated. They found that the morphology of the fungal biomass was adversely impacted in the stirred tank bioreactor which uses strong mechanical mixing. Consistent with the finding of Cao et al. [67], it has been reported that excessive agitation may lead to the rupturing of fungal hyphae [59, 68, 69]. Therefore, agitation/mixing speed in bioreactors is an important parameter governing fungal morphology and enzymatic activity.

Fluidized bed bioreactor is another type that has been used for the removal of PPCPs from municipal and synthetic wastewater [24, 58]. In this type of reactor, fungal biomass rapidly moves around the solid carrier ('bed'), allowing uniform mixing of the reaction mixture [70]. However, aggregation of fungal biomass may cause poor fluidization, resulting in spouting of bed. Biomass aggregation can be avoided by intermittent and partial purging of the biomass [71].

A bubble column reactor coupled with a microfiltration membrane has been recently explored for the removal of PPCPs [26, 27]. Such fungal membrane bioreactor was expected to offer some additional advantages over bubble column reactors [14, 72, 73] such as: (i) formation of biofilm on the membrane surface that may enhance the removal of recalcitrant PPCPs; (ii) maintenance of high fungal biomass concentration improving biodegradation rate; and (iii) effective prevention of enzyme washout. Nevertheless, bacterial contamination may hamper the growth and enzymatic activity of whole-cell WRF in any reactor configuration. Impacts of bacterial contamination on WRF are discussed in the next section.

3.1.3. Performance under non-sterile environment

The capacity of WRF for PPCP removal has been commonly investigated under sterile conditions to avoid bacterial contamination. Fungal bioreactors have also been operated under non-sterile conditions but for a short period of time [24, 26, 58]. Bacterial contamination in the bioreactor adversely affects PPCP removal efficiency by: (i) creating competition between bacteria and fungi for substrate; (ii) damaging the fungal mycelium; and (iii) disrupting the growth of fungi [26, 70]. Therefore, it is important to develop techniques for an uninterrupted growth of fungal strains and to enhance their competitiveness over bacteria. Some of the strategies to avoid bacterial contamination are outlined below:

(i) Operation under acidic pH: Optimum pH for the growth and reproduction of fungi ranges from 4.5-5. Conversely, bacteria grow at or near neutral pH. Bacterial growth can be suppressed by maintaining the pH in the range of 4.5-5. However, this is a temporary solution to the problem of bacterial contamination because some bacteria can eventually adapt to acidic environment [74].

(ii) Immobilization or attached growth of fungi: Immobilization of fungal strains onto different carriers under non-sterile conditions shows promising results. For instance, by immobilizing *C. versicolor* onto a plastic support, Hai et al. [72] was able to prevent bacterial contamination for an extended period of time while operating the reactor under non-sterile conditions for the treatment of an azo dye.

(iii) Nitrogen limited feed: Bacterial contamination can be avoided by using a media deficient of nitrogen. However, this strategy can only help during the startup of the bioreactor. Bacterial contamination would occur with the passage of operating time as bacteria would start consuming carbon and nitrogen available in fungal mycelium [74].

(iv) Coupling of bioreactor with micro-screen: Bioreactors can be coupled with a micro-screen which would retain fungal biomass but allow the washout of bacteria with effluent. Moreover, this strategy will benefit from using shorter hydraulic retention times, which will enhance the washout of bacteria from the reactor [75].

(v) Use of disinfecting agents: Inactivation of bacteria without imposing any harmful effects on fungal biomass can be a promising strategy. Depending on the wastewater characteristics, it is important to carefully select the type, dose and contact time of the disinfectant. Disinfection of wastewater using ozone has been used successfully to selectively inactivate bacteria [76, 77].

(vi) Biomass replacement: Periodic biomass replacement and purging strategy can be used to carry out long term operation of fungal bioreactors. In this strategy, biomass in the bioreactor is purged and renewed in different fractions (*e.g.*, ½ or ¼th of the initial biomass volume) at different frequencies [78, 79].

(vii) Pretreatment of wastewater: Coagulation-flocculation pretreatment of non-sterile wastewater can help to reduce the initial bacterial count which would allow an extended operation of the fungal bioreactor [79].

3.2. Removal by Extracellular LME

Individual extracellular LME have been tested for the removal of a wide range of pollutants. Use of the harvested enzyme instead of a whole-cell preparation allows decoupling of fungal growth and pollutant degradation steps and this can be a suitable strategy to avoid bacterial contamination issues. The capacity of both crude and purified/commercially available extracellular LME for PPCP removal has been reported previously [27, 41, 42, 80, 81, 82, 83, 84, 85, 86]. However, whole-cell fungi may achieve relatively better removal of a broader spectrum of pollutants than extracellular LME due to the availability of extracellular, intercellular and/or mycelium bound enzymes in addition to sorption of pollutants onto fungal biomass. In this section the performance of crude, purified or commercially available extracellular enzymes are discussed.

3.2.1. Performance of Crude LMEs

Crude extracellular enzymes have been investigated for the degradation of PPCPs in both batch and continuous mode. For instance, Wen et al. [87] achieved significant degradation of two pharmaceuticals, oxytetracycline (84%) and tetracycline (72%), by using crude MnP (40 U/L) extracted from *P. chrysosporium*. Similarly, crude enzyme solution extracted from *T. versicolor* containing MnP (30 U/L) and laccase (1500 U/L) was tested for the degradation of 10 pharmaceuticals at an initial concentration of 10 µg/L each [80]. They achieved complete degradation of five pharmaceuticals diclofenac, ibuprofen, naproxen, indomethacin and fenoprofen, while the rest were partially removed. LiP extracted from *P. chrysosporium* was tested for the degradation of diclofenac and carbamazepine at different pH [88]. It was observed that degradation of carbamazepine was mostly <10%, whereas a complete degradation was achieved for diclofenac at pH 3-4.5. Similarly, crude extract from *T. versicolor* was used for the removal of 30 TrOCs including PPCPs [81]. The results revealed that all steroid hormones were almost completely removed (>95%), while removal of two PPCPs namely diclofenac and triclosan ranged from 50-60% with poor removal of the remaining PPCPs (<20%). In another study [89], crude enzyme extract from *P. ostreatus* achieved low removal (<20 %) for two PPCPs, namely, oxybenzone and naproxen. Since WRF

also secretes natural mediators along with LMEs, crude enzyme may achieve better PPCP removal efficiency compared to purified and/or commercially available LME. For instance, Tran et al. [80] highlighted that complete removal (>99%) of some pharmaceuticals such as diclofenac, indomethacin and ibuprofen was due to the presence of some natural mediators in crude laccase solution.

Removal performance of selected PPCPs by individual crude enzymes has been systematically presented in Table 2. It can be seen that the extent of PPCP removal is different for each type of LME. Moreover, crude enzyme extract from different fungi may perform differently. For instance, Weng et al. [90] collected crude LiP from two WRF species, namely, *P. sordida* and *P. chrysosporium* for the treatment of EDCs and found that LiP from *P. sordida* was more effective than LiP from *P. chrysosporium*. Similarly, removal of DEET, an insect repellent, by *T. versicolor* was 55% in real wastewater compared to its 20% removal in acetate buffer. High removal of DEET in real wastewater was attributed to the presence of other compounds (such as phenolic compounds) which may act as redox-mediators [91], as further discussed in a following section.

[TABLE 2]

3.2.2. Performance of Purified LME

Purified LME, mostly laccase from different WRF, has been used for the treatment of PPCPs in both batch and continuous reactors. Average removal of PPCPs by purified laccase reported in recent studies is presented in Figure 1. Purified laccases are more effective for the removal of phenolic compounds such as oxybenzone, triclosan and steroid hormones. Removal of non-phenolic compounds such as carbamazepine, naproxen and ketoprofen is generally poor/unstable. Their degradation depends on their physicochemical properties such as hydrophobicity and chemical structure as well as the oxidation reduction potential (ORP) of the enzyme [31, 102]. Indeed, based on the results of the recent studies incorporated in Figure 1, removal of phenolic compounds ranges from 70-99%, while the removal of non-phenolics is generally <20%. However, relatively higher removal of some non-phenolic compounds, namely, diclofenac (40-50%), octocrylene (>80%) and ibuprofen (30-45%) has been reported because

these compounds contain both electron donating and electron withdrawing functional groups. Although both crude and purified LMEs demonstrated degradation of a range of pollutants, crude LMEs achieved better removal of some PPCPs such as diclofenac and naproxen compared to purified LMEs [27, 41, 81, 82, 83, 85], possibly due to the presence of natural mediators in crude LMEs secreted by WRF. For instance, Tran et al. [80] achieved almost complete removal of three pharmaceuticals namely diclofenac, ibuprofen and naproxen after treatment with crude enzyme extracted from *T. versicolor*, whereas purified laccase from *T. versicolor* and *A. oryzae* achieved 20-50% removal of these compounds [82, 83, 84, 103]. Additionally, utilization of crude LMEs for the treatment of PPCPs may considerably reduce the cost of the treatment process. However, extracellular extract *i.e.*, the crude enzyme solution also contains significant amount of the unspent growth media, and dosing crude enzyme means additional organic loading from this.

[FIGURE 1]

3.3. Performance of Redox-mediator Enhanced Laccase Catalyzed System

Laccase catalyzes mono-electronic oxidation of PPCPs. However, the extent of removal depends on the ORP of the enzyme and individual PPCP. Poor removal of non-phenolic PPCPs can be attributed to: (i) presence of a strong EWG in the structure of non-phenolic compounds causing steric hindrance; and (ii) higher ORP of non-phenolic compounds than laccase [102]. Removal of non-phenolic compounds can be enhanced by introducing a low-molecular weight redox-mediator. In a redox-mediator catalyzed system, highly reactive radicals are formed due to the oxidation of a mediator by laccase and these radicals then serve as an electron transfer shuttle between PPCPs and laccase, facilitating enhanced removal of recalcitrant compounds. Three oxidation mechanisms, namely hydrogen atom transfer (HAT), ionic mechanisms and electron transfer have been reported for mediators. For instance, 1-hydroxybenzotriazole (HBT) and syringaldehyde (SA) follow HAT mechanism, while 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfnoic acid (ABTS) and 2,2,6,6-tetramethylpiperidinyloxyl (TEMPO) follow electron transfer and ionic mechanisms, respectively [89, 109].

Properties of different redox-mediators used to enhance the removal of different PPCPs are summarized in Table 3. Although the mediators perform differently, two mediators namely HBT and SA have been commonly used to broaden the spectrum of compounds significantly degraded by laccase. Studies involving these mediators confirmed that they not only improved the ORP of reaction mixture but also the extent of removal. Only a few studies elucidated the performance of different mediators based on the type of substrate (i.e., phenolic *vs*. non-phenolic). For instance, N-OH type mediators (VA and HBT) were found to achieve the best removal of non-phenolic PPCPs such as clofibric acid, naproxen and carbamazepine, whereas SA and ABTS performed better for phenolic compounds such as salicylic acid and steroid hormones [82, 83, 85].

Mediator type, concentration and compound properties influence the performance of a mediator catalyzed system. For instance, removal of diclofenac improved from 40 to 80% by increasing the concentration of SA from 0.1 to 0.5 mM [103]. Similarly, Nguyen et al. [85] achieved an improvement of 35% in the removal of diclofenac in an enzymatic membrane reactor by increasing the dose of SA from 0.01 mM to 0.1 mM. However, beyond a threshold concentration increasing mediator dose may not improve the removal of PPCPs. For instance, Ashe et al. [89] observed that increasing the concentration of VA and ABTS from 0.5 to 1 mM could not enhance the removal of oxybenzone and naproxen. They also observed that removal of atrazine was reduced by 15-25 % when the concentration of VA and HBT was increased from 0.1 to 0.25 mM. This may be attributed to the complex interactions between laccase and the radicals generated due to degradation of the mediator by laccase, as discussed below.

[TABLE 3]

It has been observed in almost all the studies that enzymatic activity significantly drops with the addition of mediators. For instance, Hata et al. [111] observed 90% decline in enzymatic activity within fist 8 h of incubation in the presence of HBT. Similarly, rapid decline in enzymatic activity was also observed with the addition of VA, HBT or ABTS [89], although rate of enzyme inactivation depends on the relative stability of the radicals generated by mediators. In the absence of any known enzyme inhibitor, rapid

enzyme inactivation in mediator catalyzed system can be attributed to: (i) the blockage of active sites of the enzyme by charged radicals and metabolites; and (ii) the reaction of metabolites with enzyme-active sites to convert them into non-productive complexes [112, 113].

Despite rapid inactivation of enzymes by different mediators, mediators can compensate by enhancing the rate of reaction, eventually achieving rapid and enhanced removal of PPCPs. However, periodic replenishment of enzyme is needed to maintain the removal efficiency of PPCPs, constraining the long term operation of mediator-enzyme based wastewater treatment processes. Therefore, selection of mediator type and its concentration is vital for effective and long term operation of such systems.

4. Impacts of Physicochemical Characteristics of Wastewater

Performance of WRF in wastewater treatment depends on a number of factors such as environmental conditions and physiochemical properties of the wastewater as well as the properties of PPCPs. Influence of PPCP properties on their overall removal in a WRF mediated treatment process has been reviewed by Yang et al. [31]. Briefly, physicochemical properties of water/wastewater such as pH, temperature and the presence of dissolved organic and/or inorganic compounds may influence WRF performance. Because information about these properties are vital to design and optimize WRF mediated treatment systems, these are discussed in this section.

The operating temperature not only affects the stability of WRF enzymatic systems but also the rate of reaction. It is believed that the rate of reaction increases with increase in temperature [88]. However, depending on the WRF strain, rapid thermal inactivation of LMEs has been observed at temperature >40^{o}C [114, 115]. Only a few studies have investigated the impact of temperature on LME activity and removal efficiency in a reaction mixture. For instance, increase in temperature of the reaction mixture from 20 to 25^{o}C enhanced the removal efficiency of chlorophenols in laccase mediated treatment [116]. Similarly, Ullah et al. [117] investigated the removal of pentachlorophenol by varying the temperature from 10-45 ^{o}C and found that the optimal temperature was 25^{o}C to achieve maximum laccase activity and

pentachlorophenol removal. Temperature range of 37-40 ^{o}C has been reported to achieve optimal activity of MnP and LiP [87, 118].

Enzyme performance and substrate properties can be influenced by pH of the reaction mixture, thereby affecting the extent of PPCP removal. For instance, optimum pH for the removal of diclofenac (60-100%) by purified laccase (*T. versicolor* and *M. thermophile*) and LiP (*P. chrysosporium*) was in the range of 3.0 – 4.5 [83, 88, 103, 105]. Similarly, Wen et al. [87] observed that the removal of two antibiotics, namely tetracycline and oxytetracycline, by crude LiP and MnP was pH dependent and the best removal was achieved at pH 4.5-5.0. However, optimum pH for the removal of a personal care product triclosan was in the range of 5-5.5 during the treatment with laccase from *T. versicolor* [93, 116]. Media pH may not affect the removal of compounds highly amenable to laccase-catalyzed degradation. For instance, laccase from *T. versicolor* achieved significant removal (>70%) of steroid hormones over a pH range of 4 - 7 [103]. Similarly, changing the pH from 2.5- 6 could not improve the removal of carbamazepine by LiP [88]. In light of the above discussion, pH ranging from 4-5-5 is recommended for WRF mediated treatment processes.

The impact of dissolved organic and inorganic constituents on laccase activity and PPCP removal has been communicated in a limited number of studies. Many compounds such as sulphides, halides [119, 120], natural/synthetic organics [121, 122, 123, 124, 125, 126] and heavy metals [92, 127] can inhibit the catalytic activity of laccases [128]. Moreover, each compound may have different mode of laccase inhibition. For instance, fatty acids inhibit laccase catalytic potential by blocking the binding sites for phenolic substrates [129, 130, 131]. On the other hand, spectrophotometric assays, electron spin resonance spectroscopy and catalytic voltammetry analysis confirmed that anionic inhibitors such as halides and sulphides could block the access to the active copper sites in laccase [132, 133, 134]. Among anionic inhibitors, fluoride and azide are the most effective and can rapidly reduce the catalytic activity of laccase by 50% even at μM concentrations [135]. Although inhibition of laccases by halides can proceed in the following order: fluoride > chloride > bromide, the concentration of halides required to inhibit

laccases varies, with no fixed correlation with their inhibition potential. For instance, chloride concentration ranging from 100 µM – 100 mM may cause a 50% drop in activity of laccase from different species [35, 136].

5. Removal Mechanisms

Removal mechanisms during treatment with WRF whole-culture include (i) sorption onto the fungal biomass; (ii) degradation by extracellular enzymes; and (iii) degradation by mycelium bound or intercellular enzymes. A schematic of fungal mediated treatment process with possible removal mechanisms is presented as Figure 2. Hydrophobicity (log D) of PPCPs is a key property that governs biosorption onto fungal biomass and could facilitate enhanced removal of some compounds. For instance, a batch study to investigate the contribution of biosorption and degradation by extracellular enzymes confirmed that hydrophobic PPCPs (log D>4) were highly removed by both mechanisms [41]. Moreover this study also confirmed that biosorption of significantly hydrophobic compounds facilitated the biodegradation of these compounds. On the other hand, a few studies have reported that removal of some PPCPs such as 17β-Estradiol, 17α-Ethynylestradiol, triclosan and nonylphenol by whole-cell WRF and extracellular enzymes is comparable (see Table 1 and 2), indicating a negligible impact of biosorption on their removal.

Biodegradation by whole-cell can be due to intracellular, extracellular and mycelium-associated enzymes. This can lead to significant differences in removal by whole-cell WRF and harvested enzyme. For instance, carbamazepine, containing a strong EWG, was significantly removed by some WRF species namely *P. ostreatus* (100%) [45] and *T. versicolor* ATCC 42530 (76%) [80], whereas crude [45, 80, 81] and purified laccase [82, 85] could only achieve 5-15% carbamazepine removal. Similarly, ibuprofen and naproxen were almost completely removed by whole-cell WRF [24, 43, 58]. In contrast, their removal by crude and purified laccase was in the range of 10-40% [27, 82, 85, 103]. Since both naproxen and ibuprofen are hydrophilic compounds (log D <3), role of biosorption in their removal would be limited. However, almost complete removal of these compounds in whole-cell WRF substantiates the role of

mycelium bound and/or or intercellular enzymes. Indeed, the role of intercellular enzyme (*i.e.* cytochrome P450) in the degradation of naproxen, diclofenac and carbamazepine has been demonstrated [43, 45, 137]. These studies showed that naproxen, diclofenac and carbamazepine were partially removal (15-50%) in the presence of cytochrome P450 inhibitor during whole-cell WRF treatment. Therefore, it can be concluded that high removal of some PPCPs in whole-cell WRF treatment is due to the synergetic effects of extracellular, intercellular and/or mycelium bound enzymes. Moreover, secretion of natural mediators may also help in enhancing the removal of these compounds.

[FIGURE 2]

6. Degradation Pathways and Identification of Intermediates

PPCP degradation pathways and their intermediate products have been identified for some compounds such as carbamazepine, diclofenac, triclosan and ibuprofen. However, each WRF species may follow a different degradation pathway. Degradation pathways and intermediate products of some PPCPs are discussed in this section.

Fungal mediated treatment of diclofenac starts with the conversion of the hydroxyl group in its structure into an intermediate product, namely, hydroxy diclofenac. Hydroxy diclofenac can be amenable to further fungal biodegradation [137, 138]. In *vivo* and in *vitro* experiments for the degradation of diclofenac showed that laccase (*T. versicolor*) catalyzed degradation leads to the formation of biodegradable compounds such as: (i) hydroxelated metabolites (appeared in both *in vivo* and *in vitro* experiments); and (ii) 4-(2,6 dichlorophenylamino)-1,3-benzenedimethanol metabolite (appeared only in *in vivo* experiments) [138]. Diclofenac and its metabolites both disappeared after 24 h of incubation, reducing the ecotoxicity of the treated effluent. Degradation of ketoprofen was reported to initiate by the intercellular cytochrome P450 that converts ketoprofen into (2-[3-(4-hydroxybenzoyl)phenyl]-propanoic acid) and (2-[(3-hydroxy(phenyl)-methyl)phenyl]-propanoic acid) via hydroxylation and reduction of the ketone

group, respectively [138]. Moreover, it was also observed that the role of extracellular enzyme (laccase) in the degradation of ketoprofen was insignificant.

Both laccase and cytochrome P450 can catalyze the degradation of naproxen in WRF based treatment. Formation of two intermediates, namely 6-desmethylnaproxen and 1-(6-methoxynaphthalen-2-yl)ethanone), was observed possibly via P450-mediated desmethylation and laccase catalysis, respectively [43, 57]. Moreover, naproxen and associated intermediates were completely removed from the reaction mixture and the treated effluent was non-toxic [43].

Ibuprofen conversion predominantly starts with the formation of hydroxy-ibuprofen via hydroxylation. Marco-Urrea et al. [139] investigated the degradation pathways of ibuprofen by WRF *T. versicolor*. They observed that oxidation of isopropyl chain resulted in the formation of 1-hydroxy ibuprofen and 2-hydroxy ibuprofen. These intermediates were then degraded by *T. versicolor* to 1,2-dihydroxy ibuprofen, which was not further degraded. Hence, the ecotoxicity of treated effluent was higher than that of the initial solution [139].

Degradation pathways for carbamazepine by different whole-cell WRF including *T. versicolor* and *P. ostreatus* have also been studied. These have identified a number of stable intermediate products namely, 10,11-dihydro-10,11-dihydroxy-carbamazepine, acridine, acridone and 10,11-epoxy-carbamazepine. Interestingly, the treated effluent showed less toxicity [45, 53].

Intermediates or metabolites of triclosan during whole-cell treatment depends on WRF species. Major intermediates of triclosan during the treatment with *C. polyzona* WRF were dimer, trimer and tetramer [140], while 2-O-(2,4,4-trichlorodiphenyl ether)-b-D-xylopyranoside, 2-O-(2,4,4-trichlorodiphenyl ether)-b-D-glucopyranoside and 2,4-dichlorophenol were identified as major intermediates following its treatment with *T. versicolor* [141].

7. Future Scope of Research

Performance of whole-cell WRF has mostly been investigated in sterile batch tests. Only a few studies have been conducted in continuous flow reactors under non-sterile conditions [24, 26, 27, 58], because bacterial contamination can seriously affect WRF performance. Therefore, more efforts are required to solve this limitation.

Another limitation of WRF mediated treatment process is the washout of the enzyme and mediators with treated effluent. This is particularly problematic for enzymatic reactors where enzymes instead of whole-cell WRFs are used. Enzyme immobilization or coupling of enzymatic reactor with a membrane with suitable pore size can be used to avoid enzyme washout [27, 82, 83, 85, 142, 143]. Coupling of membrane separation with an enzymatic reactor offers several advantages over immobilized enzymatic systems such as: (i) more effective enzyme retention; (ii) reduced mass transfer limitations; (iii) ease of enzyme replenishment and (iv) ease of operation and maintenance [29, 144]. Moreover, enhanced TrOC removal by enzymatic membrane bioreactors (EMBR) has been demonstrated in a few recent studies [82, 83]. While investigating the removal of 30 TrOCs in an EMBR, Nguyen et al. [82] observed retention of PPCPs such as gemfibrozil, naproxen and ketoprofen on the enzyme gel layer formed over the membrane. It was also confirmed that the compounds retained by the enzyme gel layer were subsequently biodegraded [82]. Despite enhanced removal of some PPCPs, EMBR may not be effective to protect enzymes from thermal and chemical denaturation. In such situations, immobilized enzymatic systems have been reported to be more effective than free enzymes [145, 146, 147]. In enzyme-mediator systems, redox-mediators may still pass through the membrane with the treated effluent, thus increasing the operating costs. Washout of redox-mediators can be prevented by integrating a high retention membrane such as nanofiltration, membrane distillation or forward osmosis membrane with the enzymatic reactor, or formulating strategies to recover mediators from effluent.

Degradation pathways of only a few PPCPs such as carbamazepine, diclofenac, ibuprofen and naproxen have been reported in literature. The intermediate products in some cases are more toxic than the parent

compound. Therefore, more efforts are required to identify and elucidate the formation of intermediate products during WRF mediated treatment of PPCPs which would help to further understand the degradation mechanisms of PPCPs.

8. Conclusion

WRF mediated treatment of PPCPs is a promising and environmentally friendly technology. Varieties of PPCPs are efficiently removed by both whole-cell WRF and crude/purified LMEs. Hydrophilic and persistent PPCPs such as naproxen, ketoprofen and carbamazepine are significantly removed only in whole-cell WRF treatment because of the synergistic effects of extracellular and intercellular enzymes as well as sorption onto fungal biomass. Different redox-mediators have been introduced to enhance the removal of persistent PPCPs but continuous addition of mediators is an expensive practice. Therefore, mediator type and concentration must be selected carefully and techniques to recover mediators be optimized. Operating conditions of the enzymatic reactor including pH, temperature and interfering dissolved contaminants could influence the performance of WRF mediated treatment process. Despite the better performance of whole-cell WRF treatment than crude and purified enzymes, long term continuous operation of fungal bioreactor could not be possible due to bacterial contamination. This problems needs to be solved before their full scale applications.

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List of Tables

Table 1: Removal (%) of PPCPs by different species of WRF (whole-cell) under different operating conditions

WRF Specie	Reactor Type	Operating conditions	PPCPs	Initial concentration (mg/L)	Removal efficiency (%)	References
P. chrysosporium (LiP, MnP)	Stirred tank (Batch-fed)	Inoculum= 1.2 g/L Reactor volume = 2 L, HRT= 24 h Mixing speed = 200 rpm pH = 4.5; Temperature= 30°C Electron donor= glucose Operating time= 30 days	Diclofenac Ibuprofen Naproxen	0.8 0.8 0.8	>99 75-99 >99	[56]
	Stirred tank (Continuous)	Reactor volume = 1.5 L HRT= 24 h pH = 4.5; Temperature= 30°C Electron donor= glucose Operating time= 50 days	Diclofenac Ibuprofen Naproxen Carbamazepine Diazepam	1 1 1 0.5 0.25-0.5	92 95 95 25-60 0	[57]
T. versicolor (Laccase, LiP, MnP)	Fixed bed (Continuous)	Inoculum= 3.2 g/L Reactor volume = 0.13 L HRT= 120 days pH = 4.5; Temperature= 22°C Electron donor= glucose Operating time= 26 days	17β-estradiol (E1) 17α-ethynylestradio (EE2)	3-18.8 7.3	>95 >95	[51]
	Fluidized bed (Batch-fed)	Inoculum= 3.8 g Reactor volume = 1.5 L pH = 4.5; Temperature= 25°C Electron donor= glucose Operating time= 15 days	Carbamazepine	0.05-9	61-94	[53]
	Fluidized bed (Batch-fed)	Inoculum= 1.5 g/L Reactor volume = 10 L pH = 4.5; Temperature= 25°C Electron donor= glucose Operating time= 8 days Real hospital wastewater Non sterile conditions	Ibuprofen Acetaminophen Ketoprofen Ciprofloxacine Azithromycin Propranolol Carbamazepine	2.34 1.56 0.08 84.71 4.31 0.06 0	100 100 100 35 100 100 -50	[24]
	Fluidized bed	Inoculum= 1.4 g/L	Acetaminophen	109	100	[58]

(Continuous)	Reactor volume = 10 L pH = 4.5; Temperature= 25^{o}C Electron donor= glucose Operating time= 8 days Real hospital wastewater Non sterile conditions	Naproxen	1.62	100	
		Ibuprofen	35.5	100	
		Ketoprofen	2.17	95	
		Diclofenac	0.477	100	
		Codeine	0.606	100	
		Phenazone	0.497	96	
		Salicylic Acid	0.606	0	
		Ofloxacin	3.34	98	
		Ciprofloxacine	13.0	99	
		Sulfamethoxazole	1.41	100	
		Trimethoprim	0.853	100	
		Metronidazole	0.912	85	
		Azithromycin	1.37	26	
		Clarithromycin	2.20	80	
		Erithromycin	0.008	100	
		Tetracyclin	0.011	0	
		Caffeine	149	39	
		Carbamazepine	0.056	0	
		Atenolol	2.99	75	
		Metoprolol	0.019	95	
Membrane bioreactor (Continuous)	Inoculum= 3.5 g/L Reactor volume = 5.5 L, HRT= 1 day pH = 5.4; Temperature= 27^{o}C Operating time= 90 days Non sterile conditions	Diclofenac	0.3-1.5	55	[26]
Membrane bioreactor (Continuous)	Inoculum= 3 g/L Reactor volume = 5.5 L, HRT= 2 day pH = 4.5; Temperature= 27^{o}C Operating time= 110 days Non sterile conditions	Ibuprofen	0.005	95	[27]
		Naproxen	0.005	95	
		Ketoprofen	0.005	90	
		Diclofenac	0.005	50	
		Carbamazepine	0.005	20	
		Metronidazole	0.005	40	
		Gemfibrozil	0.005	95	
		Amitriptyline	0.005	85	
		Estriol (E3)	0.005	>95	
		17-β-estradiol	0.005	>95	

			17-β-estradiol – acetate	0.005	>95	
			17-α ethinylestradiol	0.005	>95	
			Triclosan	0.005	95	
			Benzophenone	0.005	80	
			Oxybenzone	0.005	92	
			Octocrylene	0.005	94	
	Erlenmeyer flask (Batch-fed)	Inoculum= 2-3 mg/test pH = 4.5; Temperature= 28°C Operating time= 14 days Electron donor: glucose	Triclosan	10	98	[52]
			17-α ethinylestradiol	10	94	
			Nonylphenol	10	90	
P. ostreatus (Laccase, MnP)	Erlenmeyer flask (Batch-fed)	Inoculum= 2-3 mg pH = 4.5; Temperature= 28°C Operating time= 14 days Electron donor: glucose	Triclosan	10	98	[52]
			17-α ethinylestradiol	10	62	
			Nonylphenol	10	93	
D. squalens (Laccase, MnP)	Erlenmeyer flask (Batch-fed)	Inoculum= 2-3 mg pH = 4.5; Temperature= 28°C Operating time= 14 days	Triclosan	10	98	[52]
			17-α ethinylestradiol	10	78	
			Nonylphenol	10	85	
B. adusta (Laccase, LiP, MnP)	Erlenmeyer flask (Batch-fed)	Inoculum= 2-3 mg pH = 4.5; Temperature= 28°C Operating time= 14 days	Triclosan	10	98	[52]
			17-α ethinylestradiol	10	78	
			Nonylphenol	10	85	

Table 2: Performance of crude LME for the removal (%) of PPCPs

PPCPs	**Laccase** [80, 81, 89, 91, 92, 93, 94, 95, 96, 97, 98, 99]			**LiP** [88, 90, 100]			**MnP** [80, 95, 96, 101]		
Non-phenolic compounds	Removal (%)	Initial concentration (mg/L)	Incubation time (h)	Removal (%)	Initial concentration (mg/L)	Incubation time (h)	Removal (%)	Initial concentration (mg/L)	Incubation time (h)
Carbamazepine	5 – 37	0.01-0.1	24-48	<10	5	2	14-20	0.01-4.7	24-48
Ibuprofen	<5 – 38	0.01-0.1	24-48	-	-	-	20	0.01	48
Naproxen	20-100	0.01-0.5	24-48	-	-	-	95	0.01	48
Diclofenac	60-100	0.01-0.1	3-48	>99	5	2	100	0.01	48
Gemfibrozil	20-25	0.01-0.1	24-48	-	-	-	30	0.01	48
ketoprofen	<5 -12	0.01-0.1	24-48	-	-	-	22	0.01	48
Clofebric acid	10-20	0.01-0.1	24-48	-	-	-	<10	0.01	48
Benzophenone	<5	0.1	24	-	-	-	-	-	-
DEET	20-55	0.01	4	-	-	-	-	-	-
Octocrylene	20	0.1	24	-	-	-	-	-	-
Phenolic compounds									
Estrone	70-100	0.1-27	1-24	60	27	24	>99	5	8
17β-Estradiol	>99	0.01-5	1-24	40, 85	0.6, 27	1, 24	>99	2.96	1
17α-Ethynylestradiol	>99	0.1-2.96	1-24	20, 82	6.6, 30	8, 24	>99	2.96	1
Oxybenzone	10-25	0.1-0.5	24	-	-	-	-	-	-
Nonylphenol	100	22	1	-	-	-	-	-	-
Triclosan	70-90	0.1-144	24	-	-	-	-	-	-
"-": not reported									

Table 3: Properties of redox-mediators used to improve the performance of laccase based treatment of PPCPs

Redox-mediator	Type of mediator	Free radicals	Oxidation mechanism	Application for TrOC removal	References
1-hydroxibenzotriazole (HBT)	N-OH/ synthetic	aminoxyl	Hydrogen atom transfer	PPCPs, EDCs, pesticides and industrial chemicals	[43, 81, 89, 104, 105, 110]
Violuric acid (VA)	N-OH/ natural	aminoxyl	Hydrogen atom transfer	PPCPs, pesticides and industrial chemicals	[89, 90]
N-hydroxyphthalimide (HPI)	N-OH/ synthetic	aminoxyl	Hydrogen atom transfer	PPCPs and pesticides	[89]
Syringaldehyde (SA)	$C_6H_4(OH)(OCH_3)$/ natural	phenoxyl	Hydrogen atom transfer	PPCPs, EDCs, pesticides and industrial chemicals	[82, 83, 84, 85, 89]
Vanillin (VAN)	$C_6H_4(OH)(OCH_3)$/ synthetic	phenoxyl	Hydrogen atom transfer	PPCPs, EDCs, pesticides and industrial chemicals	[89, 90, 110]
2,2,6,6-tetramethylpiperidinyloxyl (TEMPO)	N – O / synthetic	Oxoammonium	Ionic	PPCPs, EDCs, pesticides and industrial chemicals	[89, 90, 110]
2,2-azino-nis(3-ethylbenzothiazoline-6-sulfnoic acid (ABTS)	ABTS/ synthetic	$ABTS^+$ and $ABTS^{++}$	Electron transfer	PPCPs, EDCs, pesticides and industrial chemicals	[89, 90]

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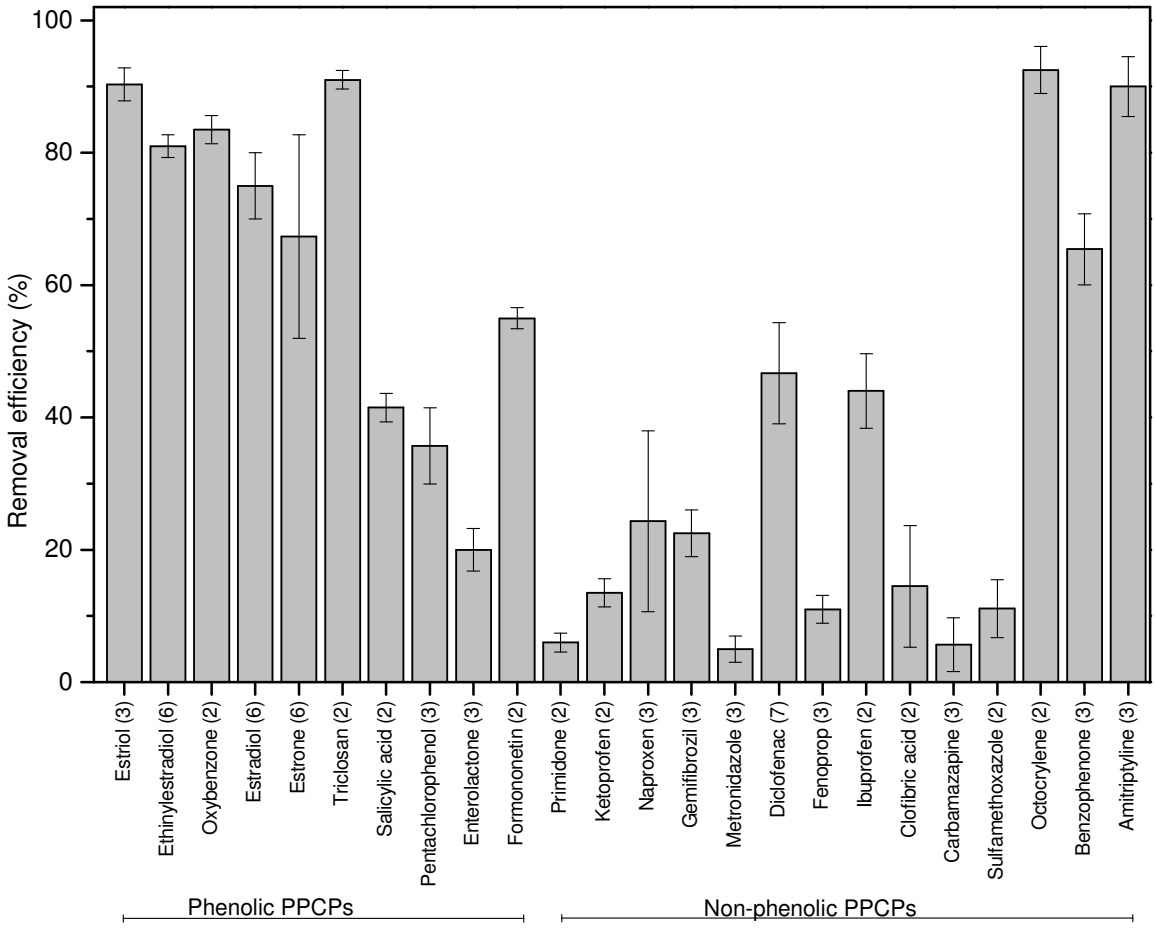

Figure 1. Average removal of phenolic and non-phenolic PPCPs after treatment with purified/commercially available laccase. Error bar indicates average±standard deviation. Numbers within parenthesis indicates number of data points. Data was collected from the following studies: [82, 83, 84, 85, 103, 104, 105, 106, 107, 108].

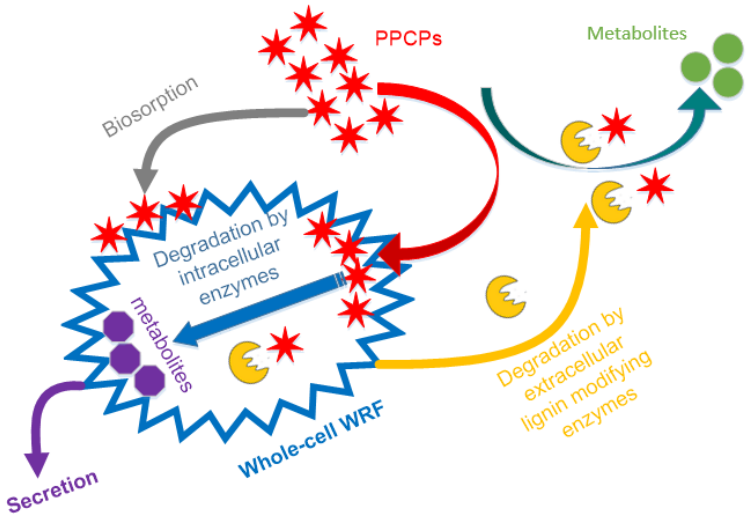

Figure 2. A schematic representation of whole-cell WRF mediated treatment process