Engineering biocatalytic material for the remediation of pollutants: A comprehensive review

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ABSTRACT

Bioremediation through biotechnological interventions has attracted more attention among researchers in field of environmental pollution control and abatement. Various cutting-edge studies in area of protein engineering and synthetic biology offer a new platform for creation of innovative, advanced biological materials for its beneficial role in environmental pollution mitigation. Biocatalysis especially receives considerable attention as sustainable approach to resource recovery from waste along with elimination of pollutants. This paper focuses on updated developments in engineering of biocatalytic substances which can degrade pollutants of emerging concern. It also explains various classes of biocatalysts, their mechanisms of immobilization, and applications in terms of environmental pollutant remediation. Opportunities and challenges for future research have also been discussed.

Keywords: Biocatalyst; Protein engineering; Pollutants; Immobilization; Bioremediation

1. Introduction

Various forms of emissions profoundly affect the environment. Manufacturing, human activities, and agricultural disposals processing make a major part in polluting air, water, and soil. A subclass of organic compounds alarmingly found in the environment enormously has labeled as emerging pollutants (EPs). These are otherwise known micropollutants (MPs) or emerging concern contaminants (Teodosiu et al., 2018; Varjani and Sudha, 2018). Such micropollutants are identified as processed synthetic chemicals without being tracked or controlled in most situations. These negatively impact people's health and many other life

forms (Sauvé and Desrosiers, 2014; Mohan et al., 2018; Bilal et al., 2019). These are otherwise called as Persistent organic pollutants (POPs). These POPs include perfluoroalkyl and polyfluoroalkyl, polycyclic aromatic hydrocarbons (PAHs), pesticides (halogenated organic compounds), and so on (Daniel et al., 1998; Mandal et al., 2015; Mandal et al., 2018; Varjani et al., 2019; Kumar et al., 2020). POPs are recognized to always have devastating health consequences concerning abnormal brain malfunction, growth, metabolic disorders, hormonal imbalance, etc. (Noakes et al., 2006; Mishra et al., 2019; Femina et al., 2020) whereas long term exposure may also lead to immunological impacts (Varjani et al., 2020b).

A huge number of environmental pollutants like dyes, nitrogen-containing chemicals, polychlorinated biphenyl compounds (PCBs), plastics, petroleum products, heavy metals, pesticides, hydrocarbons persist in the ecosystem. They are released from different industrial sectors and various agricultural resources (Benyahia et al., 2016; Varjani et al., 2017; Kumar et al., 2018; Rajmohan et al., 2019; Lakshmi et al., 2020). These pollutants are carcinogenic and highly toxic. In some cases, accumulations of these pollutants become hazardous to the also flora and fauna living in the environment (Varjani, 2017). Recently pollutant reduction and depletion are a big concern for environmental science (Do et al., 2020). Originally, waste produced from different industries was treated by incineration based on high temperature or by dumping off in a hole as biopile. Due to reduced efficiency, increased cost, and the formation of other recalcitrant derivatives such approaches were not proven quite successful. Further, bioremediation came to a picture, which provides a mechanism degradation of these pollutants by microorganisms (Vidali, 2001; Dzionek et al., 2016; Varjani et al., 2020a).

Through the use of microbes as well as their enzymes for pollutant removal is a safe, efficient, and cheaper process (Bhatnagar et al., 2017; Varjani and Upasani, 2017; Rathna et al., 2018). Progress in molecular microbiology and recombinant DNA technology can be made to improve the bioremediation process by plant and microbe genetic modification (Ramos et al., 2011; Mishra et al., 2020). Many forms of biocatalysts are deeply engaged in biological treatment, such as hydrolases, oxidoreductases, laccases, and peroxidases (Kadri et al., 2017). Various microbial sources like fungi (Example: *Pleurotus eryngii*, *Tramates versicolor*), algae (Example: *Chlamydomonas reinhardtii*, *Monoraphidium braunii*), and bacteria (Example: *Rhodococcus erythropolis*, *Pseudomonas aeruginosa*) were concluded having a catabolic process for pollutant clean up at pollution sites. Microbial lipolytic enzymes have gained attention for their ability to catalyse biotransformation reactions of ester-bond containing compounds eg. conversion of waste into high-energy products like biofuel and other value-added products via energy-efficient pathways (Kumar et al. 2020). As reported by Joutey et al. (2013), some microorganisms are capable of degrading contaminants under laboratory conditions only. This is due to environmental factors (nutrients, temperature, substrates, and electron acceptors) as they possess major role in bioremediation and influences biodegradation reactions (Varjani et al., 2019). Xu et al. (2017), have reported two bacterial strains named *P. aeruginosa* JLC1 and *Acinetobacter* sp. JLS1 for biodegradation of C16 alkane. They concluded about the temperature-sensitivity of these strains during the biodegradation process. Moreover, soil texture, occurrence of pollutants in soil matrix, indirectly affect biodegradation efficiency (Abed et al., 2015).

In the present review, groups of biocatalysts used for degradation of an array of pollutants and how they help in speedy degradation of various toxic pollutants have been summarized.

Eco-toxicological assessment for biocatalytic degradation process followed by immobilized biocatalysts and their effectiveness in removal of pollutants have also been discussed.

2. Biocatalysis as a sustainable approach

Various enzyme mechanisms have been used to effectively degrade complex organic compounds and have demonstrated that the compounds are oxidized and transformed into simpler intermediates (Chen et al., 2016; Jishao et al., 2019; Chen et al., 2020; Rajmohan et al., 2020). A relatively recent and convincing area of research is the biological approach, which uses peroxidases (oxidoreductase group) for the depletion of pollutants. The use of enzyme-based therapies provides numerous benefits, such as decreased sludge production, functioning at high and low pollutant concentrations, low energy inputs, and many others, catalytic research on a wide variety of contaminants (Bilal and Iqbal, 2019). Various studies have been reported on the biocatalytic removal of major pollutants and have been confirmed as effective ways in degradation of these compounds in a sustainable manner (Lin et al., 2017; Gonzalez-Coronel et al., 2017; Rani et al., 2017).

While enzyme-mediated cleanup has many benefits, it is important to note that there are some difficulties, such as the risk of producing hazardous dissolved by-products and the inability to handle with environmental influences (Zdarta et al., 2018a). Any of such problems can be overcome by immobilizing that enzyme on various inert surfaces. The examples for various categories of inert surfaces are silica, metal oxides (e.g. alumina, titania and magnetic iron oxides II and III), natural polymers (agar, gelatin, alginate, carrageenan, collagen, agarose), synthetic polymers (polyacrylonitrile, polystyrene, ion exchange resins) etc. (Daronch et al. 2020).

Furthermore, there are several examples of such enzymes effectively insolubilized to overcome the above-mentioned drawbacks of enzyme reusability and recycling (Alneyadi et al., 2018; Shao et al., 2019). Peroxidase and Laccase are enzymes commonly used to bioremediate polluted wastewater. Laccase is suitable for treating wastewater, as it maintains its operation across a wide range of temperature and pH. Peroxidases have a heme cofactor at their active sites and possess traces of redox-sensitive cysteine / selenocysteine. Due to the easy access to their active sites, peroxidase can help to facilitate the removal of many contaminants from wastewaters (Arca-Ramos et al. 2017).Such enzymes catalyze the oxidation-reduction of different types of harmful toxins including phenols, cresols, herbicides, synthetic clothing dyes, pesticides, chlorinated phenols, dioxins, and pharmaceuticals through assisted biodegradation process (Zdarta et al., 2018b; Muhammad et al., 2019). Although, biocatalysis is a sustainable approach in pollutant removal, process to produce biocatalyst requires high cost and some of them are not economical and sustainable. In addition to that they are unstable and most of them can't withstand several harsh experimental/environmental conditions (strong acid, high salinity, high temperature, extreme pH). The best way to find stable biocatalysts is to prospect microorganisms from extreme ecosystem capable of synthesizing stable catalysts.

3. Various groups of biocatalysts for contaminants degradation

Degradation of pollutants with the assistance of microorganisms is a sluggish method, which in actual reality reduces the viability of bioremediation (Ghosh et al., 2017; Varjani and Upasani, 2019). Microbial enzymes isolated from the cells were used for bioremediation during the last few decades along with use of microbes to address the above shortcomings

130 (Camenzuli et al., 2013; Nigam, 2013; Sonune and Garode, 2018). Biocatalysts are complex
131 groups of macromolecules that induce a variety of biochemical reactions affecting the
132 pathways to degrade the pollutants (Kalogerakis et al., 2017). The various groups of
133 biocatalysts based on their activities concerning pollutant degradation have been illustrated
134 by fig.1.

135 "[Insert Figure 1]"

136 Bioremediation based on complete and partially pure enzymes may not rely on the
137 proliferation of a single micro-organism in a contaminated ecosystem but the catalytic action
138 of the microbial produced naturally enzyme. Bioremediation can be accomplished in
139 nutrient-poor soil by using a refined enzyme. The use of biocatalytic biotransformation that is
140 harmless to the atmosphere does not contain harmful side products formed by microbial
141 biotransformation (Gianfreda and Bollag, 2002; Ruggaber and Talley, 2006). As industrial
142 scale biocatalysts production is carried out specifically by submerged fermentation, most
143 studies have concentrated on construction/application of this type of bioreactors. However,
144 production of biocatalysts through solid state fermentation process possesses higher yields
145 and less expensive (Dhakar et al. 2014). Different agro-industrial waste products have been
146 utilized for production of biocatalysts for bioremediation (Fatma et al. 2010; Debnath et al.
147 2020). In many cases, production of these biocatalysts can be enhanced by immobilizing the
148 source organisms (Lin et al. 2008; Dong-chul et al. 2019; Bera et al. 2020). It is necessary to
149 invent biocatalysts that are resistant to adverse conditions like alkaline or acidic pH, high
150 temperature, high salt concentration owing to its applications in various industries. The
151 biocatalysts used in hydrocarbon degradation have been reported to be produced by

extremophilic microorganisms (Patricia et al. 2020). Wentzel et al. (2018), have investigated production of ligninolytic enzymes, lipases, and protease from filamentous fungi and yeasts. Microorganisms such as fungi, microalgae and bacteria produce multicopper oxidase enzyme (laccase) which has versatile applications in various industries. Recombinant protein expressions have been used to increase productivity in shorter durations. Horseradish peroxidase (HRP C1A) was isolated from Horseradish plant and transferred to *E. coli* BL21 through rDNA technology which could produce more quantity of HRP C1A and could degrade phenolic compounds. Similarly, carboxylesterase was isolated from human liver and was inserted to *E.coli* could degrade pesticides, chlorine, carbamates compounds etc (Gupta et al. 2017; Gundinger, et al. 2017)

3.1. Oxidoreductases

Different groups of microorganisms and higher plants generate and secrete oxidoreductases to remove substances through oxidative coupling including the oxidation of compounds through moving electrons from reductants to oxidants resulting in the release of CO_2 and chloride ions. As a result of pollutant depletion Oxidoreductases energy or heat is produced, and for biochemical activities, microorganisms used it (Medina et al., 2017). Several pollutants such as 2,4,6-trinitrotoluene (TNT), chlorophenol, phenol, polychlorinated biphenyls (PCBs), nitroaromatic compounds, dyes (bromophenol blue, malachite green) were degraded using oxidoreductases. For example, Gram-positive bacteria *Bacillus safensis* CFA-06 produces oxidoreductase and it degrades the petroleum substances.

Different kinds of phenolic compounds are created by lignin degradation in nature binding with other compounds polymerization and co-polymerization has been transformed into

another form by oxidoreductases (Husain, 2006). The textile industry releases the color compounds into the environment which is degraded by different enzymes such as laccases and peroxidases (Jang et al., 2009). Annibale et al. (2004), have reported that *Panus tigrinus*, white-rot fungi secreted an extracellular oxidoreductase (lignin peroxidase, Mn-dependent peroxidase, and laccase) that have removed the color, phenols, and organic discharged from olive-mill wastewater.

A lot of microbial species synthesizes oxidoreductase enzymes leading to redox reactions for the removal of radioactive metals. The plant that belongs to the *Gramineae*, *Fabaceae*, and *Solanaceae* families and secretes enzymes extracellularly for soil pollutant degradation such as hydrocarbon-containing petroleum hydrocarbon and chlorinated compounds (Park et al. 2006; Edwin-Wosu et al. 2016).

3.1.1 Oxygenases

Oxygenases are the key catalyst for the degradation of aromatic compounds, catalyzing the ring's cleavage in aromatic compounds. Based on the number of oxygen molecules involved in the cleavage, oxygenases enzyme have divided into two subclasses: monooxygenase and dioxygenase. Monooxygenase has been catalyzing the addition of one oxygen atom molecule. Dioxygenase has catalyzed the addition of two oxygen atom molecules. Microorganisms like *Pseudomonas* sp. able to degrade the pesticides excreting Glyphosate oxidase (GOX). It can also catalyze chlorinated compounds of a wide variety, herbicides, and various groups of pesticides (Tangahu et al., 2011).

3.1.1.1. Monooxygenases and Dioxygenases

Through introducing one molecule of oxygen, monooxygenases catalyze the breakdown of aromatic substances and enhance their reaction and solubility. Monooxygenases have been reported to be active in desulphurization, denitrification, and dehalogenation (Arora et al., 2010). Based on their cofactor used, monooxygenases are categorized into two categories: P450 mono-oxygenases group and flavin-dependent mono-oxygenases (Gaur et al., 2018). P450 monooxygenase is a heme-containing enzyme, found both in prokaryotes and eukaryotes *Bacillus megaterium* BM3 can degrade fatty acid and aromatic compounds excreting a P450 mono-oxygenase enzyme (Gustafsson et al. 2004). Methane monooxygenase metabolizes halides including aliphatic substances, heavy metals, and aromatic hydrocarbons. As reported by Singh and Singh (2017) about the enzyme methane monooxygenase that comes in different forms. It may either occur in the cytoplasmic membrane or the cytoplasm. Monooxygenase like tetracenomycin F1 and quinol mono-oxygenase function without any cofactors isolated from *Streptomyces glauscens* bacterium and *E. Coli.,* respectively (Arora et al., 2010). Whole White-Rot Fungus Genome Sampling, *P. Chrysosporium* identified 150 genes in 16 gene clusters clustered within existing 12 cytochrome P450 (CYP) families and 11 fungal CYP clans and one single P450 reductase portion in the fungus (Tuomela and Hatakka, 2011).

Dioxygenases catalyze the oxidation of the aromatic compounds by inserting two molecules of oxygen. Aromatic dioxygeneses can be categorized according to their mechanism of action into (1) aromatic ring cleavage dioxygenases and (2) aromatic ring hydroxylation dioxygenases able to degrade the different chemicals by adding two molecules of oxygen into the ring and split the compound aromatic rings respectively (Ozer et al., 2019). *Pseudomonas putida* F1 produces Toluene dioxygenase which catalyzes toluene

degradation. It acts for several contaminants such as aromatic and aliphatic substances as dioxygenase (Muthukamalam et al., 2017). In the soil bacteria, catechol dioxygenases catalyze the conversion of aromatic precursors into aliphatic substances (Ali et al., 2017). A significant number of aromatic compounds from different chemical, medicinal, and dye factories are released into the environment. Dioxygenase breaks down the 1, 2-position of the aromatic ring to integrate two oxygen molecules into the substrate (Guzik et al., 2014). Figure 2 illustrate probable pathway of oxidoreductase (monoxygenase & dioxygenase) for the transformation of pollutants.

"[Insert Figure 2]"

3.1.2. Laccases

Oxidases and Laccases that produce copper, catalyzing the oxidation of a broad variety of aromatic substances and phenol groups found in the environment (Mai et al., 2000). By oxidizing the bonds, Laccase even decolorized azo dyes and transformed them into less toxic compounds found in the ecosystem (Legerska et al., 2016). Laccase produced by *Trametes hispida* fungus could decolorize various pollutants. *Trametes versicolor* is also a good source of laccase and it was immobilized on porous glass beads. This immobilized enzyme could degrade a wide variety of toxins, such as heterocyclic aromatic compounds, phenolic compounds, and aromatic compounds containing amines. Laccase produced by *R. Practicola* is capable of degrading and bio transforming phenolic compounds (Dodor et al., 2004; Strong & Claus, 2011). Illustration of Laccase Mediator System (LMS) and its role to detoxify organic pollutants has been shown in figure 3.

"[Insert Figure 3]"

3.1.3. Peroxidases

Peroxidases, produced by bacteria, fungi, plants, and animals are widespread. Phenolic radicals that are formed by oxidizing phenolic compounds, which quickly become less soluble (Bansal and Kanwar,2013). Peroxidases are further split into three classes: Class-1 intracellular enzyme including yeast-generated cytochrome-c peroxidase, ascorbate peroxidase (APX) formed by certain plant organisms, and bacterial catalase peroxidase. Class-2 includes lignin peroxidase (LiP) and manganese peroxidase (MnP) secreted fungal enzyme. Though Class-3 produces secreted peroxides from horseradish products, such as horseradish peroxidases (HRP) (Koua et al., 2009).

3.1.3.1. Lignin peroxidases (LiPs) and Manganese peroxidases (MnPs)

Lignin peroxidases belong to monomeric proteins that are secreted by fungi like *Trametes versicolor* and *Phanerochaete chrysosporium*. It catalyzes toxic pollutant oxidation in the presence of hydrogen peroxide and alcohol-veratryl as co-substrate and mediator respectively (Xu et al., 2014; Abdel et al., 2013). Lignin peroxidases demonstrate excellent use in wastewater treatment and bioremediation. Degradation of lignin by bacterial peroxidases is much more selective in terms of specificity and thermostability associated with fungal peroxidases (Tuomela and Hatakka, 2011; Behbahani et al., 2016).

Manganese peroxidases are known as indirectly acting to degrade lignin and xenobiotic substances are extracellular enzymes produced by fungi. This enzyme catalyzes the degradation of some phenolic groups, aromatic compounds, and coloring agents (Balaji et al.,

2019). This enzyme has a great potential to remove excess different forms of colorants such as anthraquinone, triphenylmethane, and azo dye. Zhanga et al. (2016), had identified and purified manganense peroxide from *Tremetes* sp. 48424. In *Peniophora incarnata* KUC8836 a gene (pimp1) responsible for the synthesis of manganese-dependent peroxidase was identified. Further, this gene has been expressed in the *Saccharomyces cerevisiae* fungi to estimate its potential to remove anthracene (Lee et al., 2016).

3.2. Hydrolases

Hydrolases are widely used for the bioremediation of the insecticides. These enzymes specifically break large peptide bonds, carbon-halide bonds, esters, etc. It can degrade carbazyme into 2-aminobenzimidazole. Microbe-secreted extracellular hydrolases facilitate the degradation of organic polymers which can move through cell pores (Babita et al., 2018). It is very efficient to bioremediate organophosphate and oil spills by using a hydrolytic enzyme.

3.2.1. Lipases

Lipases are used to perform inter-esterification, esterification, hydrolysis, and alcoholics reactions (Prasad and Manjunath, 2011). They are widespread in existence, catalyzing the degradation of triacylglycerols into glycerol and free fatty acids (Shukla and Gupta, 2007). Due to lipase activity the amount of hydrocarbon in the polluted soil has been reduced (Ghafil et al., 2016). Verma et al. (2012), had optimized the process for the bioremediation of crude oils with lipases extracted from *Pseudomonas aeruginosa* SL-72

3.2.2. Cellulases

Cellulase formed by microorganisms can be associated with cell envelope. These are mainly degrading the cellulose. In the textile and detergent industries, cellulose microfibrils produced during processes and pollute the environment. *Bacillus* species contain other alkaline cellulases, and *Trichoderma* and *Humicola* fungi contain neutral and acidic cellulases (Behera et al., 2017). These cellulases were used in the paper and pulp industries for the removal of ink during paper recycling (Karigar and Rao, 2011). Recently, Imran et al. (2016) had characterized cellulase from *Humicola* species which can tolerate and work in adverse conditions like extreme pH and temperature. It can be used for hydrogen bond breakdown in detergents and washing powders industries. Aslam et al. (2019) had isolated *Bacillus Amyloliquefacience*-ASK11 that was a good source of cellulase isolated from industrial leather-tanning waste.

3.2.3. Carboxylesterases

Enzyme carboxylesterases have catalyzed the degradation of ester bonds of carbamates, organophosphates, and other chlorinated organic compounds (Cummins et al., 2007). Yin et al. (2016) isolated a strain of *Pseudomonas aeruginosa* PA1 can able to synthesize carboxylesterases. This could absorb and degrade the mercury at the infected site. Carboxylesterases have hydrolyzed their ester bond using the prevalent path for the depletion of all types of pyrethroid insecticides. In a study, the active site of the carboxylesterases isolated has been modified for pyrethroid hydrolysis by *in vitro* mutagenesis (Heidari et al., 2004).

3.2.4. Phosphotriesterases

Initially isolated from soil bacteria *Pseudomonas diminuta*, hydrolyze a broad range of organophosphate (Romeh and Hendawi, 2014). Some marine bacterial species, such as *Thalassospira* tepidiphila, *Phaeobacter* sp., *Ruegeria mobilis* can degrade the coastal oceanic phosphate trimester. A bacteria *Geobacillus stearothermophilus* having the potential to hydrolyze compounds containing both lactone and organophosphate. Thermostable phosphotriesterase extracted from *Geobacillus stearothermophilus* bacteria is highly which can tolerate 100ºC (Moshe et al., 2018).

3.2.5. Haloalkane dehalogenases

Haloalkane dehalogenases are bacterial enzymes that use a hydrolytic mechanism to cleave the carbon – halogen bond of halogenated aliphatic compounds. Halogenated substances are formed anywhere in the soil as a consequence of both natural and man-made actions and can be poisonous, mutagenic (Kotik and Famerova, 2012; Koudelakova et al., 2013). Nagata et al. (2015), had identified haloalkane dehalogenase in bacterium *Xanthobacter autotrophicus* GJ10 having the ability to degrade 1, 2- dichloroethane.

3.2.6. Proteases

Proteases are found in all living forms as bacteria and fungi, plants, and animals (Kuddus and Ramteke 2012). Most of the marine microorganisms capable of producing protease (Sivaperumal et al. 2017). Kumar et al. (2014) had reported about the degradation of the diesel oil *in vitro* up to 54% by the proteases obtained from *Pseudomonas fluorescens*. Similarly, proteases from *Geotrichum candidum* and *Cladosporium cladosporioides* could decompose 55% of the nonionic ethoxylated surfactants (Jakovljević and Vrvić 2018).

4. Identification of transformation pollutants and their eco-toxicological assessment

Enzymes require multiple pathways to remove various ecological toxins, leading to the production of various metabolic compounds and the final product during the biocatalytic cycle. Researchers and scientists focus mainly on the absence of parent molecules in most degradation experiments, rather than evaluating intermediate metabolites, transformation pathways, and determining the toxicity of transforming products (Jian et al., 2020). Phenolic substrates are converted catalytically to phenoxy radicals by using peroxidases catalysts in the presence of hydrogen peroxide. The phenoxy radicals produced in the catalytic process can be coupled with each other or with other reactive substances (Torres-Duarte et al. 2010). Peroxidases group of biocatalysts could transform pentachlorophenol in a multistep pathway with an oxidative dehalogenation process to produce tetrachloro-1,4-benzoquinone. Further, tetrachloro-1,4-benzoquinone is degraded through reductive dehalogenations process. Similarly, Chloroanilines (an intermediate used in the synthesis of dyestuffs, agricultural chemicals, and pharmaceuticals) can be transformed to chlorophenol by peroxidase transformation (Torres-Duarte et al. 2010). There are many studies with respect to PAH transformation process with the application of different biocatalysts like lignin peroxidase and manganese peroxidase, PAH are generally oxidized to quinones and hydroxylated derivatives. These oxidized products are found to be more biodegradable. The pesticides belong to organophosphorus group could be transformed in to oxon (P=O) derivative by chloroperoxidase. This enzyme has a capability to replace the sulfur atom by an oxygen atom, transforming the phosphorothioate group to an oxon derivative (Torres-Duarte et al. 2010).

Various molecular methods have been used to detect the degradation of products through the catalytic reaction. These include mass spectrometry, liquid chromatography with tandem mass spectrometry gas chromatography-phase electrospray phase ionization mass spectrometry, and liquid chromatography-electrospray time-off light mass spectrometry (Alneyadi and Ashraf, 2016). Lonappan and groups (2016) had developed laser diode thermal desorption-mass spectroscopy detects the transition components. Biotoxicity assays were used to evaluate the degree of contamination in waters. A very well-known acute toxicity experiments were carried out using green algae species to categorical data on water quality levels (Cristovao et al., 2011). Cibacron Blue 3GA's toxicity and its by-products have been investigated using *Daphnia magna* as research organism. The inhibition of microalgal growth was investigated using *Chlorella vulgaris*. MALDI-ToF-MS has been used to confirm by-products of pollutant degradation (Bayramoglu et al. 2019).

A growth inhibition bioassay of *Scenedesmus obliquus* was tested with soybean peroxidase-treated triclosan solution and compared with the untreated triclosan to analyze the toxicity (Li et al., 2016). The application of triclosan to the formulations at a level of 10-µM was found to fully suppress the *S. obliquus* growth, while soybean peroxidase treatment of triclosan solution is gradually growing its growth. In another analysis complete elimination of triclosan solution toxicity was achieved within 2 h of the reaction time (Muhammad et al., 2019). Microorganisms like *B. subtilis*, *E. coli,* and *B. megaterium* were used to analyze the toxicity profile of untreated and treated substances through growth inhibition assay. For this reason, after the exposure of bacterial species to the solution for a given time, the amount of

total viable cells is counted (Muhammad et al., 2019). The toxicity reduction potential of the MnP-Tween 80 was tested using bacterial growth inhibition experiments for *B. Subtilis*. Reports revealed that a 24-hour MnP-Tween 80 treatment resulted in a total loss of *B. Subtilis* growth inhibition by Miconazole. The same treatment could decrease by 78% growth inhibition of *Pseudokirchneriella subcapitata* by sertraline (Inoue et al . 2015).To determine their toxicity with *Raphanus sativus* plants, initial as well as lacquer-treated Bisphenol-A solutions were subjected to phytotoxicity analysis. The root length and germination of the seeds were recorded after 5 days of dark incubation (Lassouane et al., 2019).

The pollutants like Diclofenac, trimethoprim, carbamazepine, and sulfamethoxazole were treated with laccase, subjected to its toxicity analysis by using bacterial luminescence method for *Photobacterium leiognathi*, and were found to be nontoxic in nature (Alharbi et al. 2019). Phytotoxicity assay was also executed by using *Lactuca sativa* seeds for sulfamethoxazole after treatment with laccase. Untreated sulfamethoxazole solution decreased the root length, the result was opposite in treated case (Al-Maqdi et al. 2018). Copete-Pertuz et al. (2018), have investigated toxicity for laccase treated oxacillin solution by MTT assay using human liver cells-hepatoma (HepG2). Other methods to analyze the toxicity of treated pollutants with biocatalysts include Yeast Estrogenic Screen (YES) assay, *Vibrio fischeri* luminescence reduction test, Microtox assay, Algal viability test using the fluorometric indicator etc. (Ji et al. 2017; Becker et al. 2016; Yousefi-Ahmadipour et al. 2016; Naghdi et al. 2018).

5. Immobilized biocatalyst for contaminants remediation

Immobilization of biocatalyst for remediation of pollutants has been reported for their speedy biodegradation. Applications of immobilized biocatalysts have been summarized in Table 1

"[Insert Table 1.]"

5.1 Immobilization with inorganic materials

Immobilized enzymes used through adsorption with a wide variety of different inorganic materials for the simultaneous removal of hazardous pollutants. Organic oxides, for example, alumina titanium, silica, and iron oxides have been applied to immobilize oxidoreductases along with selective sorption of contaminants, phenolic compounds, synthetic dyes, and antibiotics (Yu et al., 2015; Bilal et al. 2019[b]). Minerals and carbon-based materials possess good sorption properties and high stability. These were successfully applied by enzymatic oxidation and subsequent adsorption to remove pollutants in a study conducted by Ding et al. (2016).

Spherical alumina pellets and Al_2O_3 pellets were used for the adsorption covalent binding of laccase enzyme respectively. It was found that the immobilized laccase could decolorize industrial effluents rich in Reactive Black 5. The interesting part of this study was that dye was adsorbed in the support materials and it could be degraded through laccase. In this process, 80 percent of the Reactive Black 5 was adsorbed and only 4 percent was degraded by laccase (Jakub et al., 2019).In contrast, through covalent bonding, only 10 percent were adsorbed to the pellets of alumina, and 90 percent of the Reactive Black 5 were biodegraded by laccase. The major problem was the saturation of active sites in case of a covalent immobilization process. This happens due to the multipoint attachment of the biocatalysts

409 with immobilized surfaces. The efficiency of the enzyme for the removal of dye was lost in
410 this case (Osma et al., 2010).

411 The benefits of using inorganic materials for immobilization of the enzymes are having
412 exceptional mechanical and increased stability. In some cases, a functional group like
413 hydroxyl facilitates immobilization and adsorption of toxic contaminants using inorganic
414 materials simultaneously. In these cases, enzyme elution from the support can be restricted
415 owing to its multipoint attachments. However, covalent bonds formation cannot even be
416 omitted due to the existence of several functional moieties, which further decreases enzyme
417 leakage. Even successful sorption of both contaminants from the atmosphere and their
418 bioconversion products is facilitated by the existence of many functional groups. Applications
419 of inorganic oxides drawn the attention of the researchers for industrial applications. This is
420 due to its porous structure, more surface area, exceptional stability, and established
421 morphology. Additionally, applications of inorganic oxides for along with immobilization of
422 the enzyme and adsorption of contaminants results in high efficiencies in elimination.

423 5.2 Immobilization with organic materials

424 Biopolymers and synthetic polymers, apart from many inorganic materials, are also
425 applied for immobilizing the enzyme and sorption of a hazardous compound simultaneously.
426 The existence of various chemical moieties (C=O, $–NH_2$, –OH, and COOH) facilitate the
427 efficient immobilization of enzymes and the sorption of contaminants. The presence of
428 functional moieties and their natural origin exhibit very high peptide affinity concerning bind
429 the enzyme (Bilal and Iqbal, 2019). Polymers like starch, agar, carrageenan being used for
430 adsorption and biodegradation of toxic compounds simultaneously (Loffredo et al., 2014).

Apart from these, the chitosan is also most widely used as an organic polymer for immobilizing the enzyme. In a study, laccase was immobilized with chitosan and it was applied to decolorize the effluents having Sulfur Brown GD and Sulfur Blue 15. Here 70% of Sulfur Brown GD and 80% of Sulfur Blue 15 could be removed from the dye solution of 200 mg / L at pH 6.5. The interesting part of this study, it was not effective if a mixture of these two dyes was treated with the same conditions (Nguyen et al., 2016). Chitosan film has been also used to immobilize mushroom tyrosinase to degrade phenol derivatives in wastewater and to absorb quinone derivatives produced after the oxidation (Yamada et al., 2005). The synthetic polymers like polyvinyl alcohol and polystyrene are also used for the simultaneous biodegradation and adsorption of harmful compounds apart from the natural organics. There were so many studies that were carried out with these synthetic polymers for the binding of laccases or tyrosinases enzymes to degrade phenolic derivatives (Zhang et al., 2014).

In research, horseradish peroxidase was immobilized with polyacrylonitrile membranes and studied its potential for phenol degradation (Wang et al., 2016) Further, the crosslinking of horseradish peroxidase was done with glutaraldehyde to stop the elution of biomolecules from the matrix. The major problem with cross-linking was the decreasing number of chemical moieties capable of adsorbing phenol. Besides, covalent binding, adsorption, and even encapsulation using polymer supports will immobilize a wide variety of enzymes.

5.3 Immobilization with hybrid and composite materials

Hybrids materials can be synthesized by linking both organic materials and inorganic and organic precursors. These materials are having more affinity to the peptides present in the enzymes due to their biocompatibility. The two biopolymers namely chitosan and alginate were combined followed by the crosslinking of chitosan with glutaraldehyde having an

alginate-filled pore space, where *Agaricus bisporus* tyrosinase made immobilized (Ensuncho et al., 2005). The alginate beads produced possessed excellent mechanical properties. This was subjected to the enzymatic conversion to study the phenol removal from the wastewater and further sorption of quinone. About 90% of the phenol was extracted under ideal conditions after 4h of the operation. The synthetic polymers and biopolymers are very effective for pollutant adsorption and enzymatic biodegradation. As part of hybrid material, chitosan was linked to the Diaion WK10 and WK20 through the weakly acidic cation exchange resins. Tyrosinase was then covalently immobilized with this to remove alkylphenols from aqueous solutions (Jakub et al., 2019).

Zhang et al. (2020), have developed a smart microfluidic device to prepare horseradish peroxidase (HRP) and zwitterionic polymers [poly(carboxybetaine methacrylate)] in order to find a solution to enhance degradation process for bisphenol A. It was found that, this immobilized HRP could degrade 99.42% of bisphenol A in 20 minutes.

Polyacrylonitrile was combined montmorillonite to create nanofibers. This was enriched with graphene oxide to facilitate the electron transfer. The hybrid nanofibers could immobilize the *Trametes versicolor* that synthesize laccase and could extract catechol (Li et al. 2011; Wang et al., 2014). Graphene oxide was added to it to enhance the enzyme's catalytic properties. The less concentration of the immobilized enzyme and weak sorption capacity of hybrid material is the main drawback of the proposed hybrid system. Poly (D, L-lactide-co-glycolide) and multi-walled carbon nanotubes were used to fabricate hybrid fibers, that were used for the encapsulation of laccase. This system was executed to remove bisphenol A through biodegradation (Dai et al., 2016). The synthetic groups of polymers like poly(acrylic acid), poly(vinyl alcohol), and polyamine combine with inorganic precursors

(clays, iron, and silica) to produce a hybrid system and stable material for entrapment of the enzymes. This hybrid material-enzymes-system was capable of biodegradation and simultaneous adsorption of phenols (Xu et al., 2015). Similar way, a hybrid membrane system was synthesized out of chitosan and iron ions in order to degrade the color by immobilized laccase enzyme (Wen et al., 2015). Covalently immobilized enzyme (Laccase) with nanozeolite - carbon nanotube composites were synthesized and used by researchers for degradation of Direct Red 23. The activity of free laccase was found to be 60%, while the nanocomposite retained about 80% of its maximal activity after 8 days of incubation (Mahmoodi et al., 2020). Removal of the dyes (Brilliant Blue G, Procion Green H4G, and Crystal Violet) by using immobilized laccase within polypropylene chloride (PP) film and poly(glycidylmethacrylate) system was also reported (Yakup et al. 2017). Representation for immobilizing biocatalysts with polymeric materials has been illustrated by figure 4.

"[Insert Figure 4]"

6. Research needs and future directions

The lustiness of biological materials such as enzyme and microbes in the form of biosorption or biocatalytic material; under unique environmental conditions is highly desirable. Lustiness is commonly referred to as the durability and consistency of the substance against a variety of critical criteria applicable to diverse drainage and contaminated habitats. It is also essential to design well-controlled reactor units and treatment methods to mitigate the discharge of designed biological material into the surroundings. To this end, work should offer a technical foundation for risk reduction, although modern technological advancements are being processed in the production of the materials to deal with critical

environmental concerns. Finally, a key element to remember is cost, for the functional application of engineered catalysts and biosorption components. Immobilization of the desired protein onto a suitable matrix requires a pure protein that can be purified by extraction, identification, and purification steps which enhance the cost and time of a biological process.

Another essential step towards implementation is the analysis of mixture and matrix effects on biocatalytic processes, as wastewater represents a complex mixture of inorganic and organic substances. Because municipal wastewater is a dynamic mixture of various compounds, the use of enzyme combinations that function synergistically with specific selectivity should be a crucial issue in improving bio-catalytic treatment processes. The essential factor is the use of biocatalysts with optimal pH and temperature within the wastewater spectrum to maintain elevated stabilities and activities. In most experiments, a redox mediator was required to dramatically boost the efficiency of the transition (Ashe et al. 2016), that create additional pollution. Thus, research to improve the efficiency and stability of biocatalysts in wastewater is essential to make it available that do not require a mediator. In already developed industrial wastewater treatment applications, the enzymes are isolated mainly through membranes. Biofilm formation is one of the drawbacks of membrane system. Another drawback is the secreted extracellular enzymes can potentially interact with the immobilized enzymes on the membrane, leading to a loss of enzyme activity.

7. Conclusions

New developments in genetic and macromolecule engineering are unveiling enormous potential to increase the implementation of biochemical functions at the molecular level and

establish novel approaches and emerging environmental management challenges. Recent developments allowed creation of materials with new capabilities, offering a safe and cost-efficient method for handling emerging problem of remediation of recalcitrant pollutants and extracting useful goods from the product. While modern work activities have made substantial and promising strides toward this goal, important areas remain to be studied in depth until advanced biological materials can be used in action to address environmental concerns.

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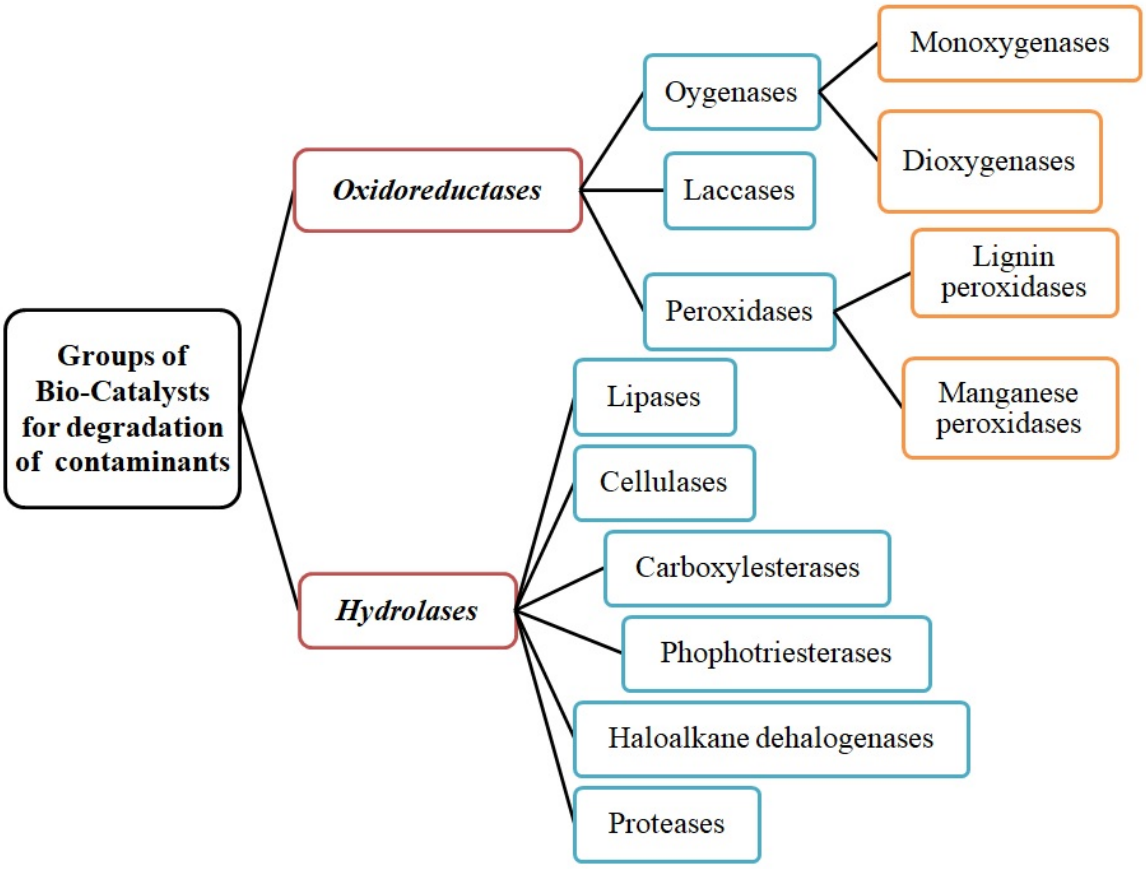
Figure Captions:

Figure 1: Classification of biocatalyst on the basis of their activity

Figure 2: Pathway to illustrate activity of oxidoreductase (monoxygenase and dioxygenase) in degradation of phenol and other aromatic pollutants

Figure 3: Illustration of Laccase Mediator System (LMS) and its possible role for bioremediation and detoxification of organic pollutants along with the active site of laccase to facilitate the catalytic cycle through electron flux

Figure 4: Schematic representation for immobilizing biocatalysts with polymeric materials and degradation of pollutants



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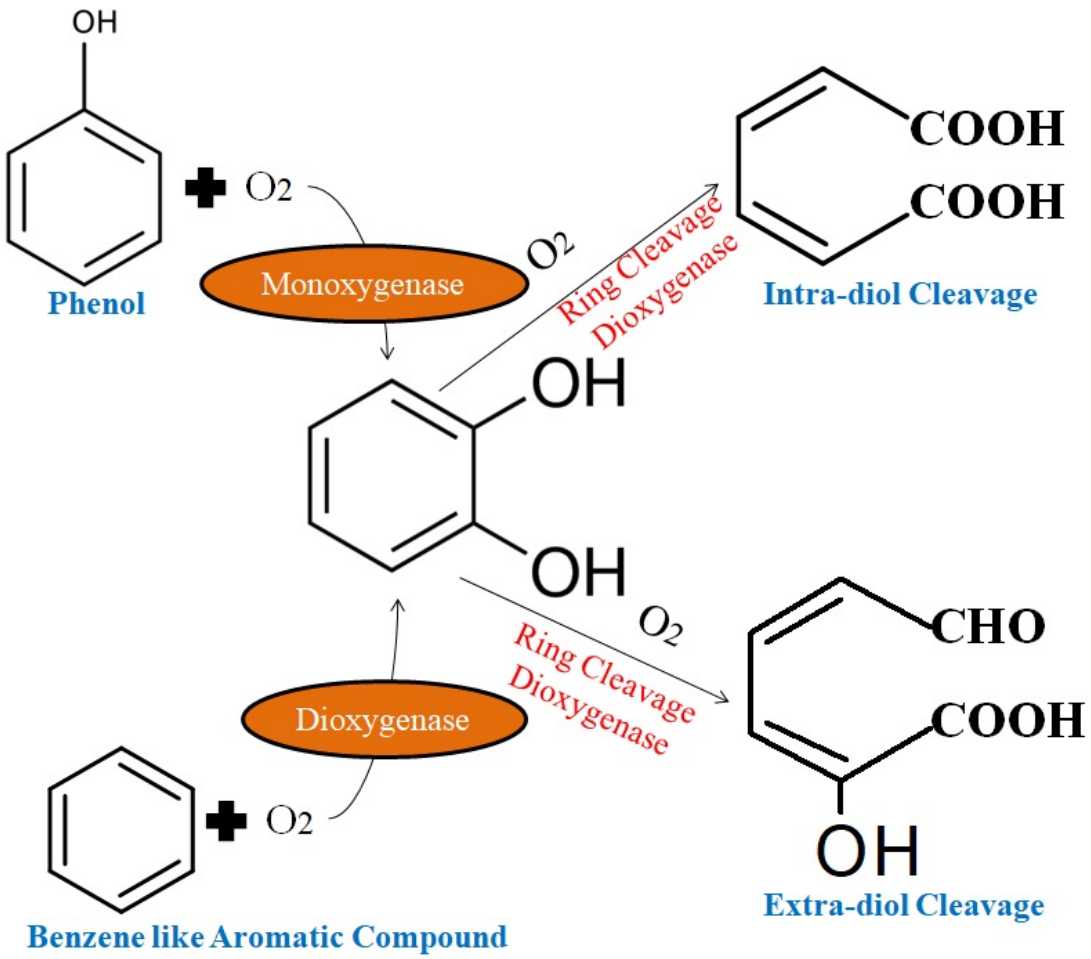

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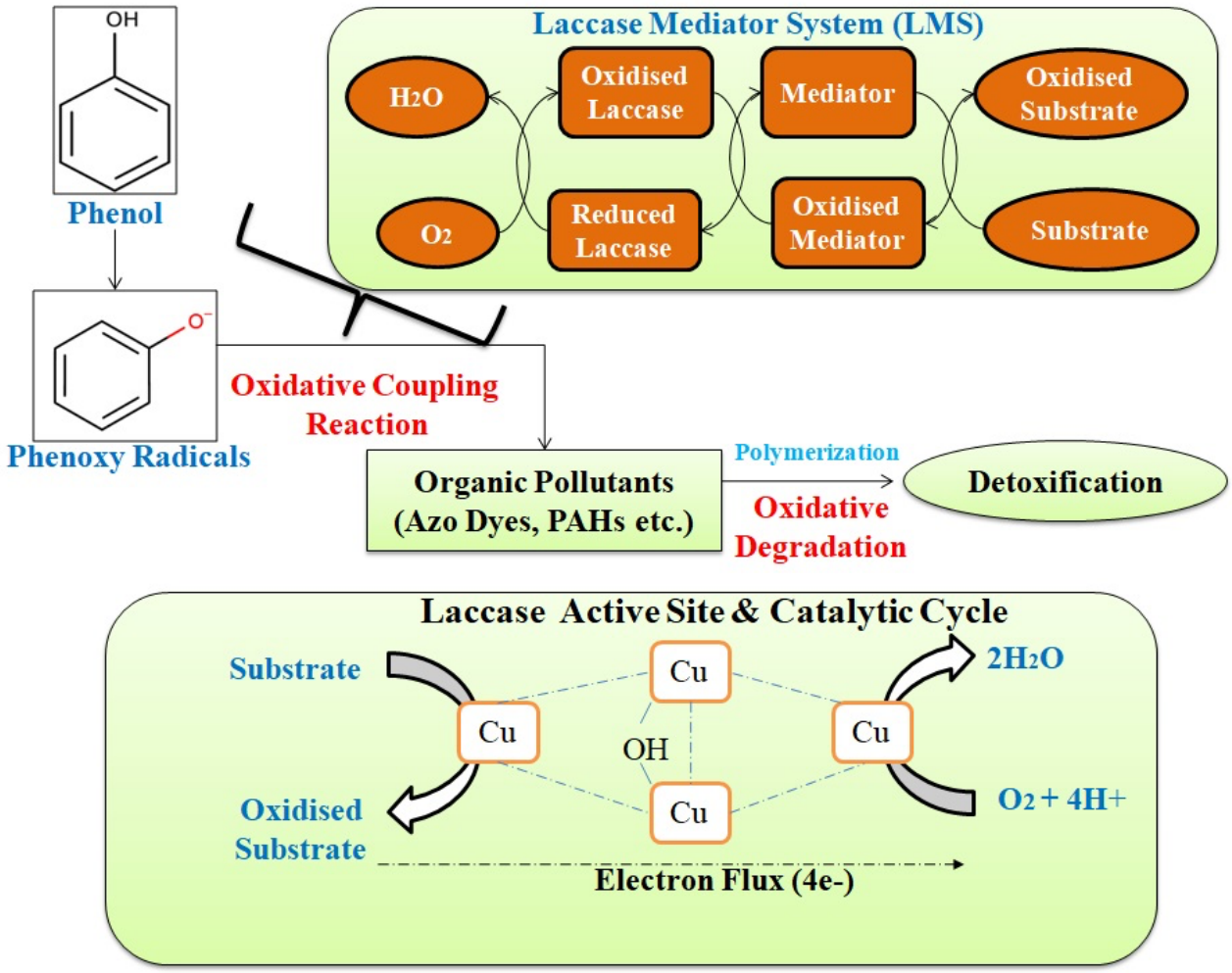

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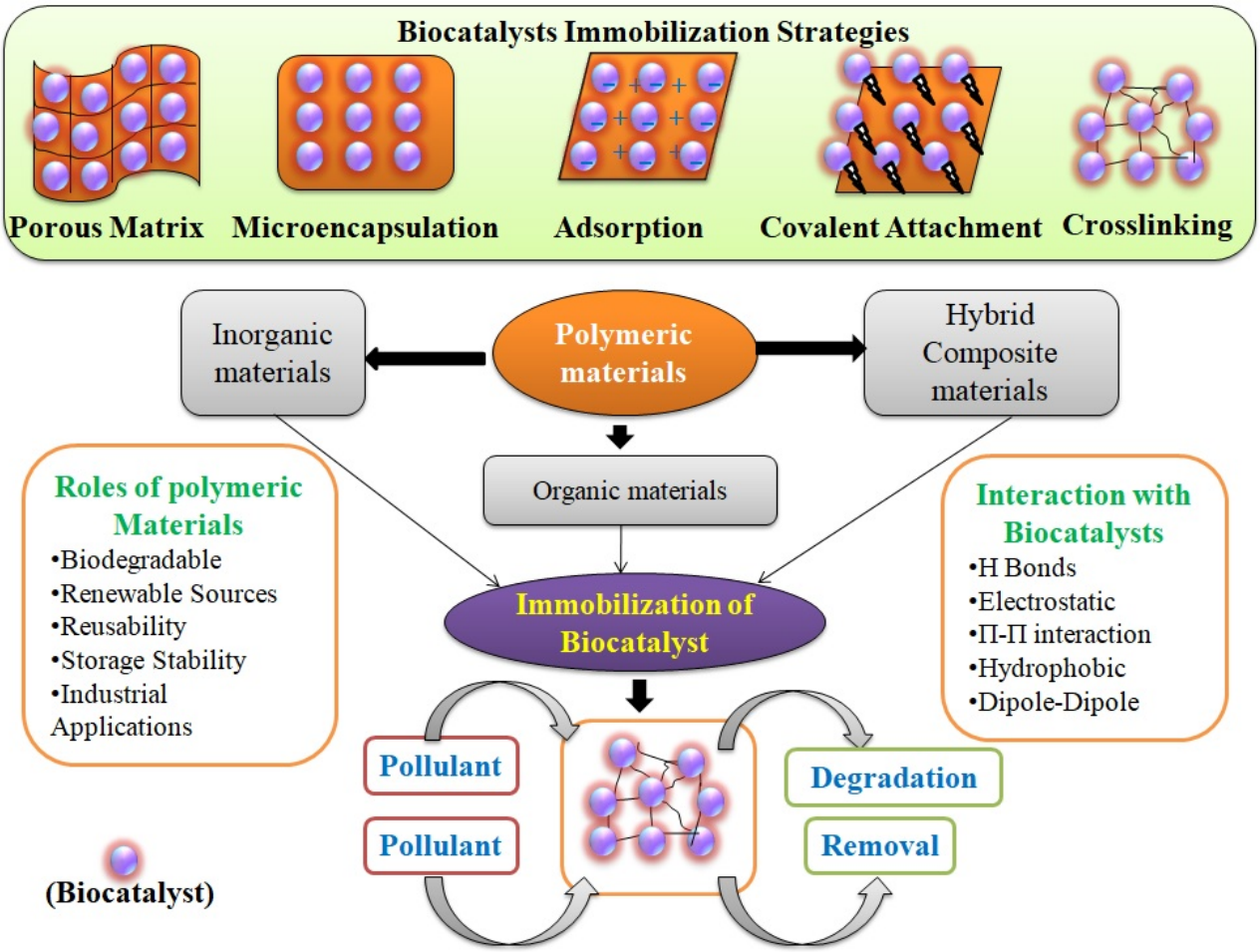

Figure 4: Schematic representation for immobilizing biocatalysts with polymeric materials and degradation of pollutants

1084 **TABLE LEGENDS**

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1086 **Table 1:** Summary of immobilized biocatalysts for pollutants remediation

Table 1: Summary of immobilized biocatalysts for pollutants remediation

Sr. No	Biocatalyst	Immobilization method	Target pollutant	Efficiency	Kinetics Parameters	Proposed methods of transformation	Reference
1	Laccase	Crosslinking with Composite nanofibers	Triclosan	65% of Triclosan was removed in 2h	K for immobilized laccase = 1.17 h^{-1} $t_{½}$ for immobilized laccase = 0.60 S First order reaction	Succesive Oxidation, dechlorination, and oligomerization of Triclosan mediated through Cu-cluster in laccase	Xu et al. 2014
2	Laccase	Crosslinking with Magnetic mesoporous silica microbeads	Mefenamic acid, Indomethacin in Municipality Wastewater	N/A	Km =64.3 ± 6.7 µM Kcat =134.6 ± 6.7 s^{-1} Kcat/Km=2.10 ± 0.11 $s^{-1}µM^{-1}$ 2nd order kinetics	Resulting radicals from oxidation interact with nonphenolic pharmaceuticals	Arca-Ramos et al. 2016
3	Laccase	Covalent binding with Halloysite nanotubes (Fe_3O_4	Sulfamethoxazole	60% of Sulfamethoxazole removed up to 7th	Km for free laccase = 80 µM Km for immobilized laccase = 90 µM	Oxidation with a redox mediator of laccase	Kadam et al. 2017

		and functionalized with g-aminopropyltrieth oxysilane)		cycle	Vmax for free laccase = 45 μM min^{-1} Vmax for immobilized laccase = 41 μM min^{-1} Lineweaver–Burk double reciprocal models		
4	Laccase	Crosslinking with magnetic nanoparticles	Acetaminophen, Diclofenac, Mefenamic acid, Atenolol, Epoxy carbamazepine, Fenofibrate, Diazepam, Trimethoprim, and Ketoprofen	Complete removal of acetaminophen, diclofenac, mefenamic acid, atenolol and epoxy carbamazepine and partial removal of fenofibrate, diazepam, trimethoprim, and ketoprofen was achieved within 12 h	Km for immobilized laccase= 0.39 mM Km for free laccase = 0.37 Mm	Direct oxidation of target pollutants by laccase	Kumar et al. 2016

5	Laccase	Covalent binding with TiO_2 nanoparticles	Bisphenol A, Carbamazepine	90 % of the Bisphenol-A was removed within 6 h and 40% of carbamazepine removed within 24 h t	For crude enzyme Km = 37.3 ± 2.5μM Kcat = 101.3 ± 11.8 μmol min^{-1} mg^{-1} For Immobilized crude enzyme Km = 42.9 ± 3.3 μM Kcat = 75.5 ± 9.4 μmol min^{-1} mg^{-1}	Reaction of radicals with carbamazepine and Direct oxidation of bisphenol A	Ji et al. 2017
6	Laccase	Entrapment, covalent binding, crosslinking with Alginate, chitosan	Malachite green	90% of Malachite green decolorized in 3 h	N/A	Direct oxidation of tetracycline and pharmaceuticals by laccase	Yang et al. 2017
7	Horseradish peroxidase	Crosslinking with crosslinked enzyme aggregates	Methyl orange dye, Basic red 9, Indigo, Rhodamine B, and Rhodamine 6G	94.26% of methyl orange, 91.73% of Basic red 9, 84.35% of indigo, 81.47% of Rhodamine B, and 73.6% of	N/A	N/A	Bilal et al. 2017

				Rhodamine 6G was removed			
8	Horseradish peroxidase	Crosslinking with chitosan–halloysite hybrid nanotubes	Tetramethyl benzidine	Crosslinked-Horseradish peroxidase showed 88 times faster catalytic activity with for Tetramethyl benzidine than that of natural Horseradish peroxidase	Vmax of immobilized enzyme=260 mol min^{-1} mg $protein^{-1}$ (88-folds higher than the free enzyme)	Direct oxidation by horseradish peroxidase in presence of H_2O_2	Kim et al. 2016
9	Horseradish peroxidase	Immobilized through adsorption with Magnetic nanoparticles (silica-coated), graphene oxide	Phenol	95% of phenol was removed from aqueous solution.	N/A	Direct oxidation by horseradish peroxidase in presence of H_2O_2. This phenomenon is probably due to different phenoxy	Chang et al. 2016

		(nanosheets), graphene oxide/Fe_3O_4, NH_2-modified magnetic Fe_3O_4/SiO_2				radicals produced by the enzyme	
10	Soybean peroxidase	Crosslinking with Silica-coated magnetic nanoparticles	Ferulic acid	99.67 ± 0.10% of ferulic was removed	Free enzyme Km=607.03 mM Vmax= 6.21 mM min^{-1} Immobilized enzyme Km=21.55 mM Vmax= 0.654 mM min^{-1}	Oxidation of phenolic compounds in presence of H_2O_2	Silva et al. 2016
11	Soybean peroxidase	Adsorption with Poly (styrene-co-maleic anhydride) (SMA) nanofiber	Diclofenac, Naproxen, Iopamidol, 2, 4-dichlorophenol, Imidacloprid, Bisphenol A	Complete removal of diclofenac and 2,4-dichloropheno, 90% removal of naproxen, 85% removal of imidacloprid and 70% removal of	Free enzyme Km (iopamidol) = 2.17×10^{-4} min^{-1} Km (imidacloprid) = 2×10^{-4} min^{-1} Km (bisphenol A) = 2×10^{-4} min^{-1} Immobilized enzyme	Photocatalytic and enzymatic oxidation	Sarro et al. 2018

				iopamidol and bisphenol A was attained in 24h	Km (iopamidol) = 12 × 10^{-4} min^{-1} Km (imidacloprid) = 9.33 × 10−4 min^{-1} Km (bisphenol A) = 7.17 × 10−4 min^{-1}		
12	Tyrosinase	Crosslinking with graphene oxide	Phenol, Bisphenol A	84.5% of phenol was removed after 2 h and 74.5 % of Bisphenol A was after 2 h	Free Tyrosinase Km=0.70 mM Vmax= 4.43 x 10^{-3} mM/s Immobilized Tyrosinase Km=3.98 mM Vmax= 0.9 x 10^{-3} mM/s Lineweaver–Burk double reciprocal models	Hydroxylation of monophenols to quinines	Liu et al. 2016
13	Organophosphorus hydrolase	Crosslinking with nonwoven fabrics	Methyl parathion	Complete removal of Methyl parathion was attained	Free enzyme Km= 331 ± 2 μM Immobilized enzyme Km= 622 ± 182 μM	P-nitrophenol is produced with cleavage of P-O bond of methyl parathion	Gao et al. 2014

14	Organophosphorus hydrolase	Covalent binding with carbon nanotube paper	Methyl paraoxon	22% of Methyl paraoxon was removed	N/A	P-nitrophenol is produced with cleavage of P-O bond of methyl parathion	Mechrez et al. 2014
15	Lipase	Entrapment with Zeolite imidazolate framework-8	p-Nitrophenyl caprylate	N/A	N/A	Hydrolysis to produce p-nitrophenyl	He et al., 2014
16	Haloalkane dehalogenase and epoxide hydrolase	Encapsulation with PVA particles, lentikats	1,2,3-Trichloropropane	97% of 1,2,3-Trichloropropane was removed converted to final product glycerol with 78% yield	The specific activities of immobilized Haloalkane dehalogenase and epoxide hydrolase were noted 29.5 and 6.5 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ respectively	Dehalogenation and hydrolysis process give glycerol	Dvorak et al. 2014
17	Laccase	Adsorption with magnetic silica microspheres	Phenolic contaminants	80% of Phenolic contaminants were removed in 5 days	For free enzyme Km= 1.0 ± 0.12 µM Kcat = 7.69 ± 0.12 s^{-1} Kcat/Km = 7.69 ± 0.19 s^{-1} μM^{-1}	Free radical chain reactions break phenolic ring	Vishnu et al. 2017

					For immobilized enzyme Km= 2.0 ± 0.19 μM Kcat 4.40 ± 0.23 s^{-1} Kcat/Km = 2.20 ± 0.16 s^{-1} μM^{-1}		
18	Formate dehydrogenase, peroxidase, and NADH oxidase	Adsorption with agarose beads	Phenol, Para-aminophenol, 2,4-dichlorophenol	95% of Phenol, 50% of Para-aminophenol, 70% of 2,4-Dichlorophenol, and 91% of Naphthol were removed	N/A	Formate dehydrogenase forms H_2O_2 through oxidation and NADH oxidase oxidize phenolic compounds	Rocha-Martin et al. 2014
19	Laccase	Covalently attached in Zeolite based nanoparticle and graphene oxide composites	Synthetic Dyes (Direct Red 23)	91% decolorization was found	N/A	Phenoxy radical formed with the oxidation of phenolic ring and followed by formation of azo linkage	Mahmoodi et al. 2020

20	Laccase	Cross linked with Sepharose-linked antibody support	Phenol red	Decolourization of phenol red dye obtained by immobilized and free laccase was 80% and 56%, respectively after 6 h of incubation	Free enzyme Km= 43.9 µM Vmax= 4938 µM.min^{-1} Immobilized enzyme Km= 55.0 µM Vmax= 408 µM.min^{-1}	Phenolic ring structures has been breakdown with free radical reactions	Zofair et al. 2020
21	Polyphenol oxidase	Crosslinked with Chitosan/montmorillonite and chitosan-gold nanoparticles /montmorillonite composites	Phenol, 4-chlorophenol, 2,4-dichlorophenol	Phenol, 4-chlorophenol (4-CP) and 2, 4-dichlorophenol (2, 4-DCP) 89.2%, 95.2% and 93.8% at 480 min respectively	For phenol Km=376.8(mg/L) Vmax= 215.5 (mg/L/h) For 4-CP Km=365.7(mg/L) Vmax= 221.7(mg/L/h) For 2,4-DCP Km=370.2 (mg/L) Vmax= 217.9 (mg/L/h)	Catalyzes the oxidation of phenolic compounds into highly reactive quinones	Wang et al. 2020