Degradation of Phenol Containing Wastewater by Advance Catalysis System – A Review

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Authors’ contributions

This work was carried out in collaboration between all authors. Author PMK designed the review article, did the literature search and wrote the first draft of the manuscript. Authors STI and NDW wrote the final drafting of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Phenols and their derivatives are broadly distributed as a characteristic pollutant due to its frequent presence in effluents of many industrial processes. Most of the phenolic compounds are toxic to living organisms as well as environment, even at low concentration. These phenol derivatives introduced into the environment, they may accumulate in soil and water. This signifies enormous environmental issues and if they enter into the food cycle through that polluted water, they can cause numerous health problems to humans. They show adverse effects on human being which are short term as well as long term effects. Enzymes are good biocatalysts. We discussed in this study about an enzymatic treatment on effluent containing phenols. Phenol degrading enzymes and their delivery systems in effluent shortly discussed. We focused only on the phenol degrading peroxidase enzyme. Numerous researchers extracted the peroxidase from various plants and their parts. Many researchers have reported that methods of biodegradation of phenols by peroxidase with additives to retain the specificity of peroxidase through their whole reaction. In conclusion, the plants having a great source of enzymes, such as horseradish roots, soybean seed hulls and turnip roots are having rich sources of enzymes. The enzymes are time saving and inexpensive catalyst.

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There are no harmful products formed after completion of reaction. Hence, enzymatic treatment is fully eco-friendly treatment.

**Keywords:** Phenol; enzyme; biocatalyst; biodegradation; peroxidase; eco-friendly.

1. **INTRODUCTION**

1.1 Phenols

Phenol (C₆H₅OH) and their derivatives are mostly distributed as a pollutant because of its common presence in effluents of several industries such as wood, resins, dye and plastic industries [1], iron, textiles, coal conversion, petroleum refining, steel as well as pulp and paper industries [2,3]. Even at low concentration, several numbers of phenolic compounds are harmful to living organisms as well as the environment. Therefore, they are classified as hazardous pollutants [4-6]. If these phenols introduced into the environment, they may accumulate in soil and water. This signifying enormous environmental issues [5] and if they enter into the food cycle through that polluted water, they can cause numerous health problems to human being. The high dose can cause paralysis, hemolytic anemia and liver damage [7]. There are some short term effects such as headache, burning eyes and respiratory irritation. Chronic effects like anorexia, weakness, fatigue, muscle pain and weight loss [1]. If they persist in the environment, they persevere to ending through bioaccumulation, transportation in living things and biomagnifications in the food chain.

![Fig. 1. Structure of phenol](image)

Conventional methods are applied to remove phenolic compounds and their derivatives from wastewater. These methods like as adsorption on activated carbon, microbial degradation, incineration, chemical oxidation, use of oxidizing agents such as UV and ozone, solvent extraction [11]. But these methods have some disadvantages like time-consuming procedures, low efficiency, high cost or generation of some products that are more harmful than the original phenolic compounds. Enzymatic treatment has been proposed by many as an alternative treatment technology to traditional methods [12]. Biological processes are achieving more important over physicochemical process, as biological systems are more effective and the end products formed are non toxic [13]. Due to these causes, more concentration has been given to the improvement of alternative methodologies for degradation of poisonous organic pollutants containing water and soil, such as phytoremediation [14].

1.3 Enzymes

Almost all enzymes are proteins, but all proteins are not enzymes. Enzymes have several beneficial characteristics. They are participating as biocatalyst in various biological reactions [15]. They are highly specific and produce only the expected products from the given reactants or substrates. Enzymes may produce extensive transformations of structural and toxicological properties of contaminants and even their
complete conversion into innocuous inorganic end products. The reaction occurs in multiple steps, as shown below:

Enzyme + \( \text{H}_2\text{O}_2 \) $\rightarrow$ Compound I + \( \text{H}_2\text{O} \) \hspace{1cm} (1)

Compound I + AH$^2_2$ $\rightarrow$ Compound II + A$^*$ \hspace{1cm} (2)

Compound II + AH$^2_2$ $\rightarrow$ Enzyme + AH$^*$ + \( \text{H}_2\text{O} \) \hspace{1cm} (3)

In first step of the process, the active site with hydrogen peroxide occurs. The oxidation of hydrogen peroxide takes place, generating compound I and water molecules. In second step of the process, compound I oxidizes a substrate molecule (AH$^2_2$), producing a substrate radical and compound II. Finally, a second substrate molecule reduces compound II and returning the enzyme to its initial form [16,17]. They may perform processes for which no efficient chemical transformations have been devised. Enzymes may present advantages over traditional technologies, and also over microbial remediation. Indeed, enzymes are not inhibited by inhibitors of microbial metabolism. All these characteristics render enzymes eco-friendly catalysts as well as enzymatic techniques, environmentally friendly processes [18]. A variety of enzymes from plants, fungi, animals and microorganisms have been reported to play important roles in an array of waste treatment applications.

Enzymes are classified into six major classes, i.e. oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases [19]. Peroxidase is one of the subclass of oxidoreductases which is segregated from other subclasses by the use of \( \text{H}_2\text{O}_2 \) (hydrogen peroxide) as an electron acceptor. Peroxidases and laccases show a wide substrate range, especially with regards to phenols and amines [20,21] and azo dyes also [22,23]. The activity of an enzyme can be influenced by a change in the conditions such as temperature, pH and change in substrate concentration or binding of specific chemicals that regulates its activity.

### 1.3.1 Phenol degrading enzymes

Many researchers work on oxidative enzymes to the degradation of phenol and their derivatives such as peroxidase, chloroperoxidase, lignin peroxidase, Manganese peroxidase, Tyrosinase, Laccase and Catechol dioxygenase. Wide applications of peroxidase in different areas of clinical biochemistry, biotechnology, environmental sciences, food industry, etc. enhance the interest for further study on the enzyme [24]. Peroxidase is the group of oxidoreductase which is the most commonly used by various researchers to the removal of phenol and their derivatives from industrial wastewater or effluent. These peroxidase group consisting enzymes, their sources and their applications are shown in Table 1.

| Enzymes                | Source                                  | Applications                                                                 |
|------------------------|-----------------------------------------|------------------------------------------------------------------------------|
| Peroxidase             | Horseradish                             | Degradation of phenols, chlorophenols                                         |
|                        | Artromyces ramosus                      | Degradation of phenols                                                       |
| Chloroperoxidase       | Caldariomyces funago                    | Oxidation of Phenolic compounds.                                              |
|                        |                                        | Biosensor chlorophenol detection                                             |
| Lignin peroxidase      | Phanerochaete chrysosporium             | Removal of aromatic compounds and phenolic materials                         |
| Manganese peroxidase   | Phanerochaete chrysosporium             | Degradation of phenols and pentachlorophenol                                 |
|                        | Lentinula edodes                        | Removal of chlorophenol                                                      |
| Tyrosinase             | Sigma                                   | Phenol biosensor. Degradation of phenols, Oxidation of catechol and polymerization of phenolic compounds |
|                        | Agaricus bisporus                       | Catechol oxidation                                                          |
| Laccase                | Trametes versicolor                     | chlorophenols and urea derivatives degradation                               |
|                        | Cerrena unicolor                        | Phenol detoxification, 2,4-dichlorophenol degradation                         |
| Catechol dioxygenase   | Comamonas testosterone                  | Chlorophenol oxidation                                                       |
2. EXTRACTION AND PURIFICATION OF PEROXIDASE

An invention reported by Lakshminarayanan in 1976 titled as Method for isolating high purity peroxidase. This invention encompasses a method for isolating a peroxidase enzyme from plant tissue containing peroxidase. The invention has as its critical step treating an aqueous extract of said plant tissue having the pH adjusted to 6 – 9 with at least 2.7 x 10^{-3} moles per litre of zinc ions. Unexpectedly at this pH the zinc ion selectively precipitates contaminating impurities from the extract. Thus, the critical step of this invention was used in conjunction with salt fractionation, solvent fractionation, dialysis, reverse osmosis, electrophoresis, column chromatography and other techniques for purifying the protein. They gave variety of eight examples, in these eight examples most common step is the deposition of various concentrations of zinc solution at different pH. They used reverse osmosis in this process to get a concentrated enzyme solution. Lastly, they converted liquid purified enzyme into solid or powder from the purified enzyme by the addition of isopropanol solvent at –10°C and mix for 30 minutes, centrifuged and solvent was discarded. Then precipitate was treated with chilled acetone 99% at –10°C, blended and filtered this mixture. Then again washed with acetone and dried at room temperature to gain the final product in the form of powder of high purity peroxidase [26].

A new method is freeze-thaw technique of recovering peroxidase from seed hulls was discovered by Pokora et al. [27]. They mentioned some examples in their article. In the first example, they prepared two sets of six different percentages of the concentration of soybean seed hull extract with water: 10%, 20%, 40%, 60%, 80% and 100%. One set is placed in the freezer (–20°C) and another set stored in the refrigerator (5°C). After 2 hours, the samples were thawed at room temperature (23°C) and took the absorbance against water in the range of 1100 nm to 200 nm. They mentioned transmittance from 1100 nm to 200 nm for both freezer and refrigerator sample. They also determine the specific activity and fold purification. Then they revealed that particulate contamination can be removed quickly (2 hours) by a freeze-thaw cycle and settling by the force of gravity. In second example, the example they determined the effect of freezing on separation. For this determination, they prepared four samples and cooled to various temperatures (–15°C, –5°C, 0°C and 5°C). After incubation the samples were equilibrated at room temperature (23°C) for 1 hour, then centrifuged at 1500 rpm for 8 minutes and the % transmittance at 700 nm was determined. Then they resulted that removal of particles indicated by % transmittance was more efficient when the sample was completely frozen.

Rehman et al. [28] concluded that the horseradish roots (HRR) were found as the best source of peroxidase among the studied vegetable sources. The optimum pH for enzyme activity was measured at 6.0 for radish whereas 6.5 for turnip, horseradish legumes (HRL) and HRR. Enzyme was found stable even at a temperature of 50°C showing relative activity from 60 to 80%. The crude enzyme was purified by DEAE cellulose chromatography after ammonium sulfate precipitation and the degree of purification were 14 folds. Enzyme extraction procedure contains following steps: 1) 100 gm cutting pieces of fresh vegetables were added to 400 ml of distilled water and then blended for 15 minutes. 2) The content was centrifuged at 6000 rpm for 15 minutes and the supernatant was passed through filter paper. 3) The extract was heated at 65°C for 3 min. to inactivate any catalase present in the extract. 4) This extract was used as a crude peroxidase enzyme.

In 2002, Alyas and co-workers studied on the extraction and purification of peroxidase from soybean seeds. The crude extract obtained by the procedure used by Ambreen et al. [29] with some modifications. The purification of peroxidase consists of partial purification by ammonium sulfate precipitation technique and purified by ion exchange and gel filtration chromatography. The activity and specific activity of crude enzyme was recorded as 17.29 U/ml and 1.586 U/mg respectively. And this crude enzyme was subject to ammonium sulfate precipitation for partial purification and the resulted activity and specific activity was 12.85 U/ml and 5.68 U/mg respectively. After ion exchange chromatography through DEAE cellulose, fraction No. 43 exhibited maximum activity of 18 U/ml and specific activity of 9.5 U/mg. This fraction was subsequently applied to sephadex G-75 column and after the election; the activity and specific activity were enhanced to 16.04 U/ml and 14.948 U/mg respectively [30].

The quantity and biological activity of peroxidase isolated from different parts of the soybean plant was compared by Sariri et al., 2003. Then they
resulted that the biological activities of peroxidase extracted from the leaves and seed coat were similar seed coat contained higher quantities of peroxidase than the leaves. In extraction procedure, seeds were soaked in distilled water at room temperature for 24 hours, after soaking grind the soaked seeds and the husk was separated by filtration. The extracts were filtered using four layers of cheesecloth to remove suspended fibrous solid particles. Crude extracts were also prepared using 10 mM phosphate buffer by exactly the same procedure. But the activity and stability of the enzyme were similar in both the extracts. Peroxidase activity was determined at room temperature with spectrophotometer following the formation of tetraguaiacol (Amax = 470 nm, ε = 26.6 mmoI cm⁻¹) in a 3 ml reaction mixture containing 1 ml of 2-methoxyphenol (guaiacol); 1 ml of 3 mM H₂O₂ and 50 ml of enzyme extract. The reaction was carried out for 3 min. One unit of peroxidase activity (U) represents the amount of enzyme catalyzing the oxidation of 1 mmol of guaiacol in 1 min. They were protein determined by Comassie brilliant blue G-250 method using bovine serum albumin as standard [31].

Liu and co-workers reported ‘A novel process of purifying soybean hull peroxidase’ in 2005. They mostly focused only on purification of peroxidase. They concluded that the RZ value (Reinheitszahl) of soybean hull peroxidase (SBP) reached 1.32 and the recovery of enzyme activity was 65% after purification. The satisfactory recovery of activity and RZ value as well as the simplicity of the procedure make this strategy a useful alternative for the purification of SBP. They used a new process is the enzyme solution was purified by ammonium sulfate–acetone cooperation precipitation, the acetone precipitation and zinc sulfate precipitation. The ammonium sulfate–acetone cooperation precipitation, namely, the two-phase system of organic solvent- inorganic salt, was adopted in their process [32].

Ghaemmaghami and co-workers in 2010 concluded that SBP extracted from soybean seed hulls is a highly robust enzyme and the enzyme exhibited the highest activity and stability at pH 6.0 and retained over 75% of the maximum activity for 12 hours. The activity of SBP was found to be 2.5 times higher at an elevated temperature of 65°C compared to the activity at room temperature. The activity retains over 95% for 30 min at 75°C. Also, this enzyme is fairly active in the presence of organic solvents such as acetone, methanol and ethanol, which widen the applicability of SBP for the treatment against a variety of organic pollutants present in industrial and petroleum waste waters and its application may be advantageous as a biosensor or for lower cost industrial wastewater treatment compared to other peroxides such as HRP. SBP is fairly active in organic solvents and exhibited optimal activity in the presence of 20% (v/v) acetone. Increasing the organic solvent content resulted in a reduction in SBP activity. Two methods were used for extraction of peroxidase from soybean seed hulls in this study: In first method 25 g of soybean hulls were soaked in 200 ml phosphate buffer (0.1M NaH₂PO₄/Na₂HPO₄, pH 6.0) in 4°C for 24 h. The extract was then filtered through four layers of cheese cloth; the filtrate was clarified by centrifugation at 6000 rpm for 15 min in 4°C to remove cell debris. The final supernatant was collected and stored at 4°C and used as a source of crude SBP enzyme. An enzyme solution was warmed to room temperature immediately prior to use. In the second method 1g of defatted soybean flakes was mixed with 0.1M phosphate buffer, pH 8.0, for 2h in 4°C (1:25, w/w) and then centrifuged at 5000 rpm for 30 min. The supernatant was maintained in 4°C. But the enzyme extracted by first method having more Activity (U/ml), Protein concentration (mg/ml) and Specific activity (U/mg) [33].

A comparative study of peroxidase purification from apple and orange seeds was studied by Zia et al. [34]. They revealed that orange seed peroxidase had more activity than apple seed peroxidase in crude extract and each step of purification. Apple and orange seeds were kept separate from the fruits, dried and soaked in 200 ml of 0.1 M phosphate buffer of pH 6.0 over night and thoroughly homogenized by blending for 15 to 20 min. The contents were centrifuged at 10,000g for 15 min to eliminate cell debris. The supernatant was removed carefully from the sediments and filtered through Whatman No. 1 filter paper to obtain more clarity of the crude enzyme extracted. Enzyme activity was determined using a UV–Vis spectrophotometer at the wavelength of 470 nm according to the method of Rad et al., 2007 with minor modifications. A mixture of pyrocatechol (170 mM) and aniline (2.5 mM) was prepared in 0.2 M phosphate buffer solution of pH 6.5. For each blank and sample cuvette, 500 µl of the earlier mentioned mixture solution and 500 µl of hydrogen peroxide (35%) was pipetted and incubated at 25°C for 3 to 4 min. Then, 50 µl of
the crude enzyme extract and 50 µl phosphate buffer solutions were added to the sample and blank cuvettes, respectively. Increase in absorbance was recorded from 4 to 5 min intervals. Protein was determined by the method of Lowry et al., 1951 using bovine serum albumin as standard. Partial purification of the crude enzyme extract was done by ammonium sulfate precipitation and ion exchange chromatography. It was observed that after partial purification, the enzyme activity was increased as compared to crude enzyme extract. Peroxidase from orange seed was purified up to 17.17 fold with specific activity of 10.17 U/mg and that from apple seed was 6.82 fold with specific activity of 7.53 U/mg after diethyl amino ethyl (DEAE) cellulose chromatography. Further purification was obtained through gel filtration chromatography by using sephadex G-75 column. Peroxidase from orange and apple seeds got purified up to 30.64 and 8.34 fold with their specific activity of 18.16 and 9.20 U/mg, respectively. It is more evident that peroxidase is the most heat stable enzyme; therefore, it is concluded that it may be potentially useful for industrial purposes [34].

Shazia et al., 2012 studied on Production and purification of horseradish peroxidase (HRP) in Pakistan. From their results, the activity of the crude HRP extracts was 6.3027 U/ml and specific activity of 0.8586 U/mg. It was shown that the activity was increased to 6.6928 U/ml and 12.77 U/mg specific activities by (NH₄)₂SO₄ precipitation. Protein contents were decreased from 7.339 mg/ml of crude extract to 0.524 mg/ml which indicate that unwanted proteins have been removed. An enzyme fraction having the highest activity after dialysis was passed through a Sephadex G-75 column for gel filtration chromatography. Maximum activity of 9.9452 U/ml was obtained in the fourth fraction during the experiment, with 0.253 mg/ml of protein contents and 39.30 U/mg of specific activity. So, the HRP enzyme was purified up to 45.77 fold [35].

Yadav et al. [36] worked on the purification and characterization of Mn-Peroxidase (MnP) from Musa paradisiacal (banana) stem juice which is an agro-waste easily available after harvest of banana fruits. Earlier MnP was isolated and purified only from fungal sources, but for the first time it was purified from available plant source. The purification procedure was simpler than that reported for the fungal MnPs. They reported that the purification procedure was simpler than that reported for the MnPs from fungal sources. The extraction procedure was same as seen earlier in this article with some modifications. The enzyme was purified from the stem juice by ultrafiltration and anion-exchange column chromatography on DEAE with 8-fold purification and purification yield of 65%. They also reported that the pH and temperature optimum of the enzyme was 4.5 and 25°C respectively. The enzyme in combination with H₂O₂ liberated bromine and iodine in the presence of KBr/KI respectively. All these enzymatic characteristics were similar to those of fungal MnP.

In 2012, Khatun et al [37] revealed that Moringa oleifera L. leaves were available in large quantities in almost all seasons, purified peroxidase from these leaves was more stable and active in acidic pH and the activity remains 90% at 60°C for 30 min incubation. Silva et al. extracts peroxidase from five vegetables and determine the enzyme activity, then they concluded that the enzyme activity more when NaCl added into the enzyme extract [16]. Peroxidase was purified 164-fold from the leaves of Moringa oleifera L. with a recovery of 28% by ammonium sulfate precipitation, DEAE-cellulose column chromatography, Sephadex G-200 column chromatography and Con-A column chromatography by them. The extraction procedure was identical as seen earlier in this article with some modifications. After Con-A column chromatography specific activity and purification fold were 346.43 and 164.18 respectively. They also studied on some metal ions out of them Ni²⁺, Pb²⁺, Zn²⁺, Al³⁺, Mg²⁺, Cu²⁺, Co²⁺ and Cd²⁺ exhibited low inhibitory effect while Fe²⁺, Fe³⁺ and Hg²⁺ exhibited strong inhibitory effects.

Anbuselvi and co-worker purified peroxidase from two varieties of Tulsi and Neem and also studied their characteristics in 2013. They were studied on Ocimum tenuiflorum, Ocimum gratissimum varieties of tulsi and Azadirachta indica, Melia azadirachta varieties of neem for peroxidase analysis. Peroxidase extracted by following procedure: 500 mg of all the four leaf samples was weighed and ground with the addition of 1 ml of Phosphate buffer (pH7). This was then centrifuged at 10000 rpm for 15 minutes at 4°C and the supernatant was passed through filter paper. It was heated in a water bath at 65°C for 3 minutes to inactivate catalase in the extract and cooled promptly by placing in an ice bucket for 10 min. The optimum pH and temperature of peroxidase were found to be 6.5 and 40°C. The
ammonium sulfate precipitation and gel filtration were used by authors for purification. Protein profiling was done in both Native PAGE and SDS-PAGE. They also concluded that from this study it was found that the protein content and its enzyme activity were different for varieties within the same species. This study was helpful in understanding the varietal difference within the same species. Local availability of these plants and reasonably high specific activity of the enzymes isolated from these medicinal plants makes it a better choice for the production of peroxidase for its use as an antioxidant [38].

Four vegetables viz. potato, carrot, eggplant and tomato were studied by Suha et al. [39] for thermostability at different pH levels of peroxidase extracted from these four vegetables. All vegetables investigated contained peroxidase enzyme. Extraction of peroxidase procedure follows by following steps: Fresh fruits of potato, carrot, eggplant and tomato were washed thoroughly with distilled water and cut into pieces. Then fruits were homogenized with ice cold 10 mM sodium phosphate buffer of pH 5, 6, 7 or 8. The ratio of quantity of fruits taken to that of buffer was maintained constant at 1:1 (w/v). The crude extract was filtered through cheese cloth and centrifuged to remove traces of fibrous particles and cell debris. Supernatant was stored at 4°C and used as a stock solution for further experiments. Then give heat treatment to an enzyme. They did not purify that crude extracted enzyme. From this study, they concluded that peroxidase of high activity was extracted at pH 5.0 from potato and tomato while those of carrot and eggplant was extracted at pH 6.0. But potato tuber contained a higher level of peroxidase whereas carrot had lower levels at all pH values. The activity of the enzyme is dependent on the pH value of the medium. The results showed that the rate of loss of peroxidase activity from the vegetables increased with both increases in temperature and heating time. Biphasic inactivation curves were observed for the enzymes extracted from all samples, where the initial heat inactivation is rapidly followed by much slower inactivation periods. The rate of loss of peroxidase activity was shown to be pH dependent. Potato peroxidase was noted to be more stable to heat. A less severe heat treatment is required to inactivate carrot, eggplant and tomato peroxidases. Complete inactivation of carrot peroxidase was accomplished within 4–10 min at 80°C and within 2–10 min at 90°C at pH 8.0, while peroxidase inactivation in eggplant required 8–10 min at 90°C at pH 8.0. Complete inactivation of tomato peroxidase required 6–10 min at 90°C at pH 6.0.

3. DELIVERY SYSTEMS FOR ENZYMES IN EFFLUENT TREATMENT

Some researchers worked on extraction of enzymes from plants, microorganisms and animals, their purification and characterization before 1980. These extracted enzymes were applied in various fields after the decade of 1980. Since last two decades, due to the increasing of awareness about the environment in the whole world, many scientists use enzymes in environmental sciences to clean the environment. Therefore, researchers studied the different techniques of the delivery of enzymes in waste water for degradation of numerous pollutants. The delivery system is selected, must be proper to the intention, easy, cost effective and efficient. However, keep in mind to ensure that the activity of the enzyme is not adversely affected due to the mode of delivery [40].

3.1 Direct Use of Biological Source

Directly introducing an enzyme into the effluent is to provide tissues or cells that produce the enzyme. This mode of enzyme delivery system is accepted when suitably adapted microbial strains are used to co-metabolize target pollutants or when the cell producing the enzyme is introduced directly into the wastewater. There are two types of direct use of biological source which are use of plant tissues or entire plant and use of microbial cells [41].

3.2 As Cell-free Enzyme Extracts

Extracted enzymes or cell-free enzymes are preferred for use over the integral organism, particularly when the effluent to be treated which cannot support growth. The extracted enzymes could be used in either pure form or crude extract. Well, it is comparatively easier to standardize optimum treatment conditions with extracted enzymes [42]. The use of extracted enzymes has simple for handling and storage over microbial cultures. The process of crude enzyme extract preparation includes grinding and homogenizing the source tissue or cells with a suitable buffer solution followed by filtration [43,44]. Crude enzyme extracts are comparatively inexpensive over pure enzymes. Crude enzyme extracts can also effectively degrade pollutants from wastewater.
3.3 In Immobilized Form

An enzyme is deemed to be immobilized when it is physically confined to a certain region of space, retaining its catalytic activity and the capacity to be used repeatedly or continuously. The use of immobilized enzymes in effluent treatment has many significant advantages over the use of free enzymes, including increased stability, localization, the ease of handling, reusability and a consequent decrease in running cost [42,45]. The HRP enzyme has proved to be an adjustable molecule that can be used in the form of cell-free crude extract [46] or in an immobilized form entrapped in calcium alginate capsules at a laboratory scale [47].

Some methods of immobilization such as adsorption, covalent binding, entrapment, encapsulation, membrane confinement and chemical coupling can adversely affect the catalytic activity of certain enzymes. Immobilization procedures need to be optimized to minimize the loss of enzyme activity and achieve maximum reusability. This method of enzyme delivery holds great potential for the continuous treatment of large volumes of effluent.

3.4 In the Form of Different Nanoparticles

Nanotechnology is one of the highly acceptable in wastewater treatment methods, which can effectively decontaminate xenobiotics in the environment. Use of nanoparticle in Reactive Remediation Technology having a great interest in wastewater treatment. Since it involves the complete degradation of pollutants into carbon dioxide and water, which are inoffensive products [41].

Remediation of polluted wastewater can be achieved by using a combination of nanotechnology and enzyme technology called as Single Enzyme Nanoparticle technology (SEN). A SEN may be elaborated as an enzyme enclosed by a protective cover which is a few nanometers thick. SENs are able to withstand drastic conditions of temperature, pH, contaminant concentration and salinity as compared to free enzymes.

Another type of novel nanoparticle is nanospheres. These are materials containing microscopic particles with nano-sized cavities. These particles can encapsulate or be embedded with various types of substances and are capable of transporting them through an aqueous medium. In contemporary times, research on carbon based nanotechnology, such as the carbon nanotube is gaining force. The nanotubes carrying oxidative enzymes such as laccases or peroxidases could be synthesized for utilization in the treatment of recalcitrant pollutants in wastewater. In the future, carbon nanotubes will widely use in water treatment. Fig. 2 (a) shows structure of nanosphere before introducing target and after treatment. The upper layer in Fig. 2 (b) indicates attached toxic material [41,48].

![Fig. 2. Structure of nanosphere: (a) Before use (b) After use](image)

4. BIODEGRADATION OF PHENOL USING FREE ENZYME AND IMMOLIZED ENZYME

In 1996, Tatsumi et al. studied on the removal of chlorophenols from wastewater by immobilized HRP. Method of Immobilization of enzyme by physical adsorption on magnetite was more effective than crosslinking method. Kalssom et al. also reported that efficient dye degradation by the SBP immobilized in polyacrylamide matrix [49]. Besides, it was discovered that HRP was adsorbed on magnetite. The enzyme had a specific activity of 100 units/mg. Crude peroxidase was prepared from horseradish and concentrated by ultrafiltration. Freeze drying method was used to obtain a crude enzyme powder. The enzyme activity was observed about 2.5 U/mg. Peroxidase was immobilized on magnetite by both chemically and physically. The immobilized protein was calculated before and after immobilization. The degradation of each chlorophenol from the wastewater by peroxidase can be seen in Table 2. They utilized the same enzyme activity (0.2 U/ml) for both soluble and immobilized enzymes. The results specify that the immobilized enzyme was more effective than the soluble and each chlorophenol was degraded to almost 100%. For soluble peroxidase, 2,4,6-trichlorophenol was more reactive than 2,4,5-
trichlorophenol. Such an effect was not observed in immobilized peroxidase. The removal of 2,4,5-trichlorophenol was observed with a lowest removal rate of only 36%.

### Table 2. Removal of chlorophenols from the wastewater by soluble and immobilized peroxidase

| Chlorophenol            | Soluble peroxidase (%) | Immobilized peroxidase (%) |
|------------------------|------------------------|----------------------------|
| p-Chlorophenol         | 58                     | 100                        |
| 2,4-Dichlorophenol     | 82                     | 100                        |
| 2,4,5-Trichlorophenol  | 36                     | 99                         |
| 2,4,6-Trichlorophenol  | 97                     | 98                         |
| 2,3,4,6-Trichlorophenol| 81                     | 99                         |
| Pentachlorophenol      | 55                     | 97                         |

In conclusion, peroxidase was very simply immobilized on magnetite by physical adsorption. HRP was immobilized from crude HRP and the enzyme was purified. The immobilized peroxidase can effectively degrade phenols because of the binding of colored reaction products to the immobilized enzyme. In the treatment of chlorophenolic wastewater, about 90% of TOC and AOX were found to be removed by immobilized peroxidase [50].

Kinsley and Nicell worked on the treatment of aqueous phenol with SBP in the presence of polyethylene glycol (PEG) in 2000. They purchased all chemicals as well as medium purity SBP as a dry powder from various places. SBP catalyzes the oxidation and polymerization of aromatic compounds in the presence of hydrogen peroxide. Studies were undertaken to characterize the use of PEG as an additive to increase the functional life of the enzyme [51]. The effectiveness of PEG increased with its molecular weight, with maximum protection accomplished with PEG of molecular weight of 35,000. Linear relationships were found between the quantity of phenol to be treated (1.0 - 10 mM) and the optimum doses of SBP and PEG required for greater than 95% removals. Observations indicate that it is the interaction between the PEG and the polymeric products that results in the protection of SBP. Following treatment, approximately 25% of the optimum PEG dose remained in the supernatant [10].

Wilberg et al. [52] reported that the SBP activity in fresh hulls was greater than in aged hulls and was preserved at -10°C. They purchased all chemicals from various places and soybean seed hulls extract obtained in two steps: 1) pH 6 phosphate buffer extractions and 2) Freeze-thaw technique. A linear relationship with a slope of 0.8 U cm$^{-3}$ mmol dm$^{-3}$ between minimum low purity SBP (LP-SBP) dosage and initial phenol concentration was found for 95% phenol removal efficiency. This relationship remained unaltered when 1000 mg dm$^{-3}$ of PEG-6000 was added to the solution. Minimum LP-SBP dosage was 1.7 times lower than those published by Kinsley and Nicell using a medium purity SBP (MP-SBP). A retention time of about 100 min was sufficient to achieve yields of 95%. This retention time decreased with increasing phenol concentration.

Cheng and co-workers deliberate on the HRP immobilized on aluminium-pillared interlayered clay for the catalytic oxidation of phenolic wastewater in 2006. HRP was immobilized on aluminium-pillared interlayered clay (Al-PILC) to obtain enzyme-clay complex for the treatment of phenolic wastewater. That immobilized HRP used for phenol removal by precipitation or transforming to other products. The addition of PEG in reaction mixture could expressively improve the phenol degrading efficiency and reduce the amount of immobilized enzyme required to attain high removal efficiency of over 90%. The complete oxidation of phenol could within the short retention time when the molar ratio of H$_2$O$_2$/phenol and the mass ratio of PEG/phenol were 1.5 and 0.4 respectively. HRP immobilized on Al-PILC had better storage stability than the free enzyme. However, the reusability of the immobilized enzyme was not satisfactory. Besides, they reported that the immobilized enzyme lost its catalytic performance in the fourth repeated test [3].

Nair and co-workers in 2008 studied on biodegradation of phenol. During the past three decades, the use of microbial strain as a catalyst in the biodegradation of organic compounds has highly developed significantly. It has been found that large numbers of microbes exist in almost all natural environments, particularly in the lithosphere. Not only natural, but also synthetic organic chemicals are casually biodegradable in a natural environment. Therefore, they produce this review article, especially on soil microorganisms and they focused only on phenol degrading enzymes secreted by microorganisms.
Biodegradation of materials involves allowing adsorption of the substrate, initial proximity, secretion of extracellular enzymes to polymerize the pollutants. The effectiveness of biodegradation of contaminants is influenced on the basis of the organic pollutant, the nature of the influencing factors, the enzyme involved, the nature of the organism and the mechanism of degradation.

Table 3 shows peroxidase enzyme involved in the biodegradation of phenol and phenolic derivatives. They also investigated the mechanism of phenol biodegradation and reported two pathways of phenol degradation viz. Meta and Ortho pathway of phenol degradation in Fig. 3 and 4 [53].

In 2009, Hejri and Saboora concluded that an increase in hydrogen peroxide up to the optimal amount leads to an eminent degradation of phenolic compounds. More concentrations of hydrogen peroxide inhibited the reaction. The effect of enzymatic removal increased in the presence of PEG as an additive. The polymerized products were in innocuous form and can be easily filtered from treated the solution. Fig. 5 containing compounds were used as substrate for study.

Table 3. Peroxidase involved in the biodegradation of phenolic compounds

| Sr. no. | Type of phenol | Enzyme              | References |
|---------|----------------|---------------------|------------|
| 1       | Phenol         | Horseradish peroxidase | [54]       |
| 2       | Phenol         | Horseradish peroxidase | [55]       |
| 3       | Phenol         | Peroxidase          | [56]       |
| 4       | Bis-phenol     | Peroxidase          | [57]       |
| 5       | Lignophenols   | Peroxidase          | [58]       |

The effect was investigated in the removal process by some parameters. The positive regression showed between enzyme concentration and degradation of phenols by the application of various concentrations of the enzyme in the reaction. To examine the optimum pH for enzyme activity resulted that removal of phenols was enhanced in neutral pH. Additionally, this study resulted that the integral soybean seed were effective in the removal of phenolic compounds in synthetic wastewater [4].

Fig. 3. Meta pathway of phenol degradation

Fig. 4. Ortho pathway of phenol degradation

Fig. 5. Chemical structures of phenol, o-cresol and m-cresol
A review article prepared by Rao et al. [18] named as Role of enzymes in the remediation of polluted environments. They reported that the environmental pollution is growing more due to the indiscriminate and frequently deliberate absolve of hazardous substances. Research efforts have been dedicated to develop new, cost effective, low-technology, eco-friendly treatments capable of reducing pollution in the atmosphere, hydrosphere and lithosphere. The biological agent, enzymes has a vast potentiality to effectively polymerize, transform and detoxify pollutants because they have been endorsed to be able to transform pollutants and are potentially suitable to restore polluted environments. This review examined some pollutants and enzymes capable of bio transforming them into innocuous products. The enzymatic processes renovated and implemented in some detoxification treatments examined in details. Not only advantages, but also drawbacks that are present in the spacious application of enzymes in the in situ restitution of polluted environments will be discussed.

Pradeep et al. [2] revealed that the phenol degraded by free enzymatic treatment. They used HRP, Radish Peroxidase and SBP for their study, their phenol removal efficiency recorded for 100 mg/L as 84%, 76% and 72% respectively. From a comparative study, peroxidase extracted from HRR was able to polymerize phenol more efficiently than the enzymes extracted from soybean hulls and radish roots. The ambient room temperature during the study period ranged from 27-32°C. The HRP also proved to be valuable in the removal of phenol at concentrations between 100 mg/L to 300 mg/L when compared with Radish Peroxidase and SBP [2].

Shruthi et al., 2012 resulted that the concentration of phenol decreased with an increase in the concentration of enzyme extract & H₂O₂. The turnip root extract (Peroxidase) degrade phenol more efficiently. Chemical methods, for instance, ozone treatment is costly and chlorine oxidation may give more toxic compounds than the phenol itself. Fig. 6 shows the extraction of peroxidase from turnip roots. The whole study performed in between 27-32°C which is the ambient room temperature. A phenol concentration of 100-500 mg/L was kept to both free and immobilized HRP. Free enzyme studies were carried out in conical flasks while immobilized HRP enzyme bead reactor used for removal of phenol. Free HRP removed 84% of 100 mg/L phenol whereas Immobilized HRP removed 62% with the same phenol concentration. Free enzyme showed a better degradation than immobilized enzyme due to the availability of the most active sites in the free enzyme than immobilized enzymes. Reduction in phenol degradation could be observed with the increase in phenol concentration. The experimental setup of immobilized enzyme bed reactor (IEBR) as shown in Fig. 7. The plastic column of 20.3 cm having a sampling port at the bottom of the column was fitted to the iron stand. Immobilized enzyme beads were filled upto

This shows that phenol degradation occurred due to enzyme action [1].

**Fig. 6. Schematic diagram of extraction of peroxidise**

Removal of Phenol has been studied using free and immobilized HRP by Pradeep et al. [13]. The experimental setup of immobilized enzyme bed reactor (IEBR) as shown in Fig. 7. The plastic column of 20.3 cm having a sampling port at the bottom of the column was fitted to the iron stand. Immobilized enzyme beads were filled upto
15.2 cm of the plastic column. Phenol and $\text{H}_2\text{O}_2$ were poured from the top of the column.

Enzyme beds prepared by the following procedure: Took 4 gm of sodium alginate and 100 ml distilled water in a beaker; that beaker was kept on a hot water bath to dissolve sodium alginate. Sodium alginate solution was cooled and 4% crude enzyme was mixed. 0.2 M of $\text{CaCl}_2$ solution was placed on a magnetic stirrer and a mixture of sodium alginate and enzyme was added drop by drop with the help of a burette. The beads have a uniform size about 8 mm in diameter and stored at 4°C prior to use [13].

Crude peroxidases from five weed plants to utilize in biodegradation of phenols in wastewater studied by Hamad and Ahmed in 2013. They were extracting crude peroxidases from *Portulaca oleracea*, *Sonchus oleraceus*, *Xanthium strumarium*, *Cyperus rotandus* and *Trianthema portulacastrum* for biodegradation of phenolic compounds in wastewater. Four models of synthetic wastewater at concentration of 10 mM were prepared in the laboratory as follows: model (A) composed of ($\alpha$-naphthol + quinol + catechol + resorcinol), model (B) composed of (resorcinol + quinol + phenol + $\beta$-naphthol), model (C) composed of (tannic acid + pyrogallol + gallic acid + $\alpha$-naphthol) and model (D) composed of (catechol + gallic acid + $\beta$-naphthol + phenol). The tested enzymes showed a wide range of substrate specificity and different rates of enzymatic activities. It is evident that the peroxidases enzymes were very active towards most of the phenolic compounds. When using pyrogallol as substrate, the peroxidase from *C. rotandus* showed high specific activity (1.75 U/mg-1) and high Km value (4.19 mM pyrogallol). All extracts showed marked ability to degrade phenolic pollutants in the tested wastewater. The highest rate of degradation was noticed when crude peroxidase from *C. rotandus* was added to both industrial and synthetic wastewater. This study revealed that *C. rotandus* is the most interesting source of peroxidase enzymes for the eliminating or reducing phenolic pollutants in wastewater [7].

Crude peroxidase extracted from fresh soybean seed hulls having more potential to degrade the phenol from synthetic wastewater this was observed by Kolhe et al. [59]. They extracted peroxidase having 6.091 U/ml activity, 2.325 mg/ml protein content and 2.62 U/mg specific activity. They examined some parameters like pH, concentration of enzyme and concentration of phenol. Then they resulted that 0.2 ml $\text{H}_2\text{O}_2$ and 0.4 ml crude peroxidase at neutral pH could be the most favourable condition for the phenol degradation in aqueous medium [59].

| Sr. no. | Phenol (ml) | Conc. (mg) | $\text{H}_2\text{O}_2$ (ml) | Enzyme extract (ml) | Incubation at room temp for 30-40 mins | FeCl$_3$ (ml) | D/W (ml) | OD at 540 nm |
|---------|-------------|------------|---------------------------|-------------------|--------------------------------------|--------------|---------|-------------|
| 1       | 1.0         | 100        | 0.50                      | 0.2               |                                      | 2.0          | 5.0     | 0.64        |
| 2       |             |            | 0.10                      | 0.4               |                                      |              |         | 0.37        |
| 3       |             |            | 0.20                      | 0.8               |                                      |              |         | 0.09        |
| 4       | 0.8         | 80         | 0.50                      | 0.2               |                                      |              |         | 0.40        |
| 5       |             |            | 0.10                      | 0.4               |                                      |              |         | 0.26        |
| 6       |             |            | 0.20                      | 0.8               |                                      |              |         | 0.04        |
5. CONCLUSION

We have concluded that the plants having a great source of enzymes, such as horseradish roots, soybean seed hulls and turnip roots are having rich sources of enzymes such as Peroxidase, Chloroperoxidase, Manganese peroxidase, Laccase and Catalase. The enzymes are having a wide range of degradation of pollutants. Some authors studied on the delivery systems for enzymes in effluent treatment. The efficiency of enzymes depends on the factors affecting parameters such as pH, temperature, retention time, purification of an enzyme, concentration of enzyme and concentration of pollutants in wastewater to degradation of phenols. But enzymes are having great efficiency to degradation of phenol and their derivatives. The enzymes are time saving and inexpensive catalyst. There are no harmful products formed after completion of reaction. Hence, enzymatic treatment is fully eco-friendly treatment.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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