

## A Review on Enzymatic Treatment of Phenols in Wastewater

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### Abstract

Tyrosinase is a natural enzyme and mainly catalyses the *o*-hydroxylation of monophenols into their corresponding *o*-diphenols. The synthesis of *o*-diphenols is a potentially valuable catalytic ability and thus tyrosinase has attracted a lot of attention with respect to industrial applications. In environmental technology, it is used for the detoxification of phenol-containing wastewaters and contaminated soils and also used in cosmetic and food industries as important catalytic enzyme. Phenols are present in effluents of a number of industries such as coal conversion, resins, plastic, petroleum refining, textiles, dyes and organic chemicals. Conventional processes for removal of phenols have drawbacks of incomplete removal of phenols. Hence, an alternative based on enzymes has been investigated. This review summarizes the current research based on removal of phenol from waste streams by enzyme polyphenoloxidase (tyrosinase).

**Keywords:** Wastewaters; Phenols; Tyrosinase; Polyphenoloxidase

### Introduction

Phenolic compounds are mainly found in wastewater of many industries such as coal conversion, resin, plastic and petroleum refineries. These compounds are toxic pollutants in industrial waste imposing risk to human health and some of them are suspected carcinogens. Phenols and cresols are highly corrosive and toxic, cause, damage to the respiratory, scarring of the skin damage to gastrointestinal tracts, kidney failure, hematological changes and nervous system depression [1,2]. Therefore, some steps should be taken to treat wastewaters [3-12] and also to degrade such environmental pollutants [13-21]. Some commonly used conventional treatment [22-29] often fails to generate final effluents at affordable cost with the required discharge quality. To overcome conventional biological treatment drawbacks, alternative technologies are sought for the treatment of phenolic wastewater such as treatment of narrow range of contaminant concentrations, generation of high sludge volumes and delays related to biological acclimatization.

Many researchers have proposed the use of peroxidase for removal of phenol and aromatic amines as a potential alternative due to following three reasons [30-35]:

- Enzymes are highly selective and can effectively treat even dilute wastes.
- They are less likely to be inhibited by substances which may be toxic to living organisms and their costs could eventually be less than that of other methods if commercially available enzymes are produced in bulk quantities.
- Enzymes operate over a broad concentration range and require low retention times with respect to other methods [36].

Peroxidases and laccases show a wide substrate range, especially with regards to phenols and amines. This suggests that these oxidative enzymes may not have specific substrate binding sites. Another problem is the susceptibility peroxidase to inactivation as it got absorbed on the final product of reaction. To remove phenols by peroxidase and laccase is expensive in comparison to tyrosinase as in case of peroxidase, hydrogen peroxide is used as an electron acceptor which is costly in comparison to oxygen which acts as an electron acceptor in case of laccase and tyrosinase. Moreover, cost of laccase isolation is a hurdle in its use to decontaminate wastewater. Therefore, use of tyrosinase is a cheaper alternative for enzymatic wastewater treatment.

Tyrosinase (EC 1.14.18.1), known as poly-phenol oxidase, is a copper-containing enzyme that catalyses an analogous phenol oxidation reaction to peroxidase [37-40]. This enzyme is widely distributed in vegetable products and fruits such as potato, mushroom, pear, tobacco, papaya, apple, avocado, banana, Florida spiny lobster, brown shrimp and others [41-43]. It is mainly responsible for browning in these food products [44]. It catalyses two different reactions. The primary reaction is the hydroxylation of monophenols resulting in *o*-diphenols, commonly known as mono-phenolase or cresolase [45]. The second reaction is the oxidation of *o*-diphenols to *o*-quinones, often known as *o*-phenolase or catecholase (Figure 1). Oxygen is used as an oxidant in the both of the oxidation reactions. This enzyme could also be used for the oxidation of many types of phenolic compounds such as chlorophenols, diphenols, methylphenols and naphthalenols [46]. Chlorinated anilines and anilines can be also oxidised to some extent and co-polymerisation with un-substituted phenol can result in good removals for these difficult-to-oxidise compounds [47].

### General characteristics and structure

Tyrosinase has been isolated and purified from a variety of plant and animal sources [48]. However, few of those are well defined, and preparations show a significant degree of heterogeneity [49]. The molecular weight of tyrosinase ranges from 13.4 KDa to 128 KDa depending on the sources [45,49] (Figure 2).

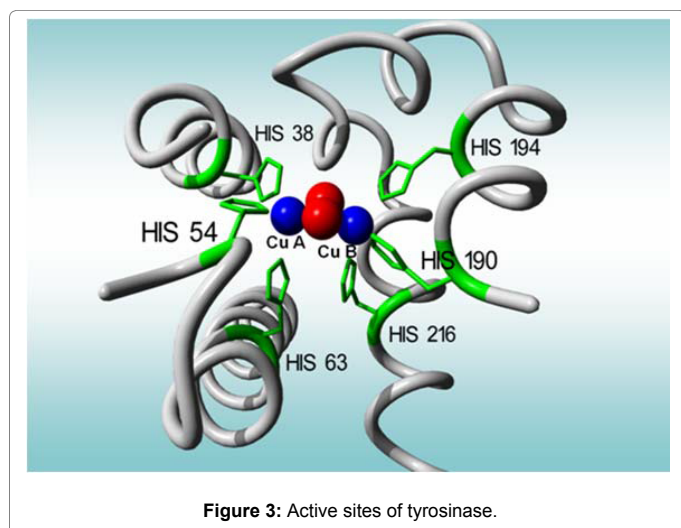
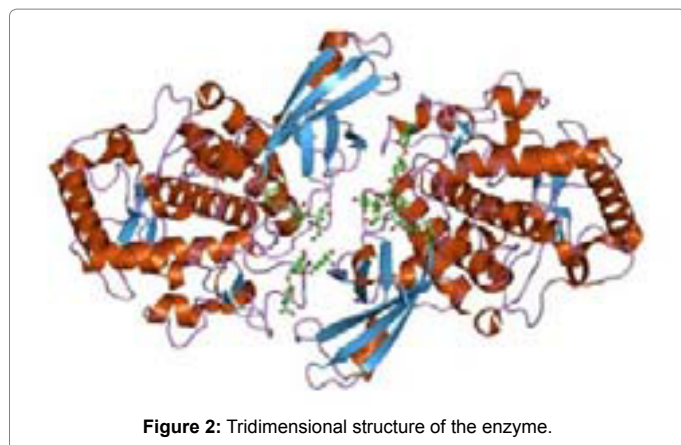
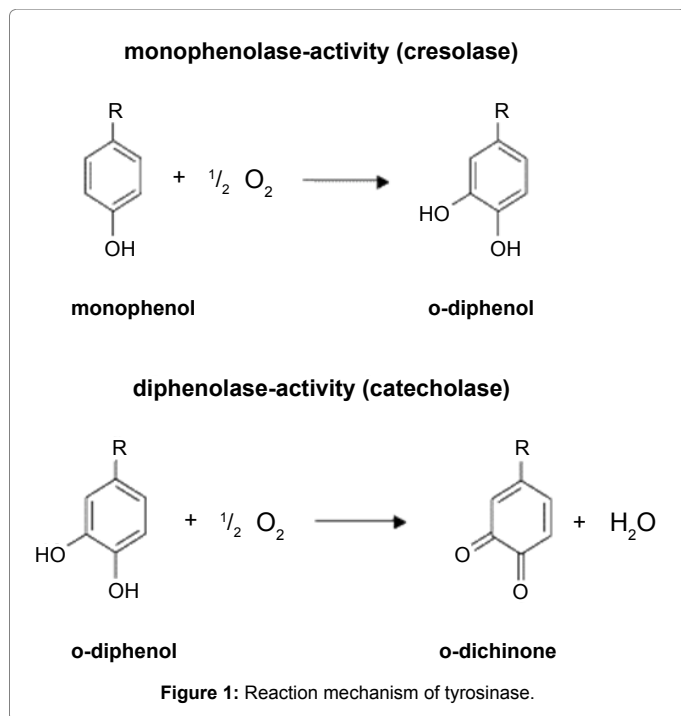
The enzymatic structure of tyrosinases has been studied by both biological and chemical approaches with respect to domain structure, primary, secondary, and tertiary structure, Cu binding sites, and activation mechanism [50-53]. It is widely accepted that the active site of tyrosinase is quite similar to that of hemocyanin, which is a respiratory copper protein, and contains coupled binuclear coppers (Figure 3).

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Source	Reference(s)
Apple	[55]
Avocado	[56]
Florida Spiny Lobster	[57]
Dog-rose -Fruit	[58]
Mushroom <i>Agaricus bisporus</i>	[59]
Neurospora	[60]
Papaya	[61]
Pear	[42]
Potato Leaf	[62]
Tobacco	[63]

**Table 1:** Sources of tyrosinase and literature sources describing methods of enzyme preparation.

Mushroom *Agaricus bisporus* tyrosinase is the only commercially available tyrosinase and is considered to be one of the most studied tyrosinases [44,45,54]. Although tyrosinase was isolated and purified from various bacteria, fungi, plants and animals, the objectives were mostly aimed at preventing food browning. The sources of tyrosinase and the authors who described preparation methods of enzymes are summarised in Table 1.

### Catalytic activity and its inhibition

**Catalytic cycle:** Tyrosinase catalytic mechanism has been studied for a long time [45,64]. This mechanism is complex and was believed to be an allosteric mechanism which involves two distinct binding sites for oxygen and aromatic compounds [45,65]. The oxidation state of copper in resting tyrosinase is mostly met derivative, which has two cupric centres [66,67]. In order to initiate the catalytic cycle, a reducing agent reacts with the two copper (II) atoms of met-tyrosinase and reduces them to de oxy-tyrosinase. However, if mono-phenol was used as a substrate, the enzyme activation could not occur without an aid of reducer [68]. Molecular oxygen binds with the deoxy tyrosinase and oxidises it to oxy-tyrosinase.

**Tyrosinase catalysed removal of phenolic compounds from wastewaters:** Atlow et al. first reported use of tyrosinase for the treatment of phenolic wastewaters [69-78] in 1984. Several monophenols and o-diphenols such as phenol [79], cresol, chlorophenol and catechol were removed very effectively with both commercially obtained and laboratory extracted tyrosinases. Tyrosinase was effective in removing phenol with initial concentrations ranging from 0.01 g/L to 1 g/L. The optimum enzyme dose to treat 50 mg/L (approximately 0.53 mM) phenol was determined to be 60 units/mL. Wada and his colleagues followed up with the tyrosinase catalysed phenol removal from wastewaters according to the procedures of Atlow et al.; however, no precipitate was formed but the reaction solution changed from colourless to dark-brown. They assumed that the enzyme purity might have an effect on the formation of precipitate: i.e., treatment with lower purity enzyme resulted in the precipitation.

### Factors affecting transformation of phenols catalysed by tyrosinase

**pH and temperature:** The optimum pH of tyrosinase activity depends on the source of enzyme and on the substrates [42]. However, only one study has been reported involving the treatment of phenol so far. Literature dealing with tyrosinase-catalysed phenolic wastewater treatment [80-84], commercially obtained or crude extract mushroom tyrosinase was used. 50 mg/L phenol was treated with 30 units/mL tyrosinase in different pH buffers and the best removal efficiency was achieved when 50 mM sodium phosphate buffer at pH 8 was used [39].

No such temperature effect has been examined for phenol treatment with tyrosinase. Probably the reason could be the indication of its instability upon exposure to high temperature conditions according to some thermal inactivation studies of tyrosinase [85].

**Enzyme concentrations:** Atlow et al. demonstrated the relationship between concentrations of phenol and tyrosinase dose required to achieve over 98% removal [46]. The required tyrosinase dose was directly proportional to initial phenol concentration over the range of 50 mg/L to 1 g/L, and the ratio was 1.2 units/ml of tyrosinase for each 1 mg/L of phenol. Wada and his colleagues however found that the optimum tyrosinase dose for 0.5 mM was 20 units/ml, which was about three times as small as that reported by Atlow et al. after following up with experiments involving the same procedures (including the use of the same tyrosinase activity assay) [86].

**Substrates:** Tyrosinase can transform many phenolic and other aromatic compounds such as phenol, 2-methylphenol, 3-methylphenol, 2-chlorophenol, 3-chlorophenol, 2-methoxyphenol, catechol, resorcinol, 2,3-dimethylphenol, 1-naphthol [46], 4-chlorophenol, 3-methoxyphenol, 4-methoxyphenol, 4-methylphenol, hydroquinone, 3,4-dichloro-aniline [47], 2-hydroxy-acetophenone and 4-hydroxy-acetophenone [87].

Aniline was quite difficult to oxidise when treated alone by tyrosinase while in the presence of two molar equivalents of phenol, this compound was transformed to high levels [47]. 0.5 mM aniline was completely removed from the solution in the presence of 1 mM phenol, whereas only 28% of aniline was removed when aniline was the only substrate. It is suggested that the removal of aniline was caused by the co-polymerisation reaction of aniline with o-quinones, which was derived from phenol oxidation with tyrosinase. In the case of aromatic compounds which are not substrate of tyrosinase such as anisole and benzyl alcohol, there was no oxidation and removal observed when in the presence of phenolic substrates [88].

The removal efficiency of substituted phenol was dependent on the type of substituent group and its positions [87,89]. Usually, para-substituted phenols were more easily oxidised than meta-substituted phenols.

**Chemical additives:** Unlike the studies involving other phenol oxidase, chemical additives were examined mostly for the purpose of removal of coloured soluble matter when tyrosinase was studied. Since precipitate has rarely been observed in phenol solutions treated with tyrosinase, the colour product remaining in the solution should be removed. Chitosan [90-98] and other natural or synthetic cationic polymers were investigated to accomplish the removal of colour from solutions [47,86,88,99-103].

Although the mechanism of the reaction between chitosan [104-112] and oxidised phenols has not yet been clearly established, the 2-amino groups of chitosan are likely to perform a nucleophilic attack on o-quinones to form covalent bonds [113,114]. The result strongly suggested that the adsorption of the quinones onto the chitosan was presumably the result of covalent interactions, which is referred to as chemisorptions. On the other hand, the interaction between phenol, pyrocatechol, or p-quinone and activated charcoal were weak and considered the result of low-energy physical forces such as hydrophobic interaction.

Wada and his colleagues investigated the use of cellulose, chitin, chitosan [115-121], hexamethylenediamine-epichlorohydrin polycondensate and polyethylenimine to remove coloured products

[86]. The first three were natural polymers and the last two were synthetic cationic polymers that had amino groups. Chitin also removed colour effectively, but cellulose had no effect on the colour removal.

**Immobilisation of tyrosinase:** The attachment of o-quinone on the amino acid residue in proximity to the active site of enzyme is associated with the inactivation of tyrosinase [122]. Therefore, in order to improve the catalytic lifetime, stability for storage and reusability of the enzyme, tyrosinase was immobilised on several support materials. Wada et al. immobilised tyrosinase on magnetite ( $\text{Fe}_3\text{O}_4$ ) activated by aminopropyltriethoxy-silane and glutaraldehyde [88]. When 1 mg of tyrosinase was used for immobilisation, immobilisation yield was about 80% and retained activity 70-80% with 500 mg of the support material. After 15 days of storage, the loss of activity of immobilised tyrosinase was about 5%. Three types of methylphenols, chlorophenols and methoxyphenols were treated with this immobilised tyrosinase. The soluble tyrosinase was inactivated rapidly, whereas immobilised tyrosinase could be used 5 times without significant reduction of activity.

Payen et al. immobilised tyrosinase into chitosan gel in many ways [87,123,124]. They prepared chitosan gel [125], by dissolving chitosan in 8% (v/v) acetic acid solution and stirred overnight. After centrifugation of the solution-undissolved chitosan was removed, then the viscous chitosan solution was added through a syringe needle into an 8% NaOH solution. The chitosan gel formed in the NaOH solution was spread on square glass slides. Highly concentrated tyrosinase was added to the central region of the square and then two gel films were combined so as to containing tyrosinase between them and were then sealed using rubber cement. Chitosan was used for the support of the enzyme as well as for the sorbent, in this way, this immobilised tyrosinase could not be reused.

## Discussion

Characterisation of tyrosinase activity was done using L-tyrosine as a substrate. At pH 7 the maximum catalytic activity was observed. Tyrosinase appeared to be unstable at low pH and at elevated temperature. According to the 5 min Microtox assay, most chlorinated phenol solutions and phenol treated with tyrosinase enzyme had lower toxicities when compared to their corresponding untreated solutions. However, toxic products and coloured products were formed during wastewaters treatment [126-131]. Chitosan was found to be effective in inducing their precipitation, resulting in detoxified and colourless solutions. The addition of chitosan was very effective in inducing the precipitation of the coloured products generated by the transformation of phenol with tyrosinase. It was found that the toxicities of the phenol solutions when treated with tyrosinase were substantially lower than the solutions treated with peroxidase enzymes. This represents a very strong advantage when considering this enzyme for applications in wastewater treatment [132-134]. In addition, tyrosinase requires oxygen as an oxidant, which is comparatively much less expensive than the hydrogen peroxide, required by peroxidase enzymes. These might be critical points of interest while considering tyrosinase for applications in wastewater treatment.

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