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ENZYMATIC TREATMENT OF WASTEWATER CONTAINING DYESTUFFS USING DIFFERENT DELIVERY SYSTEMS

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ABSTRACT

Dwindling water resources is a global problem. Effective effluent treatment is an important step towards conserving our water resources. Some pollutants such as dyestuffs resist degradation by conventional treatment methods and persist in the environment. The present article describes the use of enzymes as an alternative method for treatment of such recalcitrant pollutants. It evaluates different methods in which enzymes can be delivered to the target effluent, including nanoparticles as delivery systems. It also emphasizes the need for current and future research to focus on developing economically feasible and environmentally sustainable wastewater treatment practices.

Key words: Recalcitrant, Dyes, Enzymes, Oxidoreductase, Immobilization, Nanoparticles.

INTRODUCTION

The limited availability of fresh water is a global crisis. The growing consumption of fresh water by anthropogenic activities has taken its toll on available water resources. Unfortunately, water bodies are still used as sinks for wastewater from domestic and industrial sources. However, in recent times, the need to replenish our water resources has been receiving increasing attention. This has led to the development of strategies to return water to its source in the least toxic form possible, to enable reutilization of water. These strategies and processes may be collectively termed as 'wastewater treatment'.

An anthropogenic activity that produces large volumes of concentrated effluent is the process of dyeing. Whether applied to fabric, paper, pulp, leather etc., the processes involved in dyeing generate effluents that are rich in colorants. The presence of colorants in wastewater and eventually in receiving waters poses a threat to aquatic life forms. This article attempts to explore and evaluate the use of enzymes to degrade or decolorize the dyestuffs in effluents as an alternative to conventional treatment methods.

Need for dye removal from effluents

Synthetic dyes are preferred for use over natural dyes due to their superior performance. As compared to natural dyes, synthetic dyes impart brighter colors, show better light-fastness and are more resistant to washing. Also, synthetic dyes offer a wider variety of colors[1]. Wastewater or effluents from

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industries that manufacture paints, pigments and color cosmetics contain a variety of synthetic dyes. Industries involved in dyeing of textile, paper, leather and plastics, release effluents that are highly colored[2]. Azo dyes feature among the most widely used synthetic dyes in industry globally[3]. The fixation of azo dyes (on textile) is quite low and often, up to 50% of the applied dye may be lost in the wash stream[3].

The presence of dyestuffs in industrial effluent is more than just an aesthetic problem. The chromophores of dyes strongly absorb sunlight[1,2]. When the effluent reaches the receiving water body, the dyes hinder photosynthesis by the aquatic flora. The presence of dyes in the water body increases the Chemical and Biological Oxygen Demand (COD and BOD respectively)[4]. Additionally, effluent containing dyestuffs are found to have a large concentration of suspended solids[5]. These factors upset the ecological balance of the receiving water body. Several dyes have been found to be potentially toxic[3]. Thus, the presence of synthetic dyes is a serious environmental concern. Evidently, it is necessary to remove colorants from the effluent before it is discharged into a water body.

Causes of recalcitrance of pollutants

The removal of coloring matter from effluent is a major problem faced by industries. In general, the chemical structure of dyes contains conjugated double bonds and aromatic rings[6]. Many synthetic dyes tend to persist in the environment due to the inherent stability of their molecular structure. Azo dyes for example, have a characteristic azo (-N=N-) linkage which is electron withdrawing in nature. The presence of this linkage decreases the susceptibility of azo dyes to oxidative reactions[7] thus making them resistant to conventional degradation methods. Complex pollutants that resist degradation and tend to persist in the environment for long durations are considered to be recalcitrant pollutants[8]. Recalcitrance of a given pollutant may sometimes be attributed to unusual substitutions with halides (Cl^- or Br^-), very large molecular size, and presence of unusual bonds or highly condensed aromatic rings. The presence of tertiary and quaternary carbon atoms also contributes to recalcitrance[9].

Conventional processes for removal of dyes from effluent streams

The conventional methods used in the textile industry for color removal from effluents include physico-chemical methods like coagulation/flocculation and activated carbon adsorption. Both flocculation and adsorption generate large amounts of sludge and waste, which require separate treatment before disposal[1].

An effective means of decolorization of dye containing effluent is the application of the Advanced Oxidation Processes (AOPs). These processes are based on the generation of highly reactive species like the hydroxyl radicals ($^{\bullet}OH$) that have a strong oxidative potential ($E^0 = + 2.8$ V). These radicals can rapidly oxidize a broad range of organic pollutants in a non-selective manner. The common AOPs include Fenton and Fenton-like oxidation, ozonation, photochemical oxidation, electrochemical oxidation, photolysis using H_2O_2 and O_3, Corona Process, TiO_2 photolysis, radiolysis, wet oxidation and the use of electronic beams or γ-beams[2].

Ozonation is a method used to treat dyes in effluent. Although it effectively decolorizes dyes, it does not reduce COD adequately. It may produce exhausted ozone in the wastewater and increase the cost of treatment[2].

The Fenton process requires Fe^{2+}/Fe^{3+} and H_2O_2. It is commonly used since it is relatively inexpensive and can be easily operated. This method is capable of cleaving the azo (-N=N-) linkage, thereby bringing about the partial or complete degradation of azo dyes. The major disadvantages of the Fenton treatment are that it operates in the acidic pH range of 2-5 and that the Fe^{2+}/Fe^{3+} need to be regenerated at

the end of the treatment[2]. The conventional treatment processes have several shortcomings such as being unsuitable for use when the effluent contains high concentrations of the target pollutants, high running cost and low efficiency of removal[10].

Enzymes in wastewater treatment

In recent years, the use of living systems such as microorganisms[11,12] and plants[13] to degrade recalcitrant pollutants is gaining importance as a viable alternative to existing physico-chemical removal methods. Stringent government policies regarding permitted levels of pollutants, high costs of specialized chemical treatments for pollutant removal and the fact that some of these treatments create additional solid waste has led to the development of many effective, yet simple biological methods. These treatment processes can be collectively categorized under 'bioremediation' of wastewater[8].

Biological systems are able to bring about the degradation of the target chemicals primarily due to their enzymes. Hence enzymes, both intracellular and extracellular, are being explored as biochemical means of wastewater treatment. In general, enzymes are highly specific and extremely efficient catalysts[14]. They can selectively degrade a target pollutant without affecting the other components in the effluent. Therefore, enzymatic treatment is suitable for effluents that contain relatively large amounts of the recalcitrant target pollutants in comparison to others. More importantly, they can operate under mild reaction conditions, especially temperature and pH. In this respect, enzymes outperform the regular catalysts (transition elements like Cu, Ni etc.). From the environmental perspective, enzymes are more acceptable due to their biodegradability[15].

Considering that colorants such as azo dyes can be degraded physico-chemically by oxidation (i.e. AOPs), a majority of the enzymes that are being investigated for their dye degradation potential belong to the enzyme class Oxidoreductases. These enzymes are involved in electron transfer reactions.

In the case of reactions wherein the target pollutant is oxidized, the enzyme receives one or more electrons from the substrate and donates these electrons to an electron acceptor. Hence, at the end of the reaction the enzyme is regenerated and is available for the next catalytic cycle. Some of the oxidative enzymes such as the peroxidases require hydrogen peroxide (H_2O_2) or alkyl peroxide (R_2O_2) to act as the electron acceptor. Others such as laccases utilize molecular Oxygen for this purpose.

Peroxidases like horse radish peroxidase (HRP) (E.C. 1.11.1.7), manganese peroxidase (E.C. 1.11.1.13) and lignin peroxidase (E.C. 1.11.1.14) are ferric ion containing heme proteins and require peroxides like H_2O_2 for their functioning. Lignin peroxidase[16] and manganese peroxidase[17] are obtained from fungi. There are various plant sources of peroxidases – like horse radish[1,7,18], soyabean[19], radish[20], beetroot[21] and peanut[22]. Most of these peroxidases have been tested to determine their potential to treat synthetic and actual wastewaters.

Laccase (E.C.1.10.3.2) is a blue copper oxidase that catalyzes the four electron reduction of molecular oxygen (O_2) to water (H_2O). These enzymes are mainly obtained from lignin degrading fungi such as *Trametes versicolor*[15] and *T. Villosa*[6] as well as fungi like *Fusarium solani*[5] and *Cladospora cladosporioides*[23].

The azo groups in the azo dyes are converted to amines by reductive cleavage, a reaction catalyzed by azo reductase[24]. Azo dyes undergo reductive splitting relatively easily under anaerobic conditions[25]. The anaerobic reduction of certain azo dyes, however, yields aromatic amines that are potentially carcinogenic[26]. The degradation of different dyes by select oxidoreductases from different biological sources has been summarized in Table 1.

Table 1: Enzyme mediated decolorization of some dyes

Substrate(s)	Enzyme	Reference
3-(4 dimethyl amino-1 phenylazo) Benzene sulfonic acid	Laccase from *Trametes villosa*	Zille *et al*., 2004
Acid Orange 6, Acid Orange 7, Methyl Orange and Methyl Red	Mixture of Bacterial Oxidoreductases from Sludge Methanogens	Kalyuzhnyi *et al*., 2006
Direct Yellow	Horseradish peroxidase from *Armoracia rusticana*	Maddhinni *et al.,* 2006
Acid Blue	Laccase from *Cladosporium cladosporioides*	Vijaykumar *et al.,* 2006
Tartrazine and Ponceau	Azoreductase from Green Algae	Omar, 2008
Reactive Yellow, Reactive Black, Reactive Red and Direct Blue	Azoreductase from *Staphylococcus arlettae*	Franciscon *et al*., 2009

While peroxidases are specific to the electron acceptor, i.e. hydrogen peroxide or alkyl peroxides, they are not very specific towards the electron donor in the redox reactions that they catalyze. Consequently, a large number of electron rich chemical species can act as substrates for peroxidases[15]. 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[16]. The ability of these enzymes to act on different pollutants is affected by the molecular structure of the substrate[27], the temperature and pH of the treatment as well as the presence of intermediates[3].

Oxidoreductases catalyze redox reactions which are essentially electron transfers. The redox potential is a pivotal parameter governing enzyme mediated oxidations[15]. Sometimes, a substrate of interest may not be oxidized directly by the enzyme if the redox potential of the substrate is higher than that of the enzyme. This is observed in the case of laccases[28] which may require mediators like 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS)[29] to act as an intermediate substrate for the enzyme. Hence, the probability of a given chemical species to act as a substrate for these enzymes depends to a large extent on the difference in the redox potentials of the enzyme and the chemical species[30]. The presence of electron donating functional groups on the aromatic nuclei (substrates), such as hydroxyl groups and amino groups, facilitate oxidation by lowering the redox potential of the aromatic nucleus. It has been proposed that redox potentials of the target pollutants can be used to predict whether the pollutants can act as substrates for a given oxidoreductase of known redox potential[3].

Delivery systems for enzymes in effluent treatment

Enzymes are versatile and may be delivered to the target effluent in different ways. The delivery system selected, must be well suited to the purpose, simple, efficient and cost effective. However, special attention has to be given to ensure that the activity of the enzyme is not adversely affected due to the mode of delivery.

Enzyme delivery by direct use of biological source

One of the simplest methods of administering an enzyme to the target effluent is to introduce the cells or tissues that produce the enzyme into the effluent directly. This mode of enzyme delivery is adopted when suitably adapted strains of microorganisms are used to co-metabolize target contaminants or when the tissue producing the enzyme is introduced directly into the effluent.

Use of microbial cells

Cultures of the bacterial strain *Staphylococcus arlettae* were shown to decolorize solutions of four azo dyes (CI Reactive Yellow 107, CI Reactive Red 198, CI Reactive Black 5 and CI Direct Blue 71) in a microaerophilic/aerated sequential process. The average decolorization obtained was 97%[12]. Occasionally, a particular target compound may be degraded at a low rate by a microbial population without serving as a source of carbon or energy, i.e., it is co-metabolized. The co-metabolite does not support the growth of the concerned micro-organism(s) and the products are accumulated stoichiometrically. Co-metabolism probably occurs because some of the enzymes involved in the metabolism of the major carbon sources can use other compounds as substrates as well. For the microbial degradation of relatively recalcitrant pollutants, co-metabolism in a mixed microbial population is more advantageous than in a pure culture[9]. The drawback of using microorganisms to degrade target recalcitrant pollutants in actual effluents is that the microbes need to get acclimatized to the effluent[8] and this process can be time consuming. Also, an additional substrate may be needed to sustain the microbial culture if the effluent does not contain metabolic substrates of the selected microorganism[11].

Use of plant tissues or entire plants

The use of entire rooted plantlets of *Typhonium flagelliforme* to decolorize synthetic wastewater has been tested at the laboratory scale. The enzymes such as peroxidases, secreted by the roots of the plant bring about the degradation of dyes like malachite green, methyl orange and brilliant blue R[4]. Such methods of effluent treatment are economically feasible and offer simpler alternatives to existing methods for degradation of recalcitrant pollutants. However, adding biological material to an effluent will increase the BOD and COD of the effluent[8]. Also, if there is an uncontrolled growth of microorganisms on the biological material, the effluent could require an additional disinfection treatment before it is released into a water body[31].

Enzyme delivery as cell-free enzyme extracts

Enzymes extracted from organisms producing them are also being employed in wastewater treatment. In many cases, such cell-free or isolated enzymes are preferred for use over the intact organism, especially when the effluent to be treated contains pollutants which cannot support growth. The isolated enzymes could be used in either the pure form or as a crude extract. Enzymes need not be acclimatized to the wastewater like microbial cultures and also do not require a supply of nutrients for their growth.

When isolated enzymes are used, the growth rate of the source organism population does not affect the amount of enzyme available to treat the effluent. Besides, it is relatively easier to standardize optimum treatment conditions with isolated enzymes[32]. The use of isolated enzymes also has a definite advantage over microbial cultures in terms of ease of handling and storage.

The delivery of cell-free crude enzyme extract uses the least processed, yet functional form of the enzyme. The preparation of crude enzyme extracts typically includes simple processes such as grinding or homogenizing the source tissue in the presence of an appropriate buffer followed by filtration[19,20]. Crude enzyme extracts are preferred for use over pure enzymes, primarily because they are relatively inexpensive. Considering the scale of operation, the use of pure enzymes in effluent treatment is not economically feasible. Crude enzyme extracts can also effectively remove pollutants from effluent. A crude extract of *Cladosporium cladosporioides* containing laccase was found to decolorize solutions of the azo dye acid blue 193 upto 47% in 8 hrs[23].

Enzyme delivery in immobilized form

The functionality of enzymes depends largely on their conformation. Harsh reaction conditions like extreme temperature, very high or low pH, high ionic strength, high concentrations of reactants, and

presence of inhibitors can alter the conformation of an enzyme. Enzymes may not function optimally under such drastic conditions which are often encountered in effluent streams[32]. Immobilization methods that increase the reusability of enzymes by preventing the loss of enzyme during the course of the reaction and minimizing the loss of activity of enzymes under harsh treatment conditions have been developed.

An enzyme is said 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[31]. The use of immobilized enzymes in effluent treatment has many important advantages over the use of free enzymes including increased stability, localization, ease of handling, reusability and a consequent decrease in running cost[32]. The HRP enzyme has proved to be an adaptable molecule that can be used in the form of a cell-free crude extract[33,34] or in an immobilized form entrapped in calcium alginate capsules at a laboratory scale[7,35]. Fungal laccase immobilized using γ-aluminium oxide pellets, has been reported to decolorize solutions of azo dyes like Ponceau Red (65% decolorization), anthracinoid dyes like lanaset Blue 2R (100% decolorization) and a triphenyl methane dye like crystal violet (98% decolorization) after 24 hours[27].

However, not all enzymes are amenable to immobilization. Some of the methods of immobilization such as adsorption, covalent binding and chemical coupling can adversely affect the catalytic activity of certain enzymes. Adsorption is a widely used immobilization method which is preferred for its simplicity and ease of regeneration. But it has also been found that an immobilized enzyme that adsorbs too strongly to the supporting material may show a loss of functionality[38]. Enzymes immobilized by this method can operate in a relatively narrow range of pH, temperature and ionic strength. Drastic changes in reaction conditions cause desorption of the enzyme from the support material.

Covalent binding of enzymes on to a support or matrix may modify the conformation of the enzyme. Considering that the functionality of enzymes depends largely on their conformation, such a change can result in the loss of enzymatic activity[34]. It has been shown that enzymes immobilized by covalent binding can retain their activity more effectively if they are immobilized in the presence of their substrate or a competitive inhibitor since the active site remains protected from conformational changes in the presence of a substrate or its structural analogue[39].

Similarly, the entrapment and encapsulation of enzymes has certain advantages such as large surface area for substrate-enzyme interaction in a relatively small volume. However, the drawbacks of this method are that a high concentration of enzyme is required and that there is occasional inactivation of the enzyme on entrapment. Additionally, the pore size of the (cross linked) polymer has to be very small to retain the enzyme within the capsule[34]. HRP entrapped in calcium alginate gel showed lower decolorization (52%) of the azo dye direct yellow than the free enzyme (69%)[7].

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.

Enzyme delivery in the form of different nanoparticles

Nanotechnology is fast gaining importance in wastewater treatment. It can offer more effective methods to decontaminate xenobiotics in the environment. Nanoparticles have a very large surface area to volume ratio, high reactivity and sequestration properties all of which have immense potential for use in wastewater treatment. Remediation of waste streams containing dyestuffs, cleaning up of heavy metals from contaminated soil and water by absorption and sequestration are possible using nanoparticles. The use of nanoparticles in Reactive Remediation Technology is of great interest to wastewater treatment, since it involves the complete degradation of contaminants to harmless products such as carbon dioxide and water[40].

The remediation of contaminated wastewater can be achieved by using a combination of enzyme technology and nanotechnology known as the SEN, i.e., Single Enzyme Nanoparticle[41]. A SEN may be described as an armored enzyme surrounded by a protective 'cage' which is a few nanometers thick. The enzyme chymotrypsin has been used to create a SEN. In this case, the enzyme molecule was 'caged' by a silicate shell which was linked with its surface. While the cage covered most of the enzyme, the active site was kept chemically accessible to maintain the functionality of the enzyme[42]. The technology used to create the chymotrypsin SEN can be applied to other enzymes as well.

From the point of view of dye degradation in effluents, enzymes involved in redox reactions are of special interest for the synthesis of SENs. Enzymes involved in wastewater treatment that can be used for SEN synthesis include cell-free crude extracts or purified forms of enzymes like peroxidases, polyphenol oxidases (like laccases and tyrosinase), dehalogenases and organophosphorous hydrolases. These enzymes are capable of degrading a wide variety of recalcitrant organic contaminants such as phenols, polyaromatics, dyes, chlorinated compounds and pesticides[43]. SENs are able to withstand more drastic conditions of temperature, pH, contaminant concentration and salinity as compared to free enzymes[41].

Another type of novel nanoparticle are nanosponges. These are materials containing microscopic particles with nano-sized cavities. These particles can encapsulate or can be embedded with many types of substances and are capable of transporting them through an aqueous medium. Nanosponges with complexes consisting of nanopolymers and enzymes have been synthesized recently. These nanosponges are created by embedding the enzyme in a polymer matrix[44,45]. Novel nanoparticles such as these could be synthesized using enzymes such as peroxidase and laccase. Such nanosponges could find application in remediation of wash streams from dyeing and textile processing industries. At present, the utilization of nanopolymers in wastewater is primarily in the removal of heavy metals[41].

In recent times, research on carbon based nanotechnology such as the carbon nanotube is gaining momentum. The potential of these particles for use in remediation of soil, water and air is being evaluated. Carbon nanotubes carrying immobilized enzymes have been synthesized and incorporated into latex paints. The resulting materials can detect and eliminate hazardous chemical and biological agents[41]. Similarly, nanotubes carrying oxidative enzymes such as laccases or peroxidases could be synthesized for utilization in treatment of recalcitrant pollutants in wastewater. In the near future, carbon nanotubes are expected to be utilized to a large extent in water treatment[46].

As compared to the conventional methods used to treat complex and recalcitrant pollutants in industrial effluent, the use of nanoparticles offers relatively inexpensive alternative treatments[47]. However, nanoparticles can remain in suspension for extended periods of time, and this fact raises concerns regarding their toxicity. The toxic effects of nanoparticles on aquatic organisms are still being evaluated[41,46]. A fair evaluation of the toxic effects of nanoparticles and of the consequences of their persistence in the environment will enable the development of effective and sustainable effluent treatment strategies, which will include the judicious use of nanoparticles.

CONCLUSION

Enzymes have been employed in numerous fields primarily for their immense catalytic potential. In wastewater treatment, enzymes can be utilized to develop remediation processes that are environmentally less aggressive than conventional techniques. Their versatility and efficiency even in mild reaction conditions gives them an advantage over the conventional physico-chemical treatment methods. The biological origin of enzymes reduces their adverse impact on the environment thereby making enzymatic wastewater treatment an ecologically sustainable technique.

Despite the advantages of enzymatic wastewater treatment, the major limitation in the use of enzymes is their prohibitive cost. Currently, effluent treatment using enzymes on a large scale is not economically viable. However, if maximum reusability of enzymes is achieved through the use of standardized immobilization procedures, the running cost can be lowered considerably. The confluence of nanoscience and enzyme technology has resulted in an upcoming interdisciplinary approach to wastewater treatment. Such innovative applications of enzymes can enable the utilization of these biocatalysts to their maximum potential. Future research in this field should emphasize on the optimization of the activity of crude enzyme preparations and on the improvement of enzyme reusability to counteract the high start-up and running costs.

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