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AN OVERVIEW OF ENZYMATIC TREATMENT OF HAZARDOUS POLLUTANTS IN INDUSTRIAL WASTEWATER USING SOYBEAN PEROXIDASE

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Abstract: The potential application of the oxidoreductase-class enzyme, soybean peroxidase (SBP), extracted from soybean seed coats (hulls), to remove organic pollutants from industrial wastewater is reviewed, with an emphasis on the work completed or in progress in the authors' laboratory. The main objective of the research is to determine the technical feasibility of using crude SBP to catalyze the removal of toxic pollutants that are present in wastewater streams of various industries such as petroleum refining, coal conversion, pulp and paper, dyes, adhesives, plastics and textile manufacturing. The review will cover the advantages of enzymatic treatment over conventional treatment, factors affecting and enhancing effectiveness of enzymatic treatment, and how pre-treatment could be used to enhance the scope of enzymatic treatment. It is also expected that this could even be used as a polishing step coupled with other existing physico-chemical or biological processes to increase the overall treatment efficiency, economically.

1 Introduction

There are strict environmental laws that make it compulsory for the development and implementation of innovative, environmentally friendly wastewater treatment methods to replace existing technologies. The drawbacks of the latter processes include: high costs, low efficiency, harsh reaction conditions, high-energy demand and/or formation of hazardous by-products (Oller et al. 2011). These reasons stimulate the search for alternative treatment methods that are cheaper, faster and easier to maintain. One of the extensively demonstrated alternative methods, enzymatic treatment, combines aspects of both physico-chemical and biological processes since it uses the chemical reaction of a biological catalyst. The process is attractive due to its ease and simplicity of control, short contact time, and applicability to biorefractory compounds; there is also no limitations due to shock loading or accumulation of biomass - reduction in sludge quantity - and its effectiveness over broad ranges of pH, temperature and salinity ranges (Steevensz et al. 2014a). Peroxidases are easily obtainable from plants, microbes, and animals. This super family is classified into three groups. The soybean seed coat peroxidase (SBP) belongs to the class III of the super family consisting of secretory plant peroxidases. SBP is an inexpensive by-product of soybean seed hulls. The application of SBP ranges from medical diagnostic kits through removal of pollutants from wastewater (Ghaemmaghami et al., 2010), to polymers and resin manufacturing. SBP is also used in the baking industry as a replacement for toxic potassium bromate, which is now banned in all major markets. Because of SBP's high catalytic activity and thermostability it is also used in electrochemical biosensors. The largest market for SBP is in the treatment of contaminants present in industrial wastewater streams, sludge and soils. (Mousa Al-Ansari et al. 2011).

Phenolic compounds are considered priority pollutants with high toxicity even at low concentrations. They are present in the effluents of industries such as oil refining, petrochemicals, pharmaceuticals, coking operations, resin manufacturing, plastics, paint, pulp, paper, and wood products. Release of these compounds without treatment may lead to serious health risks to humans, animals, and aquatic systems. Anilino compounds are commonly used as precursors for the synthesis of dyes and polyamides. Phenols and anilines have an adverse effect on human health upon direct absorption by the skin, ingestion and inhalation exposure resulting in severe dermatitis, urticaria, hyperreflexia, hyporeflexia, chemosis, lacrimation, exophthalmos, ophthalmia and even permanent blindness (Mousa Al-Ansari et al. 2011).

2 Enzyme Availability

The enzyme must be cheap enough to meet the consumer's need in order to be applicable in a water treatment process. Soybean hulls, a by-product from crushing operations, are used in animal feed. It has been shown that washing these hulls with water is sufficient to extract SBP (without compromising the feed value of the hull solids) and treatment with such a low purity enzyme is as effective as using purified enzyme. There is, however, some species-variation in hull peroxidase content that must be considered (Steevensz et al. 2014a). For this study, crude SBP was obtained from seed coats with "high activity". In North America, large quantities of soybean are produced every year. In 2015-16, the United States was the leading soybean producing country with a production volume of 106.93 million metric tons. The production of soybeans in the United States is estimated to reach around 120.59 million metric tons in 2017-2018 (Statista 2018).

3 Enzymatic Treatment

In the early 1980s, Klibanov and his co-workers (1980) started enzymatic treatment of aromatics using horseradish peroxidase (HRP). This technique can be applied as a primary treatment or in combination with a biological unit. Amongst all enzymes, oxidoreductases such as laccases, tyrosinases, and peroxidases have the capability to catalyze removal of aromatic pollutants (Patapas et al., 2007). The mechanism involves polymerization of the target compounds, not breakdown, until the products reach their solubility limits and precipitate out of the solution (Cordova Villegas et al., 2016). Although HRP is the most studied heme-protein peroxidase, SBP has received lots of attention because of several advantages over HRP (Cordova Villegas et al., 2016). Peroxidases in the presence of hydrogen peroxide catalyze the oxidation of phenols and anilines to an aromatic radical. Those radicals diffuse from the active site of the enzyme into solution and non-enzymatic radical coupling formation takes place leading to dimer formation. If these dimers are soluble then they become substrates for another enzymatic cycle, forming higher oligomers. These cycles continue until the polymeric products reach their solubility limit and precipitate. One of the major drawbacks to peroxidase-based treatment is inactivation of the enzyme, primarily by the end-product polymers' adsorbing and co-precipitating the enzyme (Steevensz et al., 2014b). Tyrosinases and laccases are copper-containing enzymes. Tyrosinases catalyze *o*-hydroxylation of monophenols to *o*-diphenols followed by oxidation of *o*-diphenols to *o*-quinones. Further non-enzymatic polymerization of the quinones forms soluble products. Laccases function by a radical generation and coupling mechanism very similar to peroxidases. Laccases are activated by molecular oxygen (four electrons) and heme-protein peroxidases are activated by hydrogen peroxide (two electrons). Immobilization techniques such as support- or carrier-binding through physico-chemical interaction, encapsulation which traps enzyme inside the pores of the support, or covalent cross-linking of enzyme functional groups have also been investigated to enable large-scale enzymatic treatment (Cordova Villegas et al. 2017). Investigations to remove more than 90% of phenol over a concentration range 0.02-0.10 mM from a manufacturing industry wastewater using a peroxidase from potato pulp in a 2-h reaction time were performed by Kurnik et al. (2015).

4 Factors affecting Enzymatic Treatment

4.1 Optimum pH

Optimum pH is defined as the pH at which an enzyme possesses maximum catalytic activity. Dependence on pH indicates the important role that acid-base catalysis plays in enzymatic reactions. There are key

amino acid residues at the active site of SBP that help in catalysis. The distal histidine (His42) must be in their deprotonated form to act as catalytic base whereas an arginine (Arg38) must be in protonated form to act as a charge stabilizer. Functional groups of the substrate ionizing in the pH range in question will also play a role. An optimum pH ensures that the enzyme and substrate exist in their best states for catalysis (Mousa Al-Ansari et al. 2011). The determination of optimum pH is always conducted under "stress" conditions. The term "stress" means imposing stringency by some agent, SBP concentration in this case (insufficient SBP to effect complete conversion), in order to provide easier discerning of the optimum. Table 1 summarises the optimum pH determined for a selection of aromatic compounds recently studied.

Table 1: Optimum pH for the removal of the toxic pollutants using SBP

Aromatic Compound	Optimum pH	Reference
Remazol Brilliant Blue R (0.06 mM)	6.0	Silva et al. 2013
Crystal Ponceau 6R (0.07 mM)	3.0-5.0	Ali et al. 2013
Remazol Turquoise Blue G 133 (0.06 mM)	3.3	Marchis et al. 2011
Benzidine (0.1 mM)	5.0	Malik Altahir et al. 2015
o-Anisidine (1.0 mM)	5.0	Mazloum 2014
Crocein Orange G (1.0 mM)	8.0-8.5	Mazloum 2014
Acid Red 4 (1.0 mM)	8.0	Mazloum 2014
Acid Blue 113 (1.0 mM)	3.5-4.5	Cordova Villegas et al. 2017
Direct Black 38 (0.5 mM)	3.6-4.0	Cordova Villegas et al. 2017

4.2 Optimum Hydrogen Peroxide-to-Substrate Concentration Ratio

The peroxidase mechanism states that for every mole of hydrogen peroxide consumed, two moles of the aromatic functional groups are converted to free radicals that further couple non-enzymatically to form dimers and, through succeeding enzyme cycles, to form larger polymers. Generally, peroxidases get deactivated as hydrogen peroxide concentration increases beyond the optimum. For 1.0 mM phenylenediamines and benzenediols, the hydrogen peroxide concentration ranged from 1.5-2.5 mM (Mousa Al-Ansari et al. 2009). Whereas for removal of diaminotoluenes (DAT), the optimal hydrogen peroxide – DAT molar ratio was 2.0 for 2,4-DAT and 3.0 for 2,6-DAT (Patapas et al. 2007). Table 2 summarises the hydrogen peroxide demand for a selection of aromatic compounds recently studied.

Table 2: Optimum [H_2O_2] using SBP

Aromatic Compound	Optimum H_2O_2 (mM)	Reference
Crystal Ponceau 6R (0.07 mM)	0.175	Ali et al. 2013
Benzidine (0.1 mM)	0.150	Malik Altahir et al. 2015
o-Anisidine (1.0 mM)	1.250	Mazloum 2014
Crocein Orange G (1.0 mM)	3.500	Mazloum 2014
Acid Red 4 (1.0 mM)	3.500	Mazloum 2014
Acid Blue 113 (1.0 mM)	2.500	Cordova Villegas et al. 2017
Direct Black 38 (0.5 mM)	2.500	Cordova Villegas et al. 2017

4.3 Minimum SBP Concentrations Required

The cost of the enzyme is one of the major challenges in applying enzymatic treatment for real wastewater, so there is a need to investigate the minimum SBP concentration required to achieve at least 95% removal of the aromatic pollutants. Enzyme concentration is specified as standard units of activity (U) per volume, where, for peroxidase, 1.0 unit of activity corresponds to the consumption of 1.0 µmol of H_2O_2 min^{-1}. Table 3 summarises the minimum SBP demand for a selection of aromatic compounds recently studied.

Table 3: Minimum SBP concentration

Aromatic Compounds	Minimum SBP concentration (U/mL)	Reference
Benzidine (0.1mM)	0·00043	Malik Altahir et al. 2015
o-Anisidine (1.0 mM)	0.0116	Mazloum 2014
Crocein Orange G (1.0 mM)	0.75	Mazloum 2014
Acid Red 4 (1.0 mM)	0.75	Mazloum 2014
Acid Blue 113 (1.0 mM)	1.5	Cordova Villegas et al. 2017
Direct Black 38 (0.5 mM)	3	Cordova Villegas et al. 2017

4.4 Effect of Reaction Time

The reaction rate should be directly proportional to the enzyme concentration, thus, the reaction would progressively slow down with loss of enzyme activity over the time course (Mousa Al-Ansari et al. 2009). The reaction time also dictates the economics of the reactor used for the treatment of wastewater.

5 Improving the Effectiveness of SBP

5.1 Influence of Additives

Enzyme inactivation is the main disadvantage of the enzymatic treatment process, resulting in increased enzyme demand and thus cost (Mazloum et al. 2016). Klibanov et al. (1983) proposed that inactivation results from the return of a free radical to the active site, blocking that site and preventing further catalysis. Alternatively, it was suggested by Nakamoto and Machida (1992) that inactivation can be due to end-product polymer adsorption of enzyme, blocking the access of the substrate to the active site and/or physically removing the enzyme from solution. However, Feng et al. (2013) reported that this phenomenon, with phenol as substrate, is not inactivation, but immobilization in a form with lower specific activity. Use of additive can reduce the amount of enzyme needed in the treatment. Different additives have been used such as polyethylene glycol (PEG) or surfactants such as Triton X -100, Tween 20, sodium dodecyl sulfate (SDS) and NP-40 (Mazloum et al. 2016). It was first proposed by Nakamoto and Machida (1992) that with purified HRP, a 200-fold reduction of required peroxidase concentration can be achieved in the presence of PEG to suppress the adsorption of HRP by phenolic reaction end-product (Steevensz et al. 2012). Also, addition of PEG is known to enhance *Arthromyces ramosus* peroxidase treatment of phenol and laccase treatment of bisphenol-A. Other additives mentioned above were also effective during enzymatic treatment (Mazloum et al. 2016). The best additive among these is PEG because it has few environmental impacts and is nontoxic in concentrations remaining after separation of the precipitate (Ibrahim et al. 2001). However, it is noted that an additive could increase the carbon footprint of the process. Additive usage can be reduced or even avoided, by enhancing precipitate recycling in consecutive batch reactions, for removal of phenol. For example, the optimum SBP concentration for 1 mM phenol removal can be reduced by 2·4-fold due to precipitate recycling. Here, the active enzyme is absorbed by the precipitate during the reaction, and it has no secondary contribution of organic carbon compared to the processes where additives are used (Steevensz et al. 2014a, Feng et al. 2013).

5.2 Step Addition

Step feeding of hydrogen peroxide has been used to keep the radical concentration low and reduce the possibility of SBP inactivation by excess hydrogen peroxide thus improving removal efficiency (Ibrahim et al. 2001).

5.3 Pre-treatment to Broaden the Scope of Enzymatic Treatment

In general, pre-treatment before enzymatic contact can enable removal of non-substrate compounds using a two-step process since enzyme catalyzed oxidative polymerization is only successful with phenols and anilines. In this way, the scope of enzymatic treatment could be broadened. We have explored three variations of this concept, zero-valent iron reduction of nitro- and azo-aromatics and limited Fenton reaction of unfunctionalized aromatics.

5.3.1 Removal of Nitro-aromatics from Synthetic Wastewater Using Two-Step Zero-Valent Iron Reduction

Nitroaromatics (NACs) are produced on a large scale for use as solvents and in the manufacture of dyes, pesticides, plastics, and explosives. In addition, NACs are highly toxic and hazardous, and some of them have been recognized as human carcinogens. Degradation of NACs, which are significant environmental pollutants, is difficult to achieve. Zero-valent iron (Fe^0) reduction of NACs coupled with peroxidase-catalyzed treatment of the resulting anilines as a two-step approach for removing NACs from wastewater and process water has been investigated.

The pre-treatment step converts NACs into anilines and cleaves azo-aromatics into two anilines due to Fe^0 reduction. The second step is SBP-catalyzed oxidative coupling to completely remove the anilines thus generated in the first step. The enzymatic reaction which follows the pre-treatment process contains Fe^{2+} and sodium sulfite due to Fe^0 reduction and they need to be removed prior to enzymatic treatment. The reaction mixtures from Fe^0 treatment are generally aerated to precipitate the divalent iron as Fe^{3+} and to convert sodium sulfite (Na_2SO_3) to sodium sulfate (Na_2SO_4) (Mantha et al. 2002).

Four different NACs have been studied: nitrobenzene, *o*-, *m* and *p*-nitrotoluenes. Firstly, under anaerobic condition they were reduced to amines, and secondly, enzymatic treatment was done on the amine formed due to the reduction steps. The corresponding amines produced were aniline, *o*-toluidine, *m*-toluidine, and *p*-toluidine, respectively. Fe^0 has proven to be effective at the quantitative reduction of millimolar concentrations of NACs to their corresponding amines quickly and economically. However, enzymatic treatment was effective in treating anilines in the effluent from the Fe^0 column and converting them to insoluble polymers. Overall, it can be said that a viable two-stage approach has been developed for continuous removal of NACs from wastewater (Mantha et al. 2002). Previous studies have also been done on 2,4- and 2,6-dinitrotoluenes (DNT). The first step involved reduction of DNT to the diamino-compounds using Fe^0, followed by enzymatic treatment. More than 95% conversion of the nitroaromatic compounds to the diamino-compounds was achieved by this pre-treatment (Patapas et al. 2007).

5.3.2 Zero-valent Iron Reduction of Azo-aromatic Compounds

The zero-valent iron (Fe^0) reduction process is conducted under anaerobic conditions to limit corrosion of the iron to that arising from reduction of the azo- (or nitro-) group. Azo-dye decolourization by Fe^0 is feasible in treatment of wastewater. However, the anilino-products formed after the reductive azo-cleavage are more toxic than the parent compounds. As a result, Fe^0 reduction can only be used as a pre-treatment method in combination with amine treatment method. SBP has been used for the direct treatment of anilino azo dyes, as well as anilines produced as breakdown products of Fe^0 pre-treatment of azo-dyes, along with direct enzymatic treatment of azo dyes to determine the optimal methods of treatment (Mazloum 2014, Cordova Villegas et al. 2017). It has also been seen in comparison of one-step and two-step processes that Fe^0 allows reducing the concentrations of hydrogen peroxide and enzyme needed during treatment in certain cases. For example, with the azo dye, Direct Black 38, a Fe^0 pre-treatment allowed the enzyme concentration in the second stage to be reduced to around 80% and H_2O_2 to around 56%. This second stage was used to remove the products formed after azo cleavage of the dye with Fe^0 (Cordova Villegas et al. 2017).

5.3.2 Removal of Benzene from Wastewater by Enzyme-Catalyzed Oxidative Polymerization using Soybean Peroxidase Combined with a Modified Fenton Reaction

Benzene, a member of the BTEX family (benzene, toluene, ethylbenzene and xylenes) is a priority pollutant classified in the EPA's Toxic Release Inventory (TRI) (EPA 2008) and Environment Canada's National Pollutant Release Inventory (NPRI) list (Environment Canada 2008). The methods used to remove such aromatic contaminants from the environment include volatilization, photo- and chemical-oxidation, adsorption, bioaccumulation and biodegradation (Health Canada 2009). However, many of these treatment methods do not result in complete destruction of the chemical. As a result, previous researchers from the authors' laboratory have investigated the possibility of using a two-step process for the removal of benzene,

a non-substrate of peroxidase or laccase, from buffered synthetic wastewater through pre-treatment by limited Fenton reaction to generate the corresponding phenolic compounds. Highly reactive hydroxyl ($\dot{OH}$) radicals, from the Fenton reaction, can result in the complete destruction of BTEX to CO_2, water and inorganic salts (Saha et al. 2011).

In the first phase of the reaction, batch reactor optimization of the Fenton reaction parameters was studied. Batch reactors were set up to study the effect of pH, [Fe^{2+}], [H_2O_2] and reaction time at room temperature. The batch reactors had buffered solution of benzene (6 mM), various concentrations of Fe^{2+}, along with hydrogen peroxide added to initiate the reaction. The reactors were mixed vigorously with Teflon-coated stir bars and a magnetic stirrer. After an appropriate reaction period, reaction mixtures were quenched with sodium hydroxide and catalase. The addition of base brought the pH to 7.0 and most of the Fe^{2+} present in the solution was converted to Fe^{3+}, which precipitated out of the solution. Samples were filtered through 0.2 µm membrane filters and analyzed for benzene and phenolics by HPLC. The reaction was conducted to obtain the maximum conversion of benzene to phenolic compounds without causing major mineralization. In the second phase of the experiment, oxidative polymerization of the resultant phenolic compounds catalyzed by a laccase was carried out. The factors studied in a 3-h enzymatic treatment time were pH and laccase concentration. Under optimum Fenton reaction conditions, 80% conversion of the initial benzene concentration was achieved, giving a mixture containing oxidative dimerization product (biphenyl) and hydroxylation products (phenol, catechol, resorcinol, benzoquinone and hydroquinone). Enzymatic removal of biphenyl and benzoquinone was not possible; however, 2.5 U/mL laccase was successful in the removal of the rest of the phenolic products. It was also suggested that in order to improve overall removal efficiency, a method should be developed for benzoquinone removal (Saha et al. 2011).

6 Summary and Conclusions

The potential use of soybean peroxidase (SBP) for the treatment of industrial wastewater has gained popularity. Its availability as a by-product of the feed industry makes it easily accessible, in contrast to many other peroxidases. SBP-based treatment of selected aromatics in industrial wastewater provides a cost-effective alternative to conventional biological, chemical and physical treatments. The enzyme is effective as a crude extract rather than its purified form. However, there are many studies on synthetic wastewater compared with a limited number on real effluents. The use of additives has helped to increase enzyme economy, but the additive itself will have to be removed cheaply in a timely manner or else they will leave a carbon footprint. One limiting factor is the cost of the enzyme; however, it could be dramatically reduced by selecting an SBP-rich soybean variety or using soy hulls directly. It was also seen that in some cases, such as treatment of dyes and nitroaromatic compounds, pre-treatment is needed and/or may be beneficial prior to enzymatic treatment.

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