Additive Effect on Soybean Peroxidase-Catalyzed Removal of Anilines from Water

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Abstract

Soybean peroxidase has been shown to be effective in removal of aromatic compounds from wastewater, while the use of additives effectively reduces enzyme concentration requirement, hence overall treatment cost. Enzymatic treatment, an oxidative polymerization, was successful in removal of over 95% of both aniline and *o*-anisidine. The originality of this study lies in the findings that the additives, sodium dodecyl sulfate (SDS), sodium dodecylbenzenesulfonate (SDBS), Triton X-100, and sodium dodecanoate (SDOD), reduced enzyme concentration requirement, while polyethylene glycol (PEG, average molar mass of 3350 g/mol) had no effect on the required enzyme concentration. In addition, the presence of SDS also enhanced treatment by improving precipitation and color removal. These results are enabling advancement of soybean peroxidase-catalyzed treatment of anilines found in wastewaters as a new sustainable method.

Key words: additives; anilines; soybean peroxidase

Introduction

ANILINES ARE FOUND in various industrial effluents such as those of the rubber, herbicides, pharmaceuticals, corrosion inhibitors, dye, and pigment industries (U.S. EPA, 1994; IARC, 1999; NTP, 2014). The toxic and carcinogenic effects of certain anilines have placed them on Canada's National Pollutant Release Inventory (NPRI) list; as well as, the US Environmental Protection Agency's (US EPA) Toxics Release Inventory (TRI). Thus, the treatment of these chemicals is a major concern for industry.

Conventional treatment of anilines includes both physical and chemical methods such as, adsorption, extraction, microbial, and chemical oxidation, electrochemical techniques, and irradiation (Husain *et al.*, 2009). The problems with conventional treatments include high cost, low efficiency, and the formation of hazardous by-products (Husain *et al.*, 2009). In 1980, Klibanov *et al.* developed a new enzymatic method for the removal of aromatic pollutants from waste waters by peroxidases. Enzymatic treatment of anilines has been claimed or proposed as a preferential method of treatment over conventional treatment with microorganisms

(Nicell, 2003). Isolated enzymes can act with greater specificity and speed, are independent of bacterial growth and thus do not need acclimatizing time, can handle shock loads more readily, and are simpler to control (Mantha *et al.*, 2002; Taylor *et al.*, 1996). Overall, enzymatic treatment is viewed as a low energy chemical consumption process (Demarche *et al.*, 2012). Other studies have shown peroxidases to be successful in the treatment of anilines, including horseradish peroxidase (HRP) (Klibanov *et al.*, 1980) and bitter melon (*Momordica charantia*) peroxidase (Karim and Husain, 2009). Since the seed coats of soybeans are considered by-products, the hulls provide an inexpensive and abundant source of enzyme that could be commercially used in wastewater treatment (Nicell, 2003; Hailu *et al.*, 2010; Steevensz *et al.*, 2013).

Enzyme inactivation during treatment is one of the major disadvantages of enzymatic treatment, resulting in an increased enzyme demand and thus cost (Wu *et al.*, 1993). Klibanov *et al.* (1983) proposed that inactivation resulted 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 was a result of end-product polymer adsorption of enzymes, blocking substrate access to the active site. However, Feng *et al.* (2013) have recently shown that this phenomenon, with phenol as substrate, is not inactivation, but rather, immobilization in a form with lower specific activity.

Use of additives in enzymatic treatment has been shown to reduce the amount of enzyme needed (Nakamoto and

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Machida, 1992). Polyethylene glycol (PEG) was used in the treatment of phenol with HRP, and it reduced the amount of enzyme to 0.5% of that needed without the additive. Further studies on the effect of PEG on HRP removal of phenolic compounds were also successful (Wu *et al.*, 1993; Nicell *et al.*, 1995; Wu *et al.*, 1997). Similarly, the addition of PEG enhanced *Arthromyces ramosus* peroxidase (ARP) treatment of phenol (Ibrahim *et al.*, 2001) and laccase treatment of bisphenol-A (Modaressi *et al.*, 2005). However, it was shown to be marginally effective in the treatment of phenol using soybean peroxidase (SBP) (Caza *et al.*, 1999). Other additives shown to be effective in aiding enzymatic treatment include rhamnolipid, Triton X-100, Tween 20, sodium dodecyl sulfate (SDS), and NP-40 in the treatment of phenols by HRP (Tonegawa *et al.*, 2003); SDS, Tween 20, and Triton X-100 in the removal of phenol and 2-chlorophenol with SBP (Flock *et al.*, 1999); and SDS in the SBP-catalyzed removal of phenols in coal-tar wastewater (Al-Ansari *et al.*, 2010).

Another related application of peroxidases is their use for the controlled polymerization of aniline (e.g., Liu *et al.*, 1999), to produce a high molecular weight, electroactive, and conducting polyaniline complex in the presence of a polyelectrolyte template. The aniline monomer was electrostatically complexed to an anionic polyelectrolyte sulfonated polystyrene (SPS) template and then the enzymatic polymerization was initiated.

In this study, not template driven as in the preceding example, SBP treatment of aniline and *o*-anisidine was optimized for their removal from solution with respect to pH, H₂O₂ stoichiometry, SBP concentration and reaction time. The effect of additives, PEG, SDS, Triton X-100, and sodium dodecanoate (SDOD), on enzyme concentration and effluent quality was also investigated.

Materials and Methods

Materials

Aniline and *o*-anisidine (99% purity) were purchased from Sigma-Aldrich Chemical Company (Oakville, ON). Crude dry solid SBP (E.C. 1.11.7, Industrial Grade lot #18541NX, RZ = 0.75 ± 0.10, activity ≈ 5 U/mg) was obtained from Organic Technologies (Coshocton, OH). Dry solid bovine liver catalase (E.C. 1.11.1.6, lot #120H7060, activity ≈ 19,900 U/mg) was purchased from Sigma-Aldrich Chemical Company, Inc. The enzymes were stored at -15°C, while their substock solutions (70 U/mL for SBP and 17 U/mL of catalase) were stored at 4°C. Additives: sodium dodecyl sulfate (SDS), sodium dodecylbenzenesulfonate (SDBS), and polyethylene glycol (PEG, average molar mass of 3350 g/mole) were purchased from Sigma-Aldrich. Triton X-100 was purchased from Alphachem (Mississauga, ON). SDOD (sodium dodecanoate) (99–100% purity) was purchased from Sigma-Aldrich. Reagents: 2,4,6-trinitrobenzenesulfonic acid solution (TNBS) (1.0 M in H₂O) was purchased from Sigma-Aldrich and stored at -15°C. 4-AAP (4-amino-antipyrine) was obtained from BDH, Inc. (Toronto, ON) and stored at room temperature. Hydrogen peroxide (30% w/v) was purchased from ACP Chemicals, Inc. (Montreal, PQ) and stored at 4°C. Buffers: Analytical-grade monobasic and dibasic sodium phosphate, glacial acetic acid, and sodium acetate were purchased from ACP Chemicals.

Two spectrophotometers were used to quantify anilines through colorimetric methods. One was a Hewlett-Packard

(model 8452A) Diode Array Spectrophotometer (λ range of 190–820 nm and 2 nm resolution). The other was an Agilent 8453 UV-Visible spectrophotometer (λ range of 190–1100 nm and 1 nm resolution).

Methods

Anilines colorimetric assay. A TNBS test as per Wang *et al.* (2005) was used to measure both parent anilines. The reaction of anilines with TNBS in the presence of a phosphate buffer of pH 7.4 and sodium sulfite generated a yellow chromophore. Samples were made in a 1-mL cuvette and included 100 μ L of 10 mM TNBS, 100 μ L of 0.2 M phosphate buffer, 100 μ L of 20 mM sodium sulfite, and 700 μ L between sample and water volumes. The UV-VIS spectrophotometer was used to measure color at a wavelength of 430 nm, color development time for aniline was 30 min and that for *o*-anisidine was 50 min.

SBP activity assay. Catalytic activity of SBP was measured through a colorimetric assay as per Wu *et al.* (1997). The assay included a reagent which used 10 mM phenol, 40 mM phosphate buffer (pH 7.4), 2.4 mM 4-aminoantipyrine (4-AAP), and 0.2 mM H₂O₂ in a total volume of 950 μ L. The reagent was added to 50 μ L of diluted SBP in the cuvette to provide proper mixing. The initial rate of color formation in the first 30 s was monitored at $\lambda_{\text{max}} = 510$ nm by the UV-VIS spectrophotometer. One unit of activity is defined as the number of micromoles of H₂O₂ converted per minute at pH 7.4 and at room temperature.

Total organic carbon analysis. Total Organic Carbon (TOC) analysis was done on batch reactor experiments involving parent aniline compounds. Twenty microliter samples were collected and microfiltered. The machine was allowed to start up and run with three milli-Q water injections to make sure the system was properly purged. Each sample was then measured for both Total carbon (TC) and Inorganic carbon (IC) with the difference being TOC. Three injections were used for each reading and the average was recorded.

Experimental protocol

All experiments were conducted in batch reactors in triplicate at room temperature. The average values of these three readings are presented, with error bars representing the standard deviation.

Enzymatic treatment of anilines. Batch reactors were set up to optimize SBP treatment of both aniline and *o*-anisidine for 95% removal. Parameters investigated were as follows: pH, H₂O₂ concentration, enzyme concentration, and reaction time. All experiments were conducted at room temperature in 20 mL glass vials with each batch receiving 1 mM substrate. Acetate or phosphate buffers (40 mM) were used to cover the pH range of 3.5–7.5. H₂O₂ concentration was varied from 0.5 to 3 mM. SBP concentrations depended on the substrate. A starting point of 0.17 U/mL was used for anilines and was changed by increasing or decreasing the concentration to achieve 95% removal. A Teflon-coated magnetic stir bar was placed in each mixture and the vials were then placed on a magnetic stirrer to allow for thorough and continuous mixing.

All reactions were run for 3 h and stopped by adding 100 μL of catalase stock solution, which broke down H_2O_2 to water and oxygen. The samples were then microfiltered and analyzed by TNBS test. Once various parameters were optimized for 95% removal conditions, experiments were conducted on enzymatic treatment of anilines to determine reaction time effect and kinetics by monitoring anilines removal over a 3-h period.

Additive effect. Batch reactors were run to determine the effect of additives on enzymatic treatment. Various additives in a range of 10–200 mg/L were added to 20 mL glass vials as in the previous section and optimized and analyzed accordingly.

Total organic carbon. Batch reactors (25 mL) were run in tap water with 1 mM substrate, optimal H_2O_2 to substrate ratio, optimal SBP concentration, and optimal additive concentration determined in the previous experiments. No buffer was added and the pH was not adjusted, the pH of the water was approximately 7.0. The reaction was stopped after 3 h, the batch samples were then microfiltered and analyzed for aniline remaining and TOC remaining.

Results and Discussion

This study demonstrated the feasibility of SBP catalyzed removal of aniline and *o*-anisidine, while the addition of certain additives was able to decrease enzyme demand and improve quality of the final effluent. The optimal conditions to remove 95% of anilines were determined with and without the additives.

pH effect

SBP is known to be active over a wide range of pH, but since this study relates to wastewater treatment, it was decided to choose a pH range of 3.5–7.5 for this study. Batch reactors were run for 3 h with an initial substrate concentration of 1 mM, H_2O_2 concentration of 1.5 mM, and an enzyme concentration of 0.17 U/mL for aniline and 0.0035 U/mL for *o*-anisidine, respectively. The experiments were designed to test the effect of pH in the presence of limited SBP so that stringent conditions were created to clearly determine the pH effect. SBP showed a catalytic activity in the entire pH range studied. An optimal pH of 5.0 was observed for the removal of both aniline and *o*-anisidine, with acceptable removal in the range of 4.5–5.5, (shown in Fig. 4 later). The removal of *o*-anisidine was more affected by pH variation than the removal of aniline. There was 20% difference in the removal efficiency between optimal pH and the worst-case pH for aniline and 50% difference in the case of *o*-anisidine removal.

SBP concentration

Experiments were conducted for a 3-h duration at the previously established optimal pH of 5.0 by varying SBP concentration to determine the optimum SBP concentration (the minimum amount required for 95% removal). All other variables were held constant: H_2O_2 at 1.5 mM and substrate at 1.0 mM. Results showed 95% removal of aniline at an SBP concentration of 0.6 U/mL, while the removal of 95% of

FIG. 1. SBP optimization for removal of 1 mM aniline, in presence of 1.5 mM H_2O_2 at pH 5.0, reaction time = 3 h. Solid lines drawn simply connect the data points in a smooth manner. SBP, soybean peroxidase.

o-anisidine required 0.012 U/mL. Figure 1 shows the results for aniline (*o*-anisidine was studied analogously). It was also observed visually in both cases that, as the enzyme concentration was increased, the color intensity of the solution also increased, with aniline turning reddish brown and *o*-anisidine a purplish hue. At the optimal SBP concentration, precipitates were present in both samples; however, the *o*-anisidine-treated sample precipitates were finer and did not settle as well.

H_2O_2 effect

Peroxidase catalytic reaction stoichiometry indicates a 1 to 2 molar ratio of H_2O_2 to substrate. However, previous studies have shown that it is actually closer to 1:1 (Taylor *et al.*, 1998; Ibrahim *et al.*, 2001), while excess H_2O_2 can be limiting (Nicell, 1991). For this reason, it was important to study the effect of H_2O_2 concentration on the reaction.

SBP showed a catalytic activity in the full range of H_2O_2 concentrations studied (0.5–3 mM) for both aniline and *o*-anisidine. Figure 2 shows the results for *o*-anisidine (aniline was studied analogously, SBP range 0.17–0.70 U/mL). It is observed that the amount of H_2O_2 required increased with the amount of SBP added and the percent of substrate removed; thus, H_2O_2 demand increased with enzyme activity. At optimal conditions, the amount of H_2O_2 required for the treatment of 1 mM aniline with 0.6 U/mL SBP was 1.5 mM. In the case of *o*-anisidine, optimal treatment of 1 mM *o*-anisidine with 0.012 U/mL SBP was achieved at 1.25 mM H_2O_2 . At higher H_2O_2 concentrations, the percent substrate removal had decreased, indicating inactivation of SBP by excess H_2O_2 . The extra H_2O_2 consumption over theoretical stoichiometric requirement is attributed to its consumption by dimeric and polymeric compounds produced in the reaction, as stated by Yu *et al.* (1994). This consideration predicts an overall 1:1 substrate to H_2O_2 ratio. Biswas (1999) had stated that this increase in demand higher than a peroxide to substrate ratio of 1 could be the result of catalase activity, which is found in all plants, which can accompany the SBP catalytic reaction. Catalase decomposes hydrogen peroxide

FIG. 2. Effect of H_2O_2 concentration on removal of 1.0 mM *o*-anisidine by SBP, at pH 5.0, reaction time=3 h. Solid lines drawn simply connect the data points in a smooth manner.

to oxygen and water. Another reason for the increase in demand could be a result of hydrogen peroxide oxidation of other organic matter present in the crude SBP mixture.

Additive effect on SBP treatment of aniline and *o*-anisidine

This experiment was set up to study the effect of different additives on the removal of aniline. The additives chosen were PEG, a hydrophilic synthetic polymer; SDS, an anionic surfactant; Triton X-100, a nonionic surfactant; and SDOD, an anionic surfactant fatty acid salt. Batch reactors were set up at pH 5.0 with 1 mM aniline, 0.3 U/mL SBP, and H_2O_2 ranging from 0.5 to 2.25 mM. The optimal H_2O_2 concentration was 1.5 mM (data not shown). The concentrations of the additives were 100 mg/L except for SDOD where only 50 mg/L was used because of its low solubility. It can be seen in Fig. 3 that PEG had no effect on the SBP treatment of aniline, this is similar to findings by Patapas *et al.* (2007), in

FIG. 3. Effect of additives on treatment of 1 mM aniline with 0.3 U/mL SBP at pH 5.0. Solid lines drawn simply connect the data points in a smooth manner.

which they found the addition of PEG had no effect on *Arthromyces ramosus* peroxidase treatment of aniline, as well as a study by Steevensz *et al.* (2012) that found no PEG effect in the treatment of aniline with laccase. All other additives showed improvement of enzymatic treatment after their addition, with SDOD having the most significant impact. The only additive that also improved the quality of the treated water by removing color and improved floc settling was SDS. This is likely an electrostatic effect since the poly(aniline) would be cationic to some extent at pH 5 (an emeraldine salt).

The optimal amount of additive required is considered to be the lowest concentration added to achieve the optimal results. Batch experiments were set up at substrate concentration of 1 mM, pH 5.0, and 1.5 mM H_2O_2 and various additives in the range of 0–200 mg/L. For aniline, SBP was added at 0.3 U/mL to the batch reactors that contained SDS and Triton X-100, while 0.2 U/mL SBP was added to the samples with SDOD based on the results of previous study. The results showed that 100 mg/L of SDS or Triton X-100 and 50 mg/L of SDOD achieved the respective optimal additive effect, Fig. 4.

Similar experiments were carried out for *o*-anisidine under stringent conditions at pH 5.0 with 1 mM *o*-anisidine, 1.25 mM H_2O_2 , 0.007 U/mL SBP, and a range of additive concentrations (0–200 mg/L). Of the three additives, only SDS significantly improved SBP treatment of *o*-anisidine, starting at 50 mg/L (data not shown). However, the best color removal and precipitate formation and settling were achieved with the addition of 100 mg/L of SDS.

Additive effect on pH. The effect of an additive on optimal pH in enzymatic treatment of aniline was studied by adding Triton X-100 or SDS. Experiments were run under stringent conditions, keeping all parameters constant except for pH. Only 0.2 U/mL SBP was added along with 100 mg/L of additive where applicable. Triton X-100 did not change the pH profile and the optimal remained in the pH range of 4.5 to 5.0 as without Triton X-100 (data not shown). However, the pH effect was more pronounced with SDS addition with a slightly narrower optimal range of 4.5–5 (Fig. 5). Any change above or below this range caused significant

FIG. 4. Additive effect on SBP removal of aniline. Solid lines drawn simply connect the data points in a smooth manner.

FIG. 5. Sodium dodecyl sulfate additive effect on pH optimization in SBP-catalyzed removal of 1 mM substrate. Solid lines drawn simply connect the data points in a smooth manner.

decrease in substrate removal. The experiment was repeated for *o*-anisidine with the addition of 0.0035 U/mL SBP and 100 mg/L of SDS. The results, Fig. 5, showed an optimal pH range of 6.5–7.0, however, a pH of 6.0 was chosen as an optimal because it gave better color removal and precipitate formation.

Additive effect on H_2O_2 and required SBP concentrations. After having determined that certain additives were able to improve the treatment, it was decided to study the quantitative effects of these additives on SBP treatment of aniline and *o*-anisidine. Experiments were conducted to first find the optimal SBP concentration and then optimal H_2O_2 concentration in the presence of these additives. For optimal SBP, parameters were set at optimal pH and H_2O_2 concentration. The results showed the extent of treatment enhancement by additives: with Triton X-100, only two-thirds of SBP was needed; with SDS, the amount of SBP needed was reduced by half; and with SDOD, less than half of SBP was needed when compared to the SBP required in the absence of additives. Next, the H_2O_2 was optimized. The results indicated that, for 95% aniline removal, a H_2O_2 to substrate molar ratio of 1.5 was needed regardless of how much enzyme was reduced. Meanwhile, in the treatment of *o*-anisidine, a 12% reduction in H_2O_2 demand was achieved, whereas SBP decreased from 0.012 to 0.007 U/mL due to the presence of additive. Table 1 summarizes these results for both substrates.

FIG. 6. Effect of SDS addition on enzyme catalytic life. Initially, 1 mM aniline in presence of 100 mg/L SDS with 1.5 mM H_2O_2 , at pH 5.0 and reaction time = 3 h, 6 h. Solid lines drawn simply connect the data points in a smooth manner. SDS, sodium dodecyl sulfate.

Additive effect on reaction time. To study the effect of reaction time, experiments were set up at optimal conditions for the treatment of aniline and *o*-anisidine with SBP and various additives. Samples were taken at various time intervals during the 3-h reaction. While the presence of additives decreased the amount of SBP required to achieve 95% aniline removal, it also decreased the initial rate of reaction. The reaction in the presence of SDS showed the slowest initial reaction rate followed by Triton X-100 and SDOD. The treatment of *o*-anisidine in the presence of SDS also was slower than that without the additive. However, toward the end of the reaction, the rate was faster than the control, allowing 95% removal to be achieved within the 3-h reactions.

Next, experiments were conducted to determine how the concentration of SDS changed the optimal reaction time in the enzymatic treatment of aniline. Batch reactors were set up under stringent conditions with a range of SDS concentration from 0 to 225 mg/L, 1 mM aniline, 1.5 mM H_2O_2 , and 0.2 U/mL SBP. Samples were taken after 3 and 6 h and analyzed for percent aniline remaining. Results in Fig. 6 clearly show that SDS addition improved enzymatic treatment of aniline. With no SDS addition, an extra 3 h past the original 3-h reaction time gave only an additional 3% aniline removal, while with SDS at 100 mg/L an additional 11% of aniline reduction was achieved. This indicates that the addition of SDS protects the enzyme and increases enzyme's catalytic life. This was investigated by Flock *et al.* (1999) in the treatment of phenols with SBP, in which they showed that surfactants (SDS and Triton X-100) significantly increased the enzyme activity.

TABLE 1. ADDITIVE EFFECT ON SBP TREATMENT OF 1 mM ANILINES FOR 95% REMOVAL

Substrate	Additive	Dose mg/L	pH	[SBP] U/mL	$[H_2O_2]:[substrate]$ (mM:mM)
Aniline	None	0	5.0	0.60	1.50
	SDS	100	5.0	0.30	1.50
	Triton X-100	100	5.0	0.40	1.50
	SDOD	50	5.0	0.28	1.50
	None	0	5.0	0.012	1.25
<i>o</i> -Anisidine	SDS	100	6.0	0.007	1.10

SBP, soybean peroxidase; SDS, sodium dodecyl sulfate; SDOD, sodium dodecanoate.

FIG. 7. Total Organic Carbon of SBP-treated aniline and *o*-anisidine under optimal condition with no buffer.

Additive fate in treatment - TOC study. The use of an additive in enzymatic treatment has to take into consideration its possible negative effect on the environment if the excess amount is released with the effluent. TOC was chosen as a measure of the removal of organic content from the solutions. Batch reactors were run and analyzed for aniline and TOC remaining. Experiments, in which aniline was the substrate showed that with no additive, the amount of TOC observed, was comparable to the calculated amount, which indicated that all the enzymatically treated aniline indeed formed polymers that precipitated out of the solution, Fig. 7. The results also indicated that some of the added SDS and SDOD had been removed during the reaction since the TOC observed was less than that calculated. With Triton X-100, the TOC results showed the observed TOC value was close to the calculated value, which suggests that it remained in the solution.

In the treatment of *o*-anisidine with SBP, the amount of TOC observed was higher than that calculated for all the samples except the one with SDS, indicating that the polymers produced in SBP treatment of *o*-anisidine remained in solution, Fig. 7. The addition of SDS improved precipitate formation and, unlike the samples without additives and those with Triton X-100 and SDOD, the observed TOC was equal to the calculated TOC. This suggests that SDS addition may have precipitated all the produced floc; however, no information was collected about the fate of the additive itself. As speculated earlier, this beneficial effect of SDS may be an electrostatic effect since the poly(aniline) formed is likely to be cationic to a certain extent, hence flocculation is enhanced by the anionic SDS. It is, however, not clear what the mechanism of SDS protection is, whether it complexes with the enzyme or the poly(aniline) products—this would be a matter for future study.

Conclusions

Results of this study demonstrated the ability of SBP to treat both aniline and *o*-anisidine. Additives enhanced treatment by decreasing enzyme demand, while SDS in particular decreased the required SBP concentration by half and had the additional benefit of improving precipitate formation, settling, and color removal, while slowing initial reaction rate

and increasing SBP catalytic life. Thus, the addition of SDS was found to be superior to the other additives studied, while PEG had no effect on the treatment. The presence of SDS also affected the optimal pH range, with a change in pH having more significant impact on removal than without SDS. From TOC studies, it was concluded that, while the addition of SDS improved precipitation and better settling of products, its addition would contribute to effluent TOC.

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Author Disclosure Statement

No competing financial interests exist.

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