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CHEM-571

Horseradish Peroxidase Assays

HRP-AAP Assay

Horseradish peroxidase catalyzes the oxidation of 4-aminoantipyrine. The reaction was monitored in the UV-visible range with a spectrophotometer. A graph of absorbance versus time of the color change in the assay solutions can be used to determine the activity rates of HRP. The protocol for the HRP-AAP assay was found on Dropbox [1]. However, the lone deviation from protocol was that 4-iodophenol was dissolved in DMSO instead of water, for reasons listed later in the report.

The spectrophotometer was set to detect absorbance at 510 nm and temperature-controlled to 25°C $\pm$ 0.1°C. The spectra were collected over a time period of 300 s per assay solution. These settings were not changed for the duration of the experiment. It was also determined that the optimal assay solution volume for absorbance measurements was slightly more than 1.5 mL, when using a 3 mL cuvette (Table 1). These volumes were not varied.

Table 1: Volumes of Components of HRP-AAP Assay Solutions

Solution	Volume [µL]
4-Iodophenol	10
AAP	700
H_2O_2	750
HRP	50

The concentrations of 4-iodophenol and HRP remained constant at 18 mM and 2.3 µM, respectively, for each trial. The assay solutions were then split into two categories, in which one set of solutions had a constant concentration of H_2O_2, while the concentration of AAP was varied. The other set of assay solutions had a constant concentration of AAP and the concentration of H_2O_2 was varied (Table 2). Note that HRP was added to the cuvette immediately before absorbance measurements were taken in order to initiate the reaction.

Table 2: Initial Concentrations of Components of HRP-AAP Assay Solutions

Trial	4-Iodophenol [mM]	AAP [mM]	H_2O_2 [mM]	HRP [µM]
1	18	2.5	1.7	2.3
2	18	2.5	0.850	2.3
3	18	2.5	0.425	2.3
4	18	2.5	0.213	2.3
5	18	2.5	0.106	2.3
6	18	2.5	1.7	2.3
7	18	1.25	1.7	2.3
8	18	0.625	1.7	2.3
9	18	0.313	1.7	2.3
10	18	0.156	1.7	2.3

Two final graphs of absorbance over time were generated. One shows the absorbance of fixed [AAP] and varied [H_2O_2] (Figure 1) and one shows the results of fixed [H_2O_2] and varied [AAP] (Figure 2). Note that the absorbance of only the sodium phosphate buffer was included in both graphs to

demonstrate that it does not absorb at 510nm and did not affect the absorbance of the other components of the analyzed solutions.

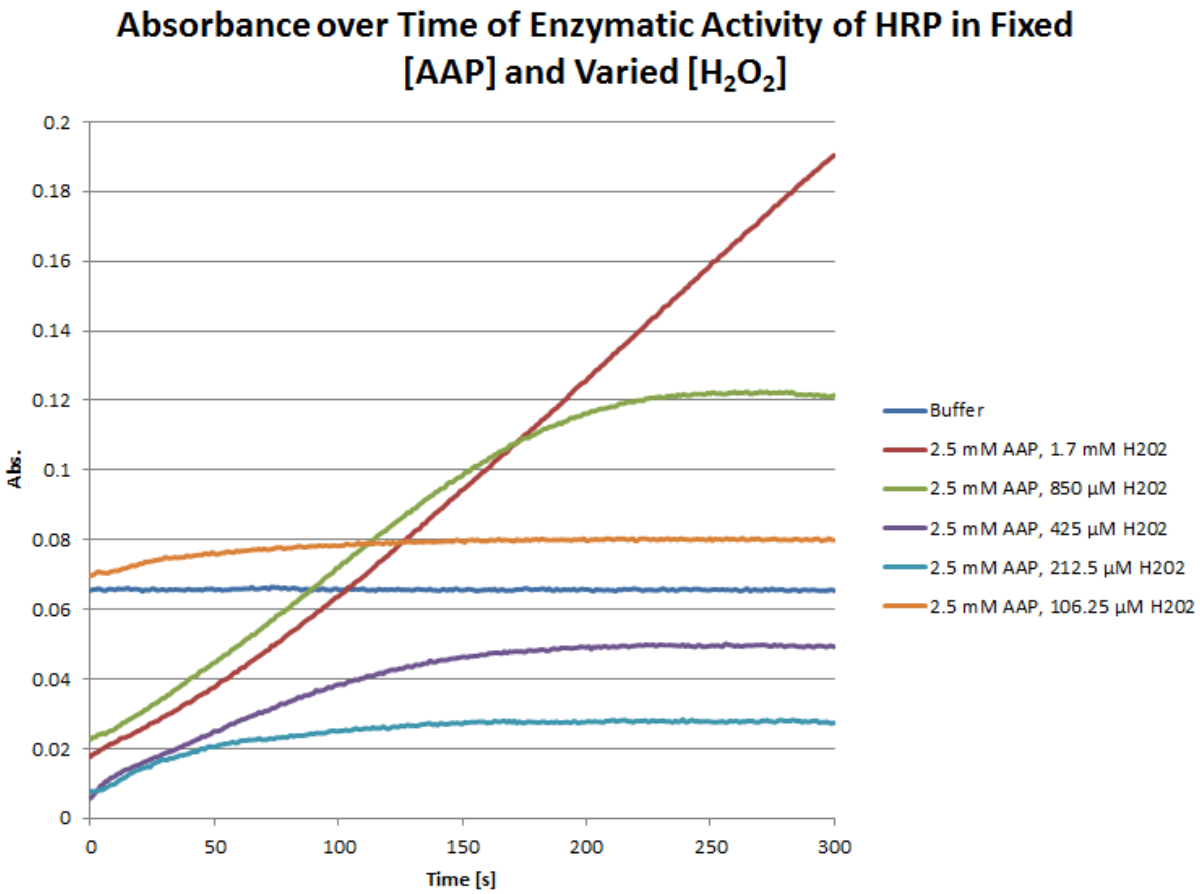

Figure 1: Absorbance over time of enzymatic activity of HRP in fixed [AAP] and varied [H_2O_2] at 510nm. The concentration of HRP in all solutions was 2.3 µM and the signals of all solutions were amplified by adding 10 µL of 18 mM 4-iodophenol in DMSO. The concentration of AAP was fixed at 2.5 mM, while the concentration of H2O2 was varied between 1.7 mM to 106.25 µM. For every 0.75 mL of H2O2 solution, 0.7 mL of AAP solution was added, for a total volume, including that of the 4-iodophenol solution, of about 1.46 mL in the cuvette.

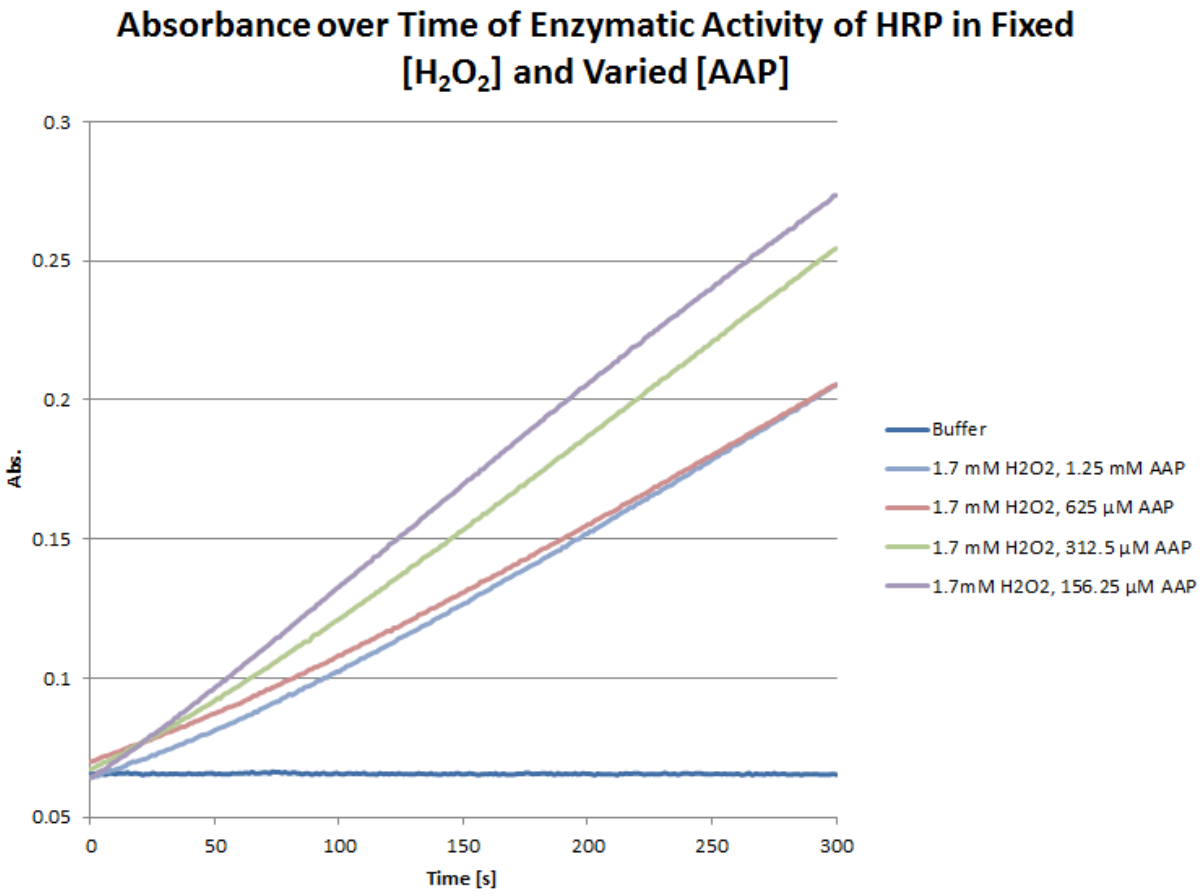

Figure 2: Absorbance over time of enzymatic activity of HRP in fixed [H_2O_2] and varied [AAP] at 510nm. The concentration of HRP in all solutions was 2.3 µM and the signals of all solutions were amplified by adding 10 µL of 18 mM 4-iodophenol in DMSO. The concentration of H2O2 was fixed at 1.7 mM, while the concentration of AAP was varied between 1.25 mM to 156.25 µM. For every 0.75 mL of H2O2 solution, 0.7 mL of AAP solution was added, for a total volume, including that of the 4-iodophenol solution, of about 1.46 mL in the cuvette.

HRP-LUMINOL ASSAY

Horseradish peroxidase catalyzes the oxidation of luminol by H_2O_2. The resultant luminescence, enhanced by adding 4-iodophenol to the solution, is monitored with a fluorimeter. A graph of intensity over time of the luminescence produced by the reaction can be used to determine the activity rates of HRP.

Initial experiments with the HRP-luminol assay were based on the luminescence procedure found on Dropbox [2]. Deviations from the protocol outlined on OpenWetWare are as follows:

- Luminol was found to be immiscible in water, so sodium carbonate was added to the solution to dissolve it completely. Then sodium bicarbonate was added to adjust the pH to 7.
- 4-Iodophenol was also found to be immiscible in water, so it was dissolved in DMSO instead. Note that the amount of DMSO in the assay solution was dilute enough that it did not affect the reaction and luminescence.

The fluorimeter was set to produce a TimeDrive spectrum over a period of 300 seconds. The excitation wavelength was 350 nm and the emission wavelength was 430 nm. The excitation slit width

was 15 nm and the emission slit width was 20 nm. These settings were not changed for the duration of the experiment. It was also determined that the optimal solution volume for fluorescence measurements was slightly less than 2 mL. This total volume determined the amounts of solutions added to the 3 mL cuvette (Table 3). These volumes were not varied. Note that the purpose of adding 4-iodophenol to the assay solution was to enhance the luminescence of the oxidation of luminol such that it can be detected by the fluorimeter.

Table 3: Volumes of Components of the HRP-Luminol Assay solution

Solution	Volume [µL]
4-Iodophenol	13.2
Luminol	920
H_2O_2	990
HRP	66

Table 4: Initial and Final Concentrations of Components of Some HRP-Luminol Assay Solution

	Initial Concentrations [mM]				Final Concentrations [mM]			
Trial	4-Iodophenol	Luminol	H_2O_2	HRP	4-Iodophenol	Luminol	H_2O_2	HRP
1	18	1.25	1.7	0.0023	0.000119	0.000578	0.000846	7.63121E-08
2	18	0.625	1.7	0.0023	0.000119	0.000289	0.000846	7.63121E-08
3	18	0.625	0.85	0.0023	0.000119	0.000289	0.000423	7.63121E-08
4	18	1.25	0.425	0.0023	0.000119	0.000578	0.000212	7.63121E-08
5	18	1.25	1.7	0.0023	0.000119	0.000578	0.000846	7.63121E-08
6	18	1.25	1.7	0.00115	0.000119	0.000578	0.000846	3.8156E-08

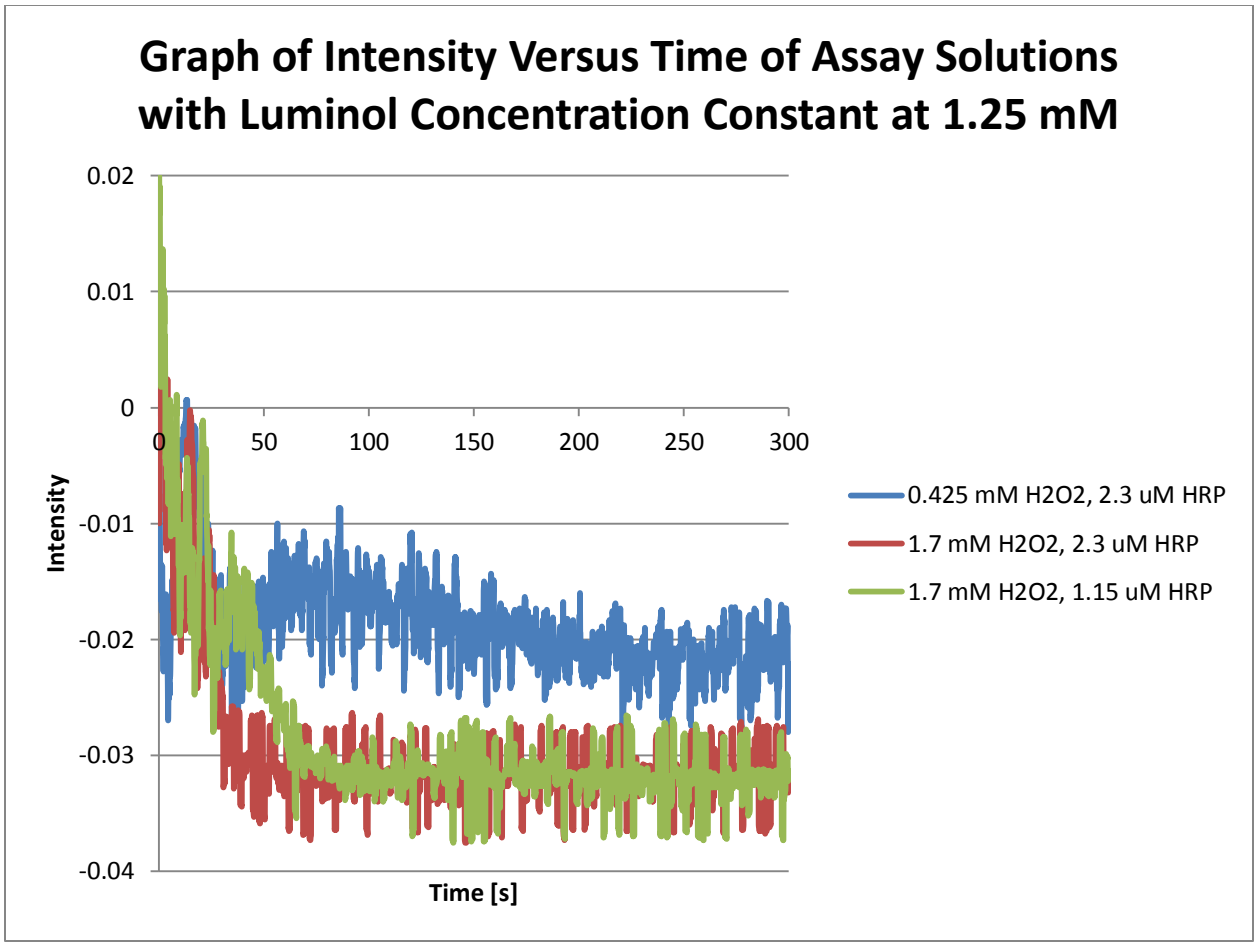

Figure 3: Graph of intensity of 4-iodophenol-enhanced solutions of luminol and H_2O_2 with horseradish peroxidase. The concentration of 4-iodophenol was constant at 18 mM and that of luminol was constant at 1.25 mM for these trials.

The original concentrations of the solutions of luminol, H_2O_2, and HRP were found to give meaningless results, so they were diluted and then recombined in various assay solutions (Table 4). From the spectra taken for assay solutions containing 2.3 µM HRP, it was hypothesized that the reaction proceeded too quickly and the luminescence did not last long enough, nor was it intense enough, to be sufficiently monitored within 300 s. For example, the spectra in Figure 1 of 0.425 mM H_2O_2 and 2.3 µM HRP shows a small peak of luminescence intensity at around 55 s, which then declines gradually. When the trial was repeated with H_2O_2 at 1.7 mM instead, the luminescence peaked quickly at around 15 s, then declined rapidly. The spectra of the assay solution containing 1.7 mM H_2O_2 and 1.15 µM HRP showed a luminescence peak of about the same intensity, but with a slightly less rapid decline (Figure 3).

The spectra taken throughout the experiment also exhibited a negative baseline. While this usually would indicate that no luminescence was produced, a blank of sodium carbonate/sodium bicarbonate buffer also produced a negative baseline (Figure 4), proving that some luminescence was indeed being produced. In addition, the HRP concentration was further varied in order to slow the reaction rate so that the reaction progress could be better observed with the fluorimeter.

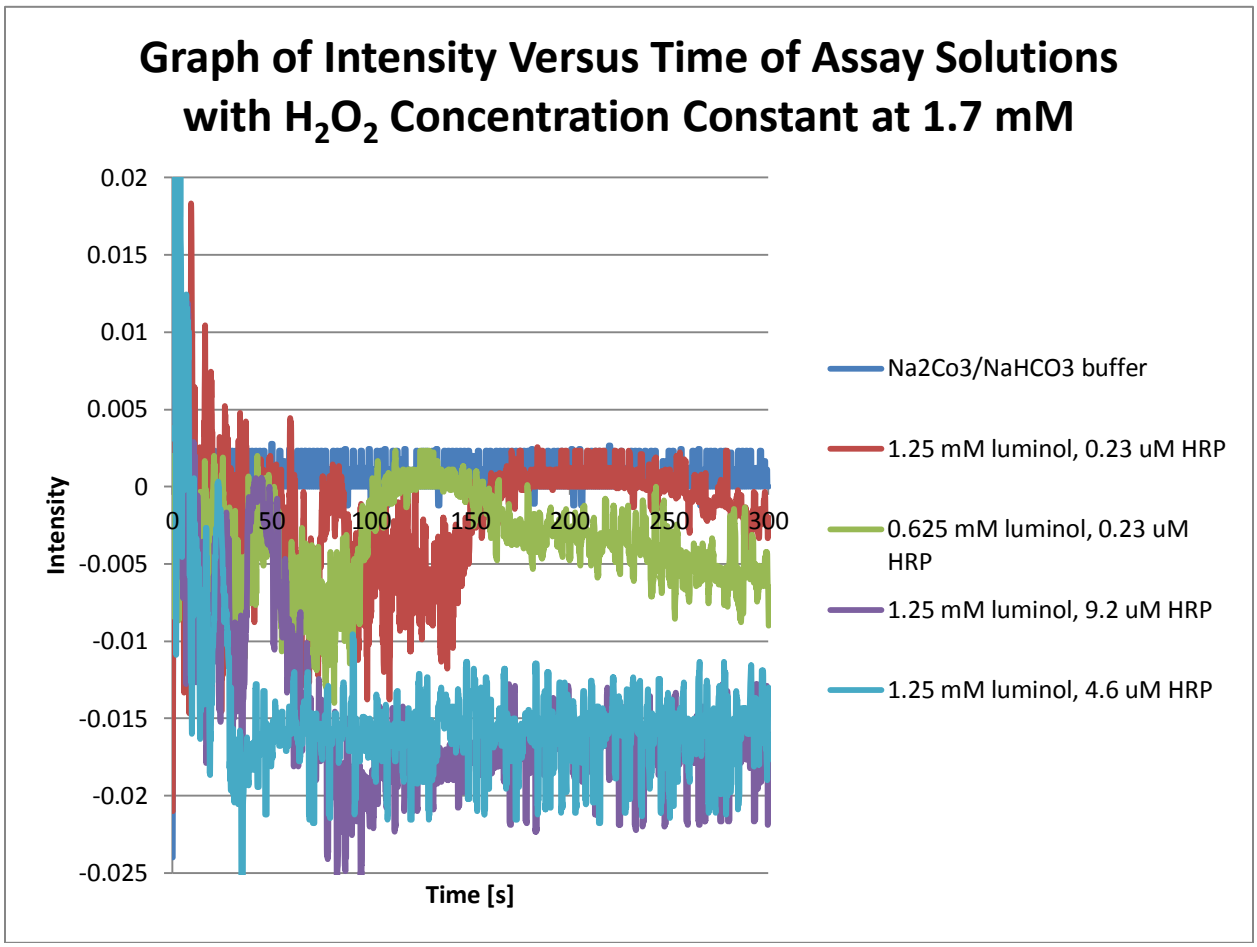

Figure 4: Graph of intensity of 4-iodophenol-enhanced solutions of luminol and H_2O_2 with horseradish peroxidase. The concentration of 4-iodophenol was constant at 18 mM and that of H_2O_2 was constant at 1.7 mM for these trials.

It was expected that as HRP concentration increases, so does the reaction rate and luminescence. However, the spectrum for 1.25 mM luminol and 9.2 µM HRP showed peak luminescence intensity at around 40 s, while the spectrum for 1.25 mM luminol and 4.6 µM HRP had a peak at 20 s (Figure 4). These nonsensical results may be a result of the diffusion of HRP as it is added to the assay solution. Since luminescence must be measured immediately afterward, the assay solution containing HRP was not mixed, and the uneven distribution of HRP due to the small volume added may have caused these differences in intensity peaks. Ultimately, lower concentrations of HRP appear to be most successful, as luminescence reaches peak intensity at later times in these assays solutions (1.25 mM luminol, 0.23 µM HRP, and 0.625 mM luminol, 0.23 µM HRP). It was also peculiar that the assay solutions were registering negative intensity, even with the blank (Na_2CO_3/$NaHCO_3$) solution, which had intensity values of around 0.001 to -0.001. Thus it was hypothesized that there may be a lag between the initialization of the fluorimeter on the computer and when the fluorimeter detector is actually activated.

Another study of HRP-luminol assays indicated that the optimal pH for the reaction is 8.5 and that the optimal mole ratio of H_2O_2 to luminol is 2.2 [3]. Although the pH of the reaction was not adjusted to 8.5, it was concluded that H_2O_2 solutions should be significantly more concentrated than those of luminol. As the various combinations of solution concentrations were analyzed, it was

determined that the optimal concentration of H_2O_2 was 1.7 mM and that of luminol was around 0.625 to 1.25 mM. Recommendations for future experiments, based on the problems discussed above are to:

- Begin the reaction by adding H_2O_2 instead of HRP. This implies that the HRP should be evenly dispersed in the assay solution and may give more consistent luminescence intensity values. In addition, the larger volume of H_2O_2 added to the solution may ensure better mixing of components. Note that the HRP-luminol reaction does not occur without H_2O_2, so HRP will not react with luminol in the assay solutions before H_2O_2 is added.
- Activate the fluorimeter detector before beginning the reaction. This may be done with the laboratory lights turned off. Since luminescence appears to occur almost immediately after the reaction is started by the addition of HRP, the lag discussed above certainly influences the spectra produced. If the detector is already turned on, then the lag will be eliminated and more accurate spectra may be obtained.
- Conduct the assay at pH 8.5. The activity of HRP may depend on solution pH, in that the binding of HRP to its substrate may occur more quickly or more slowly at various pHs. As stated above, literature has indicated that its optimal pH is 8.5. It is possible that at this pH, the assay solutions may produce more intense and uniform luminescence.

References

1. http://www.goldbio.com/pdf/10106-Protocol%201.pdf
2. Kricka, L.J. Chemiluminescent and bioluminescent techniques. *Clin. Chem.* **1991**, 37, 1472-1481.
3. Bhandari, A.; Wongee, K.; Hohn, K. Luminol-based enhanced chemiluminescence assay for quantification of peroxidase and hydrogen peroxide in aqueous solutions: Effect of reagent pH and ionic strength. *J. Environ. Eng.* **2010**, 136, 1147-1152.