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Absorption/Adsorption Properties of Peroxidase-Catalyzed Polyphenolic Precipitates Using 4-(Phenylazo) Benzoic Acid as a Model Sorbate

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By

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“Absorption/Adsorption Properties of Peroxidase-Catalyzed Polyphenolic Precipitates Using 4-(phenylazo) Benzoic Acid as a Model Sorbate”

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ABSTRACT

The adsorption or absorption of 4-(phenylazo)benzoic acid onto polyphenolic precipitates shows promise as a model system for adsorption and absorption of hydrophobic waste compounds. Using 1 mM of phenol under conditions used for enzyme conversion with soybean peroxidase and hydrogen peroxide (1 U/mL soybean peroxidase and 1.5 mM hydrogen peroxide) two conditions of ad/absorption were characterized with the Langmuir isotherm. These conditions, hereby referred to as static and dynamic, consist of ad/absorption onto phenolic precipitates either during enzymatic conversion of phenol (dynamic) or after enzymatic conversion of phenol (static). Both the dynamic and static systems showed high affinity for phenolic precipitates with Langmuir association constants of 0.088 and 0.13 L/mg, respectively. The dynamic system showed a 3-fold greater maximum ab/adsorption capacity than the static system, 51 and 16 mg/g, respectively. During the characterization process the pKa of 4-(phenylazo)benzoic acid was determined to be 2.45 and the possibility of enzyme-catalyzed reductive splitting of the azo-bond was studied. The characterization of the ad/absorption of 4-(phenylazo)benzoic acid onto phenolic precipitates showed good fit with the Langmuir isotherm. This opens the possibility of characterization of other adsorption systems with phenolic precipitates for the purpose of expanding the scope of the SBP enzymatic process as a waste-water treatment method beyond its direct substrates.

DEDICATION

To my family thanks for all the support you've given me throughout these years.

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Azo – 4-(phenylazo)benzoic acid

PABA – *para*-aminobenzoic acid

SBP – soybean peroxidase

HRP – horseradish peroxidase

HPLC – high performance liquid chromatography

BTEX – benzene, toluene, ethylbenzene and xylenes

CP6R - crystal ponceau 6R

HOBT – hydroxybenzotriazole

BET - Brunauer–Emmett–Teller

GAC – granular activated carbon

Chapter 1

1. Introduction:

Enzymatic methods have been developed in recent decades to treat wastewater as a complement to or replacement of conventional methods. One class of enzyme that has been extensively investigated is oxidative enzymes, primarily peroxidases and laccases. Soybean peroxidase (SBP) is a peroxidase that can be used as a removal agent to "clean out" organic materials from water that are substrates of SBP through oxidative means. Many studies have shown that SBP is an effective treatment method for the removal of phenols and anilines from wastewater by causing the substrate to form oligomers and polymers of sufficient size to precipitate out of the solution. A limitation of SBP as a wastewater treatment method is that it can only be used for its substrates. Many toxic compounds that are found in wastewater are hydrophobic in nature and only sparingly soluble in water. For example, the solubility of toluene, one of the BTEX group compounds which are highly toxic to humans and part of industrial waste, is 0.52 g/L[2]. Toluene is not a substrate of SBP and therefore cannot be removed from wastewater through the enzymatic process. The enzymatic process for phenol however, does create a polymer resin to which organic compounds like toluene could adhere. Previous studies were done using BTEX group compounds to show this adsorption onto the resin formed by SBP-catalyzed reaction of phenol. The BTEX group of compounds proved too volatile for reliable analysis[32] and so a model compound was chosen to characterize this interaction. The model compound 4-(phenylazo)benzoic acid was chosen because it was not considered a substrate of SBP and it is relatively hydrophobic.

1.1 BTEX Group Compounds:

BTEX group compounds (benzene, toluene, ethylbenzene and xylenes) are by-products of many industrial processes and they pose a serious problem due to their high water solubility. Benzene can be found in gasoline, toluene is used as a paint solvent and is commonly found in petroleum products, ethylbenzene is used as a gasoline fuel additive and xylenes are found in gasoline and used as industrial solvents[2]. Some physical properties of these compounds are shown in Table 1-1.

Table 1-1: Physical properties of the BTEX group compounds (benzene, toluene, ethylbenzene and xylenes).

	Benzene	Toluene	Ethylbenzene	Xylene	Reference
Density (g/mL)	0.8765	0.8669	0.8670	0.8685	2
Polarity	Non-polar	Non-polar	Non-polar	Non-polar	2
Water Solubility (g/L)	1.78	0.52	0.15	0.15	2

Exposure to BTEX group compounds in gasoline has been linked to skin irritation, dizziness, headache, sleepiness and loss of coordination in the short term where as prolonged exposure can affect the liver, kidney and blood systems.

Recent methods of BTEX group compound treatment in wastewater involves taking advantage of the non-polar structure of these compounds by providing a surface that is favourable for adsorption. One example of this is the use of carbon nanotubes to enhance BTEX group surface adsorption[3]. Methods like the use of carbon nanotubes which enhance the adsorption of these compounds are costly with prices of around $400,000 per metric tonne of carbon nanotubes[9]. Alternative methods like the use of granular activated carbon (GAC) or the use of a peroxidase like SBP along with a substrate of SBP could allow for the cheap and easy production of a surface

for BTEX group adsorption. This would make the removal of BTEX group compounds through adsorption methods more viable as the group compounds could then be removed cheaply while also treating other toxic compounds such as phenol using the enzymatic reaction. Phenol and BTEX are likely to co-occur in refinery waste streams, for example[33].

1.2 Phenol as a pollutant and phenolic polymers:

Phenolic compounds occur naturally in water and soil through decomposition of biological waste. They are also produced industrially from many different sectors and hence are present in many wastewater treatment plants. Most industrially discharged phenols are from the pulp paper and wood industry which had an average effluent concentration of 0.4 mg/L[10]. Phenols can be removed from wastewater through an enzyme-catalyzed process using peroxidases like horseradish peroxidase (HRP) or SBP. This system is well documented and everything from removal effectiveness, reaction kinetics, the chemical mechanism and many different peroxidases have been studied. Enzymatic treatment of phenol or any other substrate involves oxidation of the substrate to a free radical, non-enzymatic radical coupling and subsequent polymerization to create phenolic polymers. These are oligomer or polymer level chains of phenol compounds that get too large to remain dissolved in the aqueous solution and thus precipitate out. The polymers are particularly non-polar and make a good surface for non-polar compounds such as BTEX group compounds to adhere to. If the binding were favourable, the usefulness of SBP and other enzymes in wastewater treatment would increase due to the ability to remove non-polar toxic compounds as well as its substrates.

1.3 Soybean Peroxidase:

Soybean peroxidase is a member of the class III plant peroxidase superfamily. It can be found in the seed coat of soybeans and, like other peroxidases, it has been used to catalyze the oxidation of phenols, anilines and other aromatic substrates with hydrogen peroxide[16]. In comparison with horseradish peroxidase (HRP), the more extensively studied peroxidase, SBP shows 57% amino acid sequence homology and is more thermally stable than HRP[16]. This may be due to an increased ionic and hydrophobic interaction at the heme cavity that helps stabilize its native conformation[13]. Optimal phenol polymerization using SBP is at pH = 6.4 but greater than 90% of its catalytic activity is retained in the pH range of 5.7-7.0[17]. SBP's thermal stability is not only greater than HRP's but is also quite high in its own right, losing no activity after a 12 h incubation at 70°C[18].

When comparing SBP to HRP-C in terms of catalytic efficiency the constants used are k_{cat} (catalytic constant) and K_m (Michaelis constant) with the catalytic efficiency being defined as the k_{cat}/K_m. The comparison of catalytic efficiency at pH = 5.0 and 6.8 with hydrogen peroxide (at 0.5 mM, a non-rate-limiting concentration) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS; a common substrate for activity assay) for HRP and SBP are shown in the table below.

Table 1-2: Comparison of SBP and HRP-C catalytic efficiency at pH 5.0 and 6.8

Enzyme	k_{cat} (s^{-1})	K_M (μM^{-1})	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	pH	Reference
SBP	1230 ± 58	173 ± 9	7.1 ± 0.1	6.8	13
SBP	2663 ± 17	45 ± 1	59.2 ± 0.9	5.0	13
HRP-C	736 ± 10	178 ± 8	4.1 ± 0.1	6.8	13
HRP-C	810 ± 11	270 ± 8	3 ± 0.1	5.0	14

K constants are with ABTS and H_2O_2 as substrates

The active site of SBP contains a heme group consisting of 4 pyrrole rings connected by methine bridges coordinated to Fe (III). The entire structure of SBP is shown in Figure 1-1.

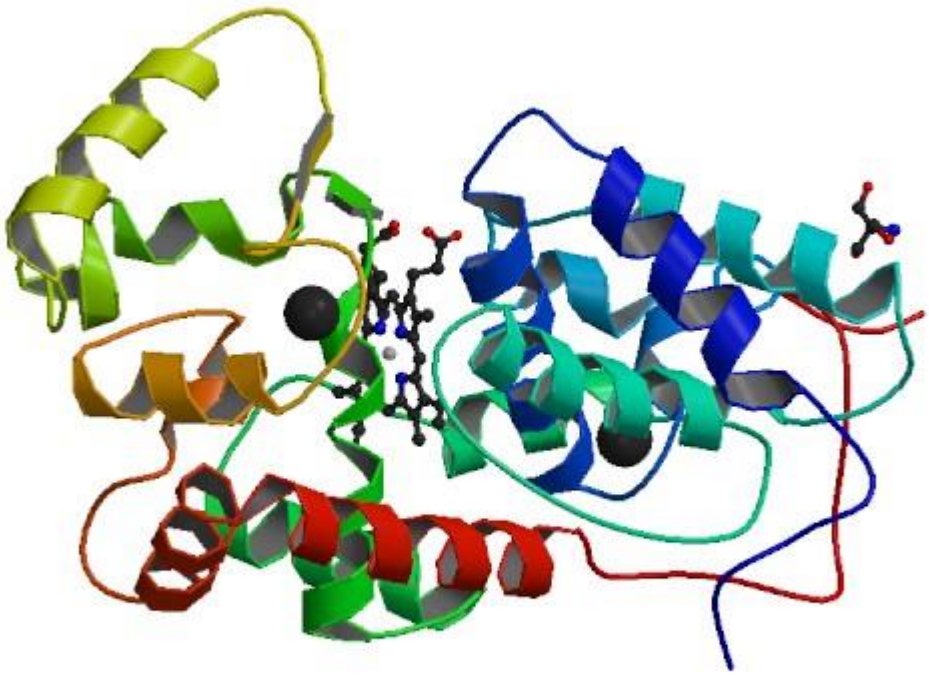

Figure 1-1: The structure of soybean peroxidase created by RCSB PDB four letter code 1FHF[34]

1.4 Peroxidase reaction mechanism:

The peroxidase mechanism comes from HRP studies though the mechanism for SBP is the same. The three-step reaction process is shown below where in the first step the native form of the

enzyme is oxidized by hydrogen peroxide for a loss of 2 electrons and becomes compound I while hydrogen peroxide is reduced to water. In the second step compound I oxidizes the substrate (AH, example phenol) and generates a free radical (A·) and becomes compound II. In step 3 compound II oxidizes another substrate molecule, generating another free radical and returns HRP to its native state[19].

$HRP[Fe^{III}] + H_2O_2 \rightarrow$ Compound I [Fe^{IV} = O, porphyrin π – cation radical] $+ H_2O$

Compound I [Fe^{IV} = O, porphyrin π – cation radical] + AH → Compound II [Fe^{IV} = O] + A· + H^+

Compound II [Fe^{IV} = O] + AH → $HRP[Fe^{III}]$ + A + OH^-

The radicals formed by the above reaction couple to form a dimer which can be further oxidized by SBP for form trimers or tetramers. This process continues until the polymer is too big to remain dissolved and precipitates out. The first two steps are very fast compared to the third, which is the rate-determining step[19]. From the reaction it would be expected that a 2:1 molar ratio of AH to hydrogen peroxide would be required to facilitate the reaction. In reality it is much higher as the oxidation of reaction intermediates consume hydrogen peroxide during subsequent cycles of the oligomerization reaction. The experimental ratios vary for different substrates but are typically 1:1 or even higher[20].

1.5 Objectives and Scope

The objectives for this study were as follows:

1. To determine the extent to which 4-(phenylazo)benzoic acid ad/absorbs onto phenolic precipitates.

2. To measure the difference between dynamic and static ad/absorption and determine their effectiveness.

3. To obtain a Langmuir analysis of any relevant ad/absorption systems.

4. To determine if ad/absorption onto hydrophobic precipitates could be a viable method for removing toxic non-substrates of SBP from wastewater.

The scope of the study includes:

1. Determining the pKa of 4-(phenylazo)benzoic acid

2. Determining if 4-(phenylazo)benzoic acid undergoes reductive azo-bond splitting in the presence of SBP and hydrogen peroxide.

3. Determining whether static or dynamic ad/absorption is more effective.

4. Determining the viability of using phenolic precipitates to remove toxic non-substrates of SBP from wastewater.

Chapter 2

2. Literature Review:

2.1 Phenol:

Phenol is a white crystalline solid at room temperature. The chemical formula is C_6H_5OH with a molecular mass of 94.11 g/mol.

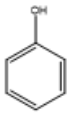

Figure 2-1: Structure of phenol.

Below is a table of chemical and physical properties of phenol.

Table 2-1: Chemical and physical properties of phenol

Property		Reference
Melting point (°C)	41	4
Boiling point (°C)	182	4
Vapour pressure (Pa)	47	5
pKa	9.99	5
Log K_{oc} (carbon water partition coefficient)	1.15–3.49	10
Log K_{ow} (octanol water partition constant)	1.46	11
Solubility in water (g/L)	88.3	12

The toxicity of phenol has been widely studied. Phenol can cause severe skin and eye and mucous membrane irritation [10]. Studies of phenol as a carcinogen show correlation between cancer mortality and exposure to phenol. Long term phenol exposure can cause damage to the heart, lungs, liver and kidneys [15].

2.2 4-(phenylazo)benzoic acid:

4-(phenylazo)benzoic acid is a relatively hydrophobic compound, Figure 4-2, that contains benzene rings, a carboxylic acid group and an azo bond linking the two rings together. The chemical formula is $C_{13}H_{10}N_2O_2$ with a molecular mass of 226.23 g/mol. It is an orange powder at room temperature, has a melting point of 242-244°C[22] and is sparsely soluble in water.

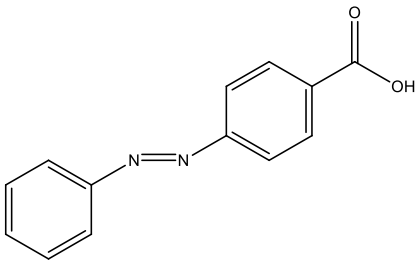

Figure 2-2: Structure of 4-(phenylazo)benzoic acid

2.3 Structure of Enzyme Polymers in Particular Phenolic Precipitates:

As mentioned in the peroxidase reaction mechanism section, peroxidases like SBP form radicals which bind together substrate molecules into oligomers and polymers. This reaction continues as long as the substrate polymer is small enough to remain dissolved in water. Once the polymer is large enough it is precipitated out removing the substrate from the water and thus ending the reaction. This reaction can be carried further if a water-miscible solvent is used such as acetone or ethanol, which would allow the polymer to remain dissolved in the solution to undergo more

enzymatic cycles and create larger polymers. Polymer syntheses of phenols using peroxidases have been found to form polymers with molecular masses from 400 to 26000 D [7]. The proposed mechanism for phenol polymerization, Figure 2-3 starts with the oxidation of phenol by peroxide in the presence of peroxidase to form the resonance-stabilized phenoxyl radical. These radicals then couple to form dimers. At the beginning almost all phenols are converted to dimers. These dimers can then be further oxidized by peroxide in the presence of peroxidase to facilitate further coupling to larger polymers. When the concentration of free radicals decreases an electron-transfer reaction from phenol becomes an alternative way to produce higher radical dimers/oligomers. This then creates radicals out of our dimers/oligomers which couple with phenol or other dimers/oligomers. This transfer of the radical from phenol to the oligomer recreates phenol which as a substrate of the peroxidase is then oxidized to its radical form. When phenoxyl radicals are not being generated fast enough oxidation to ketone structures may occur [8]. The radical coupling happens primarily through C-C and C-O coupling with *ortho*- and *para*- orientation[8]. When the polymer gets large enough for the solvent mixture in question, the polymer becomes too hydrophobic to remain in solution.

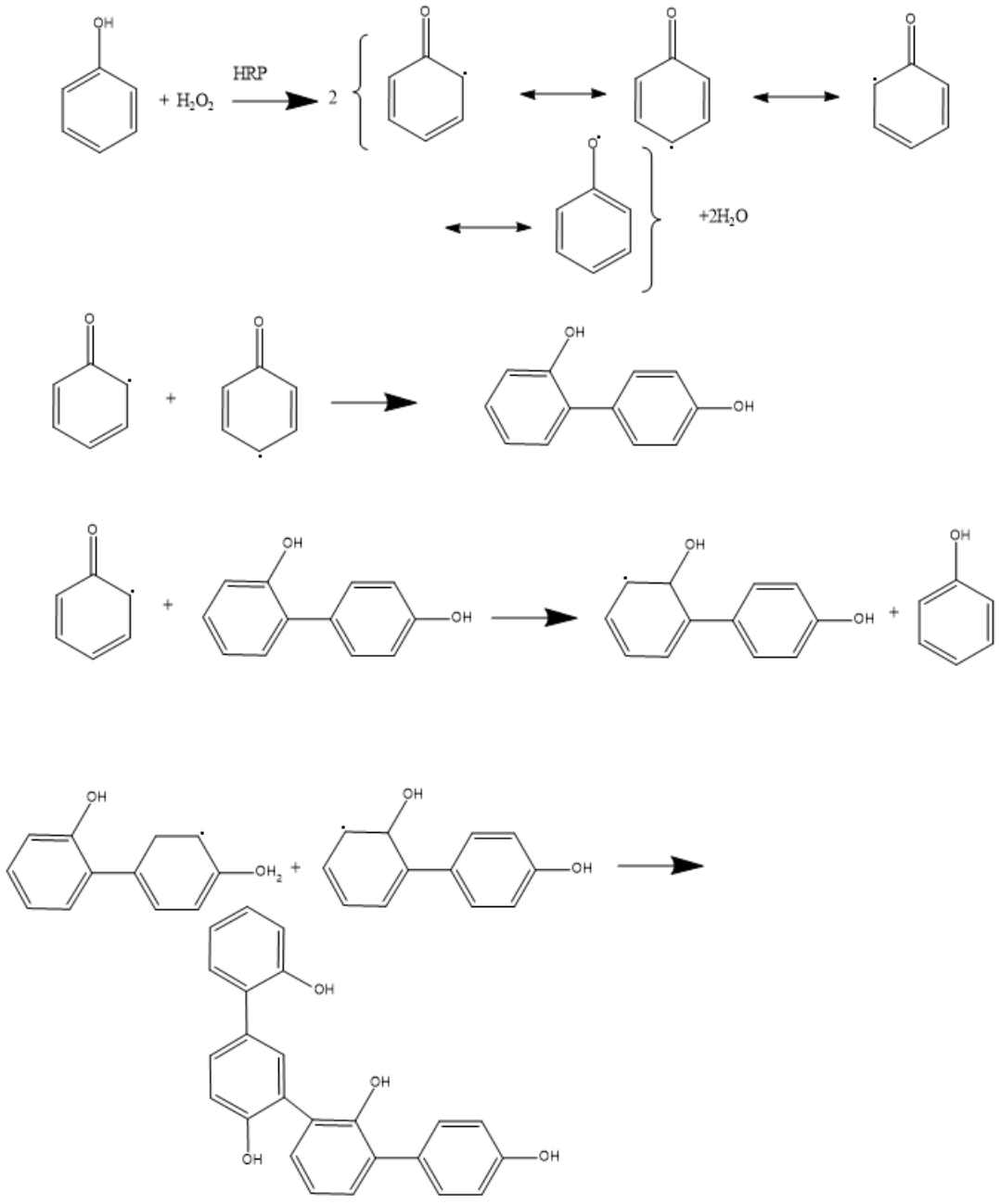

Figure 2-3: Polymerization of phenol in the presence of SBP.

2.4 Azo Bond Cleavage:

Previous studies have shown the degradation of azo-dyes using soybean peroxidase. The degradation of Crystal Ponceau 6R (CP6R) was studied in detail[21]. Among the parameters studied were optimizing H_2O_2 concentration, redox mediator amount (in this case

hydroxybenzotriazole or HOBT was used) and pH of the solution. CP6R was found to resist degradation without the addition of a redox mediator and so HOBT was used at 50 μM to ensure dye degradation. Hydrogen peroxide concentrations as well as pH were also optimized in this study. CP6R was found to undergo a reaction pathway through SBP and HOBT that involved the oxidation of CP6R to create a CP6R radical. The overall reaction is very similar to the general peroxidase reaction mechanism except it includes a transfer of the radical from HOBT to SBP. This reaction is a 4-step reaction with the first step being native SBP reacts with hydrogen peroxide to become compound I and water. The second step sees the abstraction of hydrogen from HOBT which forms compound II and a HOBT radical. The third step involves a second abstraction of hydrogen forming a second HOBT radical and the recovery of native SBP. The final reaction involves a HOBT radical attacking the CP6R and abstracting a hydrogen forming a CP6R radical. The reaction scheme is shown below.

SBP [Fe^{III}]+H_2O_2 → SBP [Fe^{IV} = O, porphyrin π – cation radical] +H_2O

Compound I (SBP [Fe^{IV} = O, porphyrin π – cation radical] +HOBT) → Compound II (SBP [Fe^{IV} = O] + ·HOBT)

Compound II (SBP [Fe^{IV} = O] +HOBT) → SBP [Fe^{III}] + ·HOBT +H_2O

·HOBT+CP6R→ HOBT+ ·CP6R

Two different reaction pathways have been proposed for azo bond cleavage, asymmetric and symmetric bond cleavage. Asymmetric reaction, Figure 2-4, cleaves at a C-N site to create an N=NH group and a ketone in the respective fragments. Degradation continues after this point to produce carboxylic acids as the end product of this reaction pathway.

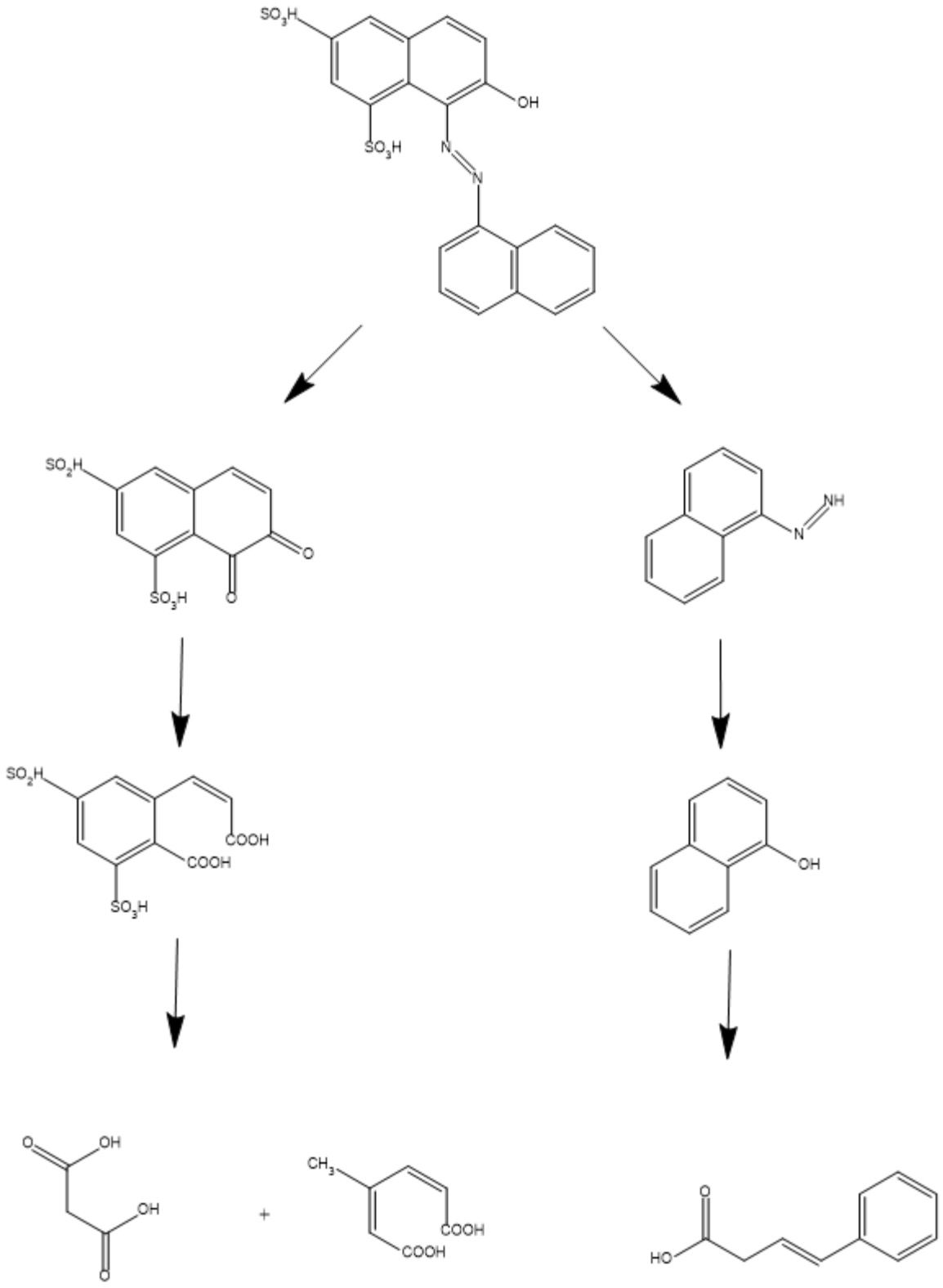

Figure 2-4: Asymmetric azo bond cleavage of CP6R[21]

This reaction occurs after the formation of a CP6R radical from the enzymatic process mentioned above. All intermediates above have been experimentally identified[21]while the mechanism in which to get from one intermediate to another has not. The above referenced study proposes a reaction mechanism involving hydroxyl radicals as an essential part of the reaction[21]. No basis is given however from the study for the presence of hydroxyl radicals and in the classical peroxidase reaction mechanism no hydroxyl radicals are formed.

Another possible pathway is the symmetric azo bond cleavage, Figure 2-5. This mechanism starts with the cleavage of the azo bond at the N=N site resulting in amine intermediates. This reaction mechanism continues to produce the final product of carboxylic acids.

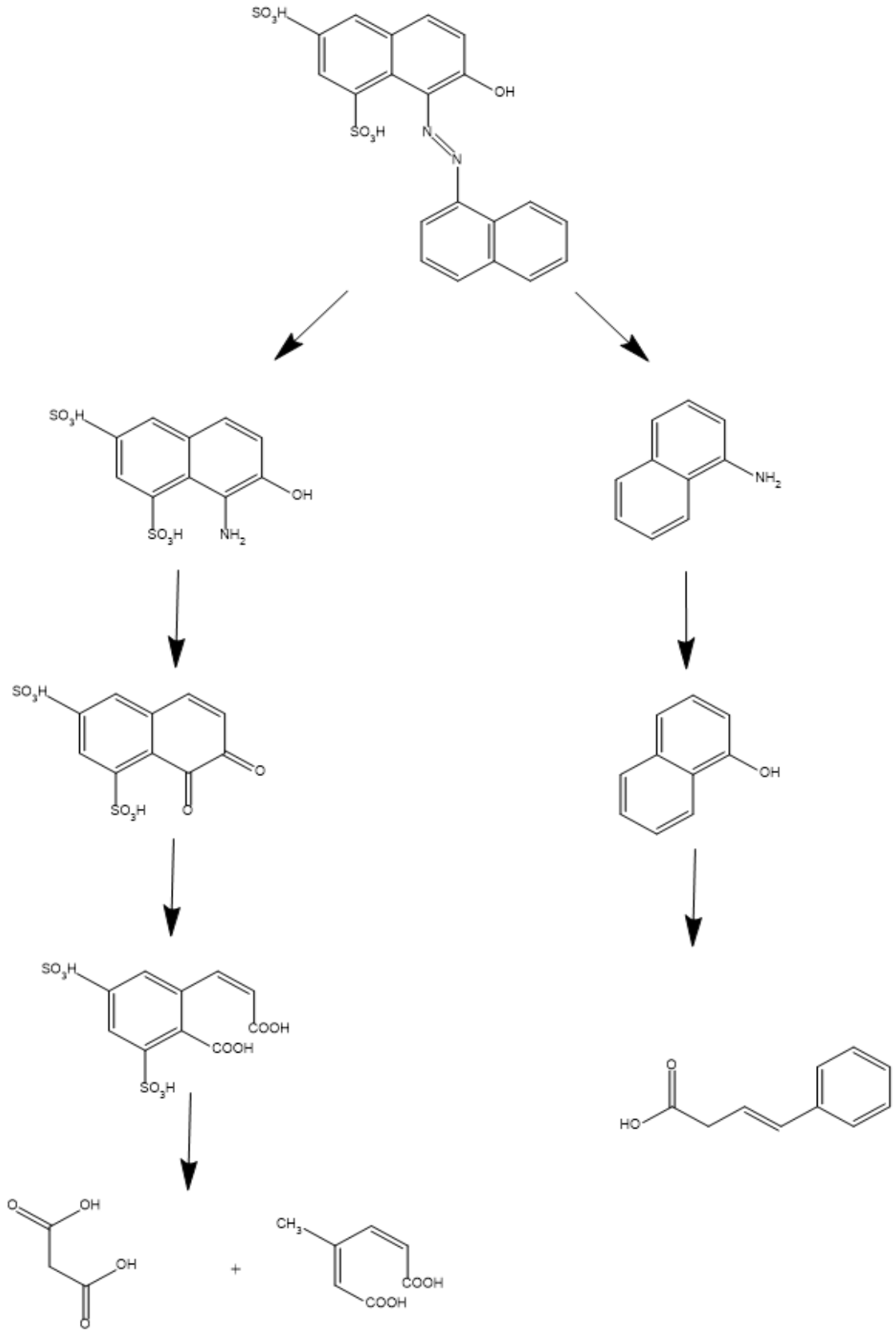

Figure 2-5: Symmetric azo bond cleavage of CP6R[21]

As mentioned previously the referenced study gives a reaction mechanism involving hydroxyl radicals but no basis for their presence. After the symmetric bond cleavage instead of continuing like the above reactions to form carboxylic acids, it is possible that SBP would oxidize the newly formed anilines to create new radicals that then couple together to form polymers. Analogously in both the asymmetric and symmetric azo bond cleavage the α-naphthol product could also undergo oxidization from SBP and form polymers from there.

2.5 Langmuir Adsorption Model:

The Langmuir adsorption model is a very common model to measure the adsorption of a substance onto a surface. The model makes three assumptions: 1) all adsorption sites on the surface are equivalent, 2) each adsorption site can only be occupied by one molecule and only a monolayer forms and 3) the adsorbed molecules do not interact with one another. This model's assumptions are obviously not applicable to every adsorption scenario as it simplifies the molecular interactions greatly but it has still been used as a starting point for characterization of adsorbed molecules. The Langmuir isotherm is given by the equation below.

$$\frac{Q_e}{Q_m} = \frac{KC_e}{1 + KC_e}$$

Where Q_e is the adsorption capacity at equilibrium (mg/g), Q_m is the maximum adsorption capacity (mg/g), K is the Langmuir adsorption constant and C_e is the equilibrium concentration of the adsorbate (mg/L). A linear form of the Langmuir constant was also used for preliminary analysis of the adsorption system.

$$\frac{C_e}{Q_e} = \frac{1}{Q_m K} + \frac{C_e}{Q_m}$$

The linear form, while useful for initial analyses, carries with it a greater amount of error. The linear form plots C_e *vs* C_e/Q_e which are not entirely independent variables as both involve the value of C_e. This increases the amount of error in the linear regression and was plotted primarily due to the ease of calculation with linear regression. All systems plotted linearly were also plotted in the more classical form of the Langmuir model as well to avoid excess error. Adsorption analysis is useful in quantifying uptake molecules by the phenolic precipitates formed by peroxidase reactions.

Chapter 3

Materials and Experimental Methods

3.1 Materials

3.1.1 Enzymes

Crude dry solid SBP (Industrial Grade lot #18541NX) was obtained from Organic Technologies (Coshocton, OH) and stored at -15℃. Liquid ARP (*Arthromyces ramosus* peroxidase) concentrate (SP-502, activity 1200 U/mL) was obtained from Novzymes (Franklinton, NC). Both enzyme stock solutions were stored at 4℃.

3.1.2 Buffers

Monobasic and dibasic sodium phosphate were purchased from BDH (Toronto, ON). HPLC grade acetonitrile and water were purchased from Fisher Scientific Co (Fair Lawn, NJ). Ammonium acetate was purchased from Sigma Aldrich (St. Louis, MO).

3.1.3 Reagents

Hydrogen peroxide (30% w/v) was purchased from ACP Chemicals Inc. and stored at 4℃. 4-aminoantipyrine (4-AAP) was purchased from BDH (Toronto, ON) and stored at room temperature.

3.1.4 Aromatic Compounds

Crystalline phenol, 4-(phenylazo)benzoic acid and aniline were purchased from Sigma Aldrich (St. Louis, MO) and stored at room temperature. *Para*-aminobenzoic acid (PABA) was purchased from Sigma Aldrich (St. Louis, MO) and stored at 4℃.

3.2 Analytical Equipment:

3.2.1 Ultraviolet-Visible Spectroscopy:

UV-Vis spectroscopy was done using an Agilent 8453 UV-Visible spectrophotometer. Cuvettes used were made of quartz with a path length of 1 cm and purchased from Hellma Analytics (Müllheim, Germany)

3.2.2 Centrifuge:

Centrifugation was done using a Corning LSE^{TM} compact centrifuge with 6*50 mL and 6*15 mL centrifuge tubes at 4000 rpm.

3.2.3 pH meter:

Oakalon PC700 pH meter with a stainless steel micro pH probe was used to measure the pH of all solutions in this study. Calibration buffers at pH =4.00, 7.00 and 10.00 were purchased from ACP inc (Slough, England).

3.2.4 High Performance Liquid Chromatography (HPLC):

HPLC data was measured using Waters HPLC system with crucial components of the HPLC being model 2489 UV/Visible detector, model 1525 binary HPLC Pump, and model 2707 auto-sampler.

3.2.5 Other Equipment:

Magnetic stir bars of various sizes were purchased from Fisher Scientific Co., syringes 10 mL and 5 mL were purchased from BD medical technologies, 0.2 µm filters were purchased from Sarstedt (North Rhine-Westphalia, Nümbrecht, Germany) and 0.45 µm filter paper from Advantec MFS (Dublin, CA).

3.3 Analytical Methods:

3.3.1 Enzyme Stock Solution Preparation:

SBP stock solution was prepared using 0.12-0.14 g of solid enzyme and 100 mL of distilled water. These two components were mixed for 24 h then centrifuged for 15 minutes at 4000 rpm. The supernatant was then separated from the pellet and stored at 4℃. The activity was measured before every experiment.

3.3.2 4-(phenylazo)benzoic acid and SBP enzymatic reaction:

Batch reactors were made up using 1 U/mL of SBP, 1.5 mM hydrogen peroxide, 5 mM phosphate buffer (pH = 7.0) and various small concentrations of 4-(phenylazo)benzoic acid ranging from 20 µM- to 80 µM. Two controls were used, one without hydrogen peroxide and one without enzyme. In place of either hydrogen peroxide of enzyme the controls were diluted with an equivalent volume of water. These solutions were left overnight then filtered and analyzed the following day using HPLC.

3.3.3 Dynamic Absorption Method:

Batch reactors were made up as above but with the inclusion of 1 mM phenol.

3.3.4 Enzymatic reaction of 4-aminobenzoic acid (PABA):

Batch reactors were made up as for 4-(phenylazo)benzoic acid, but with varying concentrations of PABA ranging from 7 µM to 1 mM, instead.

3.3.5 Acidification of PABA polymer:

To ensure that a loss in PABA concentration was due to a polymerization via the enzymatic reaction a reacted solution of PABA was acidified to precipitate any soluble polymers that may have been formed. This was accomplished by the addition of 200 µL of formic acid into a 25 mL initial volume. The acidified solution was then analyzed by HPLC and UV-VIS spectra were taken to discern changes in the absorbance.

3.3.6 pKa determination of 4-(phenylazo)benzoic acid:

In order to accurately determine the concentration of 4-(phenylazo)benzoic acid, the *p*Ka needed to be determined. The *p*Ka of 4-(phenylazo)benzoic acid was determined by titration with sodium hydroxide. The pH was measured by a Oakalon PC700 pH meter with a stainless steel micro pH probe. A titration curve was plotted and analyzed by CurTiPot (software by Ivano Gebhardt Rolf Gutz, Institute of Chemistry, University of Sao Paulo).

3.3.7 Enzyme Activity Assay:

A colourimetric kinetic assay was used to measure SBP activity. The assay measures the initial rate of formation of a pink chromophore at 510 nm when an enzymatic sample is mixed with a reagent to form a solution of SBP, 10 mM phenol, 40 mM phosphate buffer (pH=7.4), 0.2 mM hydrogen peroxide and 2.4 mM 4-AAP. The sample dilution was adjusted to give an absorbance value less than 1 and an initial absorbance before reaction of no more than 0.1. The absorbance is measured via a UV-VIS spectrophotometer. A regular assay was done via the mixing of 50 µL of diluted enzyme with 950 µL of reagent. Once the sample is mixed readings were taken for 30 s, once every 5 s. The activity is expressed in U/mL where one unit (U) is defined as the amount that catalyzes 1 µmol of hydrogen peroxide in one minute. Full details are given in Appendix A.

3.3.8 Hydrogen Peroxide Concentration Test:

A colourmietric end-point assay was used to measure hydrogen peroxide concentration. The assay measures the absorbance of a pink chromophore at 510 nm when a sample of hydrogen peroxide is mixed to form a solution of hydrogen peroxide, 10 mM phenol, 12.5 mM 4-AAP, 50 mM phosphate buffer (pH = 7.4), and 0.31 mL of Novozymes ARP concentrate. The absorbance of this solution was measured after 15 minutes. Concentrations from 0.01 mM to 0.1 mM were used to construct the standard curve. The concentration is then determined from the standard curve established under the same conditions. Full details are given in Appendix B.

3.3.9 Phenolic Precipitate Dry Mass:

The dry mass of phenolic precipitate per volume of a standard suspension (described in *Section 3.3.11*) was measured by cleaning three Buchner funnels, oven drying and cooling them in a desiccator. A known aliquot of precipitate suspension was then transferred to the funnel and vacuum filtered. The funnels were then placed in an oven at 100°C for one day to fully dry, then cooled in a desiccator and weighed. The precipitate dry mass is defined as the average change in mass of the three funnels.

3.3.10 Extraction Method:

Extractions were carried out on mixtures from both static and dynamic adsorption/absorption of 4-(phenylazo)benzoic acid on phenolic precipitate. For the dynamic method, a phenolic polymerization reaction was carried out in both the experimental batch reactor and in 3 separate batch reactors with no 4-(phenylazo)benzoic acid. The precipitates in the 3 non-experimental batch reactors were measured using the phenolic precipitate dry mass method. The experimental suspension was centrifuged for 20 minutes at 4000 rpm and the supernatant was removed. The pellet was resuspended in 100% acetonitrile and was allowed to stir for 30

minutes. The concentration of 4-(phenylazo)benzoic acid was determined for both the supernatant of the experimental suspension and the acetonitrile extract. The resulting concentrations were then compared. The static method was done similarly except the precipitate was taken from a pre-made suspension fluid of already reacted phenol. The suspension fluid is described in more detail in the static adsorption method (*Section 3.3.11)*.

3.3.11 Static Adsorption method:

To observe the adsorption of 4-(phenylazo)benzoic acid onto phenolic precipitates after enzymatic reaction, a suspension of phenolic precipitates was created. This mixture was made using 10 U/mL of SBP, 10 mM phenol and 12.5 mM hydrogen peroxide in a volume of 1 L. The resulting suspension was stirred overnight to ensure it was consistent throughout. An aliquot of 2.5 mL was then added to a 22.5 mL volume batch reactor to make a total volume of 25 mL. The batch reactor contained 4-(phenylazo)benzoic acid and 5 mM pH= 7.0 phosphate buffer. The resulting suspension was left overnight then filtered and 4-(phenylazo)benzoic acid disappearance from the supernatant was measured by HPLC.

3.3.12 Langmuir Analysis:

A Langmuir analysis was done using batch reactors made up in either the dynamic or static absorption/adsorption methods described earlier. Many concentrations of 4-(phenylazo)benzoic acid were used from 4 µM to 88 µM while the precipitate mass was kept constant. Five different points were obtained where at least 2 points lay above and below the half-saturation point so as to support the validity of the curve. Each point was done in triplicate and the curve was fitted to the Langmuir adsorption model.

$$\frac{Q_e}{Q_m} = \frac{KC_e}{1 + KC_e}$$

Q_e is the absorption capacity at equilibrium (mg/g), Q_m is the maximum absorption capacity (mg/g), K is the Langmuir absorption constant and C_e is the equilibrium concentration of the absorbate (mg/L).

3.3.13 HPLC analysis:

HPLC was done in reverse-phase mode on a C18 bonded phase with an isocratic elution for all analyses. The mobile phase for the various compounds used are shown in the table below. All methods were developed at an injection volume of 10 µL, a flow rate of 1.0 mL/min and ambient temperature, as shown in Table 3-1.

Table 3-1: HPLC Methods

Compound	Method	Wavelength (nm)
4-(phenylazo)benzoic acid	70% acetonitrile, 30% 5 mM pH = 7.0 phosphate buffer or 40% acetonitrile, 60% 5 mM pH = 7.0 phosphate buffer	325
PABA	40% acetonitrile, 60% pH = 7.0 ammonium acetate buffer	280
Aniline	40% acetonitrile, 60% 5 mM pH = 7 ammonium acetate buffer	280

3.3.14 Buffer Preparation:

Multiple buffers were used in these experiments but the main buffer used was a sodium phosphate buffer at a pH of 7.0 for batch reactors, and activity tests at a pH of 7.4. Ammonium acetate buffer, pH = 7.0 was also used for some HPLC analyses.

Chapter 4

4. Results:

4.1 pKa Determination:

Before any enzymatic reactions could be carried out, a method to quantitatively determine 4-(phenylazo)benzoic acid (hereafter occasionally abbreviated as 'Azo') concentration was needed. HPLC was used to carry out the concentration measurements, but to get a reproducible measurement it was necessary to ensure that Azo was consistently protonated or deprotonated during the analysis. The absorbance spectrum of 4-(phenylazo)benzoic acid was measured at pH = 1.03 and at pH = 12.25. The absorbance maximum for both these wavelengths was found to be 325 nm but the magnitude changed dramatically. At the pH of 1.03 the absorbance at 325 nm was found to be 0.0907 ± 0.0003, whereas for a pH of 12.25 the absorbance at 325 nm was found to be 0.1737 ± 0.0003. This is a two-fold difference between magnitude of the acidic and basic absorbance. The spectra of Azo at pH = 1.03 and pH = 12.25 are shown in Figure 4-1 and 4-2.

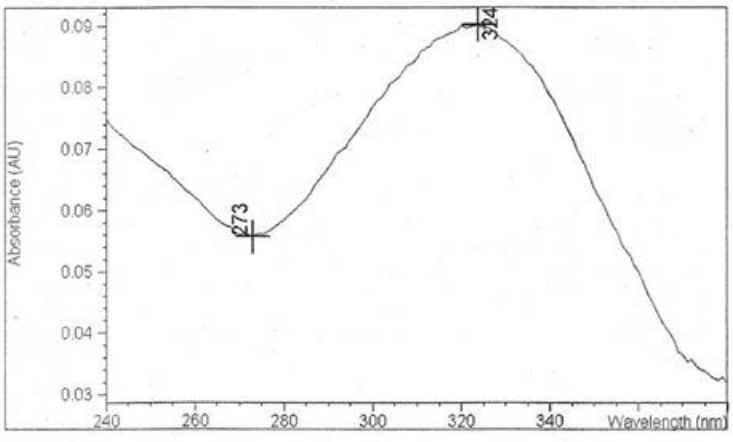

Figure 4-1: Absorption spectrum for an acidic solution of Azo. 1.5 mg of Azo was added to 250 mL (26.5 µM) but the actual dissolved concentration was not tested. The above stock solution was filtered and 25 mL was taken and acidified using 200 µL of HCl (pH = 1.03) then measured using a UV-vis spectrophotometer.

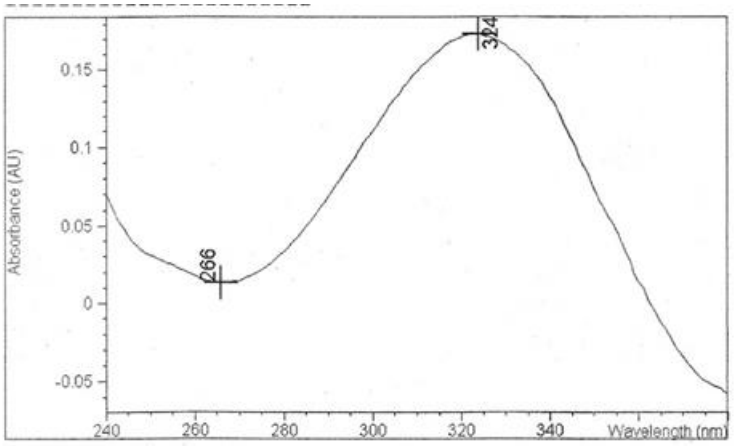

Figure 4-2: Absorption spectrum for a basic solution of Azo. 1.5 mg of Azo was added to 250 mL (26.5 µM) but the actual dissolved concentration was not tested. 25 mL of the above stock solution was filtered and made basic by adding NaOH to a final concentration of 48.2 mM (pH = 12.25) then measured using a UV-vis spectrophotometer.

Since enzymatic reactions done after this point are analyzed using HPLC to determine concentration and since HPLC uses the absorption at specific wavelengths it is necessary to make sure the extinction coefficient does not change between reactions. If Azo was protonated in some cases but not in others comparing their absorbance to a standard to determine concentration would be unreliable. To ensure that the measurements taken are reliable, the pKa of 4-(phenylazo)benzoic acid was determined by titration of the aforementioned acid against sodium hydroxide, as seen in Figure 4-3.

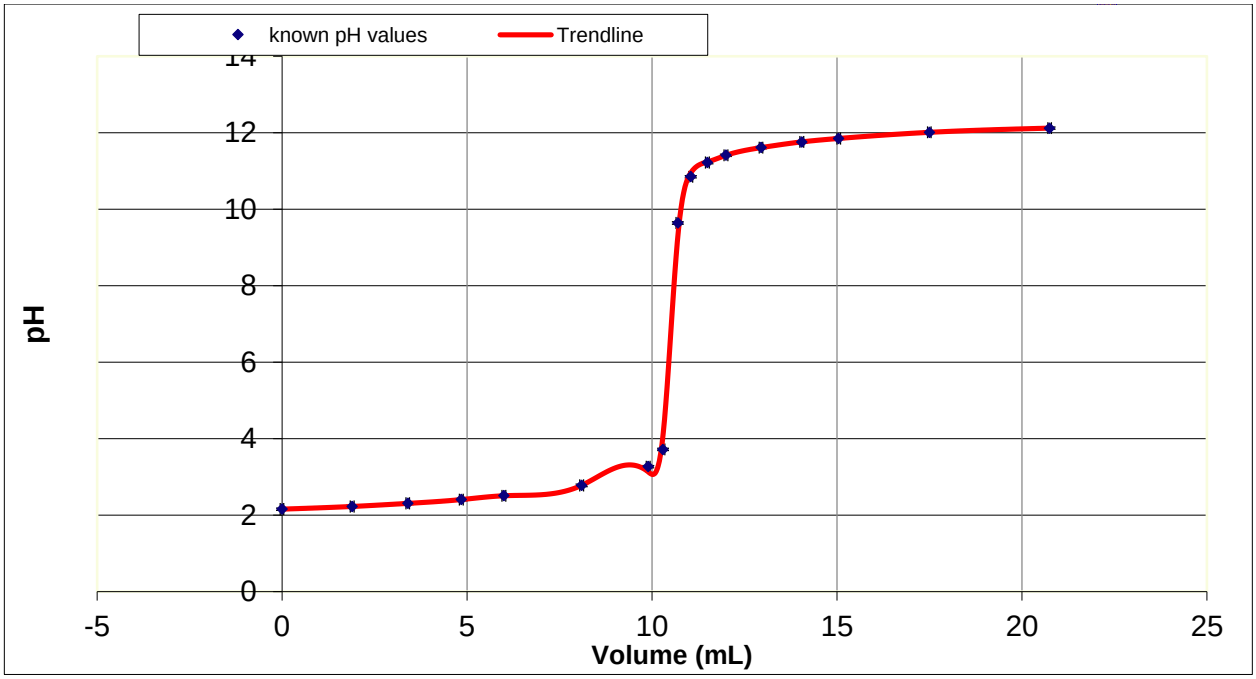

Figure 4-3: Titration of 4-(phenylazo)benzoic acid with 11mM sodium hydroxide (R^2=0.9996). The titration curve is a curve fit created using CurTiPot. There is a small blip at 8-10 mL which is due to the function being a curve fit function not a graph of the Henderson-Hasselbalch equation. Error is calculated from the standard deviation of triplicate measurements using the formula, Standard error = (Standard deviation)/ √n, where n is the number of measurements.

The first derivative of the titration curve shown in Figure 4-4 gives the equivalence point at 10.51 ± 0.05 mL. The half-equivalence point is therefore at 5.26 ± 0.05 mL which corresponds to the pKa for the compound of 2.45 ± 0.03. The error is calculated using the standard deviation of the fitted pH values of the curve from the following equation: Standard error = (Standard deviation)/ √n where n is the number of measurements. All experiments hereafter were conducted at a pH greater than 4.45. At pH values greater than 4.45, 99% of Azo can be said to be deprotonated which ensures reproducible measurements for HPLC chromatograms of Azo in the future. The pH value usually used was 7.0 because this is above 4.45 and it is a useful pH for phenol polymerization.

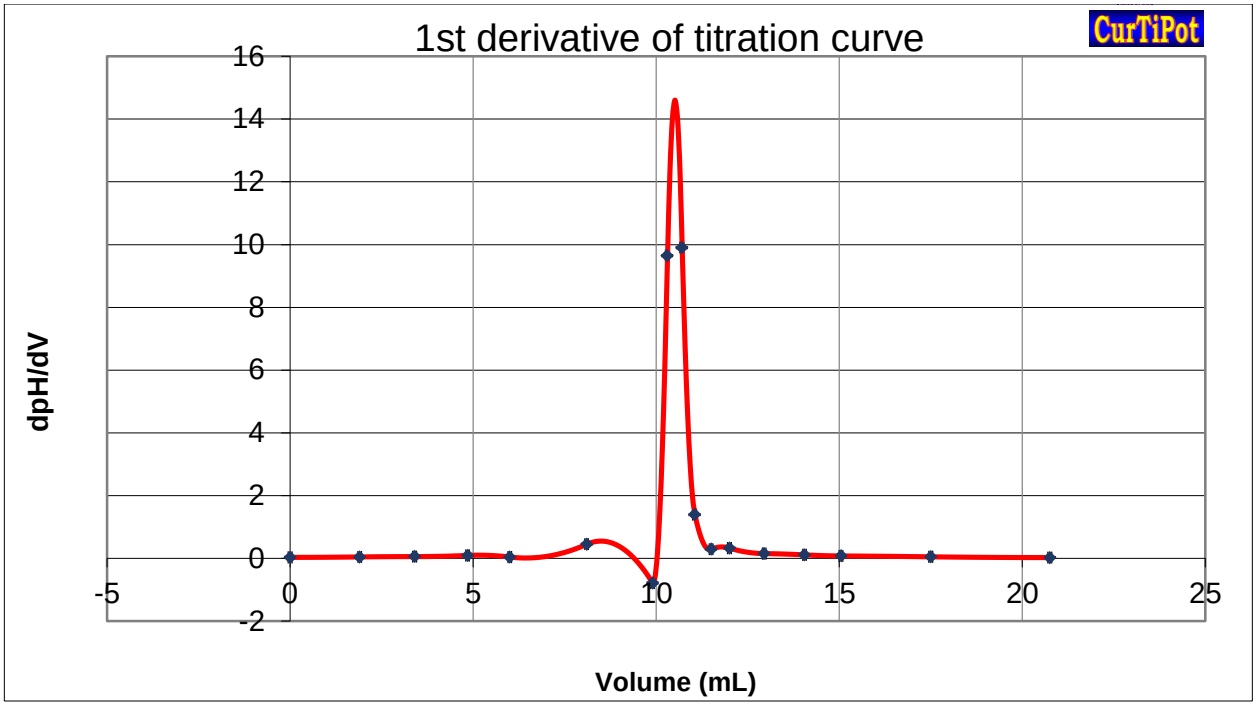

Figure 4-4: The first derivative of the titration curve in Figure 4-3. This figure shows the equivalence point at the highest value of the curve (10.51 ± 0.05 mL). Error is calculated from the standard deviation of triplicate measurements using the formula, Standard error = (Standard deviation)/ √n, where n is the number of measurements.

4.2 Initial dynamic absorption tests:

Initial tests were conducted under conditions designed to give 95% conversion for phenol polymerization using enzymatic treatment. These conditions were 1 U/mL enzyme, 1 mM phenol and 1.5 mM hydrogen peroxide at pH= 7.0 using 5 mM phosphate buffer with a reaction time of 24 hours. Due to its limited solubility, 4-(phenylazo)benzoic acid was added to a nominal concentration above saturation and excess was filtered out after stirring for 24 hours. The initial concentration determined using the no-SBP control and Azo absorbed is done as the difference between the no-phenol control and the experimental. There was an unexpected difference between the no-SBP and no-phenol controls investigated further in the following section. The errors for the controls and the experimental were calculated from the standard deviation of

triplicate measurements using the following formula, Standard Error = (Standard Deviation)/ √n, where n is the number of replicate measurements (3). The error for the difference between the two controls and Azo absorbed columns were calculated using the error propagation when subtracting values (for the difference of A-B, $\sigma_{A+B} = \sqrt{(\sigma_A^2+\sigma_B^2)}$). All error calculations are done in this manner. The data from the initial dynamic tests are shown in Table 4-1 and Figure 4-5, below.

Table 4-1: Initial dynamic absorption tests.

Azo in a control with no SBP (µM, µg)	Azo in a control with no phenol (µM, µg)	Azo in the experimental (µM, µg)	Difference between the two controls (µM, µg)	Azo absorbed (µM, µg)
27.6 ± 0.4, 156 ± 2	25.1 ± 0.4, 142 ± 2	23.5 ± 0.4, 133 ± 2	2.5 ± 0.5, 14 ± 3	1.6 ± 0.7, 9 ± 4

Reaction conditions were 1 U/mL of SBP, 1.5 mM H_2O_2, 1 mM phenol and 5 mM phosphate buffer, pH = 7.0 and reaction time of 24 h. Azo absorbed is determined by the difference between the experimental and the no-phenol control. Error in controls and the experimental is calculated from the standard deviation of triplicate HPLC injections using the formula, Standard error = (Standard deviation)/ √n, where n is the number of measurements (3). Error in the calculated values is calculated using the error propagation when subtracting values (for the difference of A-B $\sigma_{A+B} = \sqrt{(\sigma_A^2+\sigma_B^2)}$)

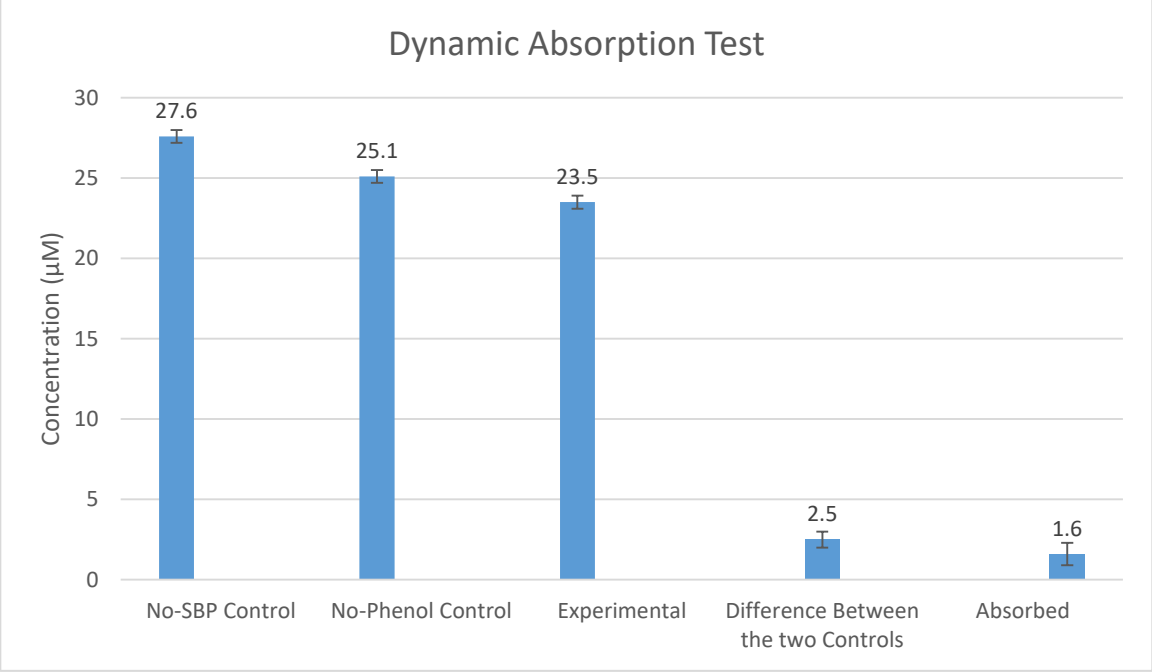

Figure 4-5: Initial dynamic absorption test results of Table 4-1.

4.3 4-(phenylazo)benzoic acid enzymatic conversion:

Although Azo was intended as a passive ad/absorbate in this work, during the initial dynamic absorption tests there was a difference in concentration between the no-phenol control and the no-SBP control, as noted above. If all of the loss of Azo measured in the experimental was due to absorption there should be no difference between the two controls. The difference of 2.5 ± 0.5 µM leads to the possibility that 4-(phenylazo)benzoic acid reacts with SBP. Because Azo does not have any functional groups that are common substrates of SBP, the likely reason for the loss in concentration is azo-bond cleavage, either symmetrical or asymmetrical. Other studies have shown azo-bond reduction in the presence of SBP[21] and its mechanisms are discussed in more detail in the literature review. Such a reaction could result in *p*-aminobenzoic acid (PABA) and aniline. Both PABA and aniline are substrates of SBP so tests were conducted to determine if this reaction takes place and if so to what extent.

An initial test was done with 4-(phenylazo)benzoic acid, SBP, hydrogen peroxide and pH= 7.0 phosphate buffer. Three sets of batch reactors were tested by HPLC analysis, one at 3 hours and the other two at 24 hours. The tests were done using a stock solution with a known mass of Azo added to make a concentration of 45 – 60 µM and 5 mM pH = 7.0 phosphate buffer. The actual concentration of 4-(phenylazo)benzoic acid was less than 45 – 60 µM since 4-(phenylazo)benzoic acid did not fully dissolve before being used and any excess was filtered out. The actual concentration was determined by HPLC. Hydrogen peroxide was added to 1.5 mM as well as 1 U/mL of SBP (the conditions for phenol polymerization) diluting the stock solution slightly. Two controls were run, one without SBP and one without hydrogen peroxide. In both controls the volume was made up with distilled water of the same volume as the missing component. The amount of Azo reacted is determined by the difference between the concentration in the

experimental reactor and the average concentration of the two controls. The resulting concentrations are shown in the Table 4-2 and Figure 4-6.

Table 4-2: Experiments to determine the possibility of 4(phenylazo)benzoic acid reacting with SBP.

	Azo in a control with no hydrogen peroxide (µM, µg)	Azo in a control with no SBP (µM, µg)	Azo in an experimental reactor (µM, µg)	Azo reacted (µM, µg)
After 3 hours	42.1 ± 0.4, 238 ± 2	41.3 ± 0.4, 234 ± 2	40.5 ± 0.4, 229 ± 2	1.2 ± 0.6, 7 ± 3
After 24 hours	39.5 ± 0.4, 223 ± 2	39.6 ± 0.4, 224 ± 2	36.9 ± 0.4, 209 ± 2	2.7 ± 0.6, 15 ± 3
After 24 hours	56.0 ± 0.7, 317 ± 4	54.6 ± 0.7, 309 ± 4	53.7 ± 0.7, 304 ± 4	2 ± 1, 11 ± 6

Experiments were done using 1.5 mM hydrogen peroxide and 1 U/mL SBP with a 5 mM phosphate buffer pH = 7.0. One experiment was stopped after 3 h and the other two after 24 h. The amount of Azo reacted is determined by the difference between the concentration in the experimental reactor and the average concentration of the two controls. Error in controls and the experimental is calculated from the standard deviation of triplicate HPLC injections using the formula, Standard error = (Standard deviation)/ √n, where n is the number of measurements (3). Error in the calculated values is calculated using the error propagation when subtracting values (for the difference of A-B $\sigma_{A+B} = \sqrt{(\sigma_A^2+\sigma_B^2)}$)

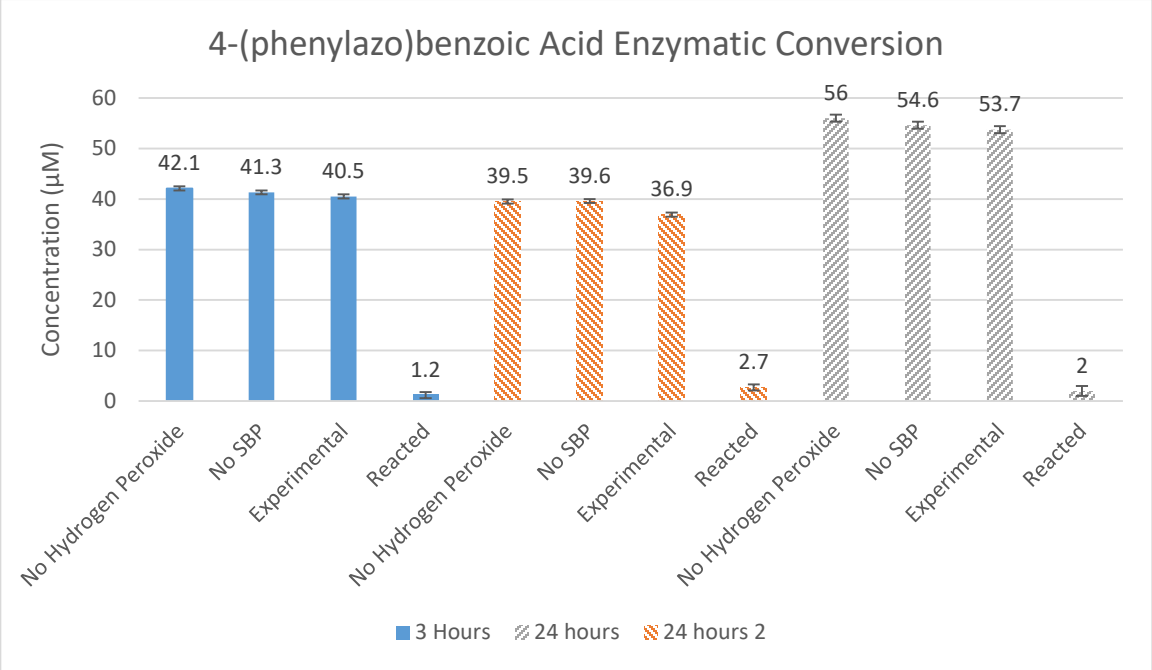

Figure 4-6: 4-(phenylazo)benzoic acid enzymatic conversion results of Table 4-2.

The experiment analyzed after 3 h shows very little if any reaction of Azo with SBP. After 24 h the amount reacted was higher but still small (< 5%). Further tests to determine if 4-(phenylazo)benzoic acid was reacting in the presence of SBP were needed.

4.4 Para-aminobenzoic acid and aniline:

The proposed mechanism for 4-(phenylazo)benzoic acid reacting with SBP involves reductive splitting of the azo-bond, which would produce PABA and aniline as daughter compounds. Thus, tests were done to attempt to find PABA and aniline as by-products of enzymatic reaction. To do this, it was necessary to be able to distinguish the peaks for aniline and PABA in a HPLC chromatogram. This means they should be well-separated from each other and from parent Azo. HPLC conditions to achieve this and a representative chromatogram are shown in Figure 4-7. PABA appears as the first peak followed by Azo and aniline, respectively.

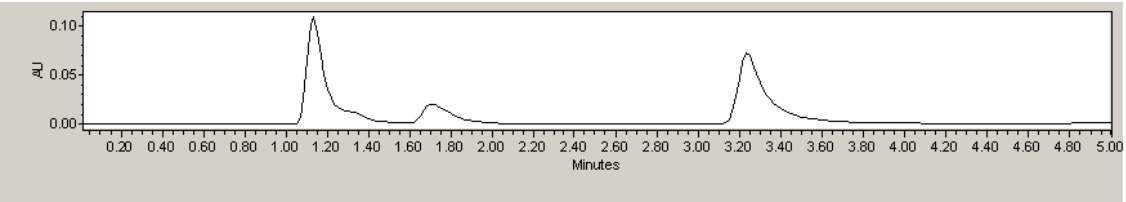

Figure 4-7: HPLC chromatogram of 4-(phenylazo)benzoic acid, PABA and aniline in the same solution. The mobile phase was 40% ACN and 60% aqueous ammonium acetate, pH = 7.0, flow rate 1.0 mL/min. This chromatogram was measured at 280 nm. PABA and Azo were added at a concentration of 117 ± 4 µM and 118.7 ± 0.7 µM respectively. Aniline was added at a nominal concentration of 0.1 mM.

The enzymatic reaction with 4-(phenylazo)benzoic acid and SBP was then run again this time looking for PABA or aniline HPLC peaks. The chromatogram for the no-enzyme control is shown in Figure 4-8.

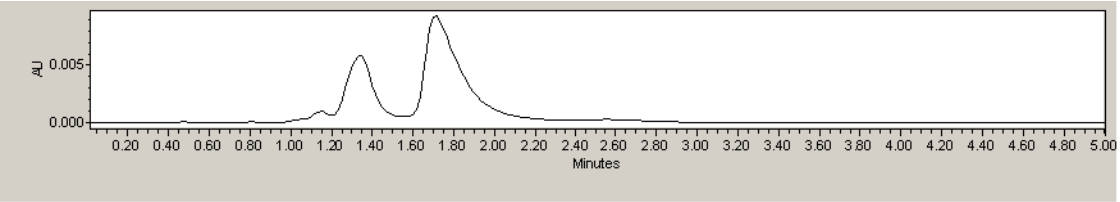

Figure 4-8: No-enzyme control for a 4-(phenylazo)benzoic acid enzymatic conversion. Reaction conditions were 68.7 ± 0.7 µM Azo and 1.5 mM H_2O_2 left for 24 h. The mobile phase was 40% ACN and 60% aqueous ammonium acetate, pH = 7.0, flow rate 1.0 mL/min. This chromatogram was measured at 280 nm.

The above chromatogram was expected to have only one peak as the only compound it contains that absorbs at 280 nm is Azo. The second peak has the correct time for Azo under these conditions and so is labeled as 4-(phenylazo)benzoic acid. The first peak is hypothesized to be the *cis*- isomer of Azo as it has a slightly different time and absorption spectrum. The *trans*-isomer of 4-(phenylazo)benzoic acid has its highest peak at 325 nm with two smaller peaks at 231 nm and 427 nm[25]. In comparison the *cis*- isomer has 3 peaks at 251, 298 and 427 nm[25]. Previous standard injections did not detect this second peak but those chromatograms were measured at 325 nm, not 280 nm as in Figure 4-8. At 325 nm the first peak disappears as the *cis*-

isomer has its absorbance shifted away from 325 nm down to 298 nm. Figure 4-9 shows the same no-enzyme control in Figure 4-8 but measured at 325 nm instead of 280 nm.

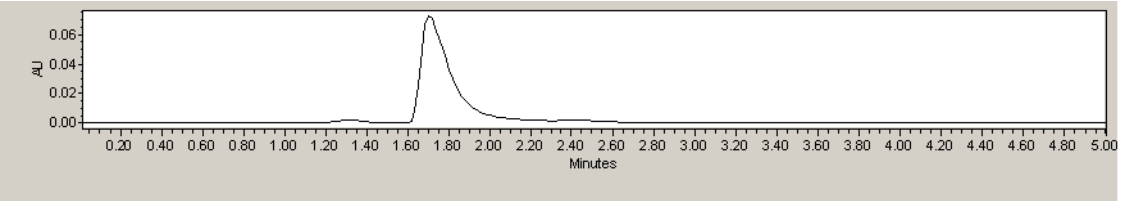

Figure 4-9: Chromatogram of the no-enzyme control in Figure 4-8 measured at 325 nm.

At 325 nm, a peak for 4-(phenylazo)benzoic acid at 1.8 minutes has an area of 436700 ± 900. A second peak appears at 1.3 minutes with an area of 13420 ± 90. In contrast these same peaks measured at 280 nm give areas of 111000 ± 700 and 51700 ± 300 for the 1.8 minute and 1.3 minute peaks, respectively. The shift in absorbance from 325 nm to 280 nm for the peak at 1.8 minutes corresponds to a loss of 75% of the peak area whereas for the peak at 1.3 minutes corresponds to an increase in peak area of 285%. This is a 3-fold increase in absorbance for the first peak and a 4-fold decrease for the second. This provides further evidence that the peak at 1.3 minutes corresponds to this *cis*- form of Azo and the peak at 1.8 minutes corresponds to the *trans*- form. Since Azo is the only compound in this solution that provides signatures in the 280-325 nm range the second peak is concluded to be the *cis*- form of Azo.

Figure 4-10 shows the chromatogram for the control with no hydrogen peroxide. The only difference between this chromatogram and the one in Figure 4-8 is the presence of SBP and absence of hydrogen peroxide. This difference has produced a third peak at around 0.99 minutes. Since SBP was absent in the previous control and present in this one this peak is likely SBP or impurities from the enzyme solution.

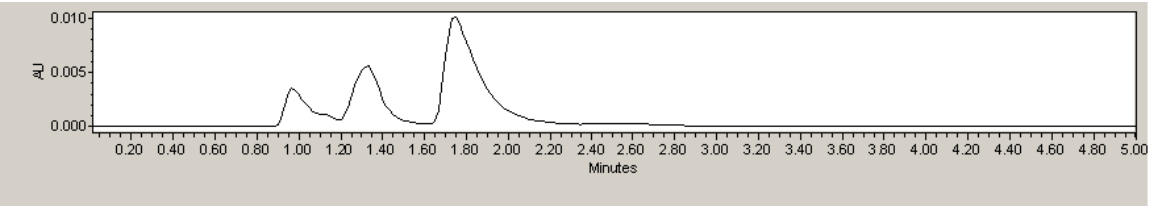

Figure 4-10: Chromatogram of a no-H_2O_2 control for a 4-(phenylazo)benzoic acid enzymatic conversion. The reaction conditions were 68.6 ± 0.7 µM Azo and 1 U/mL of SBP. The mobile phase was 40% ACN and 60% aqueous ammonium acetate, pH = 7.0, flow rate 1.0 mL/min. This chromatogram is measured at 280 nm.

The chromatogram for SBP-catalyzed reaction of Azo, Figure 4-11, shows the same 3 peaks as the no-hydrogen peroxide control. PABA if it existed would be expected to appear at 1.1 minutes and overlap with peaks at 0.99 and 1.3 minutes. A PABA peak corresponding to the expected concentration (4 ± 1 µM) should have an area of around 24000. This would be easily detectable despite the two unexpected peaks, one of enzyme impurities and the other of the *cis*- isomer of Azo. The expected concentration of PABA for this reaction is 4 ± 1 µM because 4 ± 1 µM of 4-(phenylazo)benzoic acid was lost during the reaction and azo-bond reduction is a 1:1 reaction. Aniline is also not detected at 3.3 minutes time so no direct evidence of azo-bond splitting has been found as neither product was detected. Aniline is a known substrate of SBP under the reaction conditions and is a light-sensitive substance so, at the low concentration in which it would have been produced, it is unlikely that detectable amounts of aniline would be found[24]. PABA however is very stable in water and detectable in the concentrations expected to be produced in this reaction. PABA is also expected to act as a substrate for SBP. It has not been documented, however, how good a substrate it is. Thus tests were next done to determine if PABA is a good enough substrate of SBP to polymerize under the conditions used for Azo, thus explaining its absence in the chromatogram of Figure 4-11.

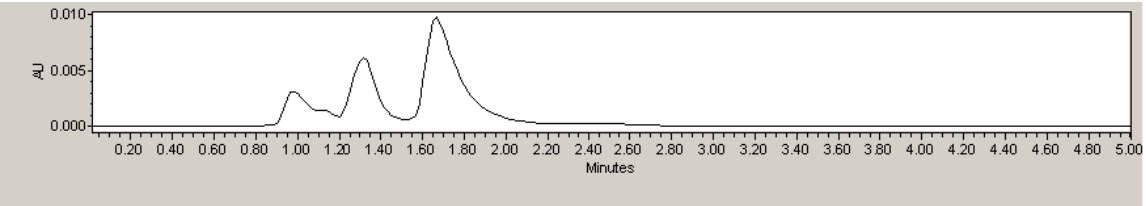

Figure 4-11: Chromatogram for a 4-(phenylazo)benzoic acid enzymatic conversion. The reaction conditions were 64.2 ± 0.7 µM Azo, 1 U/mL of SBP and 1.5 mM H_2O_2. The mobile phase was 40% ACN and 60% aqueous ammonium acetate, pH = 7.0, flow rate 1.0 mL/min. This chromatogram is measured at 280 nm.

4.5 Para-aminobenzoic acid enzymatic conversion:

PABA enzymatic conversion tests were done under the same conditions used above for Azo to determine if PABA was a substrate of the enzyme under those conditions and, if so, how much of it is polymerized over 24 h. Tests were initially done at higher concentrations of PABA than would occur in the 4-(phenylazo)benzoic acid SBP reaction. The concentration used was 0.5 mM PABA and varying amounts of enzyme and hydrogen peroxide always keeping the enzyme activity to hydrogen peroxide ratio at 1:1.5, the dimensions of which are U/mL and mM, respectively. The reaction was conducted in 5 mM pH=7.0 phosphate buffer for 24 h. The amount of PABA reacted is determined by the difference in the concentration of the experimental reactor and the average of the two controls. Results are given in Figure 4-12 and Table 4-3.

Table 4-3: Enzymatic Conversion tests for PABA using SBP and hydrogen peroxide.

	PABA in a control with no SBP (mM, mg)	PABA in a control with no hydrogen peroxide (mM, mg)	PABA in experimental reactor (mM, mg)	PABA reacted (mM, mg)
0.5 U/mL SBP and 0.75 mM H_2O_2	0.46 ± 0.05, 1.6 ± 0.2	0.45 ± 0.05, 1.5 ± 0.2	0.26 ± 0.06, 0.9 ± 0.2	0.20 ± 0.08, 0.7 ± 0.3
1 U/mL SBP and 1.5 mM H_2O_2	0.43 ± 0.04, 1.5 ± 0.1	0.47 ± 0.05, 1.6 ± 0.2	0.23 ± 0.07, 0.8 ± 0.2	0.22 ± 0.09, 0.8 ± 0.3
0.25 U/mL SBP and 0.375 mM H_2O_2	0.43 ± 0.05, 1.5 ± 0.2	0.44 ± 0.04, 1.5 ± 0.1	0.36 ± 0.04, 1.2 ± 0.1	0.08 ± 0.08, 0.3 ± 0.3

All tests had a reaction time of 24 hours and included 5 mM pH=7.0 phosphate buffer. PABA reacted is determined by the difference between the concentration in the experimental reactor and the average concentration of the two controls. Error in controls and the experimental is calculated from the standard deviation of triplicate HPLC injections using the formula, Standard error = (Standard deviation)/ √n, where n is the number of measurements (3). Error in the calculated values is calculated using the error propagation when subtracting values (for the difference of A-B $\sigma_{A+B} = \sqrt{(\sigma_A^2+\sigma_B^2)}$)

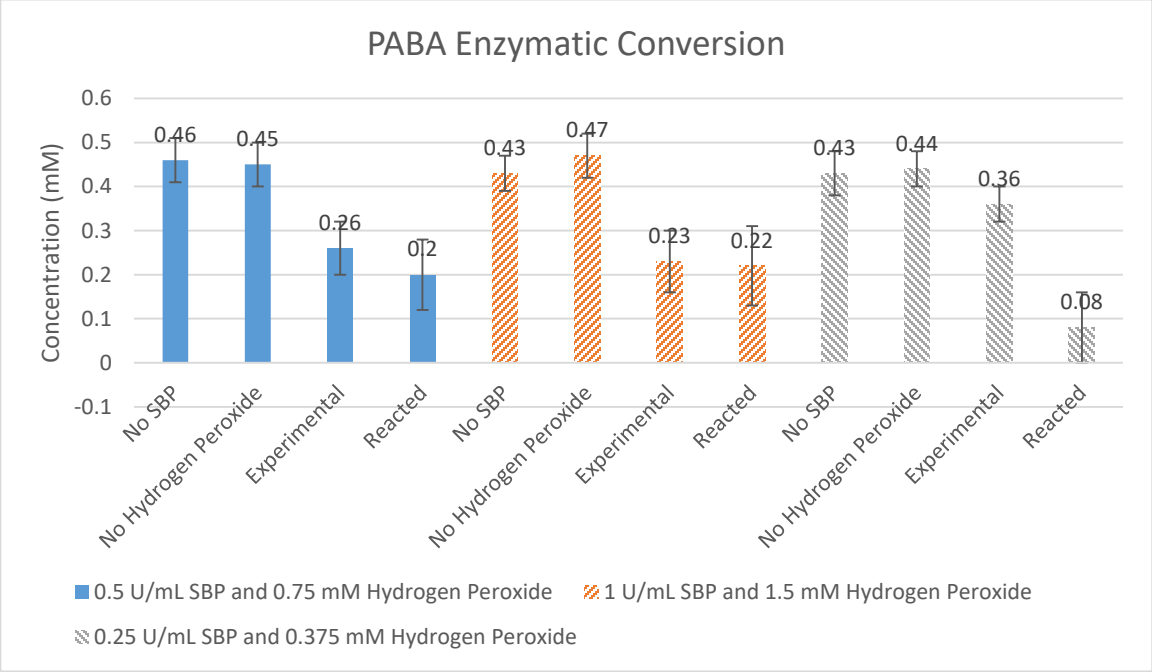

Figure 4-12: PABA enzymatic conversion test results of Table 4-3.

As seen from the above data, PABA is indeed a substrate of the enzyme. Of the concentrations tested, the best ratio of PABA concentration to SBP activity and H_2O_2 concentration gave a 49% conversion using 1 U/mL of SBP and 1.5 mM of hydrogen peroxide.

While the PABA tests did indeed show that PABA is capable of reacting under the conditions used for 4-(phenylazo)benzoic acid enzymatic conversion tests, it did not immediately produce an easily seen precipitate as SBP reactions usually do. A colour change was observed in the reaction from clear to an orange colour, however, which, with the decrease in PABA concentration, suggests that the polymers were formed and were still soluble in the reaction solution. The above test was then repeated without buffer with a starting pH of 4.29 ± 0.06 and after the reaction the solution was acidified by adding 200 uL of formic acid to give a pH of 2.24 ± 0.01 and filtered in an attempt to remove any polymers or oligomers that may have been formed during the reaction. This succeeded and almost all of the visible colour was removed and the solution looked clear. Results are shown in Table 4-4 and Figure 4-13.

Table 4-4: Experiments to confirm the polymerization of PABA by precipitating the polymer.

PABA in a control with no SBP (mM, mg)	PABA in a control with no hydrogen peroxide (mM, mg)	PABA in an experimental reactor before acidification (mM, mg)	PABA in an experimental reactor after acidification (mM, mg)	PABA reacted (mM, mg)
0.45 ± 0.05, 1.5 ± 0.2	0.42 ± 0.05, 1.4 ± 0.2	0.20 ± 0.08, 0.7 ± 0.3	0.20 ± 0.08, 0.7 ± 0.3	0.2 ± 0.1, 0.7 ± 0.3

PABA was polymerized by SBP using a 1:2:3 ratio of PABA concentration (mM) to enzyme concentration (U/mL) to hydrogen peroxide concentration (mM). The reaction was carried out for 24 h in water and then acidified with 200 uL of formic acid. The amount of PABA reacted is determined by the difference between the concentration in the experimental reactor and the average concentration of the two controls. Error in controls and the experimental is calculated from the standard deviation of triplicate HPLC injections using the formula, Standard error = (Standard deviation)/ √n, where n is the number of measurements (3). Error in the calculated values is calculated using the error propagation when subtracting values (for the difference of A-B $\sigma_{A+B} = \sqrt{(\sigma_A^2+\sigma_B^2)}$)

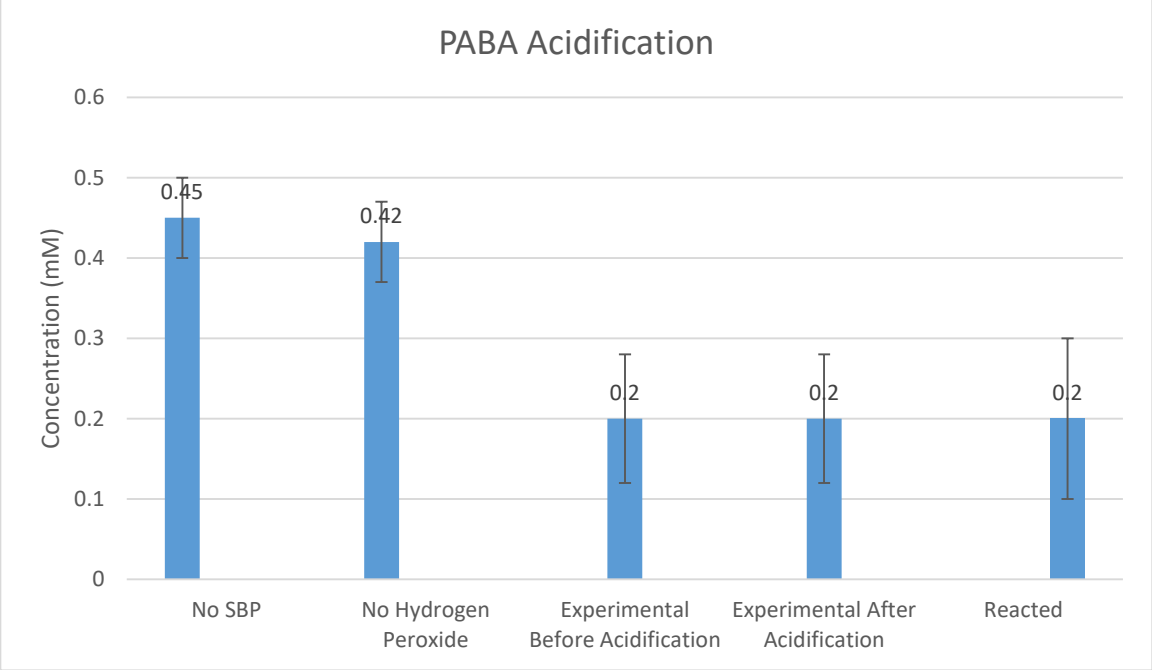

Figure 4-13: PABA acidification test results of Table 4-4.

The above evidence confirms that PABA does indeed react in the presence of SBP and hydrogen peroxide. This could explain the lack of PABA found in 4-(phenylazo)benzoic acid enzymatic conversion tests. Under the same conditions as used in the 4-(phenylazo)benzoic acid enzymatic

conversion tests (1 U/mL SBP, 1.5 mM hydrogen perozide, 5 mM pH=7.0 phosphate buffer for 24 h) PABA concentration decreased by 0.22 ± 0.09 mM. The projected maximum concentration that could be found in 4-(phenylazo)benzoic acid enzymatic conversion tests is equal to the loss in Azo due the azo-bond reduction reaction. The loss in previous tests of Azo was at most 4 µM so this small amount of PABA polymerizing before the solution was tested would explain its absence in any chromatograms.

Previous experiments done using SBP to polymerize compounds has shown some evidence that lower concentration substrates actually react worse than higher concentration substrates, the rationale being that the enzyme becomes inactivated by peroxide before the substrate is polymerized[26]. To test whether PABA acted the same way a lower concentration solution was measured. Two tests were done using a 61 and 7 µM PABA with 1 U/mL of SBP and 1.5 mM hydrogen peroxide concentrations in 5 mM phosphate buffer, pH = 7.0. The amount of PABA reacted was determined by taking the difference between the experimental reactor concentration and the average concentration of the two controls. The results from these tests are shown in Table 4-5 and Figure 4-14.

Table 4-5: Experiments to determine the extent of polymerization of PABA under lower concentration conditions.

PABA in a control with no SBP (µM, µg)	PABA in a control with no hydrogen peroxide (µM, µg)	PABA in an experimental reactor (µM, µg)	PABA reacted (µM, µg)
62 ± 1, 212 ± 3	61 ± 1, 209 ± 3	57 ± 1, 195 ± 3	5 ± 2, 17 ± 7
7 ± 2, 24 ± 7	7 ± 2, 24 ± 7	6.5 ± 2, 22 ± 7	0.5 ± 3, 2 ± 10

For each reaction 1 U/mL of SBP and 1.5 mM of H_2O_2 was used as well as 5 mM pH=7.0 phosphate buffer. The amount of PABA reacted is determined by the difference between the concentration in the experimental reactor and the average concentration of the two controls. Error in controls and the experimental is calculated from the standard deviation of triplicate HPLC injections using the formula, Standard error = (Standard deviation)/ √n, where n is the number of measurements (3). Error in the calculated values is calculated using the error propagation when subtracting values (for the difference of A-B $\sigma_{A+B} = \sqrt{(\sigma_A^2+\sigma_B^2)}$)

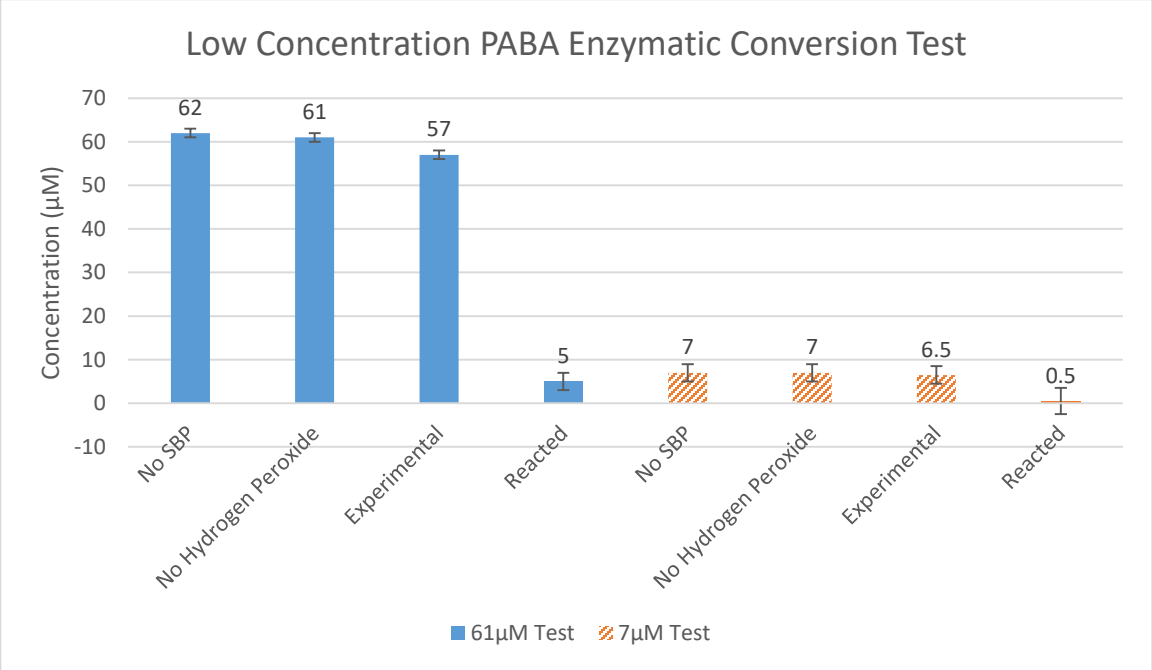

Figure 4-14: Low concentration PABA enzymatic conversion test results of Table 4-5.

It appears that under lower concentration conditions similar to the concentration of 4-(phenylazo)benzoic acid in previous tests, the percent of PABA lost throughout the reaction went down considerably. An earlier PABA test under the same conditions had an initial

concentration of 0.5 mM. The reacted concentration for this test was approximately 49% of the initial concentration compared to 8% with the 61 µM test. Likewise, the test done at 7 µM showed a 7% reaction under the same conditions. This may be due to the process of enzyme inactivation in the presence of excess hydrogen peroxide and very little reducing substrate. This small decrease in PABA concentration should mean that if Azo did react with SBP through the proposed mechanism of azo-bond reduction, PABA should be detectable in the products. This assumes that the presence of Azo does not change the conditions of the reaction. It is possible, however, that as a result of the process in which the azo-bond is reduced the reaction of its daughter compound could become more likely. This could explain its absence in the chromatogram.

The possibility of azo-bond reduction as a possible confounding factor in regards to the ad/absorption phenomenon of this study has been shown to account for little or none of the decrease of Azo concentration in the aqueous phase. Further experiments where there is a possibility of azo-bond reduction, a control without the addition of phenol have been run. This control would give the maximum loss of Azo due to azo-bond reduction possible as under the experimental reactor conditions Azo would be in competition with phenol for interaction with SBP thus decreasing the amount of azo-bond reduction occurring. Any concentration of Azo said to be ab/adsorbed in this study is corrected against any decrease of concentration in the no-phenol control.

4.6 Low concentration 4-(phenylazo)benzoic acid enzymatic conversion:

The lack of conversion in 24 h through the SBP reaction for 4-(phenylazo)benzoic acid was originally thought to be due to Azo's poor quality as a substrate for SBP. Another possibility is that it could actually be due to the low concentration compared to the relatively high enzyme

and hydrogen peroxide concentrations that were used in the preliminary tests, thus leading to SBP inactivation by peroxide. To help ascertain the quality of Azo as a substrate under reaction conditions a test was conducted with lower concentrations of SBP and hydrogen peroxide. The test was done with 32 µM 4-(phenylazo)benzoic acid, 40 mU/mL of SBP and 60 µM hydrogen peroxide in 5 mM phosphate buffer, pH = 7.0. The amount of Azo reacted is determined to be the difference between the concentration of Azo in the experimental reactor and the average of the concentrations of the two controls. The results are shown in Table 4-6 and Figure 4-15.

Table 4-6: Experiments to determine the quality of 4-(phenylazo)benzoic acid as a substrate of SBP under low enzyme and hydrogen peroxide conditions.

Azo in a control with no SBP (µM, µg)	Azo in a control with no hydrogen peroxide (µM, µg)	Azo in an experimental reactor (µM, µg)	Azo reacted (µM, µg)
32.7 ± 0.2, 184 ± 1	32.9 ± 0.2, 186 ± 1	32.8 ± 0.2, 186 ± 1	0 ± 0.3, 0 ± 2

The test contained 40 mU/mL of SBP, 60 mM of hydrogen peroxide and 5 mM of pH = 7.0 phosphate buffer. The amount of Azo reacted is determined by the difference between the concentration in the experimental reactor and the average concentration of the two controls. Error in controls and the experimental is calculated from the standard deviation of triplicate HPLC injections using the formula, Standard error = (Standard deviation)/ √n, where n is the number of measurements (3). Error in the calculated values is calculated using the error propagation when subtracting values (for the difference of A-B $\sigma_{A+B} = \sqrt{(\sigma_A^2+\sigma_B^2)}$)

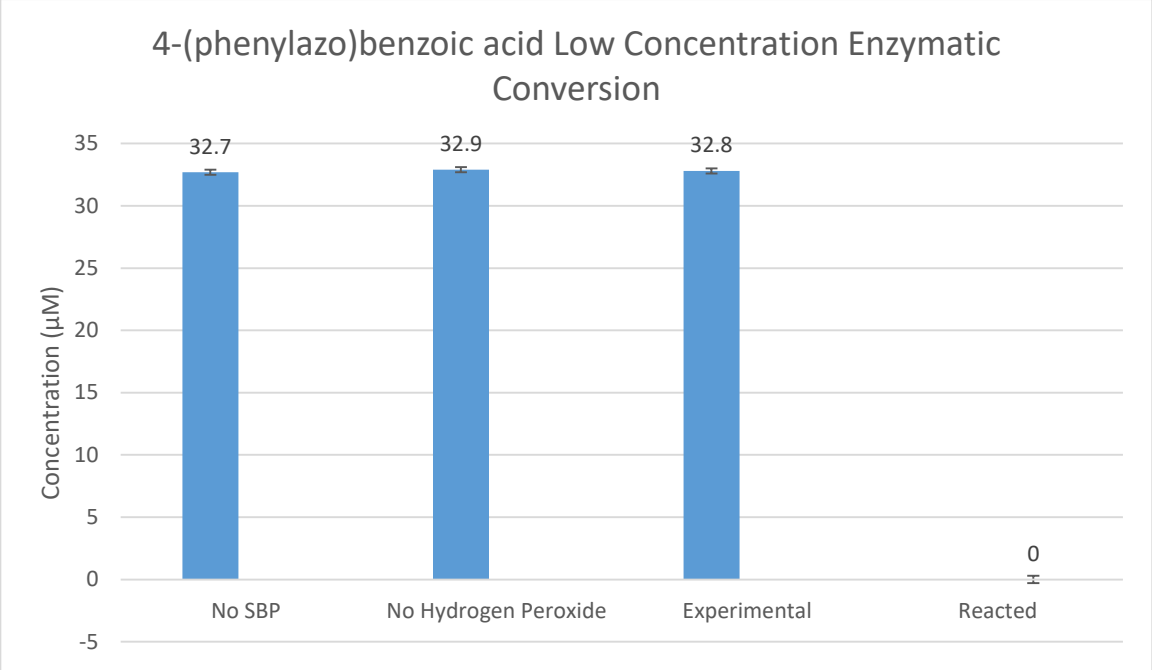

Figure 4-15: 4-(phenylazo)benzoic acid low concentration enzymatic conversion test results of Table 4-6.

As seen from the above table, the lower SBP and hydrogen peroxide concentrations did not increase the percentage of Azo that reacted. It in fact decreased to approximately 0 µM reacted. This shows that 4-(phenylazo)benzoic acid was not failing to react due to excess enzyme in previous tests and, therefore, under the reaction conditions Azo is a poor substrate of the enzyme.

4.7 Initial static adsorption tests:

To determine the effectiveness of phenolic polymer in capturing 4-(phenylazo)benzoic acid both dynamic and static tests were conducted. A dynamic absorption test is defined to be the absorption of 4-(phenylazo)benzoic acid during a phenol polymerization reaction and a static test is defined as adsorption after the phenol polymerization reaction was complete. To perform a static analysis, a large quantity of phenolic precipitates was made beforehand using 10 mM phenol, 10 U/mL of enzyme and 15 mM hydrogen peroxide with no buffer. This resulting

suspension, at 1.4 ± 0.4 mg/mL by dry weight determination, was tested for enzyme activity and hydrogen peroxide concentration 24 h after the reaction had started. Residual enzyme activity was found to be 0.12 ± 0.01 U/mL with no remaining hydrogen peroxide. Static tests were then performed by taking 22.5 mL of a known concentration of 4-(phenylazo)benzoic acid with 5 mM phosphate buffer pH=7.0 and adding 2.5 mL of suspension. With the 1 in 10 dilution, the resulting phenolic precipitate mass should be comparable to any dynamic tests done with 1 mM of phenol as performed earlier. With a residual enzyme activity of 0.012 ± 0.001 U/mL in the reaction solution and no hydrogen peroxide remaining, any loss of Azo would be due to adsorption onto the phenolic precipitates and not because of 4-(phenylazo)benzoic acid reacting with SBP. Initial static tests were conducted using the above protocol and left stirring for 24 h. The difference in concentration between the control and experimental is defined as the amount adsorbed, as shown in Table 4-7 and Figure 4-16. From the dry weight of the phenolic polymer solids found in later tests, the adsorption of Azo in this test amounts to 4 ± 2 mg/g (the theoretical adsorption is calculated using dry weights determined later in the static Langmuir analysis, since the same phenolic polymer suspension was used for both experiments).

Table 4-7: Initial static adsorption tests.

Azo in a control with no precipitate (µM, µg)	Azo in an experimental reactor (µM, µg)	Azo adsorbed (µM, µg)
36.1 ± 0.2, 204 ± 1	33.8 ± 0.2, 191 ± 1	2.3 ± 0.3, 13 ± 2

The experimental reactor contains 22.5 mL of a known concentration 4-(phenylazo)benzoic acid and 5 mM phosphate buffer pH = 7.0. 2.5 mL of phenolic precipitate suspension is then added to the solution and left for 24 h. The difference in concentration between the control and experimental is defined as the amount adsorbed. Error in controls and the experimental is calculated from the standard deviation of triplicate HPLC injections using the formula, Standard error = (Standard deviation)/ √n, where n is the number of measurements (3). Error in the calculated values is calculated using the error propagation when subtracting values (for the difference of A-B $\sigma_{A+B} = \sqrt{(\sigma_A^2+\sigma_B^2)}$)

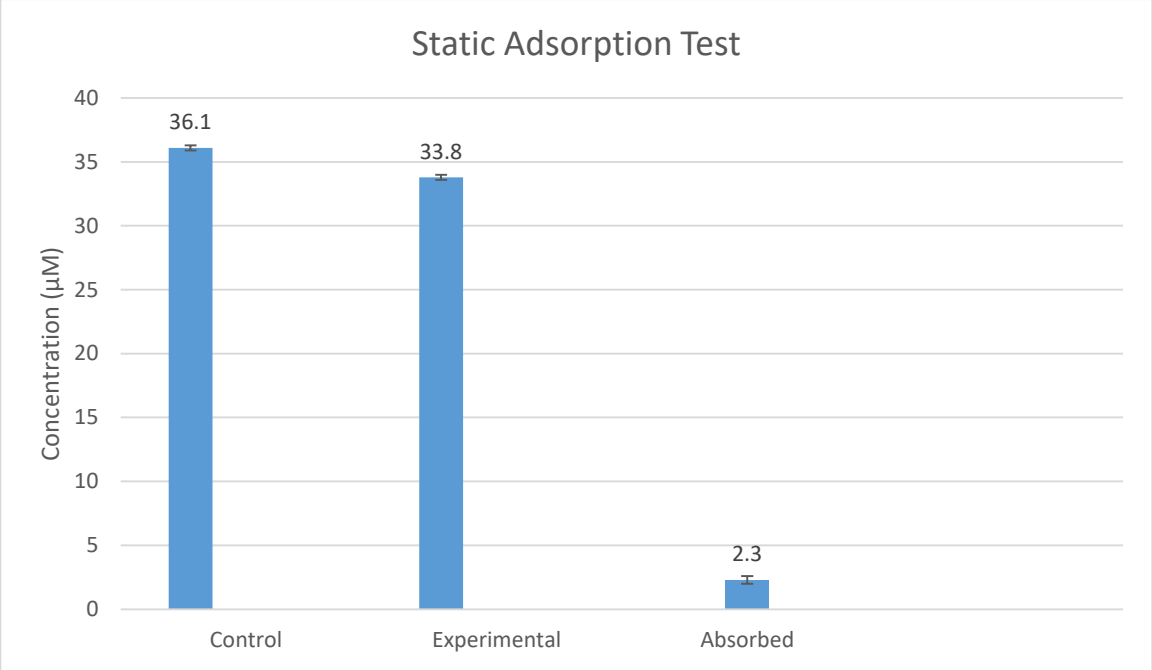

Figure 4-16: Initial static adsorption test results of Table 4-7.

4.8 Extraction of 4-(phenylazo)benzoic acid from phenolic precipitates:

Since the foregoing results are indirect, to gain further evidence of the loss of 4-(phenylazo)benzoic acid due to adsorption/absorption onto the phenolic precipitates, an extraction test was performed. This test involved attempting to extract any ab/adsorbed 4-(phenylazo)benzoic acid from the phenolic precipitate by suspending it in acetonitrile. The concentration of Azo in the acetonitrile would then be measured by HPLC. The extraction test was done by centrifuging a dynamic or static reaction suspension at 4000 rpm for 20 minutes and removing the supernatant. The pellet was then re-suspended in 25 mL of acetonitrile and allowed to stir for 30 minutes. The acetonitrile solution was then tested for Azo and any phenolic precipitates were removed. A dynamic test was done using 1 mM phenol, 1 U/mL SBP, 1.5 mM hydrogen peroxide, 5 mM phosphate buffer pH = 7.0 and 40 µM 4-(phenylazo)benzoic acid. A control with no phenol was introduced to determine any possible loss of 4-(phenylazo)benzoic acid due to reaction with SBP. The solution was left for 24 h and centrifuged

and re-suspended by the process described above. The amount of Azo lost is defined as the difference between the concentration in the experimental reactor and the control which contains no phenol. The results are in Table 4-8 and Figure 4-17.

Table 4-8: Dynamic absorption extraction test.

Azo in a control with no SBP (µM, µg)	Azo in a control with no phenol (µM, µg)	Azo in an experimental reactor (µM, µg)	Azo lost (µM, µg)	Azo recovered in acetonitrile (µM, µg)
40.7 ± 0.2, 230 ± 1	39.6 ± 0.2, 224 ± 1	35.4 ± 0.2, 200 ± 1	4.2 ± 0.3, 24 ± 2	6.5 ± 0.4, 37 ± 2

The test was done using 1 U/mL of SBP, 1.5 mM H_2O_2, 1 mM phenol and 5 mM pH = 7.0 phosphate buffer. The amount of Azo lost is defined as the difference between the concentration in the experimental reactor and the control which contains no phenol. Error in controls and the experimental is calculated from the standard deviation of triplicate HPLC injections using the formula, Standard error = (Standard deviation)/ √n, where n is the number of measurements (3). Error in the calculated values is calculated using the error propagation when subtracting values (for the difference of A-B $\sigma_{A+B} = \sqrt{(\sigma_A^2+\sigma_B^2)}$)

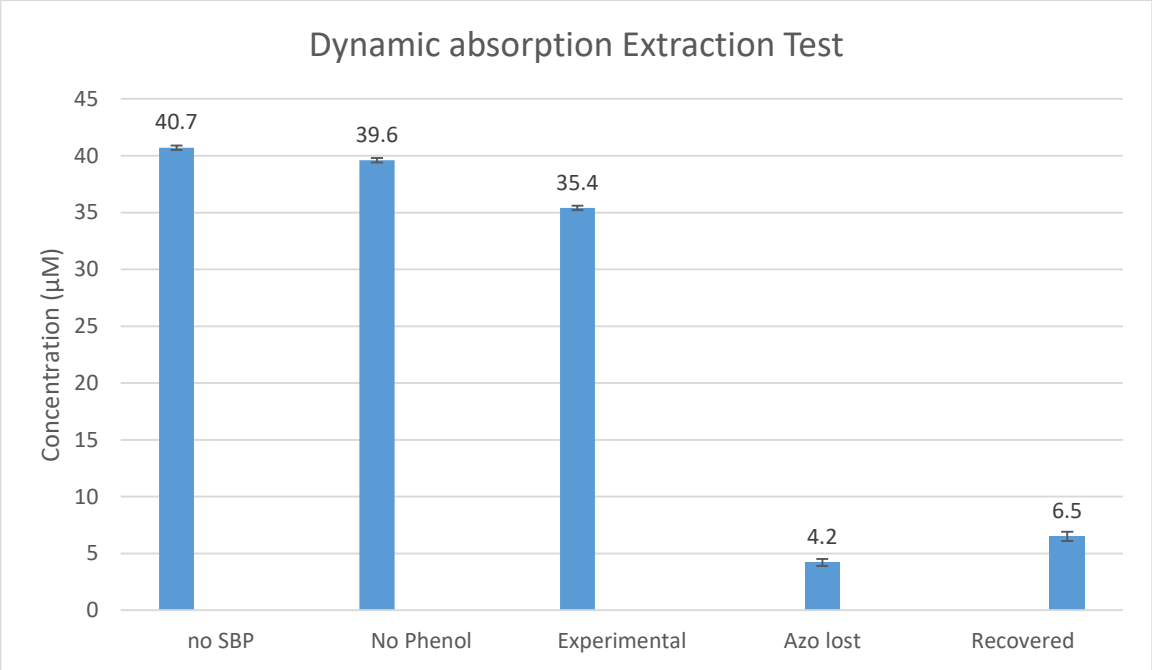

Figure 4-17: Dynamic absorption extraction test results of Table 4-8.

The concentration of Azo found in the dynamic test was too high, since it exceeded the amount of Azo concentration reacted. This may be due to the extracted pellet still being “wet” when it is suspended in acetonitrile. Some small amount of water with residual amounts of Azo would be transferred along with the pellet into the acetonitrile mixture thereby increasing the amount of Azo found in the system. The exact amount of solution transferred along with the pellet is unknown but it amounts to approximately 2 mL. 2 mL of the solution would increase the concentration of the acetonitrile solution by 2.8 µM. If the pellet contained 4.2 ± 0.3 µM of Azo the additional 2.8 µM would account for the increase to 6.5 ± 0.4 µM in recovered Azo. To deal with this excess Azo, a wash step may have been added to the extraction test where the phenolic precipitates could have been washed with water to decrease the concentration of soluble Azo. This however runs the risk of loss of phenolic precipitates with each wash step and was not conducted.

A static extraction test was also done using 2.5 mL of phenolic precipitate suspension and a solution of 22.5 mL of 5 mM phosphate buffer pH=7.0 and 86 µM 4-(phenylazo)benzoic acid. The reaction mixture was left for 24 h then the centrifugation pellet was re-suspended using the same methods as before in acetonitrile. The amount of azo lost is the difference between the concentration of the experimental and control reactors. The results are shown in Table 4-9 and Figure 4-18.

Table 4-9: Static adsorption extraction test.

Azo in a control with no suspension (µM, µg)	Azo in an experimental reactor (µM, µg)	Azo lost (µM, µg)	Azo recovered in acetonitrile (µM, µg)
86.7 ± 0.2, 490 ± 1	81.6 ± 0.2, 461 ± 1	5.1 ± 0.2, 29 ± 1	5.8 ± 0.4, 33 ± 2

The test was done using 2.5 mL of suspension and 22.5 mL of 5 mM phosphate buffer pH = 7.0 and a known concentration of 4-(phenylazo)benzoic acid. The amount of azo lost is the difference between the concentration of the experimental and control reactors. Error in controls and the experimental is calculated from the standard deviation of triplicate HPLC injections using the formula, Standard error = (Standard deviation)/ √n, where n is the number of measurements (3). Error in the calculated values is calculated using the error propagation when subtracting values (for the difference of A-B $\sigma_{A+B} = \sqrt{(\sigma_A^2+\sigma_B^2)}$)

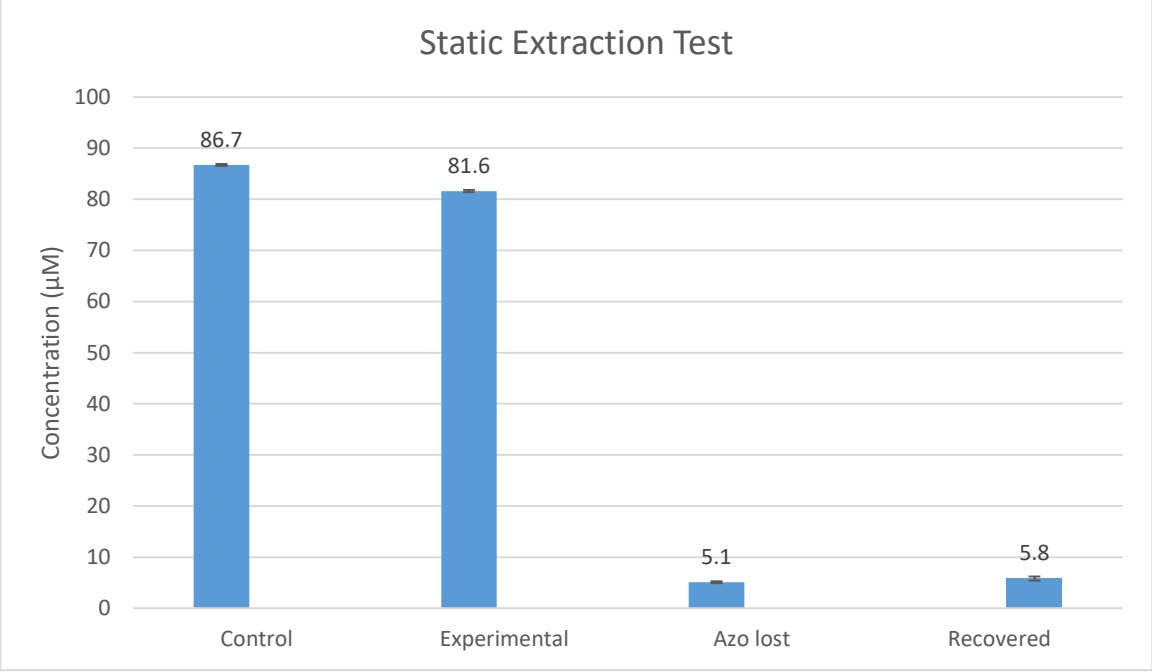

Figure 4-18: Static extraction test results of Table 4-9.

The concentration of Azo found in acetonitrile was again slightly higher than the concentration of Azo calculated to have been adsorbed onto the precipitate. This is likely due to the same reason as mentioned for the dynamic test above. The pellet for the static test was better formed

and so less water was transferred along with the pellet (less than 0.5 mL). The mass of the precipitate was measured in both the experimental and the acetonitrile solutions as some precipitate was lost during centrifugation, Table 4-10.

Table 4-10: Dry mass of the suspension before and after extraction.

Mass in experimental precipitate (mg)	Mass in acetonitrile precipitate (mg)
4.2 ± 0.6	3.4 ± 0.3

Error is calculated from the standard deviation of triplicate runs using the formula Standard error = (Standard deviation)/ √n where n is the number of measurements (3).

The theoretical mass of precipitate from 1 mM phenol at 100% conversion is 2.35 mg. This is less than the values found in the dry weight determinations above, this is likely due to impurities in the SBP solution used to make the phenolic precipitates as well as the mass of the enzyme itself. For the purposes of this study the dry weight is assumed to be the mass of the phenolic precipitates only.

As shown, some precipitate was lost during the transfer so this extraction method does not prove the ab/adsorption of 4-(phenylazo)benzoic acid onto the precipitate both due to the loss in precipitate between transfers and due to the pellet being wet as discussed earlier. The loss of precipitate would cause a decrease in the measured concentration of Azo recovered in acetonitrile while at the same time the pellet being wet would cause an increase. Both of these factors cause the measured concentration of Azo in acetonitrile to be different than the concentration of Azo suspected to have ab/adsorbed onto the phenolic precipitates. Because the concentration measured during this experiment is not equal to the concentration of Azo ab/adsorbed, this extraction cannot be used as proof of the ab/adsorption of 4-(phenyazo)benzoic acid onto phenolic precipitates. This along with the following Langmuir analysis, however, supports the hypothesis that 4-(phenylazo)benzoic acid is being ab/adsorbed in these reactions by the precipitate.

4.9 Langmuir static analysis:

To determine the favourability of the adsorption of 4-(phenylazo)benzoic acid onto phenolic precipitates a Langmuir analysis was done using variable concentrations of 4-(phenylazo)benzoic acid from 4 µM to 88 µM with an equilibration time of 24 h. A linearization of the Langmuir analysis was done first using the equation $C_e/Q_e = 1/(Q_m*K) + C_e/Q_m$ where C_e is the equilibrium concentration of the adsorbate free in solution (mg/L), Q_e is the solid phase concentration at equilibrium (mg/g), Q_m is the maximum absorption capacity (mg/g) and K is the Langmuir absorption constant. Table 4-11 below shows the values used in this linear plot and the linearization itself is shown in Figure 4-19. The half-saturation value can be determined by taking the inverse of the Langmuir constant K. The values used in the Langmuir analysis were chosen so that at least 2 values appear both above and below the half-saturation point. While the Langmuir analysis is reported for both the linear and direct-fit forms only the direct-fit was used to determine if these 2 points are in the correct locations.

Table 4-11: Data for the Langmuir analysis of static adsorption shown in Figure 4-19.

C_e (mg/L)	C_e/Q_e (g/L)
0.72 ± 0.04	0.5 ± 0.2
1.5 ± 0.04	0.7 ± 0.3
3.00 ± 0.04	0.6 ± 0.2
8.13 ± 0.04	1 ± 0.4
18.36 ± 0.04	1.6 ± 0.7

Each row corresponds to the x and y values for a single point used in the linearized Langmuir analysis in Figure 4-19. C_e is the equilibrium concentration of the adsorbate (mg/L) and Q_e is the solid phase equilibrium concentration (mg/g) which is calculated from the difference between the equilibrium concentration in solution and the initial concentration factoring in the precipitate mass. The reaction time is 24 h. Error is calculated from the standard deviation of triplicate HPLC injections using the formula Standard error = (Standard deviation)/ √n where n is the number of measurements (3).

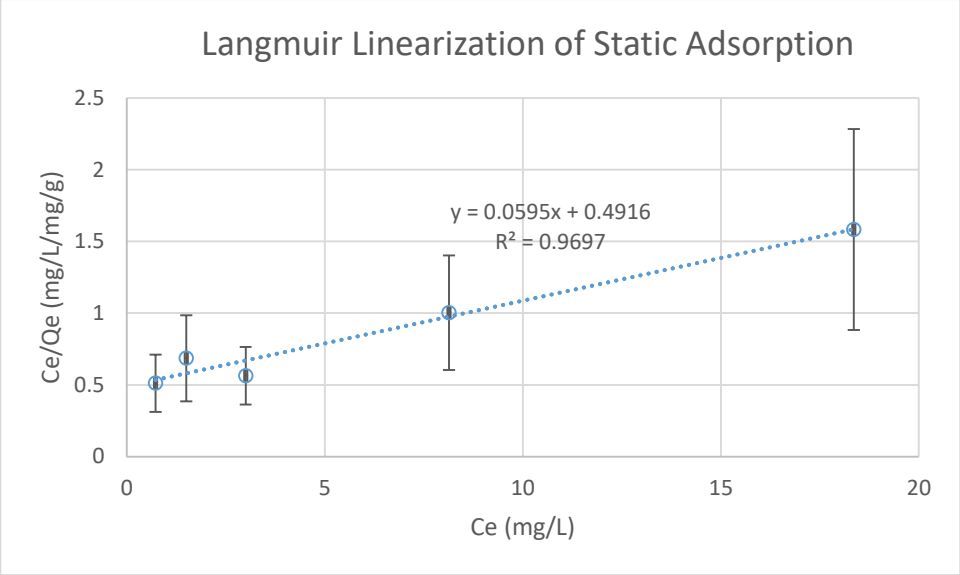

Figure 4-19: Linearized Langmuir analysis of 4-(phenylazo)benzoic acid adsorption onto phenolic precipitates under static conditions data Table 4-11.

The Langmuir isotherm can be fitted to the above linear equation where the slope is $1/Q_m$ and the y-intercept is $1/Q_m*K$. From this the values for the adsorption capacity (Q_m) and the Langmuir adsorption constant (K) were determined to be 17 ± 2 mg/g and 0.121 ± 0.002 L/mg respectively. The half-saturation point for the linearization was determined to be 8.3 ± 0.1 mg/L. After the preliminary check using a linear model, the data were directly fit to the standard Langmuir isotherm model using the equation $\Theta=KC_e/(1+KC_e)$ where theta is the adsorption capacity at equilibrium divided by the maximum adsorption capacity (Q_e/Q_m), K is the Langmuir adsorption constant and C_e is the equilibrium concentration of the adsorbate (mg/L). Table 4-12 below shows the values from the same measurements as Table 4-11 used in the direct fit to the Langmuir curve and the direct fit itself is shown in Figure 4-20.

Table 4-12: Data for the Langmuir analysis of static adsorption shown in Figure 4-20.

C_e (mg/L)	Q_e (mg/g)
0.72 ± 0.04	1.4 ± 0.6
1.50 ± 0.04	2.2 ± 0.9
3.00 ± 0.04	5 ± 2
8.13 ± 0.04	8 ± 3
18.36 ± 0.04	12 ± 4

Each row corresponds to the x and y values of a single point used in Figure 4-20. C_e is the equilibrium concentration of the adsorbate (mg/L) and Q_e is the solid phase equilibrium concentration (mg/g) which is calculated from the difference between the equilibrium concentration in solution and the initial concentration factoring in the precipitate mass. The reaction time was 24 h. Error is calculated from the standard deviation of triplicate HPLC injections using the formula Standard error = (Standard deviation)/ √n where n is the number of measurements (3).

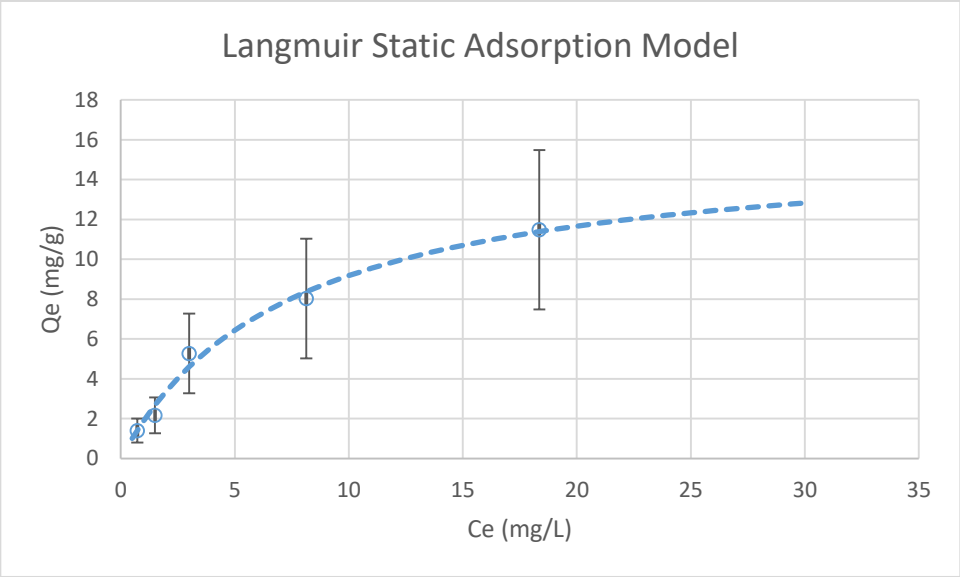

Figure 4-20: Langmuir analysis of 4-(phenylazo)benzoic acid adsorption onto phenolic precipitates under static conditions, data of Table 4-12.

From Figure 4-20, the R^2 value obtained was 0.9880 and the values for Qm and K were determined to be 16.0 ± 0.5 mg/g and 0.13 ± 0.01 L/mg, respectively. The half-saturation point for the direct fit was determined to be 7.6 ± 0.6 mg/L, thus at least 2 points are above and below this value. A better fit for the data was obtained using the direct fit to the Langmuir model than

the linearization and so the constants determined from it will be considered the more accurate ones. The equation for this Langmuir analysis of a static system is shown below.

$$\frac{Q_e}{16} = \frac{0.13C_e}{1 + 0.13C_e}$$

4.10 Langmuir dynamic analysis:

To determine the effective difference between a static and a dynamic system a Langmuir analysis was done on a dynamic system as well. The dynamic system used Azo concentrations between 4 µM and 80 µM for 4-(phenylazo)benzoic acid. A linearization of the Langmuir analysis was done first using the equation $C_e/Q_e = 1/(Q_m*K) + C_e/Q_m$ where C_e is the equilibrium concentration of the absorbate (mg/L), Q_e is the absorption capacity at equilibrium (mg/g), Q_m is the maximum absorption capacity (mg/g) and K is the Langmuir absorption constant. The standard model of the Langmuir analysis was also graphed using the equation $\Theta=KC_e/(1+KC_e)$ where theta is the adsorption capacity at equilibrium divided by the maximum adsorption capacity (Q_e/Q_m) and K and C_e are the same as above. Table 4-11 below shows the values used in this linearization and the linear plot is shown in Figure 4-21.

Table 4-13: Data for the Langmuir analysis of dynamic absorption in Figure 4-21.

C_e (mg/L)	C_e/Q_e (g/L)
0.72 ± 0.04	0.21 ± 0.03
1.39 ± 0.04	0.25 ± 0.03
2.72 ± 0.04	0.25 ± 0.03
12.56 ± 0.04	0.51 ± 0.06
18.19 ± 0.04	0.56 ± 0.06

Each row corresponds to the x and y values of a single point used in Figure 4-21 C_e is the equilibrium concentration of the adsorbate (mg/L) and Q_e is the solid phase equilibrium concentration (mg/g) which is calculated from the difference between the equilibrium concentration in solution and the initial concentration factoring in the precipitate mass. The reaction time was 24 h. Error is calculated from the standard deviation of triplicate HPLC injections using the formula Standard error = (Standard deviation)/ √n where n is the number of measurements (3).

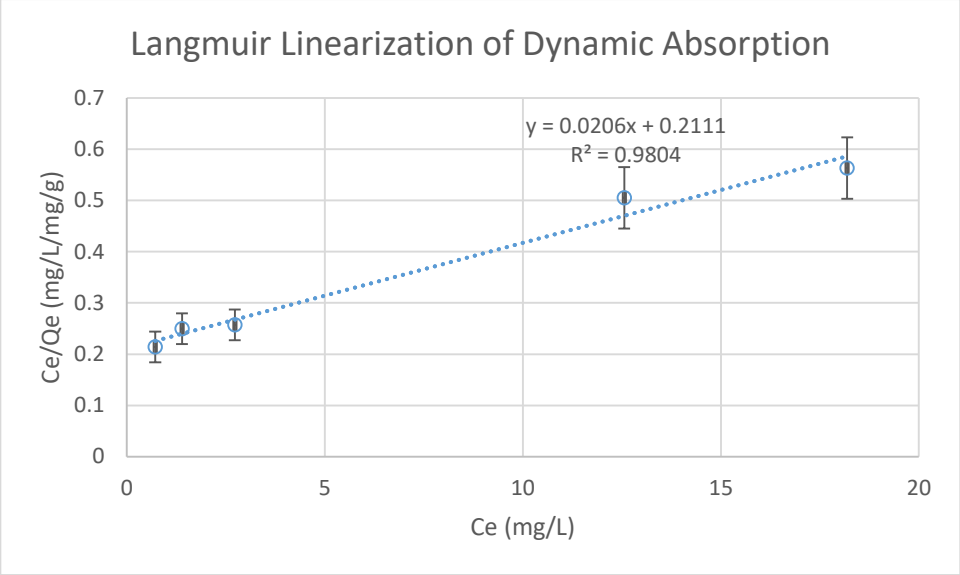

Figure 4-21: Linearized Langmuir analysis of 4-(phenylazo)benzoic acid absorption onto phenolic precipitates under dynamic conditions, data Table 4-13.

The Langmuir isotherm can be fitted to the above linear equation where $m=1/Q_m$ and $b = 1/Q_m*K$. From this the values for the adsorption capacity (Q_m) and the Langmuir adsorption constant (K) were determined to be 48 ± 4 mg/g and 0.098 ± 0.001 L/mg respectively. The half-saturation point for the linearization was determined to be 10.2 ± 0.1 mg/L thus, at least 2 values are above and below. Table 4-14 below shows the values used in this direct fit and the direct fit itself is shown in Figure 4-22.

Table 4-14: Data for the Langmuir analysis of dynamic absorption shown in Figure 4-22.

C_e (mg/L)	Q_e (mg/g)
0.71 ± 0.04	3.3 ± 0.4
1.39 ± 0.04	5.6 ± 0.6
2.72 ± 0.04	10 ± 1
12.56 ± 0.04	25 ± 3
18.19 ± 0.04	32 ± 4

Each row corresponds to the x and y values of a single point used in Figure 4-22. C_e is the equilibrium concentration of the adsorbate (mg/L) and Q_e is the solid phase equilibrium concentration (mg/g) which is calculated from the difference between the equilibrium concentration in solution and the initial concentration factoring in the precipitate mass. The reaction time was 24 hours. Error is calculated from the standard deviation of triplicate HPLC injections using the formula Standard error = (Standard deviation)/ √n where n is the number of measurements (3).

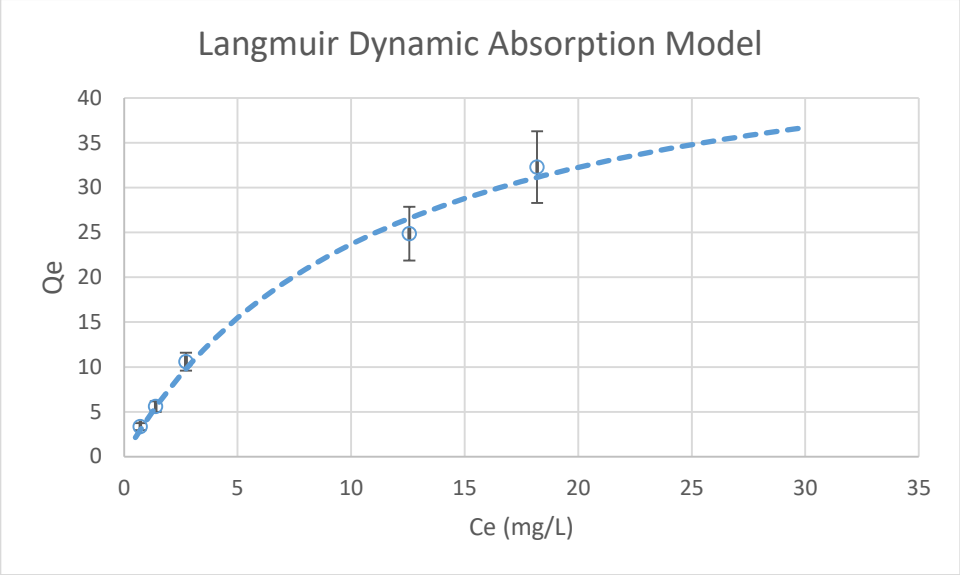

Figure 4-22: Langmuir analysis of 4-(phenylazo)benzoic acid absorption onto phenolic precipitates under dynamic conditions, data Table 4-14.

From Figure 4-22 the R^2 value obtained was 0.9922 and the values for Q_m and K were determined to be 51 ± 3 mg/g and 0.088 ± 0.008 L/mg, respectively. A better fit for the data was obtained using the direct fit to the Langmuir model than the linearization and so the constants determined from it will be used as the more accurate ones. The half saturation point is at C_e=11 ± 1 mg/L, thus at least 2 data points are above and below it. This helps verify the validity of the fit. The equation for the dynamic system is shown below.

$$\frac{Q_e}{51} = \frac{0.088C_e}{1 + 0.088C_e}$$

There is a large difference between the size of the error in the static and dynamic tests. The large amount of error in the static test comes from the measurement of precipitate dry mass. When comparing the error for the precipitate dry mass in the static test to the same error in the dynamic test the applicable numbers are ± 1 and ± 0.2, respectively. This is the only major difference in error between the two tests and so is the cause of the large difference in the final

error for the Langmuir analyses. All measured values for both tests are given in Tables C-1 and C-2 in Appendix C.

Chapter 5

5. Discussion:

5.1 pKa Determination:

The purpose of this study was to construct a model system for the adsorption of various organic compounds onto phenolic precipitates to expand the use of SBP as a wastewater treatment method. 4-(phenylazo)benzoic acid was chosen as a model compound since it was considered a non-substrate of the enzyme that was moderately hydrophobic and has a strong absorbance that could be easily detected allowing for high sensitivity in measurements. The moderate hydrophobicity of Azo was important for this study as a compound was needed that both dissolved in appreciable quantities in aqueous solution but also adsorbed in appreciable quantities to phenolic precipitates. Since the equilibrium between adsorbed and dissolved quantities of Azo was to be studied both adsorbed and dissolved quantities needed to be large enough to be accurately measured. For the purpose of concentration measurements the absorbance of the compound needed to be consistent, thus the pKa of the compound had to be determined. Knowing the pKa would allow the use of buffers to ensure either the complete protonation or deprotonation of 4-(phenylazo)benzoic acid in all subsequent tests which is important as the presence or absence of a proton would change the absorbance spectrum of Azo. Early measurements of the absorption spectra of Azo shown in Figures 4-1 and 4-2 gives the same wavelength of maximum absorption (325 nm) but with a two-fold difference in the intensity of that absorption (the conjugate base having the higher absorbance). The pKa was determined to be 2.45 ± 0.03 by the standard deviation of the titration curve fit of 4-(phenylazo)benzoic acid with concentrated sodium hydroxide shown in Figure 4-3. Comparing the pKa value for Azo to the value of the pKa for benzoic acid of 4.19 [29] it shows Azo to be more

acidic. This follows directly from the difference in structure between Azo and benzoic acid as the difference between the two is the addition of an azo-bond to the ring system. An azo-bond is a strong electron withdrawing group (EWG) which stabilizes the acceptance of a negative charge in the conjugate base. Azo would therefore have a higher acidity than benzoic acid due to the addition of the azo-bond. After the determination of the pKa, 5 mM phosphate buffer pH = 7.0 was used as the aqueous part of the mobile phase for HPLC measurements. This pH would cause Azo to be completely deprotonated thus giving consistent absorbance readings.

To ensure a consistent absorbance spectrum of Azo for these tests we might have alternatively used an isosbestic wavelength. If an isosbestic wavelength existed it would not have been at this compound's maximum wavelength of absorbance because the intensity at that wavelength varied between acidic and basic conditions (Figure 4-1 and 4-2). The maximum wavelength of absorbance was used because it gave a higher extinction coefficient allowing lower concentrations of Azo to be measured accurately.

5.2 Initial dynamic absorption tests:

Initial dynamic adsorption tests were done for the disappearance of Azo during *in situ* polymerization of phenol. These initial tests as shown in Table 4-1 and Figure 4-5 report moderate decreases in 4-(phenylazo)benzoic acid concentration of approximately 15% from 27.6 µM in the aqueous phase. Only 6% or 4.03 mg/g loss could be said to have come from absorption onto the phenolic precipitates however as a 9% or 6.26 mg/g loss was found in a reactor with no phenolic precipitates (mg of Azo per gram of phenolic precipitate). These values are theoretical values as the mass of phenolic precipitate here was not measured. The values are calculated by assuming 95% conversion of 1 mM of phenol into phenolic precipitate. A possible

reason for the loss of 4-(phenylazo)benzoic acid without the presence of phenolic precipitates could be SBP-catalyzed azo-bond reduction[21].

5.3 4-(phenylazo)benzoic acid enzymatic conversion:

Previous studies[21] have shown azo-bond reductive cleavage in the presence of SBP. How the azo-bond reduction occurs exactly is explained in more detail in the literature review section but, for the purposes of this study, the production of Azo's daughter compounds in this reaction is what were looked into. Azo-bond reduction would result in two compounds: *p*-aminobenzoic acid and aniline. Both of these compounds are substrates of SBP so the production of these compounds could lead not only to the decrease in Azo concentration without the formation of polymeric precipitates, but it could also provide a pathway for the enzymatic conversion of any PABA or aniline produced. Neither PABA nor aniline were found during 4-(phenylazo)benzoic acid enzymatic conversion reactions shown in Table 4-2 and Figure 4-6 but a small loss, 4-6% (of around 50 µM), of 4-(phenylazo)benzoic acid concentration after 24 h was observed in the presence of SBP and hydrogen peroxide.

5.4 p-aminobenzoic acid enzymatic conversion:

To justify the loss of 4-(phenylazo)benzoic acid by enzymatic conversion and the absence of any daughter compounds (PABA or aniline) in the reaction solution, a test of PABA as a substrate of SBP was conducted. Aniline was not tested as aniline is light-sensitive and for the reaction time of 24 h it was not guaranteed to remain in any measurable concentration[24]. PABA tests were conducted on high and low concentrations of PABA to determine the effectiveness of the enzymatic conversion. Initial tests were done at 0.5 mM of PABA with differing concentrations of SBP and hydrogen peroxide, shown in Table 4-3 and Figure 4-12. These tests showed a loss of 49% under the same reaction conditions that were used with 4-(phenylazo)benzoic acid

enzymatic conversion tests and phenol polymerization. These results, coupled with the expected production of a maximum of 7 µM PABA in 4-(phenylazo)benzoic acid enzymatic conversion tests, show that more than enough PABA reacted in that time period. During this test a colour change was observed but no precipitate was seen. To provide further evidence of enzymatic conversion of PABA, the solution was acidified which caused the precipitation of what is speculated to be dissolved polymers in solution. PABA concentration values for the acidification shown in Table 4-4 and Figure 4-13 show no change for the concentration of PABA before and after acidification. Acidification caused the removal of most of the colour seen in the solution, providing further evidence for the likelihood of PABA enzymatic conversion. It is possible, however, that SBP during 4-(phenylazo)benzoic acid tests was inactivated due to low concentrations of substrate compared to enzyme and hydrogen peroxide concentrations as has been shown in previous studies[26]. Tests with PABA at lower concentrations were done because of this and shown in Table 4-5 and Figure 4-14. These tests show a decrease of 8% in PABA concentration when the initial concentration was 61 µM and 7% when the original concentration was 7 µM. The latter is in the range expected to be formed from the Azo reaction. This supports the hypothesis of enzyme inactivation at low concentrations but does not explain why PABA was not found in 4-(phenylazo)benzoic acid enzymatic conversion tests. It is possible however that the presence of 4-(phenylazo)benzoic acid prevents the inactivation of the enzyme and thus allows the conversion of most of the produced PABA.

Reductive cleavage of the azo-bond in 4-(phenylazo)benzoic acid was not proven by the above tests though it still remains a plausible explanation for the loss in Azo concentration found throughout this study. Asymmetric cleavage is also still a possibility for the loss of Azo as the search for the products of asymmetric cleavage was not a part of this study and so has not been ruled out. The loss of Azo has always been in very small amount: from 3-9% of the total

concentration. This loss has always been smaller than the overall decrease in concentration seen in the presence of phenolic precipitates, which suggests that even if azo-bond reduction is occurring, it is not the only reason why Azo concentration is going down in these experiments. If azo-bond reduction occurs it does so with the use of SBP. When SBP is in the presence of phenol and 4-(phenylazo)benzoic acid there would be competition between the two substrates. This competition would lead to a smaller decrease in Azo concentration due to enzymatic conversion in the presence of phenol compared to a reaction that contained no phenol. This would mean that if a control was made that contained no phenol any decrease in Azo concentration in the control through azo-bond cleavage would be equal to or less than the loss of Azo in an experimental reactor that contained phenol *i.e* this control gives an upper limit for azo-bond cleavage. Because of the possibility of azo-bond cleavage in these reactions, a control that contained no phenol was used and any amount of Azo said to be absorbed is defined as the difference in concentration between the experimental reactor that contained phenol and the no-phenol control. It is possible that initial dynamic test values of Azo adsorption were as high as 15% but could also be as low as 6%. For the purposes of this study the lower value is used.

In general, the maximum amount of Azo lost that is potentially due to enzymatic conversion is very small (around 1.1 to 2.6 μM). While the amount of Azo lost this way is not zero, it is small enough to not significantly affect the analysis of Azo ab/adsorption and thus using a control system to correct for this small loss was deemed appropriate.

5.5 Low concentration 4-(phenylazo)benzoic acid enzymatic conversion:

With evidence of SBP inactivation at low reducing substrate concentration found in PABA enzymatic conversion tests, the question arose as to how good a substrate is 4-(phenylazo)benzoic acid. All tests of Azo's enzymatic conversion have been done with

concentrations of Azo being far lower than what would normally be used for enzymatic conversion. To confirm that Azo's small decrease in concentration is due to its poor quality as a substrate and not to enzyme inactivation in the presence of high concentration of hydrogen peroxide, a test was done with lower SBP and hydrogen peroxide concentrations while keeping the concentration of Azo close to what it was previously (32.7 μM of Azo was used in this test compared to 27.6 μM in the initial dynamic tests). This test was done with 40 mU/mL of enzyme and 60 μM of hydrogen peroxide and shown in more detail in Table 4-6 and Figure 4-15. This is a 30-fold decrease in the ratio of enzyme and hydrogen peroxide to substrate concentration when comparing this test to the initial dynamic absorption test. No change was seen in the concentration of 4-(phenylazo)benzoic acid after 24 h, which shows that Azo is indeed a poor substrate and that the lack of enzymatic conversion was not due to SBP inactivation in earlier tests.

5.6 Initial static adsorption tests:

Static adsorption tests were conducted for 4-(phenylazo)benzoic acid adsorption onto pre-formed phenolic precipitates. These phenolic precipitates were created prior to reaction and so any loss of 4-(phenylazo)benzoic acid could not be due to its enzymatic conversion. The solution containing the phenolic precipitates is referred to as the suspension. The residual activity of SBP in the suspension from preparation of the phenolic polymer was ≤ 0.01 U/mL with no hydrogen peroxide remaining in solution, Thus there is no path for enzymatic conversion of Azo and any decrease in its concentration in these tests would be due to adsorption. Compared to previous tests where absorption occurred under dynamic conditions, this static system should exhibit adsorption. Absorption is the accumulation of a molecular species throughout the bulk of a solid whereas adsorption is the accumulation of a molecular species on the surface of a solid. Static tests should result in adsorption due to the fact that the phenolic precipitates have already been

formed and only the surface is accessible. This is in contrast to dynamic tests that happen during the formation of phenolic precipitates which would result in both the surface and the bulk of the solid being available for capturing Azo.

Earlier studies of the adsorption characteristics of phenolic precipitates were done to characterize the adsorption of SBP itself onto phenolic precipitates with the goal of eluting SBP. This study showed that the minimum SBP activity to remove 1 mM of phenol could be reduced from 1.2 U/mL to 0.5 U/mL by freeing the adsorbed SBP on the phenolic precipitate itself[36]. This implies that any polymers used in this study would have adsorbed SBP effectively "blocking" possible adsorption sites. The amount of SBP blocking sites should not significantly hinder Azo adsorption however. The adsorption capacity of SBP onto phenolic precipitates for static uptake has been shown to be 3.44 U/mg with a Langmuir constant of 4.91[36]. With 1 U/mL of SBP in all experiments this would give an adsorbed amount of 2.65 U/mg which is less than the adsorption capacity of 3.44 U/mg leaving sites open for Azo. This would be the case if SBP had undergone static adsorption in this study, which it did not. SBP instead underwent dynamic adsorption as the adsorption took place when the precipitate was forming. This would greatly increase the adsorption capacity allowing for many more open "sites" for Azo to bond to thus causing it to block less sites for Azo static adsorption. On top of the relationship between dynamic and static ab/adsorption there is also the relationship between SBP and Azo specifically concerning their size. SBP as a protein molecule would occupy far more of the surface than Azo meaning many Azo molecules should be capable of "fitting" where one SBP molecule would. With the knowledge that SBP is absorbing dynamically, SBP is a larger molecule than Azo and there still being available adsorption sites if SBP had adsorbed statically, we can conclude that the presence of SBP on the surface of the phenolic precipitates should not significantly hinder Azo adsorption. The specifics of this interaction however is not explored in this study.

Initial tests shown for static adsorption in Table 4-7 and Figure 4-16 showed a decrease of 6.4% or 4 ± 2 mg of Azo/g of phenolic precipitate due to adsorption, assuming a phenolic precipitate mass equal to the phenolic precipitate mass used later in the Langmuir analysis of the static system. Both this static test and the Langmuir analysis later used the same suspension and so should have a similar dry mass loading.

5.7 Extraction of 4-(Phenylazo)benzoic Acid:

Extraction tests were carried out to provide further evidence of 4-(phenylazo)benzoic acid adsorption onto phenolic precipitates. These tests were done by conducting either a dynamic or a static ab/adsorption, then isolating the phenolic precipitates and suspending them in an equal volume of acetonitrile. The concentration of Azo in acetonitrile after 30 minutes of stirring was 16.0% of the initial available concentration for the dynamic and 6.7% for the static capture. This information is provided in detail in Table 4-8 and Figure 4-17 for the dynamic test and Table 4-9 and Figure 4-18 for the static. The recovery is comparable to the concentration of Azo lost in the static tests but shows a deviation in the dynamic test. The deviation may be due to a large amount of experimental solution being transferred with the phenolic precipitates into the amount recovered reactor. Some of the experimental solution was transferred due to a loose pellet after centrifugation which prevented complete separation of the solution and the pellet. As explained in more detail in the *Results* section, the amount of experimental solution transferred with the pellet was large enough to account for the increase in concentration observed in the acetonitrile solution used to extract Azo. Both the dynamic and static tests show a concentration in the recovered reactor that cannot be accounted for simply by any solution transferred with the pellet. A similar extraction experiment was done previously in this lab in a different study using Triton X-100 and phenolic precipitates. This extraction used ethanol in place of acetonitrile and found complete recovery of Triton X-100 from the surface of the

phenolic precipitates[1] providing support for use of such an extraction method. Because of this, the current experiments are interpreted as providing qualitative support for the hypothesis that the 4-(phenylazo)benzoic acid lost was ab/adsorbed onto the precipitates.

5.8 Langmuir Analysis:

Langmuir analyses were done for both static and dynamic systems to test the viability of 4-(phenylazo)benzoic acid ab/adsorption onto phenolic precipitates as a treatment method. The analysis gave an adsorption maximum value for static adsorption as 16.0 ± 0.5 mg of Azo/g of precipitate and a Langmuir constant of 0.13 ± 0.01 L/mg. For the dynamic test the absorption maximum was determined to be 51 ± 3 mg of Azo/g of precipitate and the Langmuir constant was determined to be 0.088 ± 0.008 L/mg. The figures that correspond to these analyses are Figures 4-19 and 4-20 for the static analysis and Figure 4-21 and 4-22 for the dynamic. Table 5-1 summarizes.

Table 5-1: Langmuir analysis for both static and dynamic systems of 4-(phenylazo)benzoic acid ad/absorption onto phenolic precipitates

	K (L/mg)	K^{-1} (mg/L)	Q_m (mg/g)
Static adsorption	0.13 ± 0.01	7.7 ± 0.6	16.0 ± 0.5
Dynamic absorption	0.088 ± 0.008	11 ± 1	51 ± 3

The dynamic system had 3-fold greater capacity for sorption 4-(phenylazo)benzoic acid compared to the static system. This could be due to more surface area being accessible in the dynamic absorption than in the static which is more easily explained using the “onion” analogy. An onion has layers of “skin” or in our case layers of surface area. The inner surface layer forms first and adsorbs some Azo, then the next layer forms on top of that and adsorbs some Azo and so on. This increases the available surface area for Azo to adsorb which thus increases the ad/absorbing capacity. The static and dynamic ab/adsorption show similar K values (0.13, 0.088

L/mg). The K constant for the Langmuir analysis corresponds to the affinity between the sorbent and the sorbate. A high K value means that most of the sorbate is ab/adsorbed at equilibrium whereas a low K value means most ab/adsorbate is left in solution at equilibrium. The higher K values for both the dynamic and static tests suggest a high affinity for Azo binding to the phenolic precipitates. This is likely due to the hydrophobicity of the compound as a whole which discourages dissolution into the aqueous phase. The half-saturation points for both static and dynamic ab/adsorption are relatively similar (7.7, 11.4 mg/L, respectively). The half-saturation point remains relatively constant because both systems involve the same ad/absorbent and ad/absorbate which has the same affinity for the surface of phenolic precipitates.

It is important to mention that the Langmuir analyses for both the static and dynamic systems are both done at pH = 7.0. This gives us the analysis of the ab/adsorption characteristics of the benzoate form of 4-(phenyazo) benzoic acid not the acidic form (pKa = 2.45). The ad/absorption is likely hampered by the presence of a negative charge on Azo which means that the analysis of the acid form would likely give a higher ab/adsorption affinity and a higher maximum ab/adsorption capacity. The entire system of course would only work for lower concentrations of Azo as the acid form is far less soluble in water than the conjugate base.

If one were to attempt to remove Azo from solution using this method where a maximum efficiency of removal was desired, a fraction of Q_e/Q_m of approximately 50%[1] would be used. This means that for a reasonable concentration of Azo for the static system around 8 mg of Azo per gram of precipitate (25.5 for the dynamic system) could be removed rather than 16 mg/g (51 mg/g for the dynamic system) as the maximum adsorption capacity implies. To get to 16 mg/g from this system removed would take a very high equilibrium concentration (C_e) that would not only take a long time to adsorb but would also exceed the solubility of Azo in solution. Because of the high equilibrium concentration required, only ~13% of the total concentration would be

removed by adsorption even if the equilibrium concentration required didn't exceed Azo's solubility. More reasonable numbers of removal from these systems therefore come from the middle of the Langmuir curve (8 and 25.5 mg/g). These ad/absorption capacities are quite small and thus if Azo was a wastewater contaminant this method would not be an effective way of removing it from wastewater. The Langmuir constants on the other hand show high affinity for ad/absorption which shows promise for using phenolic precipitates to potentially remove other compounds from wastewater.

The initial dynamic and static ad/absorption tests that were shown in Table 4-1, Figure 4-5 and Table 4-7, Figure 4-16, respectively, can be compared to the later full Langmuir analyses. The precipitate dry mass was not measured for either of these initial tests but assuming the same precipitate dry mass as measured in the Langmuir analyses (since the same suspension was used) one could find comparable numbers. For the initial dynamic test 133 µg of Azo was at equilibrium in a 25 mL solution with a measured absorbed value of 9 ± 4 µg. These values in terms of C_e and Q_e used in the Langmuir analyses (using the precipitate dry mass measured during the Langmuir analyses) gives a C_e value of 5.32 ± 0.08 mg/L and a Q_e value of 5 ± 2 mg/g. Plugging in a value of 5.32 ± 0.08 for C_e into the dynamic Langmuir equation yields a calculated Q_e of 16 ± 2 mg/g. These numbers are largely different but the initial dynamic test absorbed value was not the only mass of Azo lost from equilibrium in that experiment. An assumption was made in the initial dynamic test conditions discussed in more detail in earlier sections that the presence of phenol would not affect the enzymatic conversion of Azo. This assumption was made to simplify the problem. The addition of phenol however should affect the enzymatic conversion through a competition between phenol and Azo for SBP, one which would decrease the amount of enzymatic conversion of Azo. 9 µg is the lowest value for absorbed Azo measured in the initial dynamic test, though because of this competition it could be as high as 23 µg. If 23

μg were absorbed and none at all reacted with SBP the measured value for Q_e would be 12 ± 3 mg/g instead. Comparing this new value with a calculated value of 16 ± 2 mg/g gives us a measured and calculated value within error of one another which makes the two experiments consistent.

Doing the same comparison for the initial static test and the Langmuir static analysis give a measured C_e value of 7.64 ± 0.04 mg/L and a measured Q_e value of 4 ± 2 mg/g. These values are calculated from the equilibrium and adsorbed masses given in Table 4-7 along with the measured precipitate dry mass in Table C-1 in Appendix C. Plugging in 7.64 ± 0.04 mg/L into the static Langmuir equation a Q_e value of 8 ± 1 mg/g is found. The error on both of these number is quite high and could give us a measured value of as high as 6 mg/g and a calculated value as low as 7 mg/g. This shows a small discrepancy between the initial static adsorption test and the Langmuir analysis that is likely due to the error in both experiments.

In comparing the absorption capacity of phenolic precipitates for 4-(phenylazo)benzoic acid to the absorption capacity of a previously measured compound, the non-ionic surfactant Triton X-100, the ad/absorption capacity for Azo is significantly lower (Triton X-100 had an ad/absorption capacity of 258 mg/g[1]). This could be due to the greater hydrophobicity of the octyl- or nonyl-group in Triton X-100 compared to less hydrophobic phenylazo group of 4-(phenylazo)benzoic acid. The K value of Triton X-100 on the other hand is lower than the K value for both systems of Azo (Triton X-100 had a K value of 0.029 L/mg[1]). This shows that despite the greater hydrophobicity of Triton X-100, Azo appears to have a greater affinity for adsorption onto phenolic precipitates. This could be due to Triton X-100's tendency to form micelles in aqueous solution (critical micelle concentration 207 mg/L[35]). By forming a micelle at higher concentration levels Triton X-100 could show a preference for the micellar phase instead of

forming a monolayer onto phenolic precipitates. This would translate to a lower measured affinity for phenolic precipitates.

Another study looked at the difference between benzene and toluene adsorption onto activated carbon (named in the study as F-400 from Calgon Carbon, Pittsburgh, Pennsylvania) using a Langmuir analysis. The surface area of F-400 was 877.8 m^2/g measured using Brunauer-Emmett-Teller (BET) analysis[30]. The maximum adsorption capacity for benzene and toluene onto F-400 at pH = 7.0 were reported by the authors study to as 183.3 and 194.1 mg/g respectively. Comparing these to 16 mg/g for Azo static uptake or 51 mg/g for Azo dynamic uptake, both benzene and toluene adsorbed in far greater quantities. This is likely due to F-400's having a greater surface area than phenolic precipitates and interactions between hydrophobic surfaces and carboxylate acid groups. The surface area of phenolic precipitates have not been measured, however, so it is impossible to say for certain. The Langmuir constants for benzene and toluene under the same conditions were found to be 0.0765 and 0.0841 L/mg, respectively [30], relatively similar to 0.13 and 0.088 L/mg for the Azo static and dynamic tests of the current study.

The change in adsorption capacity and affinity of F-400 for benzene and toluene were also measured at three different pH's (3, 7 and 11)[30], Table 5-2. At these pH's the Langmuir constant for both benzene and toluene is essentially invariant. The pH of the system does not affect the structure of benzene or toluene in the same way as Azo so this unchanging Langmuir constant is unlikely to occur for Azo as well.

The adsorption capacity at across the pH range is, however, slightly different in showing a higher adsorption capacity, the higher the pH. This trend is not likely to hold for Azo and phenolic precipitates as more basic solutions cause Azo to form its conjugate base which would increase its hydrophilicity making it less likely to adsorb onto a hydrophobic surface. Furthermore, the

phenolic precipitates themselves would have significant negative charge at pH 11 due to ionization of phenolic groups.

The exact numbers for Q_m and K for benzene and toluene are summarized in Table 5-2.

Table 5-2: Langmuir analysis for benzene and toluene onto activated carbon F-400[30].

pH	3	7	11
Q_m (mg/g)	benzene: 152 toluene: 166	benzene: 183 toluene: 194	benzene: 219 toluene: 231
K (L/mg)	benzene: 0.0777 toluene: 0.0849	benzene: 0.0765 toluene: 0.0841	benzene: 0.0775 toluene: 0.0862

F-400 was oxidized with nitric acid in a second part of this study to give a sample, F-400Cox, with a more oxygenated surface which allows the analysis of that effect on the adsorption of hydrophobic compounds. For the more oxygenated surface both the adsorption capacity and the adsorption affinity decreased for both benzene and toluene (Q_m = 144, 122 mg/g and K = 0.0540, 0.0563 L/mg at pH = 7.0 for benzene and toluene, respectively[30]). The BET analysis of this new surface gave a surface area of 938 m^2/g which is slightly larger than the measured surface area for F-400. This would suggest that the decrease in both adsorption capacity and affinity is due to the addition of oxygenated groups. Phenolic precipitates have a surface made from the polymerization of phenol and will display phenolic oxygen groups similar to or greater in number than in F-400Cox. This would support the idea that phenolic precipitates act as a worse adsorption surface for unfunctionalized aromatics than activated carbon since oxygenated groups appear to decrease adsorption capacity and affinity.

For further comparison, a study constructed a Langmuir adsorption curve for benzoic acid onto granular activated carbon (GAC) at 25°C and pH = 7.0[27]. The Langmuir analysis of this system gave an adsorption capacity of Q_m = 472 mg of benzoic acid per gram of granular activated carbon and a Langmuir constant of K = 0.0325 L/mg[27]. Compared to Azo static adsorption onto

phenolic precipitates, it appears that Azo has a better affinity due to having a 4-fold greater Langmuir constant but a 30-fold lower adsorption capacity. The larger K value is likely due to the polarity of benzoic acid compared to Azo. Because Azo has a "tail" made of an azo phenyl group, it is more hydrophobic than benzoic acid and thus more likely to interact with a hydrophobic surface. The lower adsorption capacity is likely due to the specific surface area differences between GAC and phenolic precipitate, GAC having a far larger surface area per gram than phenolic precipitates. In the present study, the adsorbent's mass was measured and it was assumed that an increase in mass would cause an increase in the adsorption effects explored. The real measure of a surface's adsorption ability, however, is not its mass but its surface area. A larger surface area corresponds to a larger adsorption capacity and the large difference in adsorption capacity measured in both these studies is likely due to GAC having the larger specific surface area. This is not known for sure, GAC is a commonly used adsorbent and so its surface area has been measured many times in the literature (for example one study gives GAC a surface area of 1435 m^2/g[28] another gives surface areas of 967 and 559 m^2/g[31]) but surface area for phenolic precipitates has not been determined. The large contrast between adsorption capacities however is consistent with this rationale.

From the analysis of Azo we can conclude that the method of treating hydrophobic compounds proposed in this study is a potentially viable method. The ad/absorption does occur and fits closely to the Langmuir function. The percent concentrations ad/absorbed of 4-(phenylazo)benzoic acid are low but the effectiveness of these systems would vary among compounds.

Chapter 6

6. Conclusions:

In this study, a model compound (4-(phenylazo)benzoic acid) was used to characterize the ab/adsorption process onto a phenolic polymer created through enzymatic conversion of phenol with soybean peroxidase and hydrogen peroxide. 4-(phenyazo)benzoic acid has been shown to ab/adsorb in measurable quantities onto phenolic precipitates with high Langmuir constants showing good affinity. The ab/adsorption system has been measured and dynamic absorption has been shown to be better than static adsorption. SBP enzymatic removal has been shown to be a viable treatment method to treat toxic hydrophobic non-substrates in wastewater along with substrates of SBP like phenol. The ad/absorption does occur and fits closely to the Langmuir function, thereby enabling quantitative implementations. The percent concentrations ad/absorbed of 4-(phenylazo)benzoic acid are low but the effectiveness of these systems would vary among compounds. The sorption process investigated gives a decent ab/adsorption capacity and opens up the possibility of using phenolic precipitates and thus expands the scope of the enzymatic method for a polishing treatment of wastewater to include low concentrations of organic compounds that are not substrates of SBP.

References:

1. Feng, Wei, "Utilization of Phenolic Precipitates to Enhance Soybean Peroxidase-Catalyzed Wastewater Treatment". *Electronic Theses and Dissertations*. University of Windsor (2013) 4944.

2. Mitra S, Roy P. "BTEX: A Serious Ground-water Contaminant." Research Journal of Environmental Sciences 5(5) (2011), 394-398.

3. Pourzamani H, Masood A, Majd S, Fadaei S. "Benzene Removal by Hybrid of Nanotubes and Magnetic Nanoparticle from Aqueous Solution." Desalination and Water Treatment, (2015) 19038-19049

4. Verschueren K. "Handbook of Environmental Data on Organic Chemicals", Van Nostrand Reinhold Co., New York, (1983) 983.

5. Dean J, Lange N. "Lange's Handbook of Chemistry", McGraw-Hill, New York (1992), 30-50.

6. Hansch C, Leo A, Hoekman D. "Exploring QSAR, American Chemical Society", Washington, DC (1995), 32.

7. Dordick J, Marletta M, Klibanov A. "Polymerization of Phenols Catalyzed by Peroxidase in Nonaqueous Media". Biotechnology and Bioengineering 30(1) (1987), 31-36.

8. Ghoul M, Chebil L. "Enzymatic Polymerization of Phenolic Compounds by Oxidoreductases", Springer, New York (2012), 4-6.

9. Ren J, Licht S. "Tracking airborne CO2 mitigation and low cost transformation into valuable carbon nanotubes" Scientific Reports (2016).

10. Environment Canada "Priority Substance List Assessment Report: Phenol" (2000), 14- 32.

11. Fujita T, Iwasa J, Hansch C. "A New Substituent Constant, π, Derived from Partition Coefficients", Journal of the American Chemical Society 86(23) (1964), 5175-5180.

12. Blackman G, Parke M, Garton G. "The Physiological Activity of Substituted Phenols. I. Relationships Between Chemical Structure and Physiological Activity" Archives of Biochemistry and Biophysics 54(1) (1955), 45-54.

13. Kamal J, Behere D. "Activity, Stability and Conformational Flexibility of Seed Coat Soybean Peroxidase", Journal of Inorganic Biochemistry 94(3) (2003), 236-242.

14. Smith A, Sanders S, Thorneley R, Burke J, Bray R. "Characterisation of a Haem Active-Site Mutant of Horseradish Peroxidase, Phe41 → Val, with Altered Reactivity Towards Hydrogen Peroxide and Reducing Substrates", European Journal of Biochemistry 207(2) (1992), 507-519.

15. ATSDR Public Health Statement for Phenol, p. 4, Accessed on Oct 20th (2008), 2016

16. Henriksen A, Mirza O, Indiani C, Teilum K, Smulevich G, Welinder K, Gajhede M. "Structure of Soybean Seed Coat Peroxidase: A Plant Peroxidase with Unusual Stability and Haem-Apoprotein Interactions" Protein Science 10(1) (2001), 108-115.

17. Wright H, Nicell J "Characterization of Soybean Peroxidase for the Treatment of Aqueous Phenols", Bioresource Technology 70(1) . (1999), 69-79.

18. McEldoon J, Dordick J" Unusual Thermal Stability of Soybean Peroxidase" Biotechnology Progress 12(4) (1996), 555-558.

19. Deyhimi F, Nami F. "Peroxidase-Catalyzed Electrochemical Assay of Hydrogen Peroxide: A Ping-Pong Mechanism", International Journal of Chemical Kinetics 44(10) (2012), 699-704.

20. Yu J, Taylor K, Zou H, Biswas N, Bewtra J. "Phenol Conversion and Dimeric Intermediates in Horseradish Peroxidase-Catalyzed Phenol Removal from Water", Environmental Science and Technology 28(12) (1994), 2154-2160.

21. Ali L, Algaithi R, Habib H, Souka U, Rauf M, Ashraf S. "Soybean Peroxidase-Mediated Degradation of an Azo Dye– a Detailed Mechanistic Study." BMC Biochemistry (2013), 14-35.

22. "Benzoic acid, 4-(phenylazo)-." National Institutes of Standards and Technology (2016). Web. 29 Mar. 2017. http://webbook.nist.gov/cgi/cbook.cgi?ID=C1562932&Type=IR-SPEC&Index=1

23. "Chemical Elements Listed by Electronegativity." Lenntech. Web. 25 Nov. 2016. http://www.lenntech.com/periodic-chart-elements/electronegativity.htm

24. "Aniline." Chemical Book. Web. 8 Nov. 2016. http://www.chemicalbook.com/ChemicalProductProperty_EN_CB7169544.htm

25. Shi H, Ge W, Oh H, Pattinson S, Huggins J, Talmon Y, Hart D, Raghaven S, Zakin J. "Photoreversible Micellar Solution as a Smart Drag-Reducing Fluid for Use in District Heating/Cooling Systems." Langmuir (2012), 102-109.

26. Baynton K, Bewtra J, Biswas N, Taylor K. "Inactivation of Horseradish Peroxidase by Phenol and Hydrogen Peroxide: A Kinetic Investigation." Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology (1994), 272-278.

27. Ghorbanian S, Abolghasemi H, Radpour S, Mousavian M. "Developing a New Approach to Temperature and pH Effects in the Modelling of the Adsorption Isotherms of Benzoic Acid onto Activated Carbon." Adsorption Science & Technology. (2008), 233-250.

28. Alighardashi A, Shahali S. "Removal of Nitrate from Synthetic Aqueous Solution and Groundwater in a Continuous Pilot System using Chemical Activated Carbon Derived from Walnut Shell." Water Practice & Technology. (2016), 784-795.

29. "Table of Acids with Ka and pKa Values." At http://clas.sa.ucsb.edu/staff/Resource%20folder/Chem109ABC/Acid,%20Base%20Strength/Table%20of%20Acids%20w%20Kas%20and%20pKas.pdf

30. Wibowo N, Setyadhi L, Wibowo D, Setiawan J, Ismadji S. "Adsorption of benzene and toluene from aqueous solutions onto activated carbon and its acid and heat treated forms: Influence of surface chemistry on adsorption." Journal of Hazardous Materials. (2007), 237-242.

31. Ge¸sikiewicz-Puchalska A, Zgrzebnicki M, Michalkiewicz I, Narkiewicz U, Morawski A, and Wrobel A. "Improvement of CO_2 uptake of activated carbons by treatment with mineral acids." Chemical Engineering Journal. (2017), 159-171.

32. Beemer K. "Adsorption of BTEX group Compounds onto Phenolic Precipitates Created by Soybean Peroxidase." 59-410 undergraduate research project, Unviersity of Windsor (2014)

33. Steliga T, Jakubowicz P, and Kaputsa P. "Changes in toxicity during treatment of wastewater from oil plant contaminated with petroleum hydrocarbons." Journal of Chemical Technology and Biotechnology (2014), 1408-1418.

34. Henriksen A, Mirza O, Indiani C, Teilum K, Smulevich G, Welinder K, Gajhede M "Structure of soybean seed coat peroxidase: a plant peroxidase with unusual stability and haem-apoprotein interactions." Protein Sci. (2001), 108-115.

35. Chen, Miao, "Peroxidase-catalyzed 4-Phenylazophenol Polymerization in Micellar Solution". *Electronic Theses and Dissertations*. University of Windsor, (2015), 5457.

36. Feng W, Taylor K, Biswas N, Bewtra J. "Soybean peroxidase trapped in product precipitate during phenol polymerization retains activity and may be recycled." Journal of Chemical Technology and Biotechnology (2013), 1429-1435.

37. Feng, W, Taylor K, Biswas N, Bewtra J. "Phenolic Precipitates from Soybean Peroxidase–Catalyzed Wastewater Treatment: Concentrated Waste Serves to Concentrate Its Progenitor." Journal of Hazardous, Toxic, and Radioactive Waste (2015), 2153 - 5515.

Appendix A: SBP Activity Assay

Soybean Peroxidase (SBP) Activity Assay:

A colourimetric assay was used to measure the activity of soybean peroxidase. SBP activity is determined by a time-based measurement done on an assay reagent where the absorbance is measured at 510 nm using a spectrophotometer. One unit (U) of SBP activity is defined as the amount of SBP that catalyzes the conversion of 1 µmol of hydrogen peroxide in one minute under the assay conditions.

Assay Reagent:

The assay reagent is made using a 100 mM phenol solution with 0.5 M pH = 7.4 phosphate buffer (0.941 g of phenol, 1.31 g of monobasic sodium phosphate and 5.80 g of dibasic sodium phosphate). 5 mL of this solution is mixed with 25 mg of 4-aminoantipyrine (4-AAP) and 0.1 mL of 100 mM hydrogen peroxide and made up to 45 mL with water. Once constituted, this reagent is used within 1 hour.

Procedure:

1. SBP stock solution is diluted to around 1 U/mL.

2. A 50 µL sample of SBP is injected into the cuvette.

3. The cuvette is placed in the spectrophotometer. The spectrophotometer is blanked in advance at 510 nm with 950 uL of reagent plus 50 uL of water

4. 950 µL of the assay reagent is quickly added to the cuvette and measurements are started.

5. The computer takes measurements of the absorbance at 510 nm once every 5 seconds for 30 seconds and determines the rate.

6. SBP activity is calculated using the slope of the line and the dilution factor.

SBP Activity Calculation:

$$Activity\ in\ the\ cuvette\ \left(\frac{U}{mL}\right) = \frac{Slope\ \left(\frac{AU}{s}\right) * \left(\frac{60s}{1\text{min}}\right) * 20\ (dilution\ factor\ in\ the\ cuvette)}{6min^{-1}cm^{-1} * 1\ cm}$$

$$= slope * 200 \frac{\mu\text{mol}}{min}\ (U)$$

$$Activity\ in\ SBP\ stock = Activity\ in\ the\ cuvette * dilution\ factor$$

Appendix B: Hydrogen Peroxide Assay

A colourimetric assay was used to measure the hydrogen peroxide concentration after the reaction was complete. The combination of a known concentration of hydrogen peroxide and the assay reagent forms a pink chromophore with a wavelength max at 510 nm. A series of known concentrations of hydrogen peroxide are mixed with the assay reagent and the wavelength is measured using a spectrophotometer. From this a standard curve is constructed and the concentration of an unknown sample determined from that standard curve.

Assay Reagent:

The assay reagent is made using 12.5 mL of a 100 mM phenol 0.5 M pH = 7.4 phosphate buffer, 63.8 mg of 4-aminoantipyrine (to give 12.5 mM in the reagent), 0.313 mL of Novo ARP liquid concentrate (approximately 1200 U/mL) and is made up to a volume of 25 mL.

Procedure:

1. Prepare a stock solution of 100 mM hydrogen peroxide from 30% hydrogen peroxide (w/v) by adding 510 µL of 30% hydrogen peroxide and making the volume up to 50 mL

2. A 1 in 100 dilution is made to produce 1 mM hydrogen peroxide and a 1 in 10 dilution from 1 mM to produce 0.1 mM

3. Standard concentrations are prepared of 0.1 mM, 0.2 mM, 0.01 mM, 0.02 mM, 0.04 mM, 0.06 mM and 0.08 mM hydrogen peroxide with assay reagent. This is done by making the appropriate dilution from either the 1 mM or 0.1 mM stock solutions for a final volume of 1 mL in the assay cuvette. 200 µL of the assay reagent is added during this process and accounted for when calculating the final concentration.

4. 200 µL of assay reagent is added to the unknown solution.

5. The solutions are left to sit for 18 minutes.

6. The solutions are placed in the cuvette one by one and the absorbance is measured at 510nm against a reagent blank.

7. From the standard concentrations a linear regression curve is obtained and the unknown concentration is calculated.

Appendix C: Langmuir Isotherm

The Langmuir Isotherm has been used to characterize adsorption interactions as a function of the adsorbate concentration. The Langmuir isotherm has three assumption 1) that all adsorption sites are equivalent, 2) that each site can only one molecule and there is only a monolayer of coverage and 3) that there is no interaction between adsorbed molecules. The general equation for the Langmuir isotherm is shown below.

$$\frac{Q_e}{Q_m} = \frac{KC_e}{1 + KC_e}$$

Where Q_e is the equilibrium adsorption capacity (mg/g), Q_m is the maximum adsorption capacity (mg/g), C_e is the equilibrium concentration of the adsorbate (mg/L) and K is the Langmuir adsorption constant. The Langmuir isotherm is sometimes also written with Θ where $\Theta = Q_e/Q_m$.

To plot the Langmuir isotherm both the initial and equilibrium concentrations of the adsorbate (4-(phenylazo) benzoic acid) were measured using HPLC and the mass of the precipitate was measured by vacuum filtration and oven drying.

For the Langmuir experiments whether by static or dynamic ad/absorption, a stock solution of 4-(phenylazo)benzoic acid was used. The stock solution varied in initial concentration of Azo but tended to be around 90 μM initially. The stock solution also contained 5 mM pH = 7.0 phosphate buffer. Dilutions were made using 5 mM pH = 7.0 phosphate buffer to create 5 different concentrations of Azo somewhat equally separated from 4 μM to 88 μM. These points were chosen to ensure that at least 2 values lay above and below the half-saturation point for the system. For the static experiments phenolic precipitates were then added from a standard suspension in equal amounts to each solution (2.5 mL aliquot) such that all solutions totaled 25 mL. 2.5 mL of solution is also taken and vacuum filtered using a Buchner funnel and filter paper

(0.2 µm). This process is done in triplicate. The Buchner funnels are then oven dried overnight and the difference in weight between the initial mass of the Buchner funnel and the mass of the new system is defined as the precipitate mass.

For the dynamic experiments 250 µL of 100 mM phenol stock, 375 µL of 100 mM hydrogen peroxide and an appropriate amount of SBP stock solution were added to create a solution which contained 1 U/mL of SBP, 1.5 mM hydrogen peroxide and 1 mM phenol in a 25 mL solution. The solutions were left for 24 hours and then a 1 mL sample was taken filtered and tested in HPLC. The difference between the initial concentration of 4-(phenylazo) benzoic acid and the equilibrium concentration is defined as the adsorbed amount.

Using the equilibrium concentration, precipitate mass and adsorbed amount the Langmuir curve was then fit using Excel Solver. Tables of measured values for the static and dynamic Langmuir analyses are shown below.

Table C-1: Static Langmuir analysis

Initial Concentration (mg/L)	Equilibrium Concentration (mg/L)	Measured Precipitate Mass (mg)
0.91 ± 0.04	0.72 ± 0.04	3 ± 1
1.79 ± 0.04	1.50 ± 0.04	
3.72 ± 0.04	3.00 ± 0.04	
9.23 ± 0.04	8.13 ± 0.04	
19.92 ± 0.04	18.36 ± 0.04	

The above graph shows all measured values for the static Langmuir analysis. Concentration was determined using HPLC and precipitate mass was measured using the above method. Error is calculated from the standard deviation of triplicate runs using the formula Standard error = (Standard deviation)/ √n where n is the number of measurements (3).

Table C-2: Dynamic Langmuir analysis

Initial Concentration (mg/L)	Equilibrium Concentration (mg/L)	Measured Precipitate Mass (mg)
0.98 ± 0.04	0.72 ± 0.04	2.0 ± 0.2
1.85 ± 0.04	1.39 ± 0.04	
3.59 ± 0.04	2.73 ± 0.04	
14.58 ± 0.05	12.56 ± 0.04	
20.82 ± 0.04	18.19 ± 0.04	

The above graph shows all measured values for the dynamic Langmuir analysis. Concentration was determined using HPLC and precipitate mass was measured using the above method. Error is calculated from the standard deviation of triplicate runs using the formula Standard error = (Standard deviation)/ √n where n is the number of measurements (3).

Appendix D: Standard Curves

4-(phenylazo) benzoic acid:

4-(phenylazo) benzoic acid concentration was calculated using a standard curve of peak area *vs* concentration obtained using an HPLC. The concentration is measured in µM and the peak area at 325 nm. The standard curve was made from the dilution of a stock solution of 4-(phenylazo) benzoic acid and 5mM pH = 7.0 phosphate buffer. The dilutions were done with 5 mM pH = 7.0 phosphate buffer to maintain buffer concentration. Five different concentrations were obtained and all points are triplicates. The five values for concentration were chosen such that they corresponded to $1/10^{th}$, $2/10^{th}$s, $4/10^{th}$s, $8/10^{th}$s and $10/10^{th}$s of the stock solution. HPLC conditions were 70% ACN and 30% 5 mM pH = 7.0 phosphate buffer. A standard curve is shown in Figure D-1.

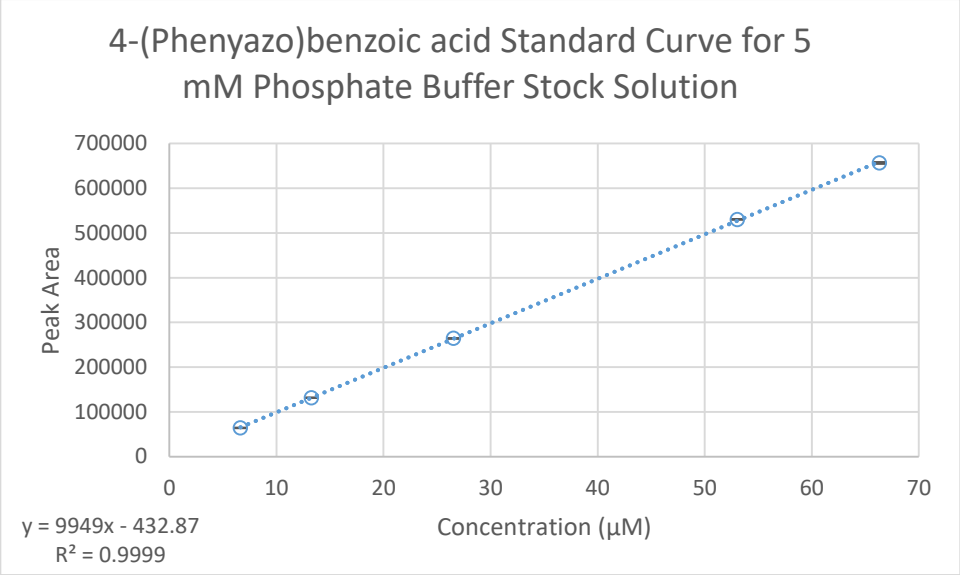

Figure D-1: 4-(phenylazo)benzoic acid standard curve for 5 mM buffer stock solution. Example standard curve for Azo concentration (µM) vs HPLC peak area measured at 325 nm. Used to determine concentration of any unknown samples. HPLC conditions used were 70% acetonitrile, 30% 5 mM phosphate buffer pH = 7.0.

A second type of standard curve for 4-(phenylazo) benzoic acid was used to calculate 4-(phenylazo) benzoic acid concentration in a solution of 100% ACN. This standard was created in the same manner as the one above except it was created from a stock solution of 4-(phenylazo) benzoic acid in 100% ACN and diluted using 100% CAN

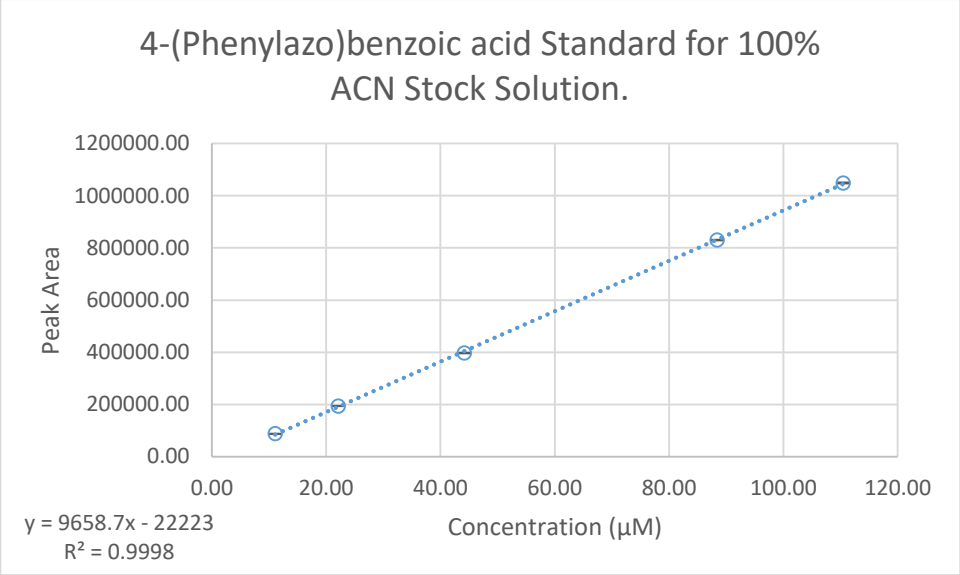

Figure D-2: 4-(phenylazo)benzoic acid standard curve for 100% acetonitrile stock solution. Example standard curve for Azo concentration (µM) vs HPLC peak area measured at 325 nm. Used to determine concentration of any unknown samples. HPLC conditions used were 70% acetonitrile, 30% 5 mM phosphate buffer pH = 7.0.

Para-aminobenzoic acid:

PABA concentration was calculated using a standard curve of peak area *vs* concentration obtained using an HPLC. The concentration is measured in both mM and µM and the peak area is measured at 280 nm. The standard curve was made from the dilution of a stock solution of PABA and 5 mM pH = 7.0 phosphate buffer. The dilutions were done with 5 mM pH = 7.0 phosphate buffer to maintain buffer concentration. Five different concentrations were obtained and all points are triplicates. The five values for concentration were chosen such that they corresponded to 1/10th, 2/10ths, 4/10ths, 8/10ths and 10/10ths of the stock solution. HPLC conditions were 40% ACN and 60% 5 mM pH = 7.0 ammonium acetate/phosphate buffer. Two standard curves one for mM levels and one for µM levels are shown below.

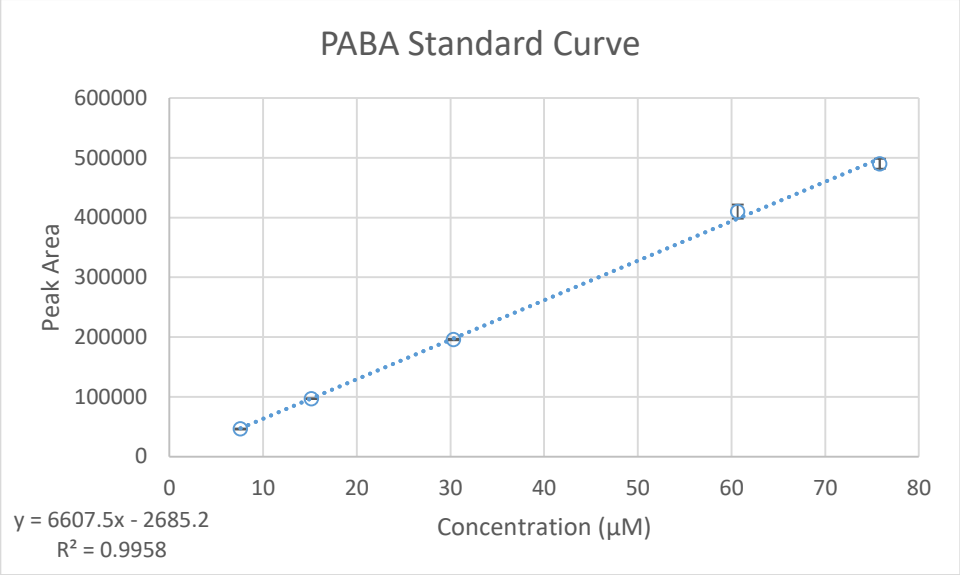

Figure D-3: PABA standard curve for concentrations from 7.58 – 75.8 µM. Example standard curve for PABA concentration (µM) vs HPLC peak area measured at 280 nm. Used to determine concentration of any unknown samples. HPLC conditions used were 40% acetonitrile, 60% 5 mM phosphate buffer pH = 7.0.

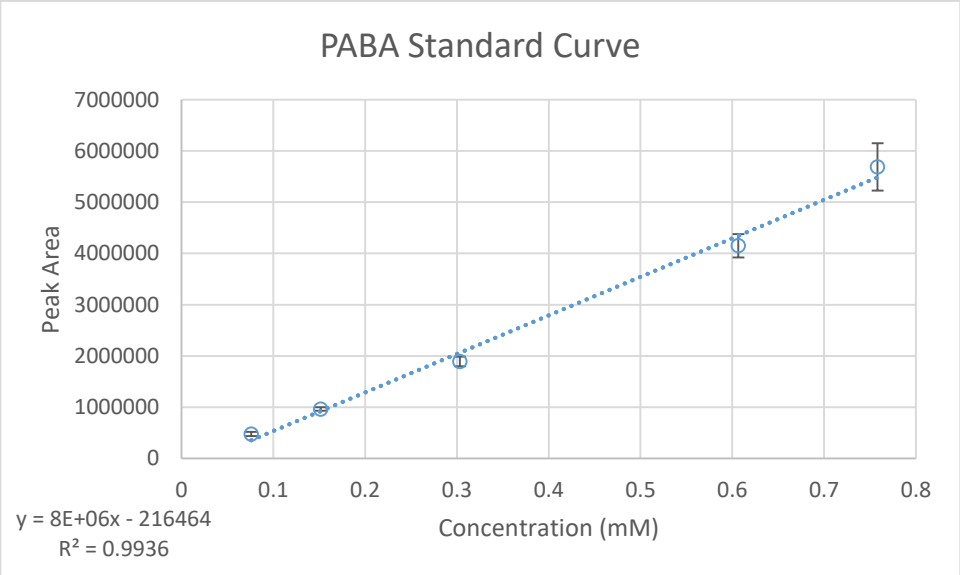

Figure D-4: PABA standard curve for concentrations from 0.076 – 0.76 mM. Example standard curve for PABA concentration (mM) vs HPLC peak area measured at 280 nm. Used to determine concentration of any unknown samples. HPLC conditions used were 40% acetonitrile, 60% 5 mM ammonium acetate buffer pH = 7.0.

Aniline:

Aniline concentration was calculated using a standard curve of peak area *vs* concentration obtained using an HPLC. The concentration is measured in µM and the peak area at 280 nm. The standard curve was made from the dilution of a stock solution of aniline and 5mM pH = 7.0 phosphate buffer. The dilutions were done with 5 mM pH = 7.0 phosphate buffer to maintain buffer concentration. Five different concentrations were obtained and all points are triplicates. The five values for concentration were chosen such that they corresponded to 1/10th, 2/10ths, 4/10ths, 8/10ths and 10/10ths of the stock solution. HPLC conditions were 40% ACN and 60% 5mM pH = 7.0 ammonium acetate buffer. The standard curve is shown below.

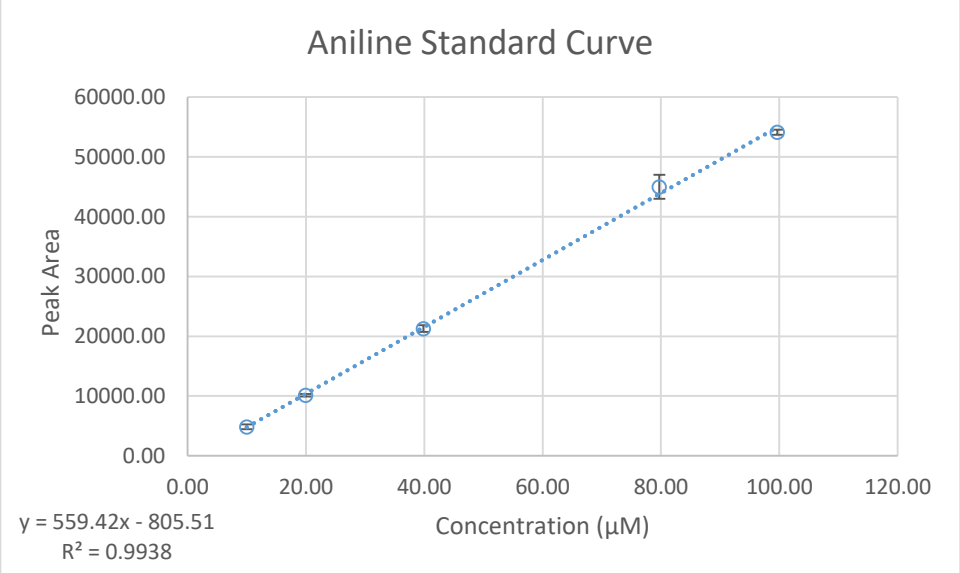

Figure D-5: Aniline standard curve. Example standard curve for aniline concentration (mM) vs HPLC peak area measured at 280 nm. Used to determine concentration of any unknown samples. HPLC conditions used were 40% acetonitrile, 60% 5 mM ammonium acetate buffer pH = 7.0.

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