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Utilization of Phenolic Precipitates to Enhance Soybean Peroxidase-Catalyzed Wastewater Treatment

Wei Feng
University of Windsor

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By

Wei Feng

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Wei Feng

APPROVED BY:

_____

A. Bassi, External Examiner
Western University

_____

A. Hubberstey,
Department of Biological Sciences

_____

R. Seth,
Department of Civil & Environmental Engineering

_____

J. Bewtra,
Department of Civil and Environmental Engineering

_____

N. Biswas, Advisor
Department of Civil and Environmental Engineering

_____

K. Taylor, Advisor
Department of Chemistry & Biochemistry

12, September 2013

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ABSTRACT

Studies on soybean peroxidase-catalyzed phenolic precipitates revealed that SBP trapped in precipitates during phenol polymerization retains activity. Contrary to the end-product inactivation model, recycling precipitates effectively utilized the active SBP. The minimum SBP concentration required for the subsequent batch reaction removal of 1 mM phenol from aqueous solution was reduced from 1.2 to 0.5 U/mL. SBP adsorption on precipitates was proven to be reversible by the addition of Triton X-100. Thus, a new explanation of the fate of SBP during the reaction is suggested: SBP is immobilized *in-situ* in an active form with reduction of specific activity rather than inactivation. Extending the study to the phenol removal reaction in the presence of polyethylene glycol, sodium dodecyl sulfate or Triton X-100 suggested some new explanations for each additive's protection mechanism. In addition, SBP and Triton X-100 adsorption was characterized by a Langmuir adsorption isotherm. Competitive adsorption between protein and non-ionic surfactant and the 'orogenic displacement mechanism' were used to explain and characterize the elution phenomenon. A quantitative relationship between SBP elution in a function of Triton X-100 concentration and precipitates concentration was established using a logistic function. A process using phenolic precipitates as the affinity matrix for concentrating SBP from dilute solution was developed. Both single-batch and consecutive cycles of operations were conducted. The process yielded a high recovery rate, and it is suitable for producing a low cost enzyme concentrate to be used in industrial wastewater treatment.

DEDICATION

This thesis is dedicated to:

My Parents – Qiang Feng and Guohua Cai

My Wife – Menghong Sun

For all the love, support and encouragement they have always given to me.

爱,信心,诚实,勇敢,骄兵必败,快意恩仇,不轻言放弃. -----七武器

Love, Confidence, Integrity, Courage, Humility, Resolve, Persistence.

-------- Seven Weapons

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1. INTRODUCTION

1.1 Background

Aromatic compounds, particularly phenol, aniline and their derivatives, have different levels of toxicity and are considered as carcinogenic and dangerous even when they exist as traces in the environment (Damborsky and Schultz 1997). Several industries including coal mining, petroleum refining, pulp and paper, synthetic resins and plastics, dyes and textiles, discharge these compounds into the environment (Hao et al. 2000, Mazzeo et al. 2010, Sarayu and Sandhya 2012). Also, overuse of herbicides and pesticides in agricultural activities contributes to the runoff of these hazardous compounds to soil, sediment and groundwater.

Many technologies, such as activated carbon adsorption, advanced oxidation processes (e.g., Fenton oxidation) aerobic/ anaerobic degradation, have been proposed, developed, commercialized and applied in the industries to meet stringent regulations of discharging wastewaters containing such pollutants. Conventional chemical and physical methods, although widely accepted, still face some serious limitations such as the formation of hazardous by-products, high cost, low efficiency and limited applicable concentration range (Husain and Ulber 2011). From an environmentally friendly perspective, biodegradation with microorganisms, which requires the least energy input and generates minimal secondary pollution, appears to be a predominant process compared with the others. However, the microbial environment requires more sophisticated maintenance to keep high removal efficiency and the biodegradation rate is highly affected by the compound toxicity and resistance to break down. Therefore it is more suitable for polishing as a secondary treatment and is not optimal for rapid removal.

The discovery of enzymatic treatment created a new opportunity for treating hazardous aromatic compounds. The high specificity of enzymes allows efficient removal of target compounds. It also has no shock loading effect, operates under mild conditions over a wide range of pH and temperature and achieves a high percentage of polymerization, thus avoiding further treatment. The scope of enzymatic treatment has been extended to a wide range of compounds in the past 30 years and many EPA high priority pollutants have been reported to be directly or indirectly removed by peroxidases (EC 1.11.1.7) and laccases (EC 1.10.3.2), the two most common classes of enzymes studied in this field to date. However, as an evolving technology, the overall cost of treatment, mainly due to enzyme cost, is still higher than many conventional treatment methods. Finding a cheaper enzyme source and establishing industrial production could help lower the enzyme cost. Also, the feasibility to treat various industrial wastewaters has not been widely proven. Hence, the cooperation between government and industry is necessary to enhance its improvement.

1.2 Phenols as Pollutants

Phenolic compounds may occur naturally in water and soil as the decomposition products of plants, vegetation and animal waste (Dobbins et al. 1987). Most of these compounds released to the environment are by-products as well as contaminants discharged from various industrial sectors and municipal wastewater treatment plants (Environment Canada 2000). In 1996, total release of 414 tonnes of phenol was reported to the Environment Canada with 58.5 tonnes being discharged into water, 76% of which was from the pulp, paper, and wood industries (Environment Canada 2000). There are over

150 pulp, paper and wood products mills in Canada. The average effluent concentration of phenol from 26 pulp, paper and wood products mills in Ontario was 0.40 mg/L in 1996 (Environment Canada 2000). The total phenol(and its salts) discharged from the petroleum and coal products refining contributed approximately 42% of the yearly discharge in 2011 (Environment Canada 2013). Phenol levels in groundwater ranged from not detected to 72.6 μg/L in 11 groundwater samples in Quebec (Lesage and Jackson 1992).

1.3 Conventional Treatment Methods

The conventional methods to remove phenol from wastewater can be divided into three categories: biological, chemical and physical, including: activated carbon/resin adsorption, wet air oxidation, ozonation/advanced oxidation, Fenton reaction, electron-oxidation, photocatalytic oxidation, and aerobic/anaerobic degradation.

In physical methods, adsorption of phenolic compounds from aqueous solution by activated carbon (AC) is one of the most investigated and frequently used methods of all liquid-phase applications (Dąbrowski et al. 2005). AC adsorption is mostly used in water treatment to remove toxic substances and a limited use of AC as post-treatment of wastewater has been reported. The adsorption capacity of AC depends on a number of factors, including physical properties of the adsorbent such as porous structure, as well as the properties of the adsorbate such as pKa, functional groups, polarity, molecular mass and molecular size and the solution conditions such as pH, ionic strength and adsorbate concentration (Dąbrowski et al. 2005). The following possible interactions between the carbon surface and phenols have been proposed: (a) electron donor–acceptor interactions

between the aromatic phenolic ring and the basic surface oxygen, (b) dispersion effect between the aromatic phenolic ring and the *pi*-electrons of the graphitic structure, (c) electrostatic attraction and repulsion when ions are present (László et al. 2003). The major drawback of AC adsorption is the regeneration of saturated carbon. The regeneration process is expensive, not complete due to a fraction of irreversible adsorption, and requires further treatment of air emissions (Ahmaruzzaman 2008). In order to reduce the cost of adsorbent material, study of AC adsorption has also been extended to low-cost natural materials, such as avocado kernels. It was found that avocado kernel AC had a maximum adsorption of 90 mg/g of phenol at the optimum pH in 4.5 - 8.5 (Rodrigues et al. 2011), whereas commercial AC only had a maximum adsorption of 30 - 45 mg/g (Stavropoulos et al. 2008) . Attempts to use physical adsorption for removing phenol also focused on various natural solid materials, such as clay, biomass, zeolite, chitin and chitosan (Ahmaruzzaman 2008).

Recently, advanced oxidation processes (AOPs) have been applied with success for removal of COD, BOD, dyes, endocrine disrupting chemicals and other persistent organic chemicals from industrial and municipal wastewaters. The *in-situ* generated hydroxyl radicals ($\cdot OH$) with high redox potential make it possible to oxidize any organic compound in wastewater. Available AOP technologies include H_2O_2/UV, ozone/H_2O_2/UV, Fenton reaction, and titanium dioxide/UV. The advantages of AOPs include effective elimination of almost all organic compounds, small footprint and, ability to handle shock loading. Disadvantages include no control on formation of intermediates, lack of oxidation selectivity, high capital cost and potential toxicity from metal oxides and metal salts to downstream biological treatment. A study of using O_3 to

remove phenol from water identified intermediates such as catechol and hydroquinone, *p*-benzoquinone and *o*-benzoquinone when using GC/MS and HPLC analysis (Turhan and Uzman 2008). A study also compared phenol removal efficiency for different ultrasound, ozone and UV combinations (Kidak and Ince 2007). For a batch reactor operation with 2.5 mM phenol, phenol decay rates at acidic and alkaline pH were as follows: at pH 2.0, $US/UV/O_3 >> O_3/UV > US/O_3 > US/UV > O_3 > US > UV$ and at pH 10.0, $US/UV/O_3 > O_3/UV > O_3 > US/O_3 > US/UV > UV > US$.

Biological treatment of phenol and phenolic compounds by bacteria, fungi, and algae has been extensively studied at biochemical, physiological and genetic levels. Activated sludge processes are traditionally utilized to treat wastewater containing phenols with a relatively low processing cost and few by-products (Al-Khalid and El-Naas 2012). However, the practical application of this technology is rather limited because of its poor adjustability to fluctuation in the loading (Al-Khalid and El-Naas 2012, Jiang et al. 2007). Pure and mixed cultures of the *Pseudomonas* genus are the most commonly utilized biomass for the biodegradation of phenols due to its high removal efficiency (El-Naas et al. 2010, Stoilova et al. 2007). Under aerobic conditions, phenol is first converted to catechol, and subsequently degraded by *ortho* or *meta* fission to intermediates of central metabolism (Gurujeyalakshmi and Oriel 1989). The two different ring cleavage pathways were used to classify catechol dioxygenases: the intradiol (such as catechol 1, 2-dioxygenase) and extradiol (such as catechol 2,3-dioxygenase) cleaving enzymes (Cai et al. 2007). Many factors could affect biological degradation efficiency, such as temperature, pH, dissolved oxygen concentration, biomass concentration and food supply. In general, phenol inhibits microbial growth and kills microorganisms at high

concentration. For synthetic wastewater phenol concentrations in the range of 1 - 500 mg/L, >95% removal of phenol was obtained at near neutral pH and temperature range of 25 - 35℃ and the time required for degradation varied from 14 - 96 hours (Al-Khalid and El-Naas 2012, Monteiro et al. 2000, Saravanan et al. 2008, Tepe and Dursun 2008). No bacterial growth could be detected at phenol concentrations above 1200 mg/L (Kumar et al. 2005). Lu et al. (2009) used immobilized *Phanerochaete chrysosporium* to treat a coking wastewater containing 313.5 mg/L phenols and 3420 mg/L COD. They found that the optimum phenol removal condition was pH 5.0 and 35℃, wherein 84% phenol and 80% COD could be removed in 3 days. Phenols also occur in an anaerobic digester during biodegradation of pesticides, amino acids, humic acids, lignins and tannins (Levén et al. 2012) . The presence of phenols can negatively affect the performance of anaerobic digestion, by inhibiting the activity of ammonia oxidizing bacteria and reducing the maximum methane potential and methane generation rates (Levén et al. 2006, Puyol et al. 2012)

For treating an industrial wastewater containing a complex organic compound matrix and high COD/BOD, the design of a phenol removal strategy to achieve the optimal discharge target requires considerations of influent physical/chemical characteristics, preliminary treatment, target compounds removal, available options of treatment units, discharge standards and potential uses of the treated wastewater. Therefore, one or more than one method might be chosen to achieve the discharge standard at the lowest cost. For example, primary treatment is essential to ensure the efficiency of secondary treatments in petroleum refinery wastewater, by which suspended solids, immiscible liquids, solid particles, salts can be significantly reduced by mechanical, physical and chemical

separation (Diya'Uddeen et al. 2011). In a case of firefighting wastewater containing 84.8 mg/L of phenol and 2322 mg/L of COD, Fenton oxidation efficiently removed 77.5% of the COD and 99% of the phenol in half an hour (Diya'uddeen et al. 2012). However, the final COD was 522 mg/L after the treatment, which indicates the wastewater still contained remaining organic intermediates, thus further biological treatment was necessary. In fact, studies on combined AOP and biological treatment have shown great success in recent years. The elimination of toxic aromatic compounds by ozone or UV/ozone helps to maintain a high efficiency of BOD/COD degradation by microorganisms in downstream treatment (Lafi et al. 2009), or, for an influent containing a readily biodegradable fraction, post-treatment AOP is more useful to avoid chemicals consumption and to eliminate the non-biodegradable portion (Oller et al. 2011). Integrated AOP/bio-treatments have been applied in various industries to remove phenol, phenolic compounds and polycyclic aromatic hydrocarbons, such as paper mill, olive mill, pharmaceutical and textile wastewaters, pesticides and herbicides and landfill leachate (Oller et al. 2011).

1.4 Enzymatic Treatment

Phenol removal by enzyme-catalyzed polymerization has been widely investigated as an alternative to conventional treatment methods. This method was first proposed by Klibanov and colleagues (Klibanov et al. 1980) using horseradish peroxidase (HRP, EC 1.11.1.7) to remove toxic phenols and anilines. Bollag, a soil chemist, also proposed the use of laccase and peroxidase to oxidize toxic aromatic compounds from a soil remediation perspective (Bollag et al. 1987, Leonowicz and Bollag 1987). Studies were

then quickly extended in various directions, such as establishing the reaction kinetics and inactivation mechanism, exploring more substrates and peroxidases, employing different reactors and examining the influence of additives. Hundreds of journal articles can now be found from any online database. Enzymatic treatment has many advantages over the conventional wastewater treatment systems. Compared with physical and chemical treatments, enzymes have high specificity for a target pollutant and result in a high efficiency of pollutant removal. The peroxidase-catalyzed oxidation ends with polymerization of the substrate, rather than having pollutant transferred to a different phase (e.g., adsorption). It also has a lower risk of forming persistent intermediates and secondary pollutants when compared with AOPs. Compared with biological treatments, enzyme treatment can deal with high concentrations of pollutant, has no shock loading effect, operates under mild conditions over a wide range of temperature, pH and salinity, has no lag-phase for biomass accumulation, is an easier process to control and maintain, is faster and therefore has a smaller footprint and has less sludge formation (Nicell et al. 1993). The main disadvantage of this method is the cost of enzyme. For peroxidase as the reaction catalyst, the lifetime is significantly reduced by several known enzyme inactivation pathways: free radical attack, H_2O_2 inactivation and reaction end-product inactivation. Different strategies have been proposed to minimize this effect. On the other hand, research attention has shifted from HRP to SBP (soybean peroxidase, EC 1.11.1.7) as a cheaper substitute in the recent years. SBP as a value-added product from soybean production has a nearly unlimited, worldwide supply of raw material. High thermal and pH stability, high reactivity and low susceptibility to H_2O_2 inactivation

combine to make it a perfect choice for application in the enzymatic treatment of industrial wastewaters.

1.5 Soybean Peroxidase

1.5.1 Soybean Peroxidase Catalytic Properties

Soybean peroxidase (oxidoreductase), a member of class III plant peroxidase superfamily, is a 37 KD glycoprotein, which can be found in the seed coat of soybeans (Henriksen et al. 2001) . Peroxidases are known for their ability to oxidize a broad range of phenols, anilines and other aromatic substrates with H_2O_2. The most extensively studied peroxidase is HRP, extracted from horseradish root. SBP, shows 57% amino acid sequence homology with HRP and has been receiving attention recently even in its crude form (Bassi et al. 2004, Henriksen et al. 2001, McEldoon and Dordick 1996). The strong heme-apo-protein interaction suggest an increased hydrophobic and ionic interaction at the heme-cavity of SBP that stabilizes its native state, which could be the major contributor to the conformational, thermal and kinetic stabilities being substantially higher than those of HRP (Kamal and Behere 2008). The optimal catalytic activity of SBP in a standard activity test with phenol and 4-AAP is at pH 6.4; more than 90% of the activity is retained in the range of pH 5.7 to 7.0 (Wright and Nicell 1999). The unusually high thermal stability allows SBP to lose virtually no activity after 12 h incubation at 70 ℃, the "melting" temperature of 90.5 ℃ is much higher than other peroxidases based on the changes in its secondary structure and SBP shows no change in the secondary structure at temperatures below 70 ℃ (McEldoon and Dordick 1996).

The k_{cat} (catalytic constant), K_M (Michaelis constant) and k_{cat}/K_M (specificity constant or, catalytic efficiency) of HRP-C and SBP with ABTS [2, 2'-azino-bis-(3-ethylbenzthiazoline-6-sulphonate)] as substrate at 25 ℃ are shown in Table 1-2. The k_{cat} and k_{cat}/K_M values for SBP are much higher than HRP-C at two different pH values, which suggests that SBP has a better catalytic performance. The k_{cat} value of SBP shows a bell-shaped curve over pH 3-10 and the maximum k_{cat} has been reported at pH between pH 5.0-5.5 (Kamal and Behere 2003).

Table 1-1 Steady-state kinetic data with ABTS as substrate at 25 ℃
(Adopted from Kamal and Behere, 2003)

Enzyme	kcat (s^{-1})	K_M (μM)	kcat/K_M (μM^{-1} s^{-1})	pH	Reference
SBP	1230 ±58	173 ±9	7.1 ±0.1	6.8	Kamal and Behere (2003)
	2663 ±17	45 ±1	59.2 ±0.9	5.0	Kamal and Behere (2003)
HRP-C	736 ±10	178 ±8	4.1 ±0.1	6.8	Kamal and Behere (2003)
	810 ±11	270 ±8	3.0 ±0.1	5.0	Smith et al. (1992)

*with 0.5 mM H_2O_2

Wright et al., (1999) had the earliest report on the comparison of oxidation ability SBP and HRP. It was concluded that SBP activity is slightly more sensitive to pH than HRP, but significantly more stable to high temperatures.

1.5.2 Active Site of Soybean Peroxidase

The active site of peroxidase commonly bears an iron protoporphyrin IX molecule, also known as heme. The iron protoporphyrin IX molecule consists of four pyrrole rings joined by methane bridges with iron (III) in the center. It carries eight side chains, four methyl groups, two vinyl groups and two propionate groups (Figure 1-1) (Dunford 1999).

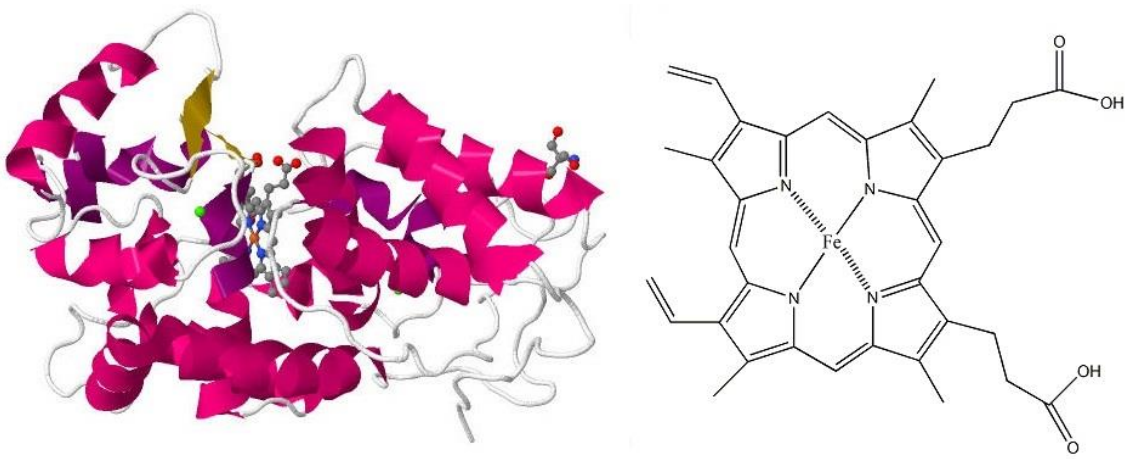


Figure 1-1 The 3-D structure of SBP and ferriprotonporphyrin IX prosthetic group.
[The 3-D structure is generated by Jmol software from RSCB-Protein Data Base, category number 1FHF; and the ferriprotonporphyrin IX prosthetic group is adopted from Van Rantwijk and Sheldon, 2000]

1.5.3 Catalytic Cycle

The peroxidase reaction mechanism was proposed from studies on HRP. Based on the steady-state kinetics of HRP/H_2O_2/AH, Dunford and coworkers developed a two substrate ping-pong mechanism model, where AH is the reducing substrate. Three step catalytic cycle can be expressed as below. In step 1, the native form of enzyme (E_0) loses two electrons to hydrogen peroxide to form an oxidized enzyme intermediate called Compound I, and hydrogen peroxide is reduced to water (Equation 1-1). In step 2, Compound I oxidizes a reducing substrate AH (phenol, for example), generates a free radical (A·) from the reducing substrate, then forms a second enzyme intermediate called Compound II (Equation 1-2). In step 3, Compound II oxidizes another reducing substrate molecule, generates another free radical and returns to its native form (Equation 1-3) (Deyhimi and Nami 2012, Dunford 1999).

$$\mathrm{HRP[Fe^{III}] + H_2O_2 \xrightarrow{k_1} Compound\ I\ [Fe^{IV} = O, Porphyrin\ \pi - cation\ radical] + H_2O}$$ Equation 1-1

$$\mathrm{Compound\ I\ [Fe^{IV} = O, Porphyrin\ \pi - cation\ radical] + AH \xrightarrow{k_2} Compound\ II\ [Fe^{IV} = O] + A\cdot + H^+}$$ Equation 1-2

$$\mathrm{Compound\ II\ [Fe^{IV} = O] + AH \xrightarrow{k_3} HRP[Fe^{III}] + A\cdot + OH^-}$$ Equation 1-3

The net HRP-catalyzed reaction is summarized as follows:

$$\mathrm{H_2O_2 + 2\ AH \xrightarrow{HRP} 2\ A\cdot + 2\ H_2O}$$ Equation 1-4

The radicals formed from the reducing substrate can couple to form a dimer product, which can be further oxidized to a dimer radical which couples analogously and the polymer chain grows by cycles of radical coupling and re-oxidation until it precipitates out. The formation of Compound I and Compound II are extremely rapid, the magnitude of the reaction rate constants k_1 and k_2 are several orders of magnitude higher than k_3, thus the third step is the rate-determining step in the mechanism (Deyhimi and Nami 2012, Dunford 1999). Theoretically, a 1:2 molar ratio of peroxide to reducing substrate is needed to completely oxidize the target compound; however, the actual H_2O_2 consumption is often in a ratio of 1:1 or even higher because oxidation of reaction intermediates would consume extra H_2O_2 above the theoretical stoichiometry (Yu et al. 1994).

1.6 Enzyme Inactivation and Additive Studies

Three possible enzyme inactivation pathways have been proposed: hydrogen peroxide inactivation (Arnao et al. 1990), inactivation due to free-radical attack during the catalytic cycle (Klibanov et al. 1983), and end-product inactivation (Nakamoto and Machida 1992). Peroxidase inactivation by H_2O_2 is a time- and H_2O_2 concentration-dependent process. As

mentioned above, peroxidase first gets activated by H_2O_2 to form Compound I under turnover conditions; however, in the absence of reducing substrate and presence of excess H_2O_2, Compound I is irreversibly converted to an inactive verdohemeoprotein, called P-670 (Baynton et al. 1994). Step addition of H_2O_2 to the reactor reduces instantaneous H_2O_2 concentration and therefore protects peroxidase from being inactivated through this path (Mousa Al-Ansari et al. 2010). The presence of reducing substrate also protects peroxidase, since the reaction rates in catalytic cycle are several orders of magnitude higher than other reaction pathways. Under turnover conditions, the free radicals generated from the reducing substrate can return to the active site and form a covalent bond thereby preventing further substrates' access to the active site. Experimental evidence of the 'free radical inactivation' hypothesis shows significant amounts of phenoxylphenol-type oligomers in solution and probably also in the heme pocket; phenoxyl radical attack results in heme macrocycle destruction (Huang et al. 2005). For end-product inactivation, earlier studies inferred that PEG (polyethylene glycol) can act as a "sacrificial polymer". It can substitute for the enzyme in adsorption onto the solid polymer products, thus preventing the apparent end-product inactivation (Nakamoto and Machida 1992). PEG is a low-cost and effective additive to extend enzyme lifetime. Reports of a "PEG effect" have covered more than 13 peroxidases and laccases with dozens of substrates (Steevensz et al. 2012). Studies to optimize PEG usage observed a threshold concentration where the minimum effective PEG concentration disappeared from the solution and the excess remained (Modaressi et al. 2005). Experiments suggested that PEG combined with the phenolic products during the reaction because of the higher partition affinity than HRP, thus protecting HRP against this type of

inactivation (Wu et al. 1998). Addition of other proteins, hydrophilic synthetic polymers, or surfactants to decrease end-product inactivation has also been reported. Surfactants such as Triton X-100 (Triton), Triton X-405, Tween 20, SDS (sodium lauryl sulfate) and DTAB (lauryl trimethylammonium bromide), have also been proven to enhance phenol removal efficiency with SBP and CIP (*Coprinus cinereus* peroxidase, EC 1.11.1.7) (Mousa Al-Ansari et al. 2010, Sakurai et al. 2003). It was reported that (a) the presence of Triton during phenol polymerization could suppress CIP adsorption by phenolic end-products; (b) Triton that was added after phenol polymerization was underway has the ability to re-activate the CIP precipitated through the phenol polymerization via its desorption (Sakurai et al. 2003). This "Triton effect" suggested a reversible immobilization of active enzyme during the reaction. However, the interaction between surfactant, enzyme and reaction products is not clear yet. Crude enzymes have shown similar phenol removal efficiency as purified enzyme with significantly less 'PEG effect'; thus, it has been suggested that the impurities serve a protective function similar to that of PEG (Mao et al. 2006, Masuda et al. 2001). SDS and Triton X are more efficient than PEG in protecting crude SBP during phenol removal (Mousa Al-Ansari et al. 2010).

1.7 Post-Treatment Toxicity

Enzymatic reaction is well-known for the effective removal of toxic aromatic compounds from water. Although enzyme-catalyzed polymerization could detoxify the target compounds, the soluble intermediate products may still have some associated toxicities. Therefore it is important to examine the toxicity before discharging it to the downstream process. Hydrogen peroxide as the co-substrate was determined to have an EC_{50}

concentration of approximately 1.46 mM or 49.6 mg/L; therefore, any remaining H_2O_2 after the treatment could contribute to the total toxicity of the effluent (Ghioureliotis and Nicell 2000). At the high end of treatment (>99% removal) by SBP and HRP, a treated 1 mM phenol sample had surprisingly higher acute toxicity than the control sample (no reaction). The toxicity was expected to be from the 2% remaining non-phenolic products, suspected to be quinones. Parallel experiments with 5 mM and 10 mM phenol showed the same residual toxicity as for 1 mM, with an even higher amount of by-products remaining. This suggested that the residual toxicity was independent of the initial substrate concentration (Ghioureliotis and Nicell 2000). Extending detention time after the treatment to 24 hours significantly reduced the toxicity level to below that of the untreated sample, especially when PEG was added to the reaction. Whereas the toxicity of PEG-treated samples after 3 h reaction was higher than that in the absence of PEG, after sitting for 24 hours, it was reduced to the same level as in the absence of PEG (Wagner and Nicell 2002a). Granular activated carbon can be used to completely remove highly toxic trace amounts of soluble products (Ghioureliotis and Nicell 1999). Other proposed strategies include addition of H_2O_2 or chitosan as post-treatment and co-polymerization of several parent and substituted phenol compounds (Ghioureliotis and Nicell 2000, Wagner and Nicell 2002a).

1.8 Peroxidases and Laccases in Wastewater Treatment Applications

Research on enzyme-catalyzed aromatic compound removal has been primarily conducted in laboratories with synthetic wastewater. SBP, as compared to HRP, laccases and microbial peroxidases, has proven its value in both catalytic efficiency on target

compound removal and feasibility for mass production. Firstly, SBP can be easily extracted from soybean hulls by soaking in water; massive worldwide production of soybeans provides an unlimited source of raw material. Secondly, SBP has higher conformational stability over the pH range of 3.0-9.0 and higher thermo-tolerance (activity remains up to 70℃) (Kamal and Behere 2003, McEldoon and Dordick 1996). Thirdly, SBP is less susceptible to irreversible inactivation by hydrogen peroxide when compared to HRP and ARP (*Arthromyces ramosus* peroxidase) (Mousa Al-Ansari 2011). Mousa Al-Ansari et al. (2010) reported the use of SBP to remove 15 mM total phenols from coal-tar wastewater. They found that 14 U/mL SBP and 20 mM H_2O_2 achieved >95% removal of phenols in 3 h reaction. A recent on-site attempt to treat an alkyd resin manufacturing wastewater with 6.8 to 27.8 mM parent phenol and >40 g/L of organic carbon by using crude SBP and Triton was successful (Steevensz et al. 2013b). The use of Triton reduced the required SBP concentration by 15-fold. Three out of four trials had >85% phenol removed with a sub-optimal Triton concentration and 30-50% excess of SBP over that predicted from laboratory studies conducted in advance. Other studies have reported the use of a fungus as an enzyme source to decolourize dyes from textile wastewater in synthetic wastewater, but little has been done to apply the strategy in real industrial wastewater treatment. Sangeeta et al (2011) reported the use of white rot fungus, *Phanerochaete chrysosporium* to treat textile industry wastewater with lignin peroxidase (LiP, EC 1.11.1.14) and manganese peroxidase (MnP, EC 1.11.1.13) produced by the fungus: 84% decolourization and 86% COD removal were achieved by the culture with glucose supplementation. Steevensz et al. (2009a) reported that refinery samples required 1.5- to 1.8-fold more enzyme than the corresponding optimum laccase

concentrations required for the synthetic samples of the same concentration in tap water (0.12 U/mL) to achieve >90% removal of phenol.

Pre-treatment is often required to ensure efficient enzymatic reaction in industrial wastewater treatment. Reducing anions consume H_2O_2 competing with enzyme and may result in insufficient H_2O_2 for substrate conversion. The reported reducing anion effects on phenol removal are F^-, Cl^-, Br^-, I^-, SO_4^-, NO_3^-, CN^-, $S_2O_3^{2-}$ and SO_3^{2-} (Wagner and Nicell 2002b). Cyanide, halide and sulfide could increase the required enzyme concentration or inactivate enzyme completely. A 'sour water' sample from an oil refinery containing phenol required 1 to 2-fold higher SBP or laccase and 2 to 3-fold higher H_2O_2 than a corresponding synthetic sample for complete phenol removal when the sample was treated directly; however, addition of H_2O_2 as a pre-treatment reduced SBP concentration by 2-fold. (Steevensz et al. 2009a). It has also been suggested that sulfite, thiosulfate, Br^- and I^-, require elevated dosage of H_2O_2 due to oxidation reactions and insufficient addition of H_2O_2 could result in apparent inhibition of the enzymatic reaction. With sufficient H_2O_2, sulfite and thiosulfate promoted the phenol conversion rate with HRP (Wagner and Nicell 2002b). The rationale was that sulfite and thiosulfate oxidization products interacted with oxidized phenolic species and thus neutralized their inactivation capability. No significant negative effect was observed with Br^- and I^- (Steevensz et al. 2009a, Wagner and Nicell 2002b). Cyanide has the strongest inhibition; thus any wastewater with cyanide should be pre-treated before enzyme treatment. High salt concentration promotes phenol polymer precipitation due to the increase in ionic strength; however, it also increases the chance of enzymes' being adsorbed on the precipitates, co-precipitating with the polymer products (Wagner and Nicell 2002b).

1.9 Initiatives and Objectives of the Study

Immobilization/adsorption of peroxidase by phenolic precipitates has been widely observed and defined as one of the negative effects in enzymatic treatment (Masuda et al. 2001, Sakurai et al. 2003, Wu et al. 1998). Early evidence had shown that the adsorption of active enzyme from the aqueous phase would result in a decrease of mixture activity, thus it was concluded to be inactivation by reaction with the end-products. The strategy to prevent the inactivation is to use a low-cost additive. For example, PEG is a low-cost polymer with an impressive protection efficiency on purified peroxidases; the use of PEG saved more than 40-fold HRP for 1 mM phenol removal, 93-fold HRP for 1 mM *m*-cresol removal and 200-fold ARP for 1 mM *p*-chlorophenol removal, and dozens of other substrates (Steevensz et al. 2012, Taylor et al. 1996, Wu et al. 1993). The PEG effect on crude SBP was barely satisfactory, the optimized SBP concentration was 1.2 U/mL in the absence of PEG and 0.9 U/mL with 125 mg/L of PEG to achieve >95% removal of 1 mM phenol removal in 3 h reaction (Mousa Al-Ansari et al. 2010). On the other hand, the use of surfactants has shown promising results on enzyme saving, but no protection mechanism has been elucidated (Mousa Al-Ansari et al. 2010, Sakurai et al. 2003). The effective ‘anti-adsorption’ phenomenon of Triton, between enzyme, surfactant and phenolic precipitates, demands more understanding. Therefore, this study chose phenolic precipitates, the reaction end-product, as an object to study its role in SBP-catalyzed phenol polymerization reaction, and to complement the understanding of additives’ protection mechanism(s).

The objectives for this study were:

I. Optimize recycling of polymerized precipitates for phenol removal in the absence of additive;

II. Study the effects of additives on SBP adsorption and phenolic precipitates formation.

III. Quantitatively characterize the SBP adsorption on and elution from phenolic precipitates.

IV. Design and optimize a process to concentrate SBP from a dilute solution using phenolic precipitates.

1.10 Scope of the Study

The scope of this study included:

I: Optimization of precipitate recycling for 1 mM phenol removal in the absence of additive:

1. Determine SBP adsorption characteristics of phenolic precipitates under turnover and static conditions;
2. Examine phenol removal efficiency by free SBP and SBP adsorbed on precipitates;
3. Determine the reversibility of SBP adsorption by the addition of Triton X-100;
4. Determine the feasibility of precipitates recycling as a consecutive batch reaction strategy for phenol removal; and
5. Optimize SBP concentration for 1 mM phenol removal with quantitatively recycled precipitates.

II: Study the effects of additives on SBP adsorption and phenolic precipitates formation:

1. Examine the additive's effect on the activity of free SBP and SBP immobilized on phenolic precipitates;
2. Investigate the PEG effect on prevention of SBP adsorption by phenolic precipitates under turnover and static conditions;
3. Examine the SDS effect on phenol polymerization and SDS fate after the reaction; and
4. Determine the feasibility to apply the precipitates recycling strategy to phenol removal in the presence of PEG, SDS or Triton;

III: Quantitative characterization of SBP adsorption on and elution from phenolic precipitates:

1. Determine SBP and Triton adsorption isotherms;
2. Examine % SBP elution in relation to % saturation, Triton concentration and phenolic precipitates concentration;
3. Develop an equation to calculate % SBP elution as a function of Triton concentration at different precipitate concentrations;
4. Examine time course of SBP elution by Triton; and
5. Adapt a competitive adsorption model for SBP and Triton adsorption on phenolic precipitates.

IV: Design and optimize a process to concentrate SBP from a dilute solution using phenolic precipitates:

1. Determine Langmuir adsorption constant, K, and adsorption capacity, Q_{max}, of phenolic precipitates under different conditions in the proposed process cycle;

2. Examine Triton mass balance and SBP activity balance in the SBP concentrating cycle;
3. Optimize ethanol wash efficiency for phenolic precipitates regeneration;
4. Conduct consecutive cycles of SBP concentration by phenolic precipitates; and
5. Propose a commercial model to use phenolic precipitates to capture SBP from SBP raw extract; and
6. Characterize SBP adsorption under turnover conditions (dynamic adsorption).

2. LITERATURE REVIEW

2.1 Phenol

2.1.1 Chemical and Physical Properties

Phenol is a white hygroscopic crystalline solid at room temperature with an acrid smell. The chemical formula of phenol is C_6H_6O, and its molecular mass is 94.11. Important physical and chemical properties of phenol are listed in Table 2-1. Phenol was first isolated from coal-tar in 1834 by the German chemist Runge (Busca et al. 2008).

Table 2-1 Physical and Chemical Properties of Phenol

Chemical	Phenol	Reference
Molecular Structure		
Chemical Abstracts Service (CAS) number	108-95-2	
Melting point (℃)	41	Verschueren (1983)
Boiling point (℃)	182	Verschueren (1983)
Vapour pressure (Pa)	47	Dean and Lange (1992)
Henry's law constant (atm•m^3/mol)	2.2×10^{-6}	Hansch et al. (1995)
pKa	9.99	Dean and Lange (1992)
Log K_{oc}	1.15–3.49	Environment Canada (2000)
Log K_{ow}	1.46	Fujita et al. (1964)
Solubility in water (g/L)	88.3	Blackman et al. (1955)

Phenol is very soluble in ethyl alcohol, ether and several polar solvents, as well as in hydrocarbons such as benzene. In water, it behaves as a weak acid (Busca et al. 2008).

2.1.2 Industrial Applications

Phenol is a common industrial chemical. In Canada, the manufacture of phenolic resins accounts for about 85% of the phenol consumption (Environment Canada 2000). Phenol

is also used in a wide range of other applications, such as a feedstock of other organic substances (including bisphenol A, caprolactam, aniline, adipic acid, alkyl phenols, chlorophenols), pharmaceutical preparations, perfumes, disinfectants and anesthetics. It is also present in a number of personal care products, including ointments, ear and nose drops, cold sore lotions and mouthwashes (Environment Canada 2000).

2.1.3 Phenol and Health

The acute and chronic toxicity of phenol to aquatic organisms and terrestrial organisms have been extensively studied (Kishino and Kobayashi 1995, McLeay 1976, Schafer Jr et al. 1983). Exposure of phenol to humans can cause severe irritation to the skin, eyes and mucous membranes (Environment Canada 2000). Study of potential carcinogenicity of phenol indicated a non-significant trend between cancer mortality and increasing duration of exposure to phenol (Blair et al. 1990, Kogevinas et al. 1995). Phenol is rapidly absorbed through skin upon contact, thence distributed to the tissues and is eliminated as metabolites, primarily in the urine. Long-term expose to phenol can cause damage to heart, skin, kidneys, liver, and lungs (ATSDR 2008).

2.1.4 Environmental Fate

In the atmosphere, phenol exists predominantly in the vapour phase with an estimated half-life of 2-23 hours depending on different weather conditions. Thus it is not expected to be transported over long distances (Environment Canada 2000). Biodegradation is a major process for the removal of phenol from surface waters (Hwang et al. 1986). The low organic carbon/water coefficient (log Koc) and low octanol/water partitioning

coefficient (log Kow) indicate that phenol is not expected to accumulate in the sediment in water (Environment Canada 2000). In soil, phenol can be removed by aerobic/anaerobic biodegradation, adsorption, volatilization and oxidation (Environment Canada 2000). The suggested half-life of phenol in soil is 170 hours (Mackay et al. 1992).

2.2 Structure of Enzymatic Polymer Products

Most attention on enzyme-catalyzed polymerization products has been from the polymer synthesis perspective, as a substitute of phenol-formaldehyde resin. Enzyme-catalyzed polymer synthesis can occur under mild conditions with good yield and avoids using toxic formaldehyde (Kobayashi 1999). In water, the polymerization reactions can only proceed to a certain stage until the polymer products precipitate out. In the presence of water-miscible solvent, such as dioxane, acetone, dimethylformamide and methyl formate, the polymer products can be kept soluble in the aqueous phase and continuously oxidized by enzyme. Polymer syntheses using peroxidases and a number of phenols have been reported to form various phenolic polymers with average molecular mass from 400 to 26000 (Dordick et al. 1987). The proposed phenol polymerization mechanism has four process steps. The first step is the formation of phenoxyl radicals by the peroxidase-catalyzed oxidation (Figure 2-1 a). The phenoxyl radicals then form dimers by recombination (for example, Figure 2-1 b). At the beginning of the reaction, practically all reacted phenols are converted to dimers. When the concentration of free phenoxyl radicals decreases, an electron-transfer reaction is more likely occur than further recombination. This leads to the third step, the formation of oligomer radicals (for example, Figure 2-1 c), which then form oligomers of even higher molecular mass by

recombination (for example, Figure 2-1 d). The radical transfer reaction of a phenoxyl radical and an oligomer regenerates a phenol monomer, which can be oxidized again by the enzyme to initiate new radical transfer reactions. When the phenoxyl radical generation is not reacting fast enough in a recombination or a radical transfer step, oxidation may take place leading to ketone structures (Ghoul and Chebil 2012). The kinetics of the polymerization has shown that the rate-determining step of the overall polymerization is the formation of the enzyme-activated monomer (Kobayashi 1999). The radical recombination happens predominantly through C – C and C – O coupling with *o*-, *p*- orientation on the benzene rings (Ghoul and Chebil 2012), thus the polymer structure is random. It is not known in what molecular mass range the polymers/oligomers start showing hydrophobicity, which is believed to be the starting point of end-product inactivation with peroxidase.

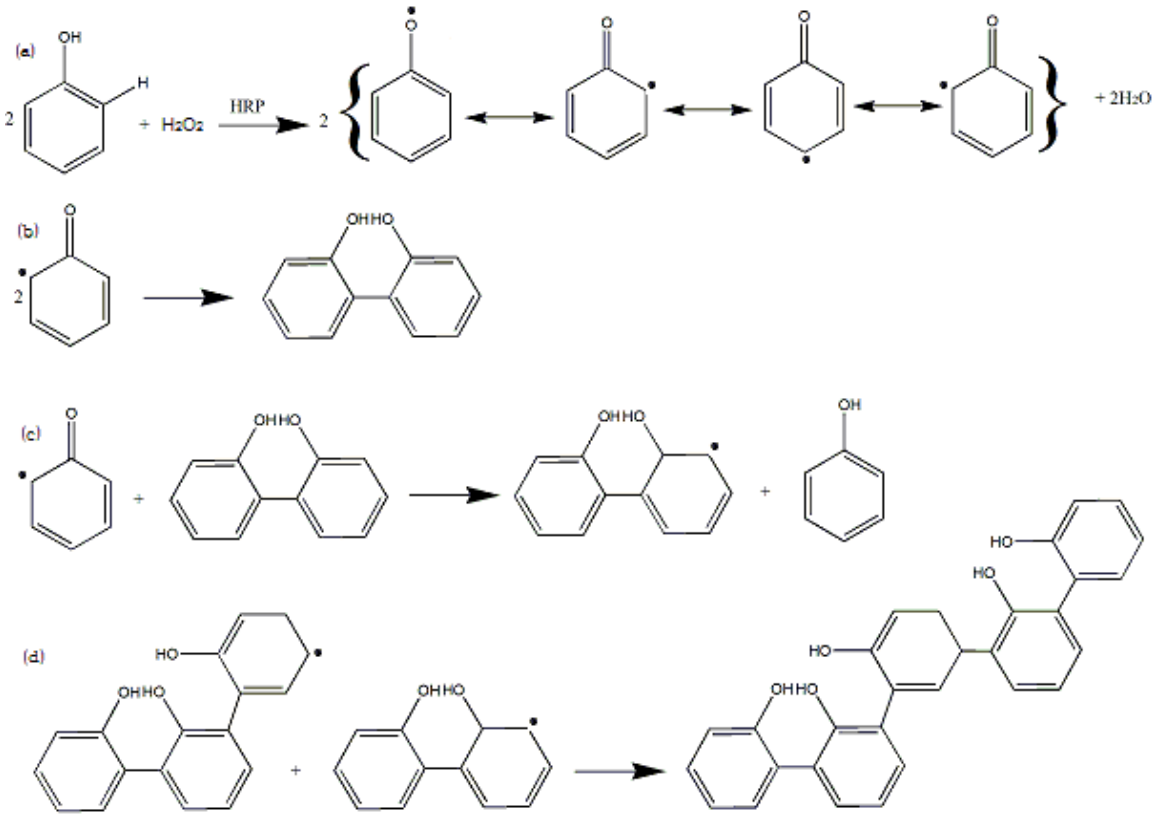


Figure 2-1 Mechanism of HRP-catalyzed phenol polymerization.

[(a) Formation of free phenoxyl radicals, (b) recombination of phenoxyl radicals, (c) radical transfer, (d) chain growth by alternating radical transfer and recombination (repetition of step 2) (adopted from Ghoul, 2012)]

For polymer chain growth, the chain initiation rate and the number of growing chains are correlated with enzyme concentration in the reaction. Higher enzyme concentration gives a lower average polymer mass with peroxidases and laccases (Hollmann and Arends 2012). At very high enzyme concentration, the initiation rate can be so dominant that only oligomers are formed; at low concentration, enzyme inactivation could become dominant and limit the substrate conversion rate thus compromising the chain growth as well (Hollmann and Arends 2012). On the other hand, the presence of an additive could affect the structure of the polymer products, thus change their physical and chemical characteristics. PEG or a PEG-based non-ionic surfactant, PEGMDE (polyethylene

glycol monododecyl ether), acts as a template for the reaction, significantly improving the regioselectivity with mainly phenylene units formed rather than a mixture of phenylene and oxyphenylene units (see Figure 2-2). The complex formation between the polyphenol and PEG by hydrogen-bonding interaction was confirmed by FT-IR (Kim et al. 2004, 2003).

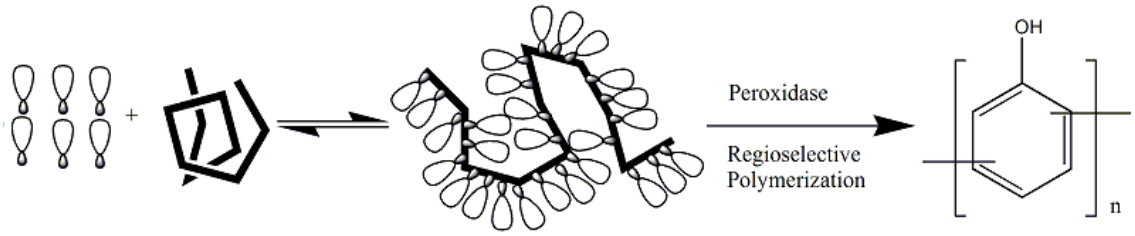


Figure 2-2 Presence of PEG to increase regioselectively of polyphenol synthesis.
(adopted from Kim et al, 2003)

Studies on the structure of random phenolic polymers in enzyme-catalyzed wastewater treatment are much fewer. HPLC analysis of phenolic products indicates that for a given phenol/H_2O_2/enzyme concentration, the product distributions could be unique and such differences could also affect toxicity after the treatment. In addition, post-enzymatic reactions, for example, phenolic radical recombination, could be influenced by the reaction pH and therefore result in a different product distribution at different pH values (Aitken et al. 1994). It has been reported that the precipitates formed are finer at neutral pH than under acidic conditions, suggesting that pH affects precipitate formation (Aitken et al. 1994, Kazunga et al. 1999). Yu et al. (1994) identified 5 stable dimers and 1 trimer as soluble products, with HRP and phenol. The use of PEG could significantly reduce the required enzyme concentration for the same level of substrate removal. However, analysis of the radioactivity after a complete treatment of radio-labelled phenol suggested

that the quantity of soluble products had increased in the presence of PEG (Ghioureliotis and Nicell 2000).

2.3 Peroxidase Extraction and Purification

HRP, the most used plant peroxidase, has been widely applied in biotechnology, in areas such as bioremediation, diagnostics and biosensors, protein engineering and cancer therapy (Ryan et al. 2006). Approximately 8000 tonnes of horseradish is produced annually in the United States, 60% of which is grown in Illinois (Kushad et al. 1999). Between 2001 to 2010, an average of seven billion units of HRP were produced and sold annually by BBI enzymes (CapeTown, South Africa). There was a yield of eight million units per tonne of horseradish root, which was not commercially viable due to the rising costs of raw materials and reagents (Barnard 2012). In recent years, as a cheaper alternative to HRP, applications and research on SBP have been steadily growing. In addition to its unusual thermal stability and catalytic efficiency, the global production of soybeans was 251.5 million metric tonnes in 2011 (SoyStats 2011). Soybean hulls, which make 8% of this total mass, are the source of SBP. Hitherto, there have only been a few companies involved in production of SBP. The feasibility of industrial scale commercialization is limited to small private companies, and the commercial viability at realistic prices has not been examined (Hailu et al. 2010). A crude SBP extract can be achieved by soaking soybean hulls in water for a few hours. It has been reported that water soaking can extract the SBP without significantly compromising the hull nutrient value (Hailu et al. 2010). Thus, recovered soybean hulls could still be sold, as usual, for animal feed. SBP yield could be as high as 90,000 units of catalytic activity (U) per kg of

hulls depending on the soybean cultivar used. Steevensz et al. (2013a) tested 65 different soybean samples from cultivars available in Ontario for the 2012 growing season. In 31 of the cultivars, the extracted SBP activity varied from 13 to 68 U/g, while the remaining 34 cultivars showed minimum or no hull activity.

Common techniques for protein concentration and purification include: ultrafiltration (UF), ion-exchange chromatography (IEC), hydrophobic-interaction chromatography (HIC), affinity chromatography (AC), gel filtration (GF), and expanded-bed adsorption (EBA). In one report, a 628-fold purification with 4% yield of SBP resulted from consecutive steps of ammonium sulfate fractionation, gel filtration, affinity chromatography and hydrophobic chromatography (Sessa and Anderson 1981). In another report, a two-step, liquid–liquid extraction process using metal ligands was developed with a yield of 64% to purify raw SBP (*Glycine max*) extract (Estela Da Silva and Teixeira Franco 2000). However, the drawbacks of these techniques such as low throughput, high cost of the solid phase and relatively short life time make it impractical for industrial scale production. As an example, the schematic for HRP production by BBI Enzymes is shown in Figure 2-3. The process is divided into primary and secondary stages. Primary processes include grinding horseradish root and extracting the enzyme from the pulp by exposing it to a slightly saline aqueous solution. The supernatant is clarified by using diatomaceous earth and then concentrated to approximately 15-30% of its original volume by using ultra-filtration with a membrane of 10 kDa molecular mass cut-off. The second stage processes

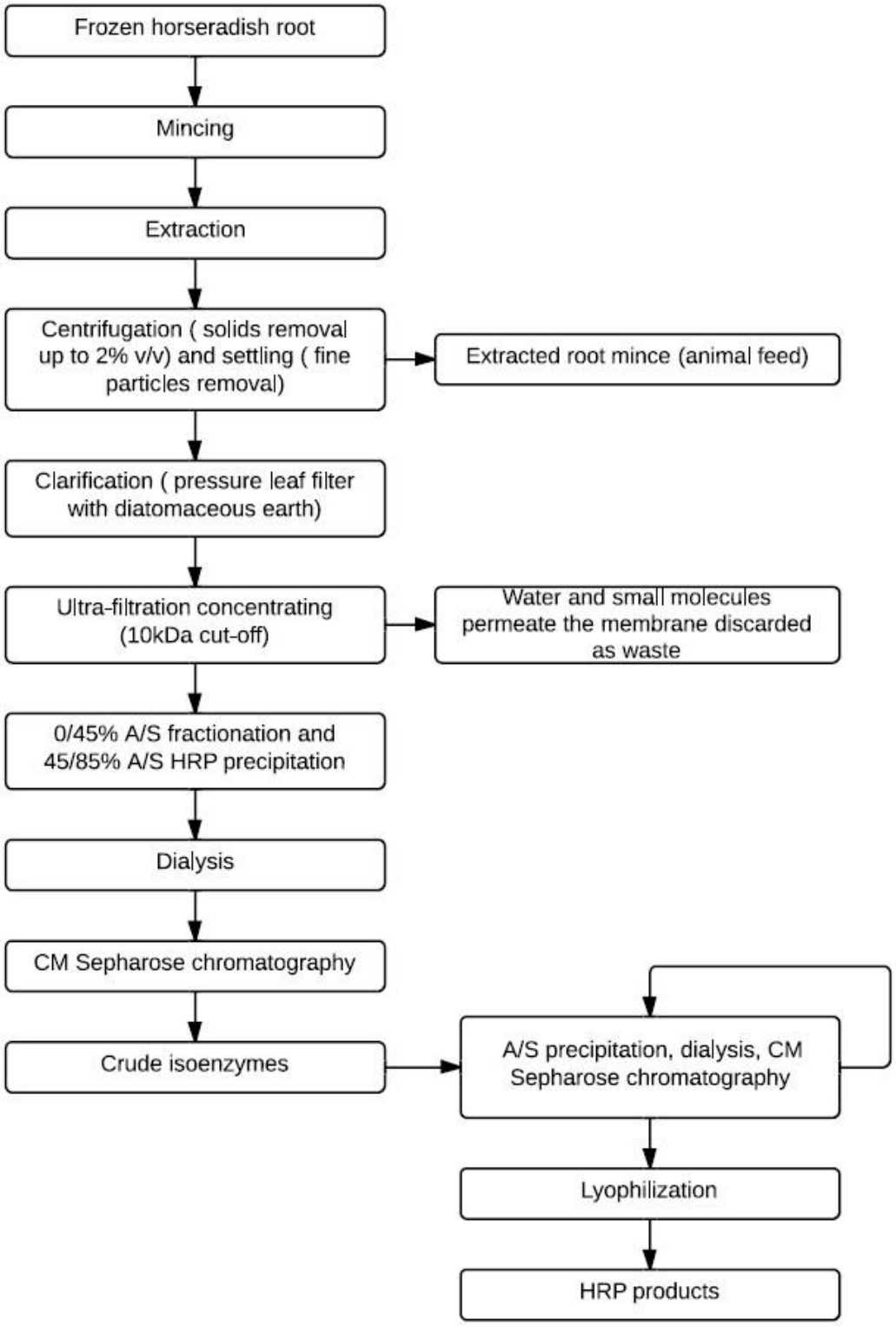


Figure 2-3 Current primary and secondary processing of HRP at BBI Enzymes.
A/S: ammonium sulfate. (Adopted from Barnard, 2012)

involve multi-stages of salt fractionation by ammonium sulfate and a series of ion-exchange and hydrophobic-interaction chromatography. The final products are salt-free lyophilized powders in different grades with RZ value (ratio the absorbance of the heme content at 403 nm to that of the aromatic amino acids at 275nm) ranging from 0.6 – 3.0 (Barnard 2012). Evidence suggests that horseradish roots left in the soil to mature for an additional growing season yields lower content of the most abundant HRP isoenzyme C (basic isoenzyme). A similar drop in SBP activity is also observed on soybean hulls, when stored in a warehouse at ambient temperature (Barnard 2012, Steevensz et al. 2013a).

Expanded-bed adsorption (EBA) has been widely studied in the past two decades for crude culture broth or broken cell slurry clarification, concentration and purification. Unlike the other chromatography technologies, which require filtration or centrifugation before sample injection, EBA allows capture of proteins from a feed-stock without prior removal of cell debris. At the adsorption stage, the flow enters the column from the bottom and expands the bed. At the designed flow rate, the undesired cells or cell debris (lighter than packing materials) pass through the column while the packing material adsorbs the protein and remains. The elution is usually carried out in a packed condition with the reversed flow, the upper adapter compresses the packing material and injects fluid to elute all adsorbed protein, and eluent is collected from the bottom of the column. The batch operation for SBP concentration in Section 4.7 can be considered as a prototype study. The major advantage in applying EBA is an efficient removal of the hull debris and isolation of the crude SBP for industrial scale production. Also, it would be a

fast and cost-effective process, avoiding time-consuming centrifugation and filtration steps.

2.4 Enzyme and Surfactant Effects on Polymer

Enzyme adsorption due to ionic binding, hydrophobic interaction and affinity binding are simple immobilization methods. Physical adsorption is a mild, easy to perform process, and usually preserves the catalytic activity of the enzyme, however, those interactions are relatively weak and may allow enzyme leakage from the adsorption matrix into the medium, with or without pH and/or temperature changes (Guisan 2006). Hydrophobic adsorption does not form chemical bonds but rather is an entropy-driven interaction, the strength of interaction relies on both the hydrophobicity of the adsorbent and the protein (Guisan 2006). One of the main problems with enzyme immobilization is the loss of catalytic activity, which could either be from a change of three-dimensional conformation of the protein or a change of substrate concentration in the microenvironment (mass-transfer effects) (Monier et al. 2010, Trevan 1981). By contrast covalent immobilization replaces the weak interactions above with strong ones and, with appropriate choice of linker 'arm', can minimize mass-transfer effects. With SBP covalent binding on aminopropyl glass beads, it was found that the SBP active site was partially modified during the immobilization process, with the enzyme retaining about 35% of its specific activity after immobilization; and a significant improvement of SBP stability over time was obtained: 50% of the immobilized activity was preserved after 70 days of storage (Marchis et al. 2012). Natural polymers, which have the advantages of being nontoxic, biocompatible and biodegradable, used as carrier materials in immobilization

technologies include: alginate, carrageenan, agarose, chitin and chitosan (Monier et al. 2010). The apparent Michaelis constant for pyrogallol, K_M (substrate concentration at which the reaction rate is half of V_{max}) was evaluated from double-reciprocal plots for HRP immobilized on modified chitosan beads. This value was higher than for free HRP, which indicated a slightly decreased affinity of HRP for substrate (Monier et al. 2010). A kinetic study of 4-chlorophenol with SBP suggested that the reaction followed a diffusion-reaction kinetic model with two stages. At time zero, the kinetic equation was reduced to the Ping-Pong bi-substrate model, which has been widely described in the literature (Dunford 1999). Intrinsic parameters of SBP can be determined by using an initial-rate procedure. However after 1-3 minutes' reaction time, the enzyme was quickly adsorbed on the polymer products, the diffusion rate and substrate concentration in the microenvironment became important for the immobilized SBP, and the Ping-Pong bi-substrate model was no longer suitable for the conditions. Extended kinetics, which incorporated an effectiveness factor, provided a good fit of the reaction's further progress. The observed effectiveness factor was reduced to 0.5 after 3-5 min of reaction and further reduced to near zero after 20 min and zero after 40 min (Gómez Carrasco et al. 2011).

Surfactant can be adsorbed onto different types of hydrophobic and hydrophilic interfaces due to the polar head and nonpolar tail groups. The adsorption of surfactants at the solid/liquid interface is strongly influenced by a number of factors, such as the functional groups of the adsorbate, the structure of the surfactant and the physical conditions of the solvent including pH, temperature, electrolyte content and presence of other solutes (Abdel-Rahem 2013). Non-ionic surfactants are physically adsorbed rather than electrostatically adsorbed or covalent binding on solid phase. Non-ionic surfactant

adsorptions on hydrophilic and hydrophobic surfaces act differently. Generally, if the hydrophobic part of the surfactant is adsorbed more strongly at the surface, this should result in the formation of monolayer or sub-monolayer structures at the surface, whereas, if the water-soluble part interacts more favourably with the surface and the hydrophobic segments are sufficiently large to trigger self-association, surface micelles or different bilayer-type aggregates can be expected at the surface (Tiberg 1996). It has been shown that surface micelles or bilayer-type aggregates were formed above a critical concentration on hydrophilic silica, and this was due to adsorbate-adsorbate and adsorbate-solvent interactions, which lead to changes in orientation and packing of adsorbed Triton at the surface, while sub-monolayer or monolayer adsorption behavior of Triton formed at a hydrophobic silica surface (Paria and Khilar 2004, Tiberg 1996). For a hydrophilic surface, when adsorbate concentration is low, the adsorbate-adsorbate interactions are minimal and adsorption follows a monolayer adsorption model. With increasing surfactant concentration, there are three possible orientations: a) when the hydrophilic group is weakly adsorbed, it will be displaced from the surface by the alkyl chains of the adjacent molecules; b) if there is a strong attraction between the hydrophilic group and a surface with hydrophilic character like silica or oxides, the alkyl chain is displaced from the surface; c) the intermediate situation is when neither type of displacement is favoured and therefore the surfactant remains flat on the surface. Finally, the adsorbed surfactants form aggregates at or beyond a critical surface aggregation concentration (CSAC), the molecules become vertically oriented and therefore a large increase in adsorption can be observed (Paria and Khilar 2004).

Triton X-100 is a widely used commercial non-ionic surfactant with 9-10 oxyethylene units (Figure 2-4). The structure contains a short hydrophobic domain for the octylphenyl group, which is expected to be adsorbed with the exposed methyl groups in close contact with the polymer surface and the water-soluble polyethyleneoxide part extending into the aqueous solution (Zhao and Brown 1996). The individual adsorptions of SBP and Triton and their competitive interactions at hydrophobic polymer surface are particularly of interest because of the observed Triton protection effect on the enzyme-catalyzed phenol polymerization reaction. The observed Triton elution of immobilized SBP is postulated to follow a competitive adsorption mechanism. Understanding adsorption behavior of each is critical to explain the surfactant protection mechanism and a useful guidance to look for better additives to extend enzyme lifetime.

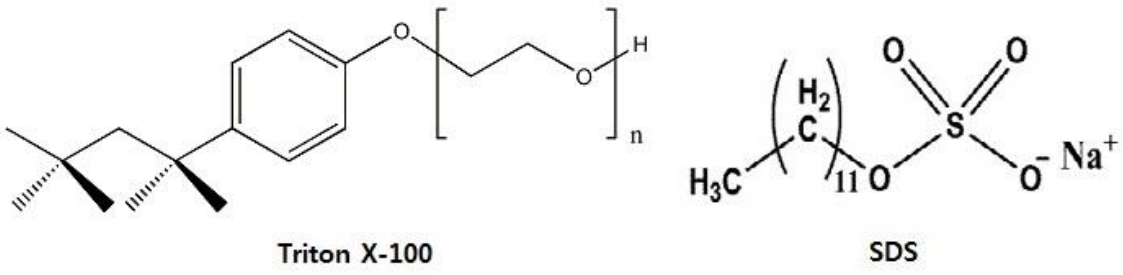


Figure 2-4 Structure of Triton X-100 (n=9-10) and sodium dodecyl sulfate (SDS)

There are two possible mechanisms of SDS interaction with the phenol polymers and SBP involved in this study. On one hand, the yield of enzymatic polymerization can be significantly increased in aqueous micelle conditions. With 100 mM phosphate buffer (pH = 7.0) and 0.45 g/L of SDBS (sodium dodecyl benzenesulfonate), the phenol conversion rate was increased over 20 times when compared with rate in the absence of SDBS at the same concentration of HRP. Also, because of the solubilization effect of micelles, the phenol polymer products can be kept soluble in aqueous solution and

therefore phenol polymer could be obtained in high yield with moderate molecular mass (Zhang et al. 2012). SDS is well known for its use in SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), where it binds in constant proportion with denatured protein molecules to impart a negative charge to linearized proteins. At low SDS concentration, the binding does not denature proteins; the interaction between SDS and protein could be by both electrostatic and hydrophobic interactions. If the hydrophilic-lipophilic balance is larger for the protein–surfactant complex than for the protein molecule on its own, a solubilization mechanism will facilitate the displacement of some of the protein from the monolayer on the interface (Pugnaloni et al. 2004).

2.5 Adsorption Models

The Langmuir adsorption isotherm is frequently used to characterize adsorption as a function of adsorbate concentration in different phases. There are three assumptions in the Langmuir model: i) all adsorption sites on the surface are equivalent, ii) each site can only hold one molecule and there is monolayer coverage only, and iii) there is no interaction between adsorbed molecules. The simplicity of the model may not be able to cover the complex properties of hydrophobic/hydrophilic or positively/ negatively charged side chains in the protein structure, but it still has been widely used as a starting point for characterization of a protein adsorption. The adsorption isotherm can be expressed by:

$$q_e = \frac{Q_{max} K C_e}{1 + K C_e} \qquad \text{Equation 2-1}$$

For SBP adsorption on phenolic precipitates, C_e is the aqueous phase SBP concentration at equilibrium (U/mL), q_e is the solid-phase SBP concentration at equilibrium (U/mg),

Q_{max} is the monolayer adsorption capacity (U/mg) of the solid and K is the Langmuir isotherm constant (an association constant, in mL/U). Different linearized forms of the Langmuir equation are also used because of the convenience of linear regression to solve for the parameters. However, the transformation of data can result in modification of the error structure, introduction of error into the independent variable, and alteration of the weight placed on each data point (Bolster and Hornberger 2007) . For example, the Hanes-Woolf form of Langmuir equation is:

$$\frac{C_e}{q_e} = \frac{1}{Q_{max}} C_e + \frac{1}{Q_{max}K} \qquad \text{Equation 2-2}$$

Because C_e (x) and C_e/q_e (y) are not independent, the correlation between x and y may be overestimated. The equation may provide good fits to data but may not conform to the Langmuir model (Bolster and Hornberger 2007). The Langmuir isotherm also can be extended to binary adsorbate systems. In addition to the single adsorbate condition, assuming both adsorbates compete for the same adsorption site on the surface and each site can hold one molecule of either adsorbate, the competitive Langmuir adsorption isotherm in a binary system can be expressed by:

$$q_{e,i} = \frac{Q_{max,i}K_i C_{e,i}}{1+\sum_{j=1}^{m} K_j C_{e,j}} \quad \text{(m=2)} \qquad \text{Equation 2-3}$$

Analogously, $q_{e,i}$ is the adsorbate i concentration on solid phase at equilibrium, $C_{e,\,i}$ is the component i concentration in aqueous phase at equilibrium, $Q_{max,i}$ is the monolayer adsorption capacity of component i and K_i is the Langmuir isotherm constant of component i in a at multicomponent solution, and m is the number of adsorbate species. For competitive adsorption, the K and Q_{max} obtained for each adsorbate are often used to predict the components' adsorption behaviour; however, this often shows large errors

with the actual experimental data due to the unknown interactions between the two adsorbates (Andriantsiferana et al. 2013, Mesquita 1998).

Different from the Langmuir model, the Freundlich model is another empirical equation based on heterogeneous adsorption. It can be accurately fit to a wide range of experimental data, but it has limitations in describing the saturation behaviour. The Freundlich equation is:

$$q_e = K_F C_e^{\frac{1}{n}} \qquad \text{Equation 2-4}$$

where the constant K_F is a function of the adsorption capacity of the specific adsorbent, and the value of 1/n is a measure of adsorption strength (LeVan and Vermeulen 1981). Another use of adsorption isotherms is to determine the change of adsorbate binding affinity with the alteration of adsorbent surface characteristics. For example, HRP adsorption on cellulosic fiber was less sensitive to a change of surface charge than hydrophobicity, and an increase of surface hydrophobicity improved both binding affinity and adsorption capacity (Di Risio and Yan 2009).

2.6 Competitive Adsorption between Surfactant and Protein

The "Vroman effect" is described as the competitive displacement of an earlier adsorbed protein by another protein with stronger binding affinity on a polymer surface. It was first observed by Vroman and Adams with dilute blood plasma protein mixtures in the 1960s (Hirsh et al. 2013). Further studies confirmed this competitive protein displacement phenomena and it has been proven to be generally present when a protein mixture competes for adsorption sties on an interface (Horbett 1984). The adsorption of proteins onto a hydrophobic polymer surface is typically irreversible; however, by adding

surfactant to the bulk phase, the adsorbed protein may desorb from the surface, which is a very similar adsorption displacement phenomenon as the "Vroman effect". For an already established protein layer, surfactant with a higher binding affinity to the interface is considered as a competing species. The displacement of adsorbed protein may occur due to the reduction in available binding sites (Mollmann et al. 2005), or it may also occur by surfactant interaction with the adsorbed protein molecules to modify the strength of the protein-surface binding forces (Tilton et al. 1993). Generally, the protein elutability can be affected by polymer type, elution agent, elution condition, protein type, protein concentration, sample age, and storage temperature (Bohnert and Horbett 1986). It was reported that the protein layers that had formed for longer time had higher resistance to being eluted by surfactant, which was called a "residence time" effect (Bohnert and Horbett 1986). Atomic force microscopy (AFM) has been used to visualize the process. Studies using AFM with Tween 20 and β-lactoglobulin at water/solid and air/water interfaces have confirmed: i) the dark domains/holes on protein film are most likely the places where Tween 20 gets adsorbed initially; ii) surfactant adsorption is a time-dependent process, while with the surfactant domain growth, the protein layer increases in thickness; iii) the compression phase has very little protein solubilization to the aqueous phase, protein layer only collapses from the interface in the final stage; iv) a more hydrophobic surface seems to require more adsorption of surfactant to reach the displacement stage; and v) the observation follows an orogenic displacement mechanism (Gunning et al. 1999, Mackie et al. 1999). The model suggests that there are three stages to desorb protein from the interface. At the first stage, surfactant starts to adsorb on the surface, the surface area that is covered by protein gets compressed but the film thickness

does not increase. In stage two, with the increasing adsorption of surfactant, when the protein film is no longer compressible, the film increases in its thickness to compensate for further decrease in area. In stage three, the protein film starts to break down when a critical surface pressure is reached, with the remaining individual molecules or small protein aggregates eventually "cleared off" from the surface at sufficiently high surface pressure (Mackie et al. 1999). It is also noted that the displacement of protein from the interface is not simply due to the exchange of individual protein molecules by surfactant (Mackie et al. 1999). At low surfactant concentrations, the expansion of the surfactant domain continues to reach an equilibrium until further addition pushes to the protein desorption stage (Mackie et al. 1999).

3. MATERIALS AND EXPERIMENTAL PROTOCOLS

3.1 Materials

Enzymes: Crude dry solid SBP (Industrial Grade lot #18541NX, RZ= 0.75±0.10, ~5U/mg) was obtained from Organic Technologies (Coshocton, OH). Liquid ARP concentrate (SP-502, activity 2000 U/mL) was a development preparation donated by Novzymes Inc. (Franklinton, NC). Dry solid bovine liver catalase (E.C. 1.11.1.6, lot#120H7060, 19,900 U/mg) was purchased from Sigma Chemical Company Inc. (St. Louis, MO). The solid enzymes were stored at -15 ℃ and the liquid ARP was stored at 4 ℃.

Aromatic Compounds: Phenol was purchased from Aldrich Chemical Corporation (Milwaukee, WI), and had purity over 99%.

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

Additives: polyethylene glycol (Average MW of 3350) and sodium dodecyl sulfate were purchased from Sigma Chemical Co. (St. Louis, MO). Triton X-100 was purchased from Alphachem (Mississauga, ON)

Buffers and Solvents: Analytical grade monobasic and dibasic sodium phosphate were purchased from BDH (Toronto, ON). Concentrated hydrochloric acid, sulphuric acid, nitric acid, glacial acetic acid, formic acid and 95% ethanol were purchased from ACP Chemicals Inc. (Montreal, QC). HPLC grade acetonitrile and methanol were obtained from Fisher Scientific Co. (Fair Lawn, NJ).

Others: Syringe filters (0.2 μm, non-sterile) were purchased from Gelman Laboratories (Mississauga, ON). Corning 15 mL and 50 mL graduated plastic centrifuge tubes and Eppendorf snap-cap micro-centrifuge tubes were purchased from Fisher Scientific Co. (Fair Lawn, NJ).

All other chemicals used for this study were analytical grade and purchased either from Aldrich Chemical Corporation (Milwaukee, WI) or BDH Inc. (Toronto, ON).

3.2 Analytical Equipment

UV-VIS Spectrophotometry: Two spectrophotometers were used: a Hewlett-Packard 8452 Diode Array Spectrophotometer (λ range of 190-820 nm and 2.0 nm resolution) controlled by a Hewlett Packard I/O card interfaced with a PC , and an Agilent 8453 UV-Visible spectrophotometer (λ range of 190-1100nm and 1.0 nm resolution) controlled by a Hewlett Packard Vectra ES/12 computer. Quartz cuvettes with 1.0 cm path length were purchased from Hellma (Concord, ON).

HPLC (High performance liquid chromatography): Triton X-100 and phenol concentrations were quantified by using a Waters HPLC system with a 1525 binary pumps, a 2487 Dual λ absorbance detector, a 717 Plus auto-sampler, equipped with a Waters Symmetry C18 reverse phase column and operated by Breeze 3.3 software.

TOC (Total organic carbon) Analysis : Triton X-100 concentration was also quantified by using a Shimadzu TOC-V CSH Total Organic Carbon Analyzer, supplied by Shimadzu Scientific Instruments (Columbia, MD). The TOC value was based on the subtraction of IC (inorganic carbon) from TC (total carbon). For TC measurement, the injected samples were carried by nitrogen gas, oxidized to CO_2 and sent to a non-

dispersive infrared spectrophotometer (NDIR) to measure the CO_2 concentration. For IC measurement, the sample was acidified with HCl or phosphoric acid to convert only IC to CO_2, and then detected by NDIR. The combustion chamber had a temperature between 680-700 ℃. All samples were micro-filtered before injection; the standard curves of TC and IC were selected from the machine database.

Centrifuges: Three centrifuges were used in this study due to different sample size and rotation speed requirements: a Jouan BR4i Refrigerated Centrifuge (Santa Fe Springs, CA) with maximum capacity of 4 * 200 mL and maximum speed of 4000 rpm, a Corning LSE^{TM} Compact Centrifuge (Tewksbury MA) with 6*50 mL or 6*15 mL centrifuge tubes and maximum speed of 6000 rpm and an Allied Fisher Scientific micro-centrifuge (Ottawa, ON) with 12*1.5 mL Eppendorf micro-centrifuge tubes and maximum speed of 10000 rpm. The centrifuge conditions are specified in the respective experimental protocols.

Other Equipment: An EA940 pH meter with a stainless steel micro pH probe was obtained from London Scientific (London, ON). Calibration buffers at pH 4.00, 7.00 and 10.00 were purchased from BDH Inc. (Toronto ON). Micro V magnetic stirrers (0-1100 rpm, model 4805-00) and VWR Magstirrers (100-1500 rpm, model 82026-764) were purchased from VWR International Inc. (Mississauga, ON). Magnetic stir bars in different sizes were obtained from Cole-Parmer Canada Inc. (Montreal, QC).

3.3 Analytical Methods

3.3.1 SBP Activity Assay

A colorimetric assay was used to measure free SBP activity in this study. The principle of the assay is to measure the initial rate of formation of a pink chromophore at 510 nm in a mixture containing SBP, 40 mM phosphate buffer (pH = 7.4), 10 mM phenol, 0.2 mM hydrogen peroxide and 2.4 mM 4-AAP (Caza et al. 1999). The sample was added to the cuvette first, followed by quick addition of reagent to provide mixing power and start the reaction. The sample dilution was adjusted according to the expected enzyme activity (to have about 0.2 absorbance change in 30 s). The regular assay was to mix a 50 μL diluted enzyme sample with 950 μL of reagent and monitor the absorbance change for 30 s, which had a detection limit around 0.1 U/mL. For a sample with activity lower than 0.1 U/mL, the sample size was increased. The different sample sizes used were: 100 μL sample with 900 μL reagent and 200 μL sample with 800 μL reagent. Also, in order to maintain the same assay condition, the concentrations of phosphate buffer, phenol, hydrogen peroxide and 4-AAP in the reagent were adjusted according to the sample size to have the same final concentrations of each component as regular test in the cuvette. One unit (U) of SBP activity was defined as that amount catalyzing 1 μmol of hydrogen peroxide conversion per minute under the assay conditions.

Three forms of SBP activities were analyzed in this study: free SBP activity in the aqueous solution, immobilized SBP apparent activity on solid phase and their mixture activity. The free SBP activity was measured by the regular technique mentioned above. Two different approaches were used to determine the immobilized SBP activity. The first approach was similar to the regular SBP activity test by taking a 50 μL suspended

phenolic precipitates sample (with active SBP immobilized on it) and mixing it with 950 μL of reagent; however, the absorbance change was monitored for 60 s to minimize the effect of light scattering by the suspended particles, which may have interfered with the readings of the colour in the cuvette. When the immobilized SBP activity was low, increase sample size significantly blocked the light path through the cuvette and compromised the accuracy of the test. Therefore, a manual enzyme kinetic test was developed to overcome this problem. In this approach, a small test tube was used instead of cuvette to have a larger samples (500 μL) mixed with reagent, and was shaken for a specified time, then the mixture was quickly filtered through a micro-filter. The absorbance at 510 nm of collected filtrate was considered as a one-point absorbance on the regular kinetic run. By repeating the test for different mixing times, a complete kinetic run was made. The slope of the linear regression line was used to calculate the immobilized SBP activity by Beer's law. The enzyme activity assay was measured on the Agilent spectrophotometer because of the built-in kinetic rate calculation function. Additional information is provided in Appendix A.

3.3.2 Phenol Colorimetric Assay

A colorimetric, end-point method was used to measure phenol concentration after enzymatic reaction (Caza et al. 1999). The reaction mixture contained 100 μL $K_3Fe(CN)_6$ (83.4 mM in 0.25 M $NaHCO_3$) 100 μL 4-AAP (20.8 mM in 0.25 M $NaHCO_3$) and 800 μL sample or sample plus distilled water. The absorbance was measured at 510 nm after a 5 minutes. The standard curve of phenol colorimetric assay can be found in Appendix B.

3.3.3 Hydrogen Peroxide Colorimetric Assay

A colorimetric method was used to determine the concentration of hydrogen peroxide (Wu et al. 1994). The presence of H_2O_2 oxidized phenol by using ARP as catalyst, and the phenolic radicals coupled with 4-AAP to form a pink quinoneimine chromophore. The absorbance of the chromophore was measured at 510 nm after 15 minutes. More information is provided in Appendix B.

3.3.4 SDS Colorimetric Assay

A colorimetric method was adopted to determine the mass of SDS in solution based on chloroform extraction (Arand et al. 1992). For quantitative measurement of SDS in aqueous solution, 150 µL of sample was mixed with 150 µL methylene blue reagent (250 mg/L of methylene blue, 50 g/L Na_2SO_4 and 10 mL concentrated H_2SO_4) in a 1.5 mL Eppendorf tube, and vortexed for 1 min. After 2 min, 0.6 mL of chloroform was added in the tube and it was vortexed again for 1 min. The lower layer colour complex of SDS and methylene blue was pipetted into a cuvette after 3 min incubation. The absorbance was measured at 651 nm against chloroform as the blank. The standard curve of SDS colorimetric assay can be found in Appendix B.

3.3.5 HPLC Analysis

Phenol and Triton samples were run under isocratic conditions, given in Table 3-1. Flow rates were controlled to be 1.0 mL/min, and the injection volume and UV-detector wavelength were adjusted according to the compound UV detection sensitivity. The standard curves of phenol and Triton can be found in Appendix. B.

Table 3-1 HPLC conditions for phenol and Triton X-100

Detecting compound	Mobile phase solvents		Ratio of solvent in mobile phase as %		Injection volume (μL)	Wavelength (nm)
	Pump A	Pump B	Pump A	Pump B		
Phenol	Acetonitrile	0.1% Acetic acid aq	40	60	10	276
Triton X-100	Acetonitrile	0.1% Formic acid	95	5	50	276

3.3.6 TOC Analysis

The Triton adsorption isotherm was initially determined by TOC analysis. A sample mixture was filtered through a micro-filter and collected in a 25 mL test tube. The machine was first blanked by milli-Q water, and then TOC of distilled water was measured prior to analyzing the samples. After sample measurement, the Triton concentration was back-calculated from TOC by assuming average repeating ethylene units in Triton to be 9.5; therefore 63.4% of the Triton contributed to TOC. Each sample was injected at least three times for TC and IC. It was assumed that all carbon sources in the sample mixture are from distilled water and Triton. The standard curve of Triton can be found in Appendix B. The Triton adsorption isotherm can be found in Appendix D.

3.3.7 Phenolic Precipitates Dry Mass Assay

To measure dry mass of a phenolic precipitates stock, three Gooch crucibles were cleaned, oven-dried, cooled in a desiccator, and then weighed before use. An aliquot of washed precipitates was transferred to the crucible, while vacuum was applied. After all supernatant was removed by filtration, the crucibles were placed in an oven at 100 ℃ for at least one day until a constant weight was obtained. Finally, the crucibles were cooled

in the desiccator again and weighed. The average mass change of three crucibles is defined as the precipitates dry mass. The precipitate solids content was specified in mg/mL of batch reactor volume.

3.3.8 Buffer Preparation

Different buffers were prepared for experiments to have the desired pH (Gomori 1955). The main buffer used in this study was sodium phosphate buffer (pH range 5.6-8.0), which was used in phenol removal experiments at pH 7.0 and the SBP activity test at pH 7.4.

3.4 Experimental Protocols

Unless otherwise stated, all experiments were run in triplicate and error bars denote respective standard deviations.

3.4.1 Phenolic Precipitates Preparation

Phenolic precipitates were prepared by SBP-catalyzed phenol polymerization in the absence of buffer. Each time, the amounts of SBP and H_2O_2 required to convert a certain amount of phenol were calculated based on the available information. Then phenol, H_2O_2 and SBP were mixed into a batch reactor. The reactions were allowed to proceed until no phenol and H_2O_2 remained before collection of the precipitates. After 24 h settling, precipitates were collected and centrifuged at 4000 rpm for 30 min, washed with fresh distilled water three times, and then transferred to a brown bottle in a suspension for storage in room temperature. The phenolic precipitates used in this study were made

several times by the same procedure but with different SBP and phenol ratios. Initially, the amount of active SBP on precipitates was intentionally kept high, so that the difference between apparent activity and inherent activity (see Section 3.4.3 for details) was higher. That was useful in determining the difference of adsorption-induced specific activity change and reversibility of SBP adsorption by the addition of Triton. Later on, during characterization of SBP and Triton adsorption on phenolic precipitates and SBP elution, an 8L batch of "cleaner" precipitates were made by the conversion of 20 g of phenol with 34 g of SBP powder. The phenolic precipitates were tested to have minimal active SBP remaining and presumably a larger specific adsorption area due to the lower amount of SBP used at the polymerization stage. It also provided consistent solid samples for the rest of the project (See Appendix C for details).

3.4.2 SBP Activity Test for 'Apparent and Inherent' Activity

In order to measure the immobilized SBP apparent and inherent activities, phenolic precipitates were incubated with and without Triton before the activity test. For a sample of precipitates taken for the SBP activity test, the term 'apparent' activity was used to express the measured activity of immobilized SBP. After incubating precipitates with a significant concentration of Triton (500 mg/L) for 30 min, the immobilized SBP had eluted from precipitates to reach desorption equilibrium. Thus, a sample of mixture where the majority of the immobilized SBP has desorbed to the aqueous phase, the term 'inherent' activity is used as an estimation of the total activity from desorbed SBP (free SBP with specific activity unchanged) in the aqueous phase plus any SBP remaining on precipitates (immobilized with a reduced specific activity).

3.4.3 Precipitates Recycling Experiments

Precipitates recycling experiments were conducted mainly to develop a process model for utilizing the remaining active SBP on the precipitates to reduce SBP cost in the overall process. It was focussed on the subsequent batch reaction after phenolic precipitates were generated in the previous batch under the optimized conditions. The phenol removal reactions were usually conducted in batch reactors continuously stirred with a Teflon-coated magnetic stir bar. Batch reactors contained 20 or 30 mL of a buffered mixture of phenol, hydrogen peroxide and SBP. Generally, SBP was added last to initiate the reaction and then the reactors were stirred for 3 h. A 50 μL sample was taken from each reactor while the precipitates were in suspension to determine the remaining enzyme activity after reaction. Another 3 mL sample was taken from the reactor, filtered through a micro-filter to measure the remaining phenolic compounds and H_2O_2 (if required) after 5 min settling to avoid a significant loss of precipitates. The rest of the sample was transferred to a centrifuge tube and centrifuged at 4000 rpm for 10 min. After centrifugation, the supernatant was removed and the precipitates pellet was carefully transferred to another reactor. The tube was rinsed three times with a fixed volume of distilled water to wash out all the solids. The washed water was also transferred to this reactor. Buffer and water were added to make up the volume. Then SBP, if required, phenol, and hydrogen peroxide were added in that order to initiate the reaction. In precipitates recycling experiments, precipitates were quantitatively transferred to the subsequent reactor. In order to avoid catalase being adsorbed on phenolic precipitates, catalase was not added to the batch reactors to quench the reaction in between reaction

cycles; instead, the reaction mixture was centrifuged to separate precipitates from phenol and H_2O_2 remaining in the solution.

3.4.4 SBP and Triton X-100 Adsorption Isotherms

SBP and Triton adsorption tests were conducted in small batch reactors with 10 mL solution. Water was usually added to the batch reactor first, then SBP/Triton were added to make sure that the adsorbate was well mixed. Precipitates were added last to initiate the adsorption. The adsorption was often checked after 30 min which was considered sufficient to reach equilibrium. After the adsorption, a 1.5 mL sample was centrifuged at 5000 rpm for 5 min. The remaining concentration in the supernatant was measured to be C_e. The q_e was calculated by the decrease in supernatant concentration divided by the concentration of adsorbent used in the test. SBP and Triton adsorption curves were obtained by plotting the remaining adsorbate in the aqueous phase at equilibrium vs. the adsorbate concentration on solid phase at equilibrium (C_e vs. q_e), and were characterized by Langmuir and Freundlich adsorption isotherms (LeVan and Vermeulen 1981). The linear form of the Langmuir isotherm (Hanes-Woolf equation) was also used to simplify the adsorption isotherm test, especially when a continuous track of Q_{max} and K change was desired (See Section 4.7.4). OriginPro 8.0 was used to fit the curves with model equations to get adsorption capacity (Q_{max}) and adsorption constant (K) and their uncertainties.

3.4.5 Concentrating SBP with Phenolic Precipitates

The SBP concentrating process was designed to have SBP adsorbed on phenolic precipitates at a low activity (6-10 U/mL) to mimic the actual SBP concentration in raw SBP extracts, collect precipitates as a concentrated suspension and then use Triton to elute all the adsorbed SBP into a small volume to achieve the goal of concentrating the enzyme. A full cycle of SBP concentration included three steps: SBP adsorption, SBP elution and precipitates regeneration, if desired. Experiments were conducted in centrifuge tubes, and the low activity SBP solution was obtained from diluted crude SBP. In the first step, phenolic precipitates were mixed with SBP extract for adsorption in a 15 mL tube. After 30 min mixing, the tube was centrifuged at 4000 rpm for 20 min to collect the precipitates. SBP activity remaining in the supernatant was measured and the difference was considered as the adsorbed SBP. After removing the supernatant, Triton solution was added in the tube and mixed with the precipitates pellet for 30 min. The volume of the injected Triton was pre-calculated according to the desired concentrating factor; the smaller the volume of Triton used, the higher would be the concentration of SBP collected. A sufficient mass of Triton was used to ensure completion of SBP elution. After elution, the tube was centrifuged again as before, supernatant was collected and the SBP activity was measured. In the final step, ethanol was added to the centrifuge tube to regenerate the precipitates in 15 mL. After 15 min mixing, the tube was centrifuged again to removal the supernatant. A 15 min water wash and another centrifugation followed to remove the residual ethanol. The precipitates then were ready to perform another cycle of concentrating. The remaining SBP activity and collected volume after the adsorption and the collected concentrated SBP activity and volume after elution were used to calculate

SBP activity balance in the cycle. In order to establish the Triton mass balance in the cycle, the remaining Triton concentration in the collected SBP and in ethanol, and the volumes of both were recorded.

3.4.6 Dynamic Adsorption of SBP on Phenolic Precipitates

Preliminary tests were conducted to determine the difference between static and dynamic adsorption. Dynamic adsorption was designed to have phenol polymerization taking place in a SBP stock solution. Here, 200 mL SBP solution at 5 U/mL was prepared to provide sufficient SBP for capturing. Then aliquots of a phenol and H_2O_2 mixture were added into SBP stock every 2 min until formation of precipitate particles was observed. The remaining SBP activity in the stock was measured and the difference between initial SBP and remaining SBP was considered as the captured and consumed SBP due to phenol polymerization. Precipitates were collected from the solution by centrifuging for 20 min at 4000 rpm. After removing the supernatant, sufficient Triton was added and mixed for 30 min to elute all the captured SBP. The concentrated SBP was collected by centrifuging for 20 min at 4000 rpm. The activity and the collected volume were used to calculate the total captured active SBP and the efficiency of dynamic immobilization.

3.4.7 Competitive Adsorption of SBP and Triton X-100 on Phenolic Precipitates

A competitive Langmuir isotherm was used to characterize the binary adsorbates' adsorption on phenolic precipitates (LeVan and Vermeulen 1981). This experiment had different concentrations of SBP (1-17 U/mL) and Triton (100-800 mg/L) mixed with 2 mg/mL precipitates. After 30 min incubation, samples were taken from the batch reactor

and centrifuged at 5000 rpm for 10 min. The supernatant SBP activity and Triton concentration were quantified by SBP activity test and HPLC, respectively. By plotting C_e vs q_e of both SBP and Triton together, the adsorption behaviours were compared with the results in the single component adsorption to determine the influence of SBP and Triton on each other.

3.5 Sources of Error

All experimental results are influenced by systematic and random errors. The standard deviations in all the experiments were in ±5% range. Sensitive experiments were repeated on different days to determine the reproducibility. All pipettes, scales and other measuring instruments were calibrated before use to minimize the systematic errors. Random errors due to human inaccuracy cannot be controlled but were minimized by conducting experiments in triplicates.

SBP activity is considered to be sensitive to the change in room temperature, reagent age and mixing conditions. In order to minimize the error, SBP stock activity was checked every day at the beginning of the experiment. All samples were pre-diluted, if necessary, to given 0.2 absorbance change in 30 s in the test. Room temperature and humidity were continuously tracked to conduct all activity tests at 20 ± 2℃ and 50 ± 10% relative humidity.

Another major source of errors is during the phenolic precipitates preparations and samplings. The entire study was conducted with three preparations of phenolic precipitates. The two smaller batches were used for experiments from Section 4.1 to 4.3, which primarily focused on the qualitative determination of SBP specific activity change

upon immobilization. The quantitative characterization of precipitates, which is from Section 4.6 to the end, used a different batch of precipitates (see details in Appendix C). Due to the random structure of phenolic precipitates and uncontrolled surface coverage during the formation, results such as the observed specific activity change of SBP via precipitates adsorption, % SBP elution as a function of Triton concentration and precipitates concentration were dependent on the specific precipitates preparations used.

4. RESULTS AND DISCUSSION

4.1 SBP Adsorption on Phenolic Precipitates under Turnover and Static Conditions

According to the end-product inactivation model, phenolic precipitates quickly adsorb HRP (purified) from the aqueous phase under both turnover and static incubation conditions, resulting in a minimum residual activity in the solution and a decrease in the mixture activity over time (Wu et al. 1998). Similar results were obtained also by using crude SBP and phenol in this study. Figure 4-1 shows that the change in SBP activity on the precipitates and in the supernatant at the initial stage of the phenol reaction (turnover conditions). A rapid decrease in supernatant SBP activity in the first minute was observed, which suggests that most of the SBP quickly disappeared from the aqueous phase. However, within 12 min, the mixture activity first doubled and then gradually diminished to half of the added activity. These results are very similar to those reported in the study mentioned above, and confirm that the end-products inactivation has played a major role in the inactivation of crude SBP. The sudden increase in enzyme activity in the mixture at the early stage of turnover conditions was also observed in the previous study (Wu et al. 1998). The dimeric intermediates, *p*-phenoxyphenol and *p,p'*-biphenol formed during phenol conversion, have respectively 207- and 63.9-fold higher reaction rates than phenol with HRP (Yu et al. 1994). Such hyper-reactivity of first-formed intermediates might account for the sudden increase of apparent SBP activity in the activity assay.

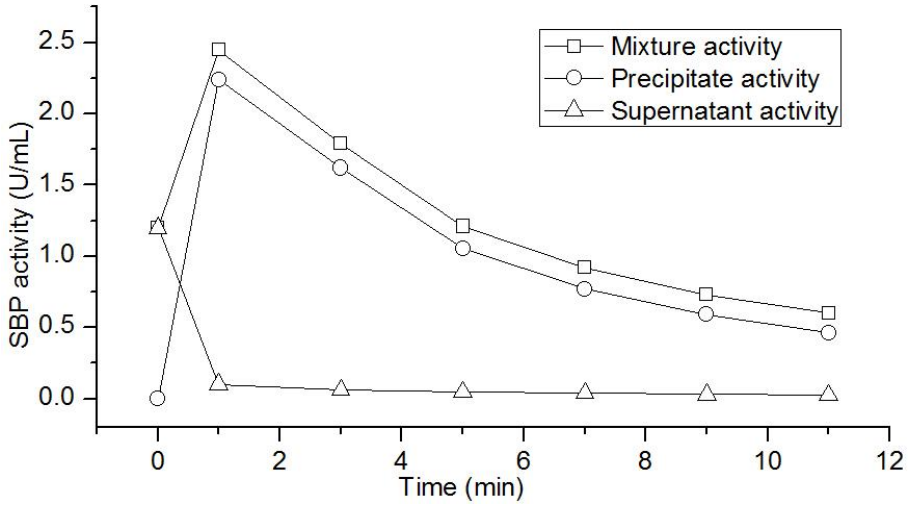


Figure 4-1 Supernatant, precipitates and mixture SBP activity changes over time under turnover conditions.

[Reaction conditions: 1 mM phenol, 1.25 mM H_2O_2, 1.2 U/mL SBP and pH = 7.0; precipitates activity was calculated by difference. Single point plot.]

For static incubation conditions, a batch of phenolic precipitates was firstly made with 4 mM phenol, 5 mM H_2O_2, 10 U/mL of SBP in 40 mM phosphate buffer at pH=7.0. After 3 h reaction, the remaining phenol was less than 0.1 mM and no H_2O_2 was detected. The precipitates were collected, centrifuged and washed 3 times with distilled water and then stored in a brown glass bottle. The precipitates had active SBP adsorbed with an apparent activity of 24.6 U/mL in the concentrated form when precipitates concentration was 8 mg/mL. Two different concentrations of precipitates were incubated with free SBP and the time course of supernatant activity was tracked as shown in Figure 4-2A. The precipitates still showed the ability to adsorb free SBP. At the higher precipitates concentration, less SBP remained in the supernatant after 3 min incubation. Also, the adsorption reached equilibrium very quickly, in less than 1 min, which is consistent with the remaining SBP activity in the supernatant under turnover conditions. An increase in the initial concentration of free SBP in the incubation resulted in an increase in the

remaining SBP activity at equilibrium (Figure 4-2 B). The decrease of mixture activity when free enzyme was incubated with phenolic precipitates was direct evidence to support the end-product inactivation model in the previous study (Wu et al. 1998). As shown in Figure 4-1 and Figure 4-3, the drop in mixture activity with time was also observed with crude SBP.

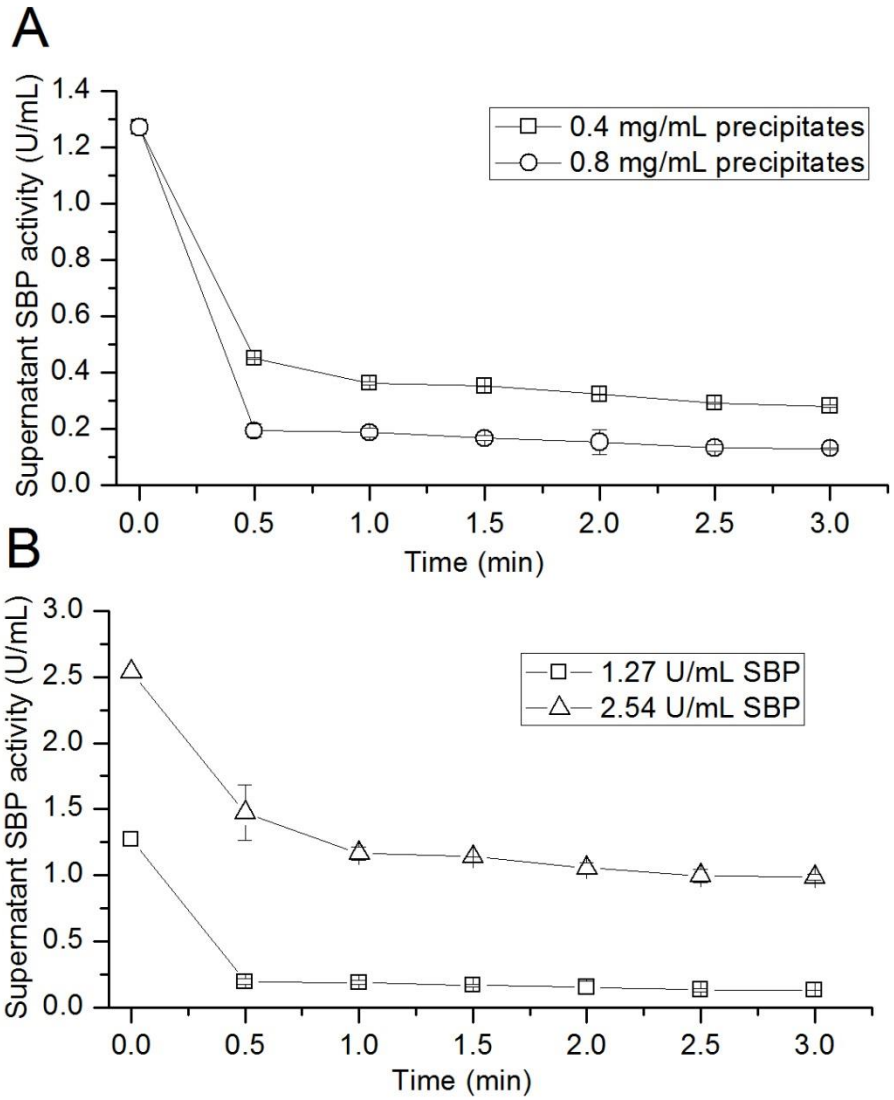


Figure 4-2 Free SBP incubation with phenolic precipitates under static conditions.

[**A**: Supernatant SBP activity change over time due to different precipitates concentrations (0.4, 0.8 mg/mL) incubated in 10 mL. Free SBP added for incubation was 1.27 U/ mL in 10 mL.

B: Supernatant SBP activity change over time at different initial SBP concentration (1.27, 2.54 U/mL) incubated in 10 mL. Precipitates concentration was 0.8 mg/mL.]

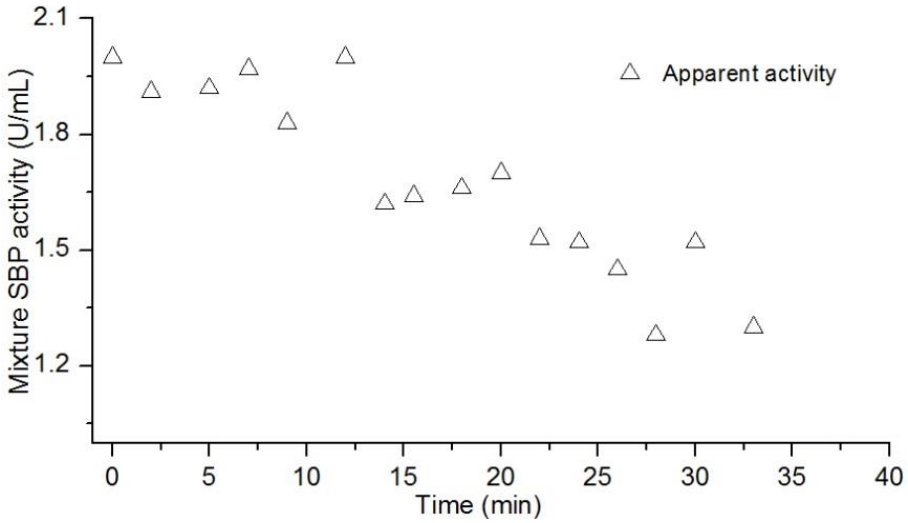


Figure 4-3 Mixture activity change over time of free SBP incubated with precipitates.

[Phenolic precipitates concentration 0.33 mg/mL, 1 U/mL free SBP and 0.4 mL of concentrated precipitates (25.4 U/mL apparent activity) were used in 10mL.]

The end-product inactivation model is based on the adsorption-induced drop in apparent activity of enzyme and consequently the reduced free enzyme catalytic efficiency. However, no further information has been provided to explain the causes in the previous studies.

If the behaviour in Figure 4-1 to Figure 4-3 is interpreted as an immobilization of free SBP on precipitates, the decrease of SBP specific activity, through the alteration of K_m and/or V_{max}, is a widely observed negative effect in enzyme immobilization studies (Monier et al. 2010, Nakamoto and Machida 1992, Trevan 1981). The phenol diffusion efficiency could be a dominant factor influencing the measured enzyme activity. For example, Figure 4-1 shows that free SBP disappeared from solution within the first minute of reaction when around 50% of the phenol was removed (see Appendix D, Figure A-9). If SBP adsorption overlaps with polymer growth, some of the earlier adsorbed SBP may be buried inside the polymer, the rest would be immobilized on the

surface with varying degrees of hindrance to substrate access. Thus, the decreasing mixture activity in Figure 4-1 and 4-3 represented an average activity of the immobilized SBP over the time period. To prove this hypothesis, the active SBP remaining on precipitates was considered as an immobilized/adsorbed enzyme, and a series of phenol removal experiments were conducted to check the catalytic efficiency of immobilized SBP.

4.2 Phenol Removal by Immobilized SBP on Precipitates

A batch of phenolic precipitates was prepared as before with excess SBP. The reaction conditions were: 3 mM phenol, 3.75 mM H_2O_2, 5 U/mL SBP in 40 mM phosphate buffer at pH 7.0. The apparent SBP activity remaining in the precipitates stock suspension was 10.4 U/mL. In order to compare phenol removal efficiency with free SBP, the active SBP on precipitates was considered to be equivalent to free SBP. A portion of the precipitates mixture was used to remove 1 mM phenol at the same nominal concentrations as free SBP and the results are shown in

Figure 4-4. The immobilized SBP remained active and was able to catalyze phenol removal, and even gave a higher phenol removal efficiency in both cases. The activity of immobilized enzyme, often expressed as specific activity, can be calculated from the disappearance of enzyme in the supernatant during immobilization divided by the mass of carrier used (Zhang et al. 2010). For the present experiment, though, the 0.4 and 0.8 U/mL activities on precipitates were not calculated this way, but by a direct measurement of the apparent activity. Because the precipitates preparation involved a carrier production stage, which had an unknown portion of the original SBP irreversibly

inactivated due to phenol turnover, the portion of active SBP remaining on precipitates was also unknown. Therefore, additional active enzyme with a lower specific activity could be used in the immobilized form rather than as free SBP. Regardless of the comparison, the general trend is clear that phenolic precipitates as the reaction end products did not inactivate enzyme in substantial proportion; rather, they immobilized the enzyme in an active form, and could be reused for further catalysis.

For better understanding the behaviour of the immobilized SBP, two more experiments were conducted using the same stock suspension of precipitates. The optimized SBP concentration for the immobilized SBP was found to be 0.8 U/mL (again, apparent activity) to achieve >95% phenol removal in 3 h (Figure 4-5). In addition, free SBP was used together with immobilized SBP and a time course of phenol remaining is shown in Figure 4-6. It is known that 0.4 U/mL of immobilized SBP gave about 20% residual phenol after 3 h reaction (Figure 4-4). Therefore, an addition of 0.3 U/mL of free SBP together with 0.4 U/mL of immobilized SBP would help to push the reaction to completion if the added free SBP remained functional. The progress curve shows a smooth approach to 95% phenol removal in a 3 h period, especially in the first few minutes, where a more rapid drop in phenol concentration would be observed when only free enzyme was used (see Appendix D, Figure A-9).

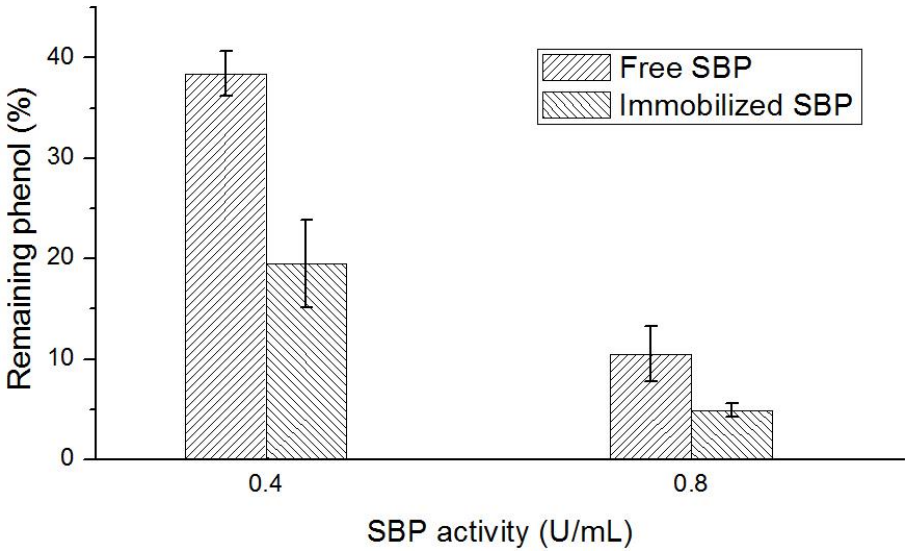


Figure 4-4 Comparison of free and immobilized SBP for phenol removal.

[Reaction conditions: 1 mM Phenol, 1.25mM H_2O_2, 0.4 and 0.8 U/mL SBP and pH=7.0]

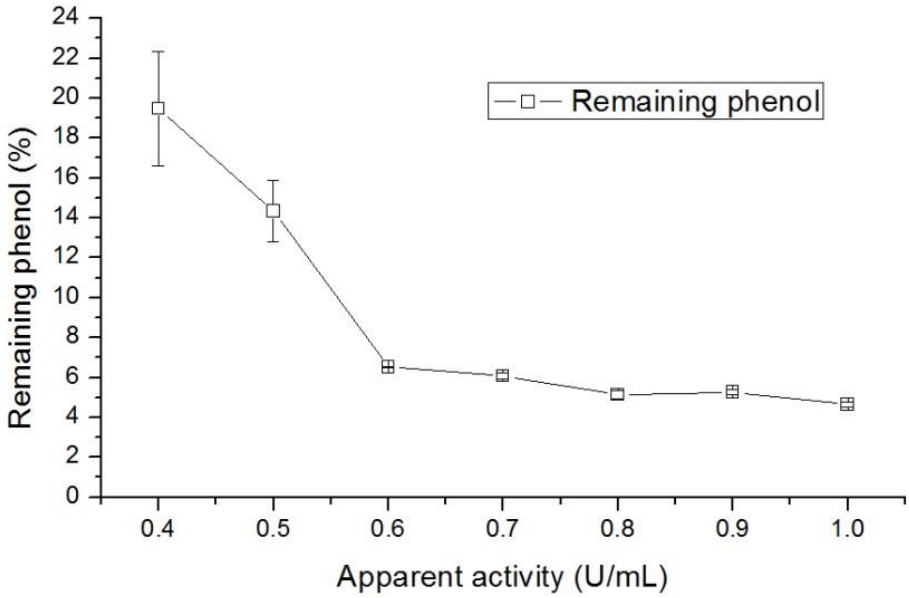


Figure 4-5 Immobilized SBP optimization for 1 mM phenol removal

[Reaction conditions: 1 mM phenol, 1.25 mM H_2O_2 and pH = 7.0]

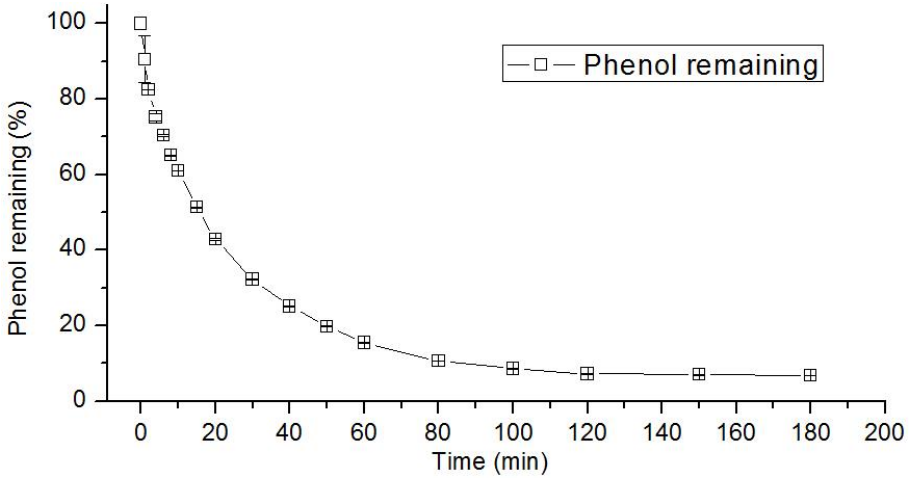


Figure 4-6 Time course phenol removal with free SBP and immobilized SBP used together.

[Reaction conditions: 1 mM phenol, 1.25 mM H_2O_2 , 0.3 U/mL free and 0.4 U/mL immobilized SBP and pH=7.0]

4.3 SBP Adsorption/desorption in the Absence/presence of Triton X-100

Adding Triton during the phenol polymerization reaction was shown either to prevent the adsorption of enzyme onto the precipitates or to promote its desorption from the precipitates, thereby re-initializing/enhancing the reaction rate and improving the removal efficiency (Sakurai et al. 2003). It was shown earlier in Section 4.1that there was a gradual decrease in SBP activity when SBP and precipitates were incubated together under turnover and static conditions. Also, active SBP on phenolic precipitates was effective as an immobilized enzyme in phenol removal. The use of Triton may free the adsorbed SBP and show evidence of specific activity change due to immobilization. Thus, an experiment was conducted to determine the effect of individual components in the following mixture: 1.16 mg/mL precipitates, 3.2 U/mL free SBP and 500 mg/L Triton. For each enzyme, precipitates and Triton combination (A to F), mixture activity and supernatant activity were measured and are shown in Figure 4-7. The difference was considered as the activity of the solid phase (precipitates). Comparison of A and C shows

that the presence of Triton slightly elevated the activity of free SBP by a few percent; however, such an influence is not considered to be significant in this experiment. B and D as controls for F, also show the change in the activity change of precipitates under normal and elution conditions. The difference of mixture activity between these two experiments suggests that the specific activity of SBP had decreased upon immobilization. Under the elution conditions, 5.0 out of 7.35 U/mL of the immobilized SBP was desorbed to the aqueous phase (supernatant activity of D) and had recovered its intrinsic parameters. It also shows that Triton elution of SBP from the precipitates was incomplete. Again, a similar change in specific activity of SBP upon immobilization is shown in E, when the same precipitates and free SBP were incubated for 30 min. The mixture activity was increased to 1.53 U/mL with 0.57 U/ mL remaining in the supernatant, which indicated that 82.2% (2.67 U/mL) of the free enzyme was immobilized by the precipitates and contributed to an increase of 0.32 U/mL of its apparent activity (E versus B mixture activity). When precipitates were incubated with free SBP in the presence of Triton, the mixture activity jumped to 10.73 U/ mL (F), close to the summation of D and C mixture activity. In addition, to elute the immobilized SBP back into the solution, Triton also prevented freshly-added SBP from getting immobilized onto the precipitates.

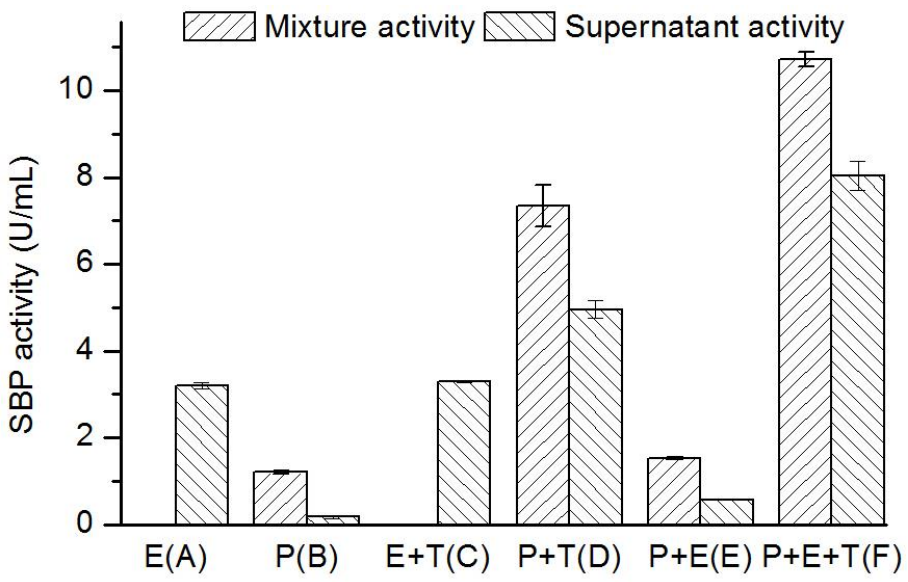


Figure 4-7 SBP adsorption by precipitates in the presence of Triton X-100

[Experiments (A to F) were conducted on various mixtures: 1.16 mg/mL of precipitates (1.25 U/mL apparent activity and 7.35 U/mL Inherent activity), 3.2 U/mL free SBP, 500 mg/L Triton X-100. E: free enzyme, P: precipitates, T: Triton X-100. Sets A, B & C are controls.]

The 'apparent' activity, estimated by the activity assay, is based on the initial rate of substrate turnover and does not accurately estimate the remaining active SBP as if it were in the free form. Since Triton elution can reverse the immobilization of SBP on phenolic precipitates, the concept of measuring 'inherent' activity, where the majority of immobilized enzyme has been desorbed, could give a better understanding of how much active SBP is available to catalyze (although slower if left immobilized) phenol turnover. This experiment supports the above reasoning. The actual SBP activity on the precipitates could be up to 5.9-fold higher after elution, which suggests the idea of precipitates recycling. Immobilization results in a new mass-transfer resistance which appears as "inhibition" through lowering of the apparent specific activity. The "loss" of SBP activity under static conditions appears to be predominantly reversible through Triton elution;

however, the immobilized enzyme was not fully eluted with Triton in this experiment and a small fraction remained on the precipitates.

A time course for desorption of immobilized SBP under static conditions was determined, Figure 4-8, to complement the results of Figure 4-7 (E and F). Mixture activity was tracked when free SBP was incubated with precipitates. The drop in the mixture activity in the first 10 min is similar to that observed in a previous study and is considered as an inactivation of SBP by mature phenolic precipitates (Wu et al. 1998). However, after the mixture activity had dropped to a certain level, the addition of 500 mg/L Triton slowly released all immobilized SBP, including the active portion originally on precipitates, back to the aqueous phase. The final activity obtained was very close to the summation of the total activity of the precipitates and free SP added (stacked bars).

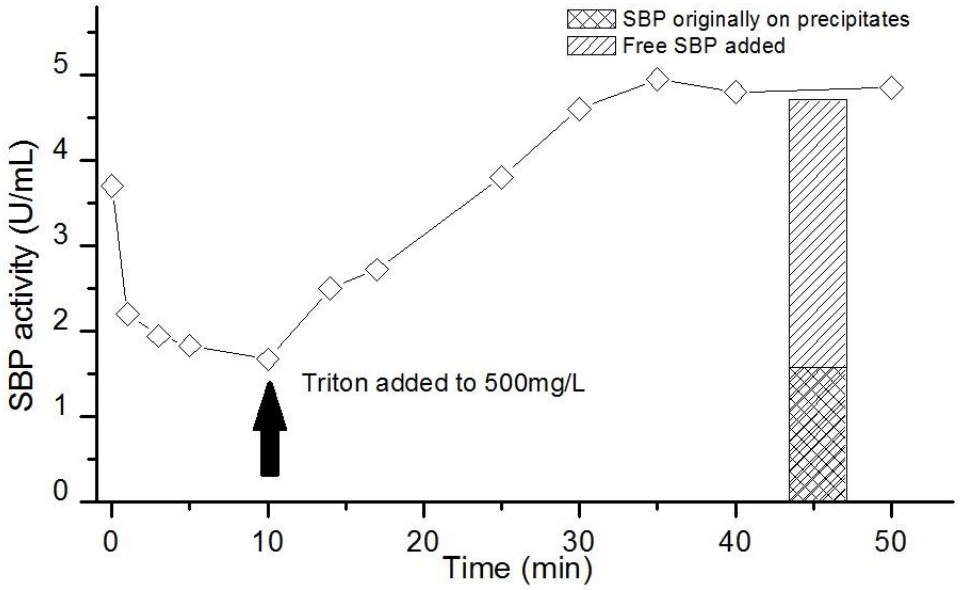


Figure 4-8 SBP immobilization on precipitates and reversal by addition of 500 mg/L of Triton*.

[Precipitates concentration was 1.5 mg/mL, pre-immobilized SBP apparent activity on precipitates was 0.44 U/mL and inherent activity was 1.60 U/mL, and free SBP added to at starting point was 3.20 U/mL. *Single point plot.]

This evidence for interaction between SBP and phenolic precipitates suggests that the adsorption of SBP does not inactivate the enzyme in substantial proportion; rather, the precipitates immobilize the enzyme in an active form, and change its specific activity, presumably by altering K_m and/or V_{max}. Under turnover conditions, phenolic precipitates formation involves multiple cycles of enzymatic oxidation and radical recombination (Ghoul and Chebil 2012). The radical recombination happens predominantly through C – C and C – O coupling with *o*-, *p*- orientation on the benzene rings (Ghoul and Chebil 2012), thus the polymer structure is random. It is not known in what molecular mass range the polymer/oligomer starts showing hydrophobicity. The hydrophobic interaction is considered the major interaction with peroxidase. A kinetic study of peroxidase immobilized on phenolic precipitates under turnover conditions has not been reported.

The end-product inactivation model was developed to provide an understanding of the "PEG effect" with horseradish peroxidase. It was demonstrated that when a precipitate was made in the presence of PEG, it was inert to the enzyme, whereas a precipitate generated in the absence of PEG had the capacity to 'inactivate' fresh enzyme (Wu et al. 1998). If extra PEG, beyond a certain threshold concentration, was used to protect enzyme, the extra PEG remained in solution (Modaressi et al. 2005). From an adsorption perspective, this can be explained as a two-adsorbate competition. If a surface area was pre-saturated with PEG, enzyme adsorption would be minimized. It may also explain why a crude enzyme appears to be less susceptible to end-product inactivation than its purified counterpart, because the hydrophobic impurities may be adsorbed on the precipitates instead of enzyme. A recent study suggested that different enzymes varied in their relative affinities to the precipitates; hence their sensitivities to the "PEG effect"

were different. In addition, the relative hydrophobicity of different polyphenolic precipitates was related to the structure of the corresponding monomer (Steevensz et al. 2012). This could imply that the end-product inactivation is not a universal occurrence. For a less hydrophobic substrate or an enzyme with no hydrophobic patches, such inactivation should be less significant, thus a less significant "PEG effect" and, by extension, "Triton effect".

The above discussion supports proposal of a new process model for recycling precipitates for the SBP-catalyzed phenol polymerization reaction. It is known that the enzyme provided would retain a portion of activity on the precipitates after the reaction. Therefore, the active enzyme could be utilized again by recycling it to a subsequent batch reaction. Also, for the active enzyme with a lowered specific activity, the recycling strategy provides a longer contact time for it with the substrate, hence the maximum utilization of enzyme might be reached.

4.4 Optimization of Precipitates Recycling for 1 mM Phenol Removal

4.4.1 Precipitates Recycling and Remaining SBP Activity under the Optimized Conditions

The active SBP remaining on the precipitates is dependent on the SBP concentration added to the reaction. The optimization of this strategy requires that the all precipitates should be prepared or generated under the same conditions, for comparison of results for reactions using precipitates to remove phenol. In Section 4.4, all precipitates were made under the optimized conditions for 1 mM phenol removal unless otherwise mentioned. Two experiments were conducted to indicate the potential of this strategy. Firstly,

precipitates generated under the optimized conditions were collected, and their apparent and inherent activities were measured as shown in Figure 4-9. The remaining apparent SBP activity was obtained from the difference between an unfiltered suspension sample and the supernatant activity from a filtered sample, which is considered as the SBP activity on the precipitates. Under the optimized conditions, SBP was previously considered as all being consumed after the reaction; however, the results suggest that the apparent activity remaining on precipitates was around 20% of the initial SBP at 1, 2 and 3 mM phenol removal, while a minimal SBP activity (less than 0.01 U/mL) remained in the supernatant (not shown). The inherent activity was obtained by taking precipitates formed in the reactor and incubating them with a high concentration of Triton. Surprisingly, the majority of the initially added SBP had remained active on the precipitates, which were 50%, 71% and 78% of the initial activity provided for 1, 2 and 3 mM phenol removal, respectively. Therefore, recycling these precipitates could significantly reduce the required SBP concentration for the subsequent batch reaction to reach 95% removal.

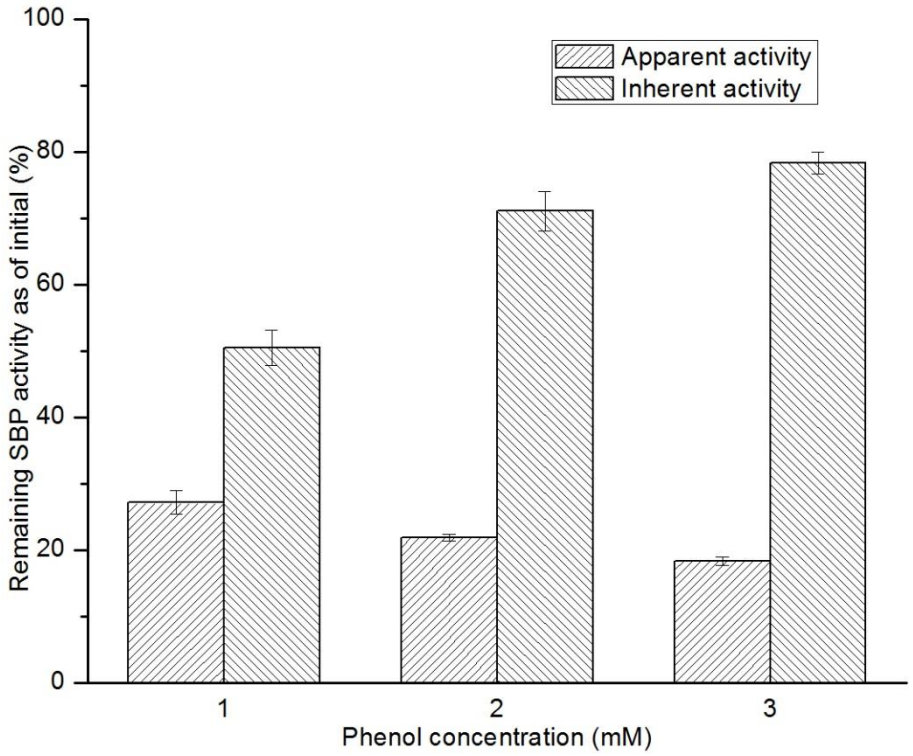


Figure 4-9 Remaining apparent and inherent SBP activity under optimized conditions of 1, 2 and 3 mM phenol removal.

[Reaction conditions: phenol (mM): SBP (U/mL): H_2O_2 (mM) = 1:1.2:1.25; pH = 7.0. Inherent activity measured after 30 min incubation with 500 mg/L of Triton X-100.]

Secondly, the potential of the recycling strategy was indicated as follows. The reaction was repeated as above and the precipitates were quantitatively transferred, without washing (to prevent SBP loss during washing), to a batch reactor to run another identical reaction without the addition of fresh SBP. Figure 4-10 shows the phenol removal after 3 h, 5 h and 20 h reaction for each scenario. In all cases, more than 60% phenol was removed by just recycling the precipitates collected from the previous batch reaction. For 1 mM phenol, there was a 23% difference between 3 h and 20 h removal, which indicated that the remaining SBP remained active for a long time on the precipitates, but because the SBP, phenol and H_2O_2 concentrations were low, SBP only slowly turned over the substrate. However, for the other two concentrations, the difference between 3 h and 20 h

removal was not significant. This could be explained by H_2O_2 inactivation of SBP (Steevensz et al. 2009b). Thus, it is inferred that proportionally less SBP was inactivated in the 1 mM reactor than in the 2 and 3 mM ones, which had longer contact time with higher H_2O_2 concentrations.

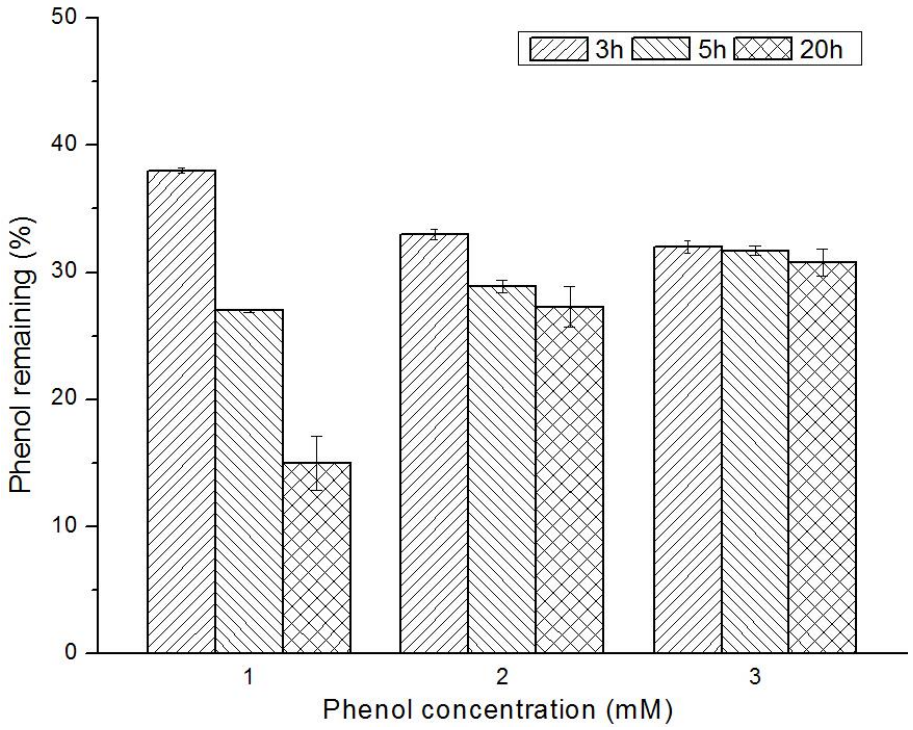


Figure 4-10 Phenol removal by quantitatively recycled precipitates in 3, 5 and 20 h reaction time.

[Reaction conditions: phenol (mM): H_2O_2 (mM) = 1:1.25; pH = 7.0, no fresh SBP was added.]

4.4.2 Consecutive Precipitates Recycling with No SBP Addition

If a SBP concentration higher than the optimized one was used to remove a given concentration of phenol (e.g., 2 U/mL SBP for 1 mM phenol), more active SBP should be obtained on the precipitates. By recycling the precipitates in consecutive reactors, the active SBP should be consumed step by step until it is all used up. Figure 4-11 shows results for such a consecutive precipitates recycling experiment to remove 1 mM phenol

under the same conditions. Precipitates generated from the previous reaction cycle were quantitatively transferred to the next cycle. The first two cycles showed >95% phenol removal. The phenol removal in the third and fourth cycles had successively decreased, presumably due to the increased irreversible inactivation of SBP. The cumulative phenol removal in four cycles was 3 mM, thus the SBP consumption was 0.67 U/mL per mM of phenol removal. Compared with the SBP consumption of 1.2 U/mL per mM of phenol removal obtained in the previous optimized condition, the SBP efficiency had increased by 81%. Hydrogen peroxide consumption in each cycle proved that the removal of phenol is due to peroxidase reaction rather than direct adsorption of phenol on precipitates.

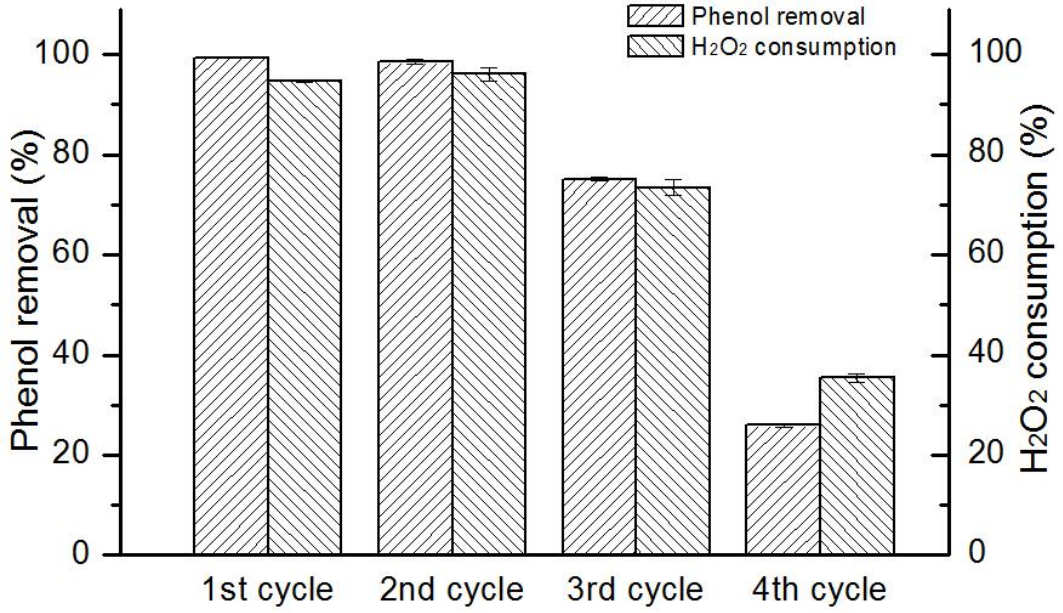


Figure 4-11 Consecutive precipitates recycling with no SBP addition.

[Cycle conditions were: 1 mM phenol, 1.25 mM H_2O_2, pH = 7.0 in 30 mL, 3 h reaction. The first cycle had 2 U/mL SBP to start the reaction; the settled precipitates from one reactor were transferred to the subsequent reactor]

4.4.3 Consecutive Precipitates Recycling with SBP Addition

The above experiment shows that, with the addition of sufficient fresh SBP, the concentration of active SBP on precipitates would not be consumed as above and it may be possible to achieve more than 95% removal of phenol at each cycle. In order to find out the minimum amount of addition SBP required to attain that level of phenol removal with quantitatively recycled precipitates, a series of seven different additions were chosen for four recycles. The precipitates were prepared under the optimized single-batch conditions in a large batch reactor, and then those were evenly distributed to smaller batch reactors to conduct the experiments. In order to get the minimum SBP addition with consecutive >95% removal of phenol, the seven additions of different SBP concentrations were gradually narrowed to two concentrations, 0.5 and 0.6 U/mL, after 4 cycles of reactions. Table 4-1A shows that 0.5 U/mL was the lowest addition of SBP to reach the above target, therefore the optimized SBP concentration is 0.5 U/mL with quantitatively recycled precipitates for 1 mM phenol removal. This observation suggests that the active SBP on the precipitates had reached a balance since free SBP added at the beginning in each cycle can be considered as the amount of SBP irreversibly inactivated during that cycle. Compared with the optimized SBP concentration for 1 mM phenol removal without precipitates recycling (Steevensz et al. 2009b), this approach could reduce SBP requirement by 2.4-fold (from 1.2 U/mL to 0.5 U/mL). The experiments were repeated under the above optimized conditions to monitor the change in the remaining SBP activity for six cycles (Table 4-1B). The results suggest that addition of 0.5 U/mL SBP could be slightly excessive, since the active SBP activity was increasing on the precipitates over successive cycles. On the other hand, the above quantitative

recycling may not be possible to apply in an actual reactor, since the portion of wasted precipitates would have active SBP on it. With this deduction, the optimum concentration of SBP addition for each cycle would depend on an increase that took into account the sludge withdrawal percentage.

Table 4-1 Optimization for consecutive precipitates recycling with SBP addition.

A. 1mM Phenol removal for different SBP additions with quantitative precipitates recycling							
	SBP addition for recycling (U/mL)*						
Recycle no.	0	0.2	0.4	0.5	0.6	0.7	0.8
1	67.4%	86.3%	94.0%	97.2%	97.7%	98.3%	98.4%
2			93.8%	96.6%	97.9%	98.3%	
3			89.0%	95.2%	96.4%	96.5%	
4				97.2%	98.5%		
B. Remaining SBP activity and phenol removal in each cycle with 0.5 U/mL SBP addition and quantitative precipitates recycling							
	Cycle no.						
	0	1	2	3	4	5	6
Phenol removal (%)	97.5	97	95.1	97.2	99	98	97
Remaining SBP activity (U/mL)**	0.21	0.22	0.26	0.29	0.44	0.62	0.72

* The precipitates were made from: 1 mM phenol, 1.2 U/mL SBP, 1.25 mM H_2O_2 and pH = 7.0 in 1L volume. The 3 h phenol removal was 98.3%. The remaining cycle conditions: 1 mM phenol, 1.25 mM H_2O_2 and pH = 7.0 with specified SBP addition. ** apparent activity

4.5 Precipitates Recycling for Phenol Removal in the Presence of Additive

It is shown above that application of the precipitates recycling strategy for 1 mM phenol removal in 3 h in the absence of additive can reduce the required SBP from 1.2 U/mL to 0.5 U/mL. The improvement was actually due to the “overdosing” of SBP to counteract an adsorption-induced specific activity drop to maintain a certain reaction rate to achieve >95% removal. In the presence of a protective additive, the required enzyme for

the same level of phenol removal was even less. For example, the requirement for crude SBP was 0.9 U/mL, 0.3 U/mL and 0.15 U/mL with PEG, SDS and Triton, respectively (Mousa Al-Ansari et al. 2010). Because of partial prevention of the end-product inactivation by the additive, a precipitates recycling strategy would not be as effective as in the absence of additive. Further, if SBP adsorption on the precipitates were completely prevented, there should be no active SBP on the precipitates, thus there would be no SBP saving by such recycling. The concerns about using a precipitates recycling strategy with additives are: 1) effect on precipitates formation in the presence of additive; 2) the fate of additive (PEG, SDS or Triton) after reaction; 3) the additive's effect on SBP activity and partitioning between the two phases. Evidence addressing these concerns is the theoretical support of the feasibility to apply such a strategy. In the following sections, PEG, SDS and Triton effects were studied separately.

4.5.1 Free and Immobilized SBP Activity Change in the Presence of PEG, SDS or Triton X-100

Free SBP was individually incubated with different concentrations of PEG, SDS or Triton, for 30 min, and the activity of SBP in each case was measured. The % difference between the measured samples and control (no additive) is plotted in Figure 4-12 to Figure 4-14. The variation of SBP activity assay was about 5% (maximum 8%) due to temperature change and the freshness of activity assay reagent. Therefore if the difference is within a 5% range, it is considered that there was no effect of additive on the SBP activity. In Figure 4-12, PEG up to 2000 mg/L is showing no significant effect on free SBP activity. It is known that high concentrations of SDS could irreversibly denature

the proteins, thus causing permanent activity loss. At pH below 3.9 (pI of SBP), SBP activity was sharply lost with low concentrations of SDS and no activity was detected when SDS concentration was above 144 mg/L (0.5 mM); however, when SBP is deprotonated above pH 3.9, it possessed the opposite charge to SDS, activity loss became less sensitive to the change in SDS concentration. At pH 5.6, SBP activity loss starts at 300 mg/L of SDS concentration, and about 20% activity disappeared with 2,000 mg/ L of SDS, and 20% of the activity was still be observed with over 20,000 mg/L of SDS (Zhang et al. 2009). Similar results were observed here at pH 6.5 when about 10% of the activity was lost with 500 mg/L of SDS, and the maximum loss was 17.5 % with 1500 mg/L of SDS (Figure 4-13). The phenomenon also happened during SDS concentration optimization for 1 mM phenol removal: less phenol removal was obtained when excessive amounts of SDS were used (Mousa Al-Ansari et al. 2010). For Triton, it is shown that SBP activity was increased 3-10 % in the presence of 100-5000 mg/L of Triton in Figure 4-14. There is a consistent increase of SBP activity but no clear pattern of % activity increase as a function of Triton concentration. On average, Triton increases free SBP activity by 6 $\pm$ 3 %. However, the detergent's effect on free SBP activity was reported differently. Sakurai et al. (2003) reported that no significant increase of CIP activity was observed when CIP was incubated with Triton and SDS, but the detergent's concentration range was not specified. And, Flock et al. (1999) reported the effect of up to 1% of SDS or Triton present in the activity assay reagent, the specific activity of SBP extract was increased 3.5-fold with 1000 mg/L SDS and 2.3-fold with 1000 mg/L Triton in oxidizing guaiacol; no negative effect on the activity was observed up to 200 g/L of SDS.

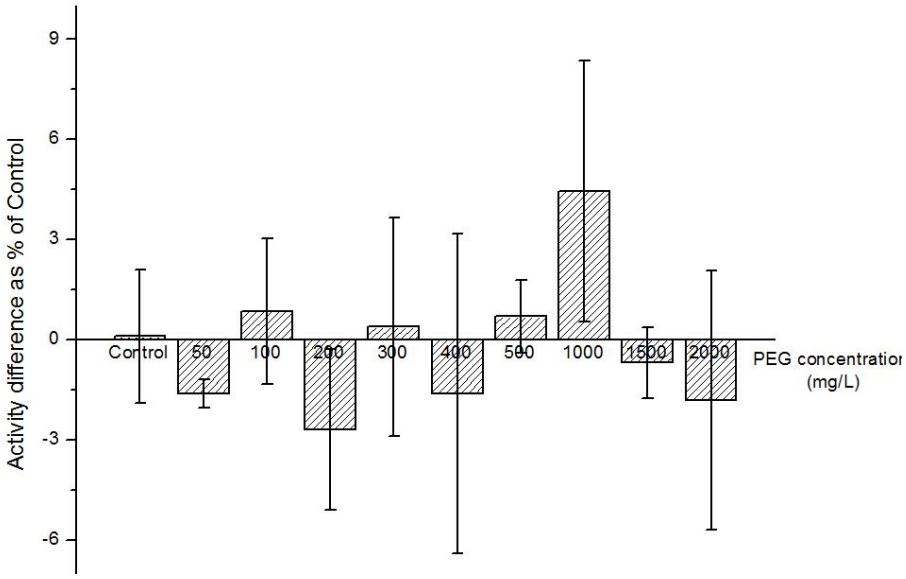


Figure 4-12 Free SBP activity change upon incubation with different PEG concentrations.

[PEG concentration: 0-2000 mg/L, SBP concentration: 1 U/mL, Incubation time 30 min, pH = 6.5 (distilled water)]

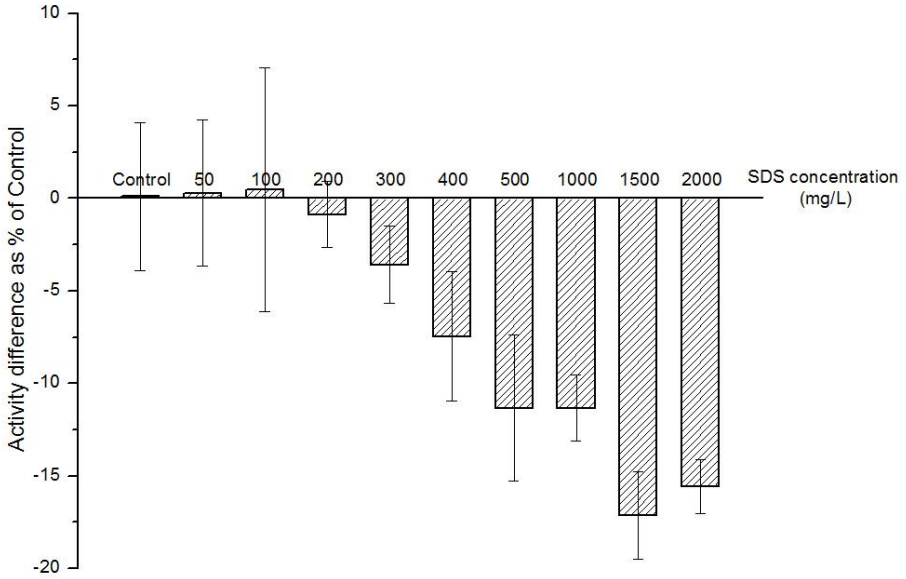


Figure 4-13 Free SBP activity change upon incubation with different SDS concentrations.

[SDS concentration: 0-2000 mg/L, SBP concentration: 1 U/mL, Incubation time 30 min, pH = 6.5 (distilled water)]

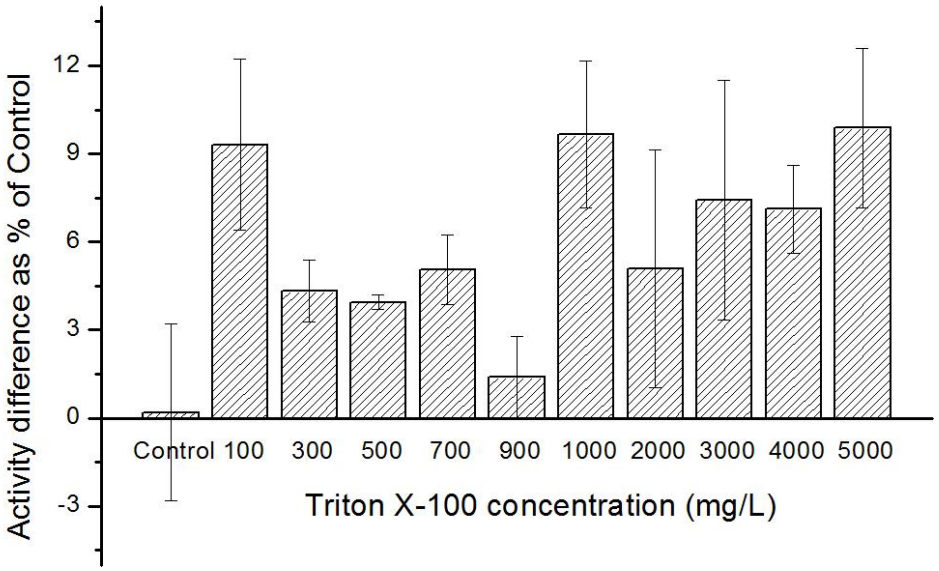


Figure 4-14 Free SBP activity change upon incubation with different Triton X-100 concentrations.

[Triton X-100 concentration: 0-5000 mg/L, SBP concentration: 1 U/mL, Incubation time 30 min, pH = 6.5 (distilled water)]

For immobilized SBP, Triton is known to elute the adsorbed SBP from phenolic precipitates, so the mixture activity would significantly increase as indicated in Figure 4-7. The effect of PEG and SDS on the immobilized SBP has not been reported. In this experiment, precipitates containing active SBP were incubated with 0-1000 mg/L PEG, SDS or Triton for 30 min, respectively, and the results are shown in Figures 4-15 to 4-17. The mixture activity was measured before and after the incubation. Both PEG and Triton also show a positive effect on mixture activity. The reported "PEG effect" to prevent SBP adsorption on precipitates has been extensively demonstrated; but for precipitates already containing active SBP, the addition of PEG as a competitive adsorbate apparently leads to re-equilibration of the adsorption, since a 30% increase of the mixture activity was observed. However, the increase is less than one-tenth of that observed with Triton. Figure 4-17 shows that Triton elution increased mixture activity nearly 5-fold at the maximum level. It also shows that the elution efficiency is related to the given Triton

concentration, and the maximum elution is observed at 300 mg/L, beyond that there is no further increase in mixture activity with additional Triton. PEG shares a similar poly-oxyethylene chain with Triton, which could be the reason that a qualitatively similar effect was observed for both. However, the hydrophilic poly-oxyethylene units should not interact with precipitates. The hydrophobic tail in Triton is believed to be directly adsorbed, while PEG does not have a hydrophobic domain – it is not a surfactant. More experiments are required to explain the drastic quantitative difference between Triton and PEG. Meanwhile, the presence of SDS showed no SBP desorption. The immobilized SBP activity seems to be sensitive to SDS since more than 10% activity loss was observed with more than 300 mg/L of SDS, and activity loss increased with an increase in SDS concentrations. This indicates that a precipitates recycling strategy is not suitable in the presence of SDS. Thus, if the immobilized SBP on precipitates has a higher potential than free SBP to be inactivated by the presence of SDS, the benefit from precipitates recycling would be limited.

It was also noted that there is a 20-fold dilution of the enzyme sample during the SBP activity assay (50 µL sample with 950 µL reagent). The effective additive concentration during the activity assay was 20-fold lower, compared with the additive concentration under the incubation conditions. Thus, the above experiments were repeated with additive present in the reagent, so that the desired concentration can be obtained in the activity assay mixture. The results for PEG and SDS are shown in appendix D (Figure A-10 to A-13). For both PEG and SDS, the influences of on SBP activity were similar to having additive present in the incubation.

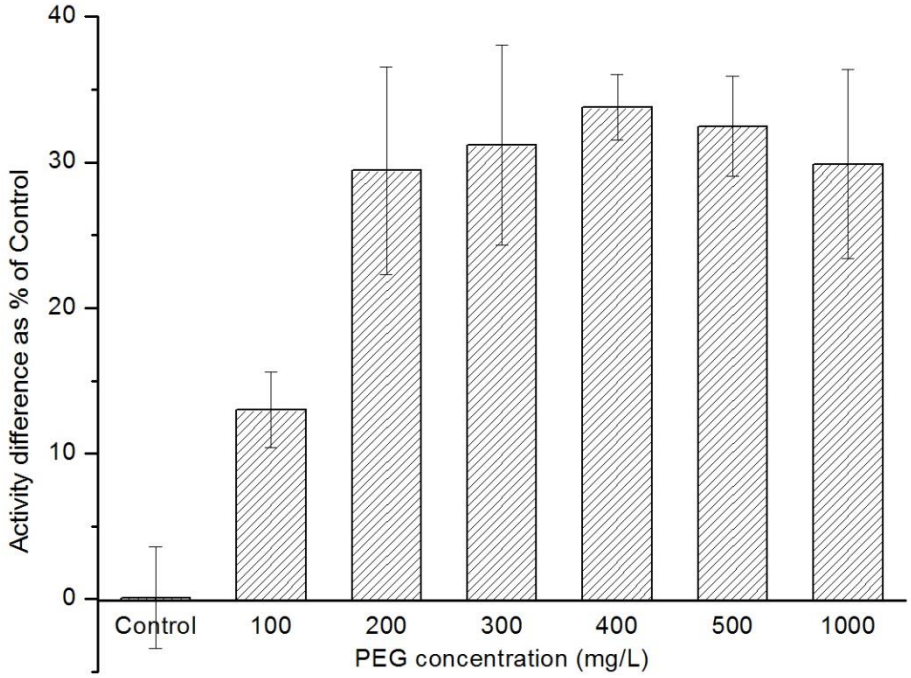


Figure 4-15 Phenolic precipitates incubated with different concentrations of PEG.
[Precipitates concentration: 0.232 mg/mL, pH = 6.5 (distilled water), 30 min incubation.]

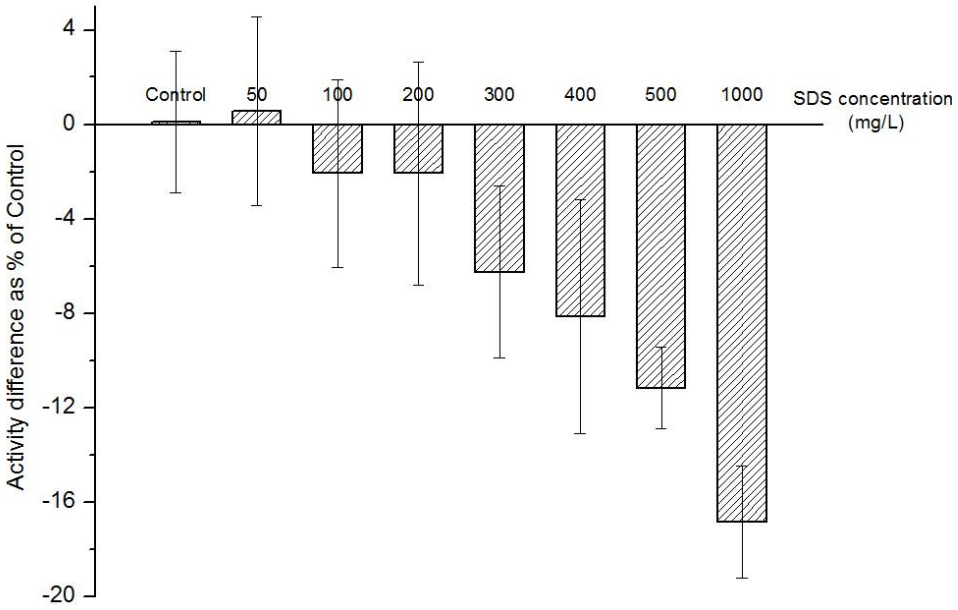


Figure 4-16 Phenolic precipitates incubated with different concentrations of SDS
[Precipitates concentration: 0.232 mg/mL, pH = 6.5 (distilled water), 30 min incubation]

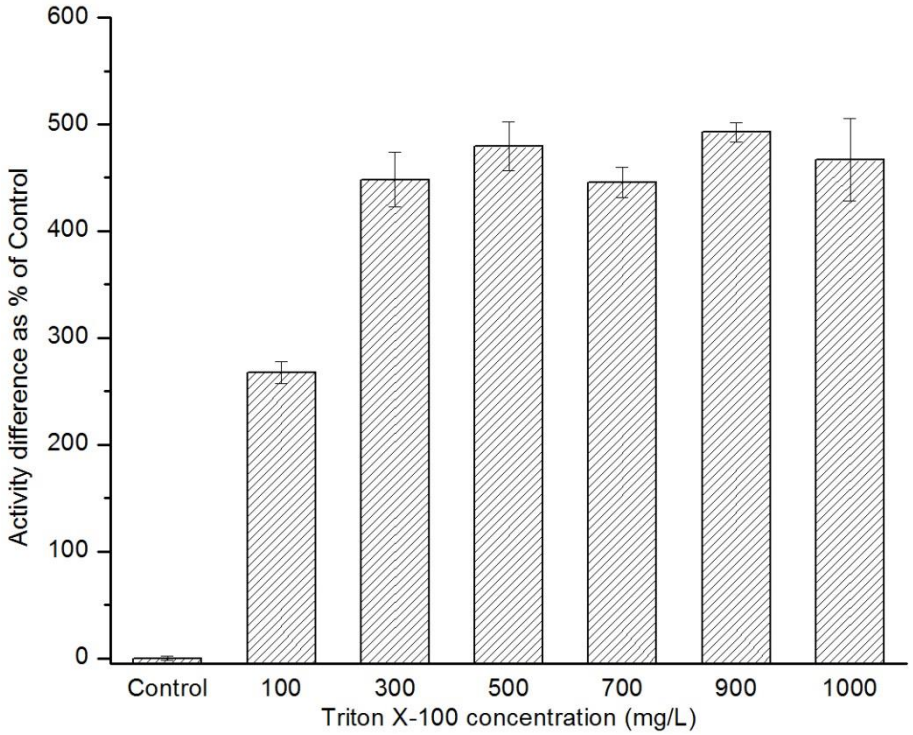


Figure 4-17 Phenolic precipitates incubated with different concentrations of Triton X- 100.

[Precipitates concentration: 0.232 mg/mL, pH = 6.5 (distilled water), 30 min incubation]

4.5.2 SBP Activity Uptake on Phenolic Precipitates under Turnover and Static Conditions in the Presence of PEG

A study of the protective effect of PEG in static incubation (no SBP inactivation due to phenoxyl radical attack or H_2O_2) reported that: 1) in the presence of PEG, adsorption of HRP by precipitates was partially prevented and no decrease in mixture activity was observed over 2.5 h; 2) phenolic precipitates formed under turnover conditions in the presence of PEG did not inactivate HRP under the above incubation conditions (Wu et al. 1998). A similar experiment was conducted with SBP, and the results are shown in Figure 4-18. SBP adsorption and inactivation (mixture activity drop) are compared under two conditions: regular precipitates incubated with 500 mg/L of PEG and precipitates formed in the presence of 500 mg/L of PEG. For supernatant activity change, in both

scenarios 1 and 2 a portion of free SBP had disappeared from the solution and was adsorbed on the precipitates. Therefore, neither PEG present during incubation nor PEG present during precipitates formation could completely prevent the adsorption. For mixture activity change, both scenarios 1 and 2 had the mixture activity equal to the theoretical total, which suggests that both scenarios prevented the adsorption-induced specific activity change. With no alteration in specific activity of SBP, the adsorption is not harmful to the catalytic efficiency. In addition, ^{1}H NMR (nuclear magnetic resonance) analysis had confirmed that PEG incorporated with phenolic precipitates during the formation. This could be the reason for the products formed in the presence of PEG to be inert to the free enzyme (Steevensz et al. 2012). On the other hand, PEG appears to have a higher partition affinity for phenolic products than enzyme, and thus prevents enzyme adsorption to the precipitates when both enzyme and PEG are present together for adsorption. However, the evidence is still not conclusive to explain the difference in the change of adsorption-induced specific activity between the two types of polymer products. Visually, precipitates formed in the presence of PEG were lighter and finer than those formed in the absence of PEG. If phenolic precipitates are considered as a porous solid carrier to the immobilize enzyme, then the substrate concentration in the microenvironment of immobilized enzyme would be affected by external and internal diffusion efficiency. To reduce the concentration gradient from the bulk solution to the microenvironment, a common approach is to reduce the carrier particle radius by grinding (Trevan 1981). Therefore, finer particles may have better diffusion efficiency and the immobilized enzyme parameters might be the same as free. More evidence is required to support such a rationale.

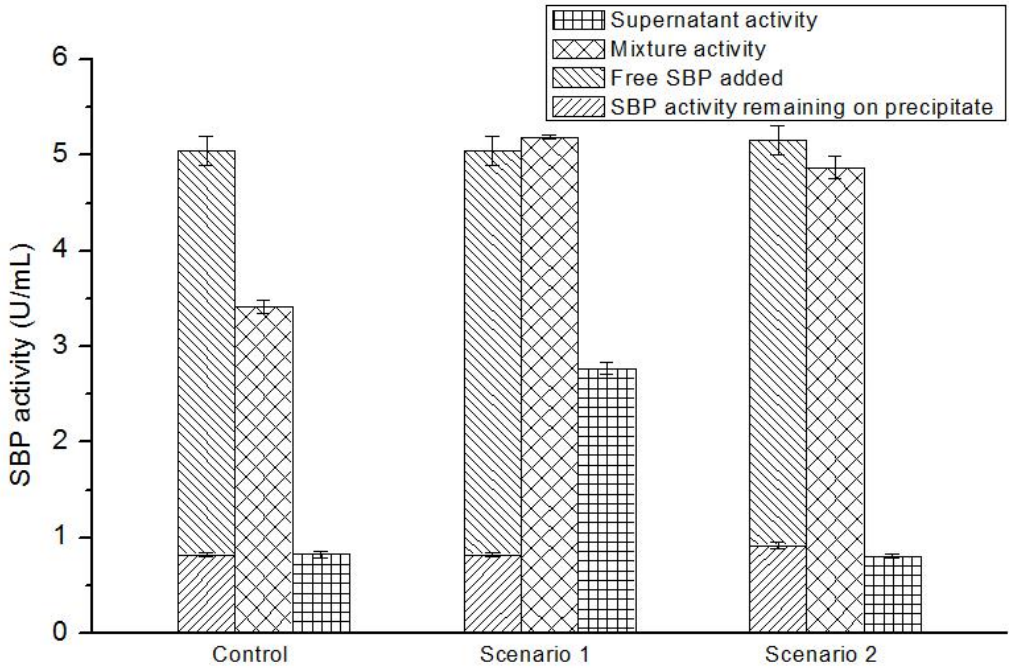


Figure 4-18 Supernatant and mixture activity change of free SBP incubated with phenolic precipitates.

[Control: precipitates made in the absence of PEG, and no PEG added during incubation; scenario 1: precipitate made in the absence of PEG, and incubated with 500 mg/L of PEG; scenario 2: precipitate made in the presence of 500 mg/L PEG. Precipitates concentration: 2 mg/mL, incubation time 30 min.]

Another way to study the PEG effect is under substrate turnover conditions. As shown in Figure 4-19, SBP was still adsorbed on phenolic precipitates in the presence of PEG, but the adsorption rate was slower than in the absence of PEG; and it gradually decreased over time. The partition of SBP between the solid phase and the aqueous phase is related to the PEG concentration in the reaction. With a higher PEG concentration, a higher supernatant SBP activity is observed. This result supports the rationale that SBP and PEG are competing in a two-adsorbate system. If the surface area was covered more by PEG due to its higher concentration in solution, fewer enzyme molecules would be adsorbed, in other words, more enzyme molecules were protected. It also indicates that

the incomplete prevention of adsorption of crude SBP could be a reason for the inefficient PEG effect.

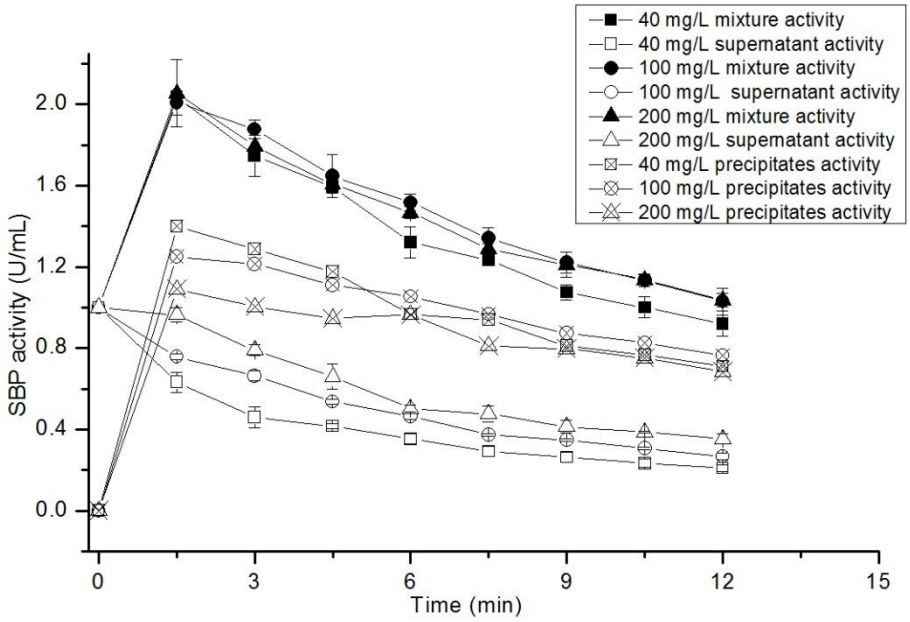


Figure 4-19 Supernatant, precipitates and mixture SBP activity change over time under turnover conditions.

[Experiment conditions: 1 mM phenol, 1.25 mM H_2O_2, 1.2 U/mL SBP and pH = 7.0 with 40, 100 and 200 mg/L PEG; precipitate activity was calculated by difference]

4.5.3 SBP Activity on Phenolic Precipitates under Turnover Conditions in the Presence of SDS

SDS as an anionic surfactant has been studied for its protective effect on phenol removal by crude SBP. The required SBP for 1 mM phenol removal can be reduced by 4-fold in the presence of 150 mg/L of SDS for synthetic wastewater, and 5.3-fold less SBP is required for coal-tar wastewater when compared with in the absence of SDS to have the same level of phenol (15 mM) removal (Mousa Al-Ansari et al. 2010). SDS is also effective in the removal of coloured polymer products after the enzymatic reaction of arylamines (Mousa Al-Ansari et al. 2009). However, little has been done to explain the protection mechanism of SDS and the fate of SDS after the reaction. By applying the

same strategy of SBP partitioning to the two phases, more information on the SDS protection mechanism could be obtained. Under turnover conditions, Figure 4-20, the measured SBP activity was nearly 3-fold higher in the early stages than the amount added, and gradually dropped to the initial value after 90 min of reaction. Similar effects were observed earlier, Figure 4-1 and Figure 4-19, but the increases of SBP activity were only about 2-fold and quickly dropped to below the initial value in 15 min. The dimeric intermediates are believed to interfere with the activity assay due to their hyper-reactivity, but those compounds should only exist at the initial stages of the reaction. Therefore, it cannot be used to explain the hyperactivity over a 90 min period. However, if SDS forms micelles in the solution, the oligomer end-products might be kept soluble in the micelles and be further oxidized in successive enzyme cycles. Because the critical molecular mass for oligomer/polymer to precipitate out in hydrophobic micelles is higher than in water, the solubilized products could remain accessible to the enzyme much longer. Since some of these soluble products are better substrates of SBP than phenol, a hyper-activity of SBP might be observed over an extended period of time. Visual observation of the reaction mixture supports the rationale; the solution had a clear brownish colour during the first 30 min (compared with 5 min in the absence of SDS) and very fine precipitates were observed afterwards.

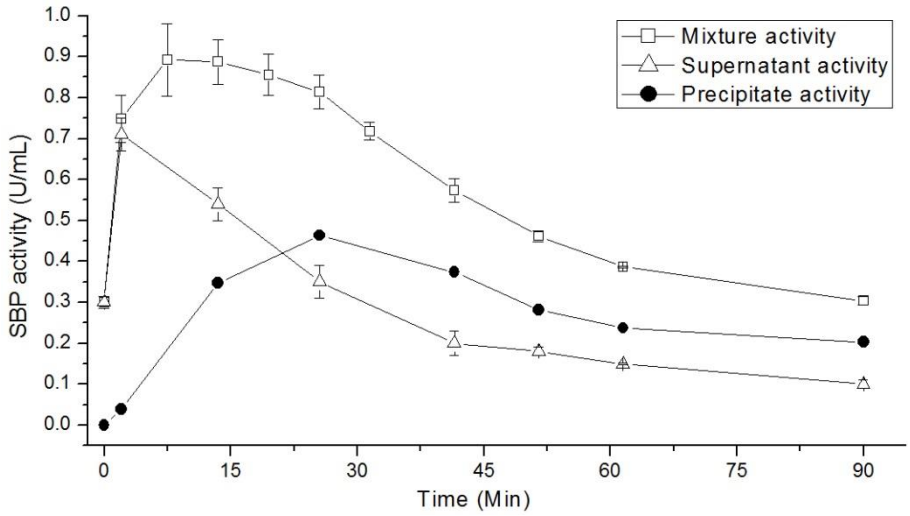


Figure 4-20 Mixture, supernatant and precipitates SBP activity change under phenol turnover conditions in the presence of SDS.

[Reaction conditions: 1mM phenol, 1.5mM H_2O_2, 125 mg/L SDS, 0.3 U/mL SBP and pH = 7.0, precipitates activity was calculated by difference.]

TOC analyses, carried out after 95% conversion of phenol in the presence of SDS, suggest that there was no removal of SDS if it is assumed that the converted carbon of phenol was completely separated. It is likely the majority of the SDS remained in the solution with SBP (Mousa Al-Ansari et al. 2010). However, the TOC test cannot distinguish SDS or soluble intermediates from all organic carbon sources. The new adsorption model suggests that compounds with hydrophobicity could be adsorbed onto precipitates. It's highly possible that the hydrophobic tail of SDS binds with precipitates and helps removal of some SDS from the solution. A new colorimetric method for the determination of SDS in aqueous solution was developed to overcome this ambiguity and, thus, provide a better understanding of the fate of SDS in the reaction (Arand et al. 1992).

4.5.4 Fate of SDS in Phenol Polymerization Reaction

There are four possible pathways for SDS to exist after the reaction: 1) adsorption on phenolic precipitates; 2) binding with immobilized SBP on precipitates; 3) binding with soluble SBP; and 4) remaining in the solution. The first two pathways are hard to distinguish, that is to say SDS could directly adsorb on phenolic precipitates or bind with inactive/active protein on precipitates. Therefore, those two pathways were considered together as SDS binding on precipitates. It is known that SDS binds with protein in a constant mass ratio of 1.4 g SDS per g of completely denatured protein in SDS-PAGE (Wikipedia 2013b). At pH 2.6 and pH 5.2 (below and above the pI of SBP), different effects on SBP activity of SDS binding were reported. A rapid drop in SBP activity was observed with 0.5 mM (144 mg/L) SDS at pH 2.6; meanwhile the same electrostatic charges on SBP and SDS at pH 5.2, protected a tryptophan residue from being exposed and the activity of SBP remained unchanged (Zhang et al. 2009). Therefore, SDS was not recommended for use in phenol removal at pH lower than 3.9. Preliminary tests confirmed the results of Zhang et al. (2009). Moreover, a trial test of crude SBP binding with SDS was conducted. SBP and SDS were mixed and then used with phenolic precipitates to remove SBP from the solution. An average of 2.77 μg of SDS disappearance per U of crude SBP was observed after the correction was made for SDS that was adsorbed by the same amount of precipitates used (data not shown). To test if SDS would bind with phenolic precipitates, different concentrations of phenolic precipitates were incubated with SDS for 30 min, and the SDS that disappeared from solution was considered as adsorbed by the precipitates. Figure 4-21 shows that with an increase in phenolic precipitates concentration, the SDS remaining in solution was

proportionally decreased. The linear regression line gives about 133 mg SDS removal per g of precipitates. Again, it is not known whether SDS is adsorbed onto the polymer surface or "coated" on active/inactive SBP protein on the polymer surface.

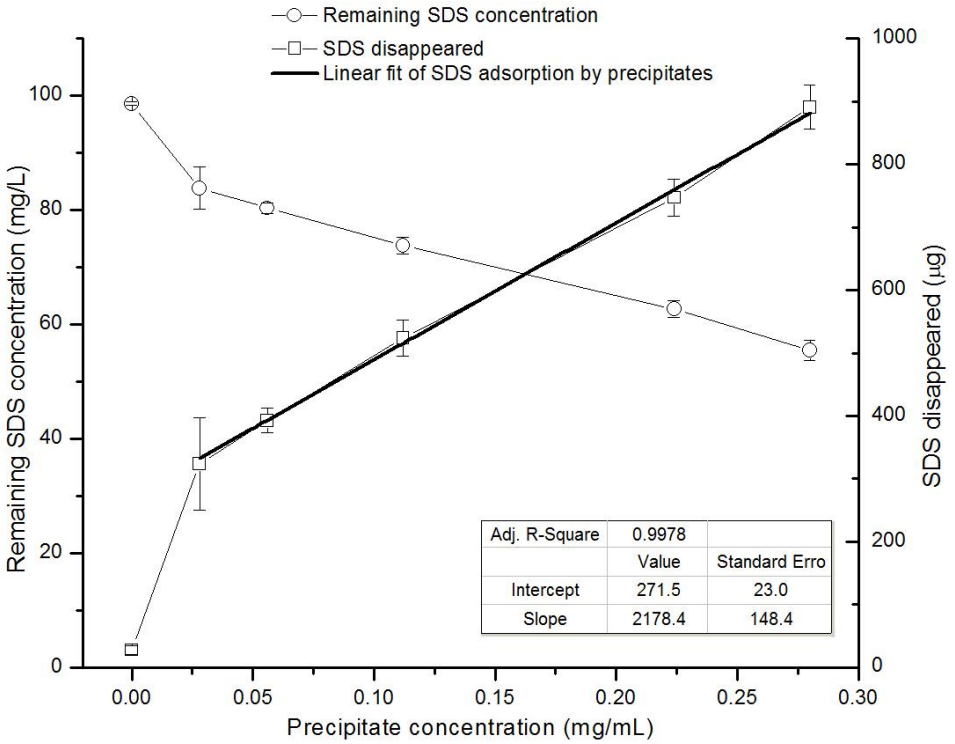


Figure 4-21 SDS adsorption by phenolic precipitates in static incubation.
[SDS initial concentration: 100 mg/L, 20 mL in volume and 30 min incubation]

The critical micelle concentration (cmc) of SDS in pure water at 25 ℃ is 8.2 mM or 2306 mg/L (Mukerjee 1972). Above the cmc, SDS molecules aggregate and form micelles; the hydrophobic inner environment and hydrophilic outer surface allows these micelles to dissolve water-insoluble substances in the micelle. In enzymatic polymer synthesis, micelles have been used to increase substrate solubility (Ghoul and Chebil 2012). The SDS concentrations involved in the current study were below the cmc. Figure 4-22A shows that the use of SDS effectively extended SBP efficiency, and phenol removal rate had increased by 65% between no-SDS and the optimum-SDS concentrations. The

maximum phenol removal (from 5 mM) was reached at 500 mg/L SDS and any further increase in SDS concentration up to 1000 mg/L did not increase phenol removal. Meanwhile, there was a sudden increase in solution colour after treatment with SDS concentrations above 200 mg/L and significantly less precipitates were collected by centrifugation of these samples.

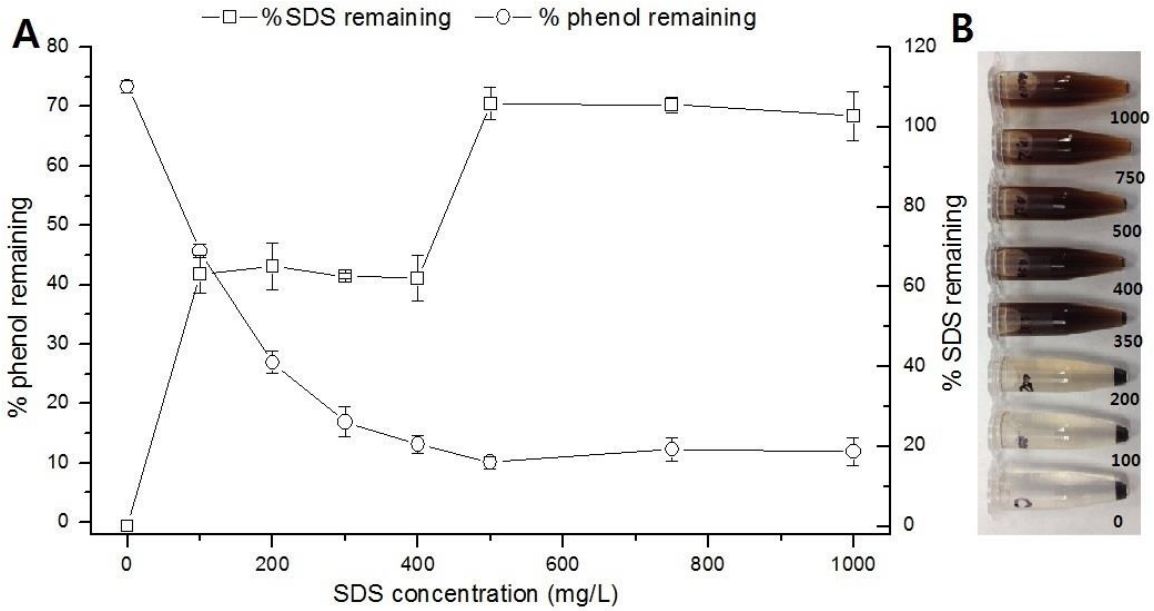


Figure 4-22 Phenol and SDS remaining after reaction with different SDS concentrations.

[Reaction conditions: 5 mM phenol, 7.5 mM H_2O_2, 0.7 U/mL SBP, 0 -1000 mg/L of SDS and pH = 7.0, 3 h reaction time; B: centrifugation (5 min x 5000 rpm) of samples in A.]

Moreover, SDS removal after the reaction was kept constant at 40% up to 400 mg/L addition of SDS, and then reduced to 0. This could be an indication of that all reaction products were soluble in the micelles above 400 mg/L of SDS, and therefore no oligomers were precipitated out and no SDS was removed. However, the cmc of SDS is much higher than the SDS used in this study, and it is unlikely that micelles would form at such a low concentration. One possible explanation is a decrease in cmc in the presence of electrolytes, which has been reported in literature. For example, 100 mM

sodium chloride (ionic strength = 100 mM), the cmc of SDS dropped to 2.1 mM (Hameed 2002). The 40 mM phosphate buffer used in all reactions had ionic strength of 240 mM, and this might further reduce the cmc to below the SDS concentration used here. On the other hand, phenol removal rate was about 2% less with 400 mg/L SDS than 500 mg/L, and SDS removal dropped from 40% to zero. Although, SDS is classified as a "readily biodegradable" surfactant, it is better to have less SDS discharged to the downstream biological treatment. The soluble intermediate products associated with SDS may also be toxic to microorganisms. Thus, it would be an acceptable trade-off to use a sub-optimal SDS concentration with a higher SBP concentration in order to have a higher degree of precipitation and overall carbon removal. A follow up experiment compared phenol and SDS removal at different SBP concentrations at 400 and 500 mg/L of SDS. Figure 4-23 shows that there is no significant difference in phenol removal with 400 or 500 mg/L of SDS, and the optimized SBP concentration was 1.2 U/mL for both. As for SDS removal, less SDS remained in solution at 400 mg/L of SDS compared with 500 mg/L until the reaction is pushed to >95% phenol removal. So, there is virtually no difference between 400 and 500 mg/L of SDS effect on phenol and SDS removal if the reaction is carried almost to completion. With the optimum SBP concentration of 1.2 U/mL, 400 mg/L of SDS gave 1% less phenol removal and about 80 mg/L higher SDS removal.

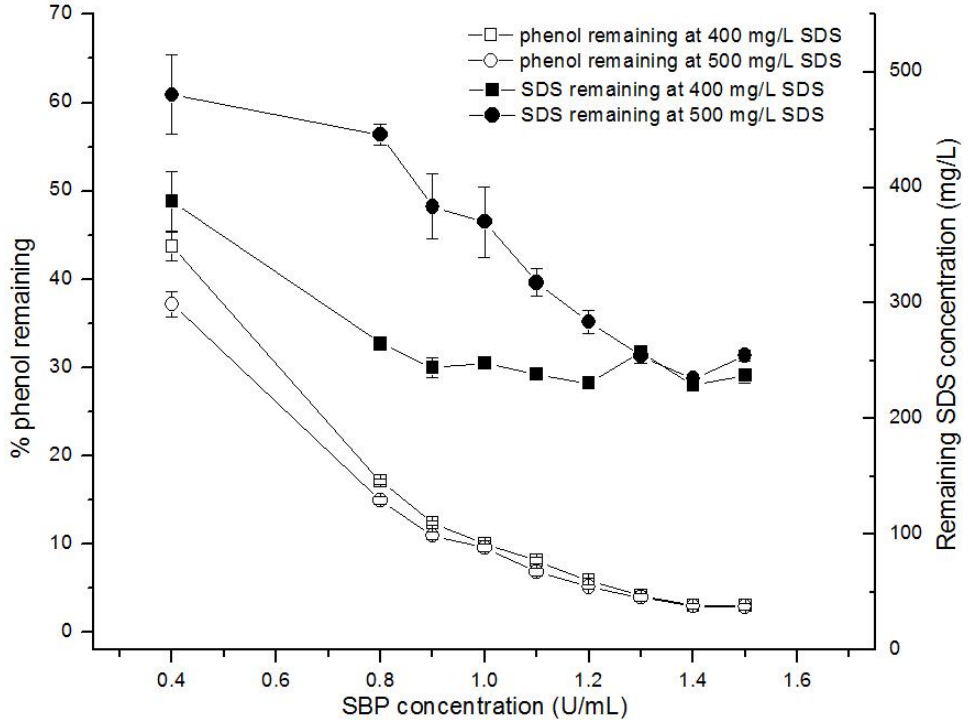


Figure 4-23 Optimization of Phenol and SDS removal at different SBP concentrations in the presence of 400 and 500 mg/L of SDS.

[Reaction conditions: 5 mM phenol, 7.5 mM H_2O_2, SBP concentration from 0.4 – 1.5 U/mL , 400 mg/L and 500 mg/L of SDS and pH = 7.0]

One additional benefit of precipitates recycling is the reduction in the remaining SDS concentration via adsorption under turnover conditions, confirm by the results from Figure 4-24. The use of 10 mg precipitates removed no phenol after 3 h reaction in the control, but did remove 10% of the added SDS. Comparing the 2 sets of phenol turnover reactions, the presence of precipitates helped in removing an extra 8% of SDS with the trade-off of 6% less phenol being removed.

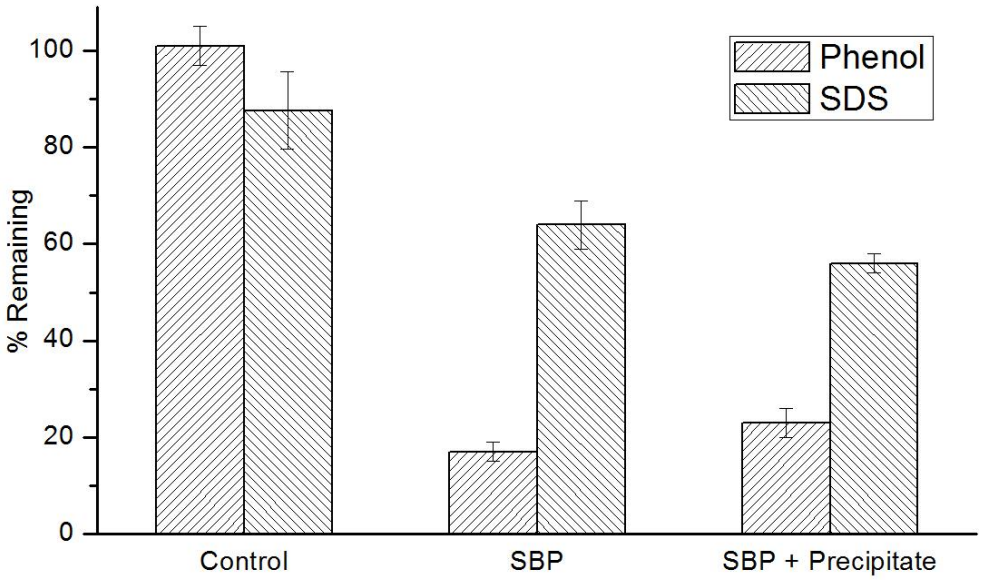


Figure 4-24 Phenol and SDS removal with extra precipitates.

[Reaction conditions for SBP + precipitate: 5mM phenol, 7.5mM H_2O_2, 500 mg/L SDS, 0.7 U/mL SBP, 10 mg of phenolic precipitates and pH = 7.0; Control: No SBP; SBP: no precipitate.]

4.5.5 Recycling Precipitates Generated in the Presence of Additive

The results on additives' protection mechanism have shown that they generally prevented SBP partitioning to phenolic precipitates, however in different ways. The PEG protection mechanism has been re-interpreted as a two-adsorbate competition on precipitates. The concentration of PEG in the aqueous phase determines the PEG tendency for adsorption to the precipitates. The protective behaviour of impurities in crude SBP can also be considered to provide an adsorption competition. On one side, it competes with PEG in the 'sacrificial' mechanism, and therefore weakens the protection efficiency of PEG; on the other side, it competes with SBP, and therefore SBP adsorption can be prevented (Mousa Al-Ansari et al. 2010). Since recycled precipitates do not carry over any solution from the previous batch to the subsequent batch, there is no expected saving on PEG dosage for each cycle of batch reactions. For SDS, it is believed that it forms micelles

under the reaction conditions; the oligomeric products are kept soluble in micelles thus avoiding the hydrophobic interaction between the products and free SBP. In addition, SDS also binds with precipitates during the reaction. By using a colorimetric test, it was confirmed that part of the SDS was removed from the solution after the reaction. The "PEG-like" surfactant Triton is believed to have similar protection behavior as PEG, but additionally, its function can be extended to elute adsorbed SBP off the polymer surface. Triton is so far the most efficient additive in terms of saving on enzyme, allowing SBP concentration to be reduced by 8-fold. Triton's elution ability suggests that precipitates formed in the presence of Triton should have no active SBP on them; thus, recycling such precipitates would have no benefit on phenol removal.

Additional concerns about applying the precipitates recycling strategy with the use of an additive are listed below:

- The amount of active SBP remaining on precipitates decides the benefit of recycling precipitates. The use of additives counteracts such effect, therefore the expected SBP saving should be less than in the absence of additive.
- Additive affects precipitates formation, finer precipitates are generally observed in the presence of additive in phenol removal. From an operational perspective, precipitates size and settling velocity are crucial to apply the strategy to a continuous process.
- The presence of additive could have a negative effect on the activity of the immobilized SBP through denaturation.

In Figure 4-25, different precipitates were made under the optimum conditions of each additive, and then quantitatively transferred, without washing, to a batch reactor to run

the subsequent reaction without the addition of fresh SBP. As predicted, precipitates generated in the presence Triton removed no phenol in 20 h of reaction, thus the use of Triton and recycling precipitates cannot be combined. Both PEG and SDS cases had active SBP carried over from the previous batch of reaction by recycled precipitates, the 3h phenol removal was 61% and 31% for SDS and PEG, respectively. The recycled precipitates formed in the presence of PEG only gave about 55% phenol removal after 20 h reaction, and no significant improvement on phenol removal was observed between 5 h to 20 h of reaction. The cumulative requirement for SBP was 0.58 U/mL per mM phenol removal in the two cycles, even higher than the required SBP in the absence of additive in consecutive cycles. The best results were obtained with SDS, when the reaction almost reached 95% removal of phenol after 20 h. However, the majority of the precipitates formed in the presence of SDS required more than 20 h to settle whereas it only took 20 min for precipitates to completely settle when no additive was used. Due to this major disadvantage for the process, the study was not continued further in this direction.

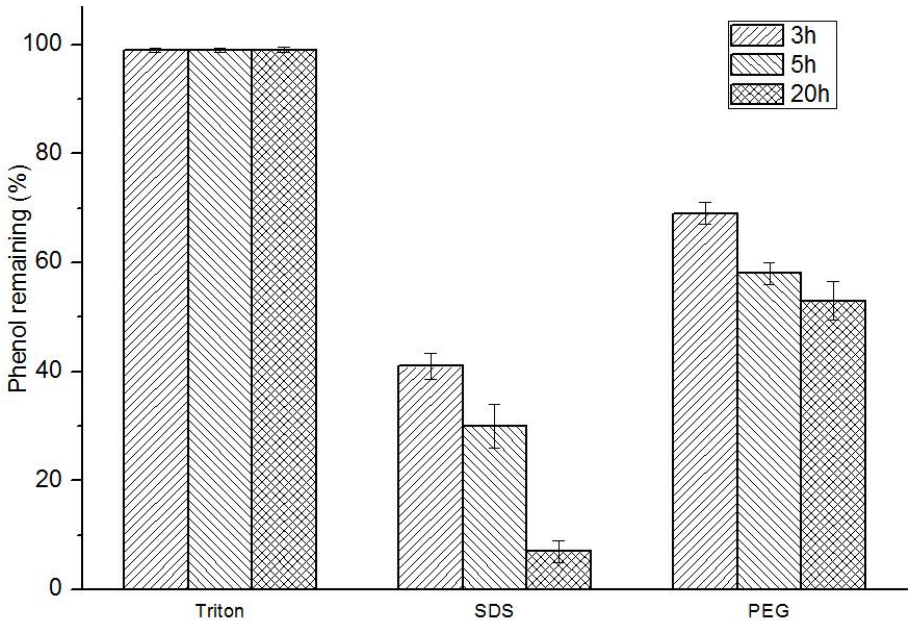


Figure 4-25 Precipitates recycling (without additional SBP) for phenol removal in the presence of Triton, SDS and PEG.

[Reaction conditions: 1 mM phenol, 1,25 mM H_2O_2, 100 mg/L PEG, 200 mg/L SDS or 200 mg/L Triton and pH = 7.0. Precipitates were generated from 1 mM phenol, 1.25 mM H_2O_2, and 0.9 U/mL SBP and 100 mg/L PEG, 0.3 U/mL SBP and 200 mg/L SDS or 0.15 U/mL SBP and 200 mg/L Triton were used in the three cases, respectively.]

4.6 Triton X-100 Elution of SBP Adsorbed on Phenolic Precipitates

Part of the Triton protection mechanism is to enhance desorption of adsorbed enzyme from polymer products. From an enzyme-saving perspective, Triton, so far, is the additive that requires the lowest enzyme concentration of crude SBP to achieve >95% phenol removal. The optimized Triton concentration has been determined for the given conditions. Use of a surfactant as a protective agent has not been widely reported in enzymatic treatment and the potential choices are numerous. Understanding of the Triton protection mechanism is useful to guide the search for a better and cheaper alternative. The elution phenomena can be mimicked by using precipitates with pre-adsorbed SBP, and then the relationship between % elution of adsorbed SBP and Triton concentration, elution time and precipitates concentration can be determined.

4.6.1 SBP Adsorption on Phenolic Precipitates under Static Conditions

The Langmuir and Freundlich adsorption isotherms are frequently used to characterize adsorption based on adsorbate concentrations in different phases. Figure 4-26 shows the original data of SBP adsorption at different precipitates concentrations. With an increase in SBP concentration, precipitates became saturated with SBP. The calculated C_e and q_e values from the above data points fitted by the two isotherms, are shown in Figure 4-27. The Freundlich isotherm fit gave a R-square value of only 0.85 vs. 0.97 for the Langmuir isotherm. The Langmuir monolayer adsorption capacity, Q_{max}, and Langmuir constant , K, were estimated to be 3.44 U/mg and 4.91 mL/U, respectively.

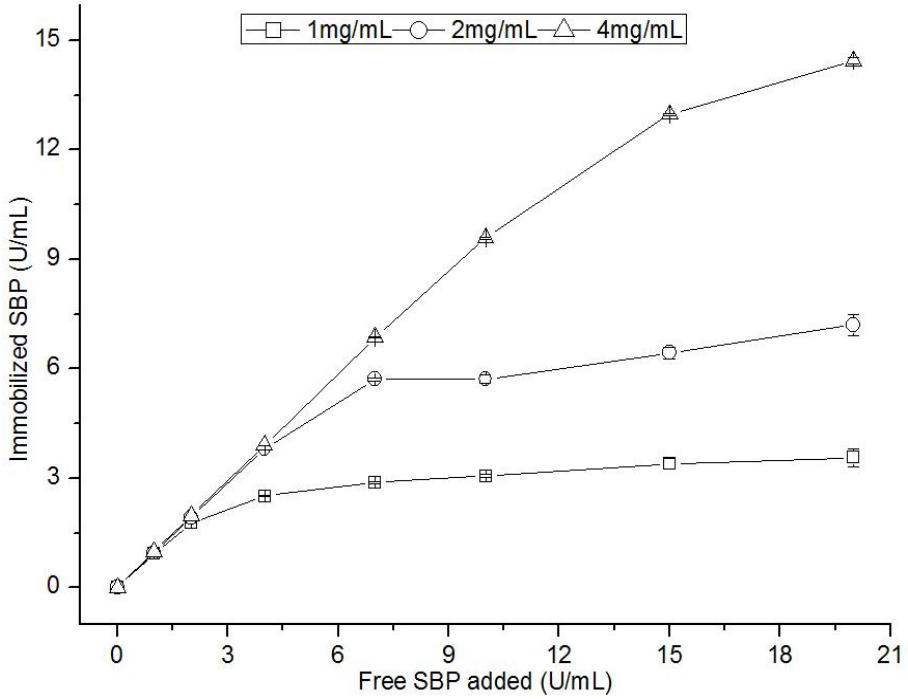


Figure 4-26 Immobilization capacity for different concentrations of precipitates.
[SBP immobilization with 1, 2 and 4 mg/mL precipitates; crude SBP concentration 0-21 U/mL; immobilization time 30 min.]

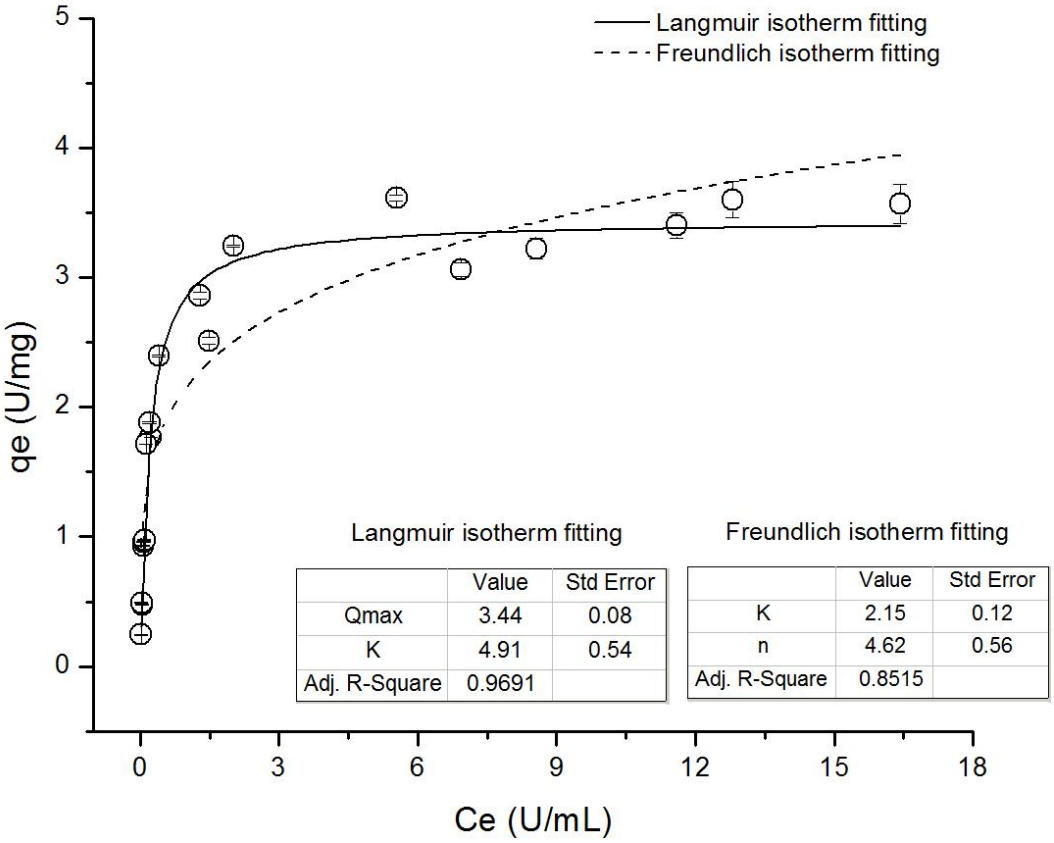


Figure 4-27 Langmuir isotherm and Freundlich isotherm plots of SBP adsorption on phenolic precipitates.

[Precipitates concentration 1-4 mg/mL, crude SBP concentration: 1-15 U/mL, incubation time 30 min]

Q_{max} provides an estimate of the capacity for enzyme to bind on solids (in U/mg of solid) as a monolayer; whereas, K, as an association constant, provides a measure of affinity bewteen enzyme and solid. The high K value suggests that the phenolic precipitates had a strong affinity to adsorb SBP, which could be an explanation for why precipitates quickly adsorbed most of the free SBP from the solution under both static and turnover conditions. It also suggests that the SBP remaining in the solution would be very low until the precipitates become saturated. In the presence of an additive, a reduction in Q_{max} and K values would be direct evidence of minimal SBP adsorption. However, the adsorption is reversible. When fresh water was used to wash/rinse precipitates with

adsorbed SBP, about 0.1-0.2 U/mL of SBP desorbed from the solids due to back-equilibration. Hydrophobic interaction is considered as the main driving force of this adsorption; however no direct evidence for the use of an artificial surface with hydrophobicity gradient to adsorb SBP over the surface, was obtained to confirm the hypothesis. The strength of hydrophobic interaction is dependent on the hydrophobicity of both phenolic precipitates and SBP. This could be an indication that some enzymes' lacking an observed 'PEG effect' with certain substrates was due to either of them lacking sufficient hydrophobicity.

4.6.2 Triton X-100 Adsorption on Phenolic Precipitates

For Triton X-100, a non-ionic surfactant, the adsorption onto a hydrophobic silica is fairly represented by a Langmuir isotherm, which indicates a non-cooperative interaction in the formation of surface aggregates and monolayer coverage characteristics (Tiberg 1996). In enzymatic phenol polymerization experiments, HPLC results showed that about 50% of the Triton had disappeared from the solution after the reaction, which is roughly 0.6 mg of Triton per 1 mg of phenol removal (Schoof, unpublished result, 2013). The only pathway by which Triton could disappear from the solution is to co-precipitate with phenolic precipitates. Like SBP, Triton adsorption may also occur when precipitates start to have enough hydrophobicity. To examine this adsorption phenomenon, Triton was incubated with different concentrations of phenolic precipitates under static conditions and the difference in Triton concentrations before and after the incubation was considered as the adsorbed Triton. Figure 4-28 shows that Triton adsorption on phenolic precipitates follows a Langmuir isotherm in the tested concentration range. The maximum adsorption

ratio is about 1 mg of Triton per 4 mg of precipitates, and the half-saturation concentration is about 33 mg/L which indicates an adsorption resistance at low Triton concentrations.

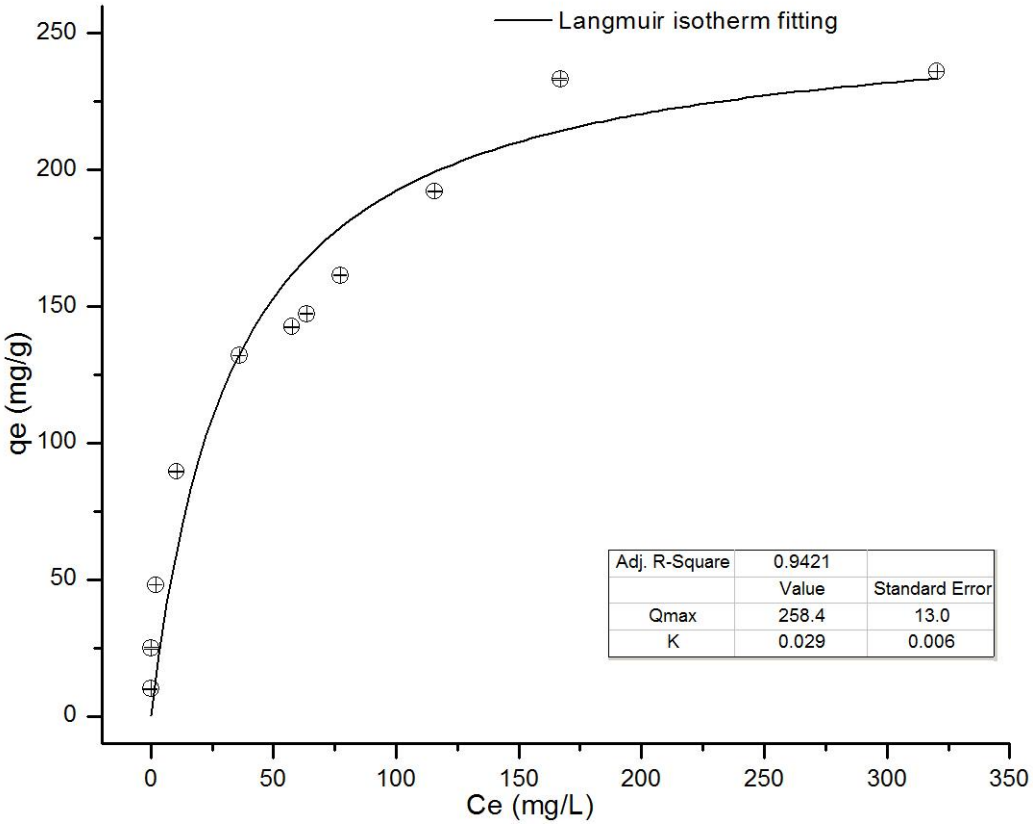


Figure 4-28 Langmuir isotherm fitting of Triton X-100 adsorption on phenolic precipitates.

[Precipitates concentration 1-5 mg/mL, Triton concentration: 10-1500 mg/L, incubation time 30 min]

4.6.3 SBP Elution at Different Precipitates and Triton X-100 Concentrations

In Section 4.3, an empirical Triton concentration of 500 mg/L was used to get the maximum elution of active SBP on phenolic precipitates and it proved to be useful at low precipitates concentrations. But, a quantitative relationship between eluted SBP as a function of total Triton concentration cannot be established under the conditions used in Figure 4-7, since the precipitates themselves carried an unknown amount of active SBP

that became immobilized during preparation. Therefore, a different precipitates preparation was used to establish % SBP elution as a function of Triton concentration at any given precipitates concentration. The preparation had a minimal remaining activity (0.09 U/mg) and a known amount of free SBP adsorbed prior to elution (details in Appendix C). The role of Triton concentration and precipitates concentration during SBP elution were first re-examined by two qualitative tests (Figure 4-29A & B). For 1.44 mg/mL of precipitates, nearly all SBP was eluted between 300 and 500 mg/L of Triton after 30 min incubation. But, when the same Triton concentration was applied on a 5-fold greater precipitates concentration, only about 10% of the adsorbed SBP was eluted.

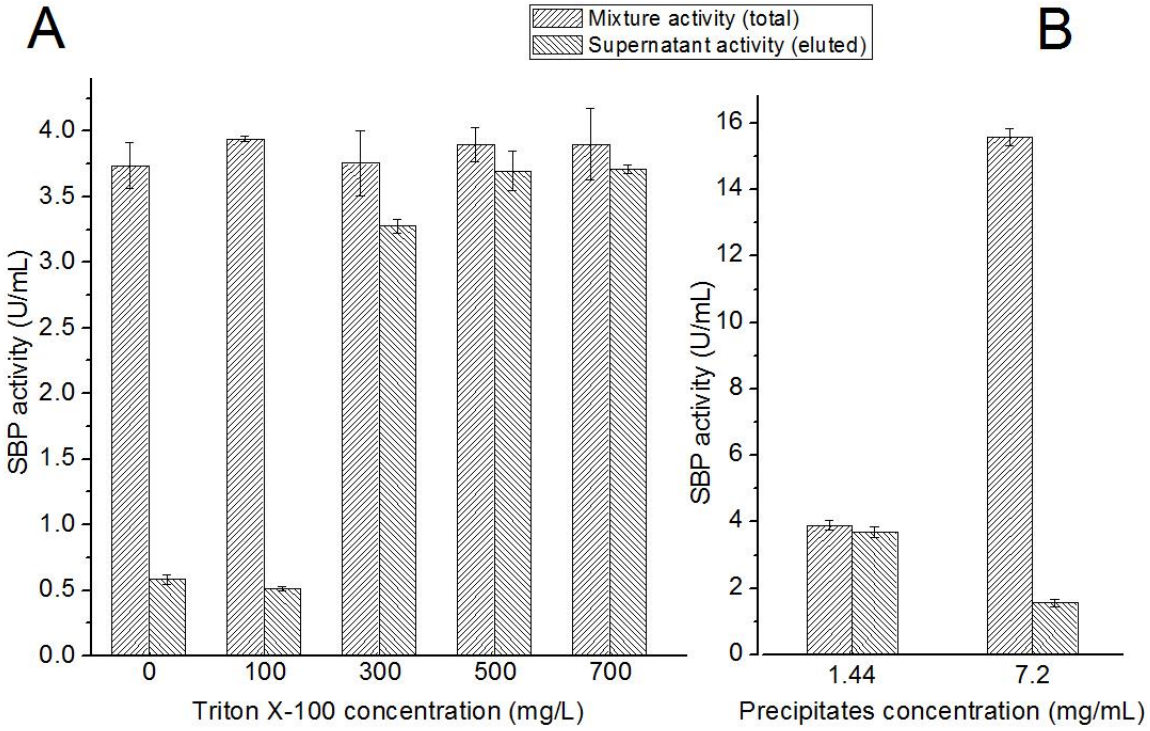


Figure 4-29 Qualitative determination of the factors influencing SBP elution.

[A: SBP elution at different Triton concentrations, 1.44 mg/mL precipitates; B: SBP elution at different precipitates concentrations, 500 mg/L Triton; both 30 min incubation]

After this qualitative experiment, the two factors were studied together in a similar manner with extension to 4 different precipitates concentrations and plotted as % SBP elution *vs.* Triton concentration, as shown in Figure 4-30.

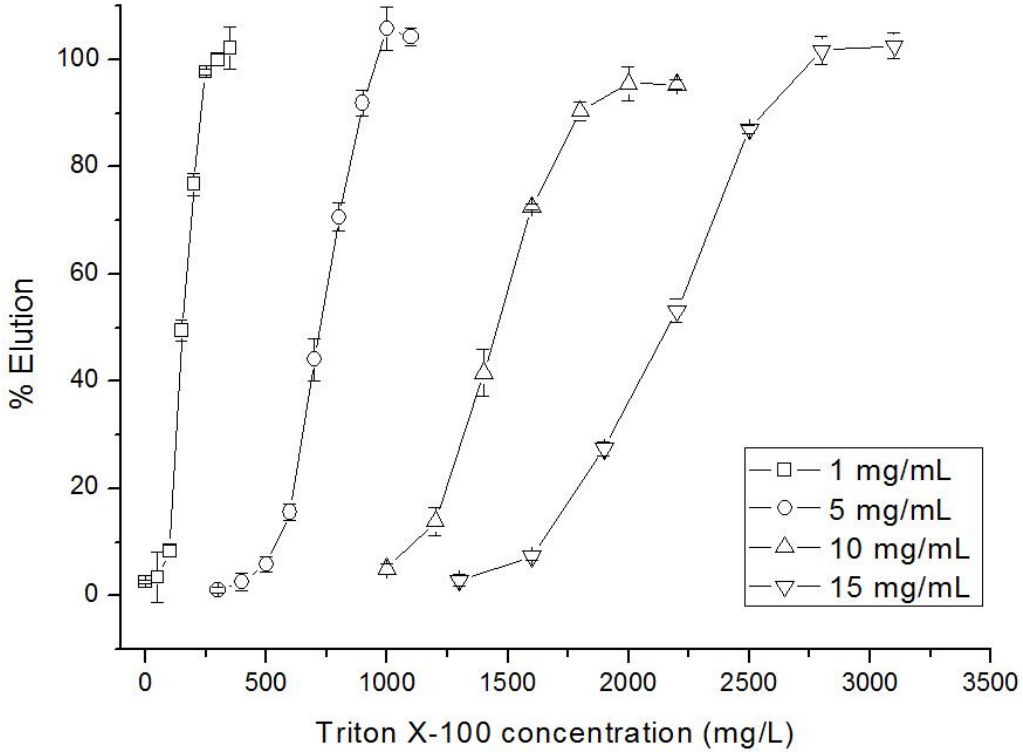


Figure 4-30 Percent SBP elution with different Triton concentrations at different precipitates concentrations.

[Experimental conditions: 1-15 mg/mL phenolic precipitates, 10-3500 mg/L of Triton, pre-adsorbed SBP 2 U/mg of precipitates; Incubation time 30 min.]

All the curves show an S-shape with a distinct breakthrough stage. On the lower end of the curves, SBP elution only happened when enough Triton was provided for a given precipitates concentration(for example, less than 10% SBP was eluted with 1600 mg/L of Triton and 15 mg/mL precipitates but nearly 80% elution could be obtained for 10 mg/mL precipitates at that Triton concentration). On the upper end of the curves, pushing elution from 90% to 100% required a significant increase of Triton when compared to the 50% to 60 % or even 50% to 80% ranges. Secondly, higher the phenolic precipitates

concentration, the higher is the Triton concentration required to reach the same percent of elution. Thirdly, over-dosage of Triton for elution did not inhibit the elution; it pushed elution to 100% and stayed. Similar studies in the literature had observed such an adsorption "replacement" phenomenon between proteins or between proteins and surfactants on a solid surface (Hirsh et al. 2013, Tiberg 1996). It is generally observed for binary competitive adsorption when the size of one component is smaller than the other (Damodaran 2004). For an already established protein layer, other proteins or surfactant with a higher binding affinity to the interface is considered as a competing species. According to the concentration and the type of competing species, the displacement of adsorbed protein may occur due to loss of competing available binding sites (Mollmann et al. 2005), or it may also occur by surfactant interaction with the adsorbed protein molecules by modifying the strength of the protein-surface binding forces (Tilton et al. 1993). The molecule of Triton is much smaller than SBP; therefore, adsorption of Triton reduces the interfacial tension more efficiently than SBP and is more favoured (Pugnaloni et al. 2004). When Triton adsorption reaches a certain level, further increases in adsorption of Triton reverse the binding of pre-adsorbed SBP on the precipitates. The S-curve behaviour of adsorption displacement has been explained as 'orogenic displacement', by analogy with the process of mountain formation(Mackie et al. 1999). The displacement does not occur simply by exchange of individual protein molecules, but is initiated with accumulation of adsorbed surfactant in the defects of the protein monolayer. With the increasing adsorption of surfactant, the interfacial pressure increases and compresses the protein film, and results in increasing of the protein film thickness. Beyond a critical surface pressure, the protein film cannot be compress anymore, and it

starts to collapse and detach from the surface in aggregates. Eventually, the continuously developed surfactant phase pushes all protein off the surface (Mackie et al. 1999, Pugnaloni et al. 2004). The proposed mechanism has been recently confirmed by AFM (Gunning et al. 1999, Mackie et al. 1999).

Besides providing an understanding of the SBP elution mechanism, the well-behaved elution curves might be fit to a mathematical equation. It is known that the percentage of SBP elution is a function of precipitates concentration and Triton concentration. A logistic function is a common S-shaped curve, which can be expressed in the first quadrant as Equation 4-1 (Wikipedia 2013a).

$$y = \frac{1}{1 + exp\{-ax + b\}} \qquad \text{Equation 4-1}$$

By taking Triton concentration from Figure 4-30 data points and dividing each by its corresponding precipitates concentration, a new set of normalized Triton concentrations can be obtained. As shown in Figure 4-31, all data points fall into a single S-curve with excellent fitting of a logistic function, where the x-axis represents a ratio of Triton concentration to precipitates concentration (C_T/C_P). By substituting C_T/C_P back into Equation 4-1, the final version of % SBP elution as a function of C_T and C_P is obtained.

$$\%\ \text{SBP elution} = \frac{1}{1 + exp\left\{-\frac{0.065}{C_p} \times\ C_T\ + 9.40\right\}} \qquad \text{Equation 4-2}$$

where C_p is precipitates concentration in the elution mixture (mg/mL) and C_T is Triton concentration in the elution mixture (mg/L). For any C_p chosen, this empirical equation could generate a curve to indicate the % SBP elution at a given C_T. The prediction is compared with the original data in Figure 4-30, and the majority of the data points except

some close to 100% elution fall on the generated curves (Figure 4-32). Also, the tested precipitates had a Triton adsorption capacity of 258 mg/g of precipitates. Thus, for any Triton concentration that is below the ratio of 100 mg/g of precipitates, there was almost no SBP eluted, whereas nearly complete elution was obtained with 200 mg/g of Triton. It is speculated that the critical interfacial pressure is related to Triton adsorption capacity and compressibility of the protein layer. Hence, for a precipitates preparation with a different Triton adsorption capacity, this ratio would vary accordingly; and for a different protein adsorbed, a different ratio would be observed. It is worth noting that the initial SBP coverage was fixed at 58% (2 U adsorbed whereas 3.44 U/mg was the maximum capacity) in all elution experiments. With a different initial coverage, more Triton would be required to reach the same level of elution; therefore, the coefficients a and b would be different in Equation 4-2. The influence of initial surface coverage at SBP elution was studied separately.

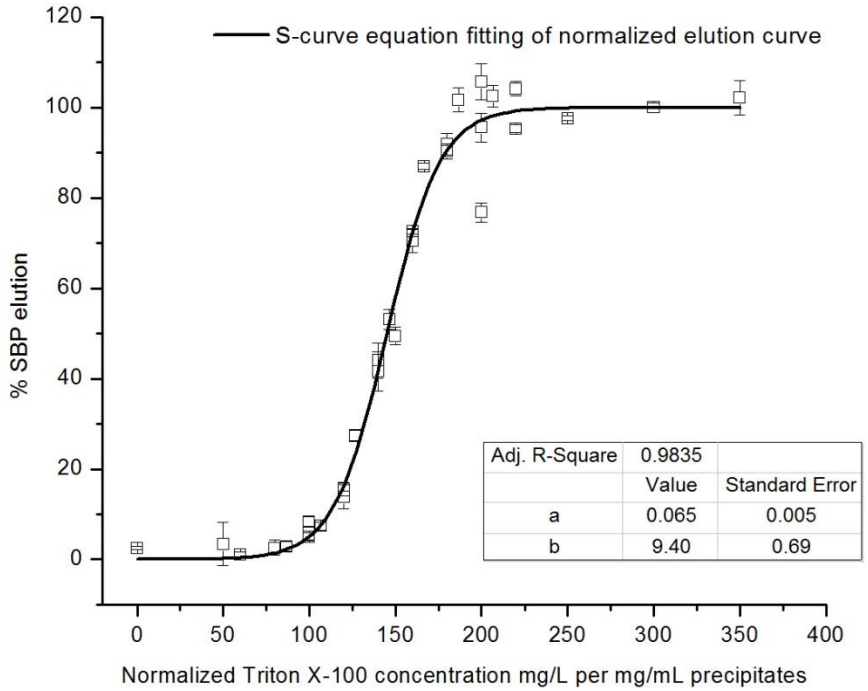


Figure 4-31 Logisitc equation fitting of % SBP elution *vs.* normalized Triton X-100 concentration.

[data taken from Figure 4-30]

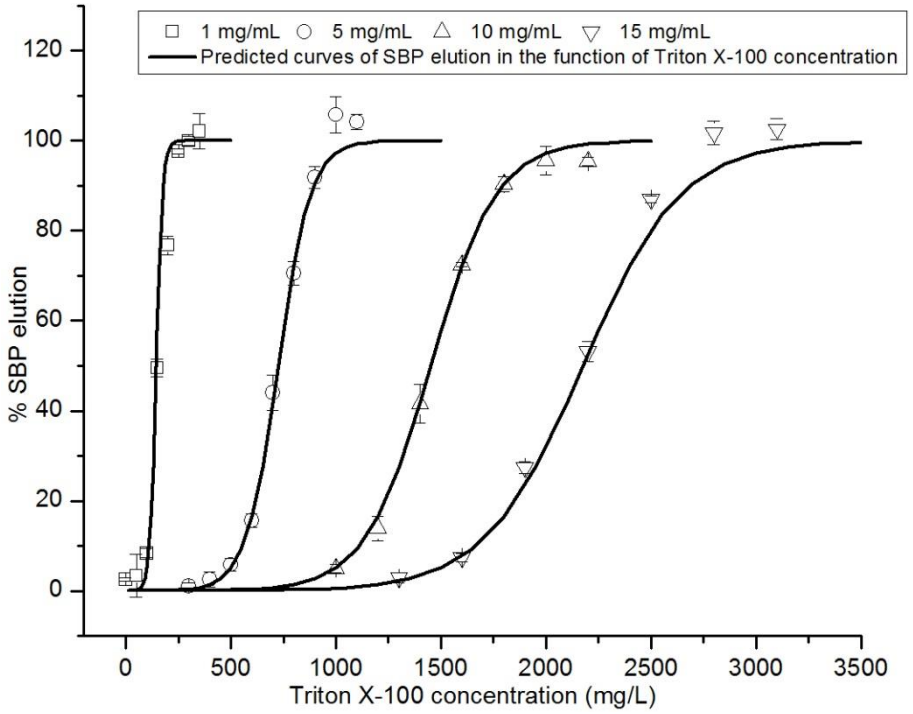


Figure 4-32 Comparison of the predicted elution curves with experimental data.

4.6.4 SBP Elution under Different Initial Adsorbed SBP and Different Triton X-100 Concentrations

According to the "orogenic displacement mechanism", the critical surface pressure, which causes the final collapse of the existing protein film, may vary due to the compressibility of the film (Mackie et al. 1999). The height of the protein film increases to compensate for the reduction in surface area while surfactant is continuously being adsorbed (Gunning et al. 1999). Different initial surface coverage by SBP could compromise the validation of the equation. Therefore, SBP elution was studied with different initial coverage (q_e/Q_{max} from 20 to 85%) at different Triton concentrations for elution. In Figure 4-33A, three different Triton concentrations were used to elute the adsorbed SBP from 5 mg/mL precipitates. The overall figure shows that elution is less sensitive to the change in initial surface coverage than to the increase in Triton concentration. The center point of each curve has the same qe/Q_{max} ratio (59%) as in Figure 4-30 and the observed % elution is very close to the predicted value. The change of surface coverage did not affect the elution significantly when 875 and 1000 mg/L of Triton were used, and therefore the predicted % elution, 87.8 and 97.3%, were quite accurate. But for 750 mg/L Triton, the optimum elution obtained at the center point, either with higher or lower surface coverage, showed lower efficiency than predicted. For elution that targeted higher completion, the effect of initial surface coverage is lower; hence, Equation 4-2 would be valid to calculate the required Triton concentration. The rationale is that when the surface pressure predominates, any size of the protein film on the surface would be "chased off" the surface. But when the surface pressure is insufficient, the collapse of the protein film may depend more on its deformability. This

could be the reason that the last 4 data points of 750 mg/L Triton gave the same SBP concentration in the aqueous phase regardless of the total SBP adsorbed on the precipitates in Figure 4-33 B. Extreme deformation can reduce surface area from 70% to 15% with an increase of the thickness from 1 to 5 nm (Mackie et al. 1999). Within the range of compressibility, the more adsorbed SBP, the higher the thickness of the protein film; thus, SBP would be only partially eluted from the surface. Due to the elevation of SBP activity in the presence of Triton, the elution under 1000 mg/L of Triton had all readings higher than 100%.

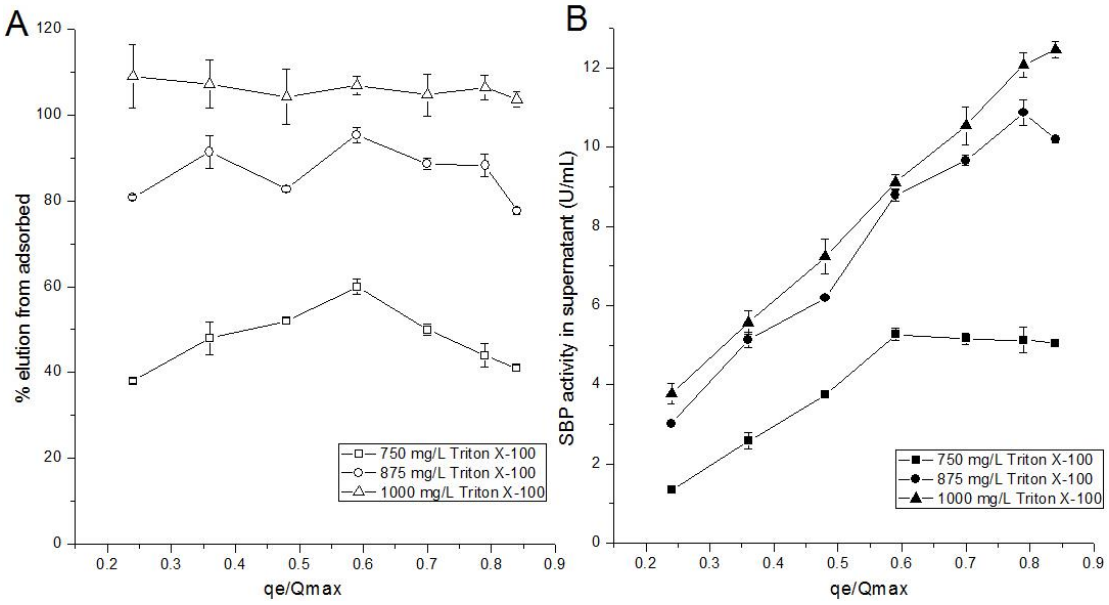


Figure 4-33 Effect of initial SBP coverage on elution by Triton X-100.
[Experimental conditions: initial SBP surface coverage from 24-84 %; Triton concentration 750, 875 and 1000 mg/L; elution time 30 min. A: % elution from adsorbed SBP; B: Actual SBP activity measured in the supernatant]

4.6.5 Time Course of SBP Elution with Different Triton X-100 Concentrations

Surfactant-induced competitive adsorption of protein has been observed to be a time-dependent process on graphite and at the air/water interface by AFM, the protein film thickness on the surface increases with time before the final stages of collapse (Gunning

et al. 1999). The above experiments had elution time fixed at 30 min to make sure equilibrium was reached, but the actual time required for desorption could be less than 30 min. The adsorption rate for Triton is dependent on the free Triton concentration. Therefore, a time course for SBP elution with different concentrations of Triton was conducted to track the elution process, and the results are shown in Figure 4-34. In this experiment, precipitates with pre-adsorbed SBP were quickly mixed with Triton in a syringe and filtered through a micro-filter. The first sample was taken after 5 sec of mixing. However, a progressively increased SBP concentration in the supernatant was not observed in the experiment; SBP desorption either reached equilibrium in less than 5 sec or spiked to a higher percentage then slowly reduced to lower percentage. With 1000 mg/L of Triton, the elution reached equilibrium almost immediately and there was no significant change in SBP concentration between 5 sec and 2 min. With 600 mg/L of Triton, the initial elution was over 70% and slowly dropped to 25% after 2 min of mixing; however, Equation 4-2 predicts % SBP elution to be 17% for 5 mg/mL precipitates and 600 mg/L of Triton; thus, it is believed that the elution would have eventually dropped to this range with a longer incubation time. The spike at the early stage could be due to insufficient mixing when Triton was added; the instantaneous high concentration could force a higher proportion of Triton to be adsorbed and then equilibrated back to the aqueous phase.

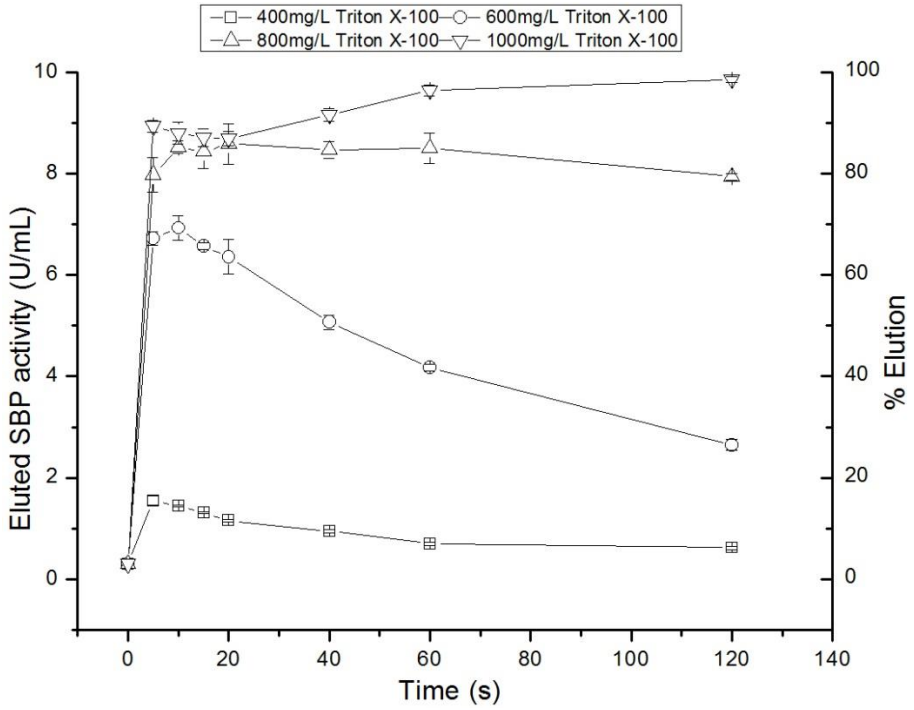


Figure 4-34 Effect of time on SBP elution with different Triton X-100 concentrations.

[Precipitates concentration 5 mg/mL, adsorbed SBP 2 U/mg of precipitates, Triton concentration 400, 600, 800, 1000 mg/L]

The interaction between Triton and SBP on a hydrophobic polymer surface is a competitive adsorption process that reverses SBP adsorption on phenolic precipitates. With pre-adsorbed SBP, the continuous adsorption of Triton builds a high surface pressure in the surfactant domain, which compresses the adsorbed protein film and eventually pushes it off the surface. The adsorption of Triton can be predominant regardless of the SBP concentration and the adsorption of SBP can be fully reversed. From the enzymatic reaction perspective, the complete prevention of SBP adsorption is an ideal condition. When SBP is kept in the aqueous phase with its intrinsic V_{max} and K_M values, the maximum catalytic efficiency is obtained. But, the ratio of 200 mg/L of Triton per mg/mL of precipitates that was needed to reach >95% elution of SBP (Figure 4-31) is not consistent with the Triton disappearance under turnover conditions. The optimized

reaction conditions for removal of 1 mM phenol is with 125 mg/L of Triton, which is equivalent to SBP elution at 0.094 mg/mL of phenolic precipitates with 125 mg/L of Triton. The Triton that disappeared was 2- to 3-fold higher than the maximum capacity expected from static incubations. This could be due to the higher available surface area at the oligomer-formation stage than its mature form, which is only the outside surface. When adsorption overlaps with polymer growth, the "onion" structure could be used to explain the adsorption. The inner surface layer forms first and adsorbs some Triton, which becomes buried inside the polymer. Therefore, the cumulative available surface area from the inner to outer layers could be significantly higher than if only the outer surface were considered. On the other hand, the competitive adsorption phenomenon is a general effect between any non-ionic surfactant and protein. The understanding of this interaction would be helpful to find a cheaper alternative surfactant to minimize the cost of the wastewater treatment.

Besides using Triton to protect SBP in enzymatic reaction, an understanding of the elution mechanism has provided a few alternative ways to utilize its advantage. As shown in Figure 4-35, phenolic precipitates, which have a high affinity and capacity to adsorb SBP, can be utilized as a carrier to adsorb SBP from a raw extract. To an SBP producer, precipitates with adsorbed SBP can be considered as a product for industrial wastewater treatment purposes. The demonstrated capacity from the above precipitates is 3.44 U/mg or 3.44 MU per kg of pure precipitates. By using these precipitates with any protective non-ionic surfactant, the adsorbed SBP can be quickly released from the precipitates under the reaction conditions and the enzyme efficiency is rejuvenated. Moreover, precipitates can also be used to concentrate a raw SBP extract instead of ultrafiltration or

chromatography. The adsorbed SBP can be eluted from precipitates with sufficient Triton added, thereby furnishing a concentrated SBP solution. With a proper regeneration method, a preparation of precipitates can recover its affinity and therefore, be reused in many cycles. The cost of membrane replacement and electricity for ultrafiltration, or packing material in a chromatography column for protein purification/concentrating is often high. Phenolic precipitates can be considered as a cost-free material to do the same job. The high affinity would result in a minimum SBP remaining in solution after the adsorption, and the reversibility with Triton can ensure a high overall yield of the process. The harvested SBP solution activity could be as high as a few hundred units per mL. Some Triton would remain in the SBP concentrate, but since Triton is beneficial for wastewater treatment anyway, it would not be harmful to have it in the enzyme solution. The next section of the study is to design and optimize the consecutive SBP concentrating process using phenolic precipitates.

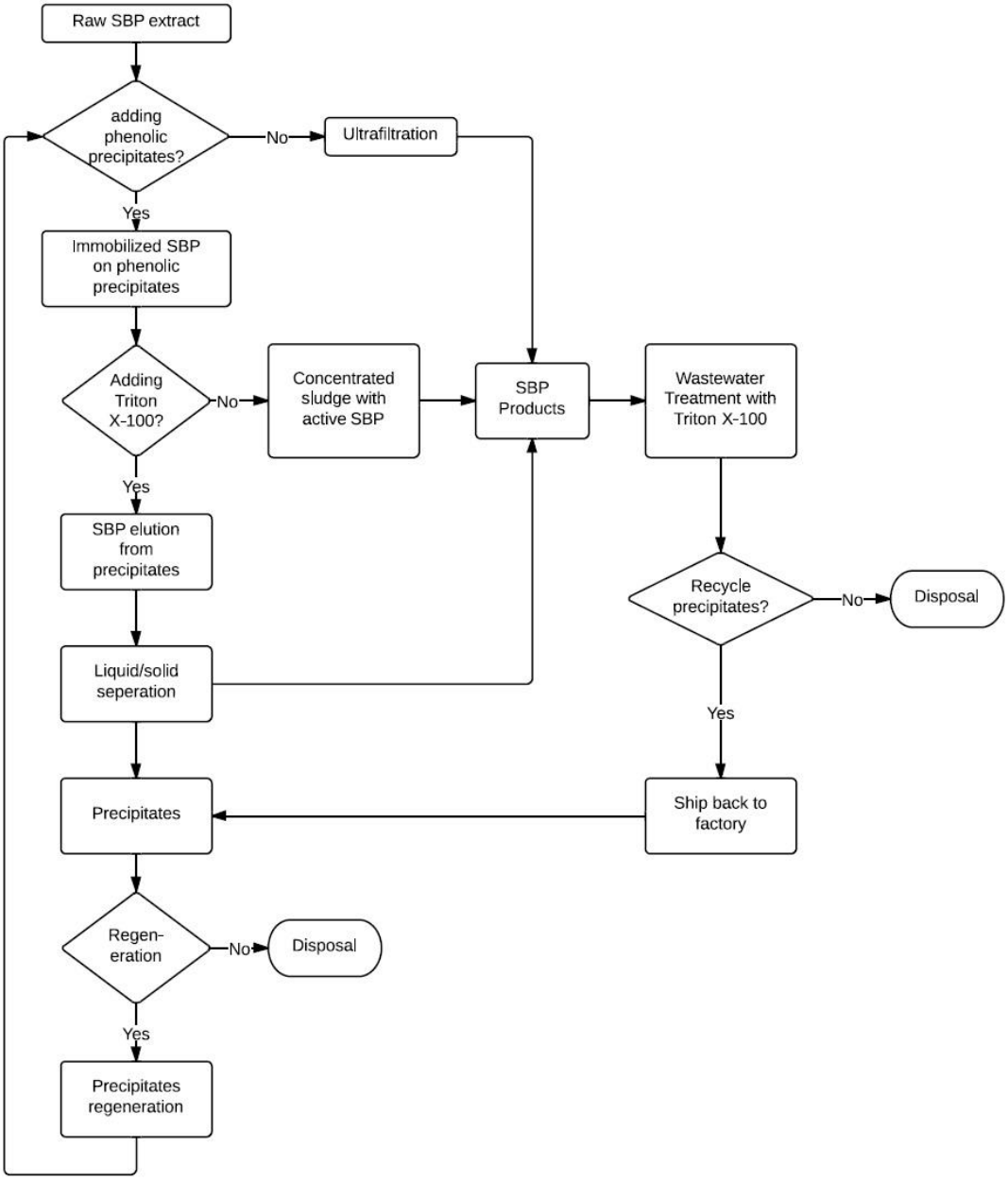


Figure 4-35 A potential flow-chart for commercial SBP production using phenolic precipitates.

4.7 Concentrating SBP using Phenolic Precipitates and Triton X-100

Crude SBP can be extracted from soybean hulls with water. The extract requires concentrating and/or purification to be further used. There are many commercialized technologies for protein purification but the overall cost is too high to be used to produce

industrial-scale SBP for wastewater treatment purposes. The major challenge is to concentrate the crude SBP extract from a low activity at the lowest price possible. Ultrafiltration and spray drying have been used to dewater enzyme extracts, however the capital cost could be significant, a cheaper SBP concentrating process would strongly improve its potential for commercialization on wastewater treatment (Hailu et al. 2010). Mixing precipitates with crude SBP extract would isolate nearly all SBP from the dilute extract. With gravity settling or centrifugation, the precipitates can be collected into a concentrated sludge form with a high concentration of active SBP. Adding Triton proportional to the weight of sludge would release all the adsorbed SBP. For batch operation, the concentrating factor is dependent on the volume difference between the SBP capturing and releasing stages. With a proper regeneration strategy, a sustainable process can be established, as shown in Figure 4-35. The change of adsorption affinity and capacity for SBP at each stage and each cycle are crucial information for the process, and will be studied separately in the next sections.

4.7.1 Change of K and Q_{max} for SBP of Phenolic Precipitates under Different Conditions

The observation of SBP elution by Triton is actually due to the alteration of phenolic precipitates' affinity for SBP when Triton is adsorbed. When the available adsorption sites are taken by Triton; it is more difficult to have SBP adsorbed even with a high bulk concentration. The Langmuir constant K of SBP adsorption for such 'modified' precipitates would be significantly lower than the original solids. Also, an ethanol wash was developed to remove the adsorbed Triton from precipitates' surface. By incubating

this modified precipitates with ethanol, the adsorbed Triton could be fully removed. Then, adsorption sites would be available again to SBP; the adsorption affinity should be regenerated as well. As mentioned in the literature review, the Langmuir isotherm could be useful to track such a change of Q_{max} and K on precipitates during the foregoing manipulations. To quickly determine the change of Q_{max} and K, the linear form of Langmuir equation was used here. Theoretically, competitive adsorption would have no effect on Q_{max} and give a decrease of K (increase of 1/K) for the primary adsorbate (SBP) compared with the single adsorption. A similar phenomenon of competitive adsorption is seen with competitive inhibition in the Michaelis-Menten enzyme kinetic model, characterized by two parameters: limiting velocity, V_{max} and half-saturation (or, Michaelis) constant, K_M: the inhibitor binds to the active site on the enzyme (with a half-saturation constant K_i) and prevents binding of the substrate; the inhibition can be overcome by increasing of the substrate concentration, and results in an elevated K_M. In Figure 4-36, precipitates with adsorbed Triton almost maintained the same Q_{max} as the original, but the affinity dropped by nearly 200-fold. In a binary adsorption system, a lower Q_{max} compared with single adsorption for both adsorbates are often observed and explained by the unknown interactions (Andriantsiferana et al. 2013, Mesquita 1998). The 200-fold affinity drop is significant, and can be used to explain why SBP is desorbed when sufficient Triton is present. Meanwhile, the change of slopes $1/Q_{max}$, between the original and regenerated precipitates suggested that there is a 17% drop of adsorption capacity during the cycle. The ideal regeneration would have affinity and capacity fully recovered. The lowered Q_{max} could be due to residual Triton on the precipitates. The effective concentration of ethanol and washing time on regeneration efficiency will be

studied later. Triton mass balance between elution and washing stages will be used to indicate completion of ethanol washing, assuming that, if all adsorbed Triton were washed off, the precipitates should fully re-gain the capacity and affinity. In fact, regenerated precipitates showed a greater affinity for SBP than the original, but the reason is not clear. The inverse of Langmuir adsorption constant (1/K, U/mL) is the half-saturation concentration of SBP at adsorption, which is an indication of the adsorption clearance. From the SBP production perspective, adsorption efficiency is a crucial factor, which determines the SBP activity remaining in solution after adsorption; a lower 1/K means less extracted SBP will be wasted.

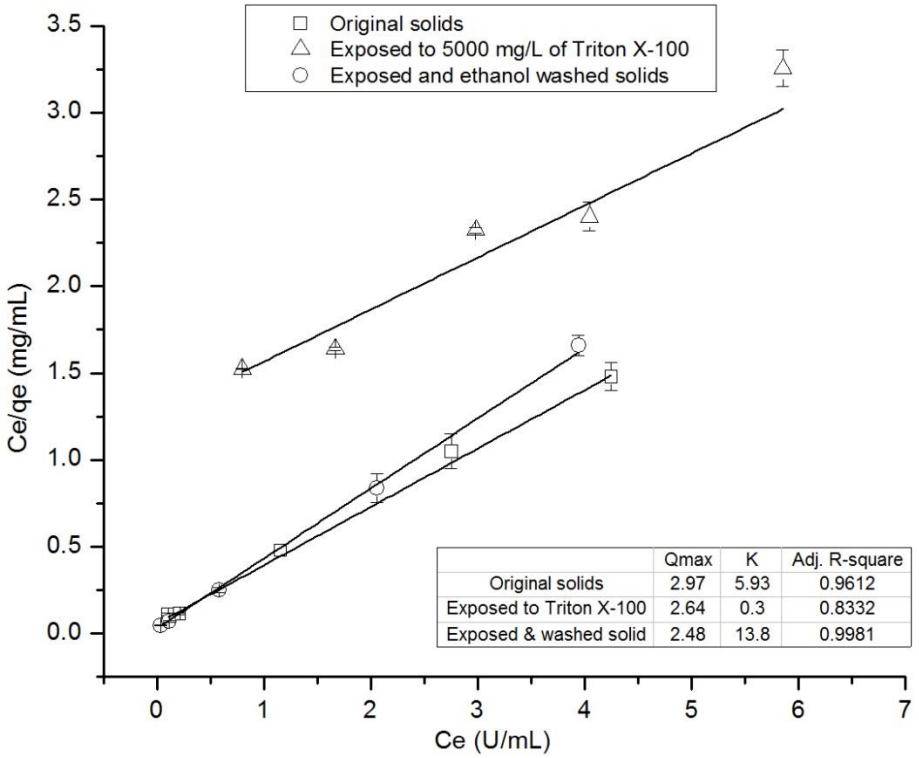


Figure 4-36 Linear Langmuir isotherm equation fitting of SBP binding to phenolic precipitates under different conditions.

[Experimental conditions: 16 mg/mL precipitates exposed to 5000 mg/L of Triton for 30 min then centrifuged, and water washed to remove the residual Triton; effective ethanol concentration at washing is 50% and 30 min washing time.]

In addition, washing phenolic precipitates with ethanol did not show any increase of SBP adsorption capacity, but Triton adsorption capacity was significantly increased. A dark brown colour was observed at the first wash of precipitates, where phenolic compound was detected in the wash by phenol colorimetric assay. Because the formation of phenolic precipitates does not involve any covalent binding, long-time washing with a solvent like ethanol could cause a small fraction of solids to be dissolved. But, this loss was not studied here. Another possible source of detected phenolic compound from ethanol wash is from the precipitates' surface, ethanol wash may re-dissolve some reaction intermediates adsorbed during the precipitates preparation conditions. An ethanol wash was also extended to precipitates formed in the presence of Triton. In reality, precipitates made in the absence of additive are not always available; generating such precipitates has an extra cost. A possible free source is to recycle the precipitates formed after the wastewater treatment as Figure 4-35 indicates. Producing precipitates in the presence of Triton reduces SBP cost by 8-fold, therefore it can also be considered as an alternative method. From Table 4-2, precipitates made in the presence of Triton have a high capacity to adsorb SBP but a low affinity as expected. The adsorbed Triton can be washed away during the regeneration, K value was increased by 17-fold.

Table 4-2 Effect of ethanol wash on SBP binding to phenolic precipitates made in the absence/presence of Triton X-100.

	Q_{max} (U/mg)	K (mL/U)	Adj. R-square
Original precipitates*	3.44 ± 0.08	4.91 ± 0.54	0.9691
Ethanol washed original precipitates	2.16 ± 0.09	10.42 ±2.03	0.9404
Precipitates made in the presence of Triton X-100	5.09 ± 0.19	0.23 ± 0.03	0.9572
Regenerated precipitates made in the presence of Triton X-100	3.25 ± 0.13	3.90 ± 0.66	0.9837

*results taken from Figure 4-27.

4.7.2 Mass Balance at the Adsorption, Elution and Washing stages

Early experiments have indicated that the adsorption of SBP only involves a specific activity change, there is no permanent denaturation of the protein upon adsorption; the loss of activity can be recovered with the addition of Triton. Low recovery rates have been reported in many peroxidase purification methods (Estela Da Silva and Teixeira Franco 2000, Miranda et al. 1995). One of the major advantages of the proposed process is that Triton elution could result in 100% desorption of the adsorbed SBP. Therefore, a SBP activity balance can be constructed from the adsorption to elution stages. The incomplete elution of SBP could cause adsorption sites to be blocked, since any remaining SBP may get denatured when exposed to high strength ethanol at washing, therefore compromising the efficiency of the next cycle. As shown in Figure 4-37, SBP activity balance was achieved in both cases. When precipitates (2 mg/mL) are overdosed with SBP, the extra enzyme remained in the solution after adsorption. The half of SBP that disappeared in the adsorption was fully available in the aqueous solution after the addition of Triton. When the SBP given is not enough to saturate the precipitates in the 4 mg/mL case, a minimal remaining activity in the solution was observed after adsorption. Likewise, the SBP that disappeared was all released at elution.

On the other hand, achieving mass balance of Triton is also critical to ensure the adsorption efficiency. The Triton that disappeared at the elution should all be recovered at the washing stage; an incomplete wash, with some Triton still remaining on precipitates would strongly compromise the affinity and capacity of precipitates to adsorb SBP in the next cycle; therefore, the adsorption efficiency over the cycles would not be maintained. All three cases in Figure 4-38 show complete regeneration of precipitates.

With 90% ethanol washing, all Triton that disappeared in the elution stage was recovered in the ethanol. The two material balance tests indicated a high recovery rate which is necessary for sustainability of the proposed concentrating process.

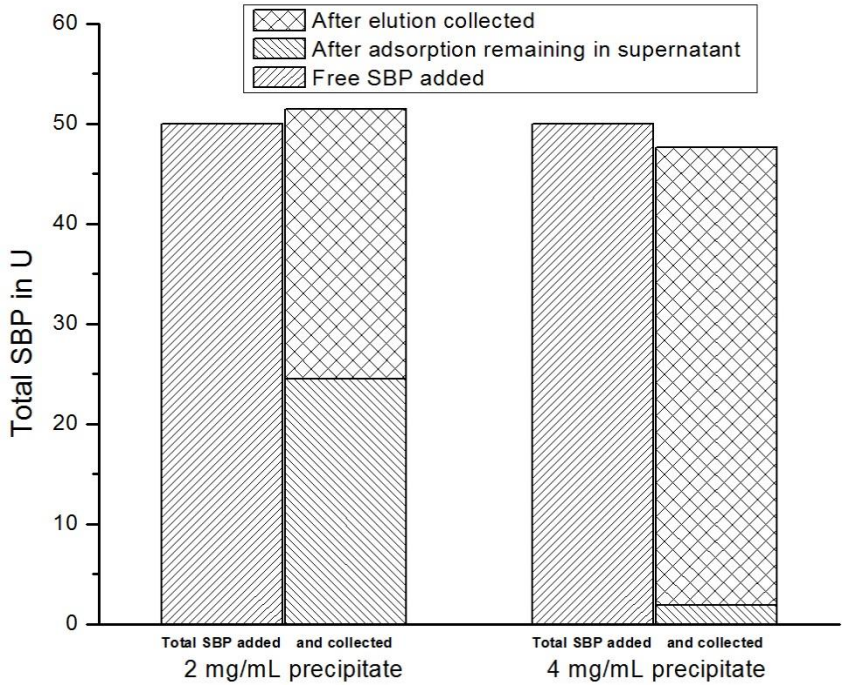


Figure 4-37 SBP activity balance at adsorption and elution stages.

[Experimental conditions: total added SBP was 10 U/mL in 5 mL; 1500 mg/L of Triton was used for elution; both adsorption and elution were 30 min.]

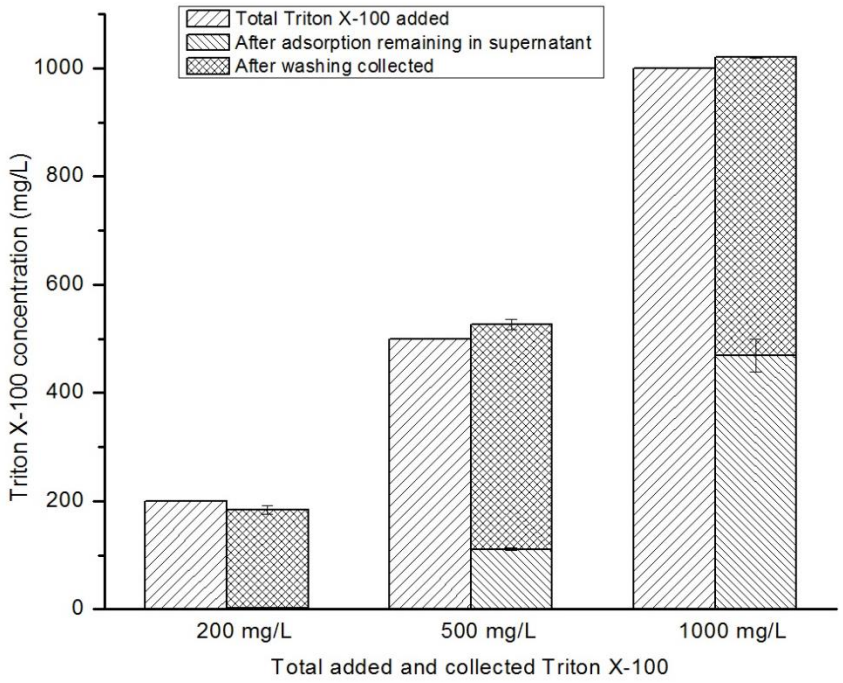


Figure 4-38 Triton X-100 mass balance at adsorption and ethanol wash stages.
[Experimental conditions: 1 mg/mL precipitates*, 90% ethanol at washing; total volume is 5 mL; *precipitates were pre-washed three times with ethanol]

4.7.3 Concentrating SBP Using Phenolic Precipitates

The idea of using phenolic precipitates to concentrate SBP came from two initiatives: 1) reducing the cost of SBP manufacturing to increase its economic feasibly; 2) taking advantage of the unusually high affinity of phenolic precipitates for SBP and the reversibility of adsorption in the presence of Triton. With no extraction, direct use of soybean hulls has been proven to effectively remove phenol and chlorophenols; however, it forms slurry which is difficult to mix, and causes disposal problem (Bassi et al. 2004). With extraction, the raw SBP extract is a clear solution containing about 6-8 U/mL activity after gravity settling (K.E. Taylor, unpublished data, 2012). A raw SBP extract could also be used directly if it was produced on the site of the wastewater treatment facility, otherwise the cost of transportation would be high due to the low the activity and

the large volume required. Ultrafiltration can be used to concentrate protein raw extract; it's a method with high yield and low denaturation of the protein. A recently proposed two-stage ultrafiltration process has the recovery of HRP from the feedstock close to 90 % (Liu et al. 2012). Our current crude SBP extract was concentrated at the Guelph Food Technology Centre (Guelph, ON) to 15-25% of the original volume by ultrafiltration(Steevensz et al. 2013b). As for any packing material to be used in a chromatography column for protein purification, the precipitates preparation developed here has a strong affinity to adsorb the target compound, SBP, and isolate it from the seed hull extraction mixture. The cost of generating or replacing precipitates is very low since it is the reaction product mixture from enzymatic phenol removal, considered as a waste. For a batch process, the proposed operation cycle has 5 stages, shown in Figure 4-35. In stage one, precipitates are added to a SBP extract for 15 min adsorption. To approach the maximum adsorption efficiency, a low fraction of q_e/Q_{max} (e.g., 50-60%) should be selected. After adsorption, the precipitates can settle by gravity then be collected from the bottom of the reactor. The water content in the sludge can be reduced to 60-70% by applying a dewatering step. At the elution stage, SBP desorption is achieved by injecting a specific competitive adsorbate, Triton in this case, to elute the previously adsorbed SBP. If 100% elution is targeted, sufficient Triton should be given to reach the critical surface pressure, hence to break down the protein film on the surface. The required time for elution is less than 5 min. The concentrating factor can be calculated by the volume ratio between the adsorption from the extract and elution by the injected Triton. The lower the water content in the sludge, the higher the concentrated SBP activity will be. However, the factor may also be affected by the rules of adsorption, a higher SBP concentration in

the aqueous phase provides a higher adsorption pressure, hence desorption could be limited. For precipitates regeneration, high strength aqueous ethanol can be added to wash all adsorbed Triton off, followed by settling, washing with water to remove all the ethanol, and dewatering. Then, the regenerated precipitates can be used for the next cycle. A Triton recovery step might be incorporated to purify the waste ethanol solution so than ethanol can be reused; but this part of the process is not included in the current study. All experiments for concentrating SBP were conducted in a batch reactor. In Table 4-3, two single-cycle SBP concentrations were performed first to test the hypothesis without regeneration.

Table 4-3 One-time SBP concentrating using phenolic precipitates

	Trial 1	Trial 2
Initial SBP Activity (U/mL)	7.5	7.5
Initial Volume (mL)	50	50
Adsorption Efficiency (%)	96.4	95.2
SBP Concentrate Activity (U/mL) *	65.4	207
SBP Concentrate Volume (mL)	5	1.1
Recovery (%) **	87.2	60.7
Concentrating Factor	8.7	27.6

*Measured SBP activity, without correction of average Triton influence on SBP activity
** Calculated from the un-corrected SBP activity
[Experimental conditions: trial 1, 180 mg precipitates, 3.5 mL of 20 g/L of Triton; trial 2, 180 mg of precipitates, 1.5 mL of 60 g/L of Triton]

In trials 1 and 2, the SBP given for adsorption was 60% of the precipitates' capacity, the remaining SBP activities after adsorption were less than 0.3 U/mL. Dilute SBP was successfully concentrated in both but at different levels depending on the volume of Triton added. The recovery rates were calculated by multiplying the collected volume with its activity. Due to a fraction of uncollectable liquid in the sludge, both of the recovery rates were not as high as expected. In batch reactor operation, a high recovery

rate and a concentrating factor cannot be obtained together; but it would be possible to achieve both on a continuous-flow column operation.

For testing the performance of consecutive SBP-concentrating, precipitates–regeneration cycles, a moderate concentrating factor 7.5 was chosen. In the 5 cycles of concentrating, both SBP and Triton concentrations were tracked at each stage, as shown in Table 4-4. Extra Triton was given to ensure the elution efficiency. From the initial SBP concentration of 8 U/mL, an average concentrating factor of 6.88 was achieved. The elution efficiencies were maintained over 90% except in the first cycle, and the overall yield is 92%. In addition, Triton washing efficiencies were also maintained over 90%, except in the first cycle, which suggested that nearly no Triton remained on the surface at the beginning of the next cycle. A small fraction of remaining Triton could be the reason that the SBP adsorption efficiency reduced slightly over the cycles.

Table 4-4 Consecutive SBP concentrating using phenolic precipitates

SBP Concentration*				
Cycles	After Adsorption (U/mL)	Adsorption Efficiency (%)	After Elution (U/mL)	Elution Efficiency (%)
1	0.1	98.9	44.5	73.3
2	0.2	98.3	57.4	93.9
3	0.3	97.0	58.6	94.8
4	0.2	97.6	57.0	92.7
5	0.4	95.9	57.8	100.4
Triton X-100 Concentration				
Cycles	After Elution in SBP (mg/L)	Adsorption Efficiency (%)	After Washing in ethanol (mg/L)	Washing Efficiency (%)
1	3437	80.7	2032	78.1
2	3935	77.9	2354	93.7
3	4742	73.3	2267	95.8
4	4641	73.9	2261	94.8
5	5406	69.6	2192	97.7

*Measured SBP activity, without correction of average Triton influence on SBP activity if involved.

[Experimental condition: 72 mg precipitates, 14 mL of 8 U/mL SBP for adsorption, 2 mL of 20 g/L Triton X-100 for elution, 15 mL of 95% ethanol for precipitates regeneration]

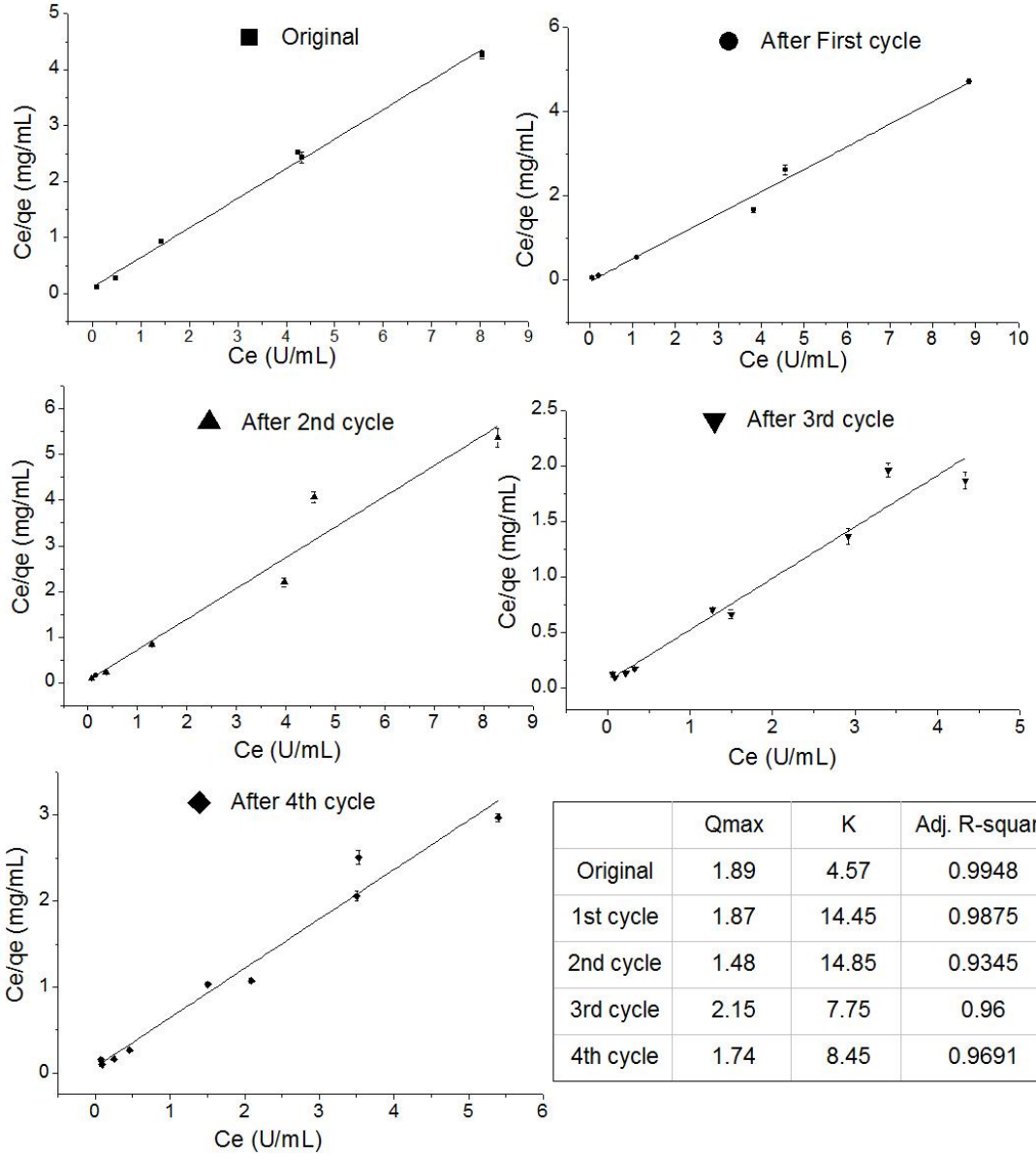
The concentrated SBP is not a purified product; in fact, it's a solution with a high concentration of Triton. The presence of Triton may limit the future use of this SBP concentrate unless it can be removed. However, the presence of Triton could be an advantage for industrial wastewater treatment. It is known that Triton is, so far, the most efficient additive to enhance phenol removal with SBP. Therefore, it is expected that Triton will be used with SBP for wastewater treatment. In addition, a reported optimum SBP-Triton system to remove phenol has two empirical relationships: a) to have 95% phenol removal in 3 h reaction, roughly 70 mg/L of Triton is required per mM phenol; b) the required SBP concentration under the above conditions follow an equation SBP(U/mL) = 0.0499 * phenol concentration (mM) + 0.1047 (Steevensz et al. 2013b). Hence, the Triton present in the concentrate is not overdosed if that SBP is used for the reaction; extra Triton is needed to reach the optimum reaction condition.

On the other hand, the proposed process gives no improvement in the RZ value from the above test. The presence of Triton significantly interfered with the measurements. After correcting the absorbance of Triton at 275nm, the concentrated SBP was shown to have RZ equal to 0.44 ± 0.02, while the un-concentrated SBP has a RZ value equal to 0.66 ± 0.05.

4.7.4 Change of K and Q_{max} of Phenolic Precipitates in Concentrating Cycles

The change of the Langmuir parameters K and Q_{max} for precipitates to adsorb SBP over the cycles are the quantitative criteria that indicate the performance of the concentrating process. A reduction of K and/or Q_{max} would result in an increase of remaining activity during SBP adsorption over the cycles; also it would be an indication of incomplete regeneration. To mimic the precipitates' surface condition at the beginning of each cycle,

a sample of precipitates was fully exposed to Triton then regenerated by ethanol wash, and this was repeated 1-4 times according to the cycle number represented, and then a Langmuir isotherm test was conducted for each to determine the Q_{max} and K. The linear form of the equation was used here again. As shown in Figure 4-39, the average Q_{max} is 1.83 ± 0.24 U/mg, no clear indication of a reducing trend. Each of the K values was higher than the original K, which indicates that the regeneration was effective. The observations here were consistent with the previous results (Figure 4-36). In fact, for any K higher than 5, the remaining activity after adsorption would be minimal. Continuous monitoring is a useful strategy to track the changes on the precipitates' surface; it provides information as to whether the precipitates are exhausted and require replacement.



	Qmax	K	Adj. R-square
Original	1.89	4.57	0.9948
1st cycle	1.87	14.45	0.9875
2nd cycle	1.48	14.85	0.9345
3rd cycle	2.15	7.75	0.96
4th cycle	1.74	8.45	0.9691

Figure 4-39 Monitoring Qmax and K change of precipitates at each cycle.
[Experimental conditions: 1-3 mg/mL precipitates concentration, 1-16 U/mL SBP concentration, incubation time 30 min.]

4.7.5 Optimization of Precipitates Regeneration by Ethanol Washing

Triton is soluble in many organic solvents, such as benzene, toluene, ethanol and ethylene glycol. Ethanol was selected to dissolve the adsorbed Triton from phenolic precipitates because it is lower priced, water miscible, non-toxic, widely applied in industry and

readily accessible. The above tests which involved precipitates regeneration all had a roughly effective ethanol concentration of 85-90%. To ensure the efficiency of regeneration, the ethanol concentrations at washing were kept as high as possible with a fixed time of 15 min. Optimization of ethanol concentration and washing time might help to reduce the cost of ethanol and accelerate the process. For optimization, precipitates with a known amount of adsorbed Triton were used. By measuring the Triton concentration in ethanol after washing, % of Triton remaining on the precipitates can be calculated. In Figure 4-40, the optimized ethanol concentration to have >95% Triton washed off is between 60 and 70%. In batch reactor operation, ethanol concentration over 70% at washing can be considered less beneficial; but for a column operation with a continuous flow, ethanol concentration at 95% would completely remove Triton from precipitates at contact.

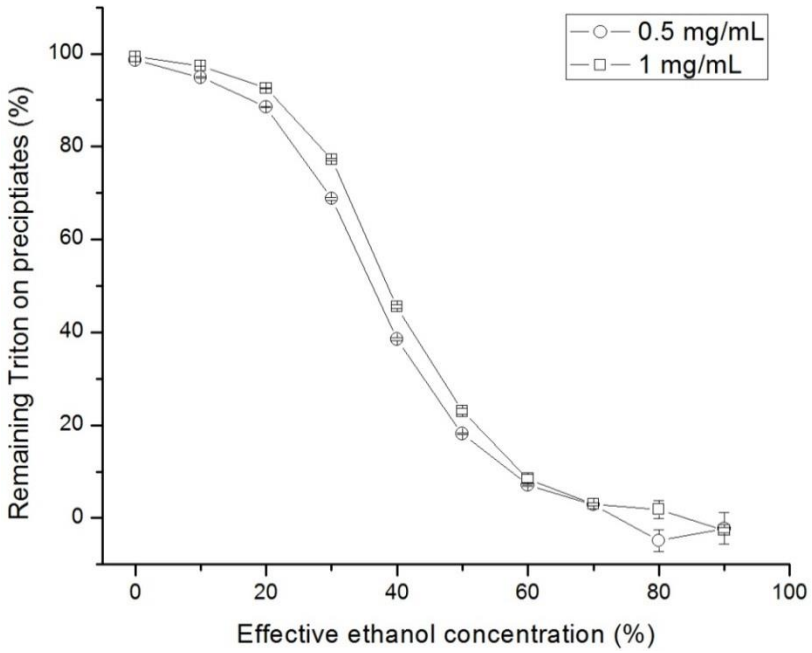


Figure 4-40 Optimization of precipitates regeneration by ethanol washing.
[Effective ethanol concentration: 0-90%, precipitates concentration: 0.5 and 1 mg/mL; mixing time 30 min]

The required contact time to reach equilibrium at washing was also studied, Figure 4-41. Precipitates were mixed with 70% ethanol in a syringe, then quickly filtered through a micro-filter. The measured Triton concentration in the filtrates suggested that the maximum washing was reached in less than 20 seconds; no further improvement on washing efficiency was seen when mixing was extended to 2 min. It is surprising that the precipitates sample reaches equilibrium very fast for SBP adsorption (Figure 4-2), SBP elution (Figure 4-34) and ethanol washing, which gives a time advantage to apply the process in a column operation. With a shorter required contact time, a higher volume of raw SBP extract can be processed.

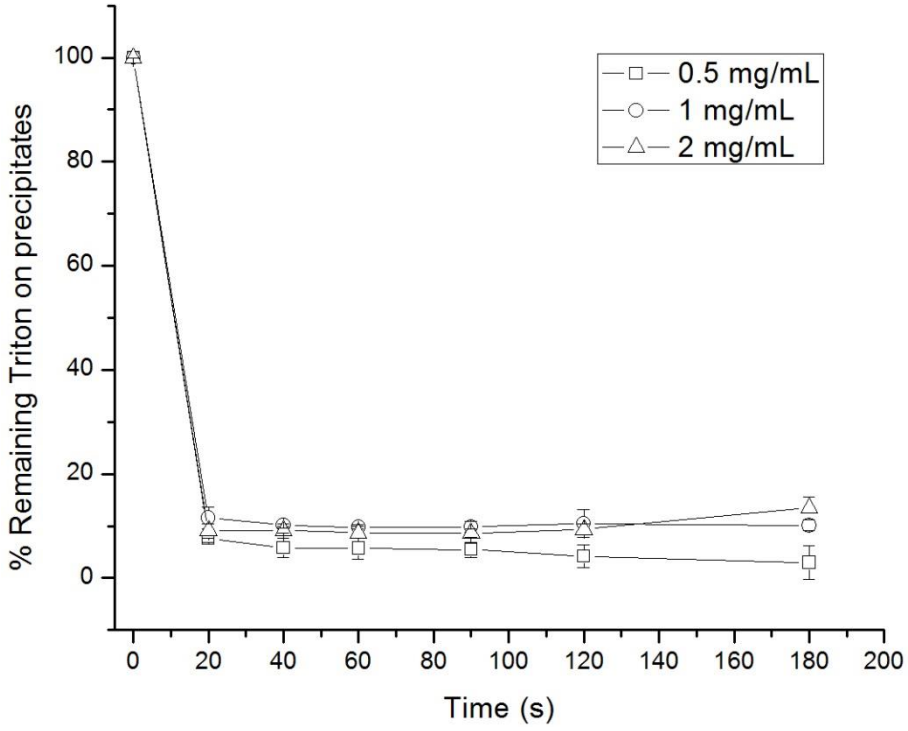


Figure 4-41 Effect of time on precipitates regeneration by ethanol washing.
[Effective ethanol concentration 70%; precipitates concentration 0.5-2 mg/mL]

In summary of section 4.7, a raw SBP extract concentrating process using phenolic precipitates and Triton has been proposed and proven on single-batch and consecutive cycles operation. Design criteria for the process include:

1. A low fraction of q_e/Q_{max} at SBP adsorption stage to ensure a high clearance rate;
2. The required Triton concentration at elution can be pre-determined with a mathematical function, Equation 4-2;
3. Ethanol concentration at washing should be higher than 60% to ensure precipitates regeneration efficiency;
4. The Langmuir isotherm can be used to quantitatively monitor the performance of the precipitates;
5. The required adsorption, elution and regeneration times are each less than 2 min.

Future work of this concentrating process should look for alternative substrates, non-ionic surfactants and solvents to further reduce the cost. This includes a determination of the specific adsorption area on phenolic precipitates and a comparison with other substrates, efficiency of SBP elution by Triton compared with other surfactants in terms of price and required concentration and a lower cost solvent for regeneration. Operation with expanded-bed adsorption requires an enhancement of precipitates settling rate, which could be solved by coating precipitates on a certain carrier. The precipitates used in the study are too fine to settle efficiently without centrifugation. The key design factor of expanded-bed adsorption would be to find a suitable inflow rate to transport the hull debris to overflow without washing away the adsorption material while still maintaining a sufficient time for adsorption.

4.8 Dynamic Adsorption of SBP by Phenolic Precipitates

As an alternative to static adsorption and elution to concentrate SBP, SBP adsorption under turnover conditions, called dynamic adsorption, was also tested because of the extremely high adsorption capacity and simple operation. The optimized 1 mM phenol removal requires 1.2 U/mL SBP in the absence of additive. Thus the process appears to consume 12.8 U of SBP per mg of phenol removal; and SBP activity in the supernatant drops to minimal after a few minutes of the reaction, which means all 12.8 U of SBP has been adsorbed on precipitates. In fact, the capacity of static adsorption is in addition to the SBP which has been adsorbed on the precipitates during the turnover conditions. As mentioned in section 4.6.5, Triton adsorption capacity was observed differently under turnover and static conditions. It was postulated that precipitates may have an "onion" structure, where the surface of inner layers is only available for dynamic adsorption during the formation stage, while the static adsorption capacity confined to the outside layer only, a much smaller area. Hence, by simply adding a small amount of phenol and H_2O_2 to an SBP extract, the maximum SBP capture could be reached during the formation of precipitates.

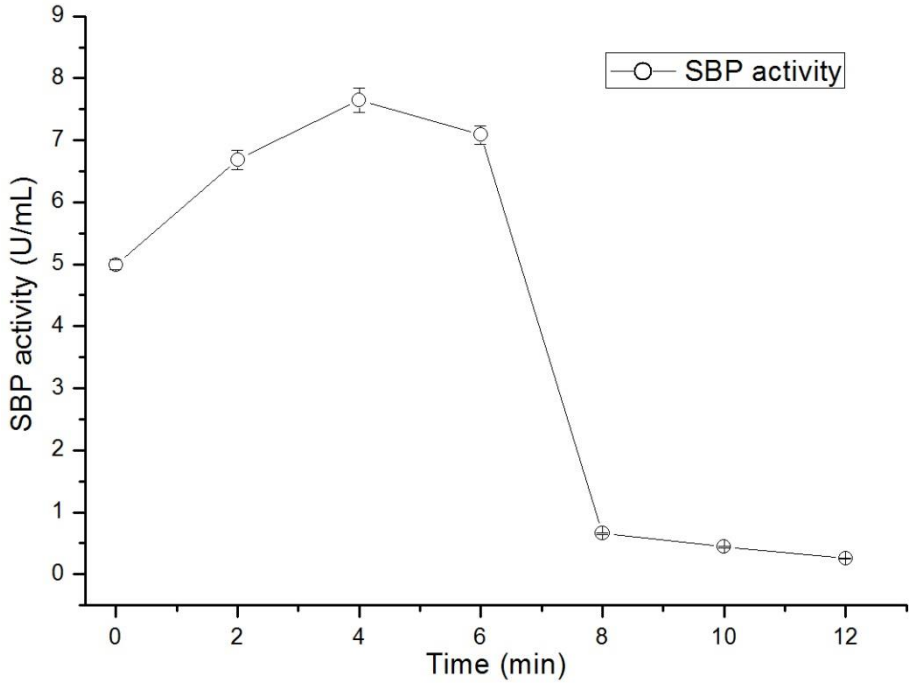


Figure 4-42 SBP activity change at dynamic adsorption.

[Experiment condition: SBP 5 U/mL in 200 mL, 6 additions of phenol & H_2O_2 mixture at 2 min intervals from time zero, 5 mg of phenol per shot; no phenol remaining at 12 min]

As shown in Figure 4-42, the addition of a phenol and H_2O_2 mixture to a SBP stock quickly had all free SBP adsorbed. With the total of 30 mg phenol added, nearly 1000 U of SBP was adsorbed on precipitates, which shows a capacity of 33 U per mg of phenol conversion. The SBP stock first became cloudy with an elevated activity in the first 4 shots, and then precipitates were observed with a significant drop of supernatant SBP activity. Compared with static adsorption, the dynamic adsorption had a 10-fold higher capacity; it is convenient to capture SBP from a stock solution, and forms precipitates with high concentration of active SBP. However, part of the SBP in the stock is irreversibly inactivated due to the conversion of phenol (free radical attack and H_2O_2 inactivation), and the inner layers might not accessible by Triton for elution; the overall SBP recovery rate is therefore lower than static concentrating. Precipitates formed in

above test were collected to examine the SBP recovery rate. With sufficient time and Triton given to reverse the adsorption, the recovery rate was around 50% of the initial SBP (data not shown). To minimize the irreversibly inactivated SBP, a substrate with lower enzyme demand, such as *o*- or *p*- cresol might be used to perform the same experiment. Future optimization of dynamic adsorption can also look for an optimum ratio of a given substrate mass to U of SBP to be adsorbed; with less substrate given for conversion, more SBP can be recovered.

4.9 Competitive Adsorption between SBP and Triton X-100 on Phenolic Precipitates

Studying of single-component adsorption of SBP and Triton has given crucial evidence to explain SBP adsorption on phenolic precipitates and the Triton protection mechanism. Langmuir isotherms were successfully used to fit and explain the adsorption behaviour. In a binary system, the presence of a second component is often expected to "inhibit" the adsorption of the first component when they compete for the same adsorption site. Such information can only be obtained in a binary system with both adsorbates' concentrations tracked. Figure 4-43 shows the SBP and Triton adsorption isotherms in a binary system. Firstly, SBP adsorption did not approach its maximum with increasing concentration in the aqueous phase; it was reversed, as expected, by the adsorption of Triton. SBP adsorption gradually approaches 0% with the increasing adsorption of Triton. Secondly, the adsorption of Triton seems unaffected by the presence of SBP; the adsorption curve shows a trend as the Langmuir adsorption model (see below). Thirdly, both SBP and

Triton were independently adsorbed at the first 5 data points, where the adsorbed SBP concentration quickly increased to 2.25 U/mg, then was "inhibited" and reversed.

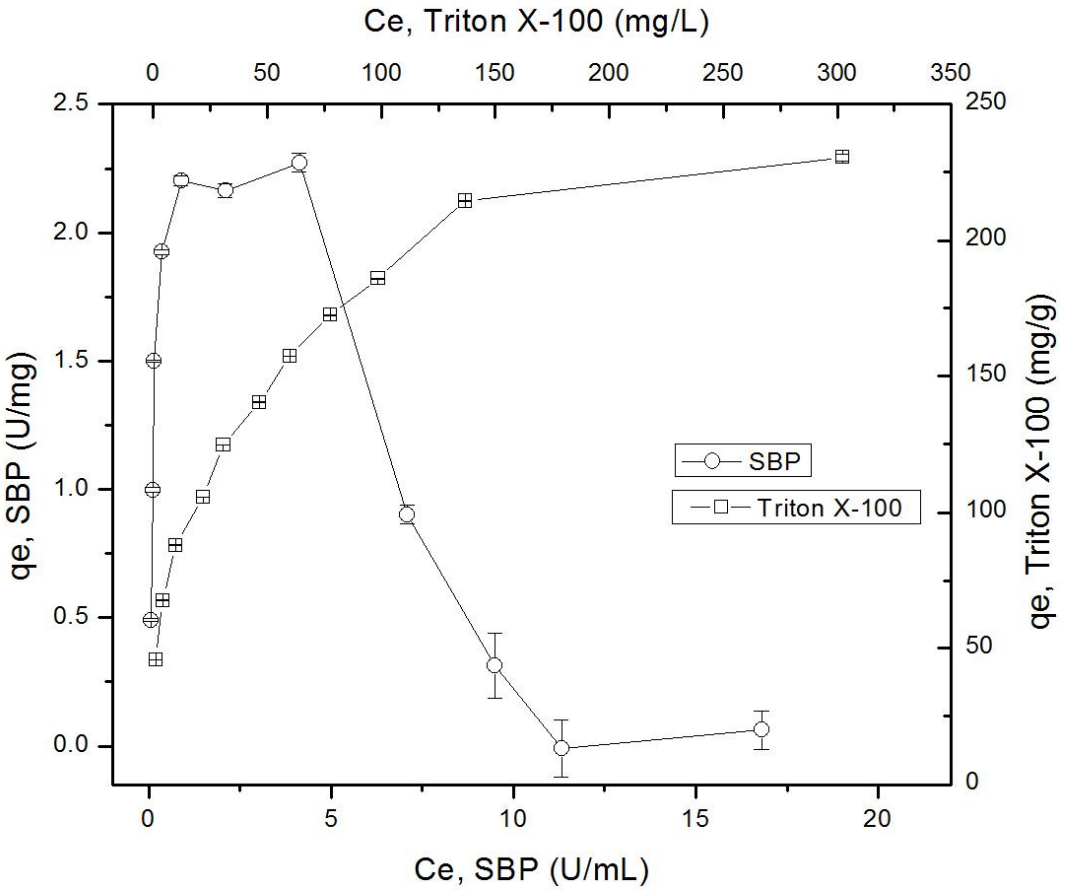


Figure 4-43 Competitive adsorption of SBP and Triton X-100 on phenolic precipitates.

[Precipitates concentration: 2 mg/mL, SBP concentration: 1- 17 U/mL; Triton concentration: 100- 800 mg/L]

To understand more of the competition, the adsorbed SBP and Triton in each scenario was converted to a theoretical surface coverage assuming the adsorption capacity was maintained as in the respective single component adsorption. In Figure 4-44, the combined surface coverage reached 100% at the fourth data point and went over 100% from the fifth to the seventh, which matches the observation that protein film on the polymer surface is compressed by the increasing surface pressure before final collapse

(Mackie et al. 1999). With the increasing adsorption of SBP and surface pressure, the protein film keeps increasing its thickness to compensate for the reduction of surface area; hence the theoretical combined surface coverage can go over 100%. After the collapse of the SBP protein film, the adsorption of Triton chased all remaining SBP off and gradually occupied 90% of the surface area.

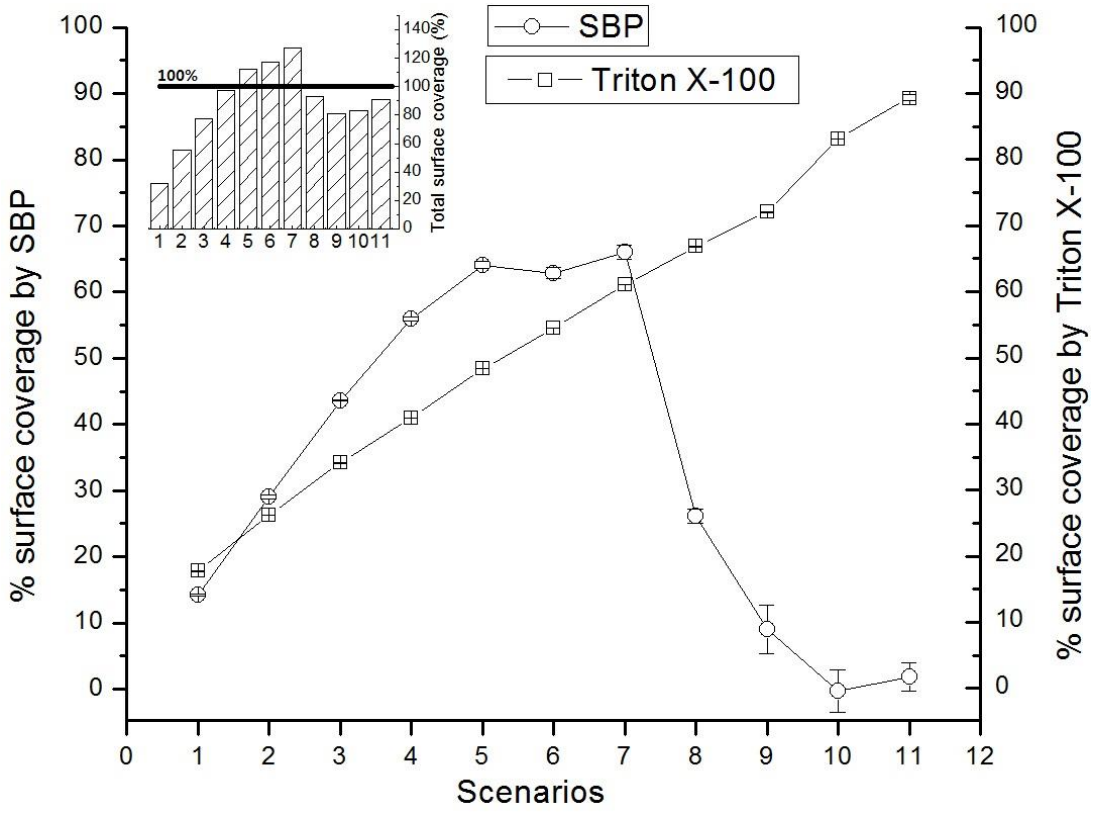


Figure 4-44 Theoretical surface coverage by SBP and Triton at adsorption.
[data taken from Figure 4-43, each scenario represents a mixture of SBP and Triton]

The Langmuir isotherm equation for the binary system can be rearranged to:

$$\frac{C_{e,i}}{q_{e,i}} = \frac{1}{Q_{max,i}K_i} + \frac{1}{Q_{max,i}}C_{e,i} + \frac{1}{Q_{max,i}}\left(\frac{K_j}{K_i}\right)C_{e,j} \qquad \text{Equation 4-3}$$

which can be solved by multiple linear regression in the form of $y = a + bx_1 + cx_2$ (Mesquita 1998). By inputting the data of C_e and q_e for both adsorbates from Figure 4-43, the equation can be solved separately for SBP and Triton, and then the obtained

adsorption affinity and capacity can be compared with the single component adsorption isotherm. The results are shown in Table 4-5.

Table 4-5 Adsorption parameters from single and competitive Langmuir isotherm

	SBP		Triton X-100	
	Single	Competitive	Single	Competitive
Q_{max} (U/mg)/ (mg/g)	3.44 ± 0.08	-0.16	258 ± 13	263.1
K_{SBP} (mL/U)	4.91 ± 0.54	0.26	n/a	0.045
K_{Triton} (L/mg)	n/a	-0.053	0.029 ± 0.006	0.04
R-square	0.9691	0.9245	0.9421	0.9844

n/a; not applicable

Due to the reversal of SBP, its adsorption curve of SBP did not follow a typical pattern of Langmuir model; the mathematically solved parameters showed two negative values, which are SBP adsorption capacity and Triton adsorption affinity. But, the data from Triton adsorption side gave a much more reasonable result compared with the single component adsorption. The adsorption capacity of Triton did not change in the binary system with a slight increase of adsorption affinity. The calculated SBP adsorption affinity in the presence Triton matches the result in Figure 4-36, where precipitates were exposed to Triton before conducting SBP adsorption isotherm. Future work could use this method to pre-determine the affinity of precipitates in the presence of an untested non-ionic surfactant; the lower the SBP adsorption affinity, the better the protective effect can be expected.

4.10 Summary of Results

In this project, SBP adsorption/desorption on phenolic precipitates in the presence/absence of additive under turnover and static conditions were extensively

studied. Two process models to utilize precipitates to enhance enzymatic wastewater treatment were proposed and proven.

4.1 SBP adsorption on phenolic precipitates under turnover and static conditions were studied. In both conditions, SBP was rapidly adsorbed by precipitates and resulted in a minimal activity remaining in the solution. The apparent activity decrease during the adsorption is suspected to be an adsorption-induced specific activity change rather than inactivation by reaction products.

4.2 Phenol removal by immobilized SBP on precipitates was proven to be feasible. The active SBP on precipitates can be used as a type of immobilized SBP alone or with free SBP together to catalyze phenol removal reaction. Also, it appeared that immobilized SBP is more efficient to remove phenol than free SBP.

4.3 By using 500 mg/L of Triton to incubate with precipitates with remaining active SBP, the majority of the adsorbed SBP was desorbed to the solution. The mixture activity increased 5.9-fold as a result of the 30 min incubation. A similar experiment that tracked the mixture activity showed the mixture activity firstly decreased and then slowly recovered after the addition of Triton. The new evidence of SBP and phenolic precipitates interactions suggests that the adsorption of SBP does not inactivate the enzyme in substantial proportion; rather, the enzyme is immobilized in an active form, the change in specific activity of SBP presumably due to alteration in K_m and/or V_{max}. Thus it appears to be SBP being inactivated. In addition, the evidence provides the theoretical support to recycle the precipitates of enzyme-catalyzed phenol

polymerization reaction. By utilizing the active enzyme on precipitates, the required SBP concentration for the subsequent reaction can be reduced.

4.4 The feasibility of precipitate recycling as a consecutive batch reaction strategy for phenol removal was first determined, then SBP concentration for 1 mM phenol removal with quantitatively recycled precipitates was optimized.

- Precipitates formed under the optimized condition to remove 1, 2 and 3 mM phenol have remaining apparent activity 20% of that initially added, and inherent activities of 50%, 71% and 78% of that initially added, respectively. By recycling the precipitates in a subsequent batch reaction with the same reaction conditions without SBP; more than 60% of the new phenol was removed in all scenarios.
- Consecutive precipitates recycling with no SBP addition was tested. The result shows that active SBP on precipitates was consumed step by step until it all used up over the cycles.
- With quantitatively recycled precipitates, the optimized SBP concentration for the subsequent batch reactions to remove 1 mM phenol is 0.5 U/mL.

4.5 Precipitates recycling for phenol removal in the presence of the additive was studied. Triton's effect on free and immobilized SBP activity was first determined. Then PEG's and SDS's protection mechanisms were studied separately for comparison.

- PEG does not have a significant effect on free SBP activity up to 2000 mg/L. At pH 6.5, nearly 10% activity loss was observed with 500 mg/L of SDS, and

17.5 % loss with 1500 mg/L of SDS. Triton increases free SBP activity by 6 ± 3 %, on average, between 100 – 5000 mg/L.

- PEG shares a similar elution effect as Triton; immobilized SBP activity increased 30% after 30 min incubation with 200-1000 mg/L of PEG. The immobilized SBP seems to be very sensitive to the presence of SDS, more than 10% activity loss was observed with higher than 300 mg/L of SDS, which further increased to 18% with 1000 mg/L of SDS. Maximum Triton elution effect was obtained at 500 mg/L with mixture activity increased 4.7-fold.
- PEG does not completely prevent crude SBP adsorption by phenolic precipitates under static incubation and turnover conditions. The presence of PEG does affect the partitioning of SBP to the two phases; with a higher PEG concentration, a higher supernatant SBP activity is observed under turnover conditions. Therefore, PEG is suggested to be acting as a competitive adsorbate with a higher adsorption affinity for the precipitates, thereby protecting SBP from being adsorbed.
- Under phenol turnover conditions with SDS, the mixture activity was increased nearly 3-fold above that initially added (0.3 U/mL), and then gradually dropped to 0.3 U/mL after 90 min of reaction. It is speculated that SDS forms micelles in the phosphate buffered solution, the oligomeric end-products were kept soluble in the micelles and oxidized by the enzyme so that a hyperactivity of SBP was observed. The solubilized products protect SBP being adsorbed. In addition, SDS can be adsorbed and removed at about 133

mg of SDS per g of precipitates. By reducing SDS concentration to a sub-optimum condition, a higher SDS removal and better precipitation can be achieved.

- The recycled precipitates formed in the presence of PEG gave about 55% phenol removal after 20 h reaction. Recycling precipitates formed in the presence of Triton did not give any phenol removal in 20 h reaction. The best result is from the SDS case, the reaction almost reached 95% removal of phenol after 20 h. However, precipitates formed in the presence of additives require a longer time for settling.

4.6 Quantitative characterization of SBP adsorption, Triton adsorption and SBP elution from phenolic precipitates were studied.

- SBP adsorption on phenolic precipitates follows a Langmuir adsorption isotherm, the monolayer adsorption capacity and Langmuir constant estimated to be 3.44 U/mg and 4.91 mL/U, respectively.
- Triton adsorption on phenolic precipitates also follows Langmuir adsorption isotherm, the monolayer adsorption capacity is roughly 1 mg of Triton per 4 mg of precipitates.
- SBP elution from precipitates is due to competitive adsorption of Triton. The elution curve shows an S-shape with a distinct breakthrough stage. The S-curve behaviour of adsorption displacement has been explained by an ‘orogenic displacement mechanism’. The adsorption of surfactant increases the surface interfacial pressure and compresses the protein film. Beyond a

critical surface pressure, the protein film cannot be compress anymore, and starts to collapse and detach from the surface in aggregates.

- The percentage of SBP elution as a function of precipitates concentration and Triton concentration was fitted by a logistic function. For any precipitates concentration chosen, the equation could generate a curve to indicate the % SBP elution at a given Triton concentration.
- The change of initial SBP coverage does not affect the use of the equation to predict % SBP elution when a high percentage of elution (>80%) is targeted. But, the equation would over-predict the % elution in the intermediate range.
- SBP desorption is a time-dependent process, and the desorption rate is significantly affected by the Triton concentration during elution. When >80% SBP elution is targeted, SBP desorption reached equilibrium in less than 20 sec.
- A flow-chart was proposed to use precipitates commercially as a carrier to adsorb SBP from raw extract. The adsorbed SBP can be eluted from precipitates with Triton, to obtain a concentrated SBP solution.

4.7 A process to concentrate SBP from a dilute solution using phenolic precipitates was designed and optimized.

- A Langmuir isotherm was used to track the change of Q_{max} and K on precipitates under the adsorption, elution and regeneration conditions. SBP adsorption affinity dropped significantly after precipitates were exposed to Triton, however the adsorption capacity was only slightly decreased.

Ethanol washing of phenolic precipitates did not show any improvement of SBP adsorption capacity but the affinity was significantly improved.

- Mass balance of SBP and Triton in adsorption, elution and regeneration stages was successfully constructed, which suggests a high recovery rate and a sustainable operation can be expected from the proposed concentrating process.
- Two single-cycle SBP concentrations had initial SBP activity increased 8.7- and 27.6-fold with a recovery rate of 87.2% and 60.7%, respectively. Due to a fraction of uncollectable liquid in the sludge, both of the recovery extents were not as high as expected.
- Consecutive SBP concentrating was conducted for 5 cycles with a designed 6.9-fold increase of initial SBP activity. Both SBP and Triton concentration were tracked throughout the experiment. The overall yield is 92%. The adsorption efficiency, elution efficiency and washing efficiency were all maintained over 90%. The concentrated SBP has no improvement in RZ value.
- By using a Langmuir isotherm to determine K and Q_{max}, the proposed process was found to have both adsorption affinity and capacity maintained well over the cycles.
- Optimization of ethanol washing of the precipitates after SBP desorption shows that 95% of adsorbed Triton can be washed off with ethanol at concentrations above 60%, and the required washing time to reach equilibrium is less than 2 min.

4.8 Dynamic adsorption as an alternative of static adsorption and elution to concentrate SBP was tested. This can be achieved by direct addition of phenol and H_2O_2 to a SBP extract. The experiment shows a 10-fold higher adsorption capacity than static adsorption. However, besides the advantage of the capacity from the "onion" structure, it might also have part of active SBP irreversibly adsorbed at the inner layers. Another part of the SBP in the stock is irreversibly inactivated due to the conversion of phenol. Therefore, the recovery rate was around 50% in the test.

4.9 A binary Langmuir isotherm equation was used to study the competitive adsorption between SBP and Triton on phenolic precipitates. The adsorption isotherm shows that SBP adsorption was reversed with the increasing adsorption of Triton. Before SBP adsorption is reversed, the theoretical combined surface coverage by SBP and Triton reached over 100%, which can be considered as an indication of SBP protein film being compressed. The binary Langmuir isotherm equation was solved by using multiple linear regression, separately for SBP and Triton. The results from Triton adsorption show a high consistency with single-component experimental observations.

5. CONCLUSIONS

Study of the phenolic precipitates formed in SBP-catalyzed phenol polymerization suggests that the adsorption of SBP does not inactivate the enzyme in substantial proportion; rather, the enzyme is immobilized in an active form, well-suited to further catalysis. The affinity and capacity of phenolic precipitates to adsorb SBP can be determined by fitting to the Langmuir isotherm equation. Recycling precipitates for phenol removal utilizes the remaining active SBP on the precipitates; the overall enzyme economy of the process can be improved. However, there is no solid evidence to support extending the strategy for phenol removal in the presence of additive. All additives involved in the study have shown different levels of abilities to prevent SBP being adsorbed on precipitates, as part of the protection mechanism. The 'Triton effect' was explained by a competitive adsorption behavior between protein and non-ionic surfactant, which causes SBP protein film collapse from the polymer surface beyond a critical surface pressure. Then, SBP elution was quantitatively characterized as a function of Triton concentration and precipitates concentration; the competitive adsorption behavior was also modelled by binary Langmuir adsorption isotherm. In addition, a process model using precipitates as an affinity matrix to concentrate raw SBP extract was proposed and proven. The high overall yield makes it suitable for producing a low cost enzyme concentrate for industrial wastewater treatment.

6. RECOMMENDATIONS

The results of this study extended the understanding of phenolic precipitates in SBP-catalyzed phenol polymerization. To implement this study from the engineering and practical perspective, future projects can be chosen from the list below.

1. Effect of ionic strength (salt concentration) on precipitates formation. It is known that precipitates form better in the buffered solution than in the tap water; however the linkage between the ionic strength and phenolic precipitates formation has not been established. An enhanced precipitation process under a high salt concentration would also affect an additive's protection efficiency.
2. Precipitates recycling in the presence of additive can be further examined with aniline and SDS. Different from phenol removal in the presence of SDS, the enhanced precipitation of aniline polymers with SDS can be considered as an advantage for the strategy.
3. Determine critical micelle concentration of SDS and Triton in the buffered solution. The literature report of cmc change under high concentration of salt was used to support the rationale in Section 4.5.4; however the study did not measure the actual cmc of SDS under the reaction conditions.
4. Determine specific adsorption area of phenolic precipitates by nitrogen gas adsorption. This would provide direct information of SBP adsorption capacity on precipitates.
5. Atomic force microscopy can used to visualize the competitive adsorption between SBP and Triton. The intermediate stage information, such as SBP protein film compression, erosion by Triton and collapse can be observed.

6. SBP elution from phenolic precipitates by different non-ionic surfactants can be determined. The strength of the interaction between SBP and non-ionic surfactant could be used as a quick determination of surfactant's protective efficiency. Surfactants with higher price and lower ability to elute SBP can be quickly screened out.
7. Operate SBP concentrating by expanded-bed adsorption with a continuous flow system.
8. Optimize the dynamic adsorption process. The current experiment with phenol has a low SBP recovery rate. This can be tested by: 1) with a lower enzyme demand substrate; 2) an optimum ratio of mg of substrate conversion for capturing U of enzyme; 3) the strategy of phenol and H_2O_2 addition.

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APPENDIX A: SBP ACTIVITY ASSAY

A colorimetric assay was used to measure SBP activity in this study. The principle of the assay is to measure the initial rate of formation of a pink chromophore at 510 nm. One unit (U) of SBP activity is defined as that amount catalyzing 1 μmol of hydrogen peroxide conversion per minute under the assay conditions.

Assay reagents:

A: 100 mM phenol in 0.5 M phosphate buffer at pH 7.4 (0.94 g of phenol, 1.3105 g of monobasic sodium phosphate and 3.7479 g of dibasic sodium phosphate in 100 mL with distilled water)

B: 100 mM H_2O_2 (113 μL of 30% (v/v) H_2O_2 in 10 mL distilled water

C: 25 mg of 4-AAP, 5 mL of A and 100 μL of B in 50 mL with distilled water

Calculation for the dilution factor:

SBP sample activity (U/mL) = SBP activity in the cuvette* A (sample pre-dilution)

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

$$= 200 * \text{initial rate (U/mL)}$$

Dilution factor = 200 * A

Procedure (for Agilent 8453 UV-Visible spectrophotometer)**:**

1. Dilute SBP sample to around 1 U/mL, and input the dilution factor for the software;
2. Take a 50 μL of SBP sample and inject it into the cuvette;

3. Put the cuvette into spectrophotometer and lock the vessel;
4. Take a 950 μL of reagent and quickly push it into the cuvette;
5. Monitor the progress line of colour formation on the computer, and take the reading of SBP activity.

APPENDIX B: STANDARD CURVES

1. Phenol Colorimetric Assay

A colorimetric assay was used to quantify the remaining phenolic compound(s) concentration after treatment of the samples. In this assay, the reaction of phenol and 4-aminoantipyrine under an alkaline conditions with $K_3F_e(CN)_6$ generates a quinone-type pink chromophore. The reaction scheme is shown in Figure A-1(Steevensz 2008). The intensity of generated colour is proportional to the phenol concentration in the reaction. Different concentrations of phenol from 0.01 mM to 0.1 mM were used to construct the standard curve.

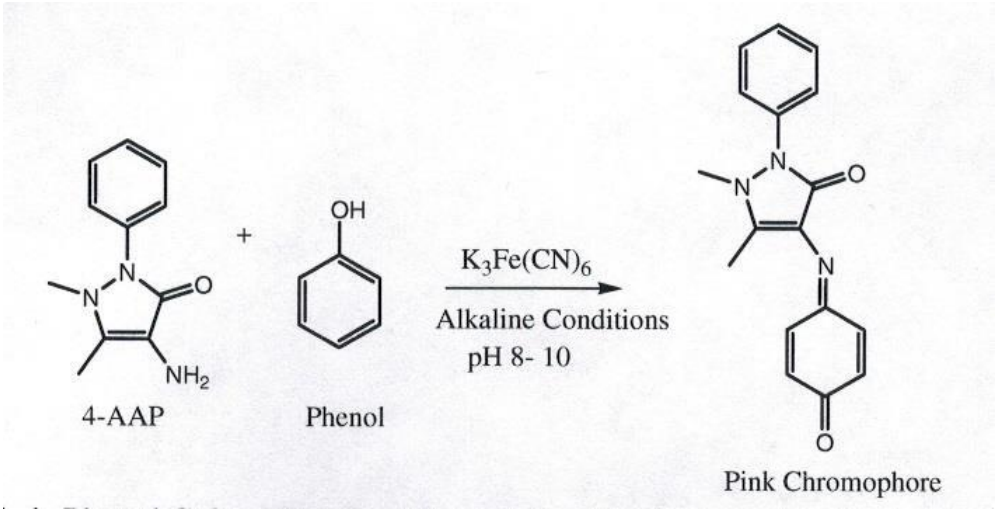

Figure A-1 Phenol colorimetric assay reaction.
[Picture adopted from Steevensz, 2008]

Assay Reagents:

A: 20 mM 4-AAP in 0.25 M N_aHCO_3 (0.2033 g of 4-AAP and 1.05 g of N_aHCO_3 mix in 50 mL of distilled water)

B: 83.4 mM $K_3F_e(CN)_6$ in 0.25 M N_aHCO_3 (1.373 g of $K_3F_e(CN)_6$ 1.05 g of N_aHCO_3 mix in 50 mL of distilled water)

Procedure:

1. Prepare phenol in different concentrations in triplicates from 0.01 to 0.1 mM in 800 µL with distilled water;
2. Add 100 µL of Reagent A, vortex 5 seconds;
3. Add 100 µL of Reagent B, vortex 5 seconds;
4. Wait for 5 minutes and measure the colour absorbance at 510 nm.

Standard curve of phenol colorimetric assay:

The standard curve is plotted with phenol concentration vs. absorbance at 510 nm. A linear equation can be obtained from the data points, which is further used to calculate the unknown phenol concentration from a measured absorbance.

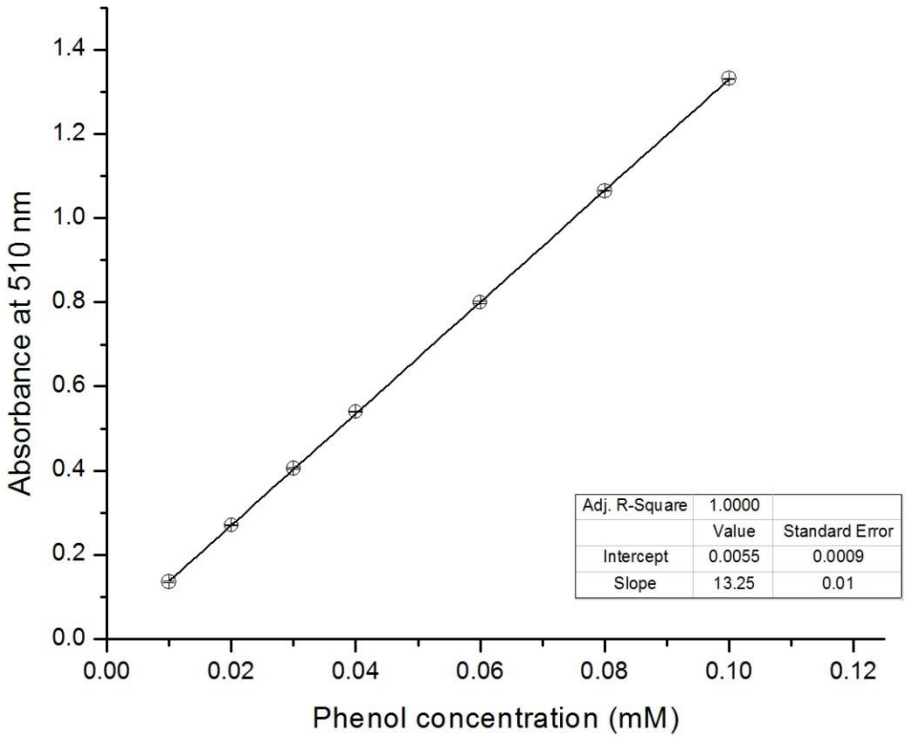

Figure A-2 Phenol colorimetric assay standard curve

2. H_2O_2 Colorimetric Assay

A colour colorimetric assay was used to quantify the remaining H_2O_2 concentration after the treatment in the samples. The same quinone-type pink chromophore as in the phenol colorimetric assay is formed here, where the H_2O_2 in the sample reacts with phenol and 4-AAP in the reagent, and catalyzed by concentrated ARP. Different concentrations of H_2O_2 from 0.01 to 0.1 mM were used to construct the standard curve.

Assay Reagents:

12.5 mM 4-AAP (63.75 mg in 25 mL), 12.5 mL 10 x concentrate (100 mM phenol in 0.5 M phosphate buffer at pH 7.4) and 0.31 mL of Novo ARP concentrate, and dilute to 25 mL with distilled water.

Procedure:

1. Prepare H_2O_2 in different concentrations in triplicates from 0.01 to 0.1 mM in 800 µL with distilled water;
2. Add 200 µL of Reagent, vortex 5 seconds;
3. Wait for 15 minute and measure the colour absorbance at 510 nm.

Standard curve of phenol colorimetric assay:

The standard curve is plotted with H_2O_2 concentration vs. absorbance at 510 nm. A linear equation can be obtained from the data points, which is further used to calculate the unknown H_2O_2 concentration from a measured absorbance.

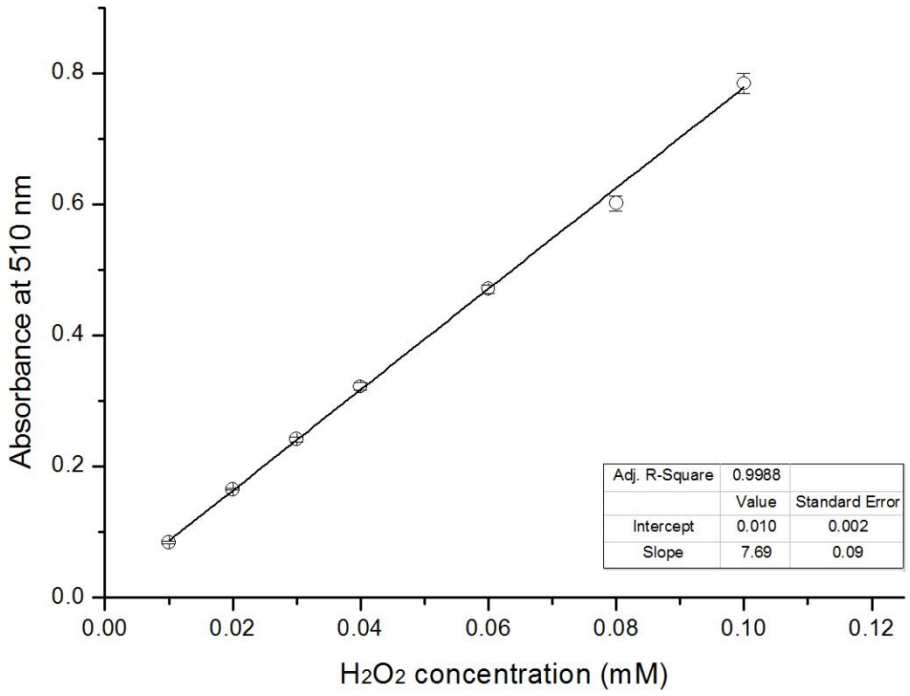

Figure A-3 H_2O_2 colorimetric assay standard curve

3. SDS Colorimetric Assay

A colorimetric method was used to determine the mass of SDS in solution based on chloroform extraction (Arand et al. 1992). In this assay, SDS and methylene blue form a chloroform-extractable ion pair in water, then the colour complex can be extracted from the water by the addition of chloroform. The absorbance of the colour complex was measured at 651 nm. Different mass of SDS from 0.2 to 1.5 μg were used to construct the standard curve.

Assay Reagents:

A: 250 mg/L of methylene blue (62.5 mg) with 50 g/L Na_2SO_4 (12.5 g) and 10 mL concentrated H_2SO_4 in 250 mL.

B: 100% chloroform

Procedure:

1. Prepare SDS in different mass in triplicates from 0.2 to 1.5 µg in 150 µL with distilled water;
2. Add 150 µL of methylene blue, vortex for 1 min, and wait for 2 min;
3. Add 600 µL chloroform, vortex for 1 min, and wait for 3 min;
4. Take the lower layer colour complex and measure the absorbance at 651 nm.
5. Resin the cuvette with acetone first then water.

Standard curve of SDS colorimetric assay:

The standard curve is plotted with SDS concentration vs. absorbance at 651 nm. A linear equation can be obtained from the data points, which is further used to calculate the unknown SDS mass from a measured absorbance.

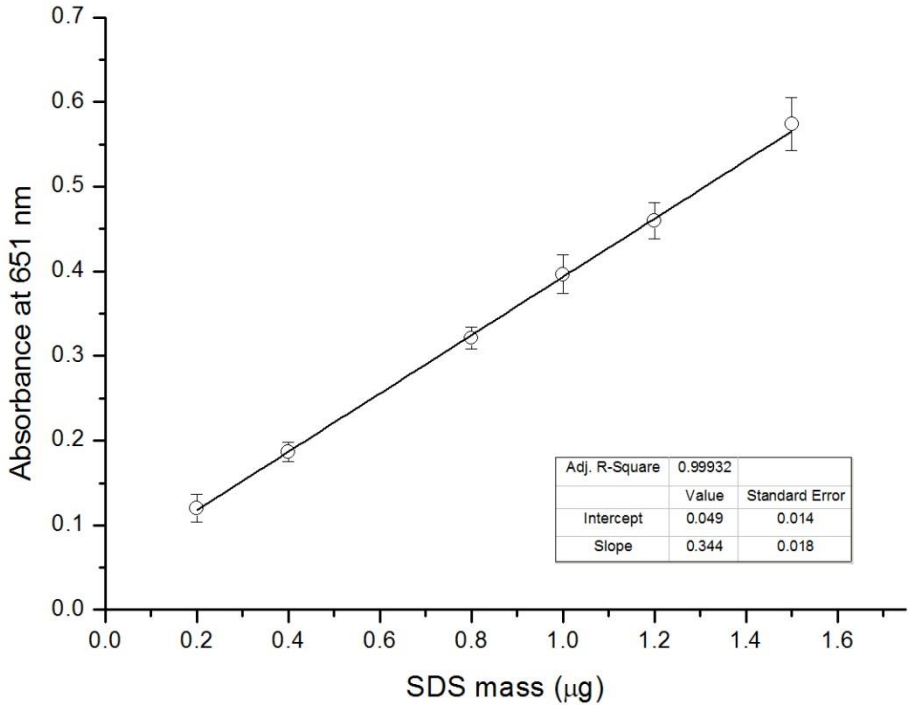

Figure A-4 SDS colorimetric assay standard curve

4. HPLC Standard Curves

The HPLC standard curve of phenol is shown in Figure A-5. The retention time is 3.73 min. The absorbance is measured at 276 nm. The mobile phase solvents are: 40% of 100% acetonitrile and 60% of 0.1% acetic acid.

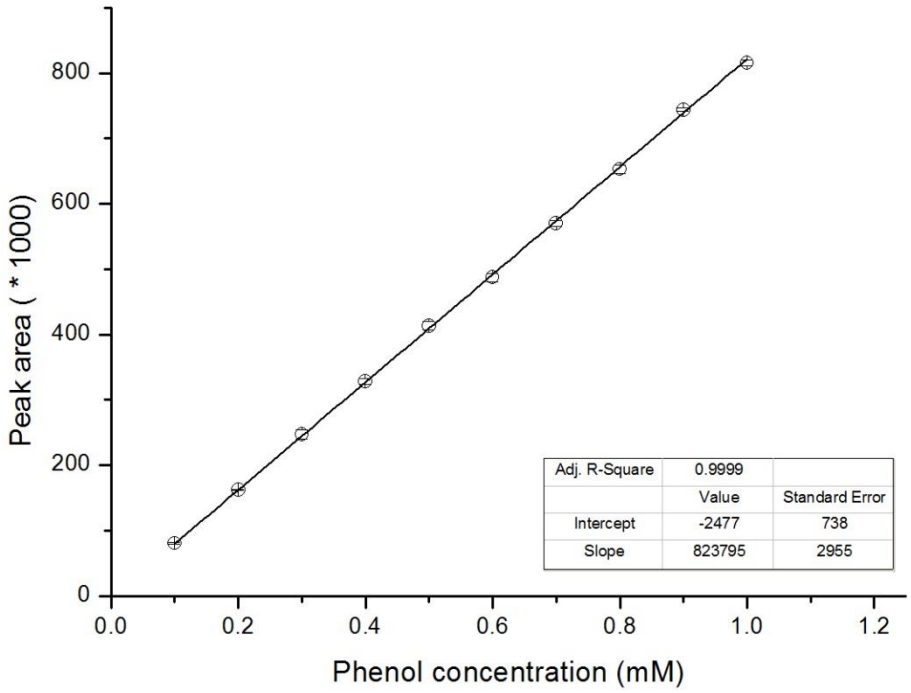

Figure A-5 HPLC standard curve for phenol

The HPLC standard curve of Triton is shown in Figure A-6. The retention time is 2.90 min. The absorbance is measured at 276 nm. The mobile phase solvents are: 95% of 100% acetonitrile and 5% of 0.1% formic acid.

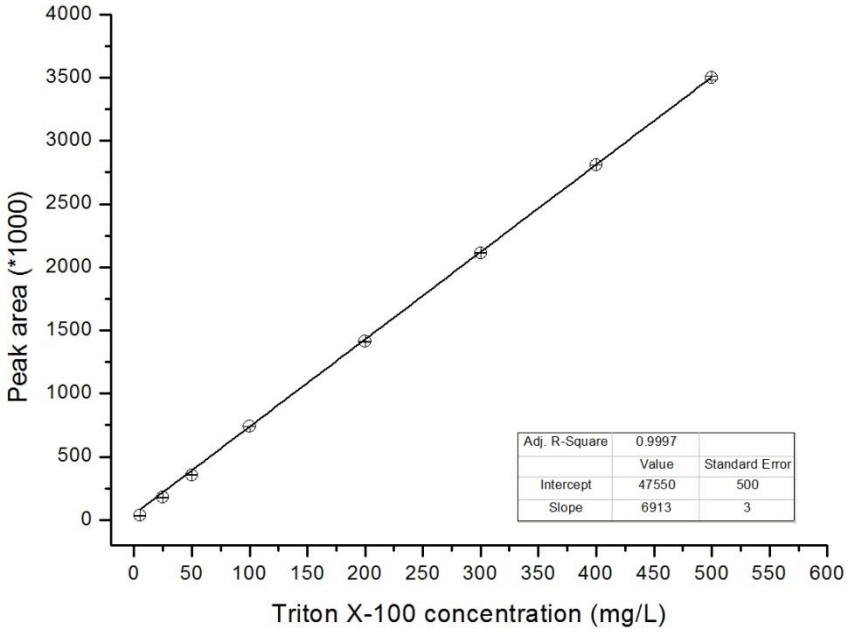

Figure A-6 HPLC standard curve for Triton X-100

5. TOC standard curve of Triton X-100

The TOC standard curve of Triton is shown in Figure B-7.

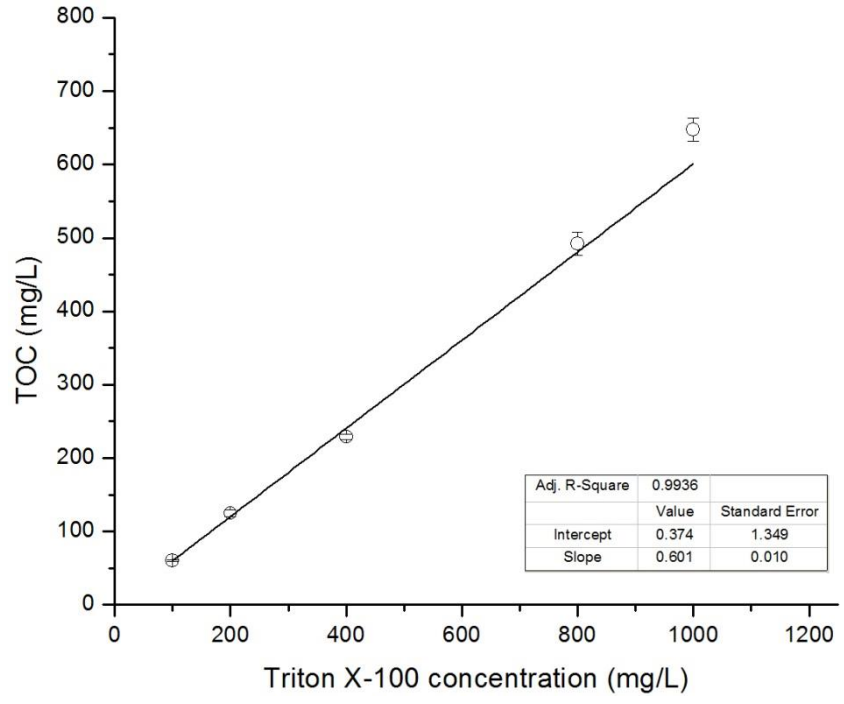

Figure A-7 TOC standard curve for Triton X-100

APPENDIX C: PHENOLIC PRECIPITATES PREPARATION

The SBP-catalyzed phenol polymerization was used to generate the phenolic precipitates for the study. In total, three batches of phenolic precipitates were prepared. At each time, the required amount of SBP and H_2O_2 to convert a given amount of phenol were calculated first, so that no significant H_2O_2 or phenol would remain after the 3 h reaction. After 24 h settling, precipitates were collected and centrifuged at 3000 rpm for 30 min, washed with fresh distilled water three times, then transferred to a brown bottle in a sludge form for storage.

In the third batch of preparation, phenol was reacted with minimum SBP given so that the minimum active SBP would remain on the precipitates after the reaction. The procedure was repeated twice to make enough precipitates for the study, in total, 20 g of phenol was converted into precipitates.

Preparation procedure:

1. Dissolve 17 g of SBP powder by in 900 mL of distilled water, and add 10 mM (0.84 g) phenol in it as the baseline phenol concentration;
2. Prepare phenol stock: take 9.16 g of phenol and dissolve it with 500 mL of distilled water;
3. Prepare H_2O_2 stock: take 13 mL of 30% (v/v) H_2O_2 and dilute it to 500 mL;
4. Take two peristaltic pumps for continuous pumping phenol and H_2O_2 into the reactor with mechanical mixing applied. The phenol feeding rate is 2.32 mL/min (0.000451 mole/min), and the H_2O_2 feeding rate is 2.05 mL/min (0.000475 mole/min). The total pumping time is 244 min;

5. Measure remaining phenol and H_2O_2 concentration in the batch reactor;
6. Pump out the supernatant after 24 h setting, collect the concentrated sludge from the bottom of reactor;
7. Wash the precipitates with distilled water and centrifuge it to remove the water, repeat the step for three times;
8. Transfer all precipitates into a brown glass bottle for storage;
9. Measure precipitates concentration in the bottle by the dry weight assay, and the remaining SBP activity on precipitates by SBP activity assay.

APPENDIX D: SUPPLYMENTARY DATA

1. TOC analysis of Triton X-100 adsorption isotherm

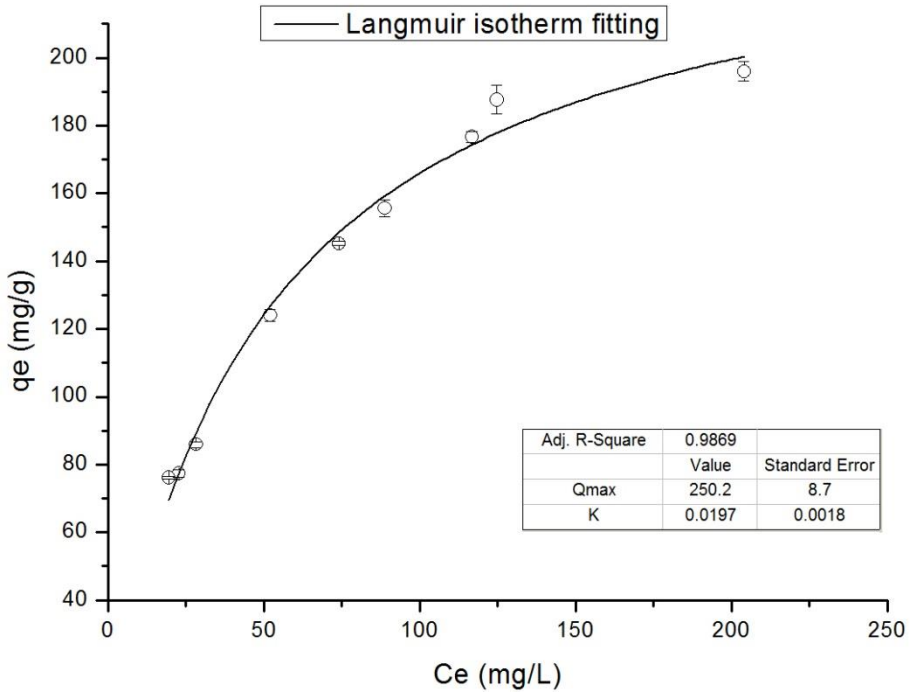

Figure A-8 Langmuir isotherm fitting of Triton X-100 adsorption on phenolic precipitates.

[[Precipitates concentration 1-5 mg/mL, Triton concentration: 10-1500 mg/L, incubation time 30 min]

2. Time course of 1 mM phenol removal with 1.2 U/mL SBP

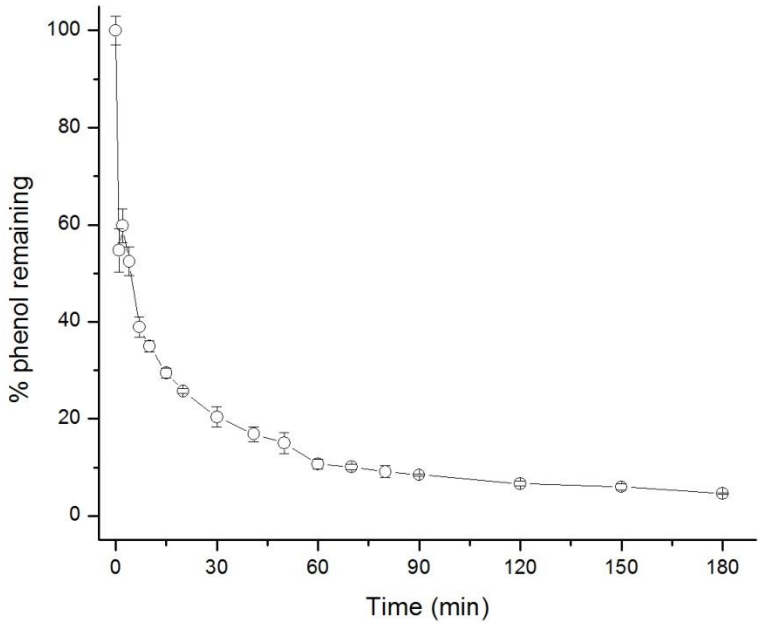

Figure A-9 Time course phenol removal with 1.2 U/mL SBP.

[Reaction conditions: 1 mM phenol, 1.25 mM H_2O_2 , 1.2 U/mL free and 0.4 U/mL immobilized SBP and pH=7.0]

3. Free and immobilized SBP activity change in the presence of PEG or SDS

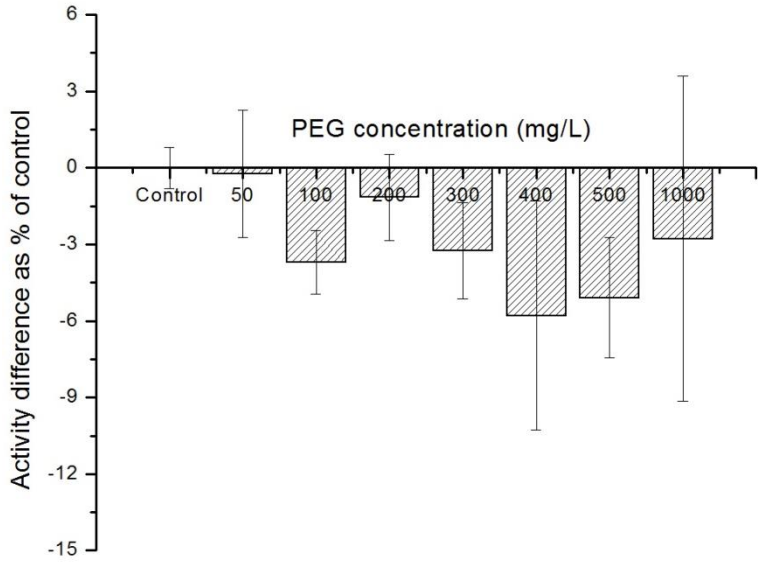

Figure A- 10 Free SBP activity change with different PEG concentrations in the reagent.

[PEG concentration: 0-1000 mg/L, SBP concentration: 1.45 U/mL]

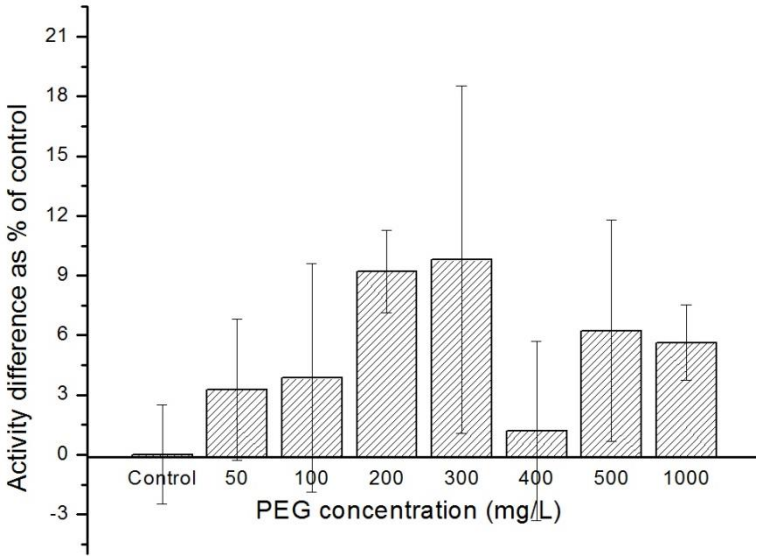

Figure A-11 Phenolic precipitates with different concentrations of PEG in the reagent.

[Precipitates concentration: 0.232 mg/mL, SBP apparent activity on precipitates: 1.15 U/mL.]

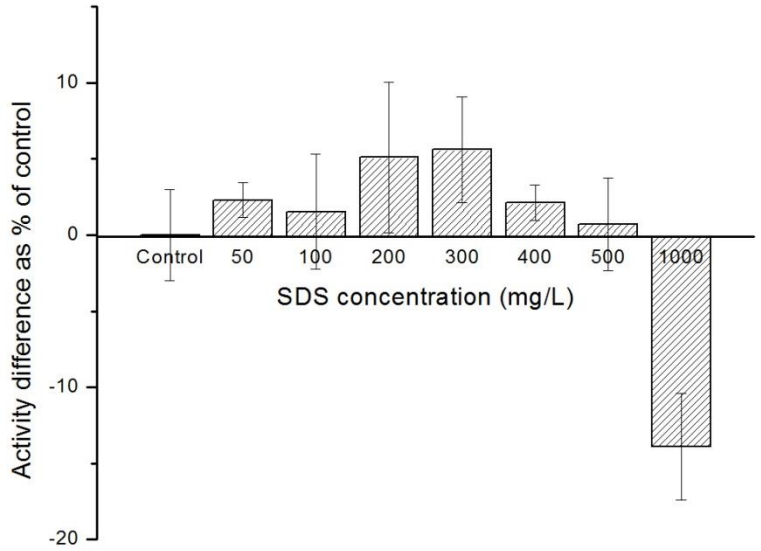

Figure A-12 Free SBP activity change with different SDS concentrations in the reagent.

[SDS concentration: 0-1000 mg/L, SBP concentration: 1.45 U/mL]

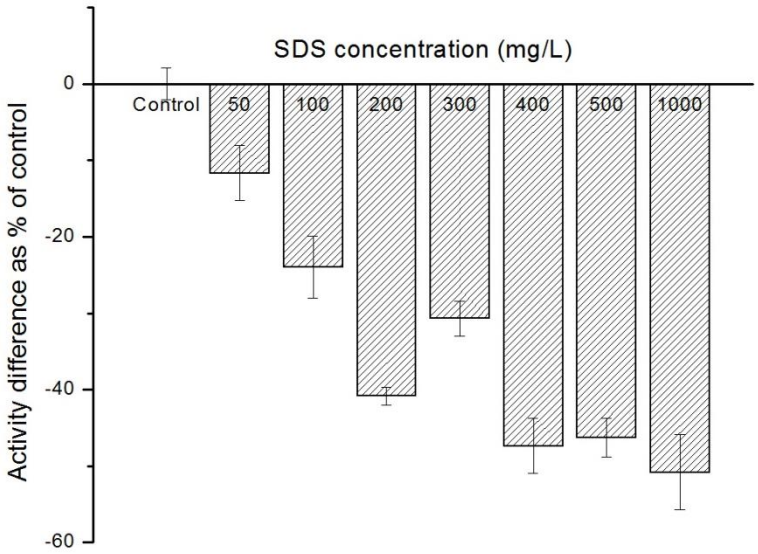

Figure A-13 Phenolic precipitates with different concentrations of SDS in the reagent.

[Precipitates concentration: 0.232 mg/mL, SBP apparent activity on precipitates: 1.25 U/mL.]

VITA AUCTORIS

Name Wei Feng

Education

Ph. D., Environmental Engineering
University of Windsor,
Windsor, Ontario, Canada
2011-2013

M.A.Sc, Environmental Engineering
University of Windsor,
Windsor, Ontario, Canada
2010-2011, promoted to Ph.D.

M. Eng, Environmental Engineering
University of Windsor,
Windsor, Ontario, Canada
2009-2010

Bachelor of Engineering, Bioengineering
Beijing Technology and Business University
Beijing, China
2002-2006

Publications

Feng, W., Taylor, K.E., Biswas, N. and Bewtra, J.K. (2013) Soybean peroxidase trapped in product precipitate during phenol polymerization retains activity and may be recycled. Journal of Chemical Technology & Biotechnology 88(8), 1429-1435.

Feng, W., Taylor, K.E., Biswas, N. and Bewtra, J.K. (2013) Phenolic precipitate utilization to enhance enzymatic wastewater treatment, Proceedings of Canadian Society for Civil Engineering 2013 General Conference, Montreal, QC

Feng, W., Taylor, K.E., Biswas, N. and Bewtra, J.K. (2011) Precipitate recycling: a new approach to wastewater treatment by peroxidase-catalyzed phenol polymerization, Poster presentation for 61st Canadian Society of Chemical Engineering Conference, London, ON

Awards

Ph.D. Entrance Scholarship, University of Windsor , 2011 - 2013
International Graduate Student Scholarship, University of Windsor, 2010 - 2011
3M Canada Company Bursary Award, University of Windsor , 2011