Optimisation of soybean peroxidase treatment of 2,4-dichlorophenol

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Abstract

In the presence of hydrogen peroxide (H_2O_2), peroxidase enzymes (PE) catalyse the oxidation of various chlorinated phenols to free radicals, which then combine to form insoluble polymers that precipitate out of solution. This study systematically characterises the treatment of 2,4-dichlorophenol (2,4-DCP) using soybean peroxidase (SBP) as an oxidising catalyst. The effects of pH, SBP concentration, polyethylene glycol (PEG) additive and initial chlorophenol concentration on 2,4-DCP treatments are reported. Optimum pH for removal of 2,4-DCP without PEG was pH 8.2. The pH operating range of SBP was from 2.5 to 9.4 which is wider than reported for horseradish peroxidase (HRP). A general equation is presented that describes the units of SBP required (without PEG) to treat a given amount of 2,4-DCP at the optimum pH of 8.2. Addition of PEG increased the effectiveness of SBP by factors of 10 and 50 for PEG-3350 and PEG-8000 respectively. A new pH optimum of 6.2 was also found when SBP was used with PEG. Batch and semi -batch enzyme delivery has also been identified as a crucial parameter for the SBP treatment process. The most effective addition scheme was based on five equal concentrations of SBP and H_2O_2 over 15 min and 30 min intervals respectively compared to a single batch addition. This protocol was the most effective as it took advantage of limiting the amount of SBP and H_2O_2 available at each step. This reduces the possible chance of SBP inactivation by excessive H_2O_2 when using a single batch concentration. Average 2,4-DCP removals achieved were 83.5%, 75.5% and 71.5% for 100, 200 and 300 mg/l 2,4-DCP concentrations respectively compared to 62%, 52% and 58% for the single batch addition control.

Introduction

In the presence of hydrogen peroxide (H_2O_2), which acts as an electron acceptor, peroxidase enzymes (PE) catalyse the oxidative polymerisation of phenols, anilines and other aromatics to insoluble oligomers (Dunford and Stillman, 1976). These insoluble oligomers can then be removed through a simple sedimentation or filtration system (Klibanov et al., 1980,1983; Dordick et al., 1980). The kinetics of the peroxidase cycle has been previously described (Dunford and Stillman, 1976; Banci 1997). To date, the majority of the experiments performed have used horseradish peroxidase (HRP) in the treatment of wastewater contaminated with phenols, cresols and chlorinated phenols (Aitken, 1993). However, researchers are currently studying PE from various sources in an effort to study the characteristics of the process and to test the validity of other PE sources (Aitken, 1993). Recently, peroxidase from soybean has been suggested as an alternative to horseradish (Al-Kassim et al., 1993a b, 1995; Nicell and Wright, 1997; Caz et al., 1999, Kinsley and Nicell, 2000). Soybean peroxidase (SBP) is derived from the soybean plant's seedcoat, which is economically advantageous because the seedcoat is a waste by-product of the soybean processing industry. Concomitantly, using SBP would help to convert a waste into a value-added product (Taylor et al., 1998).

While application of SBP is still in its infancy, exploratory studies have been reported. Taylor et al. (1996) provided a limited comparison of HRP and microbial peroxidase to SBP for treatment of phenols and reported that SBP was an effective alternative. This work was followed by two reports that studied the removal of a variety of phenols from wastewater using SBP and a comparative cost analysis of phenols treated individually and separately by SBP, HRP and microbial peroxidase (Taylor et al., 1996,1998, Caza et al., 1999). McEldoon and Dordick (1996) reported that SBP demonstrated unusually high thermal stability that could expand its industrial applications. Recently studies by Wright and Nicell (1999) and Kinsley and Nicell (2000) have also compared the benefits of using SBP over HRP for treatment of phenols as well as demonstrating the benefits of polyethylene glycol (PEG) for the protection of SBP activity.

The SBP treatment process is still in the experimental stage so researchers are continually studying and optimising treatment efficiency while studying other characteristics of the process. The influence of pH, initial chlorophenol concentration, type of chlorophenol, application of protective additives mode of addition and temperature are all factors that influence the applicability of this technology (Al-Kassim et al., 1993a b, 1994a, 1994b,1995; Caza et al., 1999). It is hypothesised that the particulate that forms and precipitates out of solution, entraps the SBP and thereby renders it inactive. To prevent such entrapment, high-molecular mass additives such as PEG can be used to bind with the forming polymers and prevent the PE from becoming entrapped (Wright and Nicell,1999 and Kinsley and Nicell, 2000). It has been reported that PEG with a molecular mass less of than 1 000 is ineffective at protecting HRP when treating phenol and that PEG with a molecular mass of 7 500 (PEG-7500) is more efficient than PEG-1000 (Nakamoto and Machida, 1992). Other researchers have continued this research using PEG-3350 (Wu et al., 1993; Ibrahim et al., 1997). However, there is no supporting documentation that suggests that PEG-3350 is more suitable than PEG-7500. A recent study has shown that SBP in the presence of PEG with higher molecular masss than 7 500 can achieve better efficiency in the removal of phenol from solution (Kinsley and Nicell, 2000).

The effect of pH on HRP catalysing phenol in the presence of PEG has been documented (Bewtra et al., 1995; Dec and Bollag, 1994a,b). The effect of pH on HRP catalysing different chlorinated

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phenols without an additive present has also been reported and the results indicate that the optimum pH of the reaction is not only dependent on the enzyme in question, but it is also dependent on the substrate involved (Dec and Bollag, 1990). Recent studies using SBP have accepted the optimum pH conditions determined for HRP (pH 6.5) as being optimum for SBP (Kinsley and Nicell, 2000). While this certainly may be the case, studies have not been performed to support this assumption. In this study the effect of a range of pHs without PEG and a range of PEGs with various molecular weighs on SBP treatment of 2,4 dichlorophenol (2,4-DCP) is reported.

The effects of reactor set-up and mode of operation have been described for HRP (Nicell et al., 1993) but information is limited for SBP (Al-Kassim et al., 1994a). By controlling the amount of enzyme and/or H_2O_2 available to the reaction by adding the SBP and/or the H_2O_2 in a semi-batch mode, there is the possibility of limiting the amount of SBP inactivation that may occur.

This study focuses on SBP and a comprehensive evaluation of optimum conditions including temperature, pH, enzyme concentration, substrate concentration, additive concentration (PEG) and molecular mass as well as mode of SBP application for treatment of 2,4-DCP.

Materials and methods

Soybean peroxidase, catalase 30% w/w, 2,4-DCP, polyethylene glycol (PEG) and H_2O_2 were purchased from Sigma Chemicals and stored at 4°C. Activity of the SBP enzyme was 70 units/mg solid (one unit decomposes 1.0 μmole of purpurogallin per min at pH 7.0 and 25°C) and the SBP had a Reinheitszahl (RZ) value of 1.3. The RZ value is a measure of heme content in the enzyme (Sigma, 1998). Catalase had an activity of 21 000 units/mg solid (one unit decomposes 1.0 μmole of H_2O_2 per min at pH 7.0 and 25°C).

A primary stock solution containing 1,000 mg/l of 2,4-DCP was prepared using Milli-Q water The final solution was transferred to a 125 ml glass bottle with a Teflon cap, and stored at 4°C. Secondary solutions containing 125 mg/l of 2,4-DCP were prepared at specific pH's. Secondary solutions with a pH of between 9.0 and 7.2 were buffered using a Tris/HCl buffer and solutions with a pH of between 7.0 and 2.6 were prepared using dibasic sodium phosphate (DSP)/citric acid buffer. (Gomori,1955).

SBP stock solutions containing 50 units/ml of SBP were made up with Mill-Q water and kept at 4°C in the dark to reduce any deactivation of the enzyme and were removed only to make dilutions for experiments. Secondary SBP solutions were prepared by diluting the primary SBP stock with Milli-Q water to the desired final concentrations.

Primary stock solutions of 50 g/l of PEG of various molecular masss were prepared by dissolving PEG in Milli-Q water . This mixture was diluted to the desired concentration in the secondary enzyme stock solutions.

Batch tests

Batch experiments were performed in 30 ml flat bottom clear glass vials with Teflon caps, with a final solution volume of 25 ml. The final reaction solutions were comprised of two parts:

- 20 ml of buffered 2,4-DCP solution
- 5 ml of enzyme solution (with or without PEG)

H_2O_2 was added to the 25 ml solution to give an equivalent 1:1 molar ratio with the 2,4-DCP, in order to activate the SBP enzyme.

TABLE 1
Batch experimental conditions

Experimental variable	Experimental conditions used unless otherwise stated	Range of variable tested during various experiments
2,4-DCP	100 mg/l	100 - 511 mg/l
SBP	0.01 units/ml	0.0005 - 10.0 units/ml
PEG*	N/A	0.002 - 2 mg/l
Time	3 h	1 min - 72 h
Temperature	22°C	4°C; 22°C
H_2O_2	0.613mM	N/A
Tris Buffer	pH 8.2	pH 7.2 - 9.2
Citrate-phosphate buffer	pH 6.2	pH 2.5 - 7.2

*PEG-1000, PEG-3350, PEG-8000

Samples were placed on a covered shaker set to 50 r/min for 3 h to ensure good mixing and to prevent light from decomposing the H_2O_2. Cooper and Nicell (1996) reported that 3 h was sufficient time to allow the reaction to go to completion. The 1:1 ratio of H_2O_2:2,4-DCP was reported to be the optimum concentration ratio (Nicell et al., 1992). However, this was verified with a time-dependent test in this study. Less H_2O_2 would be a limiting condition whereas, excessive H_2O_2 concentrations lead to the deactivation of the enzymes due to the formation of Compound III, an enzymatic state in which the enzyme does not recover and is considered to be deactivated (Arnao et al., 1990). After 3 h the reaction was halted by the addition of catalase in a 1:1 molar equivalent ratio with the substrate to decompose the H_2O_2. A 1 ml sample was drawn from the solution and filtered through a 2 μm GV13 millipore filter for high pressure liquid chromatography (HPLC) analysis. Experimental conditions used throughout the experiments (unless noted) are summarised in Table 1.

Reuse of SBP by applying additional substrate

Batch experiments were also performed to determine if SBP maintains its activity (i.e. be reused) when additional 2,4-DCP substrate is added at 1 h intervals. Experiments were performed at pH 6.2, room temperature SBP = 0.01 unit/ml; PEG-8000 = 1.0 g/l; 2,4-DCP = 100 mg/l and H_2O_2 = 1:1 molar ratio with substrate. After 1 h, a sample was drawn for HPLC analysis and an additional 20 ml of substrate (2.5 mg 2,4-DCP) was added to the mixture along with a new concentration of H_2O_2. After 1 h, this step was repeated. After 3 h a sample was drawn but no further substrate was added. A final sample was drawn after 5 h for analysis. This entire process was performed in duplicate (Test #1 and Test #2).

Mode of SBP, and H_2O_2 application

These experiments examined the effect of different modes of SBP, and H_2O_2 addition as a method of enhancing SBP removal of 2,4-DCP. Six tests were performed at pH 6.2 and 22°C and consisted of applying the same total amount of SBP and H_2O_2 in various batch and semi-batch combinations to three different initial 2,4-DCP concentrations; 100, 200 and 300 mg/l in the presence of 0.1 g/l of PEG-8000. The first test evaluated batch addition of SBP and H_2O_2 using 0.0025 units SBP/ml. This enzyme concentration was selected

TABLE 2
Modes of SBP and H_2O_2 addition

Time hours		#1	#2a	#2b	#3a	#3b	#4
0	No enzyme	All SBP, All H_2O_2	1/5 SBP, All H_2O_2	1/5 SBP, All H_2O_2	1/5 SBP, 1/5 H_2O_2	1/5 SBP, 1/5 H_2O_2	1/5 SBP, All H_2O_2
0.15			1/5 SBP		1/5 SBP, 1/5 H_2O_2		
0.30			1/5 SBP	1/5 SBP	1/5 SBP, 1/5 H_2O_2	1/5 SBP, 1/5 H_2O_2	1/5 SBP, All H_2O_2
0.45			1/5 SBP		1/5 SBP, 1/5 H_2O_2		
1.00			1/5 SBP	1/5 SBP	1/5 SBP, 1/5 H_2O_2	1/5 SBP, 1/5 H_2O_2	1/5 SBP, All H_2O_2
1.15							
1.30				1/5 SBP		1/5 SBP, 1/5 H_2O_2	1/5 SBP, All H_2O_2
1.45							
2.00				1/5 SBP		1/5 SBP, 1/5 H_2O_2	1/5 SBP, All H_2O_2
2.30							
3.00	Sample	Sample	Sample	Sample	Sample	Sample	Sample

since it gave only partial 2,4-DCP treatment under standard conditions of addition. Table 2 shows the various semi-batch addition schemes examined.

HPLC analysis

Samples were analysed for residual chlorinated phenols using a Hewlett Packard (model 1090) HPLC with a Hypersil-ODS reverse phase C18 column maintained at 40°C. The mobile phase was an isocratic mixture of HPLC grade methanol (60%) and 0.05 M sodium acetate (40%) pH 4.7 maintained at a flow rate of 0.3 ml/min. Chlorophenols were detected using a diode array detector set at a wavelength of 280 nm. The 2,4-DCP detection limit was 0.4 mg/l. In cases where no peak was detected, the detection limit was assumed.

Results and discussion

Time and temperature of reaction results

Complete removal of 100 mg/l 2,4-DCP to the detection limit occurred in approximately 1 min at 22°C and pH of either 6.2 or 7.2 when treated with 1 unit/ml of SBP. A similar time-dependent test at pH 6.2 and 4°C followed a first-order reaction that required 20 min to achieve 95% removal of 2,4-DCP and approximately 3 h to achieve 99.6% 2,4-DCP removal (Fig. 1). The average first-order reaction rate constant for these conditions at pH 6.2 and 4°C was estimated to be 8 mg/unit·min. The rate at which SBP reacts is significantly slower at lower temperatures. Comparison of the relative SBP reaction rates at 4°C and 22°C indicates that SBP is faster by a factor of about 20 at the higher temperature. This can

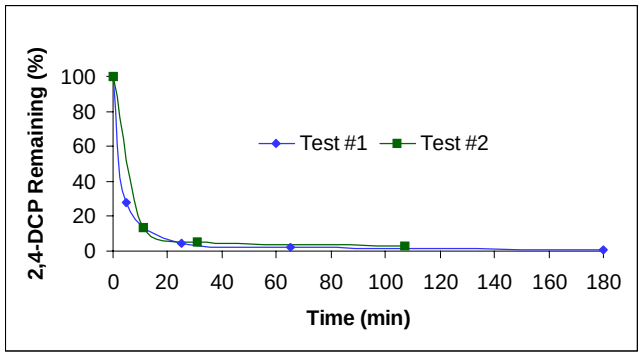


Figure 1
Removal of 100 mg/l of 2,4-DCP using 1 unit SBP/ml (no PEG) at 4°C

be significant when determining the hydraulic retention time (HRT) of a treatment process and concomitant reactor size. It is also important if considering enzyme applications for treatment of groundwater that is typically colder than 22°C.

SBP treated 2,4-DCP solutions exhibited a visual discolouration. Within seconds of injecting the H_2O_2, a milky cloud formed which became more widespread. Eventually the cloud intensified to the point where visible particles could be observed. After 3 h, these particles settled to the bottom of the reaction vessel. The 3 h time frame is important from a practical operational point of view. While 2,4-DCP removals in excess of 95% may be reported in the first few minutes of the reaction based on HPLC analysis of filtered samples, a lag time is required for the particulate to form, precipitate and settle out. Practically, this process will be affected by mixing

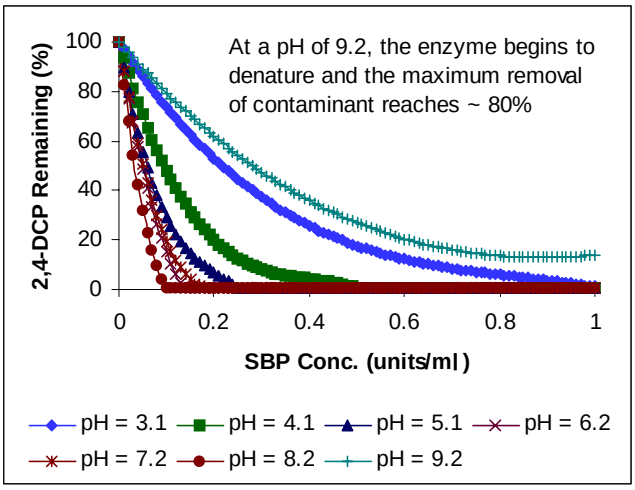


Figure 2
Effect of SBP concentration and pH on removal of 100 mg/l of 2,4-DCP (no PEG)

TABLE 3
Minimum SBP required to achieve 50% and >99% 2,4-DCP removal (no additive)

pH	SBP (units/ml) required to remove >99% of 100 mg/l 2,4-DCP	SBP (units/ml) required to remove 50% of 100 mg/l 2,4-DCP
3.1	1.02	0.215
4.1	0.50	0.095
5.1	0.25	0.060
6.2	0.14	0.049
7.2	0.18	0.051
8.2	0.10	0.045

intensity. It has been postulated that the particulate is responsible for entrapping the active enzyme within its structure thereby rendering the enzyme inactive (Kinsley and Nicell, 2000, Nakamoto and Machida, 1992).

These time-dependent tests indicated that greater than 99% removal of 2,4-DCP from aqueous solution was possible at pH 6.2 and 7.2 at 22°C using 1.0 unit/ml of SBP. This SBP concentration and concomitant efficiency served as a benchmark for future tests and comparisons in this study.

Influence of pH and SBP enzyme concentration

Batch SBP experiments were conducted to determine the optimum and sub-optimum pH ranges in which 2,4-DCP can be removed from aqueous solution and additionally to determine the effective range of enzyme concentration without PEG present. Using HRP, Dec and Bollag (1990) and Bewtra et al. (1995) determined the optimum pH for removing 2,4-DCP to be 6.5 with a working range of 3 to 10. In this study, tests were performed for pH values from 2.5 to 9.4 and for SBP concentrations between 0.01 and 9 units/ml. The data from each pH range (3.1 to 9.4) were fit to a polynomial using Statistica. The square of the correlation coefficient R^2 for each best-fit curve ranged from 0.9026 to 0.9896. Figure 2 shows the family of curves that depict the residual 2,4-DCP remaining as a function of pH and SBP concentration in the absence of PEG. The minimum amount of SBP required to remove 2,4-DCP for each pH can be determined from the plot.

A similar graph to Fig. 2 was produced for phenol removal by HRP that demonstrated catalytic ability from pH 4 to 10 (Bewtra et al. 1995). The results obtained in this experiment indicate that SBP can function in more extreme acidic conditions than HRP. Even at pH of 2.5, 1 unit/ml of SBP removed nearly 90% of the 2,4-DCP. Several other important observations relating to the effects of pH were made. The optimum pH for SBP treatment of 2,4-DCP is approximately 8.2 which is different than the optimum pH ~ 6.5 reported by Dec and Bollag (1990) for HRP. For SBP a pH slighter greater than 8.2, severely affected 2,4-DCP removal efficiency at low SBP concentrations. However, at higher SBP concentrations 1 to 7 units/ml 85% 2,4-DCP removal can still be achieved at pH 9.4. It has been speculated that peroxidase enzyme is susceptible to denaturing in slightly basic solutions. As the pH decreased and became more acidic there was a gradual loss of SBP efficiency compared to the sudden change at pHs above 8.2. Therefore, in an acidic environment it can be speculated that the SBP enzyme is denaturing but not as quickly as in a basic solution.

Table 3 shows the minimum amount of SBP required to remove 50% and >99% of 100 mg/l of 2,4-DCP at various pHs in the absence of PEG. Empirical Eqs. (1) and (2) were the best fit to the experimental data and can be used to determine the SBP requirements for 50% or >99% 2,4-DCP removal at any pH in the range tested.

99% removal:

$$SBP_{min} = -0.0173pH^3 + 0.352pH^2 - 2.3838pH + 5.54 \quad (1)$$

50% removal:

$$SBP_{min} = -0.0045pH^3 + 0.0879pH^2 - 0.5713pH + 1.2719 \quad (2)$$

where:

SBP_{min} = minimum SBP required, units/ml
pH = pH of the mixture.

Varying 2,4-DCP and SBP enzyme concentrations

Using the optimum pH 8.2, batch experiments (no additive) were performed using initial 2,4-DCP concentrations ranging from 55 mg/l to 511 mg/l to determine the relation between substrate concentration and 2,4-DCP removal efficiency per unit of SBP enzyme. Previous experiments (discussed above), indicated that the minimum amount of SBP required at pH 8.2 for greater than 99% removal of 100 mg/l 2,4-DCP was 0.01 units/ml. Consequently, SBP concentrations of less than 0.1 units/ml were used so that incomplete 2,4-DCP removal should occur.

The results of the six data sets are shown in Fig 3 . Each curve represents the concentration of 2,4-DCP remaining for the given amount of SBP used in units/ml. The curves approach linearity (R^2 values between 0.988 and 0.999) and for each specific enzyme concentration, approximately the same amount of 2,4-DCP was removed no matter what the initial 2,4-DCP concentration. The slopes of each data set (final - initial 2,4-DCP concentration excluding those that reached detection limit) were determined on a volumetric basis. The mean value of the slopes for all concentrations was determined to be 1.77 + 0.09 mg 2,4-DCP removed/unit SBP. From this information a general empirical equation (Eq. (3)) was determined that describes the units of SBP required (without PEG) to treat a given amount of 2,4-DCP at the optimum pH of 8.2. Eq. (3) indicates that the SBP reaction is zero order and independent of 2,4-DCP concentration. This would mean that the 2,4-DCP

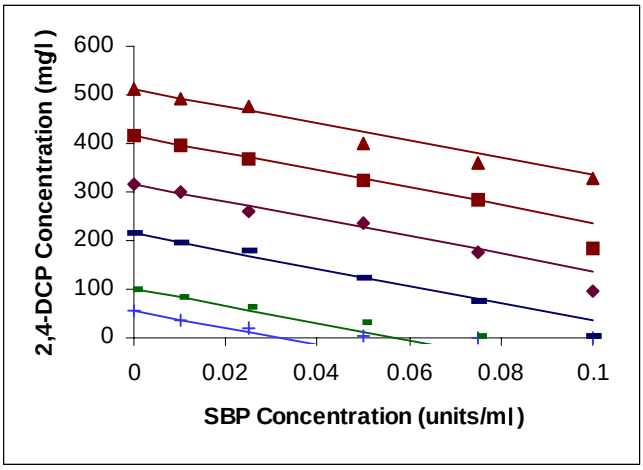


Figure 3
Relationships between SBP concentration and effective 2,4-DCP removal (no PEG) for various initial concentration of 2,4-DCP

concentrations tested are well above the half saturation concentration (K_s) of SBP.

$$\text{SBP (units)} = \text{2,4-DCP (mg)}/1.7 \qquad (3)$$

Influence of PEG concentration, molecular mass and pH

Further batch experiments were conducted to characterise the SBP reaction for removal of 2,4-DCP in the presence of the protective additive, PEG. The exact mechanism of enzyme protection afforded by PEG is not fully understood. It was hypothesised that, when dissolved into solution with the substrate, PEG offers the free radicals that form a binding site to attach to rather than to another free radical. However, it is not clear how radicals attaching to other radicals decrease enzyme activity (Wu et al., 1993; Ibrahim et al., 1997).

Using PEG-3350 and PEG-8000 the effect of pH on SBP activity was evaluated. The optimum pH for removal of 2,4-DCP for SBP in the presence of PEG-3350 is shown in Fig. 4. Addition of PEG-3350 resulted in little to no improvement (and even worse removal at low acidic pH) for pH 2.5, 3.1, 4.1, 8.6, and 9.2. Marked improvement was observed between pH 5.1 and 8.2. The optimum was pH 6.2, greater than 99% removal of the 2,4-DCP using 0.01 units/ml of SBP with a PEG-3350 concentration of 1.2 g/l. This result was about a 10-fold improvement when compared to tests without PEG. Fig. 4 also shows that with increasing PEG-3350 concentration, the removal of 2,4-DCP increases for certain pHs. A 3rd degree polynomial was fit to all the data (best fit to data) to produce the contour plot shown in Fig. 5 which shows the relationship between pH, PEG-3350 concentration and 2,4-DCP removal. The contour plot shows that increasing the concentration of PEG-3350 can improve the 2,4-DCP removal efficiency by about a factor of 10 near the optimum, pH 6.2. Similar trends and pH optimum of 6.2 related to the effect of PEG-8000 concentration were observed (not shown). However, 2,4-DCP removals were higher at lower SBP concentrations by a factor of about 5-10 than those obtained with PEG-3350.

The results of PEG molecular mass and initial phenol concentration experiments on 2,4-DCP removal (pH 6.2) are shown in Fig. 6. PEG-1000's ineffectiveness to improve SBP efficiency for 2,4-DCP removal was confirmed. However, PEG-3350 (Fig. 6) showed a significant increase in enzyme efficiency when compared to the control (no PEG). With the highest concentration of PEG-3350 additive used (2 g/l) greater than 95% removal for all three initial 2,4-DCP concentrations was observed. However, at lower PEG-3350 concentrations, less efficient 2,4-DCP removal was observed. Using PEG-8000, the results indicted a significant increase in 2,4-DCP removal at lower SBP concentration by a factor of about 100 compared to SBP treatment of 2,4-DCP without PEG. Greater than 95% removal was achieved for every concentration of PEG-8000 tested (Fig. 6c). These results indicate that not only can greater 2,4-DCP removal be achieved using PEG-8000 at lower enzyme concentration but that it can be done using a lower concentration of PEG. This is important when considering the effects of residual PEG in water, since as the molecular mass of PEG increases, it becomes more recalcitrant (Kinsley and Nicell, 2000). Therefore, there is a tradeoff between the beneficial effects of high molecular mass PEG on SBP reactions and the negative effects of residual PEG in the environment. Kinsley and Nicell (2000) also reported on the effects of PEG on the removal of phenol using SBP. Kinsley and Nicell (2000) reported that when using 0.2 units/ml SBP to treat 1 mM phenol in the presence of 100 mg/l of PEG-3350, PEG-8000 and PEG-35000 SBP removed approx. 50%, 84% and 98% of the phenol respectively. Therefore by doubling the molecular mass from 3 350 to 8 000, the removal efficiency was increased by 34%. By increasing the molecular mass by a factor of ten to 35 000 (4.4 times higher than 8 000), the removal increased by 48% which is only 14% more than PEG-8000. Kinsley and Nicell (2000) also studied residual PEG remaining in solution and determined that COD increases rapidly with increasing PEG molecular mass. They recommended that optimum PEG concentrations should be determined in order to minimise residual PEG. Wu et al. (1993, 1998) also addressed the use and fate of PEG when applied for the protection of HRP treating phenol. Based on environmental concerns, increasing the molecular mass but achieving only marginally better enzyme efficiency, the use of PEG-35000 may not be justified in terms of treating residual PEG in the environment. Further investigations with high molecular mass PEG are needed.

Additional batch tests were carried out to determine the degree of treatment that could be obtained by decreasing the concentration of SBP below 0.01 units/ml (0.0005, 0.001 and 0.005 units/ml) at various concentrations of PEG-8000 (0, 0.2, 1.0 and 2.0 g/l) using initial 2,4-DCP concentrations of 100 mg/l or 300 mg/l. When no PEG was added , there was no removal of 2,4-DCP. When PEG-8000 was used, there was a significant increase in 2,4-DCP removal for all SBP concentrations. Figure 7 shows that a SBP concentration of 0.01 units/ml can successfully treat up to 300 mg/l of 2,4-DCP (pH 6.2) using various concentrations of PEG-8000. Applying 0.005 units of SBP/ml of 2,4-DCP resulted in removals between 85 to 95% and 70 to 85% for initial 2,4-DCP concentration of 100 or 300 mg/l, respectively.

Decreasing the SBP concentration proportionally decreased the maximum enzyme efficiency. For example, using 0.0005 units SBP/ml, 100 mg 2,4-DCP/l and various concentrations of PEG-8000, up to 30% removal of 2,4-DCP was obtained. Likewise, when treating 300 mg/l 2,4-DCP with 0.001 units SBP/ml, 35 % removal of 2,4-DCP was attained and when using 0.005 units SBP/ml, up to 80% removal was achieved. A general observation to note is that for both initial 2,4-DCP concentrations at higher SBP concentrations the per cent removal using 2.0 g/l of PEG-8000 is only approximately 10% greater then the removal using 0.2 g/l PEG-8000 indicating that a tenfold increase in PEG-8000 concentration results only in a slight increase in 2,4-DCP removal

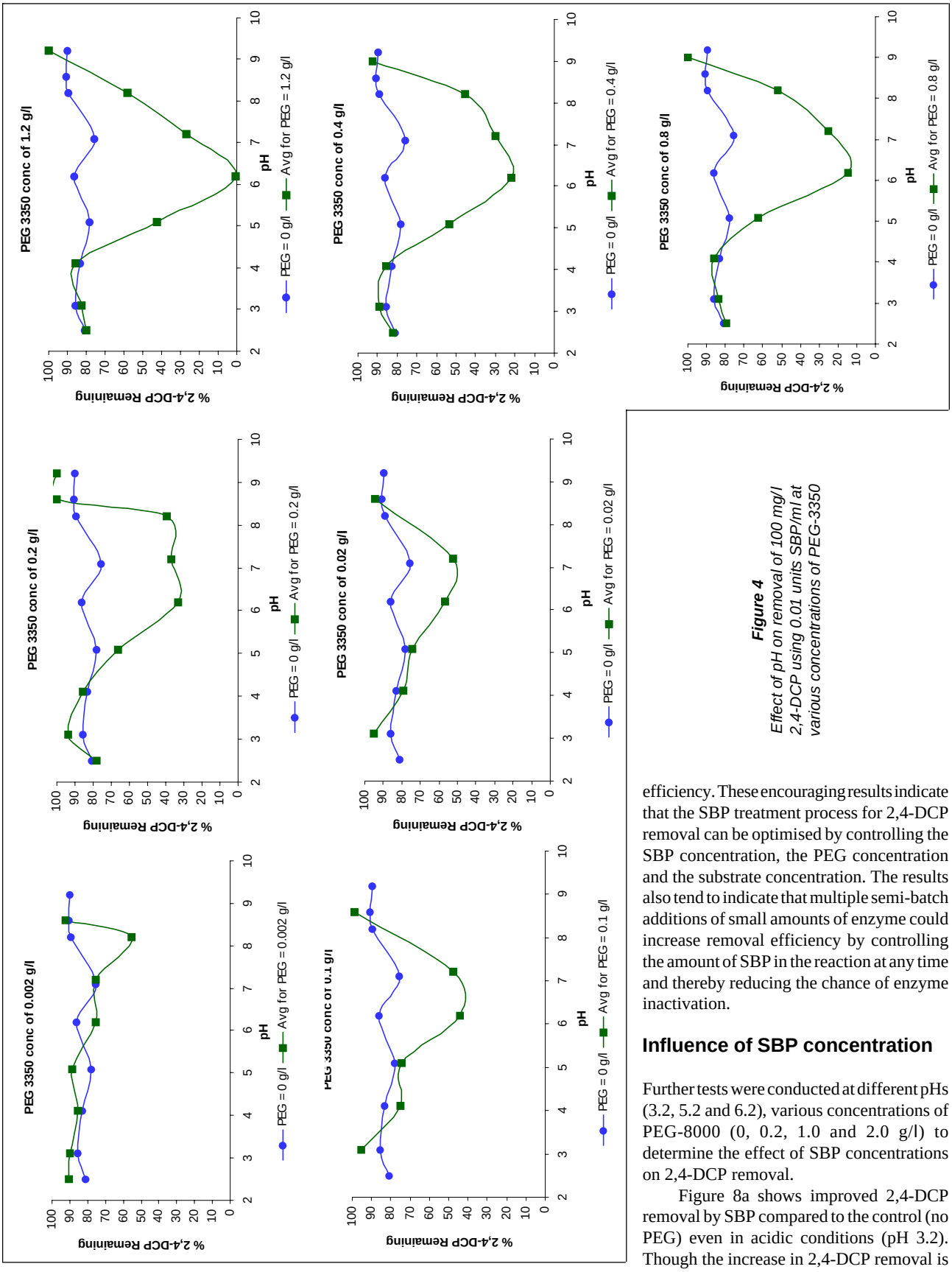


Figure 4
Effect of pH on removal of 100 mg/l 2,4-DCP using 0.01 units SBP/ml at various concentrations of PEG-3350

efficiency. These encouraging results indicate that the SBP treatment process for 2,4-DCP removal can be optimised by controlling the SBP concentration, the PEG concentration and the substrate concentration. The results also tend to indicate that multiple semi-batch additions of small amounts of enzyme could increase removal efficiency by controlling the amount of SBP in the reaction at any time and thereby reducing the chance of enzyme inactivation.

Influence of SBP concentration

Further tests were conducted at different pHs (3.2, 5.2 and 6.2), various concentrations of PEG-8000 (0, 0.2, 1.0 and 2.0 g/l) to determine the effect of SBP concentrations on 2,4-DCP removal.

Figure 8a shows improved 2,4-DCP removal by SBP compared to the control (no PEG) even in acidic conditions (pH 3.2). Though the increase in 2,4-DCP removal is not as good as under more optimum pH, it does require 25% less SBP (0.75 units/ml) with PEG-8000 to achieve >99% 2,4-DCP

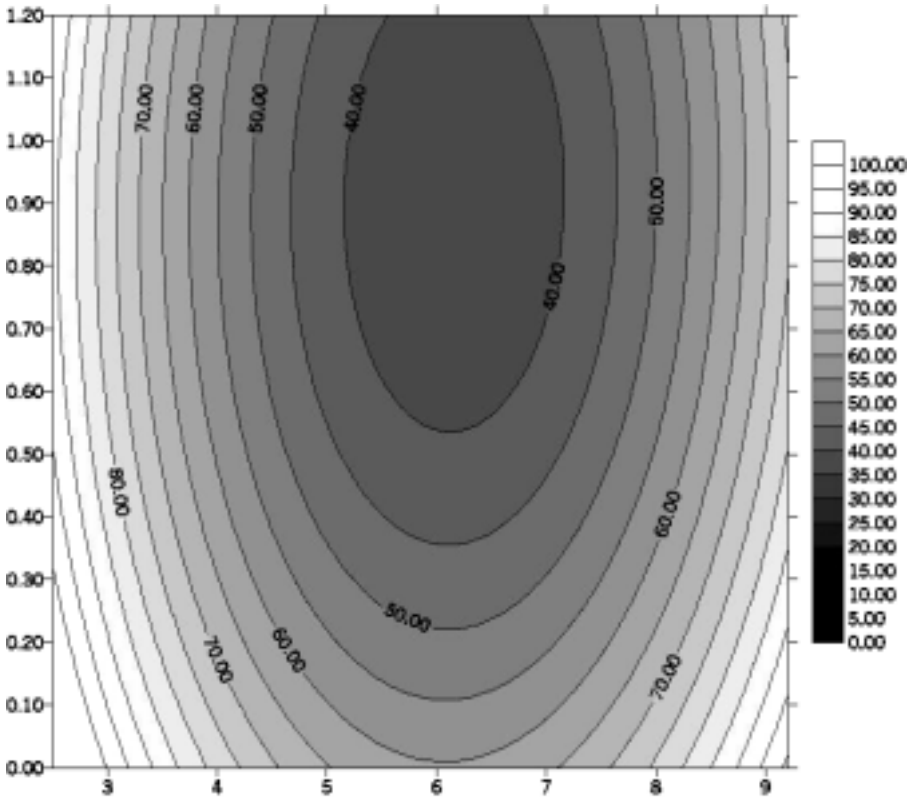
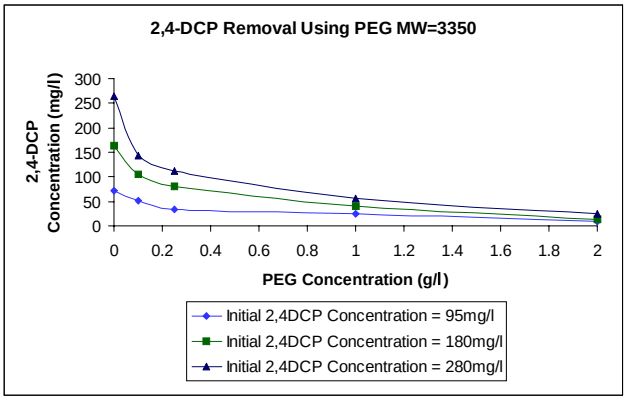


Figure 5
Contour plot showing per cent 2,4-DCP remaining (initial concentration 100 mg/l) at various pH (x-axis) and PEG-3350 concentrations (y axis g/l)



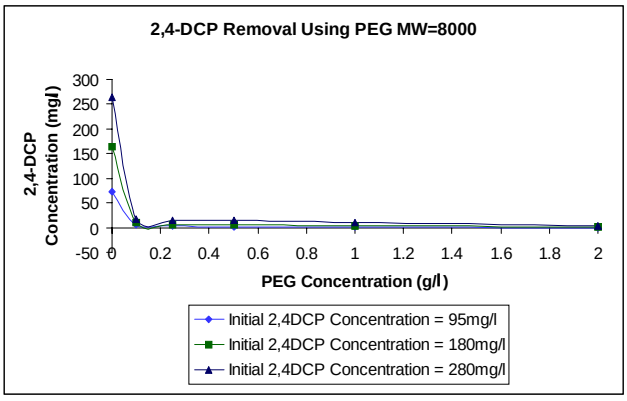


Figure 6
Effect of PEG molecular mass on removal of 2,4-DCP at various initial concentrations of 2,4-DCP using 0.01 units SBP/ml with increasing PEG concentration (PEG-1000, PEG-3350, PEG-8000)

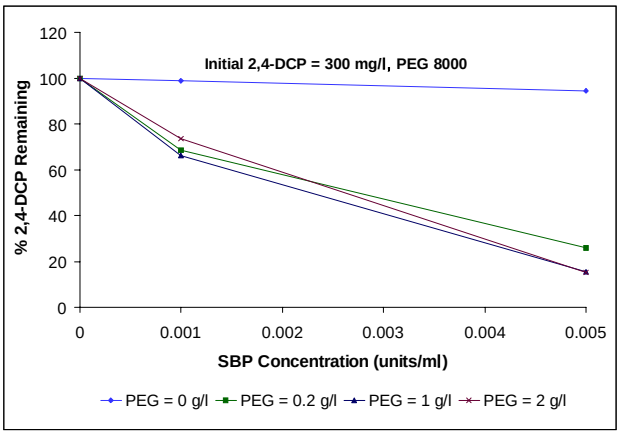
removal than it does without PEG (1.0 units/ml). Using the same SBP concentration with no PEG, only 90% removal is achieved. It is important to note that the same 2,4-DCP removal efficiencies (pH 3.2) were observed when adding 0.2 or 2.0 g/l of PEG-8000. Excess residual PEG remaining (2.0 g/l PEG-8000) could be discharged to the environment and could have a negative effect.

At pH 5.2 (Fig. 8b), a significant increase in removal efficiency occurs in the presence of PEG-8000 at low SBP concentrations. In the presence of PEG-8000, only 0.025 units/ml of SBP are required to achieve >99% 2,4-DCP removal. Approximately ten times that amount of SBP (0.25 units/ml) is required to attain >99% removal without PEG-8000. For comparison, using 0.05 units/ml SBP without PEG present, removed only 60% (compared to >99% with PEG-8000). Again there was not a large advantage to using 2 g/l PEG-8000 over 0.2 g/l in terms of 2,4-DCP removal.

At pH 6.2 (Fig. 8c), similar results to pH 5.2 were obtained but at lower SBP concentrations. What was significant was the extent of 2,4-DCP removal achieved with and without PEG-8000 at SBP concentrations of 0.005-0.01 units/ml. With any concentration of PEG-8000, 2,4-DCP removal was >88% while without PEG, there was only 5 to 11% 2,4-DCP removal. Under more optimum pH conditions less PEG-8000 was required to achieve the same level of enzyme efficiency.

Reuse of SBP by semi-batch 2,4-DCP addition

Figure 9 shows duplicate results of semi-batch 2,4-DCP additions that indicate that multiple use of SBP is possible. The amount of 2,4-DCP removed from the mixture after 1 h was between 90 to 98%. A second chlorophenol spike injected at 1 h achieved 34 to 45% removal of the remaining 2,4-DCP. After the third 2,4-DCP addition at 2 h only 8 to 13% of the 2,4-DCP was removed and it was concluded that SBP activity terminated. The decrease in activity may be explained by the kinetics of the reaction. If



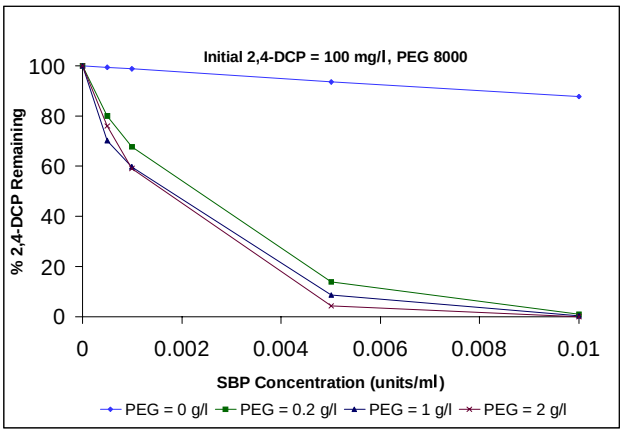


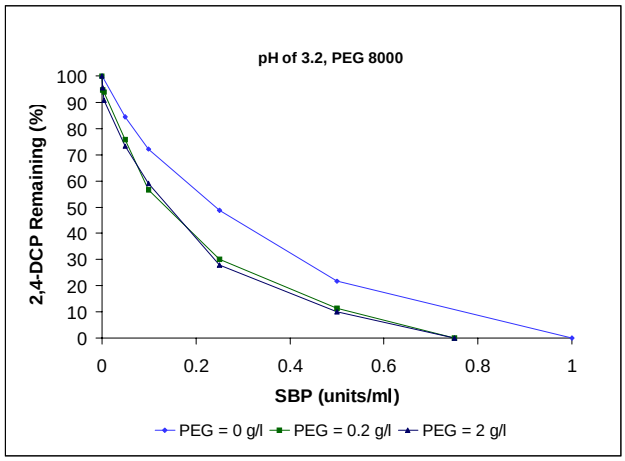
Figure 7
Per cent 2,4-DCP remaining vs. SBP concentration for various concentrations of PEG-8000 at initial pH 6.2 and 2,4-DCP concentrations of 100 and 300 mg/l.

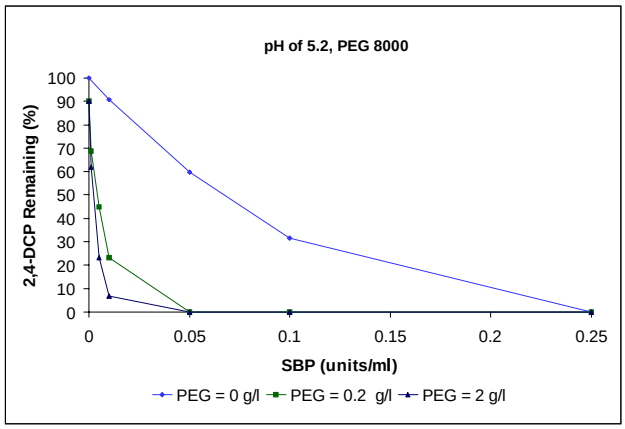
substrate is limited as it was in this case (first h), excessive H_2O_2 may be present in solution and the enzyme may follow the inactivation path to P-670 or Compound III. Therefore, reversing the process and limiting the enzyme or H_2O_2 instead of the substrate may be more efficient in optimising the reaction. This was studied in the final set of tests.

Mode of SBP, and H_2O_2 application

Based on the experimental plan outlined in Table 2 the results in terms of 2,4-DCP removal efficiency are summarised in Table 4 (no PEG present). The schemes that were least to most efficient in terms of 2,4-DCP removal were as follows: #4 (least efficient), #1, #2a, #2b, #3a and #3b (most efficient). Method #4, used a semi-batch addition of 1/5 the total SBP and a full concentration of H_2O_2 every 30 min. Ideally, splitting up the SBP concentration should improve the reaction efficiency, however, it was counteracted by too much H_2O_2. As previously discussed, such conditions create what is called the suicide mechanism. Excessive amounts of H_2O_2 compared to substrate availability cause the enzyme to become catalytically inactivated as SBP follows the inactivation path to P-670 or Compound III. It is clear that in this case, excessive H_2O_2 is preventing the reaction from achieving the batch test benchmark results.

The remaining schemes achieved better results than the control (single batch addition) test #1. Test #2a and #2b produced relatively





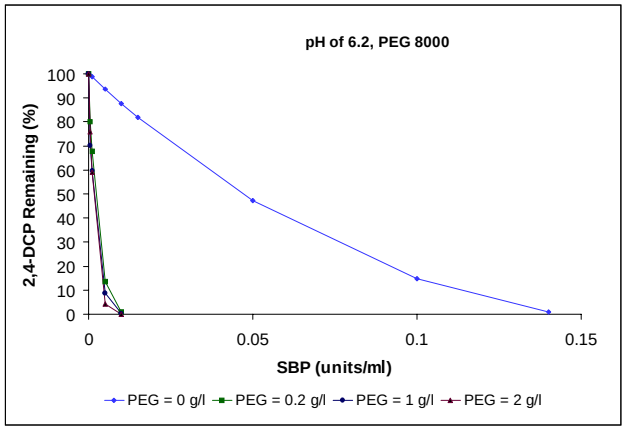


Figure 8
Per cent 2,4-DCP remaining (initial concentration 100 mg/l) vs. SBP concentration for various concentrations of PEG-8000 at pH 3.2, 5.2 and 6.2

similar results. Scheme 2 was based on distributing the SBP concentration over five equal time intervals (#2a – 15 min, #2b – 30 min. intervals) while using a single initial batch concentration of H_2O_2 to initiate the reaction. The most effective addition schemes were #3a and #3b that were based on five equal concentrations of SBP and H_2O_2 over 15 min and 30 min intervals respectively. This protocol was the most effective as it took advantage of limiting the amount of SBP and H_2O_2 available at each step. This reduces the possible chance of SBP inactivation by

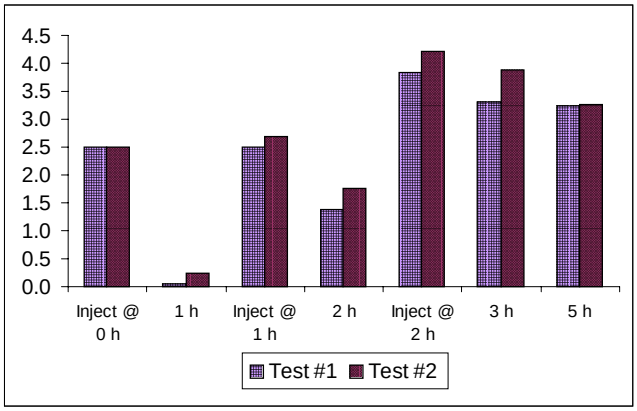


Figure 9
Mass of 2,4-DCP remaining after 3 equal injections of $2x10^{-5}$ moles of 2,4-DCP (SBP concentration remained constant at 0.25 units[initial concentration]; mass of PEG-8000 remained constant at 25 mg; H_2O_2 was added in 3 equal concentrations in a 1:1 molar ratio to the 2,4-DCP added)

TABLE 4
Mode of operation and percent 2,4-DCP removal

Test #	% 2,4-DCP removal		
	100 mg/l	200 mg/l	300 mg/l
1 (control)	62	52	58
2a	78	72	60
2b	76	64	62
3a	83	75	70
3b	84	76	72
4	55	47	30

excessive H_2O_2 when using a single batch concentration. The average 2,4-DCP removals achieved in #3a and #3b were 83.5%, 75.5% and 71.5% for 100, 200 and 300 mg/l 2,4-DCP concentrations respectively compared to 62%, 52% and 58% for the single batch addition control (#1).

Conclusions

Time and temperature are important factors to be quantified when applying SBP for treatment of 2,4-DCP. The rate at which SBP removed 2,4-DCP at room temperature compared to 4°C was about 20 times faster. Practically, it is important to realise that conversion precedes precipitation and that a lag phase exists. While 2,4-DCP removal in excess of 95% may be reported in the first few minutes based on filtered HPLC analysis, time is required for the particulate to form, precipitate and settle out. The optimum pH for SBP treatment of 2,4-DCP with and without PEG was determined to be approximately pH 6.2 and pH 8.2 respectively. The operating range was from pH 2.5 to 9.4. At the optimum pH of 8.2 (without PEG), the removal efficiency of various initial concentrations of 2,4-DCP (50-500 mg/l) is zero order within the SBP range of 0.001 to 0.1 units/ml and can be summarised by stating that 1.77 mg of 2,4-DCP is removed per unit of SBP activity. In the presence of the additive PEG- 3350 and PEG-8000 2,4-DCP removal efficiency of SBP increases by factor of 10 and 50 respectivly compared to no PEG addition. PEG-8000 as a protective additive for SBP treatment of 2,4-DCP was successfully demonstrated. By using optimum pH conditions, PEG-8000 concentration and SBP concentration can be optimised coincidentally to minimise the amount of SBP needed while simultaneously limiting the residual PEG that might be released to the environment. Substrate addition strategies indicate that SBP enzyme can be reused.

Batch and semi-batch enzyme delivery has also been identified as a crucial parameter for the SBP treatment process. The most effective addition scheme was based on five equal concentrations of SBP and H_2O_2 over 15 min and 30 min intervals respectively compared to a single batch addition. This protocol was the most effective as it took advantage of limiting the amount of SBP and H_2O_2 available at each step. This reduces the possible chance of SBP inactivation by excessive H_2O_2 when using a single batch concentration. Average 2,4-DCP removals achieved were 83.5%, 75.5% and 71.5% for 100, 200 and 300 mg/l 2,4-DCP concentrations respectively compared to 62%, 52% and 58% for the single batch addition control

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