Enzyme-catalyzed oxidation of 17ß-estradiol using immobilized laccase from *T. Versicolor*

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Abstract

Endocrine disruption is a problem of increasing environmental significance, as anomalies continue to be discovered in wildlife exposed to a variety of exogenous toxic compounds released into the aquatic environment through municipal and industrial effluents and agricultural runoff. The estrogens excreted by humans and entering aquatic systems via sewage treatment plants are of particular interest, as estrogen excretion cannot be feasibly controlled at the source and estrogens are among the most potent endocrine disruptors known. As phenolic compounds, estrogens are amenable to oxidation through the catalytic action of oxidative enzymes. Earlier work was directed toward characterizing the removal of estrogens using peroxidase enzymes as well as the fungal laccase *Trametes versicolor* in batch reactions. The ability of this laccase enzyme has been studied extensively and has demonstrated a very good ability to remove substrates such as phenol, bisphenol A and 17-β estradiol (E_2) from aqueous solutions. In order to minimize the amount of enzyme required to achieve effective treatment, this study focuses on characterizing the removal of E_2 using immobilized laccase. Through this approach, it is anticipated that treatment costs will be reduced since immobilization permits the re-use of the active enzyme, rather than discarding the enzyme with treated solutions. The enzyme was immobilized by covalent bonding onto silica beads and the reactions were conducted in a bench-scale continuous-flow packed bed reactor. The influent concentration of E_2 was 10 μM for most studies.

The effects of mean residence time were determined for several influent E_2 doses, and observable E_2 transformation occurred under the reaction conditions employed. The stability and reactivity of the immobilized enzyme were observed over varying temperature and pH. As expected, conversion declined when the temperature of the system was changed from room temperature to near freezing at pH 5. However, this decline reflected a change in the rate of reaction and not an instability of the enzyme since it was found that conversion was restored to its original level when the system was brought back to room temperature. Likewise, conversion increased when the

system was brought to warmer temperatures, and conversion levels were restored when the system was brought back to room temperature. Previous work conducted with aqueous laccase had demonstrated that the enzyme is more reactive toward E_2 at pH 5, but the enzyme is slightly more stable over the long term at pH 7. As expected, in the present study, E_2 removal increased when the pH of the influent to the immobilized laccase reactor was changed from pH 7 to pH 5. Also, studies aimed at observing the more long-term changes in reactivity for the system which is used only periodically for E_2 removal and is stored at either pH 5 or pH 7 confirmed the enzyme to be more stable at pH 7 in the immobilized system. Studies also showed that the immobilized enzyme maintained a constant level of activity when treating a constant supply of aqueous E_2 at a low mean residence time over a 12 hours period and when treating a constant supply of aqueous E_2 at high mean residence time over a period of 9 days. Results from tracer studies suggest that the reactor used in this study was far from optimal; thus, the transformation of E_2 under the studied conditions might be increased significantly simply by optimizing the reactor and its flow characteristics.

Résumé

La perturbation des systèmes endocriniens est un problème qui gagne de plus en plus d'importance dans l'environnement naturel. Les scientifiques continuent de trouver bien des effets néfastes dans les poissons et la faune qui ont été exposé à une grande variété de composants toxiques ayant été rejetés dans les systèmes aquatiques à travers les effluents municipaux et industriels et le ruissellement agricole. Les estrogènes qu'excrètent les humains et qui se retrouvent ensuite dans les systèmes aquatiques après avoir passé à travers les usines de traitement d'eaux usées sont particulièrement intéressantes d'un point de vue de recherche car cette excrétion ne peut être contrôlé et les estrogènes comptent parmi les substances perturbatrices des systèmes endocriniens les plus néfastes. En tant que composés phénoliques, les estrogènes peuvent être oxydés et polymérisés dans des réactions catalysés par des enzymes oxydatifs. Des études précédentes avaient comme objectif de caractériser la transformation d'estrogènes catalysé par les enzymes peroxydase, ainsi que l'enzyme fongique laccase de *Trametes versicolor* dans la phase aqueuse. La capacité de l'enzyme laccase a déjà été étudié intensivement et celui-ci a démontré une bonne réduction de substrats nocifs tels que le phénol, le bisphénol A et le 17β- œstradiol (E_2) dans les solutions aqueuses. Afin de minimiser les quantités d'enzyme requises pour effectuer le traitement de E_2, la présente étude a comme objectif de caractériser la réduction de E_2 en utilisant le laccase immobilisé par liaisons covalentes sur de petites perles de silice dans un réacteur de taille laboratoire. Ainsi, il est prévu que les couts de traitement pourraient être réduits puisque l'immobilisation permet de réutiliser l'enzyme plutôt que de le jeter avec les effluents traités.

Les effets du temps de résidence moyen dans le réacteur ont été étudiés pour quelques différentes doses de E_2. Une transformation de E_2 a été observe sous des conditions de réactions variées. Les résultats ont démontrés que le temps de contact nécessaire afin de réduire de façon significative le E_2 est de moins de dix minutes. La stabilité et la réactivité de l'enzyme immobilisé ont été observées contre des pHs et des températures variées. Comme prévu, la transformation de E_2 est diminué quand la température

du système a tombé de 21°C à 3°C au pH 5. Cette diminution reflète un changement dans le niveau de réaction et non d'une instabilité de l'enzyme, puisque l'on a pu observer que la transformation a atteint son niveau original lorsque la température a été remontée à 21°C. De même, la transformation de E_2 a augmenté lorsque la température a été monté de 21°C à 33°C. Des études précédentes dans la phase aqueuse avaient démontrées que le laccase est plus réactif au pH 5 mais plus stable au pH 7. Les différents pHs ont eu les mêmes effets dans le cas du laccase immobilisé. La réactivité était plus élevé au pH 5, mais lorsque l'on a rangé deux réacteurs pendant trois mois, celui entreposé au pH 7 a démontré beaucoup plus de stabilité que celui entreposé au pH 5. Cette étude a aussi démontré que le laccase est stable au cours d'une longue durée de réactions. Lors du traitement de E_2 avec un bas temps de contact au pH 5 pendant douze heures, ainsi qu'avec un haut temps de contact au pH 7 pendant neuf jours, le laccase n'a perdu de son activité. Les résultats obtenus d'études de traceurs suggèrent que le réacteur utilisé dans ces expériences est loin d'être optimal. Il est donc fortement possible que la transformation de E_2 sous les conditions prescrites serait augmentée significativement en améliorant la forme du réacteur et les conditions de flux.

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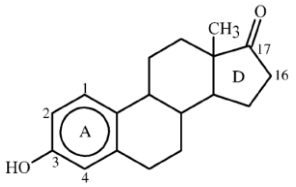
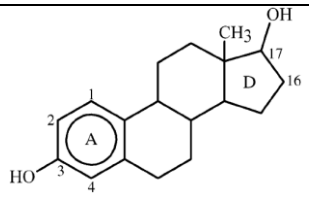
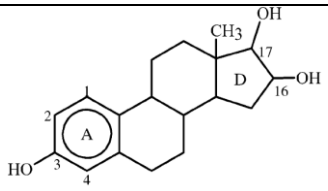
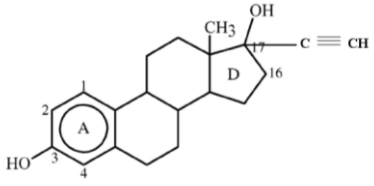
1. Introduction

Endocrine disruption is a problem of increasing environmental importance, as made evident by anomalies that continue to be discovered in wildlife exposed to a variety of exogenous toxic compounds. These compounds are released into the aquatic environment via municipal and industrial effluents and agricultural runoff (Sumpter, 2005). Estrogens excreted by humans and entering aquatic systems via sewage treatment plants are of particular interest, as estrogen excretion cannot be feasibly controlled at the source and estrogens are among the most potent known endocrine disruptors (Birkett and Lester, 2003).

Particularly problematic estrogens include the natural estrogens estradiol (E_2), estriol (E_3) and estrone (E_1) and also the synthetic estrogen ethinylestradiol (EE_2), which is the active ingredient in birth control pills and other therapeutic reagents. Such compounds are frequently found in wastewaters and in surface waters in quantities that are in excess of their predicted no-effect concentrations (Khan and Nicell, 2007). Given that these estrogens are phenolic compounds (see Table 1), they are amenable to oxidation through the catalytic action of a variety of oxidative enzymes (Khan and Nicell, 2007). Thus, it has been postulated that there is potential for applying enzymes to treat problematic estrogens commonly found in waste streams (Petrenko et al., 1999; Suzuki et al., 2003; Auriol et al, 2006; Khan and Nicell, 2007).

The recognition of this potential has recently prompted numerous researchers to explore the technical feasibility of this process. Much of this work was directed toward evaluating the ability of plant peroxidases and fungal laccases to treat aqueous-phase estrogens in bench-scale batch reactors (Khan and Nicell, 2007; Fang et al., in preparation). While peroxidase enzymes have demonstrated relatively high redox potentials and can achieve appreciable transformation of their target substrates, even at very low concentrations in the nanomolar to micromolar range (Hayashi and Yamazaki, 1979; Auriol et al., 2006; Khan and Nicell, 2007), their catalytic activity requires

Table 1: Chemical structures of problematic estrogens.

Compound	Structure
Estrone (E_1)	
17β-Estradiol (E_2)	
Estriol (E_3)	
17α-Ethinylestradiol (EE_2)	

the use of hydrogen peroxide as an oxidant (Nicell et al., 1992). In contrast, laccase enzymes have a lower redox potential, but have the advantage of using readily available molecular oxygen as an oxidant (Kurniawati and Nicell, under review). Also, substrate conversion can be enhanced when laccase is used in conjunction with various non-catalytic co-oxidants known as mediators (Kurniawati and Nicell, 2007, a). Of the many laccases that can be selected as candidate enzymes for treatment processes, laccase from *Trametes versicolor* exhibits amongst the highest redox potentials (Yaropolov et al., 1994) and has demonstrated a very good ability to remove substrates such as phenol, bisphenol A, and estrogens, including estradiol (E_2), 17-α ethinylastradiol (EE_2), and estriol (E_3), from aqueous solutions (Kurniawati and Nicell, under review; Kim and Nicell, 2006; Fang et al., in preparation).

The use of enzymes for wastewater treatment has many potential advantages including the substrate specificity of enzymes, no susceptibility of the biocatalyst to shock-loading effects (i.e., since it is a biochemical system rather than a biological system), high rates of reaction, efficient use of oxidants, and effective treatment at low substrate concentrations (Aitken, 1993; Karam and Nicell, 1997). While the use of enzymes for transforming waste compounds has many advantages, there are also some drawbacks that could limit their application, such as the gradual inactivation of the enzyme over time, inactivation of enzymes due to side reactions in reacting mixtures, and interference of wastewater contaminants with enzymatic reactions (Nicell, 2003). Moreover, when the aqueous enzyme is used to treat contaminants in waste streams, the high cost of continually discarding the enzyme with treated solutions may be particularly prohibitive.

In earlier work with peroxidase and laccase, the enzymes were found to be quite robust in terms of their ability to catalyze the oxidation of substrates in the presence of many wastewater contaminants and under wide ranges of pH and temperature (Nicell, 2003; Kim and Nicell, 2006; Wagner and Nicell, 2000). Inactivation was found to occur with both types of oxidase enzymes, but their rates of inactivation were found to be low when substrate concentrations were low (Buchanan and Nicell, 1999; Kurniawati, 2006; Khan and Nicell, 2007). All of these results are quite promising in terms of the treatment of estrogens that are present in wastewaters at very low concentrations. However, the problems associated with the release of enzymes in the treated effluents still represent a significant limitation that must be addressed. Thus, it is hypothesized that immobilizing the enzymes onto a solid support for continual use in a reactor system could mitigate some of these problems. Immobilization is a process whereby enzyme is attached or adsorbed onto some water-insoluble matrix. The enzymes can thus be retained in a reactor and exposed to a continuous flow of substrate over time.

In order to attempt to minimize the amount of enzyme required to achieve effective treatment of estrogens, the objective of the present study was to characterize the removal of E_2 using *Trametes versicolor* laccase immobilized onto controlled porosity silica beads

and used in a small-scale flow-through reactor. E_2 was chosen as a model substrate because it is the most potent of the natural female hormones, and previous studies found very promising trends in the removal of this and other estrogens (Arcand-Hoy et al., 1998; Khan and Nicell, 2007; Fang et al., in preparation). The impacts of the following system conditions on the transformation of E_2 by laccase immobilized in a packed bed reactor were investigated: pH, contact time, initial E_2 concentration, and temperature. The stability of the enzyme over time and under varying conditions of pH and temperature were also studied.

It should be noted that all experiments were conducted using influent E_2 concentrations much higher than that which are typical of wastewater treatment plant influents; i.e., experiments were conducted in the mg/L range whereas E_2 has been found in treatment plant effluents in the ng/L range (Aerni et al., 2004). This was done in order to establish clear and informative trends, while working within the confines of available analytical equipment. However, the influent E_2 concentrations used in experiments are quite close to those found in human female urine and so the results may be relevant if treatment were to be performed at the source (Escher et al., 2006).

2. Literature Review

2.1 Laccase Enzyme and Its Applications

Laccase (EC 1.10.3.2) is one of the oldest known enzymes, having been the subject of studies beginning at the end of the nineteenth century when it was first demonstrated in the discharge of a Japanese lacquer tree (Yoshida, 1883) and a few years later in fungi (Bertrand, 1896). Although it was discovered a long time ago, laccase only began garnering considerable attention once its role in the degradation of wood by fungi came to light (Baldrian, 2005). Today, this enzyme is being studied for use in many applications, including delignification in the pulp and paper industry, improvement in the production of fuel ethanol, drug analysis, wine clarification, and bioremediation of wastes containing trichlorophenols, alkenes, synthetic dyes, herbicides, and many other phenolic and non-phenolic pollutants (Mayer, 2002). This widespread use is due to the exceptional versatility of laccase (Mayer, 2002), as well as its high rates of catalysis and its use of the readily available molecular oxygen as its co-factor (Kurniawati and Nicell, under review).

Laccase is a polyphenoloxidase that catalyses the oxidation of phenols and aromatic or aliphatic amines (Riva, 2006). This redox reaction is carried out by a cluster of four copper atoms in the catalytic centre of the enzyme (Yaropolov et al., 1994). As shown in Figure 1, in a typical laccase reaction, one molecule of oxygen is reduced to two molecules of water, while simultaneously oxidizing four substrate molecules to their respective radicals (Riva, 2006). It should be noted that this stoichiometry has been shown to vary depending on the initial substrate concentration (Kurniawati and Nicell, 2007, b). The radicals produced from the catalytic reaction can then undergo non-enzymatic reactions leading to the production of polymers (Duran et al., 2002). In the context of the treatment of waste, it is important to determine the characteristics of the formed polymers. For example, these products should not be more toxic or, in the case of

estrogens, more estrogenic than the original substrate. In the case of 17β-estradiol, it was found several decades ago in rat studies that the polymers formed from enzymatic oxidation of the estrogen were non-estrogenic, meaning they did not interact with estrogen receptors in cells (Klebanoff and Segal, 1960).

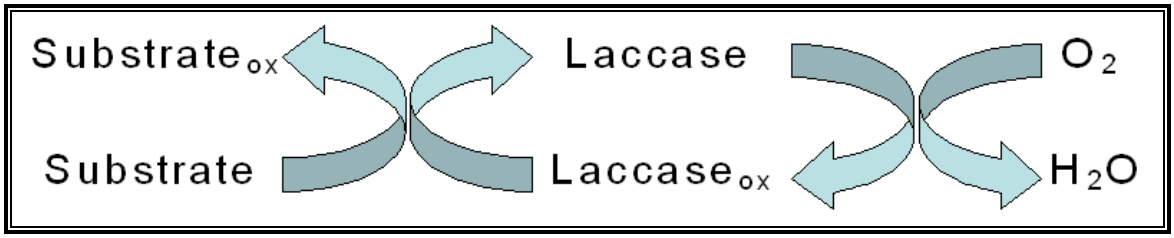


Figure 1: Catalytic cycle of laccase.

Generally, laccases isolated from fungi and higher plants have been studied for use in remediation applications; however, laccases are also naturally produced for various functional reasons in bacteria and insects as well (Baldrian, 2006; Kurniawati and Nicell, under review). It has been observed that the activity of laccase is related to its redox potential (Xu, 1996). Thus, fungal laccases may be more effective in remediation systems, as they generally have higher redox potentials than plant laccases (Yaropolov et al., 1994). Of the fungal laccases which have been studied, the laccase from *Trametes versicolor*, whose tertiary ribbon structure is shown in Figure 2, has been shown to have the highest redox potential (Yaropolov et al., 1994), from which it is assumed to have a relatively high activity. The fungus *Trametes versicolor*, shown in Figure 3, is a polypore mushroom found practically all over the world (Shirley, 2001). Its natural prevalence and the long-time interest shown toward it for industrial applications have driven industries to mass produce this laccase at a relatively low cost (Ikehata et al., 2004).

Many studies have studied the transformation of phenolic environmental pollutants using this species of laccase (Roy-Arcand and Archibald, 1991; Majcherczyk et al., 1998; Wong and Yu, 1999; Jolivalt et al., 2000; Peralta-Zamora et al., 2003; Dodor et al.,

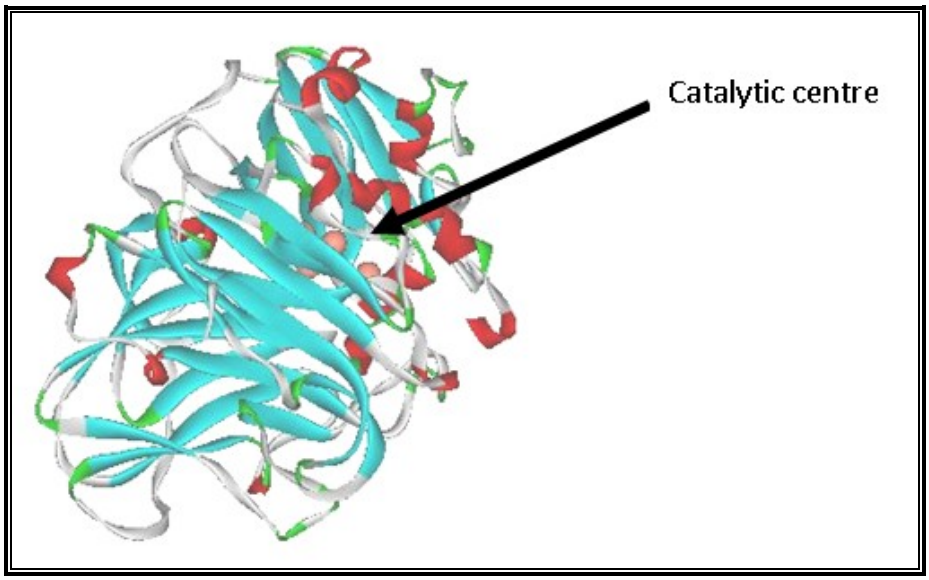


Figure 2: Tertiary ribbon structure of laccase from *Trametes versicolor* (adapted from Piontek et al., 2002).

Figure 3: Photograph of *Trametes versicolor* fungus (Shirley, 2001).

2004; Lee et al., 2004; Keum and Li, 2004; Kim and Nicell, 2006; Kurniawati and Nicell, under review; Fang et al.,. in preparation; Ryan et al., 2007). Notably, laccase from *Trametes versicolor* has been shown to oxidize phenol, bisphenol A, and various natural and synthetic estrogens in batch reactions at relevant environmental conditions, albeit with artificially high substrate concentrations in the case of estrogens (Kurniawati and Nicell, under review; Kim and Nicell, 2006; Fang et al., in preparation). Kurniawati and Nicell (under review) and Kim and Nicell (2006) found that reactions were catalyzed optimally by laccase around pH 5 to 6, but that the stability of the enzyme under non-reacting storage conditions was optimal around pH 7. They also observed that increasing temperature resulted in higher enzyme activity (i.e., rate of reaction) but adversely impacted enzyme stability. It has also been observed that step changes in temperature or pH generally entail an enzyme activity recovery period on the order of several minutes to several hours (Kurniawati and Nicell, under review), during which the tertiary structure of the enzyme untangles itself and drives to an equilibrium state corresponding to its pH- or temperature-specific conformation (Palmer, 1995). In general, treatment effectiveness was demonstrated by the researchers, but a significant disadvantage of using "free" laccase in all of these studies was that the enzyme was continually discarded with the treated solutions.

2.2 Immobilization of Enzymes

From the onset of research into using enzymes for industrial and waste treatment functions, it was clear that the full-scale application of these methods would be hampered by the high cost and limited availability of the required enzymes. The idea of attaching an enzyme to an insoluble matrix such that the enzyme can be used over its active life is not novel. In fact, the first attempt to immobilize an enzyme dates back to the early 1950s (Grubhofer and Schleith, 1954). Then, in the late 1960s, the first industrial applications of immobilized enzymes were achieved in Japan (Marconi, 1989; Chibata, 1978). Interest in immobilization of enzymes then waned for some years, until the development

of many new genetic engineering techniques in the 1980s promised to provide large quantities of enzymes at high purity (Marconi, 1989).

The main benefit of "insolubilizing" enzymes through the use of immobilization techniques is the anticipated very significant reduction in cost, since the enzymes can be used again and again over the course of their active lives for catalyzing desired reactions of substrates. The costs of using immobilized enzymes can still be very high, however, because of the potentially high costs of equipment and control of operating conditions (Marconi,1989). Other limitations faced by a reactor of immobilized enzymes include mass transfer limitations, enzyme inactivation, loss of enzyme from the support media, loss of the support media itself, compaction of the support structure, plugging of the reactor matrix by suspended solids, reactions of substrates with residual binding agents, and microbial growth (Kay, 1968; Siddique, 1992).

There exist numerous chemical and physical methods for immobilizing enzymes onto a wide range of supports. The four principal means of immobilizing enzymes onto polymeric supports are (Marconi, 1989):

- creating covalent bonds between the enzymes and polymeric material through a reaction with functional groups that are not involved in the redox reactions;
- cross-linking with natural polymers or synthetic macromolecules;
- adsorption onto insoluble matrices; and
- physical entrapment within cross-linked polymer matrices and fibres.

Physical immobilization techniques tend to result in higher initial enzyme activities but lower activity retention over time, whereas chemical methods tend to result in slightly lower initial enzyme activities but with greater stability over time (Duran et al, 2002). The most common method on the laboratory scale is covalent binding onto inorganic polymeric material (Marconi, 1989). The chemical linkages between the enzyme

and its polymeric support are known to be stable with varying temperature, pH, ionic strength, substrate concentration, and other variables (Marconi, 1989). On the other hand, the disadvantages of this method are that the immobilization process is laborious and requires the use of reagents which are often environmentally toxic and need to be carefully discarded (Duran et al., 2002).

The protocol for covalent immobilization of enzymes generally involves three steps. The first step is to modify the surface of the inorganic support by adding organic functional groups. This is often done through silanization, and supports that have already undergone this initial step can be purchased for laboratory use. These coating organic functional groups are then derivatized either to arylamine groups using *p*-nitrobenzoyl chloride or to aldehyde groups using glutaraldehyde (Duran et al., 2002). The support material can then be exposed to the enzymes, at which point the derivatized functional groups on the media will form covalent bonds with the amino, carboxyl, phenol, sulphydryl, hydroxyl, imidazole, and/or indole groups present in the protein (Marconi, 1989). The two main supports used in laboratories are controlled porosity glass and controlled porosity silica (Girelli and Mattei, 2005). Glass exhibits greater stability over a wider range of temperatures and is more resistant to acid, while silica exhibits a higher specific surface area (Girelli and Mattei, 2005).

There exist numerous examples in the literature of laccase from *Trametes versicolor* immobilized in this way onto silanized porous glass or silica beads treated with glutaraldehyde (Duran et al., 2002). In various studies, the following observations were made concerning immobilized enzymes (Duran et al., 2002; Leonowicz et al., 1988):

- the immobilized enzyme showed a higher specific activity (rate of reaction measured using an oxygen probe) than its equivalent in the aqueous phase;
- its kinetic parameters were not affected;

- it was generally very stable over long-term applications; and
- it was less sensitive to high temperatures than the free enzyme.

Often, the properties of an immobilized enzyme are significantly different from those of the free enzyme because of the changes in the enzyme's structure resulting from immobilization and because of the modified environment within which the enzyme must catalyse reactions (Siddique, 1992); thus, the properties of the immobilized enzyme cannot be completely inferred from those of the free enzyme. For example, the nature of the surface charges on the carrier medium has been found to shift the pH curve of the immobilized enzyme with respect to that of its free counterpart: e.g., media with polycationic functional groups tend to enhance activity in the acid range, while media with polyanionic functional groups tend to enhance activity in the alkaline range (Goldstein, 1970; Levin et al., 1964).

The operational and storage stability of an immobilized enzyme reactor are critical engineering considerations. Operational and storage stability are most significantly affected by duration, pH, ionic strength, and temperature (Siddique, 1992). Additionally, operational stability can be affected by extent of use (Duran et al., 2002). In his review of fifty stored immobilized enzyme systems, Melrose found that thirty were more stable than the free enzyme, eight were less stable, and twelve were similar (Melrose, 1971). Using free *Trametes versicolor* laccase to transform phenol, Kurniawati found that the enzyme was most stable over time when in non-reacting aqueous solutions at pH 6 to 7 (Kurniawati and Nicell, under review). In a study of laccase from the fungal species *Pleurotus ostratus* that had been immobilized by covalent bonding to a solid support, it was found that storage stability over time was much higher in the alkaline range, especially from pH 9 to 11, whereas enzyme activity decreased sharply over time throughout the acid range (Hublik and Schinner, 2000).

The type of reactor chosen to hold the immobilized enzymes is also a very important engineering consideration. There are basically three different types of reactors from

which to choose: completely-mixed tank reactors, packed-bed reactors, and fluidized-bed reactors. Of these, packed-bed reactors most closely approximate plug flow, which under ideal conditions could achieve the highest conversion for a given residence time (Fogler, 2005). The choice of reactor type and its operational characteristics depends on many factors, including: the form of the immobilized enzyme (i.e., particulate, membranous, fibrous), the nature of the substrate (i.e., soluble, particulate, colloidal), surface to volume ratio of the carrier medium, mass transfer characteristics, ease of fabrication and operation, and reactor costs (Vieth and Venkatasubramanian, 1974).

3. Materials and Methods

3.1 Chemicals and Reagents

Laccase from *Trametes versicolor* (EC 1.10.3.2) was purchased from Fluka (Ronkonkona, NY). The nominal activity quoted by the manufacturer was 0.72 U/mg, where 1 U corresponds to the amount of enzyme which converts 1 μmol of catechol per minute at pH 6.0 and 25 °C. The dry enzyme powder was stored in a sealed container in a refrigerator at 4°C until needed. 17ß-estradiol (E_2) (> 98% purity) was purchased as a dry powder from Sigma-Aldrich (St-Louis, MO). Concentrated stock solutions of E_2 were prepared in methanol and stored in a refrigerator at 4°C until needed. Glutaraldehyde solution (25%, Grade II), glycine, and American Chemical Society (ACS) grade phosphoric acid (85% in water) were purchased from Sigma-Aldrich (St-Louis, MO). Sodium chloride and boric acid powder were purchased from Fluka (Ronkonkona, NY). Sodium hydroxide (1 N) and sulphuric acid (1 N) were purchased from Fisher (Fair Lawn, NJ). Glacial acetic acid (HPLC grade) was purchased from Caledon Laboratories (Georgetown, ON). Britton-Robinson buffer was used to maintain the desired pHs of all reaction solutions. The buffer was made up of a solution of 0.1 M acetic acid, 0.1 M boric acid, and 0.1 M phosphoric acid, which was then adjusted to the desired pH by addition of 1 N sodium hydroxide. All reagents and buffers were prepared using distilled-deionized water produced using a D4741 Nanopure Water System from Barnstead/Thermolyne (Dubuque, IA).

Methylene blue powder purchased from Anachemia (St-Laurent, QC) was used in all tracer studies. A concentrated methylene blue solution was prepared in distilled water and stored. More dilute solutions of 4.8 mg/L concentration were prepared in distilled water on the day of the tracer study.

CPC (controlled porosity carrier)-silica carrier silane-coated beads were purchased from

Fluka (Ronkonkona, NY). As indicated by the manufacturer, these beads were derivatized with 3-aminopropyl-triethoxysilane and were reported as 30 to 45 mesh with a pore size of 375 Å. Since the beads are sensitive to humidity, they were stored in sealed containers in a cool, dry place until use.

3.2 Immobilization

Laccase was immobilized on the CPC-silica beads using a method adapted from Champagne (2005). First, the 8 g of beads were immersed in 2.5% glutaraldehyde in a stoppered filtration flask and put under light vacuum for 2 hours in order to degas the beads and allow as much bead surface area as possible to be coated with aldehyde groups. Subsequently, the beads were thoroughly washed with buffer and were then immersed in a stock laccase solution (i.e., 200 mg of laccase in 200 mL of buffer prepared the same day) for at least 18 hours at 4^{o}C. After thoroughly washing the beads with buffer again, the beads were rinsed with 0.5 M sodium chloride in order to prevent sorption of the enzyme onto the beads, as only the measurement of reactions by covalently bonded enzyme was desired. The beads were again thoroughly washed with buffer and then immersed in 2.5 mg/mL glycine for at least 18 hours at 4^{o}C. Once again, they were thoroughly washed, first with buffer, then 0.5 M sodium chloride, then buffer, and finally stored in buffer at 4^{o}C until use. All reagents used in the immobilization process were made in pH 5 buffer.

3.3 Chemical Analyses

Tracer studies were conducted in order to determine the mean-residence time of the immobilized-enzyme reactor system (see below) and also to determine the time it took for the reactor effluent to reach a steady-state concentration. During tracer studies, the absorbance of the methylene blue in the flow leaving the reactors was measured using a Hewlett Packard 8453 diode array spectrophotometer set to a wavelength of 624 nm.

Initially, a calibration curve, seen in Figure 4, was plotted to ensure that the proposed influent methylene blue concentration of 6.4 mg/L fell within the linear portion of such a curve.

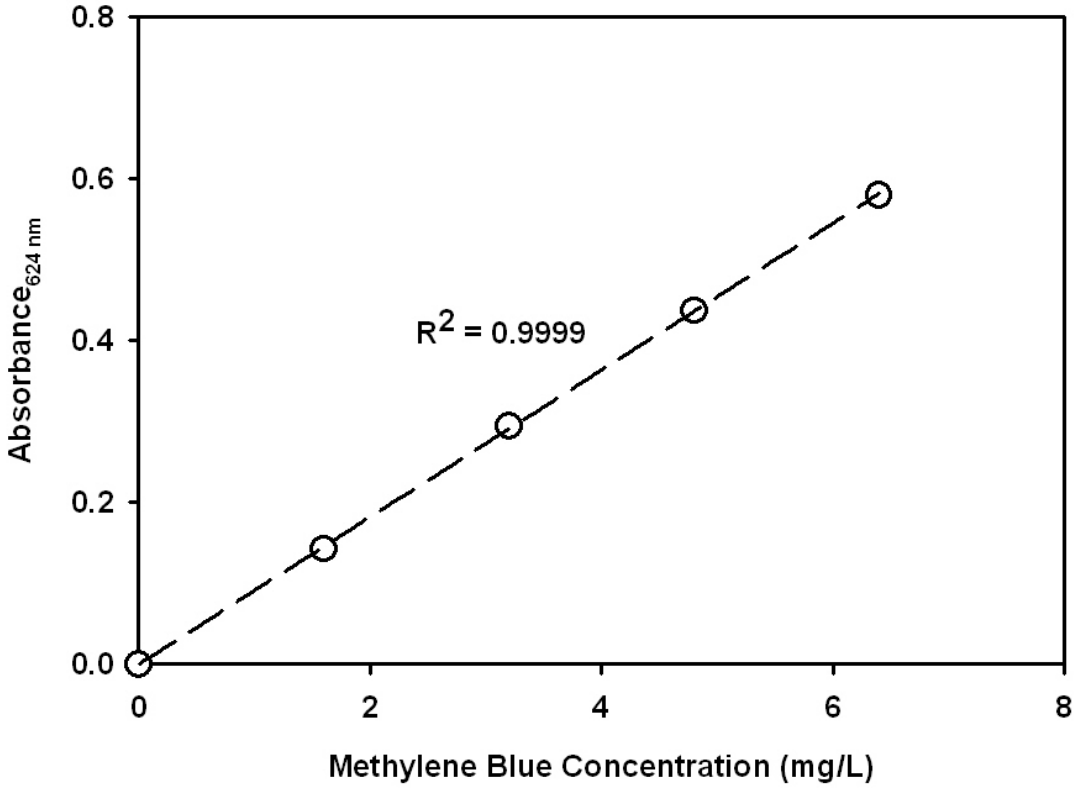


Figure 4: Calibration curve showing the linear relationship between concentration and absorbance in the entire range of concentrations observed in subsequent tracer studies.

The concentration of E_2 in samples was determined using a High Performance Liquid Chromatograph (Agilent HPLC 1100 Series) fitted with a reverse-phase column (Zorbax SB-C18 Column). A carrier phase consisting of 60% HPLC-grade acetonitrile and 40% distilled-deionized water was maintained at a flowrate of 0.8 mL/min, and an injection volume of 20 µL was used. This isocratic flow was maintained at 39^{o}C and was analyzed by the fluorescence detector (FLD) for 8 minutes. The FLD was set at an excitation wavelength of 230 nm and an emission wavelength of 310 nm. Because a linear trend relates the area under a substrate's FLD peak to the concentration of that substrate, a

calibration curve was prepared in order to translate FLD data to E_2 concentration.

3.4 Experimental Protocols

The immobilized enzyme reactor system used throughout this study is described in detail in Appendix C. Kontes Chromaflex 2.5 cm inner diameter, 15 cm length columns, with their accessory fittings, flow adapters, and1/8 inch inner diameter PTFE tubing were purchased from Fisher (Fair Lawn, NJ). Tygon 3/32 inch inner diameter tubing was also purchased from Fisher (Pittsburgh, PA). Additional fittings to join the two types of tubing were purchased from Mandel (Guelph, ON). The syringe pumps used were Harvard Apparatus 22 models, with which 50-mL capacity glass syringes were used. The continuous flow pump used was a Waters Millipore Model 510.

The beads onto which laccase had been immobilized were placed into the bottom of a column. A flow adapter was inserted into the column and placed above the beads so that the beads were immersed in buffer and also that a layer of 2 – 3 mm of buffer and 2 – 3 mm of air headspace existed above the bed. This resulted in a reactor media volume of 14.7 cm^3, and liquid and air headspace volumes of 1.0 – 1.5 cm^3 each. 85 cm of PTFE tubing was attached to the inlet of the reactor. When a syringe pump was being used to inject flow into the reactor, the end of the PTFE tubing was further attached by a fitting to 10 cm of Tygon tubing which could easily be attached to a 50-mL syringe. Otherwise, when using a continuous flow pump, the tubing was left as previously described.

Before any experiments with E_2 were undertaken, tracer studies were performed using silica beads (i.e., in their original state without immobilized enzyme) set up in a reactor exactly as described above. For each of the different flowrates that were to be used in subsequent experiments, water was passed through the column, and at a time zero, a constant flow of water at the desired flowrate containing 6.4 mg/L methylene blue was introduced. Samples of effluent were taken intermittently and analysed using the spectrophotometer.

For reaction experiments, an influent with the desired concentration of E_2 and pH was prepared and continuously stirred in its partially-covered container during an entire experiment. Once the pump was started, samples of reactor effluent were taken only after the system had reached near-equilibrium, as had been pre-determined by tracer studies with methylene blue passing through an equivalent reactor with enzyme-less beads. For all samples, 1 mL of effluent was collected and 100 μL of sulphuric acid was added to the sample to reduce the pH to below 2.5. The samples were acidified to halt any catalytic reaction that would be caused if some of the immobilized laccase had leached into the effluent, thereby causing more oxidation reactions to occur before the samples could be analysed by HPLC.

4. Results

In order to explore the use of immobilized laccase for removal of E_2 from aqueous solutions, experiments were conducted to determine the effects of pH, temperature, mean residence time, and initial substrate concentration on E_2 transformation. Studies were also conducted to study the short- and long-term stability of immobilized laccase under various working conditions.

In order to compare the efficacy of the enzyme at achieving E_2 conversion under various conditions such as pH and temperature and over time, it was necessary to ensure that the same quantity of enzyme was available for each experiment. At present, it is not possible to ensure that the quantity of enzyme immobilized on a given quantity of solid media in a reactor column is the same between experiments. Thus, it was not possible to directly compare results achieved using different columns. Therefore, in experiments conducted to measure the effect of selected variables, all experiments were run using the same column. However, when studying the effect of a control variable such as pH on substrate conversion, it was necessary to ensure that the enzyme activity in the reactor did not decrease during or between successive experiments. At present, no enzyme activity assay is available to monitor the activity of immobilized laccase (Note that this is discussed in greater detail in Appendix A). However, it is possible to measure the activity indirectly by passing a solution with fixed pH, temperature, initial substrate concentration and flowrate through the column and assessing the conversion of that substrate achieved under these conditions. If the quantity of substrate transformed declines over time, this is an indication that the activity in the column is also declining. This indirect measurement of activity will be used throughout experiments discussed below.

4.1 Tracer Studies

A tracer study with step injection of the tracer compound was performed at each of the

flow rates at which subsequent experiments would be performed in order to characterize the flow through the reactor and to determine the time needed under each flow condition for the reactor to reach equilibrium. This time to equilibrium gave an indication for future experiments of the minimum time the influent should flow through the reactor before a sample should be taken. The results from these tracer studies for influent flowrates ranging from 0.2 mL/min to 8 mL/min are shown in Figure 5 (a)-(e). A summary of flow characteristics arising from the tracer studies is reported in Table 2.

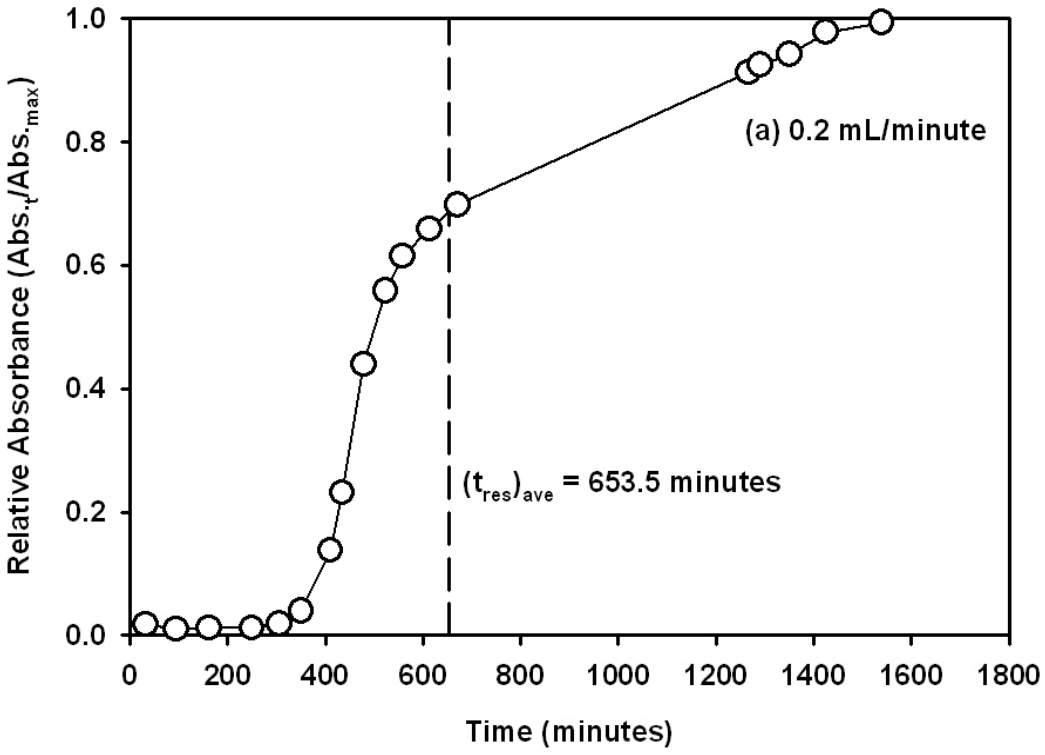
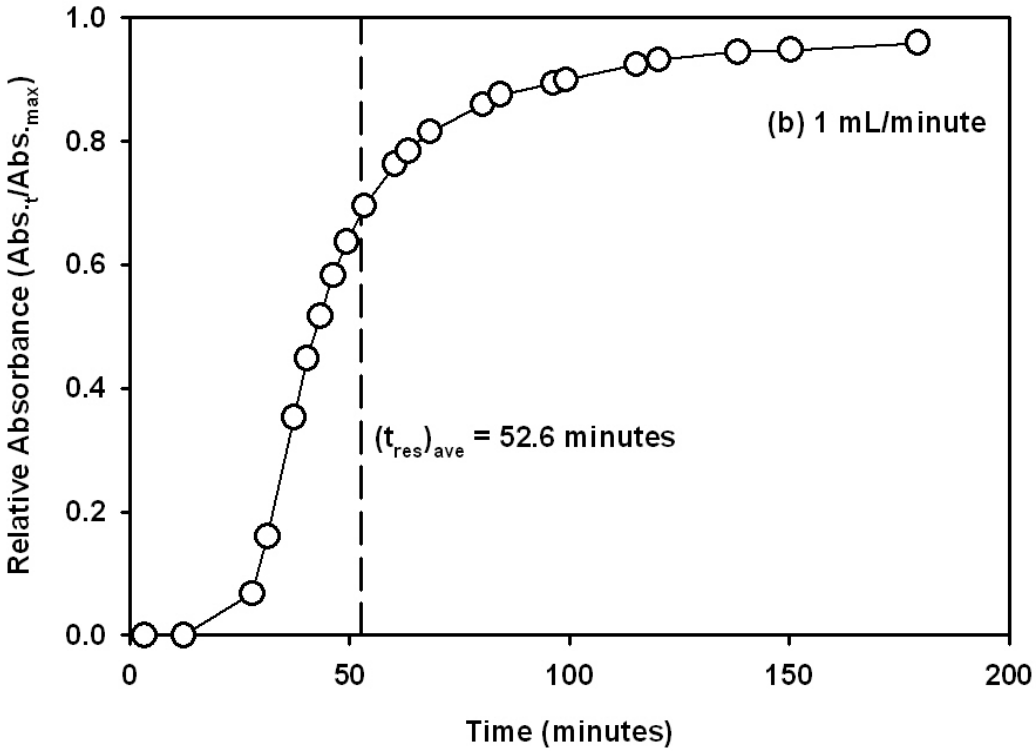
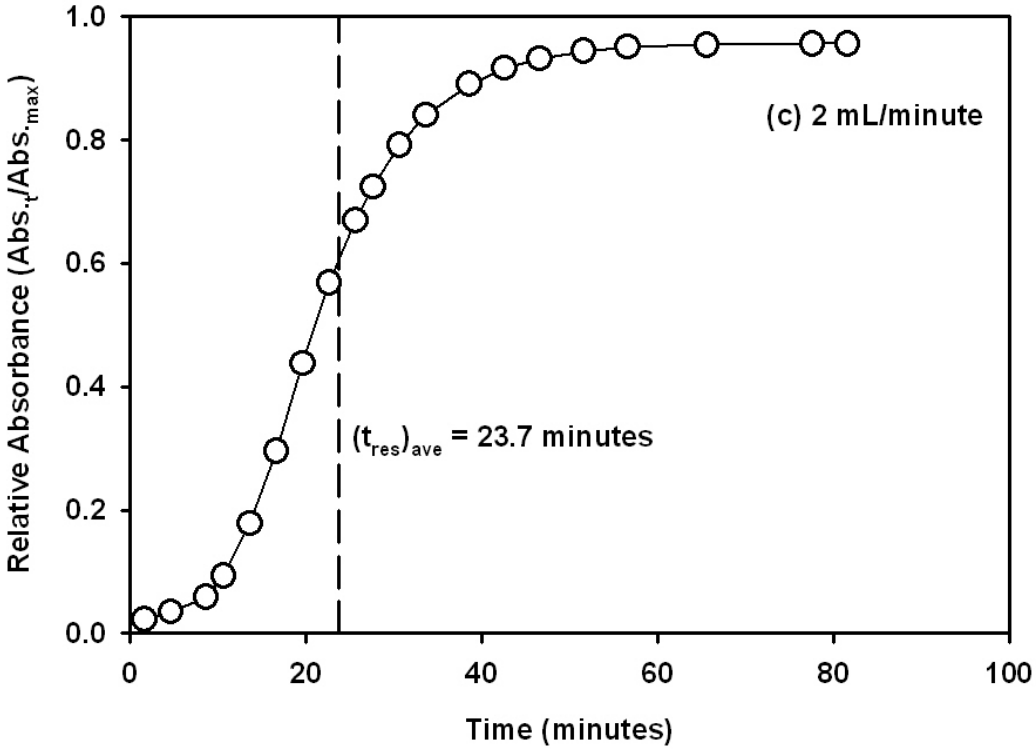
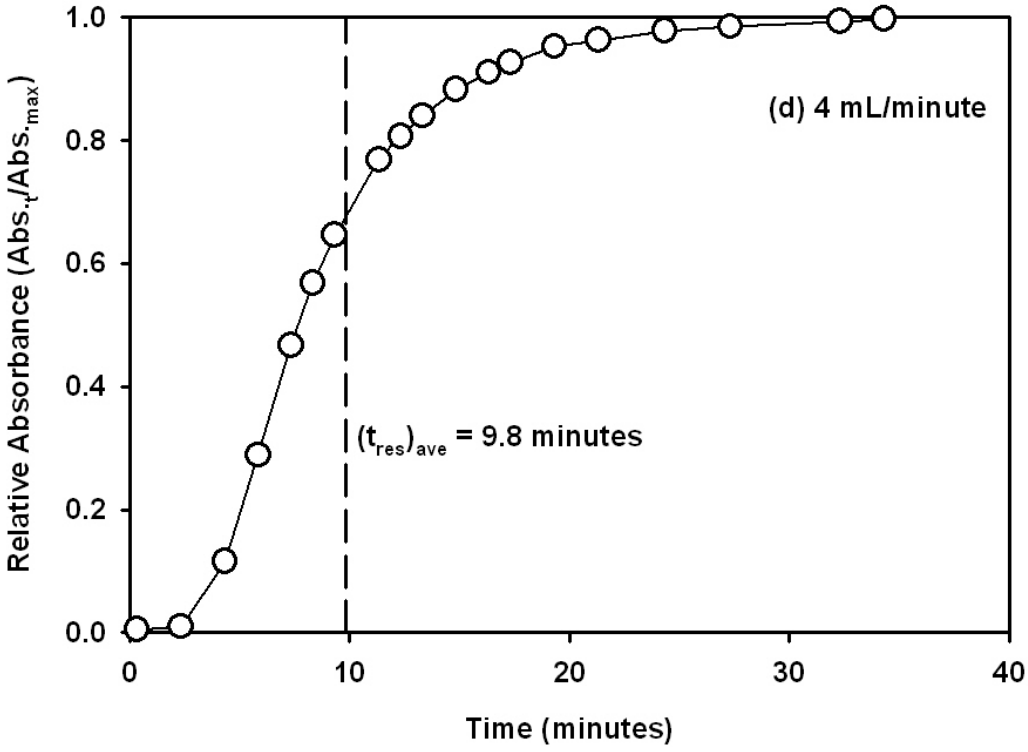


Figure 5: Tracer studies showing the absorbance of the tracer compound, methylene blue, over time in the effluent (Abs_t) from the packed bed reactor relative to the influent absorbance (Abs_{max}) at flowrates of (a) 0.2, (b) 1.0, (c) 2.0, (d) 4.0 and (e) 8.0 mL/min. Experimental conditions: $[Methylene\ blue]_i$ = 6.4 mg/L, 21°C, 8 g. (Figures 5(b)-(e) follow on subsequent pages.


1.0
0.8
0.6
0.4
0.2
0.0
Relative Absorbance (Abs.t/Abs.max)
(b) 1 mL/minute
(tres)ave = 52.6 minutes
0
50
100
150
200
Time (minutes)


1.0
0.8
0.6
0.4
0.2
0.0
Relative Absorbance (Abs.t/Abs.max)
(c) 2 mL/minute
(tres)ave = 23.7 minutes
0
20
40
60
80
100
Time (minutes)


1.0
0.8
0.6
0.4
0.2
0.0
Relative Absorbance (Abs.t/Abs.max)
(d) 4 mL/minute
(tres)ave = 9.8 minutes
0
10
20
30
40
Time (minutes)

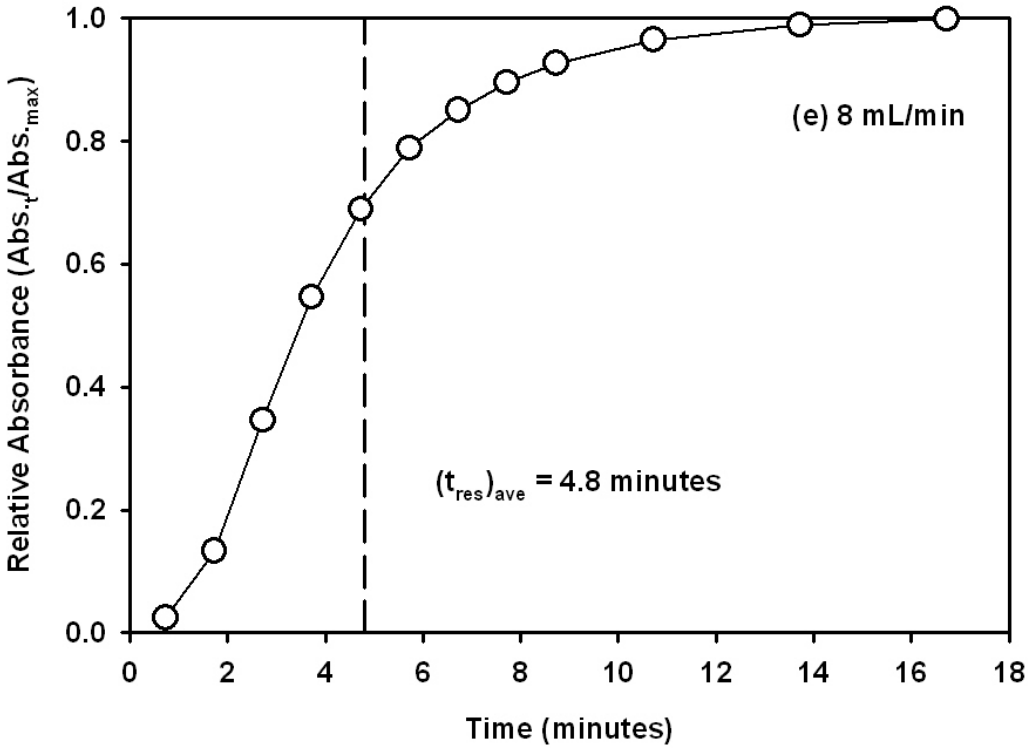

1.0
0.8
0.6
0.4
0.2
0.0
Relative Absorbance (Abs.t/Abs.max)
(e) 8 mL/min
(tres)ave = 4.8 minutes
0
2
4
6
8
10
12
14
16
18
Time (minutes)

Figure 5 (a) shows data arising from the tracer study conducted at 0.2 mL/min. In this case, a data gap is present because the data could not be collected continuously over the time due to the lengthy conditions of the experiment. The method of tracer data interpretation by Levenspiel (1979) requires data which is not too spaced out, however, it was apparent that the trend within the gap was probably very close to linear, and so the method was used with the data as is, with the assumption that this would not introduce significant error. In Figures 5 (b) and (c) it is not clear why the relative absorbance plateaued but did not reach 1. This is attributed to experimental error.

The porosity, n, of the packed bed of silane-coated silica beads was determined according to the water saturation method. Three trials gave an average porosity of 0.68 ± 0.03. The mean residence time, $(t_{res})_{ave}$, and variance, σ^2, of the reactor under each flow condition were evaluated according to the method of tracer study data interpretation presented by Levenspiel (1979). These characteristics, along with each empty bed contact time (EBCT), the ideal contact time (CT, assuming plug flow conditions), and the time to reach 95% of the equilibrium (i.e., the effluent tracer concentration reached 95% of the influent value) are presented in Table 2.

Table 2: Summary of tracer study results and characteristics of the packed bed reactor system with a porosity (n) of 0.68 for each of the flowrates used in all subsequent immobilized-enzyme experiments.

Flowrate (mL/min)	EBCT (Note 1) (min)	CT (Note 2) (min)	$(t_{res})_{ave}$ (min)	σ^2 (min^2)	Time to 95% equilibrium (min)
0.2	73.5	50.0	655	103 000	1400
1.0	14.7	10.0	52.6	730	160
2.0	7.3	5.0	23.7	112	65
4.0	3.7	2.5	9.8	27.4	19
8.0	1.8	1.2	4.8	8.3	11

Note 1: EBCT = Empty bed contact time;

Note 2: CT = ideal contact time assuming plug flow conditions

The time to reach 95% of equilibrium was determined and used as a basis for sampling in subsequent experiments because in reactor systems with low flow rates, and hence high mean residence times, the time to reach full (100%) equilibrium was inordinately long. In experiments in which time is the independent variable, this was not a concern, as the experiments were conducted for much longer than this time to 95% equilibrium. Otherwise, in experiments where samples were taken after the time to 95% equilibrium, triplicate samples, with lags between each sampling time, were taken. The values in Table 2 were calculated as follows (Levenspiel, 1979):

$$EBCT = \frac{Volume}{Flow\ rate} \tag{4.1}$$

$$CT = EBCT \times n \tag{4.2}$$

$$(t_{res})_{ave} = \frac{\sum_{i=1}^{n} t_i \Delta Abs._i}{\Delta Abs._{\max}} \quad where\ \Delta Abs._i = Abs._i - Abs._{i-1} \tag{4.3}$$

$$\sigma^2 = \frac{\sum_{i=1}^{n} t_i^{\,2} \Delta Abs._i}{\Delta Abs._{\max}} - \bar{t}^{\,2} \tag{4.4}$$

As seen in Table 2, the variance became much greater as the mean residence time increased, indicating that the flow characteristics deviated more and more from plug flow as the mean residence time increased.

4.2 Effect of pH on Substrate Transformation

In order to be effective as a catalyst in applications involving the conversion of a target

substrate, laccase must not only be stable under various environmental conditions such as pH, it must also be able to exert its catalytic action on a target substrate. Therefore, studies were conducted to optimize the transformation of E_2 over a range of pHs. However, given earlier findings that the optimal conditions for laccase stability as well as transformation of phenolic substrates in the aqueous phase are in the slightly acidic to neutral conditions (Kurniawati and Nicell, under review; Kim and Nicell, 2006; Fang et al., in preparation), the effect of pH on immobilized laccase was studied over a limited pH range of 4 to 7.

An experiment was conducted in which 10-μM solutions of E_2 were prepared in buffers ranging from pH 4 to 7 and passed through the reactor in order to assess transformation of E_2. The initial concentration of the stock solution was measured using HPLC. Between runs at each pH, a solution of E_2 in pH 5 buffer was passed through the reactor and a sample of the effluent E_2 concentration was measured in order to ensure that enzyme activity had remained relatively constant over the course of the experiment. Thus, the pH experiments were conducted in the following order: pH 5, pH 7, pH 5, pH 6, pH 5, and pH 4. Each part of the flow sequence was run for 40 minutes, and at each pH, a series of three samples were taken, each two minutes apart. The effect of pH on the transformation of E_2 by immobilized laccase is shown in Figure 6.

The effect of pH on the removal of E_2 by immobilized laccase follows the same trend as was observed previously for laccase in the aqueous phase (Fang et al., in preparation); i.e., transformation was optimal at pH 5, followed closely by high removal at pH 6. Though the pH optimum of 5 does not fall into the typical range of pHs for domestic wastewaters, which is from pH 6.7 to pH 7.5 (Qasim, 1999), this pH was used for most subsequent experiments in order to optimize reaction conditions for the use of laccase. However, some experiments that involved the measurement of enzyme stability over time were performed at pH 7, as it has been found that, although transformation rates tend to be lower at pH 7, the enzyme was observed to be more stable in the aqueous phase over a longer period at this pH (Kurniawati and Nicell, under review).

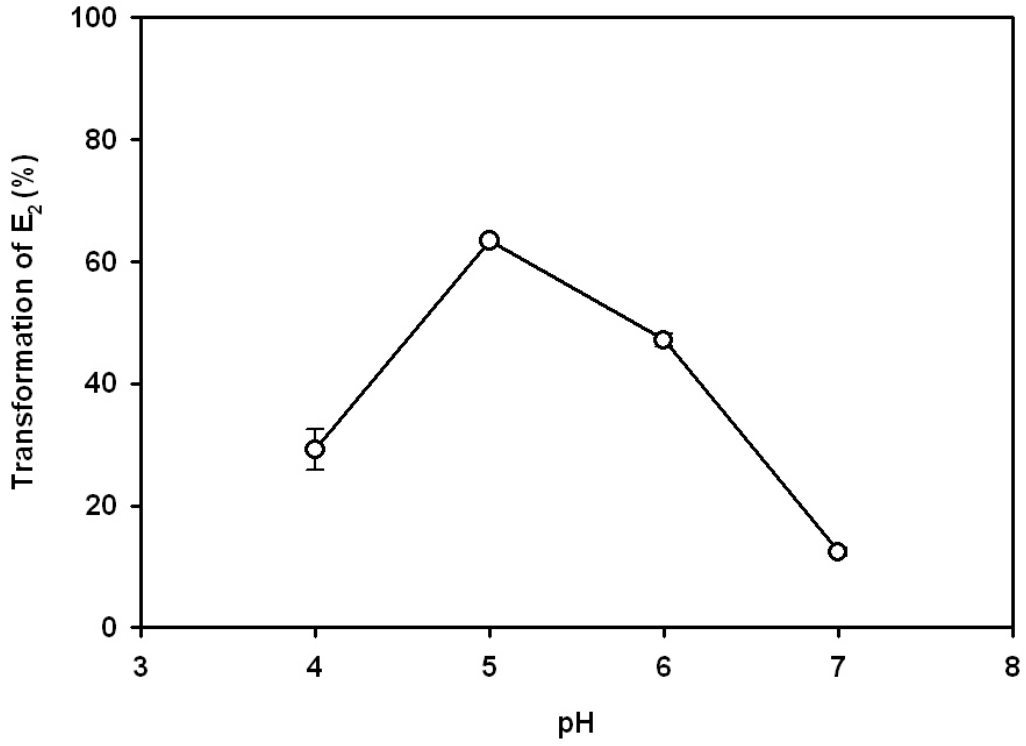


Figure 6: Effect of pH on the transformation of E_2 by the packed bed reactor. Reaction conditions: 4 g dry media, 19.2 U/g nominal activity, $[E_2]_i = 10.0 \pm 0.5$ µM, 21°C, EBCT = 4.2 minutes. Error bars represent one standard deviation from the mean of three samples taken at 2-minute intervals during the same experiment.

A subsequent experiment was run in which approximately 10 µM E_2 in pH 5 buffer was passed through the reactor, followed by a solution of the same concentration at pH 7, and then again at pH 5. For each period of different pHs, the effluent E_2 concentration was measured over time. The purpose of this experiment was to assess the impact of varying the pH in the reactor on the activity of the enzyme. The results are shown in Figure 7. Seveal trials of the experiment were run, and all showed a similar trend. The results of this experiment demonstrate that much more transformation occurs at pH 5 than at pH 7, which is consistent with the results reported above in Figure 6. They also show that, although step changes in pH of the solution through the reactor did not have a large impact on transformation ability of the enzyme, transformation declined somewhat after

the pH was brought back to pH 5 from pH 7. In this experiment, the removal of E_2 achieved after equilibrium was established at pH 5 was approximately 35%, whereas after the system was exposed to pH 7 and then returned to pH 7, the removal declined to approximately 28%. The results demonstrate that the step changes to which the enzyme was exposed caused a slight decrease in activity.

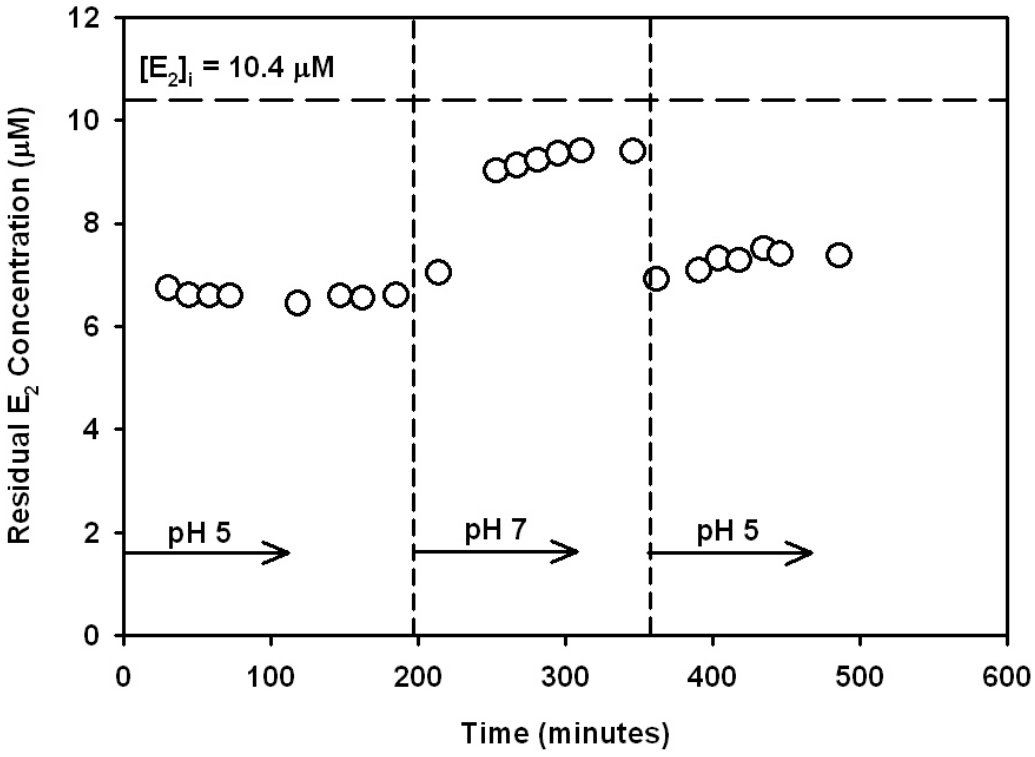


Figure 7: Effect of changing pH on the transformation of E_2. Reaction conditions: 8 g dry media, 18.0 U/g nominal activity, $(t_{res})_{ave}$ = 9.8 minutes, $[E_2]_i$ = 10.4 µM, 21°C.

4.3 Reaction Kinetics

Three separate experiments using different influent concentrations of E_2 (i.e., 5, 10 and 20 µM) were conducted in which substrate transformation was assessed as a function of mean residence time. The results are shown in Figure 8. As expected, as the mean residence time increased, the amount of E_2 transformed also increased. Only a short

residence time of approximately five minutes was required to achieve moderate removal of E_2 (e.g., greater than 50% in the case where $[E_2]_i = 20$ µM). This residence time was used in subsequent experiments because a moderate level of conversion in the control was deemed desirable when evaluating the impacts of process variables on treatment effectiveness; i.e., under this fixed condition it is possible to observe both positive and negative impacts of other variables (e.g., pH, temperature, time) on treatment. Figure 9 presents the relative transformation of E_2 at a mean residence time of 4.8 minutes for each tested value of initial substrate concentration. From this graph, it is evident that relative transformation of E_2 increases as its initial concentration increases.

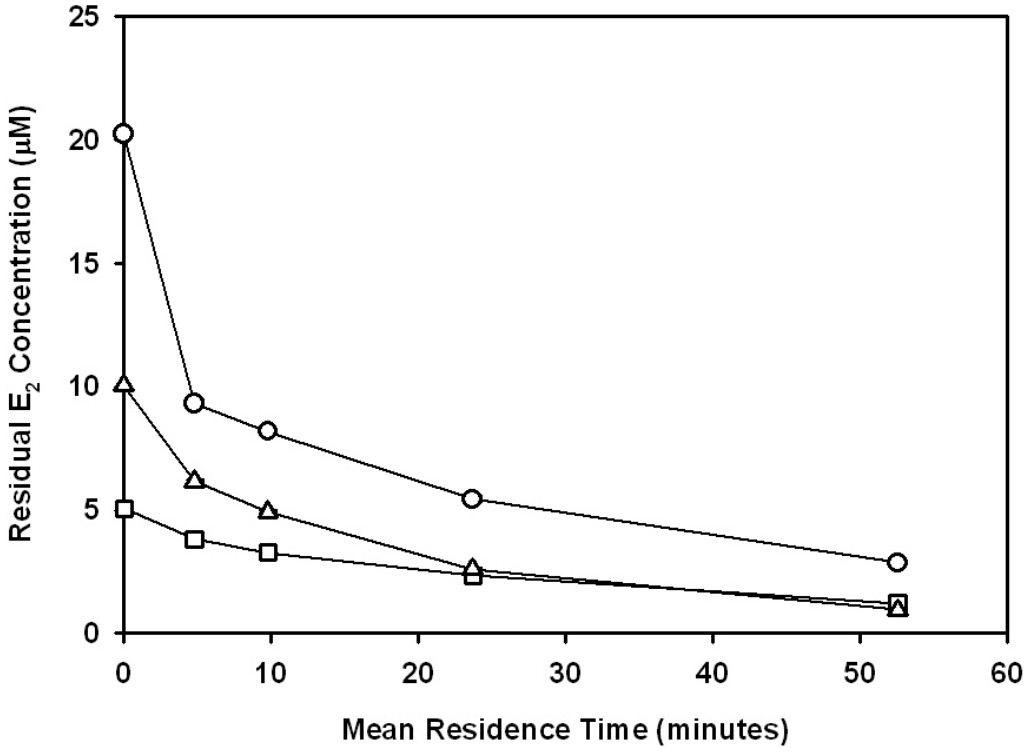


Figure 8: Transformation of E_2 as a function of mean residence time for three influent concentrations. Reaction conditions: 8 g dry media, 18.0 U/g nominal activity, at 21°C and pH 5 with initial estrogen concentrations, $[E_2]_i$, of (□) 5 µM, (○) 10 µM, and (Δ) 20 µM. Error bars represent one standard deviation from the mean of three samples taken at 5-minute intervals during the same experiment,and are not visible because of their small size.

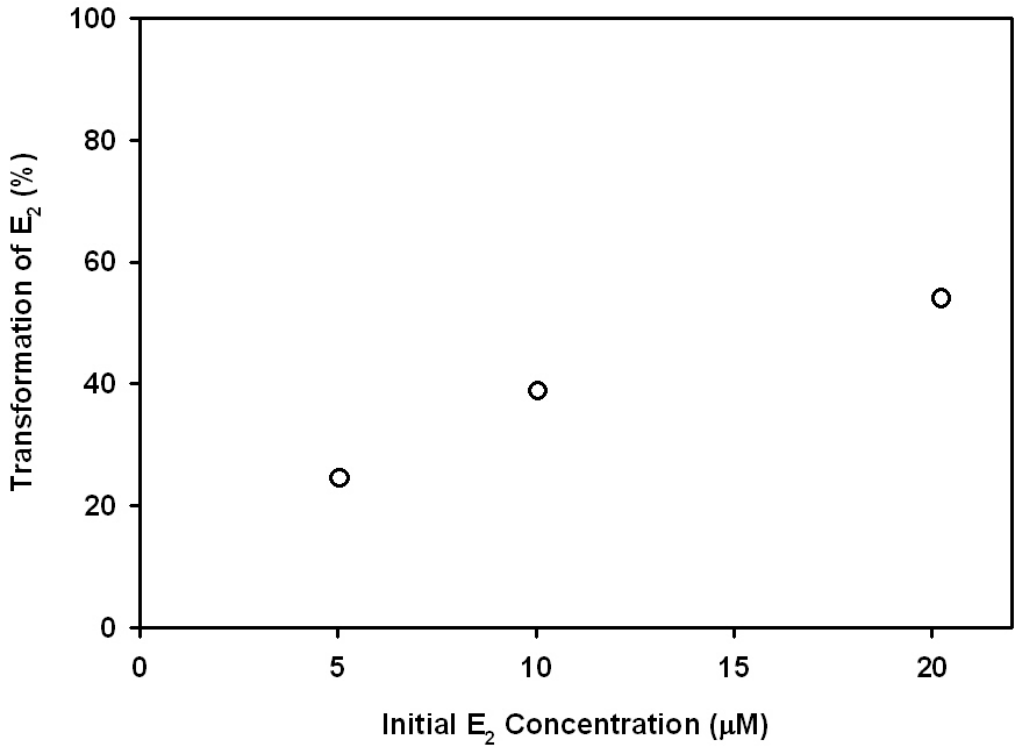


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Figure 9: Transformation of E_2 as a function of initial E_2 concentration at a mean residence time of 4.8 minutes. Reaction conditions: 8 g dry media, 18.0 U/g nominal activity, at 21°C and pH 5. Error bars represent one standard deviation from the mean of three samples taken at 5-minute intervals during the same experiment, and are not visible because of their small size.

4.4 Temperature

The effect of temperature is an important variable to be examined in laccase-catalyzed reactions because it can have a two-fold effect. Firstly, most enzymes are inactivated at elevated or even moderate conditions of temperature and some can be deactivated at temperatures at or below 10^{o}C (Palmer, 1995; Kurniawati and Nicell, under review). Thermal inactivation is believed to be mainly caused by denaturing of the tertiary structure of the enzyme either through protein unfolding or disruption of the active site of the enzyme (D'Amico et al., 2003; Palmer, 1995). Secondly, according to the Arrhenius Law, the rate of reaction should tend to increase with temperature. Therefore, there are

two simultaneous effects associated with changes in temperature in a reacting system: (1) a change in the rate of reaction over time caused by thermal inactivation of the enzyme; and (2) a change in the reaction rate due to Arrhenius effects. Therefore, experiments were conducted to characterize the ability of immobilized enzyme to transform E_2 at different temperatures, as well as to assess the impact of step changes in temperature on the enzyme's stability. The ambient temperature of the reactor was 21°C. In one experiment, the reactor was operated at the ambient temperature for several hours after which a step change in the temperature of the flowing fluid was made to 3°C and the conversion of E_2 was then monitored over time for approximately three hours. Thereafter, the system was returned to the ambient temperature and the system was again monitored for an additional period of several hours. This experiment was then repeated but with an intervening step-change of reaction temperature to 33°C. In both cases, the step change in temperature involved quickly transferring the reactor to a room set at the desired temperature and supplying the reactor with a portion of the same E_2 stock solution that had previously been equilibrated at the desired temperature. The results of these experiments are shown in Figure 10.

As can be seen in the figure, the time for the enzyme reactor to respond to step changes in temperature was quite short. In both cases, whether reverting from 3°C back to 21°C or reverting from 33°C back to 21°C, the enzyme appeared to return to a level of activity similar to that measured before the step change in temperature. Figure 10 (a) shows that reducing the temperature from 21°C to 3°C reduced the activity of the immobilized enzyme by approximately 20%, whereas Figure 10 (b) shows that increasing the temperature from 21°C to 33°C increased its activity by approximately 20%. Given that the enzyme was quite stable under these temperature conditions, it is evident that within the range of temperatures observed, Arrhenius effects dominate in the reacting system.

4.5 Stability of Laccase

The use of enzymes immobilized on a support for removal of substrates is being studied primarily for its potential as a means for reducing the total amount of enzyme that is required to treat a continuous flow of contaminated water. As such, an important criterion for reducing the amount of enzyme required to accomplish treatment is whether the enzymes immobilized on a support remain active for a substantial period of time. To satisfy this criterion, it is essential that (1) the enzyme be stable when stored on the support for extended periods of time prior to or between uses; and (2) the enzyme be stable under reaction conditions. Thus, these two aspects of laccase stability were studied, as shown below.

4.5.1 Stability under Storage Conditions

The results shown above demonstrated that the pH optimum for laccase-catalyzed transformation of E_2 is at pH 5, but it is also expected that treatment of E_2 would often be required at pH 7. In prior studies in which soluble laccase (i.e., non-immobilized) was used to transform phenol in aqueous solution, it was found that although the enzyme's catalytic activity was higher at pH 5 than at pH 7, its stability was much greater at pH 7 (Kurniawati and Nicell, under review). Thus, an experiment was conducted using two separate reactors where one was always subjected to pH 5 solutions and the other always subjected to pH 7 solutions. Each reactor was used periodically for 3-hour runs over a 3-month period, and was stored under conditions of no flow at 25°C, with one reactor stored with its media in pH 5 buffer and the other with its media in pH 7 buffer. The results of this experiment are shown in Figure 11. As expected, higher transformation was initially achieved in the reactor runs conducted at pH 5 due to the greater activity of the enzyme toward the target substrate under this condition. However, a much larger decline in activity over time is seen in the reactor runs at pH 5, shown in Figure 11 (a), than in the reactor runs at pH 7, shown in Figure 11 (b).

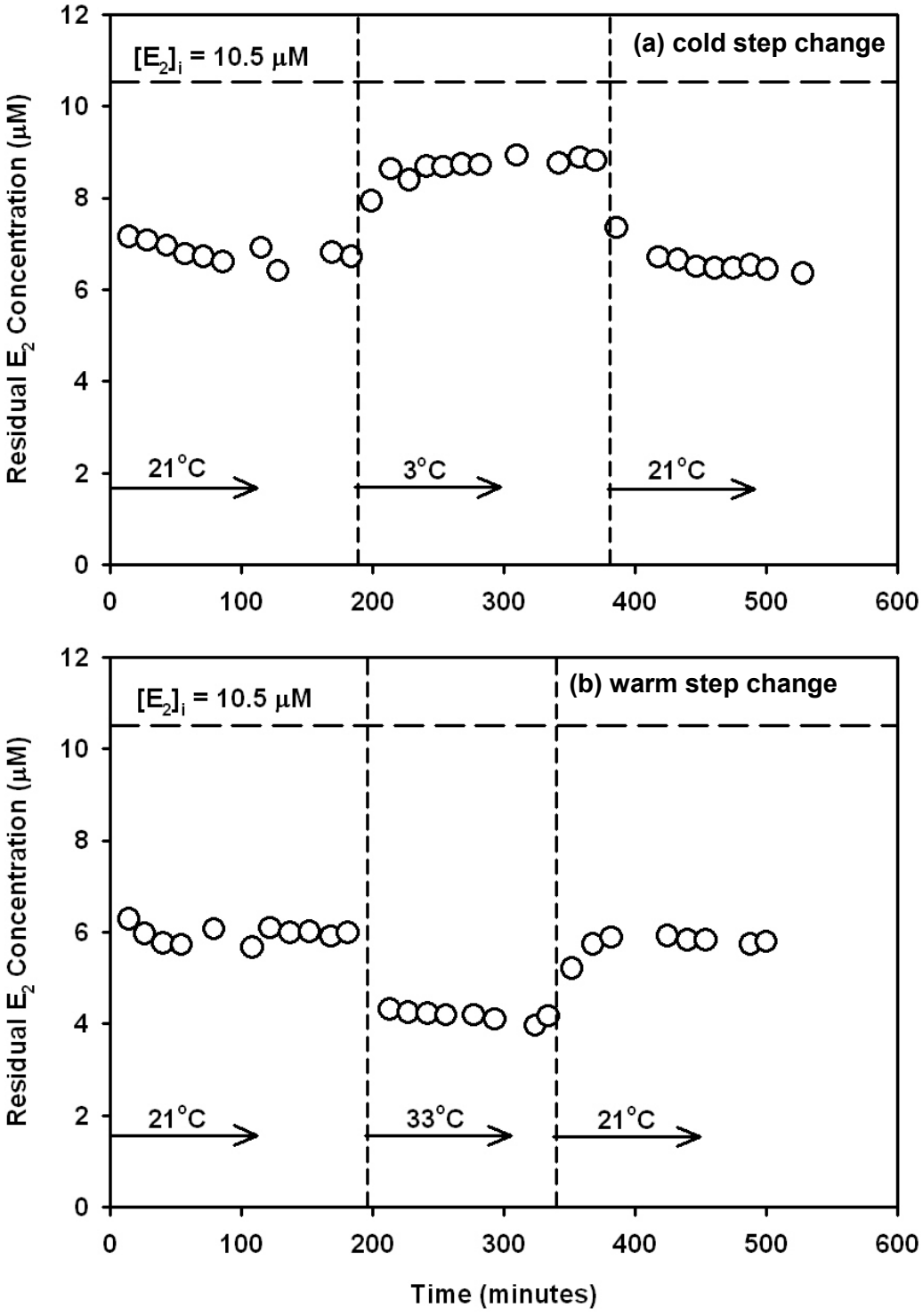

Figure 10: Effect of changing temperature on E_2 transformation accomplished by immobilized enzyme for temperature changes (a) from ambient (21ºC) to cold (3ºC) and back to ambient, and (b) from ambient to warm (33ºC) and back to ambient. Reaction conditions: 8 g, 18.0 U/g nominal activity, pH 5.

4.5.2 Inactivation of Immobilized Laccase under Reacting Conditions

An experiment was conducted to assess the stability of the immobilized enzyme over a 12-hour period of continuous flow of substrate through the reactor. The results shown above in Figure 11 indicate that the enzyme was more stable (retained its activity better) under storage conditions at pH 5 than at pH 7. While it is more likely to be practical to run an immobilized enzyme treatment system over the long term at pH 7, it might also be desirable to operate the system at pH 5 for moderate lengths of time. Two experiments were therefore conducted to assess the stability of laccase under these conditions.

Firstly, the reactor was run for a period of approximately 12 hours at pH 5. The results are shown in Figure 12. A linear regression fit of the data gives a slope of -8.9×10^{-6} μM E_2/minute, with a regression coefficient (R^2) of 0.9997 The very low slope, which is essential zero given the precision of E_2 measurements and other sources of experimental error, indicates that insignificant inactivation of the enzyme occurred over this period of operation at pH 5.

Secondly, because the results from Figure 11 suggest that it might be more feasible to operate a reactor of immobilized laccase over the long-term at pH 7, a final experiment was undertaken to assess a reactor's ability to transform E_2 with continuous flow of influent at pH 7 over a longer period of 9 days. A very long mean residence time of approximately 655 minutes was chosen for this study. This high residence time was chosen in order to determine if a high degee of removal of the target substrate could be achieved and maintained at pH 7, which is not optimal for the enzyme's catalytic activity, as was demonstrated above. The results of this experiment shown in Figure 13 demonstrate that the significant increase in mean residence time dramatically increased the transformation of E_2 achieved by the reactor at pH 7. Moreover, the results show that under the specified conditions, the immobilized enzyme in the reactor was very stable over a nine-day period. A linear regression fit to the data gives a slope of -1.2×10^{-3} μM

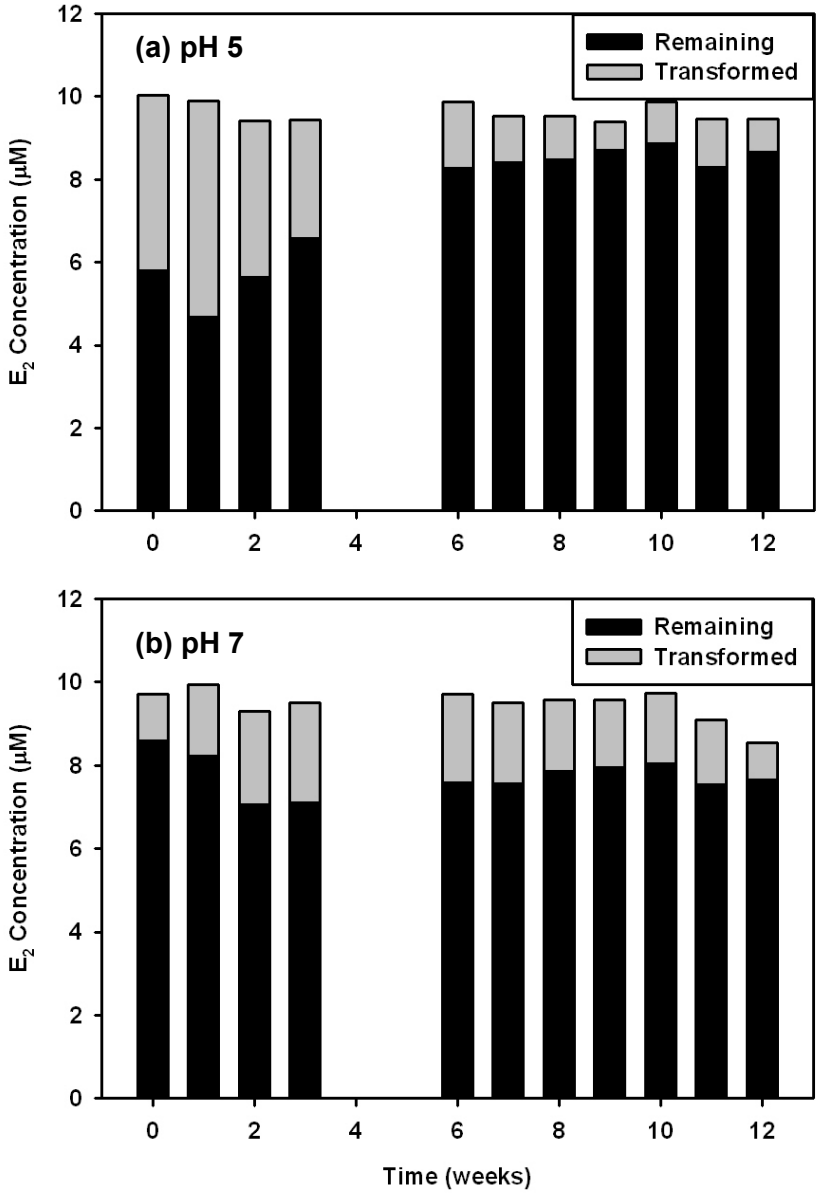

Figure 11: Residual and transformed E_2 resulting from periodic 3-hour runs of the substrate through an immobilized-enzyme packed bed reactor stored at 25°C and (a) pH 5 or (b) pH 7 for extended periods of time. Reaction conditions: 8 g dry media, 18.0 U/g nominal.

E_2/minute, with an R^2 value of 0.988. Given the precision of the measurements and sources of experimental error, the slope is so close to zero as to seem insignificant.

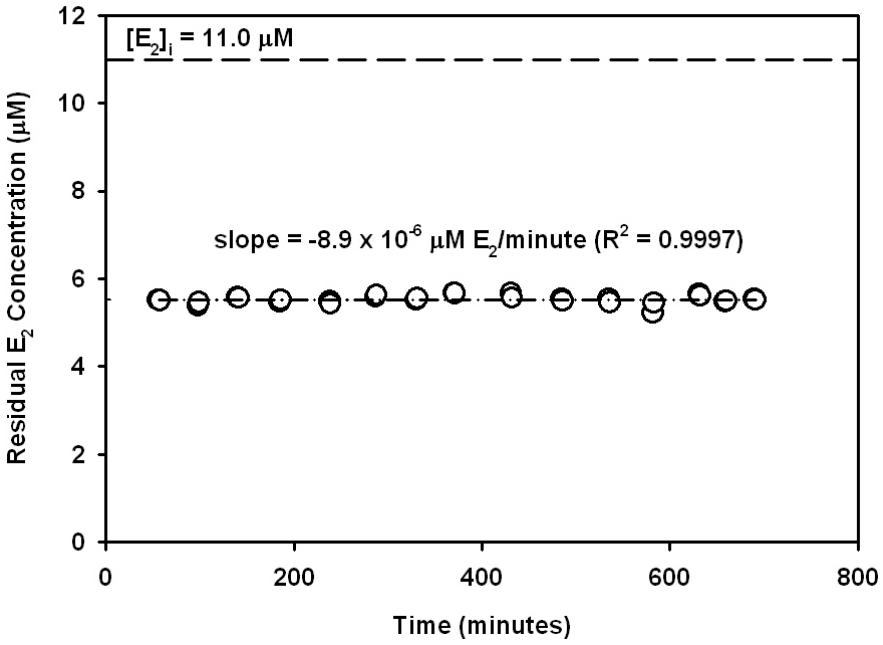

Figure 12: Stability of immobilized enzyme over a 12-hour period at pH 5 with continuous flow of E_2. Reaction conditions: 8 g dry media, 18.0 U/g nominal activity, $(t_{res})_{ave}$ = 23.7 minutes, $[E_2]_i$ = 10.98 µM at 21°C. Overlapping data points are not replicates;

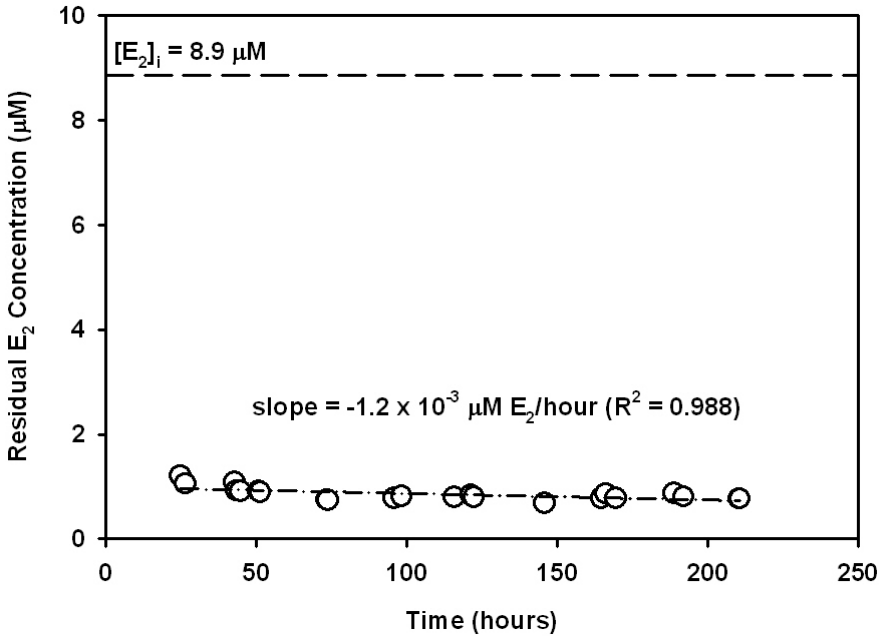

Figure 13: Stability of immobilized enzyme over a 9-day period with continuous flow of E_2. Reaction conditions: 8 g dry media, 18.0 U/g nominal activity, $(t_{res})_{ave}$ = 653.5 minutes, $[E_2]_i$ = 8.86 µM, pH 7, 25°C.

5. Discussion

The tracer studies for all flow rates used in this study were presented in Figure 5 and Table 2. In all cases, the mean residence time of the non-reactive dye was longer than the ideal contact time (CT) for an ideal plug-flow reactor, with the greatest deviation occurring for the lowest flow rate, as seen in Table 3. These results suggest that there are many dead zones within the reactor, and that the volume of these dead zones and time spent by reacting solutions within them increases as the flow rate decreases. The occurrence of dead zones as well as channelling effects greatly impacts the quality of the reactor effluent, given that much of the enzyme in dead zones was not being used to its fullest capacity. If the reactor were optimized with respect to flow conditions, it is anticipated that the treated effluent could be of much greater quality. Thus, the data presented in this study is conservative and the transformation of E_2 at any stated conditions could be greater if the reactor were fully optimized.

Table 3: Disparity between mean residence time and ideal PFR contact time for different flow rates.

Flowrate (mL/min)	Ratio of mean residence time to ideal contact time; $(t_{res})_{ave}$ / CT (dimensionless)
0.2	13.0
1.0	5.2
2.0	4.7
4.0	3.9
8.0	3.9

There exist many ways to optimize a packed bed reactor so as to minimize dead zones. For example, the inlet structure could be redesigned such that flow would be more evenly distributed as it is introduced into the top of the packed bed. Alternatively, the geometry

and the capacity of the reactor could be changed. A reactor with a higher flowrate will tend to decrease the effect of dead zones. A wider and longer column could also minimize wall effects. Therefore, for a desired residence time, the reactor could be optimized by increasing the flowrate and proportionately increasing the depth and/or width of the packed bed to achieve the desired residence time. Dead zones could also be reduced by employing a medium with different size and shape.

The denaturation and consequent decrease in activity of enzymes at extremes of pH occurs because of folding of the tertiary structure containing pH-affected ionisable side-chains (Palmer, 1995). In an earlier study, the optimal pH for the aqueous-phase laccase-catalyzed transformation of phenol was found to occur at pH 6, with comparable transformation also occurring at pH 5 (Kurniawati and Nicell, under review). In the case of BPA, the optimum pH was at 5 (Kim and Nicell, 2006). Similarly, the transformation of E_2 and ethynylestradiol (EE_2) by laccase in the aqueous phase has been found to occur optimally at pH 5 (Fang et al., in preparation). A third estrogen, estriol (E_3), follows a pattern closer to that of phenol (Fang et al, in preparation). In all cases, the conversion of the target substrates was optimal under slightly acidic conditions with reasonable conversion being achieved relative to the optimum in the pH range of 4 to 7.

Similar results to those quoted above were found in this study: i.e., the conversion of E_2 occurred optimally at a pH of 5, followed closely by good conversion at a pH of 6. From this, it is evident that optimal performance by laccase would not be achieved within the typical range of pHs for domestic wastewaters, which is from pH 6.7 to pH 7.5 (Qasim, 1999). Thus, in order to maximize performance, the pH of the influent solutions would either need to be decreased or other treatment parameters would need to be more adjusted to compensate for the lower activity of the enzyme under near-neutral conditions. The latter of these two options would seem more feasible and, based on results obtained pertaining to enzyme stability, preferable, since it was found that the enzyme does exert significant catalytic ability under neutral conditions (see Figure 13) and the enzyme system stored over the long term with only short intermittent uses is much more stable at

pH 7 than at pH 5 (see Figure 11).

The greater stability of laccase at pH 7 has been noted elsewhere in the literature. Kurniawati and Nicell (under review) found that aqueous laccase at pH 5 was moderately stable with an activity loss of approximately 10% after being stored for 6 hours, while at pH 7 it was very stable with an activity loss of approximately 3% in the same period. Kim and Nicell (2006) also observed that the stability of stored laccase was highest at pH 6 and 7. Under the storage conditions of the present study, which lasted three months, a much greater discrepancy between stability at pH 5 and 7 was observed. In practical applications, where immobilized enzyme would most probably be stored for some extended periods, perhaps on the order of days or weeks than hours, storage at pH 7 would therefore be highly preferable to storage at pH 5.

Kurniawati and Nicell (under review) also noted moderate recovery times (i.e., 5 minutes to 1 hour) for aqueous phase laccase exposed to extremes of pH and then brought back to neutral pH. This has been thought to occur because of tangles formed during denaturation, which render the refolding of the tertiary structure more difficult (Palmer, 2005). As seen in Figure 7, there appears to be a moderate recovery period in the case of immobilized laccase as well. After the expected to time to equilibrium from tracer studies (see Table 2), the transformation of E_2 continues to show a sloping trend, possibly indicating a gradual return to its original conformation, when pH is brought from 5 to 7 and is then returned to pH 5.

In addition to pH, the effects of another important influent parameter, temperature, on the reactivity and stability of laccase were studied. It was found that within the range of temperature from 3 to 33^{o}C, immobilized laccase activity is positively correlated with temperature (see Figure 10). Kim and Nicell (2006), in the case of conversion of BPA by the same species of laccase in the aqueous phase, found that activity of the enzyme was positively correlated with temperature in the range of 25 to 45^{o}C, and above 45^{o}C the two became negatively correlated. This seems to suggest that over a very wide range of

temperatures, Arrhenius' Law, stating that reactions increase with increasing temperatures, has a greater impact than the temperature-induced unfolding of the enzyme's tertiary structure. Only when a very high temperature is reached or exceeded (e.g., 45^{o}C), does the negative impact of temperature become apparent.

While, in general, the positive correlation with temperature exists for the reactivity of the enzyme, the opposite effect is observed when measuring the effect of temperature on enzyme's stability. For example, Kurniawati and Nicell (under review) and Kim and Nicell (2006) noted dramatic decreases in storage stability with increasing temperature. Also, Kim and Nicell (2006) determined that temperature-induced inactivation was greater in a reacting system than in a stored system. While the stability of the enzyme based on temperature was not assessed in the present study, the practical implications of such results are very important because the relationship is very pronounced. For example, due to seasonal variations, most influent wastewaters in the United States fall within the range of 3 to 27^{o}C (Metcalfe and Eddy, 2003). Thus, though reactivity would be slightly higher at higher temperatures, the stability of the enzyme would be much greater during the tertiary treatment of typical domestic wastewaters.

Though the stability of the immobilized enzyme system with respect to temperature was not studied over the long term, the recovery time for step changes in temperature was indirectly investigated. Kurniawati and Nicell (under review) found that recovery times when the non-reacting enzyme undergoes a step change in temperature were relatively long, ranging from 3 to 12 hours. Similar to the case of pH, this recovery time would be due to the steady untangling of side chains in the tertiary structure of the enzyme (Palmer, 1995). In this study, however, as seen in Figure 10, there was no clear evidence of a recovery time period. When step changes in temperature were introduced to an operating system, the enzyme appeared to achieve a steady activity after the expected time to steady-state determined by tracer studies and no later. This could be due to a stabilizing effect of the enzyme in a chemically immobilized state. Alternatively, the data in the present study may not have been sufficiently precise to reliably observe a small change

(i.e., < 5%) in activity over time.

The importance of storage stability has been discussed, but even more important in practical applications is operational stability. As seen in Figure 12, operating a reactor of immobilized laccase at the optimal (in terms of reactivity) pH of 5 did not result in any inactivation under the employed conditions. Because treatment is more likely to occur near neutral pH and over longer periods of time, the results in Figure 13 are more representative of what is more likely in practice. At pH 7 and over 9 days, albeit with a high mean residence time of approximately 655 minutes (EBCT = 73.5 minutes), no enzyme inactivation was observed when continuously treating 10 μM of E_2.

A major limitation to using an immobilized enzyme system for tertiary treatment of domestic wastewaters was illustrated in Figure 9. Under the studied conditions, the lower the influent substrate concentration, the lower the transformation within the reactor. This likely arises from lower rates of reaction and slower rates of mass transfer at low substrate concentrations. Fortunately, significant treatment was accomplished in the immobilized-enzyme packed reactor with reasonable retention times. However, the influent concentrations used in these experiments were between 1 and 10 mg/L, whereas wastewater treatment plant effluents have been found to have E_2 concentrations between 1 and 24 ng/L (Aerni et al., 2004), which are six orders of magnitude lower. Thus, some other operational parameters would need to be greatly enhanced to compensate for the expected reduced reaction rates with lower estrogen concentrations; i.e., the quantity of enzyme immobilized per unit volume of reactor would need to be significantly increased. In addition, the rate of mass transfer from the bulk solution to the immobilized enzyme would be much slower for environmentally relevant substrate concentrations. Thus, enhanced mixing and dense distribution of the enzyme over greater surface areas would be required to offset these mass transfer limitations. These issues represent very significant challenges that must be overcome before the tertiary treatment of estrogens using immobilized laccase can be considered practical. As an alternative, source treatment could be considered as an option, though this would represent a very significant

change in waste management paradigms for urban communities. Such a paradigm shift is already being considered and promoted due to a variety of advantages and opportunities associated with the source separation, collection, and treatment of urine (Escher et al., 2006). Human urine has an average E_2 concentration of around 3 μg/L (Johnson and Williams, 2004), or three orders of magnitude lower than the experimental influent concentrations. Therefore, source treatment might improve the reaction kinetics dramatically over what would be possible with tertiary treatment, but mass transfer limitations may still prove to be extremely restrictive.

Despite the limitations noted above for the treatment of estrogens, the results of this work are quite encouraging since it has been demonstrated that immobilized laccase is very stable when used to treat low concentrations of substrates. Therefore, this technology could also be considered for the treatment of a variety of problematic phenolic pollutants in various wastewaters.

6. Conclusions and recommendations

The objective of this research was to address the growing problem of estrogens and other endocrine disruptors which pass unchanged through wastewater treatment plants and make their way into natural aquatic environments. Previous work has focused on treating these compounds using laccase in the aqueous phase. The main objective here was to explore enzymatic treatment a step further by immobilizing the enzyme onto a support such that it could be used retained in a continuous flow reactor instead of being discarded with treated effluents. The estrogen estradiol, E_2, was used as a model substrate. Flow characteristics within the reactor were investigated using dye tracer studies, and the effects of pH, temperature, contact time, and influent substrate concentration on the transformation of E_2 within the reactor were studied. Also, the storage and operational stability of the system were assessed.

The transformation of E_2 was found to occur optimally at pH 5, whereas long-term storage stability was much greater at pH 7 than at pH 5. Because wastewaters are typically at or near neutral pH, real applications of the system could be used under the conditions which confer greatest stability. For the immobilized laccase system, E_2 conversion was positively correlated with temperature in the range from 3 to 33^{o}C, and for step changes in temperature, the system did not show any perceptible recovery period typical of an enzyme slowly regaining its conformation.

Kinetic experiments revealed that the contact time required for significant E_2 conversion was relatively low, even in a non-optimal reactor configuration, which is promising in terms of larger-scale operations. However, when treatment was conducted using a variety of influent concentrations of E_2, it was observed that the rates of reaction slowed significantly with lower substrate concentrations. Given that the E_2 concentrations used in these experiments were 3 to 6 orders of magnitude greater than what might be found in a real treatment applications, this is a significant problem which needs to be addressed before the feasibility of this approach can be established. It should also be noted

that the reactor used in all experiments exhibited fairly poor flow characteristics. Therefore, improving the reactor design could lead to a significant increase in substrate conversion under all studied conditions.

The monitoring of the operational stability of the enzyme yielded extremely promising results. In particular, a reactor run continuously at typical treatment conditions of pH 7 and at 25^{o}C, albeit with high influent E_2 concentration and relatively long contact times (i.e., 10 μM, with an EBCT of 73.5 minutes and mean residence time of 655 minutes), resulted in high substrate conversion with no inactivation over a 9-day period. To further assess the feasibility of the treatment process, the length of time that the reactor can be used before the immobilized enzyme needs to be replaced would need to be assessed. Studies are also needed to determine the magnitude of the impact of other contaminants in wastewater matrices on enzyme stability.

The results of this study demonstrate that a reactor of immobilized laccase has potential to be used in the treatment of E_2 and possibly many other phenolic substrates. The feasibility of using this type of system in full-scale applications will depend on many factors, which have yet to be studied. In particular, the following recommendations are suggested for future work:

- The maximum silica bead capacity for enzyme should be determined as discussed in Appendix B.
- The reactor system should be changed and optimized in an attempt to achieve significant transformation of lower influent substrate concentrations. In particular, the reactor geometry and immobilized enzyme distribution should be optimized.
- The ability of the reactor system to catalyze the oxidation of other important phenolic contaminants, such as pharmaceuticals and other forms of estrogen, as well as aromatic amines should be investigated. Furthermore, the use of immobilized laccase in industrial applications other than waste treatment should

be explored.

- The fate, toxicity and estrogenicity of reaction end-products should be assessed.
- The effects of mediators, which are enzyme co-factors that can be used to enhance substrate conversion rates in the aqueous phase, should be investigated in the immobilized system.
- Tracer studies using another tracer dye, such as fluorescent orange, should be performed to validate the results obtained using methylene blue. Although the beads which had been used in tracer studies regained their whiteness after being washed with water, Levenspiel (1979) notes that when mean residence time is significantly higher than ideal contact time (as observed in this work), the cause is sometimes an interaction between the media and the dye.
- The effects of different temperatures on the long-term storage and operational stability of the system should be determined.
- A complete kinetic model of the immobilized enzyme system should be developed to aid in the design and optimization of reactor systems.

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Appendix A

Two methods exist for the determination of laccase activity; one is the measurement of oxygen uptake in a closed system, as laccase uses oxygen as a co-factor, and the other is photometric analysis when coloured oxidation products are formed (Johannes and Majcherczyk, 2000). In the study presented here, the measurement of oxygen uptake by laccase could not be employed to monitor enzyme activity because parts of the immobilized system employed in this study were open to the atmosphere. In addition, an oxygen probe could not feasibly be inserted into the pressurized reactor, and, finally, it was determined that the monitoring of the mass balance of oxygen in the system would be complex. The most common substrates used in colorimetric tests are the phenolic compounds guaicol, 2, 6-dimethoxyphenol, and syringaldazine, which form quinones, and the non-phenolic 2, 2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) which becomes a coloured radical cation ($ABTS^{+\bullet}$) upon oxidation by laccase (Johannes and Majcherczyk, 2000). As opposed to the phenolic substrates, the oxidation potential of ABTS has been found to be pH independent in the range of pH 2 – 11 (Johannes and Majcherczyk, 2000). Thus, for an immobilized-laccase system, the use of a colourimetric assay, preferably using the pH-independent ABTS as a substrate, is most suitable.

When laccase activity is assessed based on $ABTS^{+\bullet}$ formation, one unit of enzyme activity is generally defined as the amount of enzyme that converts 1 μmol of ABTS to $ABTS^{+\bullet}$ per minute in a mixture containing dilute laccase, an excess amount of ABTS (1 to 2 mM), and pH 4.5 buffer (Dodor et al., 2004; Kurniawati and Nicell, under review). The colour generation caused by formation of ABTS radicals is monitored in a spectrophotometer at a wavelength of 420 nm and with an extinction coefficient of 36 000 $M^{-1} \cdot cm^{-1}$ (Wolfenden and Wilson, 1982).

Two methods for employing ABTS as a colour-generating substrate for immobilized laccase are described in the literature. The first is an indirect measure of activity undertaken during the immobilization process and the second involves taking a

small sample of immobilized enzyme, measuring its colour-generating ability, and then extrapolating this to the entire batch of immobilized enzyme.

In the first method, the laccase stock solution to which the reactor media will be exposed is made and a small sample is taken. Protein content is measured and ABTS is added to the sample and colour generation is measured as explained above (Rogalski et al., 1994; Champagne, 2005). After the beads have been exposed to the laccase solution for 24 hours, as part of the process described in the Materials and Methods section, the beads are washed and all washings are collected. Protein content is then measured and ABTS is added to a sample of the washings and the reaction is analyzed in the spectrophotometer as described above (Rogalski et al., 1994; Champagne, 2005). A mass-balance can be used to estimate how much enzyme activity has been retained by the beads. This accuracy of this method, however, hinges on several assumptions. First, it is assumed that all laccase which has not become covalently bonded to the beads is washed out during this washing. Secondly, it is assumed that covalent bonding of the laccase onto the beads does not affect in any way the activity of the enzyme. Unfortunately, this approach does not provide an *in situ* method for the measurement of activity of the immobilized enzyme. This is an important drawback since this approach cannot be used to monitor the activity of the enzyme in a reactor system over time.

In the second method, when the immobilization method is complete, a small sample of the beads covalently coated with laccase is sectioned away from the batch and is inserted into a small vial with buffer and ABTS. After a pre-determined length of time (less than 30 minutes) under mild shaking, acid is added to stop all reactions, the mixture is centrifuged and decanted, and the decanted sample is analyzed in the spectrophotometer (Dodor et al., 2004). Using the method described above, the activity of the sample can be calculated and extrapolated to the whole batch. The limitations of this method include the assumptions that the very small sample of beads adequately represents the whole batch, and that shaking the sample does not disrupt the immobilized laccase matrix. Also, because the colour generation is not measured over the course of the reaction but

rather after a discrete time interval, one must ensure that the colour yield falls in the linear portion of a plot of $ABTS^{+\bullet}$ colour formation with respect to time. To do this, at least two samples of different mass or volume of beads would need to be taken to verify direct proportionality. In this situation, the accurate measurement of mass or volume of so many tiny amounts would be very challenging. This method, on the other hand, as opposed to that previously described, makes it easier to track activity over time, as small samples of the reactor media could be taken periodically, so long as the sum of their volumes is significantly smaller than the volume of the batch of immobilized enzyme.

The method initially attempted in this study involved passing a constant flow of ABTS solution through a reactor of immobilized enzyme and measuring the absorbance of triplicate effluent samples after steady-state had been reached (according to tracer studies). This approach had been used before; e.g., by Hublik and Schinner (2000), who used this assay in a reactor of laccase immobilized on a different support. If every new reactor could be evaluated in this way, and if this activity measure could be repeated over time on a same column, it would be an effective way to compare results based on activity. The following equation could be used to determine a measure of activity within the reactor:

$$Activity\ \left[mol/\min\right] = \frac{Q\left[mL/\min\right] \times \Delta Abs.\left[cm^{-1}\right]}{\varepsilon\left[M^{-1}\cdot cm^{-1}\right]} \times \frac{1L}{1000mL}$$

where $\varepsilon = 3.6 \times 10^4\ M^{-1}\cdot cm^{-1}$ at 420 nm.

All trials were performed on a reactor with 8 g of dry media and 18.0 U/g nominal activity at a flow rate of 4 mL/min. A first trial was performed using 2 mM of ABTS as an influent. This concentration, however, was deemed too high, as the effluent had an absorbance value over 3. Also, the reactor media was stained a grey/purple colour which could not be removed with thorough washing. A new batch of reactor media was made, and three separate activity trials were run through the reactor, with thorough washing

with buffer between trials. Using an ABTS concentration of 0.02 mM, all effluent samples fell within the linear portion of a spectrophotometer calibration curve for the compound. However, while the triplicate samples for each trial were very precise, the average effluent absorbance for each trial was very imprecise, as seen in Table 4. Thus, under the studied reactor conditions, this method did not appear to provide an acceptable measure of activity.

Table 4: Results of three trials of the attempted activity assay. Absorbance of influent and effluent ABTS was measured at 420 nm.

Trial	Influent Absorbance	Mean Effluent Absorbance
1	0.000	0.360 ± 0.000
2	0.001	0.234 ± 0.001
3	0.000	0.422 ± 0.001

It was decided that a fairly simple and unobtrusive way to monitor changes in activity in the immobilized-enzyme packed bed reactor over time would be to use one set of influent reaction conditions as a basis upon which all other results could be compared. In this way, an indirect measure of activity changes would be based on the quantity of substrate converted by the immobilized enzyme system. The base set of influent conditions chosen was as follows: $[E_2]_i$ = 10 μM, pH = 5, Temperature = 21 or 25°C, EBCT = 3.7 minutes, and conditions were varied depending on what trend the experiment intended to explore. Also, all batches of immobilized laccase prepared over the course of the study employed the same method, exposing the beads to the same nominal activity. Experiments whose aim was to determine the effects of varying conditions used this set of conditions as a comparison measure. Thus, though the results of experimental runs under these precise conditions were not always exactly the same (i.e., because a new reactor was used or because the reactor had undergone some inactivation over time), the results of varying conditions in a single column could be compared.

Appendix B

As discussed in Materials and Methods section, a fixed mass of silica beads was always exposed to a fixed reported activity of laccase in a fixed volume of buffer for 24 hours; i.e., in this study, 8 grams of silica beads were exposed to 200 mg of 0.72-U/mg laccase in 200 mL of buffer. This, however, does not necessarily represent the condition that would achieve maximum capacity of activity of laccase on the beads. An important engineering study would be to determine the optimum laccase activity on the beads, which would be a compromise between substrate conversion ability and the cost of adding more and more enzyme. In a short but incomplete study, the results of which are shown in Figure 14, it was found that the addition of enzyme follows a diminishing returns trend. As the beads are exposed to increasing amounts of enzyme, there are possibly mass transfer limitations which result in decreasing marginal treatment abilities. The results shown in Figure 14 are for reactors containing 4 g of dry media. Most experiments undertaken in this study used reactors with 8 g of dry media and a nominal activity of 18.0 U/g.

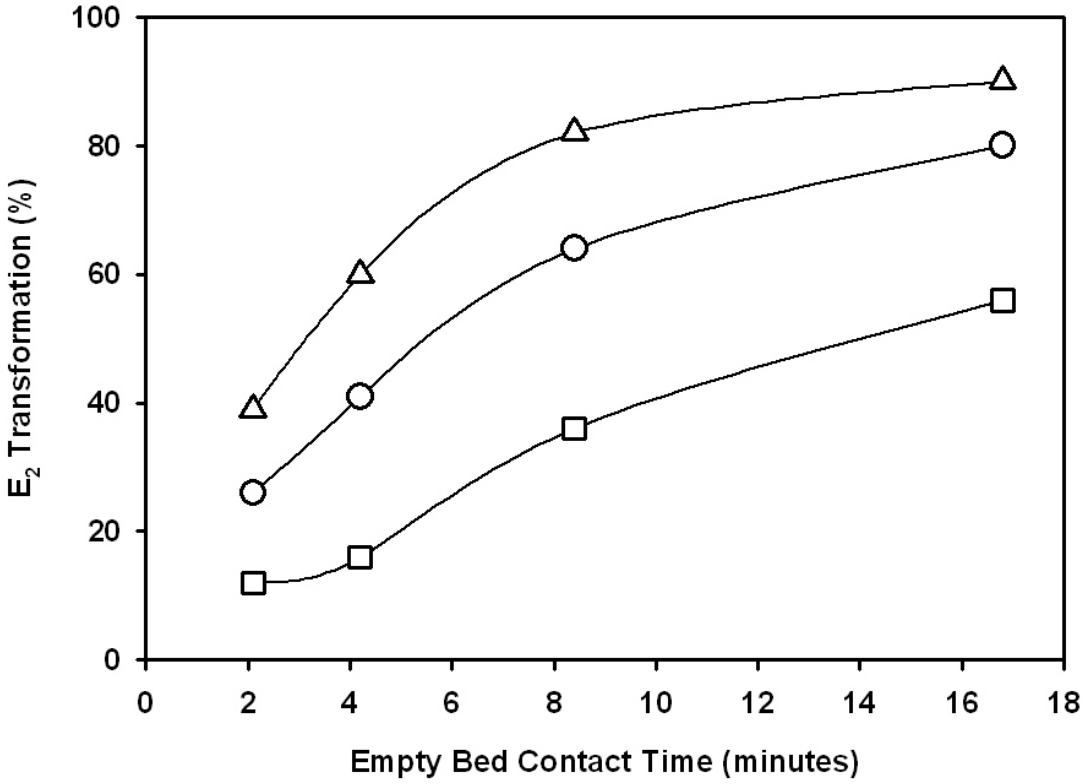

Figure 14: Effects of enzyme dose (also referred to as nominal activity) on E_2 treatment ability. Reaction conditions: 4 g dry media, pH 5, 21°C, (□) Nominal activity = 2.4 U/g, (○) Nominal activity = 9.6 U/g, (Δ) Nominal activity = 19.2 U/g.

Appendix C

Figure 15 depicts the system set-up. Influent was pumped from a stirred and covered source and into a flow adapter and through the packed bed. Temperature was controlled by setting up the system in a constant temperature room. **Influent samples** were taken from the covered source and **effluent samples** were taken from the final tube end.

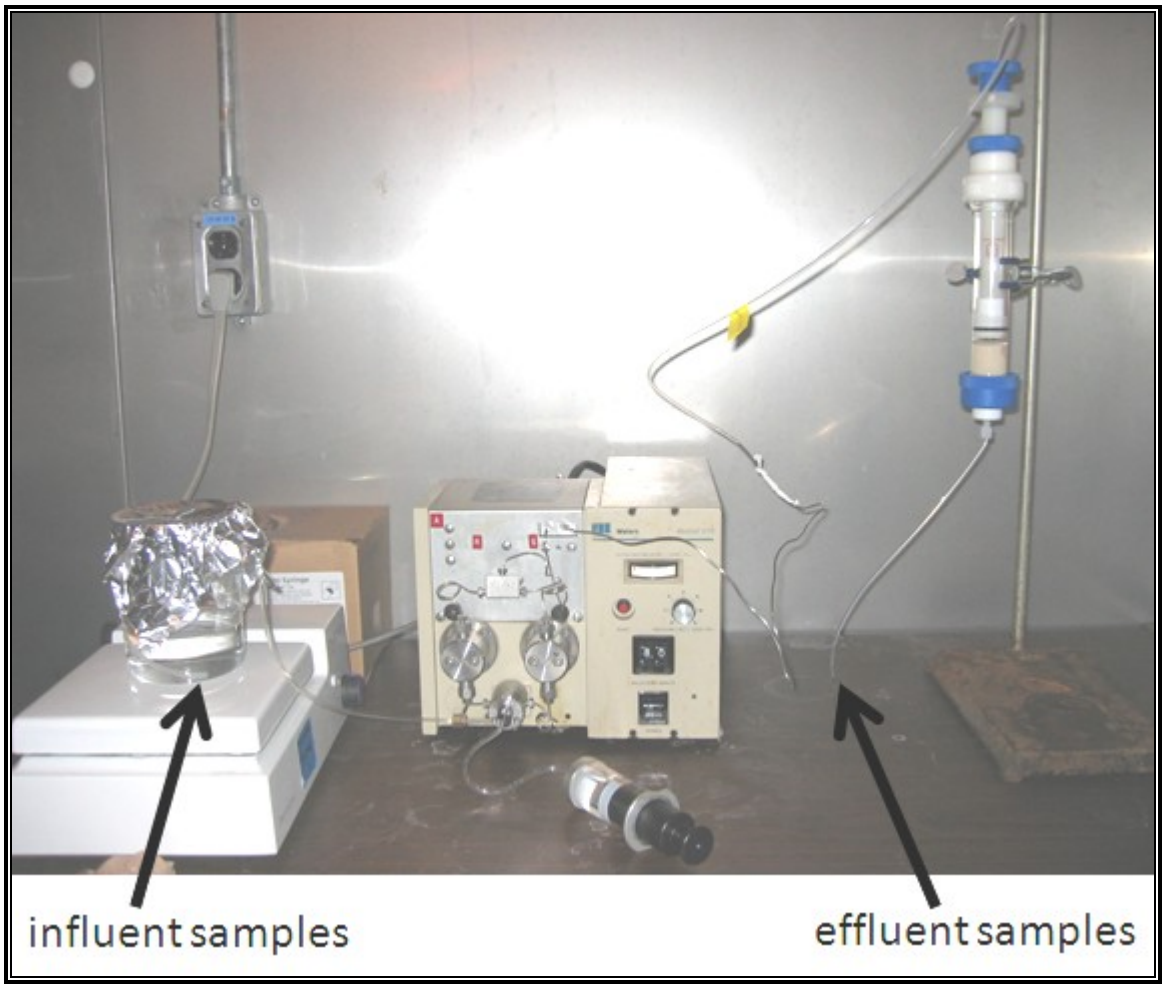

Figure 15: System set-up when Waters Millipore Model 510 pump was used, highlighting where influent and effluent samples were taken.

Washing the pump

When the pump has been stored away or when it has been used to pump unknown or incompatible substances, it must be thoroughly washed before use. This was done by pumping a solvent, in this case the polar protic isopropanol from Fischer (Fair Lawn, NJ), such that it passed through all unchangeable metallic pump parts. Approximately 300 mL of isopropanol were pumped, half at a low flowrate (1 – 2 mL/min) and half at a high flowrate (8 – 9 mL/min), followed by approximately 300 mL of distilled deionized water.

Priming the pump

When the pump has been idle or when it has entrained air, it must be primed before use. The free end of the **influent tube** shown in Figure 16 must be immersed in some liquid and the **valve** must be screwed open. At this point, a syringe attached to a short stretch of **syringe tube** must be pulled open until liquid is seen filling the syringe. The **valve** must then be screwed shut. The pump is then ready to be turned on for operation.

Pressurizing the column

The Kontes Chromaflex flow adapter does not come with a useful user's manual, so the instructions for pressurizing the column/flow adapter set-up are discussed here, with Figure 17 as a reference, for ease of future use. First, the column should be fixed in a vertical position. The free end of the tube leading up to the flow adapter must be attached to the exit of the pump. The **top cap** and **O-ring** portion of the **flow adapter** can be tightened with respect to each other; these should be tightened (default position) and then loosened only slightly (approximately a half turn) so that the **O-ring** can slide within the column. With the appropriate liquid (buffer or distilled water) as an influent, the pump should be turned on and run until all tubing and the entire **flow adapter** are filled with liquid, at which point the pump must be turned off. Taking care that the reactor media remains free of large air bubbles and keeps a level surface, the column should be filled

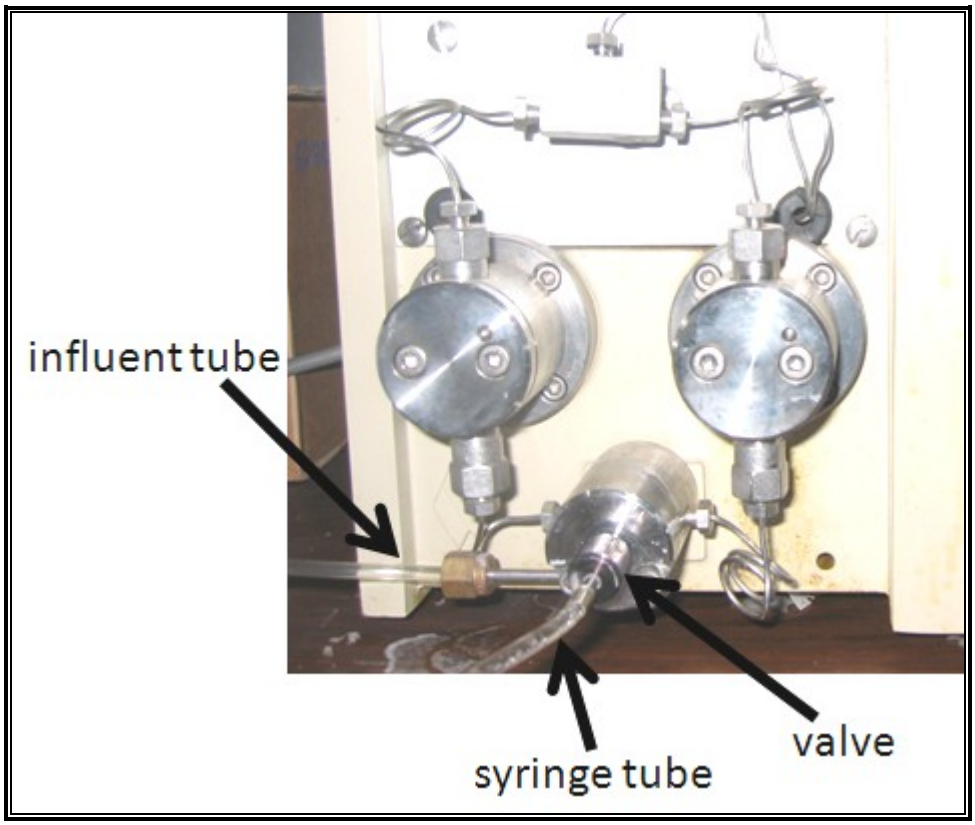

Figure 16: Part of the Waters Millipore Model 510 pump interface, highlighting parts required for priming.

with liquid from a separate receptacle; this liquid will remain in the column if the effluent tube is held upward beside the column. Once the column has been filled with liquid, the effluent tube can be let down and the **flow adapter** can be slowly pushed into the column; liquid should leave at the same rate that the adapter is being pushed in. When the adapter has reached the desired position, the effluent tube should once again be held upward beside the column, preventing any further liquid from leaving the column. At this point, the **screw cap** must be screwed down onto the glass column's helical groove. Then, the **tightening nut** should be descended until it rests directly above the **screw cap**. Finally, the **pressure nut** should be screwed upward until it touches the **top cap**; these two should fit snugly together but not too tightly. The effluent tube can then be let down, and the liquid level in the column should remain constant. When the pump is turned on, the pressure within the reactor should not change.

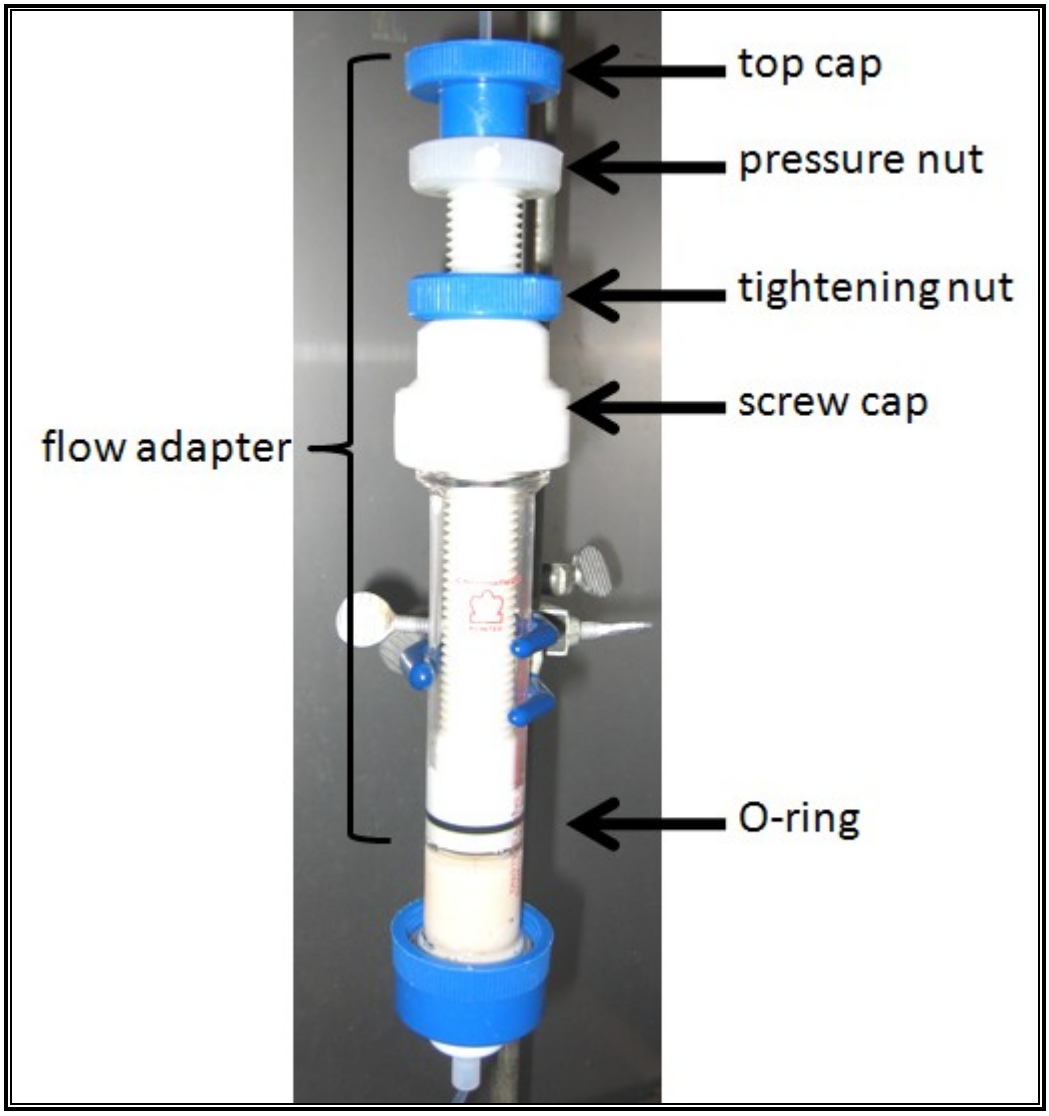

Figure 17: Kontes Chromaflex column and flow adapter.