CHAPTER 1

INTRODUCTION

Chlorophenols are a group of chemicals in which chlorine molecules have been added to phenol structure. There are five basic types of chlorophenols: monochlorophenols, dichlorophenols, trichlorophenols, tetrachlorophenols, and pentachlorophenols. Except for 2-chlorophenol, which is a liquid at room temperature, all of the chlorophenols are solids. The chlorophenols have a strong medicinal taste and odor; small amounts (at parts per billion [ppb] to parts per million [ppm] concentrations) can be tasted in water. Small amounts of mono- and dichlorophenols are produced when wastewater or drinking water is disinfected with chlorine or if certain contaminants are present in the raw water and during bleaching of wood pulp in the paper manufacturing. The contamination of these chlorophenols has been found to a greater extent in water, soil and also in the air. Ingestion of chlorophenols has been shown to pose health hazards and this has necessitated the need to degrade these compounds prior to releasing into the environment.

Many methods have been suggested for the removal of these compounds from the water samples e.g. – removal through the adsorption on bituminous shale (Tutem *et al,* 1998), removal using red mud (Gupta *et al.,* 2004), removal using microalgae *Tetraselmis marina* (Petroutsos *et.al.* 2007). Apart from using conventional methods for removal, various biological methods have been studied using enzymes for efficient removal of these compounds. Due to the inefficiency of the conventional methods in the removal of these compounds, biological methods have gained more attention. (Bergbauer *et al,* 1991). Very few microorganisms have been found to degrade these compounds. Of all the biological

methods studied, the application of laccase from white rot fungi has gain tremendous importance in this field due to its ability to degrade chlorophenols. These fungi, which are capable to degrade lignin in wood, show also potency to degrade many structurally diverse organic pollutants (Kang and Stevens, 1994). The ability of white rot fungi to attack persistent chemical structures, including chlorophenols, in many cases depends on the expression of the ligninolytic enzyme system (Eaton 1985; Spadaro *et al.,* 1992; Hawari *et al.,* 1999).

Laccase which is one of the ligninolytic enzymes of the white rot fungi has been implicated in the dechlorination of chlorophenols compounds. Dechlorination can be achieved with laccase (Lyr, 1963; Konishi and Inoue, 1972; Ruggiero *et al.,* 1989; Roy–Arcand and Archibald, 1991; Cho *et al.,* 2001) in the presence of some lignin residues from coupling process (Roy–Arcand and Archibald, 1991). This oxidative coupling process can function in two steps: firstly chlorophenols with aromatic co–substrates (hydrogen donors) are enzymatically oxidized to free radicals or reactive quinines, and then subsequent oxidative coupling of the products is completed without further involvement of the enzyme (Dec and Bollag, 1994, 1995).

In the current study, 2-chlorophenol was chosen as a model chlorophenol compound for the enzymatic degradation using free and immobilized laccase derived from a white rot fungus i.e. *Trametes versicolor*. Degradation of 2-chlorophenol by laccase was optimized for selected parameters i.e. temperature, pH and enzyme loading. The kinetics of the reaction was studied and laccase activity in its free and immobilized form was compared.

Substrate assay was performed based on the reaction of the degraded product of 2-chlorophenol with Methylene Blue dye. The possible products of degradation are quinone/phenoxy radicals. Hence, by reacting this anionic radical with a cationic dye would result in a coupling reaction, thus rendering decolorization of the dye. In this study, Methylene Blue was used as a cationic dye to couple with the quinone radicals obtained from 2-chlorophenol degradation by laccase. Based on a 1:1 stoichiometry between the phenoxy radical and Methylene Blue molecules, the measure of change (reduction) in the concentration of the dye was equated to the amount of phenoxy radicals formed from 2-chlorophenol. Hence, this is an indirect assay method to determine the activity of laccase in the degradation of 2-chlorophenol.

In this study, statistical method of Response Surface Methodology (RSM) was applied to optimize the degradation of 2-chlorophenol using free and immobilized laccase.

The objectives of the study were

1. to study the biodegradation of 2-chlorophenol by free and immobilized laccase from *Trametes versicolor*;
2. to optimize selected variables for the biodegradation of 2-chlorophenol by free and immobilized laccase using statistical design method;
3. to optimize selected variables for efficient immobilization of laccase in sodium alginate beads;
4. to calculate kinetic parameters' values for free and immobilized laccase;
5. to propose mechanism(s) of action for 2-chlorophenol biodegradation by laccase;
6. to develop a rapid assay protocol for 2-chlorophenol biodegradation by laccase;

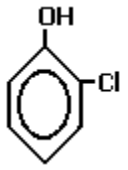
7. to determine using calculation the extent of external and internal mass transfer limitation on the substrate diffusion in the sodium alginate beads.

CHAPTER 2

LITERATURE REVIEW

2.1 Chlorophenols

2-chlorophenol (Figure 2.1) is a commercially produced chemical used entirely as an intermediate in the production of other chemicals. It represents a basic chemical feedstock in the manufacture of higher chlorophenols for such as fungicides, slimicides, bactericides, antiseptics, disinfectants, and wood and glue preservatives. 2-chlorophenol is also used to form intermediates in the production of phenolic resins, and has been utilized in a process for extracting sulfur and nitrogen compounds from coal.



2-chlorophenol (o-chlorophenol),

Figure 2.1 2-chlorophenol

2-chlorophenol (ortho- or o-chlorophenol) is a substituted phenol having the empirical formula C_6H_5OCl. It has a molecular weight of 128.56, a density of 1.2573 at 25°C, and a vapor pressure of 1 mm Hg at 12.1°C (Sax, 1975; Stecher, 1968). 2-chlorophenol melts at 8.7°C and exhibits a boiling point range of 175 to 176°C.

Sources of 2-chlorophenol: 2-chlorophenols are found in the waste water effluents during the commercial production of 2-chlorophenol, its chemically derived products and the inadvertent synthesis of 2-chlorophenol and due to chlorination of phenol in effluents and drinking water sources.

Hazardous effects of 2-chlorophenol: In case of aquatic life, 2-chlorophenol does not appear to be extremely toxic but has been found to impair the flavor of the fish at low concentrations (Ambient Water Quality Criteria for 2-chlorophenol, U.S EPA). In case of human, its toxic effect includes carcinogenicity, reproductive and developmental toxicity, neurotoxicity, and acute toxicity. Depending on the routes of exposure, the symptoms of 2-chlorophenol exposure/poisoning vary. A selected list of such symptoms is shown in Table 2.1, which is derived from the International Chemical Safety Cards (ICSC):

Table 2.1 Symptoms of 2-chlorophenol poisoning

Route of Exposure	Symptoms
1. Inhalation	Cough, Shortness of breath, Sore throat.
2. Skin	May be absorbed. Redness and pain
3. Eyes	Redness, pain and blurred vision
4. Ingestion	Abdominal pain, convulsions, drowsiness and weakness.

As 2-Clorophenol is a Cresol or Phenol compound the symptoms of poisoning given by U.S. EPA are as follows:

i) Eye, skin, mouth and gastrointestinal injuries.

ii) Nausea, vomiting and diarrhea.

iii) Hypotension, myocardial failure, pulmonary edema, neurological changes, liver and renal toxicity, methemoglobinemia and hemolysis.

Methods of removal from water: Conventional methods to remove this compound from water were based on adsorption phenomena. The substances used to adsorb this

compound were bituminous shale (Tutem *et al.,* 1998), red mud (Gupta *et al.,* 2004), microalgae *Tetraselmis marina* (Petroutsos *et.al,,* 2007), coir pith carbon (Namasivayam and Kavitha, 2003) etc. Due to the inefficiency of removal of this compound using conventional methods, biological methods gained importance.

In biological method, until very recently, the literature concerning biodegradation and bioremediation of organic chemical wastes (xenobiotics) dealt almost exclusively with bacteria. Studies reveal that a few bacterial species like *Rhodococcus erythropolis* co-cultured with *Pseudomonas fluorescens* P1 (Goswami *et al.,* 2005), *Pseudomonas putida* CP1 (Farrell and Quilty, 2002) and *Pseudomonas pickettii* strain (Fava *et al.,* 1995) are capable of degrading 2-chlorophenol. It is now becoming apparent that fungi also play an important role in degrading organic materials in the ecosystem, and that they have the potential for remediating contaminated soil and water. Although bacteria are fast growing and can respond to a changing environment by population utilizing the energy source present, there are important advantages of using fungi instead of bacteria for biodegradation. Many of the pollutants are toxic to the organisms that are supposed to degrade them. The extracellular enzyme system of fungi enables them to tolerate considerably higher concentrations of certain xenobiotics than bacteria that have their enzymes inside the cell. Also, many of these chemicals have low water solubility and are therefore not available to the same extent to bacteria. Among fungal species the white rot fungi are most widely studied due to their tremendous efficiency in degrading phenolic compounds like chlorinated phenols. Within this group, the widely studied species are *Trametes versicolor* (Sedarati *et.al*, 2003) and *Phanerochaete chrysosporium* (Zouari *et al.,* 2002). The ability of these white rot fungi to degrade complex compounds is due to the lignin-degrading enzyme(s) system. During recent

years, it has become clear that the lignin-degrading enzyme(s) system of these fungi is quite nonspecific. Due to their non-specific nature, these white rot fungi are found to degrade broad range of structurally diverse xenobiotics (Table 2.2).

Table 2.2 Xenobiotics mineralized by white-rot fungi

Xenobiotics Mineralized	Reference
1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane (DDT)	Bumpus *et al.*, 1985
Lindane	
2,3,7,8-TCDD	
3,4,3',4-TCB	
Aroclor 1254	Eaton 1985
4-Chloroaniline and Chloroaniline-lignin conjugates	Arjmand and Sandermann 1985
Benzo(a)pyrene	Haemmerli *et al.*, 1986
Triphenylmethane dyes	
Crystal violet	
Pararosaniline	
Cresol red	Bumpus and Brock 1988
Bromphenol blue	
Ethyl violet	
Malachite green	

Continued from the previous page…

Brilliant green	
2,4,5-Trichlorophenoxyacetic acid	Ryan and Bumpus 1989
Polycyclic aromatics	
Anthracene	
Fluorantherne	Huttermann *et al.,* 1989
Benzoperylen	
Azo and Heterocyclic dyes	
Orange II	
Tropaeolin O	Cripps *et al.,* 1990
Congo Red	
Azure B	
Trinitrotoluene	Fernando *et al.,* 1990

Lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase from white rot fungi can dehalogenate polychlorinated phenols. Peroxidases and laccases have long been known to oxidize a wide variety of phenols. Lyr demonstrated in 1963 that a crude laccase from *T. versicolor* oxidized chlorophenols to unknown products with the release of chloride,

and subsequent studies with plant peroxidases showed that chlorophenols were enzymatically dechlorinated to give uncharacterized products (Saunders and Stark, 1967).

2.2 Laccase

Laccases (EC 1.10.3.2) are glycosylated multicopper-oxidase enzymes that are found in many plants, fungi, and microorganisms. The copper is bound in several sites *viz.* Type 1, Type 2, and/or Type 3 copper. The ensemble of types 2 and 3 copper is called a trinuclear cluster. Laccases act on phenols and similar molecules, performing a one-electron oxidation and they reduce O_2 to water. Figure 2.2 is an example of structure of laccase obtained from *T. versicolor.*

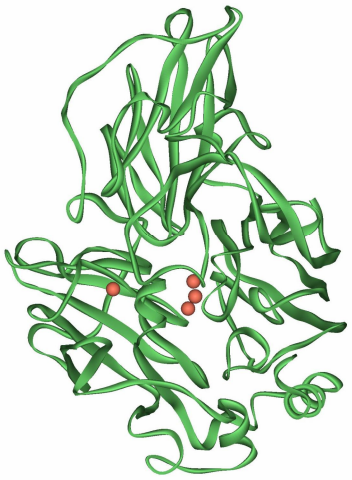


Figure 2.2 Laccase from *T. versicolor*
(Image Source: University of Oxford, Department of Chemistry, UK.)

Laccase is classified as one of the lignin modifying enzymes (LMEs) together with lignin peroxidase and manganese peroxidase. The enzyme was discovered in the exudates of *Rhus vernicifera*, the Japanese lacquer tree (Yoshida, 1883), and was described for the first time by Yoshida in 1883 and characterized by Bertrand in 1985 (Mayer and Staples, 2002; Thurston, 1994). They were discovered to be present in insects, bacteria and widely diffused in basidiomyceteous and ascomyceteous fungi (Claus, 2004).

Laccase has a broad specificity towards aromatic compounds with hydroxyl and amine groups (Young *et al.,* 1995). Hence, this enzyme can carry out a one electron oxidation of various aromatic substrates such as diphenols and aromatic amines (Wesenberg *et al.,* 2003; Thurston, 1994). In addition, laccase can also catalyze the oxidation of methoxy – substituted monophenols, ortho and para diphenols as well as non–phenolic substances in the presence of an artificial primary laccase substrate for example 2,2`-azino-bis (3-ethylbenz-thiazoline-6-sulfonate) (ABTS) (Thurston, 1994; Bourbonnais and Paice, 1990). Due to their very broad substrate range they are implicated in an extensive series of functions such as pathogenesis, immunogenesis and morphogenesis of organisms and in the metabolic turnover of complex organic substances such as lignin, humic matter, and toxic xenobiotics (McGuirl and Dooley, 1999). The simplest reactions catalyzed by laccases are those in which a vast set of substrates, typically phenols and arylamino compounds, are oxidized to the corresponding radical species by direct interaction with their active site (Claus, 2004) and accompanied by the reduction of molecular oxygen to water. More recently it was discovered that white-rot fungi can expand their substrate range and are then able to oxidize compounds with a redox potential exceeding their own, such as non-phenolic benzylalcohols (Johannes and Majcherczyk, 2000; Xu *et al.,* 1999; Bourbonnais and Paice, 1990). In the presence of specific mediators, the catalytic competencies of the enzyme towards the substrates, which are not oxidized directly, either because the larger size or higher redox-potential than laccase could be extended. These mediators act as intermediate substrates for laccase, the oxidized forms of which are sufficiently stable to leave the enzyme site and react with the bulky or high redox-potential substrate targets. This finding led to the discovery that laccase-mediator systems effectively play a major role in the biodegradation of lignin and recalcitrant aromatic

pollutants (Murugesan, 2003; Mayer and Staples, 2002; Bourbonnais and Paice, 1990). Owing to their high and non-specific oxidation capacities, to the lack of a requirement for cofactors and to the use of readily available oxygen as an electron acceptor, laccases are useful biocatalysts with some established and many more emerging biotechnological applications such as biobleaching, xenobiotics bioremediation, textile dyes decolorization, biosensors, food industry etc. (Couto and Herrera, 2006; Aust and Benson, 1993).

2.2.1 Laccase Structure

These multicopper oxidases are generally monomeric glycoproteins containing about 500 amino acids arranged in 3 β-barrel domains assembled to model three spectroscopically distinct copper binding catalytic sites: one type 1 (T1, blue copper, characterized by a strong absorption at 600 nm), one type 2 (T2, normal copper) and one type 3 (T3, EPR-silent antiferromagnetically coupled dinuclear coppers) (Ducros *et.al.,* 1998; Malmstrom, 1982). Four one-electron oxidations of the reducing substrates mentioned above which occur at the T1 site on the protein surface are coupled to the four-electrons reduction of dioxygen to water which occurs at the internal T2/T3 cluster (Solomon *et al.*, 1996, 2001).

$$O_2 + 4\ e^- + 4\ H^+ \rightarrow 2\ H_2O$$

The most commonly used substrates for laccase activity determination are syringaldazine, 2,2`-azino-bis (3-ethylbenz-thiazoline-6-sulfonate) (ABTS) (Wolfenden, 1982) and 2,6-dimethoxy phenol (de Jong *et. al*., 1994).

Figure 2.3 showed the varying stages of laccase during its reaction with compatible compounds:

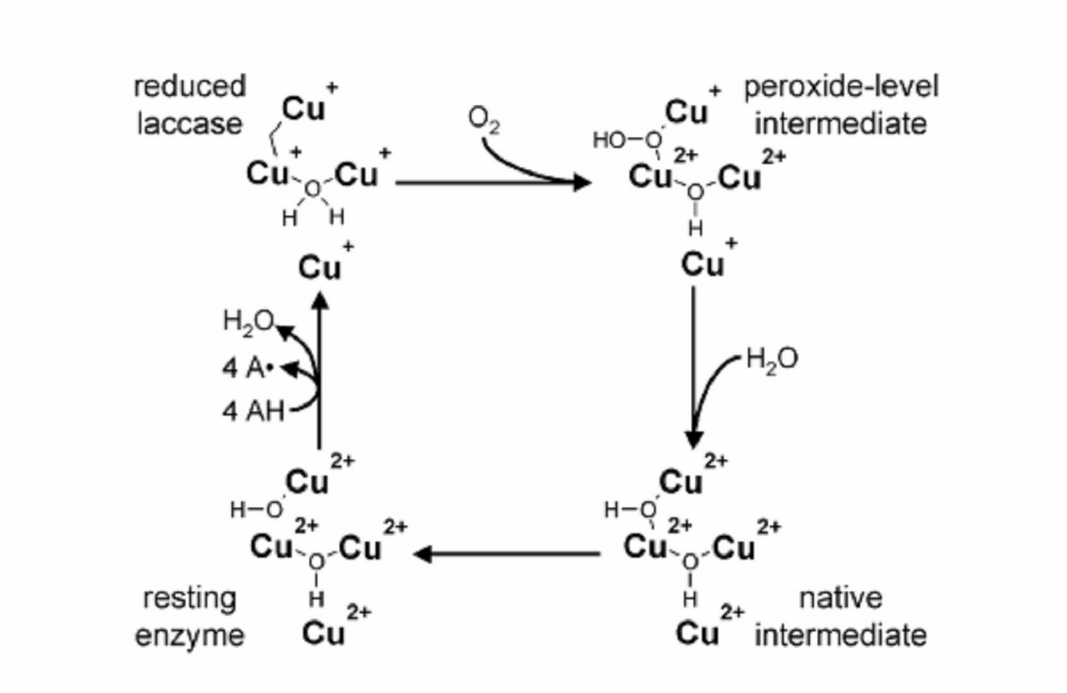


Figure 2.3: Catalytic cycle of laccase during reaction with suitable compounds

(Wesenberg *et al.*, 2003)

2.2.2. Biotechnological and Industrial Applications of Laccase

Due to their ability to oxidize both phenolic and nonphenolic lignin related compounds, as well as highly recalcitrant environmental pollutants, laccase has become a preferred choice for several biotechnological processes. Such applications include the detoxification of industrial effluents, mostly from the paper and pulp, textile and petrochemical industries, use as a tool for medical diagnostics and as a bioremediation agent to clean up herbicides, pesticides and certain explosives in soil. Laccases are also used as cleaning agents for certain water purification systems, as catalysts for the manufacture of anti-cancer drugs and even as ingredients in cosmetics. In addition, their capacity to remove xenobiotic substances and produce polymeric products makes them a useful tool for bioremediation purposes.

Laccase-mediated systems have been applied successfully to numerous processes such as pulp delignification (Xu *et al.,* 1999; Bourbonnais *et al.,* 1998, 1997; Crestini and Argyropoulos, 1998), oxidation of organic pollutants (Collins *et al.,* 1996), and the development of biosensors (Kulys *et al.,* 1997) or biofuel cells (Palmore and Kim, 1999). Table 2.3 summarized a list of potential applications of laccase in various industries:

Table 2.3 Industrial and environmental applications of laccases

Application	**Laccase Source**	**Reference**
Decolorization	*Aspergillus niger*	Soares *et al.,* (2002)
of dyes	*Pleurotus ostreatus*	Hou *et al.,* (2004)
	Trametes hirsuta	Domínguez *et al.,* (2005)
Degradation of	*Cladosporium sphaerospermum*	Potin *et al.,* (2004)
xenobiotics	*Trametes sp.*	Tanaka *et al.,*(2003)
Biosensors	*Agaricus bisporus, Aspergillus niger,*	Timur *et al.,*(2004)
	Trametes versicolor	Jarosz-Wilkołazka *et al.,*(2005)
	Coriolus unicolor	Ferry and Leech (2005)
Effluent	*Gliocladium virens*	Murugesan (2003)
Treatment	*Lentinula edodes*	Casa *et al.,* (2003)
	Trametes versicolor	Lucas *et al.,* (2003)
Biopulping	*Coriolus versicolor*	Call and Mücke (1997)
	Peniophora sp., Pycnoporus sanguineus,	Kandioller and Christov (2001)

Continued from the previous page…

	Trametes hirsuta, Trametes versicolor	Archibald *et al.,* (1997)
	Trametes versicolor	
Organic	*C. hirsuta*	Baker *et al.,* (1996)
synthesis	*Trametes versicolor*	Akta *et al.,* (2001)
	T. villosa	Uchida *et al.,* (2001)
Food industry	*Chinese rhus lacquer*	Huang *et al.,* (1995)
	Trametes hirsuta	Kuuva *et al.,* (2003)
	Pycnoporus cinnabarinus	Georis *et al.,* (2003)
Biobleaching	*Coriolus versicolor*	Balakshin *et al.,* (2001)
	Pycnoporus cinnabarinus	Georis *et al.,* (2003)
	Trametes versicolor	Paice *et al.,* (1995)
Denim	*Trametes versicolor*	Pazarlıoglu *et al.,* (2005)
bleaching		

2.3 Chlorophenol degradation by free and immobilized laccase

Several studies have been conducted to compare the activity of free and immobilized laccase in the degradation of chlorophenols. In one study transformation of chlorophenols by free and immobilized laccase from *Trametes versicolor* was examined. (Sedarati *et al.,* 2003). Leontievsky *et al.,* (2001) demonstrated the transformation of 2,4,6-trichlorophenol by free and immobilized fungal laccase. The detoxifying effects of laccase in

reaction with xenobiotics was shown using intact fungal cultures (Kadhim *et al.,* 1999; Milstein *et al.,* 1992), isolated laccases in soluble form (Ullah *et al.,* 2000a ; Arcand and Archibald, 1991) and with immobilized laccase from different sources (Ruggiero *et al.,* 1989). Of these, the immobilized enzyme had an obvious advantage because of increased stability, resistance to unfavorable conditions of reaction and shifted catalytic properties.

2.4 Enzyme Kinetics

2.4.1. Free enzyme kinetics

The rate of reaction catalyzed by an enzyme increases linearly with the substrate concentration up to a point where it reaches the maximum rate value called V_{max} beyond which there is no further increase in reaction rate; this is called substrate saturation kinetics. The phenomenon of substrate saturation is described by the Michaelis-Menten equation given below.

$$v = \frac{V_{max}[S]}{[S] + K_m} \qquad \text{Eq. (1)}$$

where, V_{max} is the maximum rate of reaction, which occurs when the enzyme is saturated with substrate, v is the rate of reaction, [S] is the substrate concentration and K_m is Michaelis constant. This equation can be used to predict the rate of reaction catalyzed by an enzyme at any substrate concentration provided the values of V_{max} and K_m, are known. K_m is the substrate concentration needed to obtain a reaction rate equal to 1/2 V_{max}. The substrate concentrations and reaction rates considered in the above equation are always the initial values, i.e., the values at the start of reaction. The value of K_m is characteristic for each enzyme substrate combination. K_m may be influenced by pH, temperature, ionic strength and other factors.

Figure 2.4 showed a typical Michaelis-Menten plot.

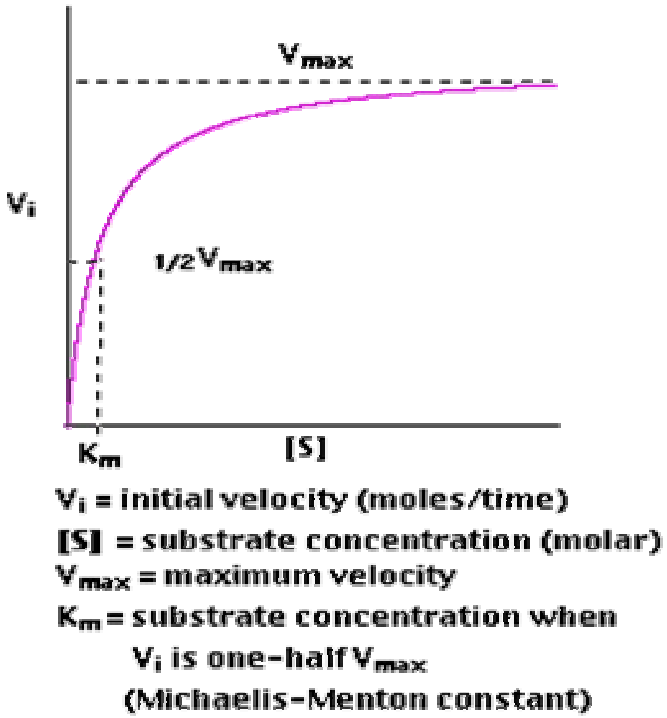


Figure 2.4 Michaelis-Menten plot

(Doran, 1995)

Following are the features of the above plot:

- At low values of [S], the initial velocity, V_i, rises almost linearly with increasing [S].
- But as [S] increases, the gains in V_i level off (forming a rectangular hyperbola).
- The asymptote represents the maximum velocity of the reaction, designated $V_{max.}$
- The substrate concentration that produces a V_i that is one-half of V_{max} is designated the Michaelis-Menten constant, K_m.

K_m is (roughly) an inverse measure of the affinity or strength of binding between the enzyme and its substrate. The lower the K_m, the greater is the affinity (so the lower the concentration of substrate needed to achieve a given rate).

Several graphical methods are available for estimating kinetic parameters for Michaelis-Menten reaction. The first step in the kinetic analysis of enzyme reactions is to obtain data for initial rate of reaction V as a function of substrate concentration [S]. These data can then be plotted in various ways for determination of V_{max} and K_m. Initial reaction rate is preferred because experimental conditions such as enzyme and substrate concentrations are known most accurately at the start of the reaction.

In order to obtain a linear plot for the estimation of V_{max} and K_m, Lineweaver-Burk plot can be used in which $1/V$ plotted against 1/[S] gives a straight line with slope K_m / V_{max} and intercept $1/ V_{max}$. Figure 2.5 showed a Lineweaver-Burk plot.

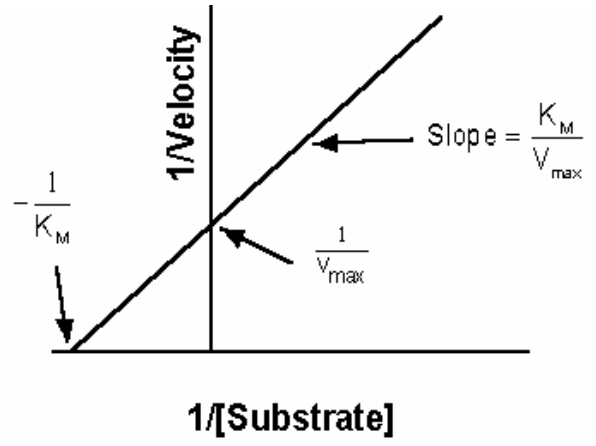


Figure 2.5 Lineweaver-Burk plot

A linear plot called Eadie-Hofstee plot can also be used. In this plot, $V/$[S] is plotted against V to give a straight line with slope $-K_m$ and y-intercept is V_{max}. Figure 2.6 showed an Eadie-Hofstee plot.

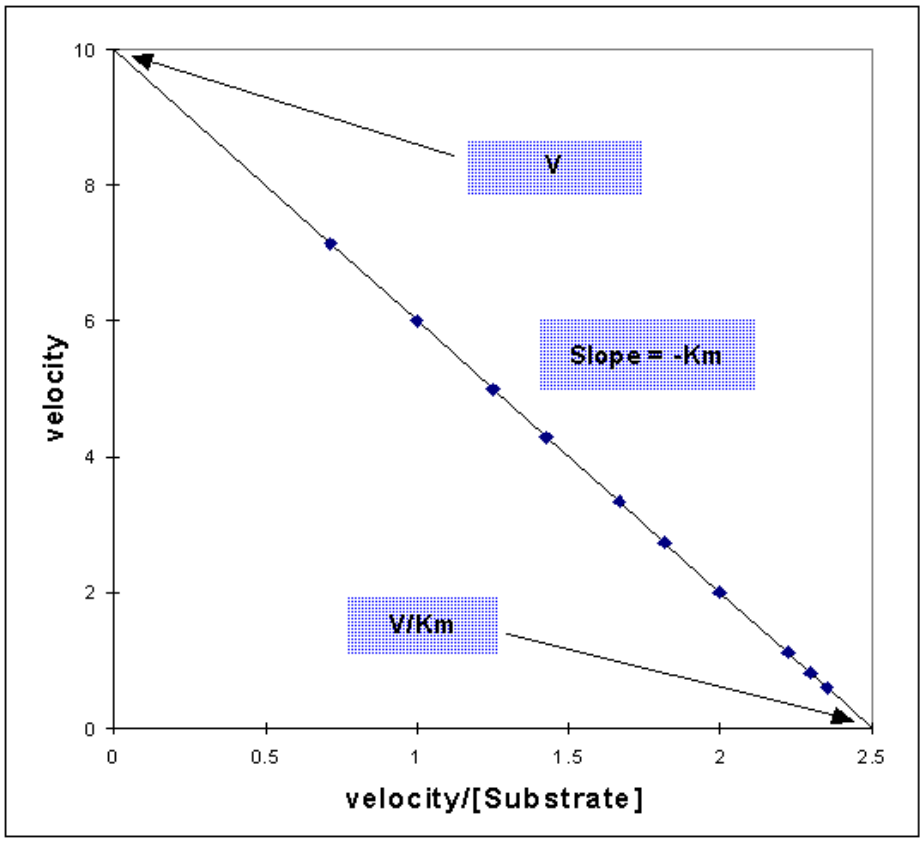


Figure 2.6 Eadie-Hofstee plot

Another linearised plot was proposed by Langmuir, in which [S]/*V* plotted against [S] gives a straight line with slope 1/ V_{max} and intercept K_m / V_{max}.

Direct linear plot can also be used for the determination of kinetic parameters. This was proposed by Eisenthal and Cornish-Bowden. In this, *V* is plotted against [S] on the negative horizontal axis. A straight line is then drawn joining corresponding (-[S], V) points. In the absence of experimental error, lines for each pair intersect at a unique point (K_m, V_{max}). Figure 2.7 represents the direct linear plot.

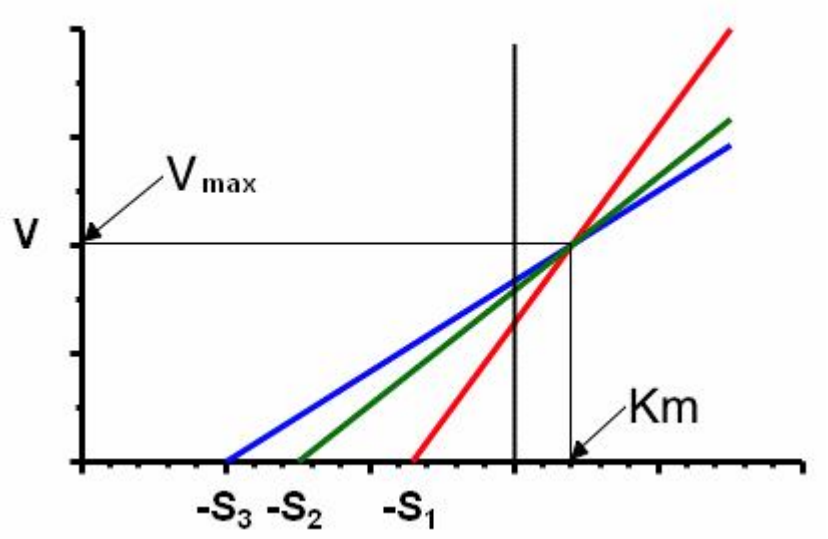


Figure 2.7 Direct linear plot

In order to obtain accurate kinetic parameter values, non-linear regression should be used and not the slopes and intercepts of the linear regression plot. This is because the transformations (reciprocals) distort the experimental error, so the double-reciprocal plot does not necessarily obey the assumptions of linear regression. However, linear plots can be used to provide an initial estimate of the kinetic parameters value for use in the non-linear regression.

2.4.2. Immobilized enzyme kinetics

The kinetics of immobilized enzyme would be different from that of the free enzyme. Many factors are responsible for these differences. The mechanisms responsible for the difference in the kinetics of free and immobilized enzyme may be classified as follows:

(1) *conformational effect:* the conformation of the enzyme may be altered by immobilization.

(2) *electrostatic and partitioning effect:* the concentration of important chemical species (for example, hydrogen ions, substrate molecules and product molecules) in the immediate environment of the immobilized enzyme may be different from their concentration in the free bulk solution due to the physicochemical properties of the support.

(3) *diffusional or mass transfer effect:* the observed kinetics of the immobilized enzyme may not be solely governed by interactions between the enzyme and substrate, but instead may be limited to some extent by the rate of substrate diffusion to the external surface of the support, and/or by the rate of substrate diffusion through the internal pores of the support.

In cases where effect (1) is significant, the true or intrinsic kinetic parameters (like V_{max} and K_m) of the immobilized enzyme may be different from those of the soluble enzyme (Harvey *et al.*, 1996)).

For example, when an enzyme is immobilized in the spherical beads of sodium alginate and if the reaction in the particles follows Michaelis-Menten kinetics, the rate of reaction, r_A is given by:

$$D_{Ae}\left(r^2\left(d^2C_A/dr^2\right)+2r\left(dC_A/dr\right)\right)-\frac{r^2V_{max}C_A}{K_m+C_A}=0 \qquad \text{Eq. (2)}$$

D_{Ae} is the effective diffusibility of the substrate in the solid,

C_A is the concentration of the substrate in the particle,

r is the distance measured radially from the center of the particle and

K_m is the Michaelis-Menten constant.

(Doran, 1995)

2.5 Enzyme Immobilization

Enzymes display great specificity and are not permanently modified by their participation in reactions. Since they are not changed during the reactions, it is cost-effective to use them more than once. When the enzymes are in solution with the reactants and/or products it is difficult to separate them. Therefore, if they can be attached to the reactor in some way, they can be used again after the products have been removed. The term "immobilized" means unable to move or stationary. Thus, an immobilized enzyme is an enzyme that is physically attached to a solid support over which a substrate is passed and converted to product. There are a number of advantages to attaching enzymes to a solid support and some of the major reasons are listed below (Chibata, 1978; Henry, 1989):

- Multiple or repetitive use of a single batch of enzymes.
- The ability to stop the reaction rapidly by removing the enzyme from the reaction solution (or *vice versa*).
- Enzymes are usually stabilized by binding.
- Product is not contaminated with the enzyme (especially useful in the food and pharmaceutical industries).
- Analytical purposes - long half-life, predictable decay rates, elimination of reagent preparation, *etc*.

The most important benefit derived from immobilization is the easy separation of the enzyme from the products of the catalyzed reaction. This prevents the enzyme contaminating the product, minimizing downstream processing costs and possible effluent handling problems, particularly if the enzyme is noticeably toxic or antigenic. It also allows

continuous processes to be practicable, with a considerable saving in enzyme, labour and overhead costs. The plant size needed for continuous process is half than that required for batch processes using free enzymes. The capital costs are, therefore, considerably smaller. Table 2.4 listed some of important industrial uses of immobilized enzymes*:*

Table 2.4 Industrially important immobilized enzymes (http://www.lsbu.ac.uk/biology/enztech/improcess.html*)*

Enzyme	EC number	Product
Aminoacylase	3.5.1.14	L-Amino acids
Aspartate ammonia-lyase	4.3.1.1	L-Aspartic acid
Aspartate 4-decarboxylase	4.1.1.12	L-Alanine
Cyanidase	3.5.5.x	Formic acid (from waste cyanide)
Glucoamylase	3.2.1.3	D-Glucose
Glucose isomerase	5.3.1.5	High -fructose corn syrup
Histidine ammonia-lyase	4.3.1.3	Urocanic acid
Hydantoinase	3.5.2.2	D- and L-amino acids
Invertase	3.2.1.26	Invert sugar
Lactase	3.2.1.23	Lactose-free milk and whey
Lipase	3.1.1.3	Cocoa butter substitutes
Nitrile hydratase	4.2.I.x	Acrylamide
Penicillin amidases	3.5.1.11	Penicillins
Raffinase	3.2.1.22	Raffinose-free solutions
Thermolysin	3.2.24.4	Aspartame

Immobilized enzymes had been successfully used in a number of biotechnological processes. Dulik and Fenselau demonstrated their use in drug metabolism studies (Dulik and Fenselau 1988), wherein the immobilization of drug-metabolizing enzymes onto polymeric support offered several advantages like increasing storage stability, separation of products from the incubation mixture, the ability to recover and reuse the enzyme catalysts and stabilization of the tertiary structure of membrane-bound enzymes. In another study by Chirillo *et al.,* immobilized enzyme reactors were used in a continuous flow analyzer for detection of urea, glucose and uric acid (Chirillo *et al.,* 1979). The reactors used in this study consisted of plastic cartridges containing spirally wound nylon tubes, on the inner walls of which specific enzymes have been immobilized by covalent binding. Enzyme had been immobilized for the biotransformation reactions as well for the production of new and safer drug intermediates (Hickey *et al.,* 2007). In this study, use of miniaturized flow reactors for thermophilic enzymes has been demonstrated. Various types of reactors have been designed for specific enzymatic reactions. Fluidized bed reactors (Bodalo *et al.,* 1995), immobilized capillary enzyme reactor (Tang *et al.,* 2007), stirred tank reactors (Bodalo *et al.,* 1993) are a few types of reactors that have been used.

Methods used for the immobilization of enzymes fall into four main categories (Figure 2.8):

1. physical adsorption onto an inert carrier;
2. covalent binding to a reactive insoluble support;
3. cross-linking of the protein with a bifunctional reagent and
4. inclusion in the lattices of a polymerized gel.

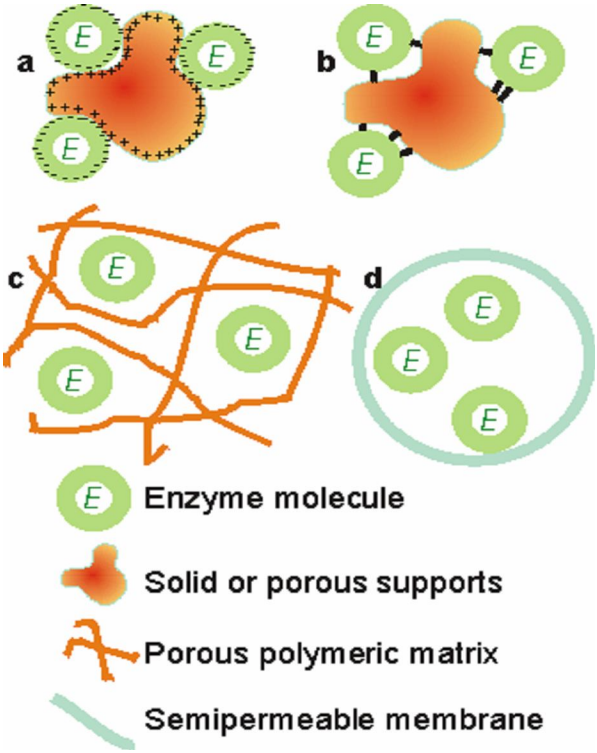


Figure 2.8 Immobilized enzyme systems (a) enzyme non-covalently adsorbed to an insoluble particle; (b) enzyme covalently attached to an insoluble particle; (c) enzyme entrapped within an insoluble particle by a cross-linked polymer; (d) enzyme confined within a semipermeable membrane

(Image source: London South Bank University, Faculty of Engineering, Science and The Built Environment)

Entrapment of enzymes and/or cells in alginate is one of the simplest methods of immobilization. Alginates are available commercially as water-soluble sodium alginates, and have been used for more than 65 years in the food and pharmaceutical industries as thickening, emulsifying, film forming, and gelling agents. Entrapment in insoluble calcium alginate gel is recognized as a rapid, nontoxic, inexpensive, and versatile method for immobilization of enzymes and cells (Park *et al.,* 1995; Palmieri *et al.,* 1994; Nilsson, 1987).

2.6 Response Surface Methodology (RSM)

Many statistical experimental design methods have been employed in bioprocess optimization. Among them, RSM is the one suitable for identifying the effect of individual variables and for seeking the optimum conditions for a multivariable system efficiently.

Response surface methodology (RSM) is an effective statistical technique for the investigation of complex processes. The method was introduced by G. E. P. Box and K. B. Wilson in 1951. The main advantage of RSM is to reduce number of experimental runs needed to provide sufficient information for statistically acceptable result. It is a faster and less expensive method for gathering research result than the classical method (Shieh *et al.*, 1995). RSM had been successfully used for various optimization process like enzymatic synthesis of sorbitan methacrylate (Jeong and Park, 2006), chitinase production (Navani and Kapadnis, 2005), biopolymerization rate of catechol using laccase (Nahit, 2005).

It is common to begin a process model for design of experiment (DOE) with a "black box" type with several discrete or continuous input factors and one or more measured output responses. Experimental data are used to derive an empirical model linking the outputs and inputs. These empirical models generally contain first and second order terms. The most common empirical models fit to the experimental data take either a *linear* form or *quadratic* form.

A linear model with two factors, X_1 and X_2, can be written as

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \text{Experimental error} \qquad \text{Eq. (3)}$$

where Y is the response for given levels of the main effects X_1 and X_2 and the X_1X_2 term is included to account for a possible interaction effect between X_1 and X_2. The constant β_0 is the response of Y when both main effects are 0.

For a linear model with three factors X_1, X_2, X_3 and one response, Y, would look like (if all possible terms were included in the model)

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{123} X_1 X_2 X_3 +$$ Exerimental error

Eq. (4)

The three terms with single "X's" are the *main effects* terms. There are three two-way interaction terms and one three-way interaction term (which are often omitted, for simplicity). When the experimental data are analyzed, the entire unknown "β" parameters are estimated and the coefficients of the "X" terms are tested to see which ones are significantly different from 0.

A second-order (quadratic) model (typically used in *response surface* DOE's with suspected curvature) does not include the three-way interaction term but adds three more terms to the linear model, namely

$$\beta_{11} X_1^{\,2} + \beta_{22} X_2^{\,2} + \beta_{33} X_3^{\,2}$$

Hence, equation 4 can be modified as:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^{\,2} + \beta_{22} X_2^{\,2} + \beta_{33} X_3^{\,2}$$ + Experimental error

Eq. (5)

In RSM the experiment is designed to allow us to estimate interaction and even quadratic effects, and therefore give us an idea of the (local) shape of the response surface we are investigating. RSM designs are used to:

- find improved or optimal process settings
- troubleshoot process problems and weak points
- make a product or process more *robust* against external and non-controllable influences. "Robust" means relatively insensitive to these influences.

Box-Behnken designs are experimental designs of RSM, devised by George E. P. Box and Donald Behnken in 1960, to achieve the following goals:

- each factor, or independent variable, is placed at one of three equally spaced values (at least three levels are needed.);
- the design should be sufficient to fit a quadratic model, that is, one containing squared terms and products of two factors;
- the ratio of the number of experimental points to the number of coefficients in the quadratic model should be reasonable (in fact, their designs kept it in the range of 1.5 to 2.6);
- the estimation variance should more or less depend only on the distance from the centre (this is achieved exactly for the designs with 4 and 7 factors), and should not vary too much inside the smallest (hyper) cube containing the experimental point;

Box-Behnken designs are constructed by first combining two-level factorial designs with incomplete block designs (IBD) and then adding a specified number of replicated center points. For example Box-Behnken design for three factors involves three

blocks, in each of which 2 factors are varied through the four (4) possible combinations of high and low. It is necessary to include centre points as well (in which all factors are at their central values).

Figure 2.9 showed a visualization of BBD on three factors.

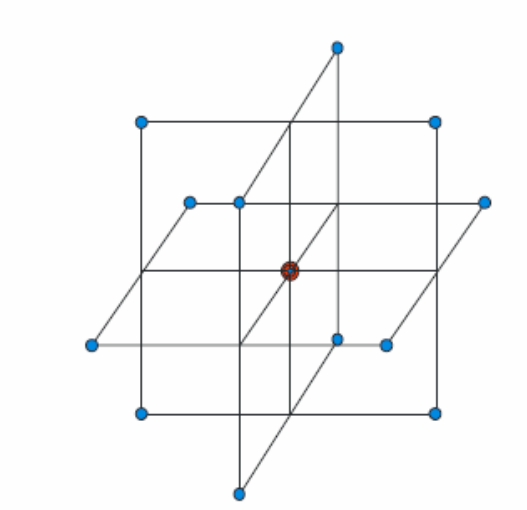


Figure 2.9 Box-Behnken design on three factors

Box-Behnken design has been successfully used for optimization of various processes like enzymatic hydrolysis of mango kernel starch (Chowdary *et al.,* 2000), carotenoid production by *Aspergillus carbonarius* in submerged fermentation (Sanjay *et al.,* 2007) and in determining the hydroxyl fatty acid content in castor oil (Turner *et al.,* 2004).

2.7 Assay methods for 2-chlorophenol degradation by laccase

Chlorophenols and their products of degradation by laccase have been assayed by using methods like high performance liquid chromatography (HPLC) (Sedarati *et al.,* 2003; Farnet *et al.,* 2002), mass spectrometry (Eberlin and Cesar, 2008). They can also be determined by coupling the degraded products with specific compounds, which enable the

formation of dyes, which can then be spectrophotometrically determined and assayed (Abdullah *et al.*, 2007). The higher the intensity of the dye, the higher is the coupling reaction, which indirectly implies the stochiometrically proportionate amount of degraded product formed. The assay of degradation products of chlorophenols can also be determined by using chloride sensors. The major disadvantage of using chloride sensors for assaying degraded products of chlorophenols is an underestimation of the result in cases when the degradation is not through the de-chlorination mechanism alone.

CHAPTER 3

MATERIALS AND METHODS

The experiments conducted in this research were as follows:

- Linearity assay of laccase enzyme using syringaldazine as substrate;
- Determination of kinetic parameters (V_{max} and K_m) for free and immobilized enzyme using syringaldazine as substrate under optimal condition;
- Development of indirect assay method for degradation of 2-chlorophenol by laccase;
- Determination of 2-chlorophenol concentration to be used in subsequent studies;
- Optimization of pH, temperature and enzyme loading for the reaction of free laccase with 2-chlorophenol;
- Enzyme immobilization and immobilization optimization;
- Determination of immobilized enzyme activity using syringaldazine;
- Optimization of temperature, enzyme loading and reaction time for the reaction of immobilized laccase with 2-chlorophenol;

3.1 MATERIALS

3.1.1

Laccase from *Trametes versicolor* (Sigma). The enzyme activity was 0.8 U of enzyme in 1 mg powder. A 0.2 U ml^{-1} enzyme solution was prepared by dissolving 0.25 mg of powder in 1 ml distilled water. Similarly, enzyme solutions were prepared with concentrations (U ml^{-1}): 0.006, 0.008, 0.01, 0.03, 0.05, 0.1, 0.3, 0.4 and 0.6.

3.1.2

Phosphate buffer solution: For 50 mM solution with pH7, the buffer was prepared by following the calculation by Henderson-Hasselbalch equation which is as follows:

$pH = pK_a + \log ([\text{conjugate base}]/[\text{acid}])$ Eq. (6)

Hence, to prepare a phosphate buffer with pH 7, the following calculation was used.

$pH = 6.86 + \log ([Na_2HPO_4]/[NaH_2PO_4])$

$7 = 6.86 + \log ([Na_2HPO_4]/[NaH_2PO_4])$

$\log ([Na_2HPO_4]/[NaH_2PO_4]) = 7 – 6.86 = 0.14$

$([Na_2HPO_4]/[NaH_2PO_4]) = \text{antilog } (0.14) = 1.380/1$

$[Na_2HPO_4] = 1.38/2.38 \times 50\text{mM} = 29 \text{ mM}$

$[NaH_2PO_4] = 1/2.38 \times 50\text{mM} = 21 \text{ mM}$

where 50mM is strength of buffer. Once the number of moles was known, mass was calculated as follows:

Number of moles (N) = weight of compound / molecular weight.

Hence, for preparing 1 L of buffer solution

Mass of Na_2HPO_4 used = $0.029 \times 141.98 = 4.1$ g

Mass of NaH_2PO_4 used = $0.021 \times 119.98 = 2.5$ g

Hence, 4.1 g Na_2HPO_4 and 2.5 g of NaH_2PO_4 was dissolved in 1 L of distilled water.

3.1.3

2-chlorophenol (C_6H_5ClO, molecular weight 128.56 gm mol^{-1}) (Sigma). 100 mL of stock solution of 1mM was prepared in 99% ethyl alcohol, which was further diluted to obtain 6, 7, 8, 9 and 10 µM solutions. The stock solution was prepared as follows:

1 M = 128.56 g L^{-1} ethyl alcohol (99%)

1mM = 0.12856 g L^{-1} ethyl alcohol (99%)

To prepare in 100 mL,

1 mM = 0.013 g / 100 ml ethyl alcohol (99%)

Density of 2-chlorophenol is 1.26 g mL^{-1}.

Hence for 0.0013 g the equivalent volume would be 0.013 / 1.26 = 10.3 µL. 100 ml of stock solution was thus prepared by dissolving 10.3 µL of 2-chlorophenol in 100 ml of 99% ethyl alcohol. Using the stock solution, various dilutions were prepared by using the formula $m_1v_1 = m_2v_2$, where m_1 is the molarity of the stock solution, v_1 is the volume of the stock solution, m_2 is the desired molarity and v_2 is desired volume.

3.1.4

Methylene Blue dye ($C_{16}H_{18}N_3ClS$, molecular weight 373.91 gm mol^{-1}) (Sigma). A concentration of 0.1mM was used. 0.1mM solution was prepared in distilled water as follows:

1 M = 373.91 g L^{-1} distilled water.

1 mM = 0.374 g L^{-1} distilled water

0.1 mM = 0.0374 g L^{-1} distilled water = 3.74 mg / 100 mL distilled water

Hence, 3.74 g of Methylene Blue dye powder was dissolved in 100 mL distilled water to obtain 0.1mM solution.

3.1.5

Sodium Alginate (molecular weight 216 gm/mol) (Fluka). 1 % sodium alginate solution was prepared by dissolving 1 g of the powder in 100 mL of distilled water.

0.5% calcium chloride solution was prepared by dissolving 0.5 g of calcium chloride in 100 mL of distilled water.

3.1.6

Syringaldazine ($C_{18}H_{20}N_2O_6$, molecular weight 360.36 g mol^{-1}) (Fluka). 1 mM stock solution was prepared in 95% ethyl alcohol. From this stock solution 0.1, 0.2, 0.3, 0.4 and 0.5 mM solutions were prepared using the formula $m_1v_1 = m_2v_2$. The 1 mM stock solution was prepared as follows:

1 M = 360.36 g L^{-1} ethyl alcohol (95%)

1mM = 0.36 g L^{-1} ethyl alcohol (95%)

To prepare in 100 mL,

1 mM = 0.036 g / 100 ml ethyl alcohol (95%).

Hence, 1mM stock solution was prepared by dissolving 0.036g of syringaldazine in 100 mL 95% ethyl alcohol.

3.1.7

Equipments: UV/VIS Spectrophotometer Jasco V-630 (Japan) with 10 mm light path, quartz cuvettes with 10 mm light path.

3.2 METHODS

3.2.1 Linearity assay for laccase enzyme using syringaldazine as substrate

To determine the enzyme activity of free laccase 1.0 ml 0.006 U ml^{-1} of enzyme was pipetted into a test tube and to it 1.0 ml distilled water and 1.0 ml of phosphate buffer pH 5.5 were added. To this mixture, 0.2 ml of 0.5 mM syringaldazine solution was added and a time course measurement was performed in the spectrophotometer at a wavelength of 525 nm for 20 minutes at 25^{o}C. The same procedure was carried repeated using different enzyme concentrations (0.008, 0.01, 0.03 and 0.05 U ml^{-1}). Three replicates measurement were made

for each enzyme concentration. The initial reaction rate i.e. a change in absorbance per minute was obtained using the slope analysis function of the spectrophotometer's software as change in absorbance per minute. This value was converted into concentration of syringaldazine by using Beer-Lambert's relationship.

$$A = k \bullet c \bullet L \qquad \text{Eq. (7)}$$

whereby A = absorbance

k = extinction coefficient of laccase (65000 $M^{-1}cm^{-1}$)

c = concentration of the substrate

L = light path length (10 mm).

t = time (minutes)

$$\frac{dA}{dt} = k \bullet L \bullet \frac{dC}{dt} \qquad \text{Eq. (8)}$$

where dA/dt is the rate of change in absorbance dc/dt is the rate of change for the substrate.

Therefore,

$$\frac{dC}{dt} = \frac{dA}{dt} \bullet \frac{1}{k \bullet L} \qquad \text{Eq. (9)}$$

for syringadazine: k = 65000 M^{-1} cm^{-1}, L = 1 cm. A does not have units but dA/dt is measured in min^{-1}.

Therefore, dc/dt = dA/dt • 1/65000 M^{-1} cm^{-1} • 1cm = M min^{-1}

To convert Mmin^{-1} into mMmin^{-1} the following relationship was used:

M min^{-1} = 1000 mM min^{-1} Eq. (10)

The concentration thus obtained in M min^{-1} was converted to mM min^{-1} and this value was plotted against different enzyme concentration. From the linear range of the plot, an appropriate enzyme concentration range was selected for the further experiments. Similar

calculation was used to obtain initial rate of reaction at different syringaldazine concentrations.

3.2.2. Determination of kinetic parameters (V_{max} and K_m) for free laccase using syringaldazine as substrate

To determine the kinetic parameters of free laccase, 1.0 ml 0.1 U ml^{-1} of enzyme was pipetted into a test tube and to it 1.0 ml distilled water and 1.0 ml of phosphate buffer pH 5.5 were added. To this mixture, 0.2 ml of 0.5 mM syringaldazine solution was added and a time course measurement was performed using spectrophotometer at a wavelength of 525 nm for 20 minutes at 25°C. The same procedure was repeated using different syringaldazine concentrations. Three replicates measurement were made for each syringaldazine concentration. The initial reaction rate was calculated as the change in absorbance per minute. This value was converted into concentration of syringaldazine by using Beer Lambert's relationship (Eq.s 7 – 10). From the rate data against syringaldazine concentration, the V_{max} and $K_m$values were calculated using non-linear regression technique (Polymath® 6 software).

3.2.3. Development of indirect assay method for degradation of 2-chlorophenol by laccase.

Laccase decolorization of Methylene Blue dye

The spectrum for Methylene Blue solution (Methylene Blue dye dissolved in water) was obtained within the wavelength range of 400 – 900 nm. The maximum absorbance was found to be at 665 nm (specific to Methylene Blue dye).

In order to evaluate the possible reaction between laccase enzyme and Methylene Blue dye, 1.0 ml of buffer solution, 1.0 ml of 0.03 U ml^{-1} of laccase, 1.0 ml distilled water and 0.4 ml of 0.1 mM Methylene Blue solution were added to a test tube and incubated at 25°C for 10 minutes. Meanwhile, a blank was prepared by adding 0.4 ml of 0.1 mM Methylene Blue solution to 1.0 ml of buffer solution and 2.0 ml distilled water in a test tube which was incubated at 25°C. Total reaction volume for both sample and blank were 3.4 ml. After 10 minutes the absorbance values of blank and sample were recorded at 665 nm. Three replicates measurement were made and the difference in the absorbance values of blank and samples were recorded.

Laccase degradation of 2-chlorophenol

To 1.0 ml of buffer, 1.0 ml of 0.03 Uml^{-1} of laccase solution and 1.0 ml of distilled water were added in a test tube. To this mixture, 0.2 ml of 6 μM 2-chlorophenol solution was added and incubated at 25°C for 10 minutes. Then, the test tube was boiled at 100°C for 5 minutes to inactivate the enzyme. Subsequently, the reaction mixture was cooled to room temperature and 0.4 ml of 0.1 mM Methylene Blue solution was added and further incubated at 25°C for 10 minutes (**Solution 1**). Meanwhile, a blank was prepared by adding 1.0 ml buffer solution, 2.0 ml distilled water, 0.2 ml 99% ethanol and 0.4 ml Methylene Blue to a test tube which was also incubated at 25°C. Total reaction volume for both sample and blank were 3.6 ml. Three replicates were made for Solution 1 reaction. The absorbance of the blank and samples of Solution 1 were measured at 665 nm and the difference between the absorbance of the blank and the individual sample value was calculated. A *t*-test was performed at 95% confidence level (n=3).

Visible light spectrum of 2-chlorophenol and Methylene Blue dye mixture in liquid solution

To 1.0 ml of buffer solution and 2.0 ml of distilled water, 0.2 ml of 8 μM 2-chlorophenol and 0.4 ml of 0.1 mM Methylene Blue solution were added in a test tube and incubated at 25°C for 10 minutes. In another test tube, 1.0 ml of buffer solution, 2.0 ml of distilled water, 0.2 ml of 99% ethanol and 0.4 ml of 0.1 mM Methylene Blue were added and incubated at 25°C for 10 minutes. Total reaction volume for the two different mixtures was 3.6 ml.

Subsequently, the spectra for the two solutions were obtained within the wavelength range of 400 – 900 nm.

3.2.4 Determination of 2-chlorophenol concentration to be used in subsequent studies

The reaction for Solution 1 (Section 3.2.3) was repeated using different enzyme (0.1, 0.2, 0.3, 0.4 and 0.5 U ml^{-1}) and 2-chlorophenol (6, 7, 8, 9 and 10 mM) concentrations and the absorbance was recorded at 665 nm. Three replicates were prepared for each enzyme and 2-chlorophenol concentration. The values of absorbance were plotted against different enzyme concentrations and in another plot, the values of absorbance was plotted against different 2-chlorophenol concentrations. Using Beer-Lambert's equation (k for Methylene Blue = 74028 M^{-1} cm^{-1} at 665 nm) the change in absorbance with time were converted into the respective rate of change in Methylene Blue concentrations. Blank was prepared for each different concentration of enzyme and 2-chlorophenol used. These mixtures were incubated at 25°C for 10 minutes. Then, they were boiled at 100°C for 5 minutes to inactivate the

enzyme. Subsequently, the reaction mixture was cooled to room temperature and to this 0.4 ml of 0.1 mM Methylene Blue solution was added and further incubated at 25°C for 10 minutes. The absorbance value for blank was also converted into the rate of change of Methylene Blue concentration and using this value, the actual time change in the concentration of Methylene Blue was calculated by subtracting the concentration value of samples from that of blank. These rates of change in concentration of Methylene Blue with different enzyme and 2-chlorophenol concentrations were plotted against different enzyme and 2-chlorophenol concentrations. From the plot, the linear portion was determined and an appropriate concentration of 2-chlorophenol and enzyme concentration was used for all subsequent experiments.

3.2.5 Optimization of pH, temperature and enzyme loading for the reaction of free laccase with 2-chlorophenol

Using Response Surface Methodology, two levels of pH (5 and 7), temperature (30°C and 60°C) and enzyme concentration (0.1 U ml^{-1} and 0.2 U ml^{-1}) representing low and high values were selected. Using MINITAB®14 software, a Box-Behnken design consisted of 45 experiments (including center points) was constructed at three pH values specified at 5, 6 and 7, temperatures at 30°C, 45°C and 60°C, enzyme concentration at 0.1, 0.15 and 0.2 U ml^{-1}.

Table 3.1 showed the randomized run design table generated by MINITAB®14 software using Box-Behnken design.

Table 3.1 Experimental runs for free laccase degradation of 2-chlorophenol using Box-Behnken design

Run	Block	A	B	C
1	1	+	0	+
2	1	-	0	+
3	1	-	0	+
4	1	0	+	+
5	1	0	0	0
6	1	-	0	-
7	1	+	0	+
8	1	+	-	0
9	1	0	+	-
10	1	0	0	0
11	1	0	0	0
12	1	-	+	0
13	1	-	0	+
14	1	0	-	+
15	1	-	-	0
16	1	-	-	0
17	1	0	0	0
18	1	+	-	0
19	1	+	+	0

20	1	-	+	0
21	1	0	+	-
22	1	0	0	0
23	1	-	0	-
24	1	0	-	-
25	1	+	+	0
26	1	+	-	0
27	1	+	0	-
28	1	-	0	-
29	1	0	+	+
30	1	0	-	-
31	1	+	0	-
32	1	0	-	-
33	1	0	+	-
34	1	+	+	0
35	1	+	0	+
36	1	0	-	+
37	1	0	0	0
38	1	0	0	0
39	1	0	0	0
40	1	0	0	0
41	1	-	+	0
42	1	0	+	+

43	1	0	-	+
44	1	-	-	0
45	1	+	0	-

where A, B and C represents the main effects and +, 0 and – refers to the upper, medium and lower values for the main effects. In the experiment, A is pH, B is temperature and C is enzyme concentration.

1.0 ml of buffer, 1.0 ml of enzyme solution and 1.0 ml of distilled water were added to a test tube. To this mixture, 0.2 ml of 0.008 mM 2-chlorophenol solution was added and incubated at 25°C for 10 minutes. Subsequently, the test tube was boiled at 100°C for 5 minutes to inactivate the enzyme. Then, the reaction mixture was cooled to room temperature and to this 0.4 ml of 0.1 mM Methylene Blue solution was added and further incubated at 25°C for 10 minutes. A blank was prepared by adding 1.0 ml buffer solution, 2.0 ml distilled water, 0.2 ml 99% ethanol and 0.4 ml Methylene Blue solution to a test tube. Total reaction volume was 3.6 ml. The absorbance value was recorded for blank and the sample after 10 minutes at 665 nm. This was repeated for all 45 sets of experiment combination obtained from the software and the results recorded. Using Beer Lambert's relationship, the change in absorbance with time was converted into the rate of change of Methylene Blue concentration. The actual rate of change in Methylene Blue concentration was calculated from the concentration difference between blank and individual samples. Based on the experimental data gathered, optimization of pH, temperature and enzyme concentration for free laccase degradation of 2-chlorophenol was performed using the MINITAB®14 software.

3.2.6 Enzyme immobilization and immobilization optimization

The enzyme immobilization method used was *via* entrapment inside calcium alginate beads.***Laccase immobilization***

Sodium alginate solution and enzyme solution were mixed thoroughly in the ratio of 8:1 (8 ml of 1.0% sodium alginate solution and 1.0 ml of enzyme solution) and using a 1.0 ml disposable syringe, the solution was added drop-wise into a beaker containing 20 ml of 0.5% calcium chloride solution. The beaker was then covered with an aluminum foil and incubated for half an hour at 4°C. The beads were recovered by filtration. The recovered beads were then washed with 30 ml distilled water and 5.0 ml buffer.

Optimization of laccase immobilization parameters

The volume ratio of sodium alginate and enzyme solutions, concentration of calcium chloride used and the drying time for beads were optimized. Beads were prepared as outlined in above section with the following combinations as shown in Table 3.2.

Table 3.2 Optimization of immobilization parameters

Concentration of $CaCl_2$ (%)	Volume of sodium alginate solution : volume of enzyme solution	Drying time at 4°C (hr)
0.1	8 : 1	1
0.5	8 : 1	1
0.75	8 : 1	1
1	1 : 1	1
1	1 : 1	0.5
0.75	8 : 1	0.5
0.5	8 : 1	0.5
0.1	1 : 1	1

The activity of the immobilized enzyme was determined using syringaldazine. Immobilized laccase activity was used as the response in the optimization of immobilization condition. Three replicates of each enzyme concentration (0.01, 0.02, 0.03, 0.04 and 0.06 U ml^{-1}) were used for each set of combination listed in Table 3.2.

3.2.7 Determination of immobilized enzyme activity using syringaldazine

The enzyme activity of the immobilized beads was determined using syringaldazine as the substrate. Prior to the determination, standard calibration plot was prepared relating the initial rate of reaction for free enzyme at different enzyme concentrations *viz.* 0.006, 0.008, 0.01, 0.03 and 0.05 U ml^{-1}

The immobilized enzyme beads were prepared as outlined in section 3.2.6. Three replicates were prepared for each enzyme concentration. After the beads were incubated for half an hour at 4°C, they were separated from the calcium chloride solution *via* filtration using Whatman No. 1 filter paper. The filtrate was collected in another fresh beaker. The beads were washed thoroughly with 30 ml distilled water and 5.0 ml buffer solution. The washings were also collected in the same beaker. The beads were then transferred to another beaker containing 5.0 ml buffer solution. To this, 1.0 ml of 0.5 mM syringaldazine solution was added and absorbance was recorded for every 5 minutes for an hour. The absorbance data against time was recorded for each replicate. The average of the three replicates' absorbance was calculated and plotted against time for different laccase concentrations.

The filtrate and the washings were subjected to enzyme activity determination by taking 3.0 ml of the filtrate plus washings solution in a cuvette and adding 0.2 ml syringadazine solution to it. The solution was mixed properly and was subjected to the time course measurement at 525 nm in the spectrometer for 15 minutes. The initial rate of reaction

was obtained and from the standard calibration plots for free laccase activity, the enzyme activity of the filtrate plus washings can be determined. Three replicates were made for this determination.

Protein assay was performed for the filtrate and washings using Bradford method. To 0.1 ml of known mass of bovine serum albumin (BSA) in solution, 3.0 ml of Bradford reagent was added and incubated for 15 minutes. A blank was prepared using 0.1 ml distilled water and 3.0 ml of Bradford reagent. After 15 minutes of incubation, the absorbance reading was recorded for blank and sample at 595 nm. Three replicates were used for each BSA concentration (0.1, 0.2, 0.4, 0.6, 0.8 and 1ppm). A standard curve for the protein assay was constructed using different concentrations of bovine serum albumin (BSA) as standard protein.

To determine the protein content in the filtrate and washings, 0.1 ml of the filtrate solution was taken and to it 3.0 ml of Bradford reagent was added in a cuvette and incubated for 15 minutes. A blank was prepared using 0.1 ml distilled water and 3.0 ml of Bradford reagent. After 15 minutes of incubation, the absorbance reading was recorded for blank and sample at 595 nm. Three replicates were prepared for each laccase concentration used.

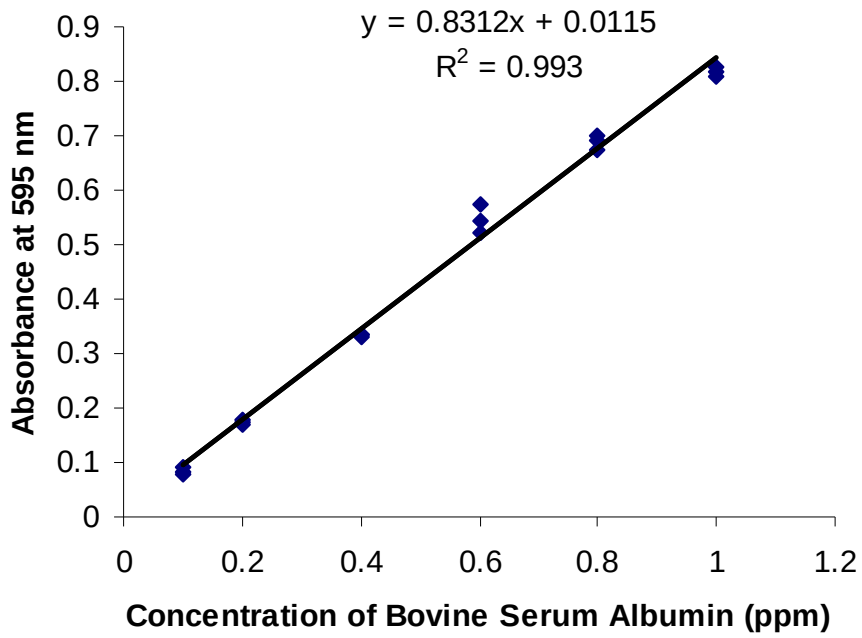


Figure 3.1 Standard curve for protein assay

The enzyme activity retained was calculated by the following equation:

$$E_{ret} = E_{ini} - E_{sw} \qquad \text{Eq. (11)}$$

where E_{ret} is the enzyme activity retained in the beads after the immobilization process (U)

E_{ini} is the initial enzyme activity (U)

E_{sw} is the enzyme activity in the supernatant and washings (U)

The absorbance value obtained during the protein content determination was converted into microgram (μg) by using the following equation:

$$\text{Protein content } (\mu g) = A \bullet 31 \bullet 0.1 \bullet 0.001 \bullet 1000 \bullet V_{sup} \qquad \text{Eq. (12)}$$

where A is the absorbance

31 is the total dilution of the sample (3.0 ml Bradford reagent and 0.1 ml sample)

0.1 is the volume of the sample

The concentration of the standard protein is mg L^{-1} (or ppm), hence to convert it to mg/ml a factor of 0.001 is multiplied.

Since the concentration is required in ug, the mg value is multiplied with 1000.

V_{sup} is the total volume of the supernatant plus washings.

From the immobilized enzyme activity value, immobilization efficiency was calculated using the following equation:

Efficiency of Immobilization = $E_{ret} \bullet 100 / E_{ini}$ Eq. (13)

where E_{ret} is the enzyme activity retained in the beads after the immobilization process (U ml^{-1}) and E_{ini} is the initial enzyme concentration (U ml^{-1}).

3.2.8 Determination of kinetic parameters (V_{max} and K_m) for immobilized enzyme using syringaldazine as substrate

To determine the kinetic parameters of immobilized laccase, 9.0 ml (total volume of beads) of 0.003 U ml^{-1} of immobilized enzyme was taken in a beaker and to it 5.0 ml of phosphate buffer pH 5.5 was added. To this mixture, 1.0 ml of 0.5 mM Syringaldazine solution was added and absorbance value was determined at two minutes interval using the spectrophotometer at a wavelength of 525 nm for 30 minutes. The same procedure was repeated using different syringaldazine concentrations (0.4 mM, 0.3 mM, 0.2 mM and 0.1mM). Three replicates were prepared for each syringaldazine concentration. The initial reaction rate was calculated as the change in absorbance per minute. This value was converted into concentration of syringaldazine using Beer Lambert's relationship (Eqs. 7 – 10). From the rate data against syringaldazine concentration, the V_{max} and K_m values were calculated using non-linear regression technique.

3.2.9 Optimization of temperature, enzyme loading and reaction time for the reaction of immobilized enzyme with 2-chlorophenol.

Using Response Surface Methodology, two levels of reaction time (10 and 20 minutes), temperature (30°C and 60°C) and enzyme concentration (0.2 U and 0.4 U ml^{-1}) representing the low and high values were selected. With the aid of MINITAB®14 software, Box-Behnken design comprising of 45 experiments (including center points) with three reaction times *viz.* 10, 15 and 20 minutes, temperatures at 30°C, 45°C and 60°C, and enzyme concentration at 0.2, 0.3 and 0.4 U ml^{-1}.

Table 3.3 showed the randomized run design table generated by MINITAB®14 using Box-Behnken design:

Table 3.3 Experimental runs for immobilized laccase degradation of 2-chlorophenol using Box-Behnken design

Run	Block	A	B	C
1	1	+	0	-
2	1	0	+	-
3	1	-	-	0
4	1	+	0	-
5	1	0	+	-
6	1	0	0	0
7	1	0	+	+
8	1	0	-	-
9	1	-	0	+

10	1	-	-	0
11	1	0	0	0
12	1	+	-	0
13	1	+	+	0
14	1	0	+	+
15	1	-	0	-
16	1	-	0	+
17	1	0	-	-
18	1	0	0	0
19	1	+	0	+
20	1	0	-	+
21	1	0	-	+
22	1	-	+	0
23	1	+	0	-
24	1	-	-	0
25	1	0	0	0
26	1	-	+	0
27	1	-	0	-
28	1	-	0	+
29	1	+	-	0
30	1	+	0	+
31	1	0	-	-
32	1	-	0	+

33	1	-	+	0
34	1	0	+	-
35	1	+	+	0
36	1	-	0	-
37	1	+	+	0
38	1	0	0	0
39	1	0	0	0
40	1	+	-	0
41	1	0	0	0
42	1	0	+	+
43	1	0	0	0
44	1	0	-	+
45	1	0	0	0

where A, B and C represents the main effects and +, 0 and – refers to the upper, medium and lower values for the main effects. In the experiment, A is enzyme concentration, B is temperature and C is reaction time.

Enzyme was immobilized in sodium alginate beads by using the method outlined in section 3.2.6. The beads were stored at 4°C for half an hour and then filtered to recover them. The beads were then washed thoroughly with 30 ml distilled water and 5.0 ml buffer solution. The washed beads were now transferred into a fresh beaker containing 5.0 ml buffer. To this mixture, 1.0 ml of 8 μM 2-chlorophenol solution was added and the beaker was shaken for a few seconds and then covered with a parafilm. This reaction mixture was placed in a water bath at specified temperature for a specified period of time. After that, the

beaker was removed from the water bath and the beads separated by filtration. The filtrate was collected in a test tube and was boiled at 100°C for a few minutes to inactivate any free enzyme that might be present. The reaction solution was then cooled to room temperature and to this 1.0 ml of 0.1 mM Methylene Blue solution was added. Meanwhile, a blank was prepared by adding 5.0 ml buffer solution, 1.0 ml 99% ethanol and 1.0 ml 0.1 mM Methylene Blue dye. Both blank and the sample were incubated at 25°C for 10 minutes. The absorbance value was recorded for blank and the sample after 10 minutes at 665nm. The reaction was repeated for all 45 sets of experimental combinations generated by the software and the results were recorded. Using Beer Lambert's relationship, the time change in absorbance values were converted into the respective change of Methylene Blue concentrations with time. The actual time change in Methylene Blue concentration was calculated from the concentration difference between blank and individual samples. Based on the results obtained, optimization of reaction time, temperature and enzyme concentration was performed for immobilized laccase degradation of 2-chlorophenol using MINITAB®14 software.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Linearity assay of laccase enzyme using 0.5 mM syringaldazine as substrate

The time course measurement obtained for different enzyme concentrations is shown in Figure 4.1. Linearity assay was performed to determine the range of enzyme concentration within which, the initial rate of reaction is directly proportional to the enzyme concentration. Thus, any enzyme concentration chosen within this linear range was expected to give a predictable initial rate of reaction. From Figure 4.1, it was clear that enzyme concentration from 0.03 to 0.05 U ml^{-1} would give a very short assay time *viz.* ~ 1.6 minutes, which made it difficult to adopt in practice for the assay purposes. On the other hand, enzyme concentration from 0.006 to 0.01 U ml^{-1} resulted in assay time of 6.6 to 10 minutes, which was deemed to be more practical. Hence, assay time for free enzyme reaction was arbitrarily chosen to be 10 minutes.

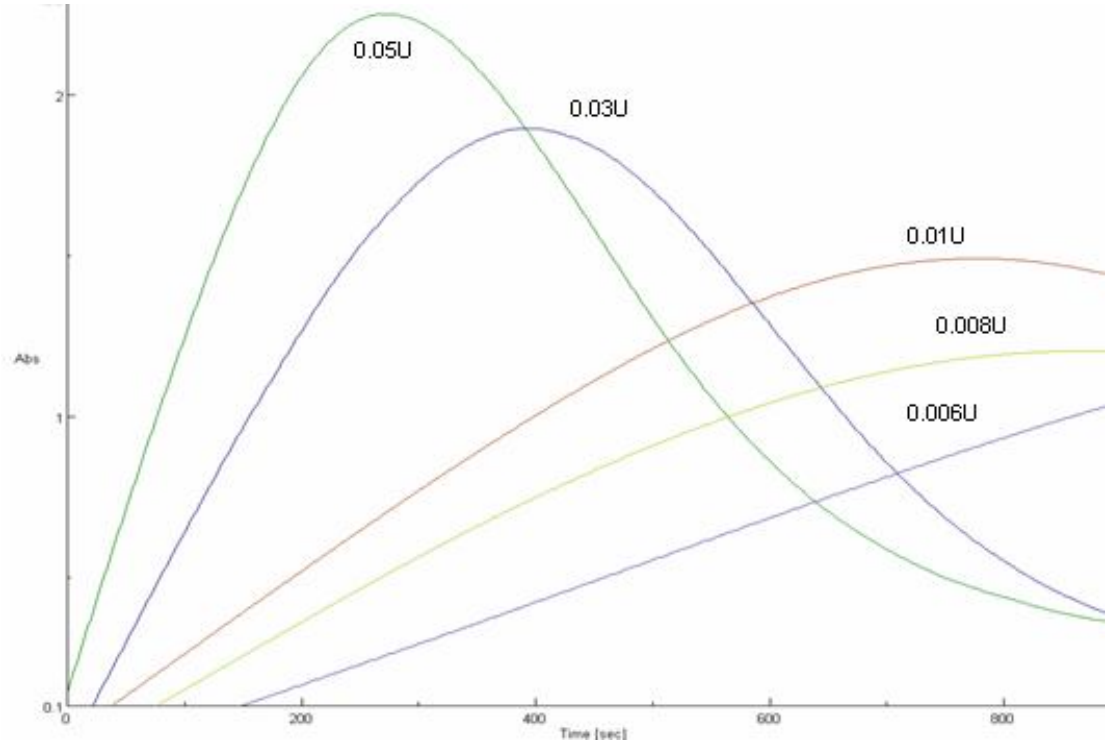


Figure 4.1 Enzyme activity with different laccase concentrations using 0.5mM syringaldazine as substrate at 25^{o}C

Table 4.1 showed the change in initial rate of reaction with changes in enzyme concentration.

Table 4.1 Reaction rate data as a function of syringaldazine concentrations

Enzyme concentration (U ml^{-1})	**Initial Rate of absorbance change (dA min^{-1})**	**Initial Rate of Reaction (μmol min^{-1})**
0.003	0.001	1.200
0.004	0.002	1.723
0.005	0.003	2.308
0.015	0.007	6.062
0.025	0.011	10.215

Plotting the above data separately for change in substrate concentration with time is shown in the plot below (Figure 4.2).

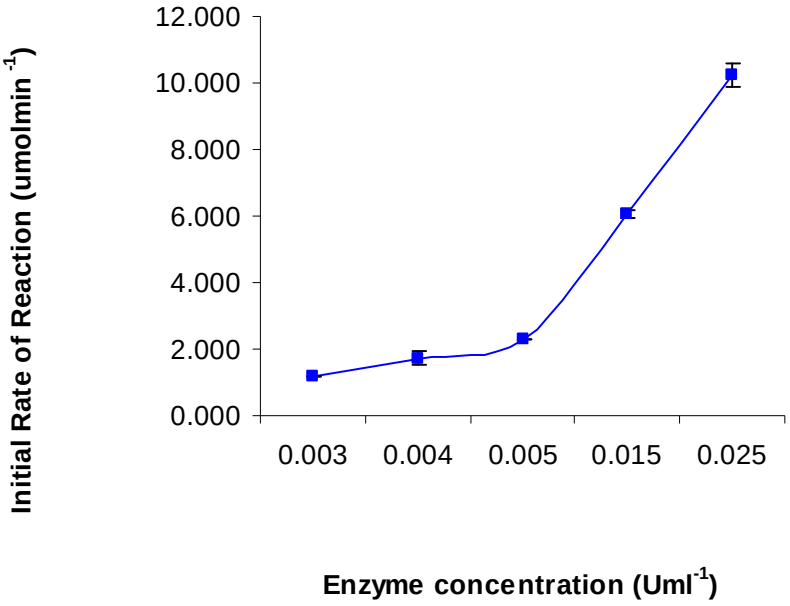


Figure 4.2 Initial reaction rates for various enzyme concentrations

By plotting two different standard curves from the Figure 4.2, the following two plots were obtained: (Figure 4.3 and 4.4)

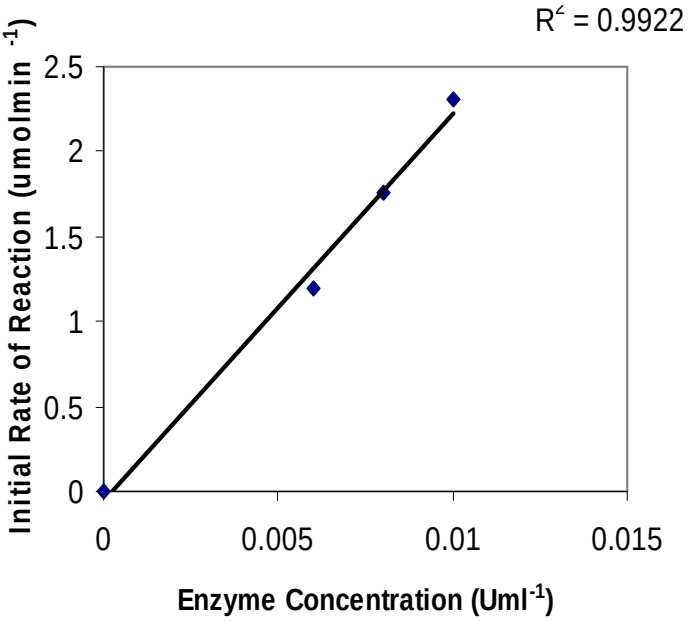


Figure 4.3 Calibration plot for the free enzyme activity for the enzyme concentration range 0.006 to 0.01Uml^{-1}

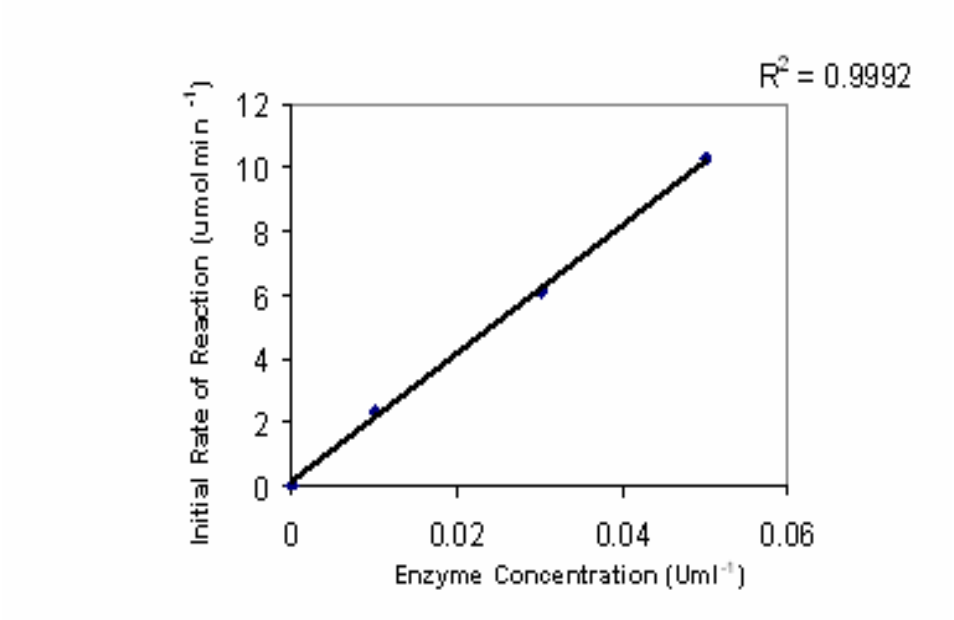


Figure 4.4 Calibration plot for the free enzyme activity for the enzyme concentration range 0.01 to 0.05 U ml^{-1}

These two calibration plots were used in the free enzyme activity determination in the supernatant and washings during the immobilization process.

The plot in Figure 4.2 showed a linear relationship between the enzyme concentration and initial rate of reaction within two different concentrations range i.e. 0.003 to 0.005 U ml^{-1} and 0.005 to 0.025 U ml^{-1}; the two different linearities range observed with the increment of enzyme concentrations are shown separately in Figures 4.3 and 4.4, respectively. Hence, as the enzyme concentration increases, the initial rate of reaction also increases. With an R^2 value of more than 0.99 for both plots and Figures 4.3 and 4.4 showing a near perfect linear fitting, consequently this allowed the possibility of selecting any enzyme concentration within these ranges.

4.2 Determination of kinetic parameters (V_{max} and K_m) for free and immobilized enzyme using syringaldazine as substrate.

The kinetic parameters of the free and immobilized enzyme were determined by using different concentrations of substrate (syringaldazine) for the enzyme reaction. The time course measurement obtained for different substrate concentrations was shown in Figures 4.5 and 4.6, respectively, for free and immobilized laccase.

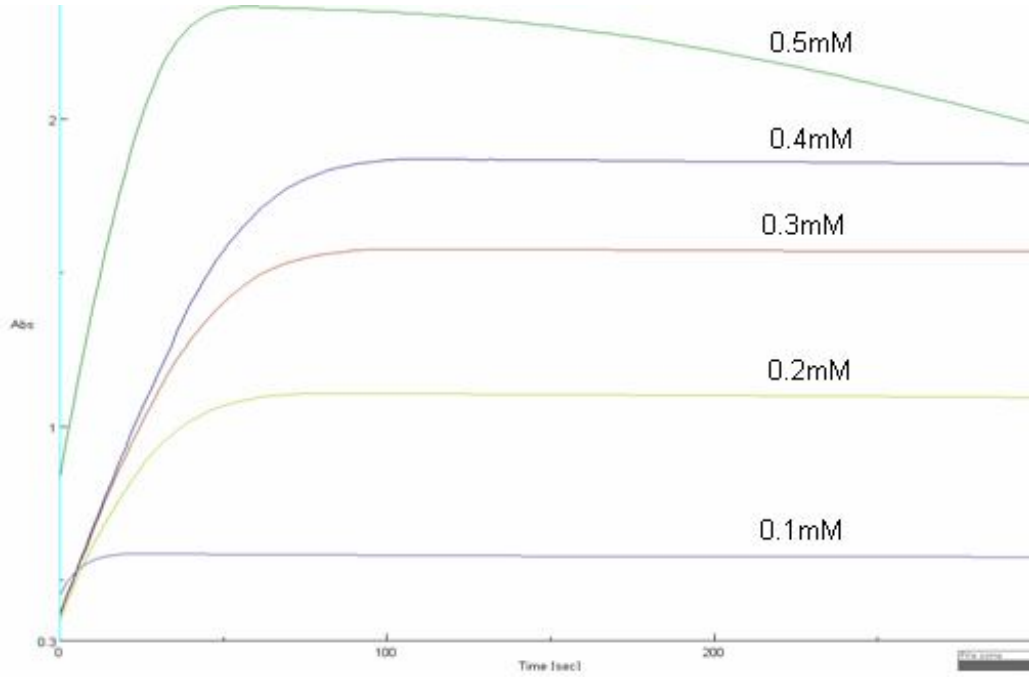


Figure 4.5 Free enzyme activity with different syringaldazine concentrations at 0.1 U ml^{-1} enzyme

For free enzyme system, Figure 4.5 showed that with an increase in substrate concentration, the initial rate of reaction also increased as indicated by the slope of absorbance curve against time.

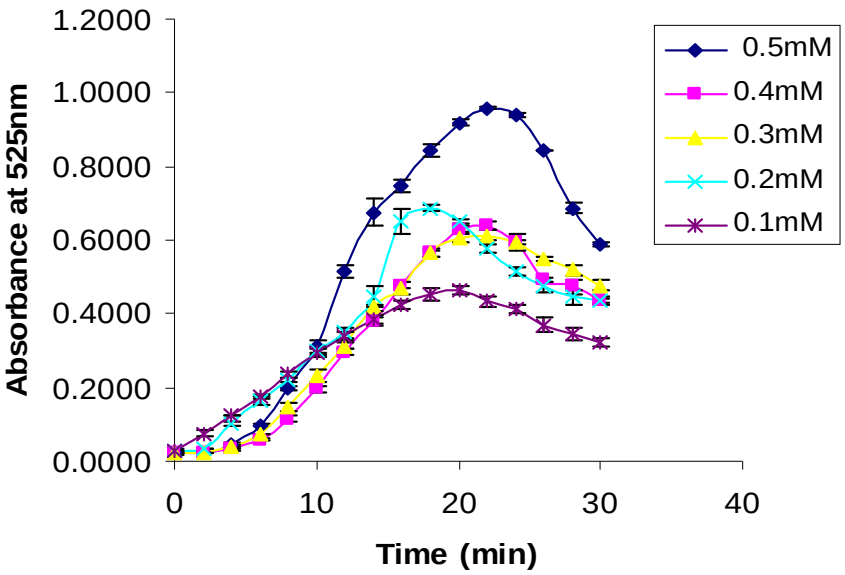


Figure 4.6 Immobilized laccase activities with different syringaldazine concentrations at 0.003 U ml^{-1}

Similarly for immobilized enzyme system, Figure 4.6 showed that with an increase in substrate concentration, the initial rate of reaction also increase as indicated by the slope of absorbance curve against time. From Figure 4.6, a minimum possible enzyme assay time was found to be 10 minutes as the enzyme reaction gained its pace only after 5 minutes for most of the enzyme concentrations used and the maximum reaction was seen after 15 minutes in all cases. Thus, for immobilized enzyme reaction, an assay time within a range of 10 to 20 minutes was deemed to be practical for the subsequent studies. Figure 4.6 also indicated a slight decline in the rate of reaction after passing through the maximum reaction rate and before reaching the constant reaction rate. The possible reason for this could be that during the enzymatic reaction, due to the interaction of various components of the reaction mixture, other products could have formed, which may have exhibited an inhibitory affect on the enzyme activity.

Plotting the initial reaction rate (i.e. change in concentration of substrate with time) against substrate concentration for the free enzyme system resulted in the plot below (Figure 4.7).

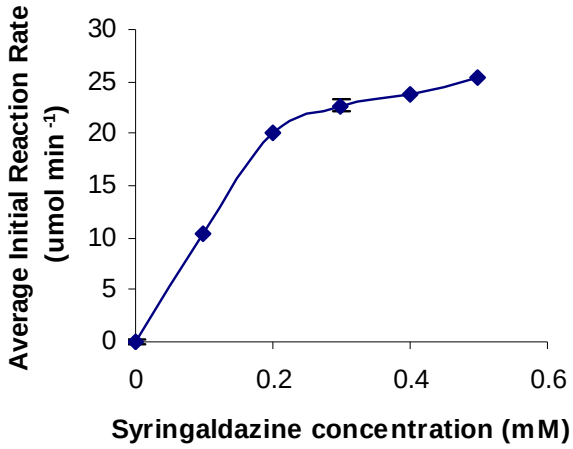


Figure 4.7 Initial rate of reaction as a function of syringaldazine concentrations for free enzyme system

(each data point was based on triplicates measurement, standard deviation < 5%)

It was clear from Figure 4.7 that there was a steep escalation in the initial rate of reaction within the narrow concentration of 0.1 mM to 0.2 mM syringaldazine for the free enzyme system. From 0.2 mM to 0.5 mM syringaldazine, the initial rate of reaction was changing only within a narrow range as compared to the lower syringaldazine concentrations. This indicated that the active site of the laccase enzyme may be saturated with the substrate. Hence, any concentration between 0.2 mM to 0.5 mM of syringaldazine can be chosen for subsequent studies.

Plotting the initial reaction rate (i.e. change in concentration of substrate with time) against substrate concentration for the immobilized enzyme system resulted in the plot below (Figure 4.8). Contrary to the free enzyme system, the reaction rates calculated for immobilized system were much lower. It was also evident that the rates were increasing regularly with the substrate concentration, although the substrate concentrations used were in a similar range to the free enzyme system. The increase in the initial rate of reaction with syringaldazine concentration in the immobilized enzyme case was attributed to the increased concentration driving force for substrate diffusion into the alginate beads. Consequently, syringaldazine concentration at 0.5 mM which gave the highest initial rate within the substrate range studied would be used for subsequent enzyme assay in the immobilized system.

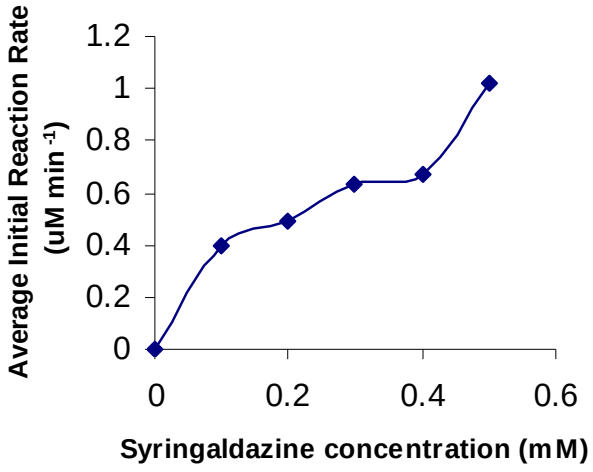


Figure 4.8 Initial rate of reaction as a function of syringaldazine concentrations for immobilized enzyme system (each data point was based on triplicates measurement, standard deviation < 5%)

From Figure 4.7, it was clear that the reaction rate profile in the free enzyme system followed a saturation kinetics (or rectangular hyperbolic). Thus, the reaction kinetics for laccase enzyme with syringaldazine as substrate can be modeled using Michaelis-Menten kinetics. Subsequently, the kinetic parameters V_{max} and K_m were calculated using a non-linear regression technique (Polymath® 6 software). The kinetic parameters' values obtained were:

For free enzyme:

$V_{max} = 40.4 \pm 0.2$ uM min^{-1}

$K_m = 0.253 \pm 0.078$ mM

For immobilized enzyme:

$V_{max}^{app} = 1.34 \pm 0.25$ uM min^{-1}

$K_m^{app} = 0.275 \pm 0.138$ mM

where V_{max}^{app} is apparent maximum rate of reaction and K_m^{app} is apparent Michaelis-Menten constant.

The calculations showed that the K_m value for both free and immobilized enzyme are almost similar, indicating that the immobilization of laccase using alginate beads had minimal effect on the enzyme affinity towards its substrate. This indicates that the active site of the enzyme was not significantly altered by immobilization. This was not surprising since the mechanism of immobilization in this case was *via* entrapment of enzyme molecule within the gel milieu rather than chemical bonding and/or modification based attachment.

On the other hand, the calculations showed that the apparent V_{max} value for immobilized enzyme was about 96% lower than that of the free enzyme resulted in a much slower rate of reaction. This lower apparent V_{max} value for immobilized enzyme must be interpreted with caution since two different total laccase activity units were used in the

experiments to determine the kinetic parameters *viz.* 0.1 U and 0.027 U for free and immobilized enzyme systems, respectively. Since V_{max} is a linear function of total active enzyme present, which can be expressed in the form of $V_{max} = K_{cat} . [E]_t$, where K_{cat} and $[E]_t$ are catalytic constant (or turnover number) and total active enzyme amount, respectively, it can be argued that one of the factors that contributed to a lower V_{max} for immobilized system was the enzyme amount used which was 3.7 times lower than the free enzyme system. A closer examination revealed that this V_{max} value for immobilized enzyme at this particular total enzyme unit (0.027 U) was much lower than the theoretical value if the internal and external mass transfer limitations were absent. With free enzyme system's V_{max} as a reference (i.e. 40.4 ± 0.2 uM min^{-1}) and at a constant K_{cat}, the V_{max} for immobilized system should have been 10.9 uM min^{-1} instead of 1.34 uM min^{-1} as was determined from experimental data. This represented an approximately ten times reduction in rate. It is shown in subsequent analyses (section 4.11) that internal mass transfer within the alginate bead could have been limited by the rate of internal diffusion and hence lowered maximum reaction rate of the immobilized enzyme. For the free enzyme, the reaction rate depends almost entirely on the speed of diffusibility of the substrate to the free enzyme's active site.

4.3 Development of a rapid assay method for biodegradation of 2-chlorophenol by laccase.

Since the method chosen for the assay of products of 2-chlorophenol degradation by laccase was an indirect method, wherein the ability of the degraded products (anionic quinone or phenoxy radicals) to decolorize the cationic dye like Methylene Blue as a result of coupling reaction is exploited, it is essential to determine various interactions between the

components of the entire reaction. Methylene Blue was chosen due to its well known spectrum and plausible reaction with the anionic radicals that could be generated from 2-chlorophenol biodegradation by laccase. The possible reactions within the mixture were identified and tested. The following possible reactions were identified:

1. reaction between laccase and Methylene Blue
2. reaction between Methylene Blue and 2-chlorophenol
3. reaction between laccase and 2-chlorophenol.

The spectrum for Methylene Blue was obtained (Figure 4.9), which indicated a sharp peak at 665 nm.

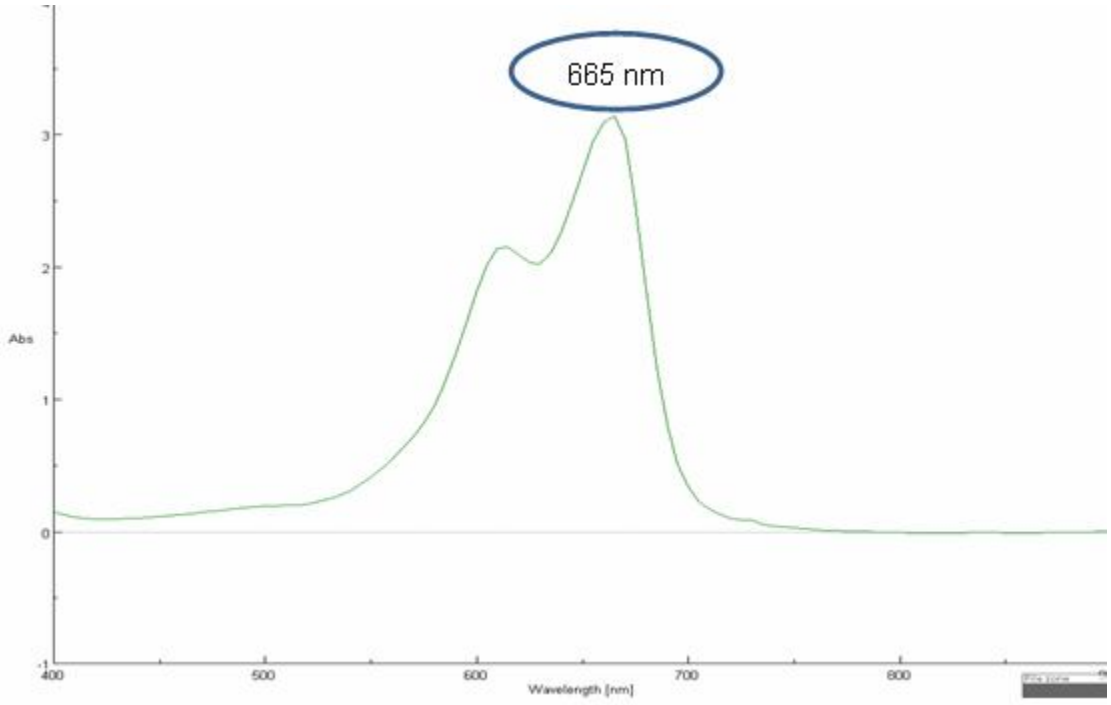


Figure 4.9 Methylene Blue spectrum

The three possible interactions above were confirmed by comparing the spectra obtained from each combination. Interactions 1 and 3 were further confirmed by running a *t*-test.

Figure 4.10 showed the spectra comparison for each of the above three combinations.

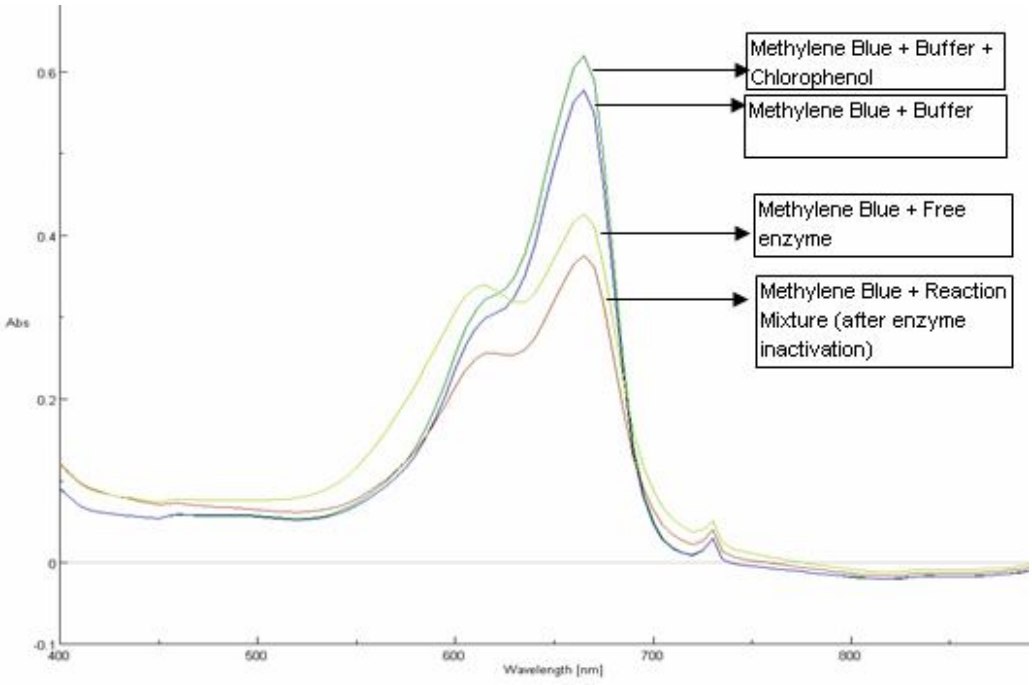


Figure 4.10 Spectra comparison for various interactions

In Figure 4.10, a significant drop in the spectrum peak height for the Methylene Blue plus free enzyme (laccase) mixture was observed, thus indicating possible degradation of the dye by the enzyme. This required the enzyme inactivation step in the assay of the degraded product of 2-chlorophenol by laccase in order to prevent the reaction of residual free enzyme with the dye.

The spectra also indicated that the peak height for Methylene Blue solution alone appeared at exactly the same wavelength to that of Methylene Blue plus 2-chlorophenol solution indicating that the presence of 2-chlorophenol inside the Methylene Blue solution

has no effect on the maximum light absorptivity by Methylene Blue at 665 nm. . 2-chlorophenol is known to absorb light strongly within the ultra-violet range at 276 nm (Navarro *et al.,* 2008). The interpretation of this observation was the absence of any degradation reaction between Methylene Blue and 2-chlorophenol in the liquid mixture. A *t*-test was performed to further confirm this using three replicates; the results were tabulated below in Table 4.2

Table 4.2 Interaction between Methylene Blue and 2-chlorophenol

Component/s	Replicate #	Absorbance at 665nm	Average	Std Dev	Std Dev^2
Methylene Blue + buffer (Run 1)	1	0.4354	0.4398	0.0176	0.000309
	2	0.4592			
	3	0.4249			
Methylene Blue + buffer + 2-chlorophenol (Run 2)	1	0.4371	0.4321	0.0155	0.00024
	2	0.4444			
	3	0.4147			

N.B. : Std Dev = standard deviation

Figure 4.11 showed the two tailed unpaired *t*-test determination result for the above two components.

Using a two tail unpaired t-test for runs 1 and 2 we get

$$t = \frac{\overline{x_1} - \overline{x_2}}{\sqrt{S_p^2(1/n_1 + 1/n_2)}}$$

where $S_p^2 = (S_1^2 + S_2^2)/2$ (since $n_1 = n_2$)

$\overline{x_1}$ = Mean of determinations for run 1.

$\overline{x_2}$ = Mean of determinations for run 2.

S_1 = Standard Deviation of run 1.

S_2 = Standard Deviation of run 2.

$S_1^2 = 0.000309$ $Sp^2 = (0.000309 + 0.00024)/2 = 0.000274$

$S_2^2 = 0.00024$ $1/n_1 + 1/n_2 = 1/3 + 1/3 = 0.6667$

$t = 0.0077/0.0135 = 0.57$

Critical value for t from the t-table at (3+3-2) 4 degrees of freedom is 2.7765.

Conclusion: There is no significance difference in the absorbance value obtained by blank (methylene blue) and chlorophenol + methylene blue.

Inference: Chlorophenol does not degrade methylene blue dye.

Figure 4.11 *t*-test for Runs 1 and 2

A third observation from Figure 4.10 was that that there was a significant drop in the peak height for the spectrum for Methylene Blue plus reaction mixture consisting of un-reacted 2-chlorophenol, degraded product of chlorophenol, laccase and buffer (after inactivation of enzyme) as compared to Methylene Blue alone. This indicates that there could be a significant interaction between the degraded products of 2-chlorophenol and the dye. To further confirm this, a *t*-test was conducted using three replicates, the results of which were tabulated in Table 4.3.

Table 4.3 Interaction between Methylene Blue and degraded product of 2-chlorophenol

Components	Replicate #	Absorbance at 665nm	Average	Std Dev	Std Dev^2
Methylene Blue + buffer (Run 1)	1	0.4354	0.439833	0.01757	0.000309
	2	0.4592			
	3	0.4249			
Reaction mixture after enzyme inactivation + Methylene Blue (Run 3)	1	0.3336	0.336933	0.00535	2.86E-05
	2	0.3341			
	3	0.3431			

Figure 4.12 showed the two tailed unpaired *t*-test determination result for the above two components.

Using a two tail unpaired t-test for runs 1 and 3 we get

$$t = \frac{\overline{x_1} - \overline{x_2}}{\sqrt{S_p^2(1/n_1 + 1/n_2)}}$$

where $S_p^2 = (S_1^2 + S_2^2)/2$ (since $n_1 = n_2$)

$\overline{x_1}$ = Mean of determinations for run 1.

$\overline{x_2}$ = Mean of determinations for run 3.

S_1 = Standard Deviation of run 1.

S_2 = Standard Deviation of run 3.

$S_1^2 = 0.000309$ $Sp^2 = (0.000309 + 0.0000286)/2 = 0.0001688$

$S_2^2 = 0.0000286$ $1/n_1 + 1/n_2 = 1/3 + 1/3 = 0.6667$

$t = 0.103/0.01 = 10.3$

Critical value for t from the t-table at (3+3-2) 4 degrees of freedom is 2.7765.

Conclusion: There is a significant difference between the absorbance values obtained by blank (methylene blue) and degraded product of the enzyme.

Inference: Chlorophenol degradation product degrades methylene blue dye.

Figure 4.12 *t*-test for Runs 1 and 3

The most likely mechanism for the chemical reaction between Methylene Blue and the laccase-degraded by-products of 2-chlorophenol was proposed in section 4.4.

In order to check the stability of the reaction products between 2-chlorophenol by-products from laccase degradation and Methylene Blue dye, a time course measurement was run in the spectrophotometer after the addition of Methylene Blue to the reaction mixture (after enzyme inactivation) for 1 hour. The time course measurement plot obtained is shown in Figure 4.13.

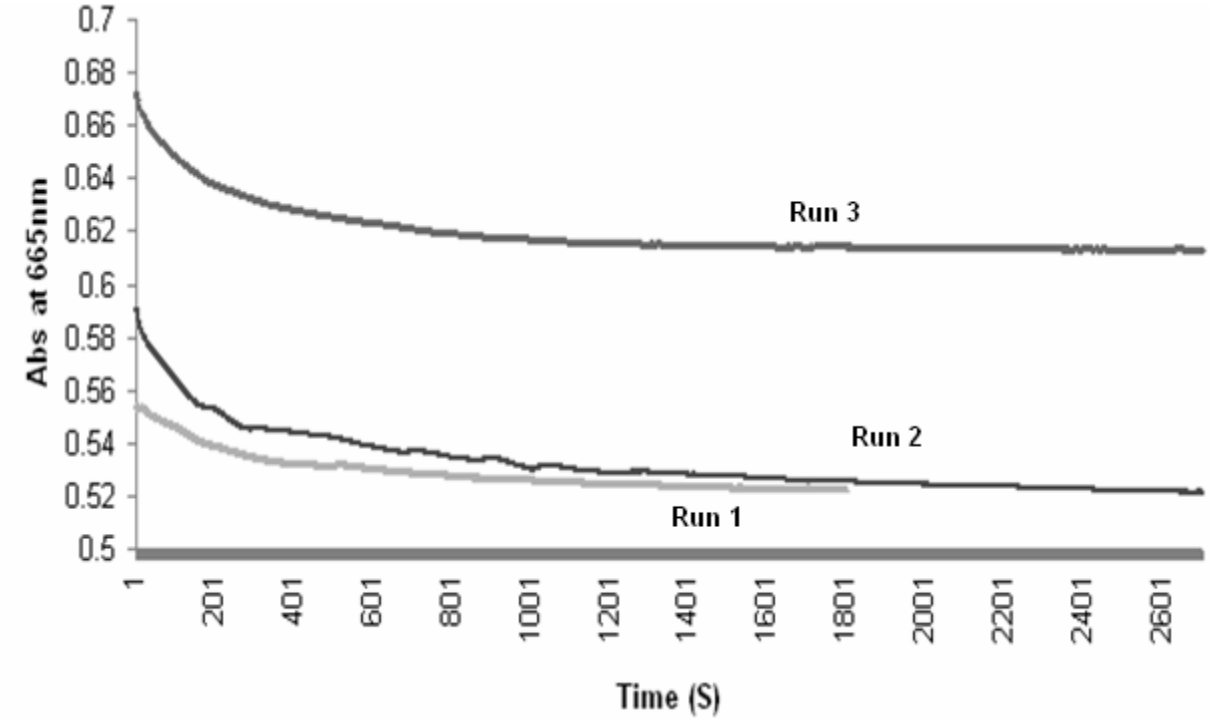


Figure 4.13 Stability of the reaction product between 2-chlorophenol laccase-degraded by-products and Methylene Blue dye

From the Figure 4.13 it was evident that the reaction product was stable even after one (1) hour. The mixture containing the products of 2-chlorophenol degradation by laccase and Methylene Blue was mixed before subjecting it to the time course measurement in

spectrophotometer. At different initial level of assay, it was observed that for all three determinations (Runs 1, 2 and 3), there were no more significant change in absorbance at 10 minutes and beyond. Since the reaction time chosen for the assay of enzymatic degradation of 2-chlorophenol was only 10 to 20 minutes throughout the experiment, this chemical stability was considered fair and the proposed assay method can be put into a practical use.

A 1:1 stoichiometry was proposed for the reaction between the anionic quinone or phenoxy radicals by-product(s) from laccase degraded 2-chlorophenol and the cationic Methylene Blue dye based on the following points:

- Figure 4.10 presented the spectra for all the various combinations of components. The spectra showed similar absorption maxima at 665 nm with respect to the Methylene Blue spectrum, hence indicating there were no additional products formed apart from the coupling product of Methylene Blue and product from laccase degraded 2-chlorophenol, which resulted in the reduction of the absorption peak height.
- The pKa value of phosphate buffer was 6.86 and that of Methylene Blue was 0 to 1 (Disanto and Wagner, 1972). Hence, anions generated were more likely to couple with Methylene Blue rather than buffer.

The following section discussed the proposed mechanism underlying this chemical reaction.

4.4 Proposed Mechanism of Action of 2-chlorophenol degradation of laccase and its assay

Oxidation of phenolic compounds with laccase gives a resonance-stabilized radical intermediate (Roy-Archand and Archibald, 1991). Figure 4.14 illustrated the resonance-stabilized radical intermediate formation.

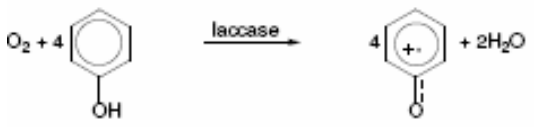


Figure 4.14 The initial reaction of phenolic compound with laccase (Roy-Archand and Archibald, 1991)

In one study by Leontievsky *et al.***,** after the degradation of trichlorophenol by laccase a combination of products were formed (Leontievsky *et al.***,** 2001) indicating that the aromatic ring was not cleaved. Figure 4.15 illustrated the mechanism proposed in the above study.

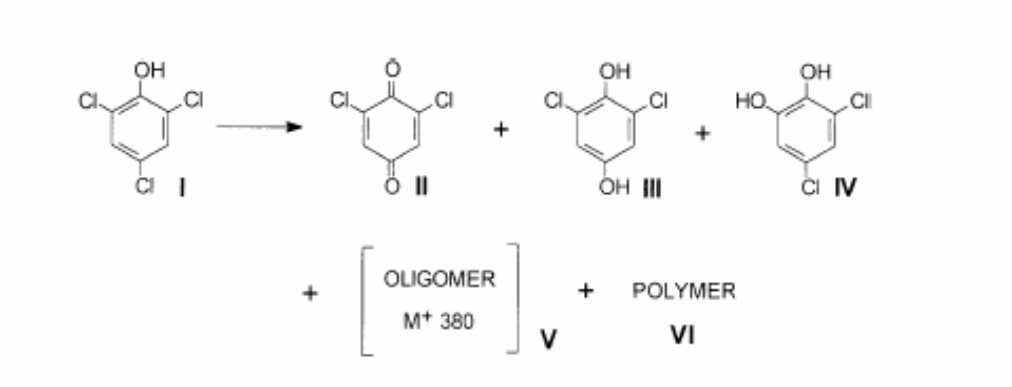


Figure 4.15 Mechanism proposed by Leontievsky *et al.*, in the degradation of trichlorophenol by laccase

In another study by Schultz *et al.,* degradation of chlorinated hydroxybiphenyls by laccase yielded an intact aromatic biphenyl ring with an incorporation of double bond between them (Schultz *et al.,* 2001). Figure 4.16 illustrated the mechanism proposed in the above study.

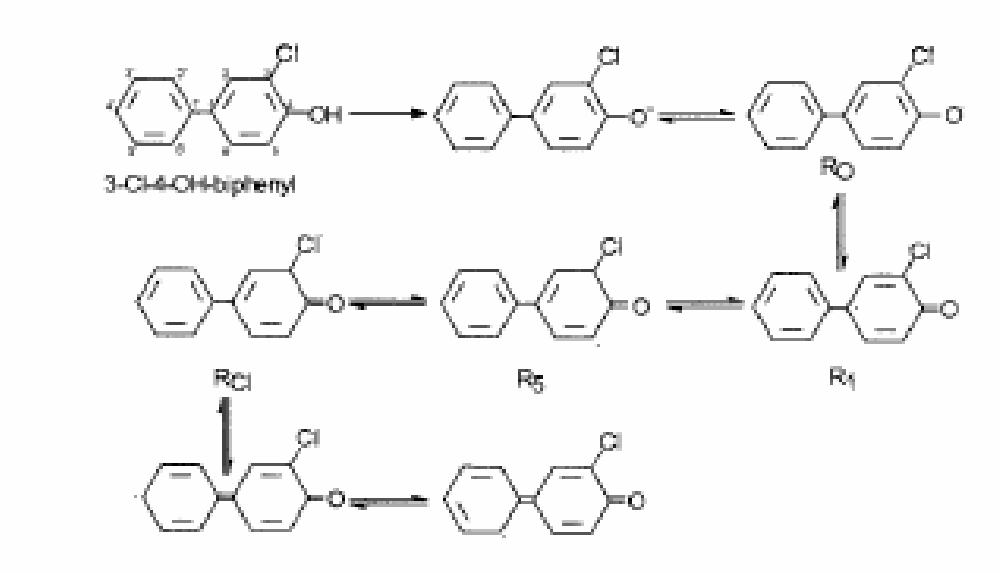


Figure 4.16 Mechanism proposed by Schultz *et al.*, in the degradation of chlorinated hydroxybiphenyls by laccase

In relation to the two studies above, Figure 4.17 illustrated the possible radical generation and coupling reaction of these radicals with Methylene Blue dye. Two possible mechanisms of action were proposed for the reaction of laccase with 2-chlorophenol as illustrated in Figure 4.17.

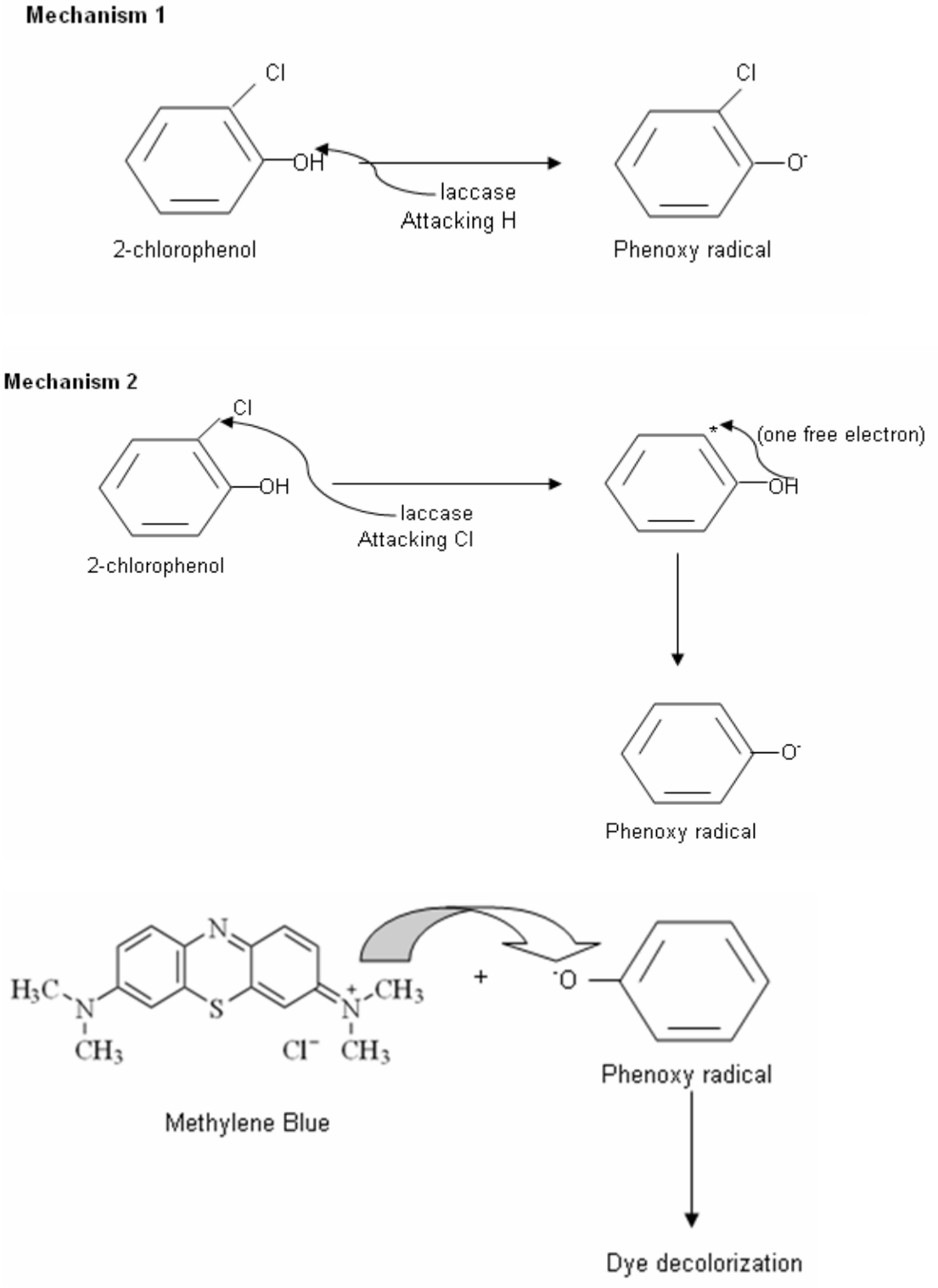


Figure 4.17 Proposed mechanisms for generation of 2-chlorophenol radicals and coupling reaction of the radical(s) with Methylene Blue

4.5 Determination of 2-chlorophenol concentration to be used in subsequent studies

The concentration of 2-chlorophenol to be used in the study was determined by initially screening between five different initial concentrations. The initial concentrations selected were 6, 7, 8, 9 and 10 μM. The change in Methylene Blue concentration was plotted against the different initial substrate (2-chlorophenol) concentrations (Figure 4.18).

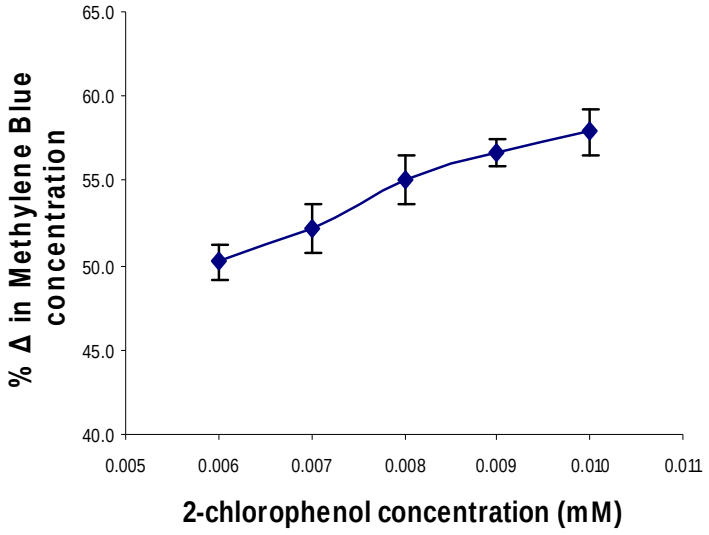


Figure 4.18 The percentage ΔMethylene Blue concentration with respect to change in 2-chlorophenol concentration

Figure 4.18 showed that the percentage ΔMethylene Blue concentration with respect to 2-chlorophenol concentration was directly proportional and hence the plot obtained was practically a linear plot. Figure 4.19 represents a linear plot plotted for the above data.

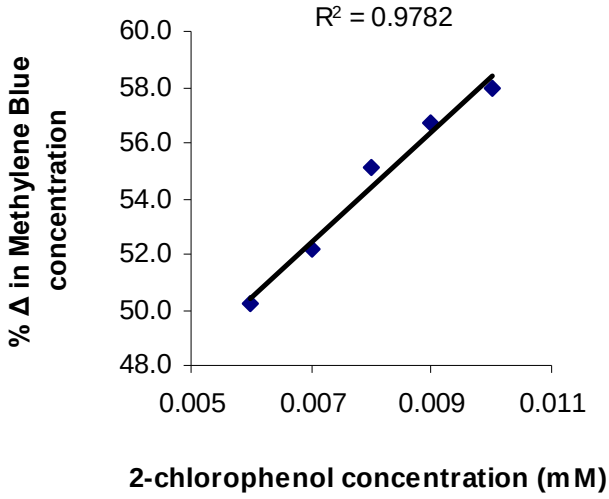


Figure 4.19 Linear plot for the ΔMethylene Blue concentration with initial 2-chlorophenol concentration

An R^2 value of 0.9782 indicates a good fit for the linear relationship between initial concentration of 2-chlorophenol and ΔMethylene Blue concentration. That is, with every 1 μM increase in 2-chlorophenol concentration, there was an average of 2% increase in ΔMethylene Blue concentration. Thus, following this linearity, the middle point of initial 2-chlorophenol concentration range tested i.e. 8 μM was arbitrarily chosen to be used in the subsequent experiments.

4.6 Optimization of pH, temperature and enzyme loading for the reaction of free laccase with 2-chlorophenol.

The factors chosen for optimization of enzymatic reaction were pH, temperature and enzyme loading. It is important to optimize the key factors that influence the free laccase

reaction with 2-chlorophenol if a comparison is to be made later with the immobilized laccase system degrading 2-chlorophenol which should also be under optimal condition.

The results of the 45 experimental runs based on MINITAB®14 design were tabulated in Table 4.4 below.

Table 4.4 Results of 45 experimental runs for free laccase system

C1 Randomized Order	C2 Run Order	C3 Point Type	C4 Blocks	C5 pH	C6 Temperature (°C)	C7 Enzyme Loading ($U\ ml^{-1}$)	C8 ΔMethylene Blue concentration ($mol\ L^{-1}$)
8	1	2	1	7	45	0.2	2.86×10^{-6}
7	2	2	1	5	45	0.2	2.84×10^{-6}
22	3	2	1	5	45	0.2	2.74×10^{-6}
12	4	2	1	6	60	0.2	2.44×10^{-6}
14	5	0	1	6	45	0.15	3.18×10^{-6}
35	6	2	1	5	45	0.1	2.24×10^{-6}
38	7	2	1	7	45	0.2	2.85×10^{-6}
17	8	2	1	7	30	0.15	1.72×10^{-6}
25	9	2	1	6	60	0.1	1.62×10^{-6}
28	10	0	1	6	45	0.15	2.79×10^{-6}
30	11	0	1	6	45	0.15	2.96×10^{-6}
33	12	2	1	5	60	0.15	2.26×10^{-6}
37	13	2	1	5	45	0.2	3.07×10^{-6}

41	14	2	1	6	30	0.2	1.95×10^{-6}
1	15	2	1	5	30	0.15	1.53×10^{-6}
31	16	2	1	5	30	0.15	1.54×10^{-6}
45	17	0	1	6	45	0.15	3.03×10^{-6}
2	18	2	1	7	30	0.15	1.66×10^{-6}
19	19	2	1	7	60	0.15	2.41×10^{-6}
3	20	2	1	5	60	0.15	2.09×10^{-6}
10	21	2	1	6	60	0.1	1.62×10^{-6}
29	22	0	1	6	45	0.15	2.97×10^{-6}
20	23	2	1	5	45	0.1	2.12×10^{-6}
39	24	2	1	6	30	0.1	1.46×10^{-6}
4	25	2	1	7	60	0.15	2.14×10^{-6}
32	26	2	1	7	30	0.15	1.61×10^{-6}
21	27	2	1	7	45	0.1	1.70×10^{-6}
5	28	2	1	5	45	0.1	1.92×10^{-6}
27	29	2	1	6	60	0.2	2.32×10^{-6}
9	30	2	1	6	30	0.1	1.64×10^{-6}
36	31	2	1	7	45	0.1	1.7×10^{-6}
24	32	2	1	6	30	0.1	1.38×10^{-6}
40	33	2	1	6	60	0.1	1.66×10^{-6}
34	34	2	1	7	60	0.15	2.43×10^{-6}
23	35	2	1	7	45	0.2	2.82×10^{-6}
26	36	2	1	6	30	0.2	2.04×10^{-6}

13	37	0	1	6	45	0.15	2.82×10^{-6}
43	38	0	1	6	45	0.15	2.81×10^{-6}
15	39	0	1	6	45	0.15	2.88×10^{-6}
44	40	0	1	6	45	0.15	2.98×10^{-6}
18	41	2	1	5	60	0.15	2.07×10^{-6}
42	42	2	1	6	60	0.2	2.42×10^{-6}
11	43	2	1	6	30	0.2	1.97×10^{-6}
16	44	2	1	5	30	0.15	1.39×10^{-6}
6	45	2	1	7	45	0.1	1.83×10^{-6}

Analysis of Variance (ANOVA) and Response Surface Regression for the above data were performed by MINITAB®14 software and the following results were obtained:

Term	Coefficient	Standard Error Coefficient	T	P
Constant	-0.000019	0.000003	-5.616	0.000
pH	0.000004	0.000001	4.072	0.000
temperature	0.000000	0.000000	6.968	0.000
enzyme loading	0.000045	0.000013	3.597	0.001
pH*pH	-0.000000	0.000000	-4.317	0.000
temperature*temperature	-0.000000	0.000000	-10.228	0.000
enzyme loading * enzyme loading	-0.000134	0.000028	-4.725	0.000
pH*temperature	0.000000	0.000000	0.036	0.971
pH* enzyme loading	-0.000000	0.000001	-0.246	0.807
temperature* enzyme loading	0.000000	0.000000	1.003	0.323

S = 0.0000002359 R^2 = 85.6% R^2 (adjusted) = 81.9%

P value < 0.05 for main effects and interactions between pH*pH, enzyme loading*enzyme loading, temperature*temperature indicates that the effects are significant and a *P* value > 0.05 for interactions between pH, temperature and enzyme loading indicates that the there were no significant effect of interaction between any two different main effects. Post analyses plots were constructed and shown as follows:

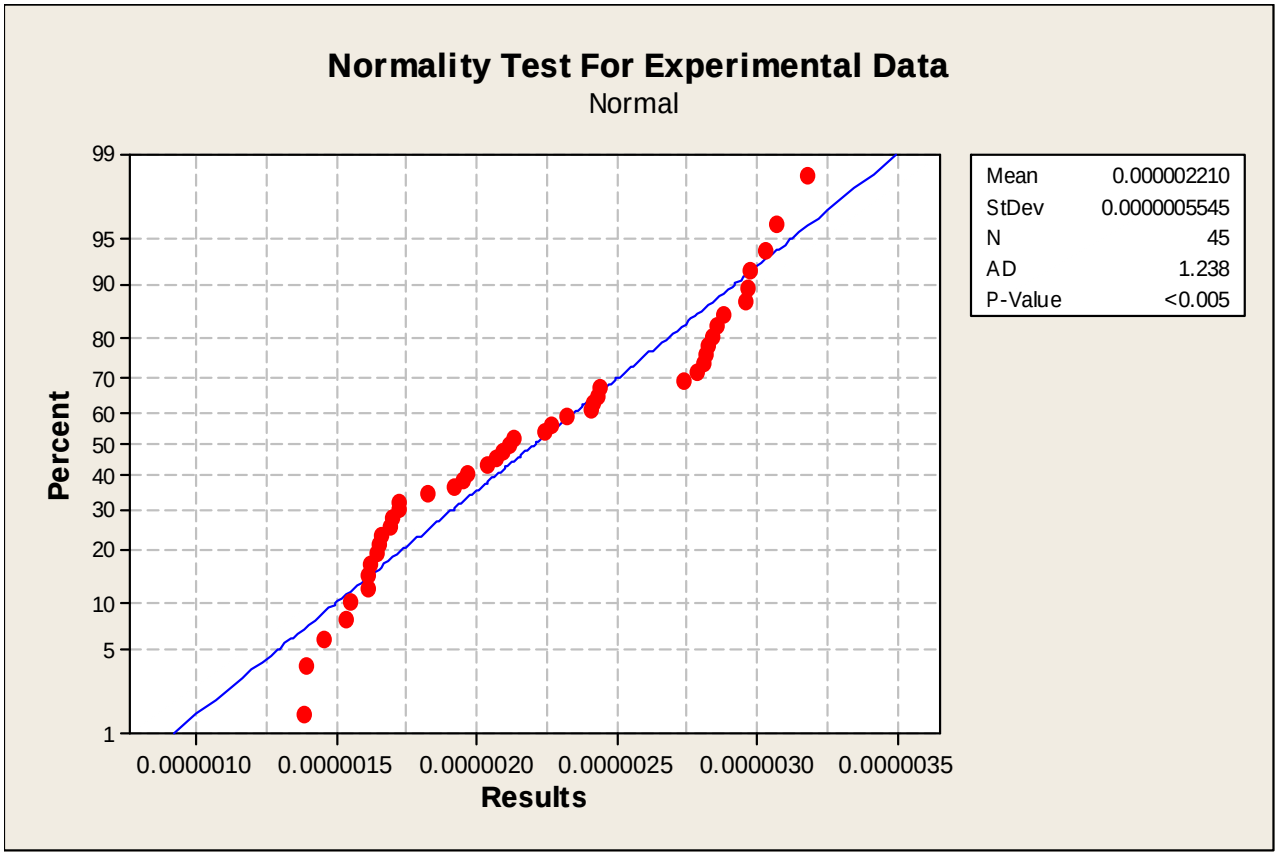


Figure 4.20 Normality plot for experimental data

The normality graph plotted for the experimental data has shown the *P*-value to be < 0.05, which shows a slight deviation from a normal distribution. This slight deviation was attributed to a relatively small number of the experimental runs (n = 45). This slight

deviation is usually acceptable and do not present a major concern on the assumption of the normality of the data collected.

Figure 4.21 showed the normal probability of the residual values (errors).

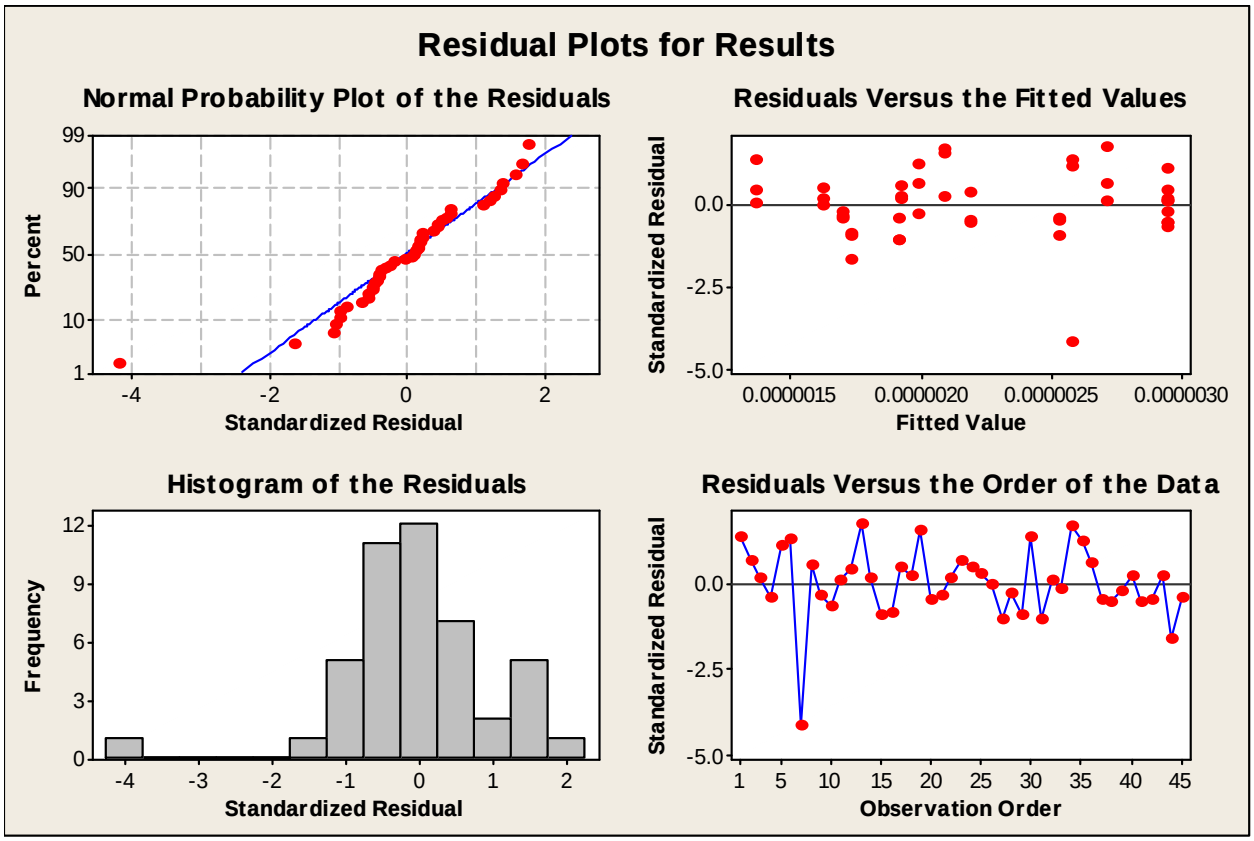


Figure 4.21 Residual plots for the results

The normal probability plot of the residuals showed that the residuals were closely distributed following the normal distribution (top left panel) (residual = experimental value – model fitted value). This supported by the shape of the histogram of the residuals (bottom left panel) which retained the bell-shape curve albeit with a minor skew to the left of the distribution graph. The slight deviation of the experimental data observed earlier (Fig. 4.20) contributed to this minor skewness. This indicated that the errors associated with the experimental data collected were purely due to random error. When the standardized

residuals were plotted against fitted values (top right panel), a random pattern of data distribution was observed indicating the absence of systematic error. The order of the experiments execution has no influence on the data collected, as shown by the random occurrence of data in the standardized residual plot against observation order (bottom right panel).

The main effects plot was shown in Figure 4.22.

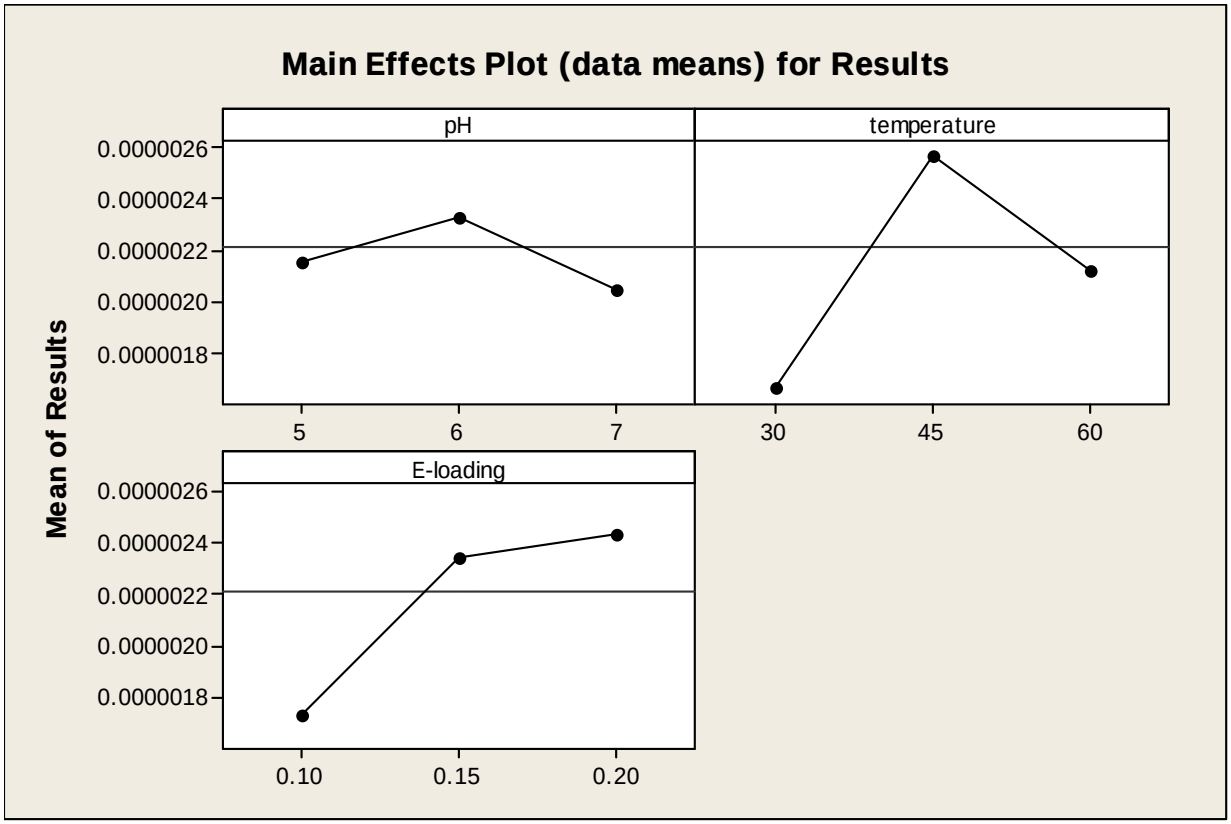


Figure 4.22: Main effects plot

The above figure showed the change in the response i.e. ΔMethylene Blue concentration after reaction with the 2-chlorophenol degradation products with respect to pH, temperature and enzyme loading variables. It could be seen that as these three variables were varied, the response also changed. Based on the difference between the lowest value and

highest value obtained for the result, the significance of the effect of that particular factor on the response can be determined.

From the above figure it was evident that the pH has a little significant effect on the enzyme reaction within the selected pH range in this study since the corresponding change in the response was only within a very narrow range ($2.0 \times 10^{-6} – 2.3 \times 10^{-6}$). On the other hand, temperature and enzyme concentration had significant effect on the response as the responses at different levels of temperature and enzyme-loading were changing within a relatively broad range ($1.0 \times 10^{-6} – 2.5 \times 10^{-6}$ for temperature and $1.6 \times 10^{-6} – 2.5 \times 10^{-6}$ for enzyme-loading). That means, when the temperature was increased from 30 to 45 °C, there was a drastic increase in the ΔMethylene Blue concentration and when it was further increased to 65 °C, there was a drop in the result. This indicated that the optimum temperature for the enzymatic reaction may be 45 °C. Similarly, when laccase concentration was increased to 0.15 U ml^{-1}, there was a steep rise in ΔMethylene Blue concentration. An increment of another 0.05 U ml^{-1} of laccase had also increased the response.

The interaction plot for the variables was shown in Figure 4.23.

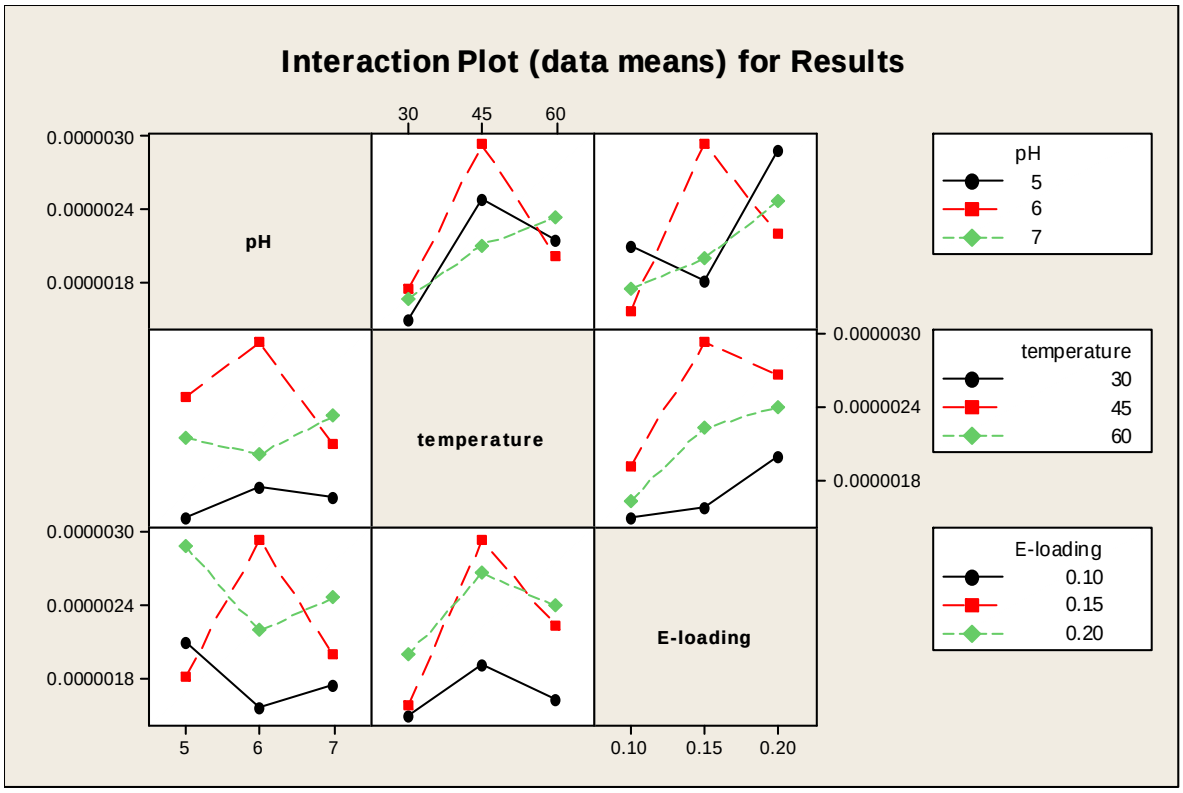


Figure 4.23 Interactions plot

The interactions plot is used to indicate the possible interactions between the three selected variables i.e. pH, temperature and enzyme concentration on the process response i.e. ΔMethylene Blue concentration. Intersection of the three legends indicated possible interaction between any two chosen parameters on the response. In the first row, the interactions between pH and temperature and pH and enzyme concentration were shown. In the second row, the interactions between temperature and pH and temperature and enzyme concentration; and in the third row, the interactions between enzyme concentration and pH and enzyme concentration and temperature were shown.

It is observed that the interactions between pH, temperature and enzyme-loading may be present. These possible interactions were further examined using contour plots as shown in the Figure 4.24.

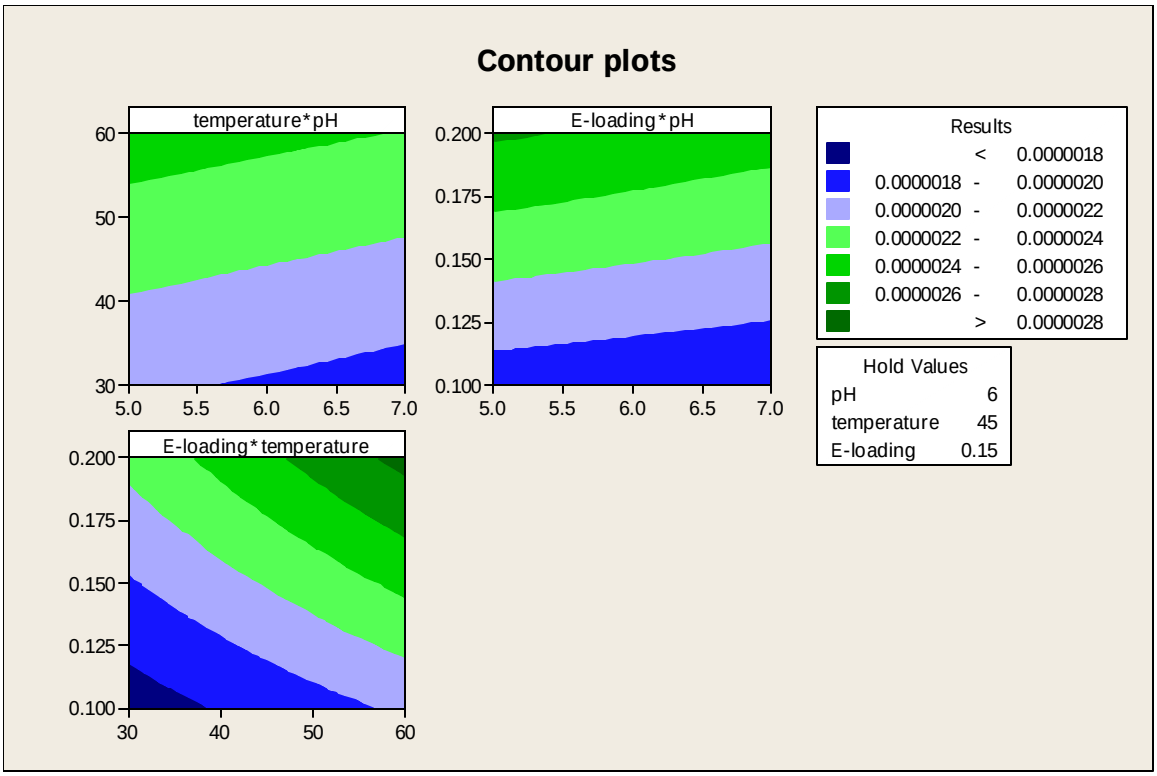


Figure 4.24 Contour plots for various interactions

In the contour plots of these variables (Fig. 4.24), only very minor curvatures were present indicating insignificant interactions between the selected parameters. Hence the contour plots confirmed the earlier analyses of variance (ANOVA) on the significance of the main and interaction effects.

Optimization was carried out by "Response Optimization" program of the MINITAB®14 software. Details of the optimization routine were shown below:

Optimization goal: To maximize the response

Upper value : 3.0 x 10^{-6}

Lower value : 2.5 x 10^{-6}

Optimization starting point:

pH : 5.0
temperature : 55 °C

enzyme-loading : 0.2 U ml^{-1}

The optimal values thus obtained were shown in Table 4.5:

Table 4.5 Optimization values

Local Solution			**Predicted Responses**		
pH	Temperature (°C)	Enzyme-loading (U ml^{-1})	ΔMethylene Blue concentration (mol L^{-1})	Desirability	Composite Desirability
5.0	55	0.2	2.8 x 10^{-6}	0.87571	0.87571

Global Solution			**Predicted Responses**		
pH	Temperature (°C)	Enzyme-loading (U ml^{-1})	ΔMethylene Blue concentration (mol L^{-1})	Desirability	Composite Desirability
5.0	55	0.2	2.8 x 10^{-6}	0.87571	0.87571

For pH 5, temperature 55°C and enzyme concentration 0.02 U ml^{-1}, the global solution was the same as that of the local solution showing that there was only one optimal solution. The desirability of the optimum solution was found to be about 88%. Hence, the optimization results obtained were:

pH : 5

temperature : 55°C and

enzyme concentration : 0.2 U ml^{-1}.

A confirmatory run using optimized conditions resulted in the following results (Table 4.6):

Table 4.6: Results of confirmatory test using optimized conditions

Replicate #	Δ Methylene Blue concentration (mol L^{-1})
1	2.87×10^{-6}
2	2.76×10^{-6}
3	2.86×10^{-6}
Average	$2.83 \times 10^{-6} \pm 0.061 \times 10^{-6}$

The average value was found to be very close to the maximum predicted response i.e. 2.8×10^{-6}, thus confirming the optimization results. The reduction in the Methylene Blue concentration under optimized conditions was about 34.3%, corresponding to the equivalent amount of 2-chlorophenol degraded products formed and hence 2-chlorophenol degradation by laccase assuming one phenoxy radical generated from each molecule of 2-chlorophenol and a 1:1 stoichiometric relation between chlorophenol degraded product and Methylene Blue as per proposed mechanisms of action (Section 4.4, Figure 4.17).

4.7 Enzyme Immobilization and Immobilization Optimization.

4.7.1 Enzyme Immobilization

Entrapment method was employed for immobilization. Enzyme was immobilized in insoluble calcium alginate beads. Entrapment methods are based on the physical occlusion of enzyme molecules within a "caged" gel structure such that the diffusion of enzyme molecules to the surrounding medium is severely limited, if not rendered totally impossible. What creates the "wires" of the cage is the cross-linking of polymers. A highly cross-linked

gel has a fine "wire mesh" structure and can more effectively hold smaller enzymes in its cages. The degree of cross-linking depends on the condition at which polymerization is carried out.

Gelation of calcium alginate does not depend on the formation of permanent covalent bonds between polymer chains; instead polymer molecules are cross-linked by calcium ions. Because of this, calcium alginate beads can be formed in extremely mild conditions, which ensure that enzyme activity yield of over 80% can be routinely achieved (Park *et al.,* 1995; Palmieri *et al.,* 1994; Nilsson, 1987).

4.7.2 Immobilization Optimization

Since the required reaction time for all the immobilized enzyme reactions was 10 to 20 minutes, the parameters like the mesh size (which depends on the number of cross-linking which in turn depends on the concentration of calcium chloride used) and stability and strength of the beads (which depends on the drying time and the consistency of the enzyme solution, which again depends on the ratio of sodium alginate solution and enzyme solution) were identified as important variables to be optimized in order to achieve maximum immobilization efficiency, while allowing easy diffusion of the substrate through the barrier (bead). This is expected to cause a small lag within the reaction assay time of 10 to 20 minutes. The optimization was performed by adding buffer and syringaldazine solutions to the beads and measuring the absorbance of the supernatant at 525 nm after 20 minutes. All the various combinations were tested (3 replicates for each combination) and the average results were tabulated in Table 4.7.

Table 4.7: Various combinations tested for immobilization optimization

Concentration of $CaCl_2$ (%w/v)	Volume of sodium alginate solution : volume of enzyme solution	Drying time (hrs at 4°C)	Average Absorbance at 525 nm after 20 min.
0.1	8 : 1	1	0.621
0.5	8 : 1	1	0.452
0.75	8 : 1	1	0.201
1	1 : 1	1	No reaction
1	1 : 1	0.5	No reaction
0.75	8 : 1	0.5	0.353
0.5	8 : 1	0.5	0.525
0.1	1 : 1	1	0.685

From Table 4.7, it was evident that a higher calcium chloride concentration (1% w/v) could have encouraged the formation of a more rigid beads by developing many cross linking despite using a less viscous enzyme solution in sodium alginate (1:1 ratio), thus possibly lowering the diffusion efficiency of substrate through the bead. Hence, despite different drying times, there was no reaction seen after 20 minutes. The beads formed using 0.1% calcium chloride were found to be too fragile and not sufficiently rigid despite using a more viscous enzyme solution in sodium alginate (1:8 ratio). Hence, a high absorbance value for the supernatant could indicate enzyme leakage through the bead barrier due to poor cross-linking by calcium. For 0.75% w/v calcium chloride, it was observed that as the drying time increased, the absorbance value decreased. For a consistent bead formation, the ratio of volumes of sodium alginate and enzyme solution was maintained at 8:1 for both drying

times. It is suggested that longer drying time may encouraged highly extensive cross-linking within the beads. Hence, if the drying time is reduced, the cross-linking may not be so extensive, thus enabling the substrate to diffuse relatively easier through the bead barrier. This was further supported by the supernatant absorbance value which was higher for lower drying time and *vice versa*. The absorbance values for 0.5% w/v calcium chloride were found to be higher than that of 0.75% w/v. The reason could be formation of a comparatively weaker cross-linking due to a lesser concentration of calcium chloride. The difference in drying time was again found to influence the absorbance value similar to that of 0.75% w/v.

Based on the analyses, the usage of 0.1% w/v and 1% w/v calcium chloride was ruled out. In order to decide between 0.75% w/v and 0.5% w/v calcium chloride solutions, the lag time and the efficiency of immobilization were determined. For this, the beads were placed in 5.0 ml of 50 mM phosphate buffer solution (pH 7) and 1.0 ml of 0.5 mM syringaldazine solution was added to it. The absorbance of the reaction mixture was measured every 5 minutes and the values recorded were plotted against time for each set of combinations of drying time with 0.5% w/v and 0.75% w/v calcium chloride. Figure 4.25 showed the comparison between different combinations of calcium chloride concentration and drying time.

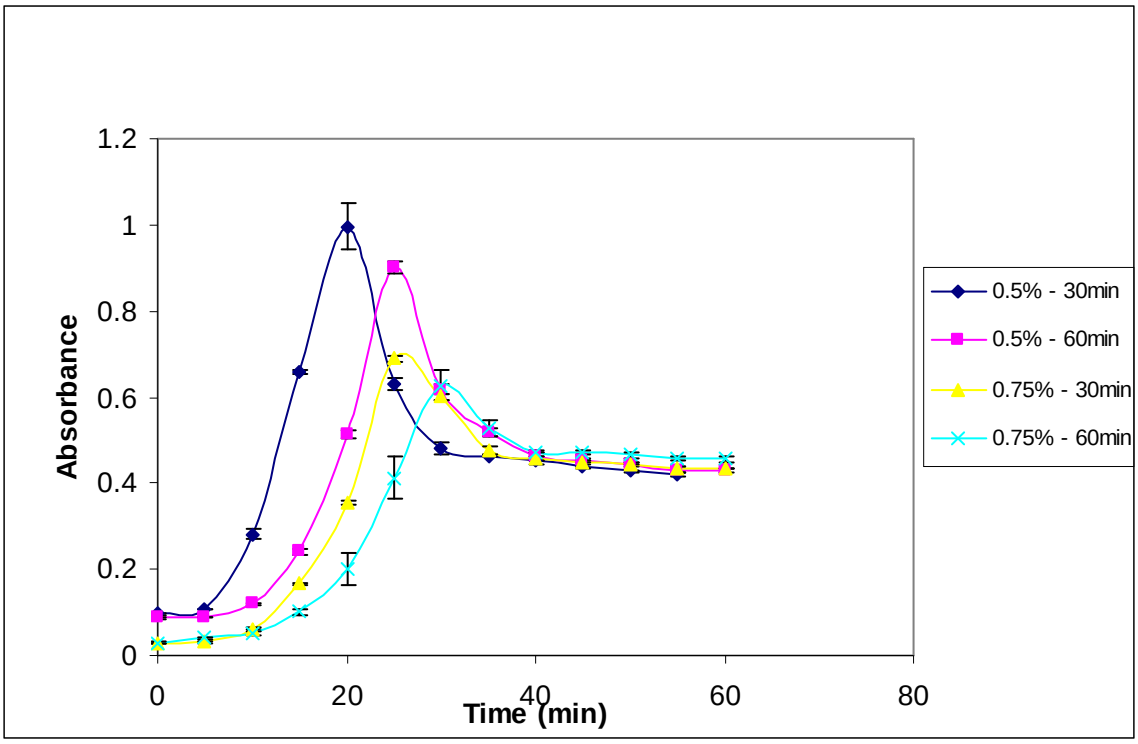


Figure 4.25 Comparison of each set of combinations of drying time with 0.5% and 0.75% calcium chloride solution

All four sets of combinations were tested using 0.02 U ml^{-1} enzyme concentration. From Figure 4.25, it can be observed that the lag time is the minimum for 0.5% w/v calcium chloride and drying time 30 minutes followed by for 0.5% w/v calcium chloride and drying time 60 minutes, 0.75% w/v calcium chloride and drying time 30 minutes and 0.75% w/v calcium chloride and drying time 60 minutes. The immobilization efficiency for each set was illustrated in the following Table 4.8:

Table 4.8 Immobilization efficiency for various combinations of calcium chloride solution and drying time

Calcium chloride solution (%w/v)	Drying Time (min)	Immobilization Efficiency (%)
0.5	30	91
0.5	60	95
0.75	30	97.5
0.75	60	98

Based on above results, the optimum parameters for enzyme immobilization were found to be 0.5% w/v calcium chloride solution, 8:1 sodium alginate and enzyme solution ratio and 30 minutes drying time for the beads. The selection was based on the fact that the enzyme reaction time was desired to be maximum 20 minutes and hence the combination with least lag time was considered optimum. The lag time is the time required for the enzyme to access the substrate after crossing the barrier of beads. Calcium alginate matrix and liquid-film layer around the beads formed significant barriers for substrate to enter the bead. Two factors affect the strength of the calcium carbonate layer *viz.* concentration of calcium chloride solution and drying time. The higher the concentration of calcium chloride solution and drying time for beads, the more dense is the matrices. Once the matrices become very solid and compact, , the lag time increases as the time required by the substrate to diffuse through will also increases. From Figure 4.25, it was evident that the combination with a lower calcium chloride solution concentration and a lower drying time for beads gave a lower lag time and hence the lower reaction time. But again, if the calcium layer is not strong, the enzyme can be leaked through the beads. This will hamper the immobilization

efficiency. Hence, the optimal parameters need to be selected in such a way that a reasonable amount of immobilization efficiency is maintained. For the optimum parameters chosen above, the immobilization efficiency was considered acceptable at 91%.

4.8 Determination of immobilized enzyme activity using syringaldazine

Activity of immobilized enzyme was determined using syringaldazine as substrate. The immobilization efficiency was determined by estimating the protein content and enzyme activity in the supernatant and washings solutions of immobilized beads. The activity in immobilized beads was then calculated by the difference between the initial enzyme concentration (before immobilization) chosen and the enzyme concentration in the supernatant and washing solution of the immobilized beads. Protein concentration was determined using the Bio-Rad Protein Assay, based on the Bradford (1976) dye-binding procedure, with bovine serum albumin (BSA) as standard. Assay of enzyme activity in the supernatant can give only enzyme activity but cannot give the estimate of actual enzyme loss as not all the un-immobilized enzyme may exhibit activity. Hence, to estimate the enzyme loss, a parallel protein assay was performed.

Using the standard curves in Figures 4.3 and 4.4, the enzyme concentration was determined based on the initial rate of reaction. The protein content quantitation was performed and the results of the two tests and determination of enzyme immobilization efficiency was tabulated in Table 4.9.

Table 4.9 Determination of enzyme immobilization efficiency

Enzyme Concentration (U ml^{-1})	Activity in supernatant+ washings (U)	Protein content in the supernatant + washings		Actual Enzyme used (µg)	Activity loss (U)	Activity retained (U)	Immobilization efficiency %
		Absorbance	µg				
0.12	Not detected	0.004	0.7	150	0.0005	0.1195	99.6
0.2	Not detected	0.089	13.8	250	0.0110	0.1890	94.5
0.3	Not detected	0.201	31.2	375	0.0249	0.2751	91.7
0.4	0.0013	0.353	54.7	500	0.0438	0.3562	89.1
0.6	0.0002	0.152	23.6	750	0.0188	0.5812	96.9

Calculations:

The protein content value was converted from absorbance to the micrograms by using Eq. (12).

The activity of stock enzyme powder was 0.8U of enzyme per 1mg of powder. Hence from this relationship, the actual enzyme quantity used was calculated using equation (14).

Actual enzyme quantity used (µg) = enzyme units used • 1000 / 0.8 Eq. (14)

Activity loss was calculated using equation (15).

Activity loss (U) =

Enzyme (µg) in supernatant • enzyme (U) used / enzyme (µg) used Eq. (15)

Activity retained in beads (U) = Enzyme (U) used – Activity loss (U) Eq. (16)

Immobilization efficiency =

Activity retained in beads (U) • 100 / Enzyme (U) used. Eq. (17)

From the results shown in Table 4.9, it can be concluded that the immobilization efficiency was above 89% for all the concentrations chosen. The comparative activity of different enzyme concentrations chosen was shown in Figure 4.26

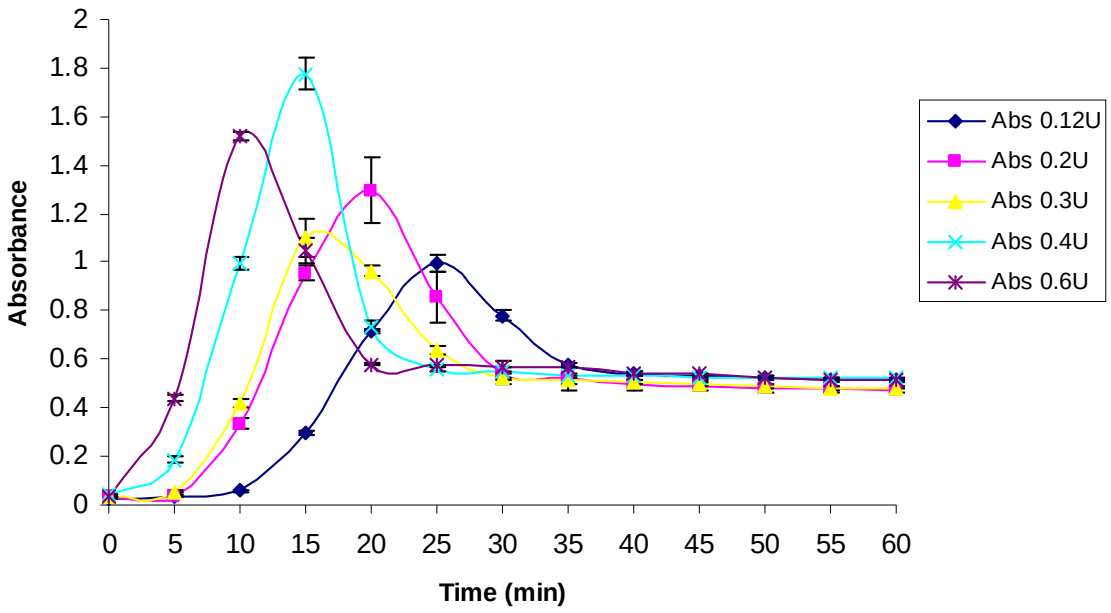


Figure 4.26 Activity of different immobilized enzyme concentrations

Figure 4.26 illustrates that with increasing total active enzyme units, the lag time decreased. Also it was observed that at higher total active enzyme units, the beads had immediately turned pink on its surface, thus indicating that some of the enzyme could have probably been adsorbed onto the surface of beads and the amount of enzyme adsorbed onto the beads increased with increase in enzyme concentration. Thus, as the diffusion of substrate inside the beads takes place the enzyme adsorbed onto the beads surface could have initiated the reaction with the external soluble substrate resulting in a reduced lag time. It was observed that the maximum absorbance peaks for syringaldazine oxidation by 0.3 U and 0.6 U of immobilized laccase were slightly lower relative to 0.2 U and 0.4 U immobilized enzymes, respectively. The exact reason(s) for these observations were not known at this stage.

4.9 Optimization of temperature, enzyme loading and reaction time for the reaction of immobilized enzyme with 2-chlorophenol.

The factors which were chosen to be optimized were temperature, enzyme loading and reaction time. The reason for not choosing pH was that it was found in the free enzyme reaction pH did not have significant effect on the enzymatic reaction. Since the optimum enzyme concentration in the free enzyme reaction in section 4.6 was found to be 0.2 U ml^{-1}, which is the upper limit of the three enzyme concentrations studied, concentrations with a higher number of total active units than 0.2 U were chosen for immobilized enzyme reaction optimization taking into account the possible losses due to denaturation during immobilization procedure and failure to entrap the enzyme within the beads. Hence, the concentrations chosen for immobilized enzyme reaction were 0.02 U ml^{-1}, 0.03 U ml^{-1} and 0.04 U ml^{-1}, the lag time of all of which were around 5 minutes. Since diffusion rate of substrate is an important factor when using immobilized enzyme, the factor affecting this rate i.e temperature was chosen. It is hypothesized that once an optimum temperature is determined, this would simultaneously allow the determination of optimum reaction time, hence the inclusion of the reaction time factor in the optimization program.

The results of the 45 experimental runs following the statistical design from MINITAB®14 were tabulated in Table 4.10.

Table 4.10 Results of 45 experimental runs

Run Order	Point Type	Blocks	Enzyme Loading (U ml^{-1})	Temperature (°C)	Reaction time (min)	ΔMethylene Blue concentration (mol L^{-1})
1	2	1	0.04	45	10	2.45×10^{-6}
2	2	1	0.03	60	10	7.73×10^{-7}
3	2	1	0.02	30	15	9.66×10^{-7}
4	2	1	0.04	45	10	1.91×10^{-6}
5	2	1	0.03	60	10	1.23×10^{-6}
6	0	1	0.03	45	15	2.14×10^{-6}
7	2	1	0.03	60	20	2.73×10^{-6}
8	2	1	0.03	30	10	3.12×10^{-7}
9	2	1	0.04	45	20	3.09×10^{-6}
10	10	1	0.02	30	15	1.04×10^{-6}
11	0	1	0.03	45	15	3.22×10^{-6}
12	2	1	0.04	30	15	2.19×10^{-6}
13	2	1	0.04	60	15	2.26×10^{-6}
14	2	1	0.03	60	20	2.38×10^{-6}
15	2	1	0.02	45	10	1.30×10^{-6}
16	2	1	0.02	45	20	2.57×10^{-6}
17	2	1	0.03	30	10	5.69×10^{-7}
18	0	1	0.03	45	15	3.40×10^{-6}

19	2	1	0.04	45	20	3.0×10^{-6}
20	2	1	0.03	30	20	5.65×10^{-7}
21	2	1	0.03	30	20	6.47×10^{-7}
22	0	1	0.02	60	15	1.53×10^{-6}
23	2	1	0.04	45	10	2.30×10^{-6}
24	2	1	0.02	30	15	7.12×10^{-7}
25	0	1	0.03	45	15	2.77×10^{-6}
26	2	1	0.02	60	15	1.91×10^{-6}
27	2	1	0.02	45	10	1.28×10^{-6}
28	2	1	0.02	45	20	2.76×10^{-6}
29	2	1	0.04	30	15	1.66×10^{-6}
30	2	1	0.04	45	20	3.08×10^{-6}
31	2	1	0.03	30	10	4.39×10^{-7}
32	2	1	0.02	45	20	2.44×10^{-6}
33	2	1	0.02	60	15	1.97×10^{-6}
34	2	1	0.03	60	10	8.56×10^{-7}
35	2	1	0.04	60	15	3.13×10^{-6}
36	2	1	0.02	45	10	1.22×10^{-6}
37	2	1	0.04	60	15	2.79×10^{-6}
38	0	1	0.03	45	15	2.65×10^{-6}
39	0	1	0.03	45	15	2.64×10^{-6}
40	2	1	0.04	30	15	2.03×10^{-6}
41	0	1	0.03	45	15	2.38×10^{-6}

42	2	1	0.03	60	20	2.52×10^{-6}
43	0	1	0.03	45	15	2.75×10^{-6}
44	2	1	0.03	30	20	7.77×10^{-7}
45	0	1	0.03	45	15	2.37×10^{-6}

Analysis of variance (ANOVA) and response surface regression for the above data were performed by MINITAB®14 software and showed the following results:

Term	Coefficient	Standard Error Coefficient	T	*P*
Constant	-0.000013	0.000002	-6.704	0.000
enzyme-loading	0.000000	0.000006	0.079	0.937
temperature	0.000000	0.000000	8.900	0.000
Reaction Time	0.000001	0.000000	5.133	0.000
enzyme-loading*enzyme-loading	-0.000014	0.000009	1.636	0.111
temperature*temperature	-0.000000	0.000000	-11.638	0.000
reaction time*reaction time	-0.000000	0.000028	-6.555	0.000
enzyme-load*temperature	-0.000000	0.000000	-0.390	0.699
enzyme-loading*reaction time	-0.000000	0.000001	-1.490	0.145
temperature*reaction time	0.000000	0.000000	4.181	0.000

S = 0.0000002842 R^2 = 91.9% R^2 (adjusted) = 89.9%.

P value < 0.05 for main effects temperature and reaction time and interactions between reaction time*reaction time and temperature*temperature indicates that the effects of temperature and reaction time are significant and a *P* value > 0.05 for main effect enzyme-loading and interactions between enzyme-loading*temperature and enzyme-loading*reaction

time indicates that enzyme loading has no significant effect on the optimization. The same is true for interactions between enzyme loading with either reaction time or temperature. Hence, the enzyme loading was fixed at the lowest concentration i.e. 0.02 U ml^{-1} for the optimization program.

The significant effect shown by temperature and reaction time factors on the optimization is explained as follows: in the degradation of 2-chlorophenol by immobilized laccase in spherical alginate beads, the rate of substrate diffusion increases with increasing temperature and when rate of diffusion increases, the reaction time would also be reduced.

Statistical plots for the analyses of the experimental data were presented below.

Using above results, various plots were plotted as follows:

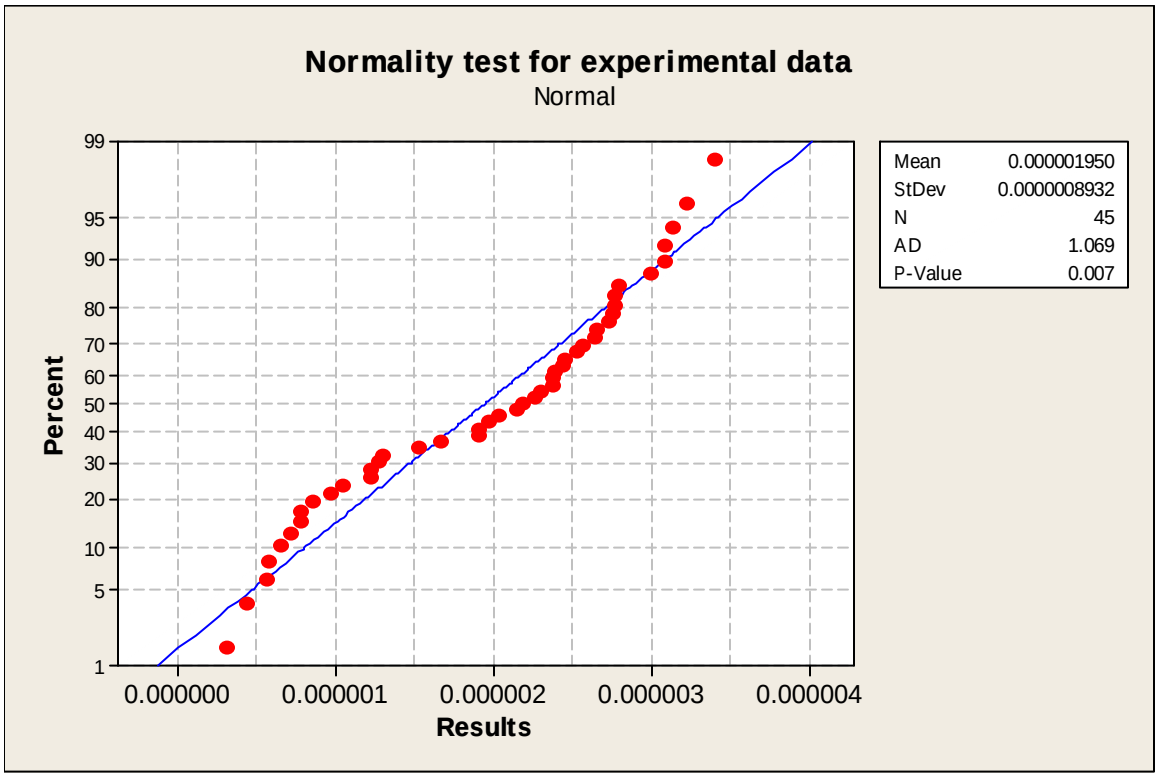


Figure 4.27 Normality plot for experimental data

The normality graph plotted for the experimental data has shown the *P*-value to be < 0.05, which shows a slight deviation from a normal distribution. This slight deviation was attributed to a relatively small number of the experimental runs (n = 45). This slight deviation is usually acceptable and do not present a major concern on the assumption of the normality of the data collected.

Figure 4.28 showed the normal probability of the residual values (errors)

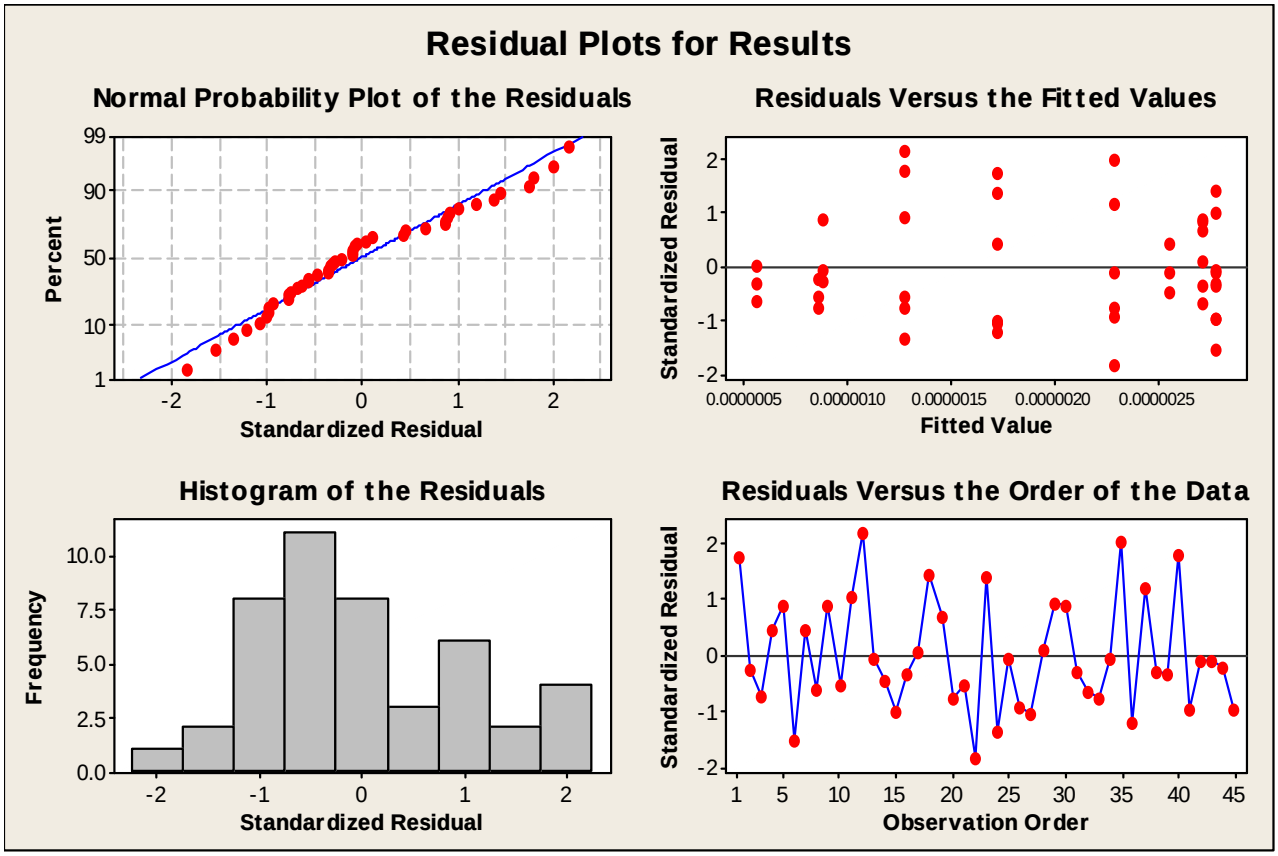


Figure 4.28 normal probability of the residual values

The normal probability plot of the residuals showed that the residuals were closely distributed following the normal distribution (top left panel) (residual = experimental value – model fitted value). This supported by the shape of the histogram of the residuals (bottom left panel) which retained the bell-shape curve albeit with a minor skew to the left of

the distribution graph. The slight deviation of the experimental data observed earlier (Fig. 4.27) contributed to this minor skewness. This indicated that the errors associated with the experimental data collected were purely due to random error. When the standardized residuals were plotted against fitted values (top right panel), a random pattern of data distribution was observed indicating the absence of systematic error. The order of the experiments execution has no influence on the data collected, as shown by the random occurrence of data in the standardized residual plot against observation order (bottom right panel).

Figure 4.29 represents the main effects plot

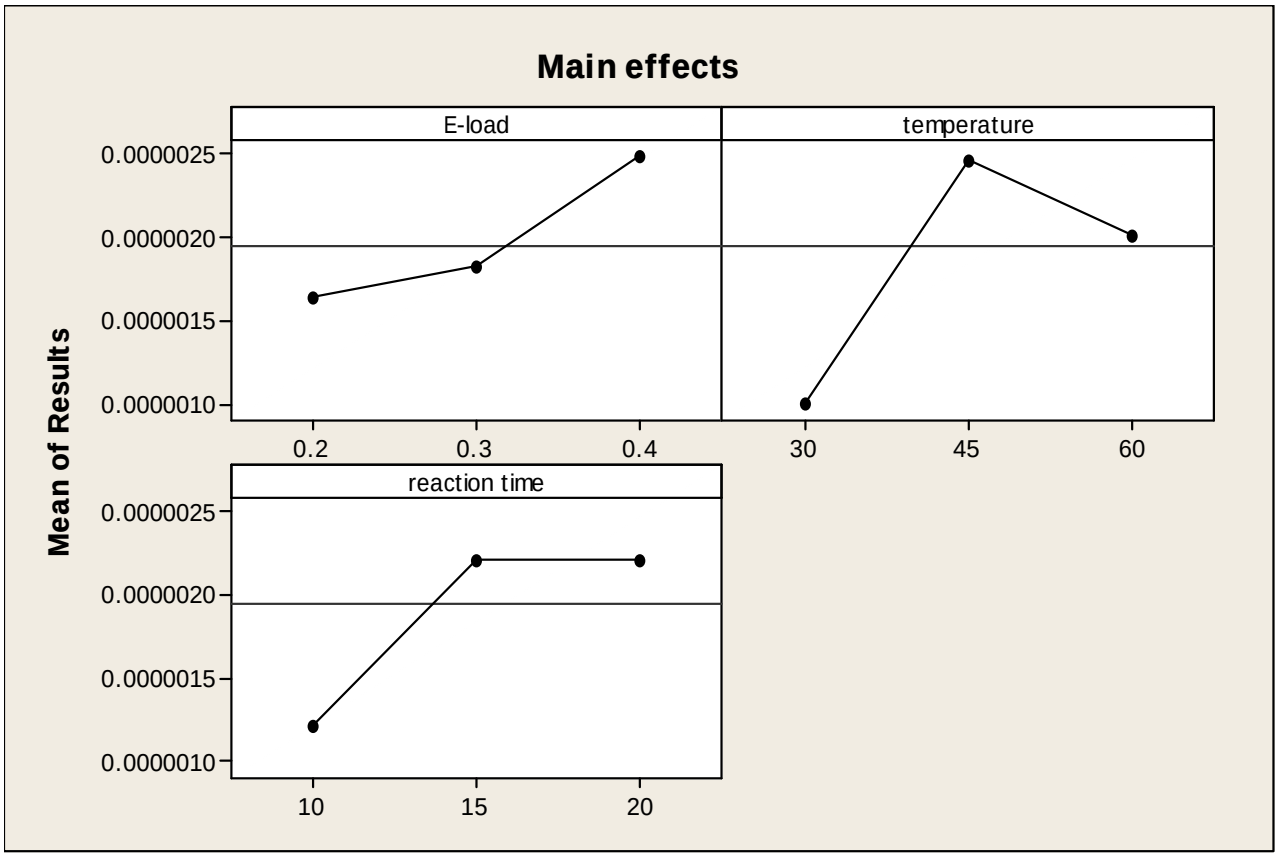


Figure 4.29 Main effects' plot

The above figure showed the change in Methylene Blue concentration after reaction with the 2-chlorophenol degradation products with respect to factors like temperature, enzyme loading and reaction time. It could be seen that as these three variables were varied, the response i.e. ΔMethylene Blue concentration also changed. Based on the difference between the lowest and highest value obtained for the response, the significance of the effect of that particular variable on the factor can be determined.

It was evident that the enzyme concentration has no significant effect on the enzyme reaction within the selected range of values since the corresponding change in the response was only within a very narrow range (1.6×10^{-6} to 2.4×10^{-6}). On the other hand, temperature and reaction time have significant effect as the responses were distributed within a relatively broad range (1.0×10^{-6} to $2.4\ 10^{-6}$ for temperature and 1.2×10^{-6} to 2.3×10^{-6} for reaction time).

Figure 4.30 showed the interactions plot for the variables

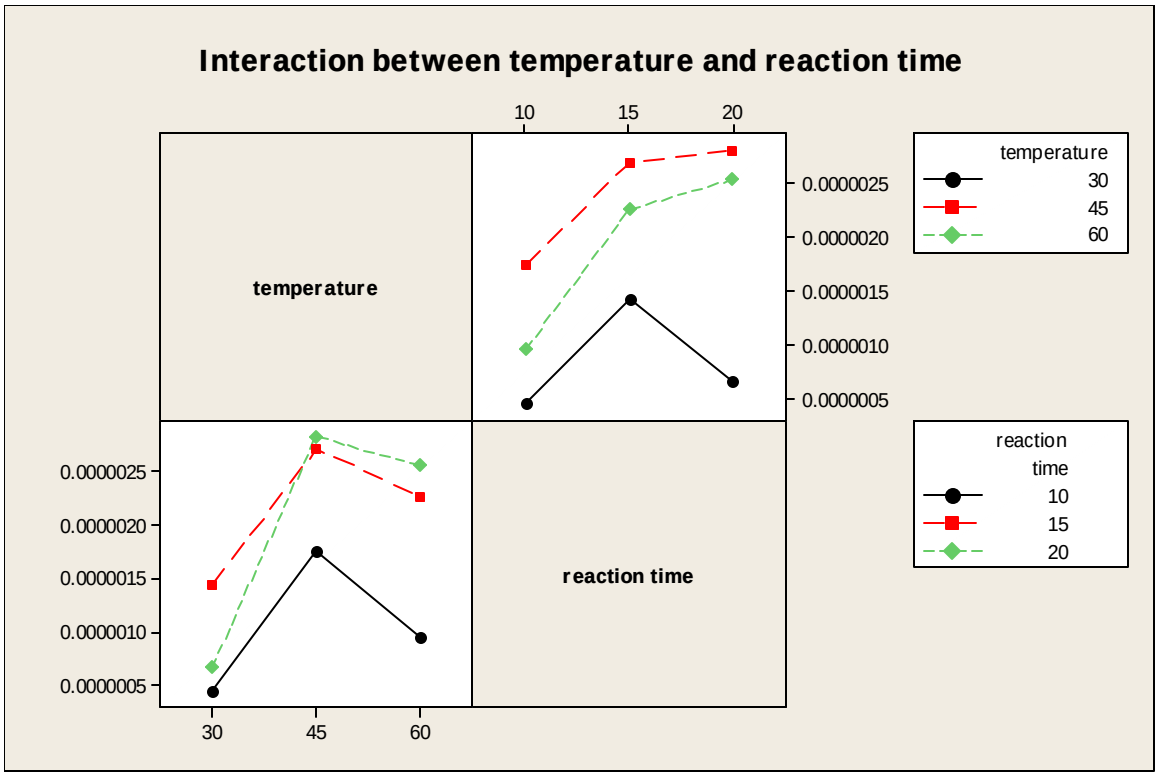


Figure 4.30 Interactions plot

The above plot showed the possible interactions between the two factors selected *viz.* temperature and reaction time on the ΔMethylene Blue concentration. Intersection of the three legends may indicate possible interaction between any two chosen parameters on the response. The interaction plot indicated that any possible interactions were not likely to be strong between reaction time and temperature since the general trend observed was the change across the variables' values resulted in a corresponding change in the responses. For example, within the range of reaction time tested, the effect of different temperatures behaved in a similar manner (top right hand panel), and similarly for the variables shown in the bottom left hand panel. This is further confirmed by the presence of minor curvature in the contour plot as shown in Figure 4.31.

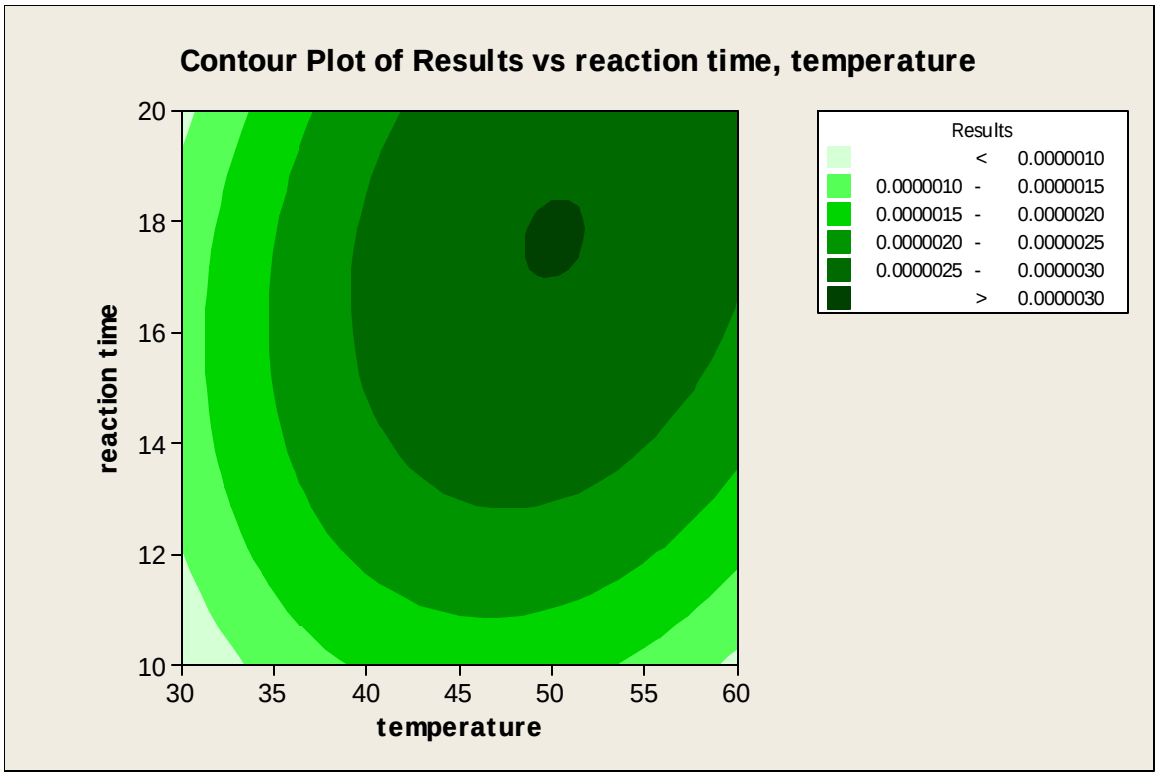


Figure 4.31 Contour plots for interaction between reaction time and temperature

Figure 4.32 showed the overlaid contour plot in which the enclosed white-colored region in was considered the feasible region for optimization of temperature and reaction time.

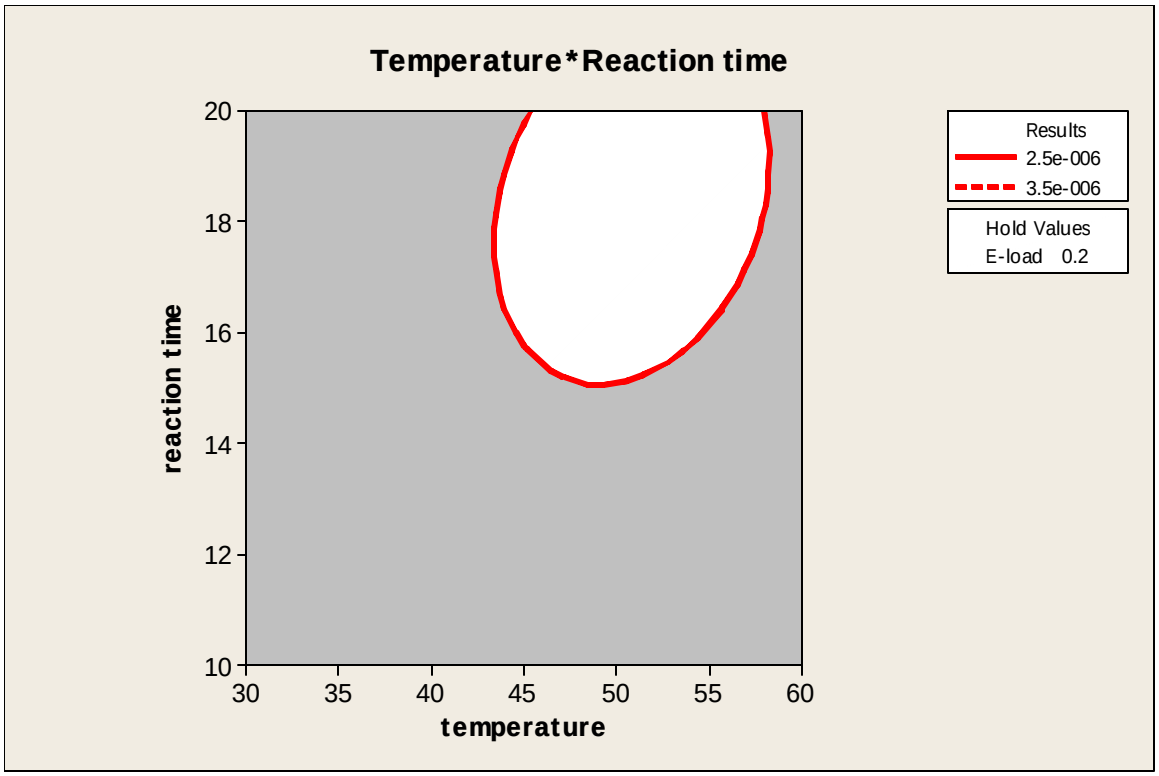


Figure 4.32 Overlaid contour plot showing feasible optimization region (white color) for reaction time and temperature

The enclosed white-colored region in Figure 4.32 is considered the feasible region for optimization of temperature and reaction time. The possible range shown in the plot for reaction time was from 15 to 20 min and for temperature was from 45°C to 55°C. Since it was evident from the response surface regression data and Figure 4.29 that enzyme loading did not have significant effect on the immobilized enzyme reaction, this variable was fixed at 0.2 U ml^{-1} to simplify the model while plotting the interactions and contour plots as shown in Figures 4.30 and 4.31.

Optimization was carried out by "Response Optimization" program of the MINITAB®14 software. Details of the optimization routine were shown below:

Optimization goal: Target: 3.0×10^{-6}

Upper value: 3.5×10^{-6}

Lower value: 2.5×10^{-6}

Temperature: 45°C

Reaction time: 15 min

The optimal values thus obtained were shown in Table 4.11

Table 4.11 Optimization values

Local Solution		**Predicted Responses**		
Reaction time	Temperature	ΔMethylene Blue concentration (mol L^{-1})	Desirability	Composite Desirability
15	45	3.1×10^{-6}	0.57918	0.57918

Global Solution		**Predicted Responses**		
Reaction time	Temperature	ΔMethylene Blue concentration (mol L^{-1})	Desirability	Composite Desirability
15	45	3.1×10^{-6}	0.57918	0.57918

The global solution was found to be the same as that of local solution indicating that there was only one possible optimal solution. The desirability of the optimum solution was found to be approximately 60%. Hence, the optimization results obtained were: reaction time 15 minutes, temperature 45°C and enzyme loading 0.02 U ml^{-1}.

A confirmatory run using optimized conditions gave the following results (Table 4.12):

Table 4.12 Results of confirmatory test using optimized conditions

Replicate #	ΔMethylene Blue concentration (mol L^{-1})
1	2.91×10^{-6}
2	2.76×10^{-6}
3	2.90×10^{-6}
Average	$2.86 \times 10^{-6} \pm 0.084$

The average value was found to be very close to the target response i.e. 3.0×10^{-6}, thus confirming the optimization. The reduction in the Methylene Blue concentration under optimized conditions was about 20.7%, corresponding to the equivalent amount of 2-chlorophenol degraded product formed and hence 2-chlorophenol degradation by laccase assuming one phenoxy radical generated from each molecule of 2-chlorophenol and a 1:1 stoichiometric relation between chlorophenol degraded product and Methylene Blue as per proposed mechanisms of action (Figure 4.17).

The comparison of reduction in Methylene Blue concentration by both free and immobilized laccase reactions under their respective optimized conditions was shown in Table 4.13.

Table 4.13 Comparison between free and immobilized laccase systems under their respective optimized reaction conditions

Free laccase		**Immobilized laccase**	
ΔMethylene Blue concentration by total active enzyme in 10 minutes reaction time (μmol L^{-1})	**ΔMethylene Blue concentration per unit enzyme per unit time (μmol L^{-1} U^{-1} min^{-1})**	**ΔMethylene Blue concentration by total active enzyme in 15 minutes reaction time (μmol L^{-1})**	**ΔMethylene Blue concentration per unit enzyme per unit time (μmol L^{-1} U^{-1} min^{-1})**
2.83	1.415	2.86	0.953

Table 4.13 showed that the rate of 2-chlorophenol degradation per unit of free laccase was approximately 33% more than that of immobilized laccase. This reduction in 2-chlorophenol degradation capacity by immobilized laccase could be attributed to the diffusion barrier due to external and internal mass transfer at the surface and within the bead. When an enzyme is entrapped, the additional resistance due to the entrapment matrix will pose more resistance to the diffusion of substrate to the active site of the enzyme. This is in contrary to the free enzyme where the reaction velocity is limited only by diffusion of substrate into the free enzyme active site.

Hence, another likely reason for the lower V_{max} value, and a comparatively lower rate of reaction for immobilized laccase (33%) was the presence of substrate gradient internal and external of the alginate beads. Thus, section 4.10 described the determination of the extent of external and internal mass transfer limitation on the alginate-bead immobilized laccase.

4.10 External and internal mass transfer determination for immobilized laccase

To determine the distribution of substrate within an alginate bead, a concentration profile was calculated for a bead with average dimension. In order to determine if external mass transfer was significant, the observable modulus for external mass transfer (Ω) for a bead with average dimension was calculated.

External mass transfer

Observable modulus for external mass transfer, Ω, for a bead was calculated as follows:

$$\Omega = \frac{R}{3}\left(\frac{r_{obs}}{K_s C_{bulk}}\right) \qquad \text{Eq. (18)}$$

where R is the average radius of the bead (m), r_{obs} is the rate of substrate consumption per unit volume of beads at steady-state (g L^{-1} s^{-1}), K_S is the liquid-phase mass transfer coefficient (m s^{-1}), and C_{bulk} is the substrate concentration in the bulk liquid (g L^{-1}). Because no data was available for the liquid-phase mass transfer coefficient of syringaldazine, the K_S value for phenol in water (Vinod and Reddy, 2006) was used in Eq. (18) as being representative for syringaldazine and 2-chlorophenol. This K_S value was 20.12 x 10^{-7} m s^{-1}.

External mass transfer of the substrate to the beads did not limit the rate of reaction in this study as observable modulus for external mass transfer, Ω, was found to be 0.02, which is very much less than 1. Because the Ω-value was much smaller than 1, it can be inferred that the concentration of the substrate (syringaldazine or 2-chlorophenol) at the surface of the bead was nearly the same as the concentration in the bulk liquid. Table 4.14 showed the calculation data for the Ω.

Table 4.14 Observable modulus for external mass transfer calculation

r_{obs} (g L^{-1} s^{-1})	Average radius of the beads, R (m)	K_s(m s^{-1})	C_{bulk}(g L^{-1})	R/3	K_s*C_{bulk}	r_{obs}/K_s*C_{bulk}	Ω
$1.75x10^{-6}$	0.0015	$20.12x10^{-7}$	0.18	0.0005	$3.6x10^{-7}$	4.86	0.00243

Concentration profile within the bead

Assuming a uniform distribution of laccase in the bead, a spherical shape of the bead, isothermal conditions, a first order reaction and no partitioning of the substrate between the exterior and interior of the bead, the rate of diffusion of the substrate in the bead at steady-state was equated to the rate of the consumption, as follows:

$$D_e \frac{d^2 C_{syr}}{dr^2} + \frac{2}{r}\frac{dC_{syr}}{dr} = k_1' C_{syr} \qquad \text{Eq. (19)}$$

where C_{syr} is the concentration of syringaldazine in the beads, D_e is the effective diffusion coefficient of syringaldazine in the beads (m^2 s^{-1}), k_1' is the apparent first-order rate constant of syringaldazine oxidation (s^{-1}) and r is the radial position in the bead (m). In the absence of a D_e value for the system under investigation, the average D_e value for phenol in Ca-alginate beads was used. This value was 1.21 x 10^{-11} m^2 s^{-1} (Dursun and Tepe, 2005).

The boundary conditions used for solving Eq. (19) were: $C_{syr} = C_{syr,s}$ at $r = R$ and $dC_{syr}/dr = 0$ at $r = 0$, where R is the radius of the bead and $C_{syr,s}$ is the concentration of syringaldazine on the surface of the bead. In the absence of an external mass transfer limitation, C_{syr} is equal to the bulk concentration of syringaldazine, i.e. C_{bulk}. Integration of Eq. (19) gives the following equation:

$$C_{syr} = C_{syr,s} \frac{\sinh\left(r\sqrt{\left(k_1'/D_e\right)}\right)}{\left(\frac{r}{R}\right)\sinh\left(R\sqrt{\left(k_1'/D_e\right)}\right)} \qquad \text{Eq. (20)}$$

The substrate concentration profile within a bead as a function of its local radial position was estimated using Eq. (20). Although it was shown earlier that the kinetics of syringaldazine oxidation by immobilized laccase can be approximated using Michaelis-Menten model, for simplicity first-order reaction kinetics was used in this concentration profile calculation. Owing to the non-linearity of the Michaelis-Menten expression, simple analytical integration of Eq. (19) when this rate expression is used will not be possible. Because Michaelis-Menten kinetics lie somewhere between zero- and first-order kinetics, explicit solutions found for these two expressions can be used instead to estimate the extreme limits for Michaelis-Menten reactions.

In order to estimate the value of apparent first-order rate constant, (K_1), for the enzymatic reaction, the volumetric rate of reaction was plotted against various initial syringaldazine concentrations. The rate constant, k_1 (s^{-1}), was calculated as the slope of the plot.

The volumetric rate of reaction was calculated using the following equation:

$$r_{vol} = \frac{\Delta C(t)}{\Delta t} \qquad \text{Eq. (21)}$$

where r_{vol} (g L^{-1} s^{-1}) is the volumetric rate of reaction, ΔC (g L^{-1}) is the change in concentration of substrate over the time Δt (s).

Figure 4.33 showed the determination of the apparent first-order rate constant, k_1'.

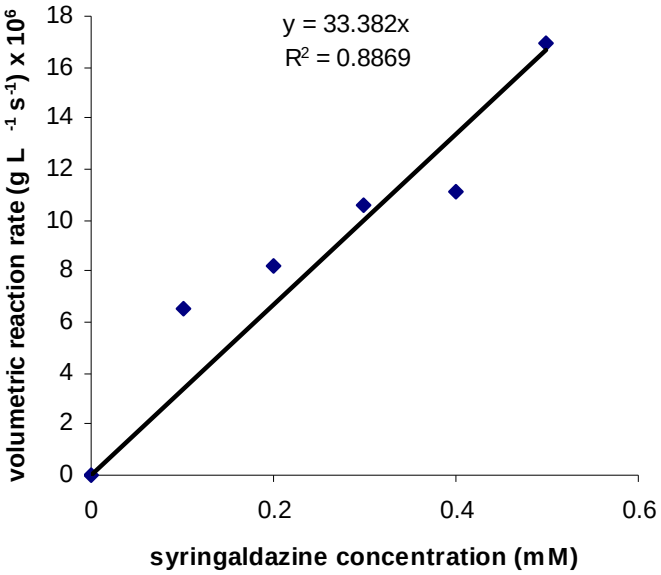


Figure 4.33 Determination of apparent first-order rate constant k_1

From the slope of the line in Figure 4.33, the value of k_1 was found to be 3.34 x 10^{-5} s^{-1}.

Substituting this value in the Eq. (20), the concentration profile of syringaldazine in the beads was calculated at various radial positions within the beads starting from 0 m to 0.0015 m (average radius of the bead). Figure 4.34 showed the concentration profile as a function of various radial positions within the bead.

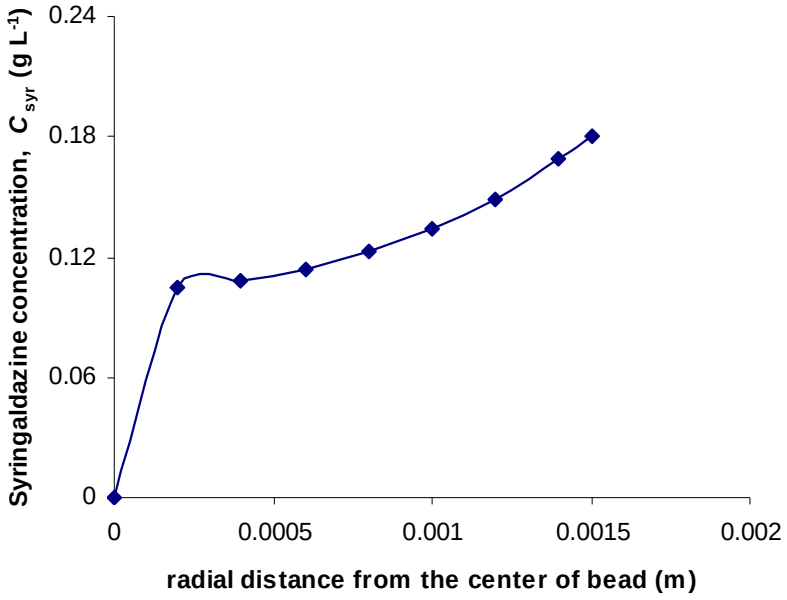


Figure 4.34 Syringaldazine concentration profile in beads with average diameter of 0.003 m

Bulk concentration for syringaldazine and its concentration changes were determined using Eq. (7), which were subsequently used for the calculation of observed and volumetric rates. Then, these values were used alongside other previously explained values to complete the solutions of Eq. 18 to 21.

It was observed that there exists significant substrate concentration gradient throughout the radius of the alginate bead. The substrate concentration decreased in an almost regular manner as it is moving away from the surface of the bead towards the center, with substrate concentration reduced to 0 g L^{-1} at the center. Thus, although external mass transfer may posed only a small finite limitation on the substrate diffusion from bulk liquid to the surface of the bead, internal mass transfer on the other hand could have posed significant limitation on the diffusion of 2-chlorophenol inside the alginate bead, and thus on the rate of degradation of 2-chlorophenol by laccase enzyme inside the bead. Consequently, this may

partly explain the slightly lower level of 2-chlorophenol degradation by immobilized laccase as compared to the free enzyme.

Despite this observation, the above studies indicated that the immobilized laccase system's efficiency with respect to 2-chlorophenol degradation was comparable with that of the free laccase system, despite a limitation on the diffusion of 2-chlorophenol inside the alginate bead. Keeping in view the advantage of immobilized system (recovery and re-usability of the enzyme), immobilized laccase system may be considered an option comparable to the free laccase system in 2-chlorophenol degradation.

CHAPTER 5

CONCLUSIONS

- Assay time for free and immobilized laccase was determined at 10 minutes and 15 minutes, respectively.

- Kinetics of free and immobilized laccase were studied using syringaldazine as substrate. The kinetic parameters were found to be as follows for free and immobilized enzyme.

  Free laccase:

  $K_m = 0.253 \pm 0.078$ mM

  $V_{max} = 40.4 \pm 0.2$ uM min^{-1}

  Immobilized laccase:

  $K_m^{app} = 0.275 \pm 0.138$ mM

  $V_{max}^{app} = 1.34 \pm 0.25$ uM min^{-1}

- Factors like pH, temperature and enzyme concentration were optimized for the free enzyme reaction and reaction time, temperature and enzyme concentration were optimized for the immobilized enzyme reaction using a statistical tool *viz.* Box-Behnken method of Response Surface Methodology. The optimum conditions were found to be as follows for free and immobilized laccase.

Free laccase:

pH : 5;

temperature: 45°C;

enzyme concentration: 0.2 U ml^{-1}

Immobilized laccase:

temperature: 55°C;

enzyme concentration: 0.02 U ml^{-1};

reaction time: 15 minutes.

- The effect of immobilization on the enzymatic degradation capability was studied. Enzyme was immobilized in sodium alginate beads and the factors affecting immobilization and the reaction time (rate of diffusion) like the concentration of calcium chloride used, the ratio of enzyme solution and sodium alginate solution and the drying time for the beads were optimized to obtain maximum response within shortest possible time, without compromising the immobilization efficiency. The optimum conditions were found to be as follows:

  0.5% calcium chloride solution;

  1:8 ratio of enzyme solution and sodium alginate solution and

  30 minutes of drying time.

  The immobilization efficiency was found to be around 90%.

- A rapid assay method was developed for the assay of degraded products of 2-chlorophenol, wherein the anionic phenoxy radicals, possibly generated during 2-chlorophenol degradation, couples with a cationic dye, Methylene Blue and renders it

colorless. The extent of decolorization indicated the extent of product formation and hence the extent of 2-chlorophenol degradation assuming a 1:1 stoichiometric reaction between degraded products of 2-chlorophenol and the dye.

- 2-chlorophenol was successfully degraded by both free and immobilized laccase under optimum conditions with efficiency of immobilized laccase comparable to that of free laccase

- External mass transfer was found to exert a negligible limitation on the substrate diffusion from the bulk liquid to the sodium alginate bead.

- Internal mass transfer posed significant limitation on the substrate diffusion from the surface of the sodium alginate bead to its radial interior.

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