CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction.

Laccases are extracellular enzymes, non-haem, copper containing proteins that catalyze the one-electron oxidation of phenols to phenoxy radicals (Kirk and Shimada, 1985 and Farrell, 1987). Laccases (EC 1.10.3.2) catalyse the reaction below:

\[
\begin{align*}
\text{OH} & \quad \text{OH} \\
\text{or} & \quad \text{or} \\
\text{O} & \quad + \quad \text{H}_2\text{O}
\end{align*}
\]

This reaction is characteristic of laccase and indeed is the main criterion according to which the enzyme is classified. Laccase oxidizes a very wide range of substrates
including monophenols, triphenols and ascorbic acid as well as o-diphenols and p-diphenols.

**Occurrence of laccase**

Laccase was first detected by Yoshida in 1883 from the *Rhus vernicifera* (lacquer tree). The latex of the lacquer tree called “laccol” by Bertrand, contains a catechol derivative. It is the reaction catalysed by laccase that darkens the lacquer upon oxidation.

Laccase was later detected in a number of basidiomycetes and fungi imperfecti including *Lactarius, Polyporus, Aspergillus, Pleurotus, Polystictus, Psalliota, Glomerella, Podospora, Botrytis, Neurospora* and *Russula* (Turner, 1974; Wood and Goodenough, 1977). It is also present in actinomycetes (Kirk and Farrell, 1987). The occurrence of laccase in higher plants is, however, much more limited.

The genus *Rhus* has been investigated in considerable detail (Nakamura and Ogura, 1966). Joel et al (1978) reported that laccase occurs in the cavity of the secretory ducts of all the members of the Anacardeaceae, but, at least in mango fruit, it is absent from the rest of the tissues. The other authentic proof of laccase in higher plants is its presence in peaches (Lehman et al., 1974) and in *Aesculus* (Wosilait et al., 1954).
Molecular weight of laccases

The molecular weight of various laccases differ considerably. That of *Rhus* laccase has been reported to vary between 101,000 Daltons and 140,000 Daltons depending on the species and even variety, as for example in Japanese *Rhus vernicifera* (Reinhammar, 1970; Holwerda *et al*., 1976). Peach laccase has a molecular weight of 73,500 Daltons (Lehman *et al*., 1974). Even the molecular weight of the fungal laccases vary appreciably. For example the molecular weight is 67,000 Daltons for *Lactarius* (Iwasaki *et al*., 1967); 60,000 to 65,000 Daltons for the main *Polyporus* laccase (Fahraeus and Reinhammar, 1967); 71,000 Daltons for *Aspergillus* (Clutterback, 1972) and 56,000 Daltons for *Botrytis* (Mayer *et al*., 1977). *Podospora* laccase exist in more than one form of varying molecular weight (Jonsson *et al*., 1968; Cheung and Marshal, 1969). *Podospora* laccase I has a molecular weight of 390,000 Daltons. Laccase from *Agaricus bisporus* has molecular weight of 100,000 Daltons (Wood, 1980). Laccase from the white-rot fungus *Trametes trogii* excretes laccase with a molecular weight of 70,000 Daltons (Garzillo *et al*., 1998). *Chaetomium thermophilium*, a cellulolytic fungus from composting municipal solid waste exhibited laccase activity with a molecular mass 77,000 Daltons (Chefetz *et al*., 1998). The basidiomycete *Marasmius quercophilus* is commonly found during autumn on the decaying litter of the evergreen oak. This white-rot fungus has strong laccase activity which consists of a monomeric glycoprotein of approximately 63,000 Daltons (Dedeyan *et al*., 2000).
Copper content in laccase

All laccases are made up of a single polypeptide chain containing four atoms of copper per polypeptide. Purified laccases characteristically contain a blue copper centre. Copper may be present in laccase in at least three different forms. One form is detectable by electron paramagnetic resonance (EPR) and is intensely blue (Type I). The other is EPR detectable but not blue (Type II) and a third not detectable at all by EPR (Type III).

It is generally accepted today that all three types of copper are present in laccases, that is, one atom each of types I and II and two atoms of type III (Holwerda et al., 1976). A significant difference exists between Rhus and fungal laccases. Rhus laccase can be reconstituted from copper and apoprotein, while fungal laccases cannot be reconstituted readily. However, in Lactarius laccase apparently such reconstitution was possible (Iwasaki et al., 1967). PM1 laccase from the basidiomycete strain (CECT 2971) was found to contain four copper ions of three different types which formed the redox center of some eukaryotic species (Reinhammar, 1984). The N terminus of the PM1 laccase is very similar to the N terminus of Coriolus hirsutus and Phlebia radiata laccases (Kojima et al., 1990 and Saloheimo et al., 1991). It is interesting to note that copper has been reported to be a strong laccase inducer in the fungal species Trametes versicolor (Collins et al., 1997), Phanerochaete
chrysosporium (Ditter et al., 1997) and the ligninolytic fungus Pleurotus ostreatus (Palmieri et al., 2000).

Optimum pH for activity of laccase

The pH optimum for the activity of the different laccases show quite considerable variation, being pH 6.4 or pH 7.5 for Rhus depending on the species and 4.5 for the Burmese lacquer tree Melanorrhea usitata laccase (Omura, 1961). The pH optimum of Neurospora and Podospora laccase towards quinol is around pH 6.0; that of Ganoderma is around pH 5.4; Botrytis laccase shows optimal activity at pH 4.7 and Polyporus around pH 4.0 (Fahraeus and Ljunggren, 1961; Froehner and Eriksson, 1974; Dubernet et al., 1977). The optimum pH for the laccase activity of Chaetomium thermophilum (Chefetz et al., 1998) was 6.0 and that of Marasmius quercophilus was 4.5 (Dedeyan et al., 2000).

The pH optimum also differs depending on the substrate oxidized. Stability of laccases from various sources is reported to be pH dependent and changes with the buffer and other factors in the medium (Molitoris and Esser, 1970). Most laccases from fungi are unstable at alkaline pH, the most extreme case of unstability being Botrytis laccase which is rapidly inactivated above pH 7.0 (Dubernet et al., 1977). Rhus laccase seems to be more stable to changes in pH than the fungal laccase (Malmstrom et al., 1970).
The role of laccase in biological function

Several laccases have been purified and characterized biochemically (Wood, 1980, Sugiura et al., 1987 and Karhunen et al., 1990) and some genes encoding the laccases have also been cloned and genetically characterized (Kojima et al., 1990 and Saloheimo et al., 1991). The biological function of laccase has been implicated in different processes such as sporulation (Leatham and Stahman, 1981); pigment production (Clutterback, 1990) and rhizomorph formation (Worrel et al., 1986). Chaetomium thermophillum produces laccase during the thermophilic stage of composting and remains active for a long period of time at high temperature and alkaline pH values (Chefetz et al., 1998). This suggests that the enzyme is involved in the humification process during composting.

The best documented role of laccase is in wood rotting. Lignin-degrading ability of white-rot fungi is associated with production of extracellular phenol oxidases (Kirk and Kelman, 1965). Laccase, a p-diphenol-oxidase (E.C.1.10.3.2), catalyzes the oxidation of phenols to quinones and phenoxyradicals which spontaneously polymerize. Lignin peroxidases (LiP), manganese peroxidases (MnP) and laccases are three families of enzymes that were implicated in the biological degradation of lignin. Galliano et al. (1991) studied lignin degradation by Rigidoporous lignosus, a fungus that synthesizes manganese peroxidase (MnP) and laccase. When both enzymes were added to the
reaction mixture at the same time, lignin solubilization was extensive. Here, the MnP and laccase acted synergistically. Laccase can depolymerize and solubilize lignin. It is presumed that the main role of peroxidases is the conversion of the soluble lignin fragments into smaller molecules.

Chemical degradation of lignin has been the only method used for pulping in the paper-making process. In addition, the pulp and paper industry generates effluents containing lignin derivatives and other phenolic compounds. These are often toxic and give effluents their characteristic brown colour. Such compounds are difficult to remove by currently available technology (Cripps et al., 1990).

For some time now biological pre-treatment has been considered a viable option for upgrading the huge quantities of lignocellulose waste generated annually through the activities of the agricultural and forest industries (Bushwell, 1991). Many such lignocellulosic materials might serve as a feedstock for ruminants but the high lignin content inhibits digestion of the polysaccharide components within the rumen. One potential method for the biological removal of this lignin involves the use of white-rot fungi.

Phenols are present in the wastewaters from a number of industries, including coal conversion, petroleum refining, resins and plastics, dyes and other organic chemicals, textiles, timber, mining and dressing and pulp and paper. Virtually all phenols and
many of its derivatives are considered to be hazardous pollutants (Baker et al., 1978). Hence, removal of phenols from industrial aqueous effluents is an important practical problem. Conventional methods for dephenolization of industrial waste waters include solvent extraction, microbial degradation (Cripps et al., 1990), adsorption on activated carbon and chemical oxidation (Lanouette, 1977). These methods, although effective and useful, suffer from serious drawbacks, such as high cost, incompleteness of purification, formation of hazardous by-products and applicability to only a limited phenol concentration range.

Therefore, alternative technologies are desirable. Several polyphenoloxidases catalyse the oxidative coupling of phenolic compounds, resulting in the formation of polymeric compounds (Sjoblad and Bollag, 1981). Laccase from *Pyricularia oryzae* is capable of oxidizing phenolic azo dyes (Chivukula and Renganathan, 1995).

Monitoring of environmental pollution and determination of polyphenolic compounds is of great importance for various industries. Phenol-oxidizing enzymes as biosensors looks very promising to measure the total content of polyphenolic compounds. According to Ghindilis et al. (1992) laccase is the best for use in analytical systems for the determination of polyphenolic compounds. Laccase can catalyse oxidation of both para and ortho-substituted phenols.
Malovik et al. (1984) reported a method of lignin determination based on the use of laccase immobilized on the surface of glass bearing amino groups. However, in this case there is practically no improvement in the stability of the immobilized enzyme as compared to the native laccase.

Polycyclic aromatic hydrocarbons (PAHs) are pollutants that are found in most terrestrial and aquatic environments (Ceniglia, 1993). They represent a health risk to animals and humans because of their toxic, mutagenic and carcinogenic properties. Laccase produced from Trametes versicolor (Collins et al., 1996) and Pycnoporus cinnabarinus (Rama et al., 1998) were efficient degraders of PAHs. Pickard et al. (1999), reported that laccase from Coriolopsis gallica exhibited similar PAHs oxidation properties as other white rot fungi laccases.

2.2 Enzyme Immobilization

Why immobilization?

Immobilization refers to the localization or the confinement of a biocatalyst on a support material. Enzymes are biocatalysts which catalyse chemical reactions under very mild conditions, usually at room temperature and atmospheric pressure, which help to avoid thermal degradation of substrates and products. However, the uses of enzymes are usually limited by their relative instability, high cost and difficulty in recovery for reuse (Ivan and Robert, 1994).
Immobilization of the enzymes eliminates some of these problems, though there are inevitably some limitations, as summarised below in Tables 2.1, 2.2 and 2.3.

**Table 2.1: Advantages of Immobilized Enzymes (Mosbach, 1987)**

<table>
<thead>
<tr>
<th>No</th>
<th>Advantages of Immobilized Enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Recovery and reuse of the enzyme</td>
</tr>
<tr>
<td>2.</td>
<td>Increased stability with respect to temperature, time and self-degradation (Leonowicz <em>et al.</em>, 1988)</td>
</tr>
<tr>
<td>3.</td>
<td>Product obtained is enzyme free</td>
</tr>
<tr>
<td>4.</td>
<td>New medical and industrial uses (Cheetham, 1985)</td>
</tr>
<tr>
<td>5.</td>
<td>Use of multi enzyme system</td>
</tr>
<tr>
<td>6.</td>
<td>Greater control of catalytic process, permits continuous operation</td>
</tr>
<tr>
<td>7.</td>
<td>Modification of properties such as pH optimum and attraction for substrates</td>
</tr>
</tbody>
</table>

**Table 2.2: Limitations of immobilized enzymes (Mosbach, 1987)**

<table>
<thead>
<tr>
<th>Limitations of Immobilized Enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Loss of activity during immobilization due to blockage of active sites</td>
</tr>
<tr>
<td>2. Mass transfer problems which is due to steric restrictions on substrate accessability</td>
</tr>
<tr>
<td>3. Enzyme inactivation</td>
</tr>
<tr>
<td>4. Empirical nature of immobilization technology makes it extremely difficult to estimate quantitatively</td>
</tr>
<tr>
<td>5. Operational characteristics for optimisation</td>
</tr>
<tr>
<td>6. Possible requirement of higher quality feedstock</td>
</tr>
</tbody>
</table>
2.2 Supports for enzyme immobilization

In an immobilized enzyme system, the nature of the support material and the interactions between the enzyme and the support are the major components that influence its performance. A suitable support will enhance the performance of an immobilized enzyme system. Though there are no perfect support materials, certain desirable characteristics are recognised (Ivan and Robert, 1994) as shown in Fig. 2.2. Supports for enzyme immobilization may be classified according to their morphology or chemical composition (Ivan and Robert, 1994). General methods of enzyme immobilization may be classified as shown in Fig. 2.3.
Figure 2.2: Classification of supports for enzyme immobilization according to their morphology or chemical composition (Ivan and Robert, 1994)

Classification of supports

Morphological

Non-porous
- Natural Polymers
  - Cellulose
  - Starch
  - Dextran
  - Agar and agarose
  - Alginate
  - Carrageenan
  - Chitin and chitosan

Porous
- Synthetic Polymer
  - Polystyrenes
  - Polymethacrylates
  - Polyacrylamide
  - Glycidyl
  - Vinyl and allyl polymers
  - Polyamides

Chemical

Organic
- Protein
  - Collagen
  - Gelatin
  - Albumin
  - Silk

Inorganic
- Minerals
  - Attapulgiteday
  - Bentourite
  - Kieselguhr
  - Punic stone
  - Vermiculite
- Fabricated Materials
  - Nonporous glass
  - Controlled pore glass
Figure 2.3: Classification of immobilization methods for enzyme (Ivan and Robert, 1994)
Table 2.3: Desirable characteristics of an enzyme or protein carrier (Mosbach, 1987)

<table>
<thead>
<tr>
<th>Desirable Characteristics of an Enzyme or Protein Carrier</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Large surface area per unit volume</td>
</tr>
<tr>
<td>2. Permeability</td>
</tr>
<tr>
<td>3. Hydrophilic character</td>
</tr>
<tr>
<td>4. Insolubility in all the solvents involved in the processes</td>
</tr>
<tr>
<td>5. High chemical, mechanical and thermal stability</td>
</tr>
<tr>
<td>6. High rigidity</td>
</tr>
<tr>
<td>7. Suitable particle size and shape</td>
</tr>
<tr>
<td>8. Resistant to microbial attack</td>
</tr>
<tr>
<td>9. Low cost and easy availability</td>
</tr>
</tbody>
</table>

2.3.1 Cross-linking

This carrier-free method of immobilization is based on the formation of covalent bonds between enzyme molecules by means of bifunctional or multifunctional reagents. This leads to the formation of a cross-linked biocatalyst which is insoluble in water but still retains the bulk of its enzyme activity.

Reagents with two identical functional groups have been most widely used to produce insoluble aggregates, with glutaraldehyde being the most commonly used reagent. Carboxypeptidase (Quiocio and Richards, 1964), catechol-1,2-dioxygenase (Neujahr, 1980) and penicillin amidase (Carleysmith et al., 1980) were cross-linked with glutaraldehyde. Examples of other multifunctional reagents are diazobenzidine,
hexamethylene diisocyanate, 1,5-difluoro-2,4-dinitrobenzene and trichloro-s-triazine. The cross-linking of enzymes depends on factors such as the concentration of enzyme and the cross-linking agent, pH and ionic strength of the solution, temperature and the time of reaction.

2.3.2 Physical adsorption

The physical adsorption method is the earliest and simplest immobilization method. Nelson and Griffin (1916) immobilized β-D-fructofuranosidase (invertase) by adsorption onto aluminium hydroxide. This method is based on the interactions of hydrogen bonds, Van der Waals forces or affinity binding between an enzyme and the support surface. The usual procedure consists of mixing the enzyme and support together for a period of time under appropriate conditions, followed by separation and washing to remove unadsorbed materials. Adsorption is highly dependent on pH, solvent nature, ionic strength, quantity of enzyme and adsorbent, temperature and time for interaction (Mclaren, 1954). Trypsin, α-chymotrypsin (Nemat and Karimian, 1986) tyrosinase and glucose oxidase have been immobilized on alumina by this method.

Advantages of this method include simplicity in coupling procedure, the large choice of differently charged and shaped supports available, little or no overall enzyme inactivation and its reversibility by permitting reuse of both the support and enzyme.
2.3.3 Ionic binding

Ionic binding involves formation of salt-like linkages between the enzymes charged groups and the opposite charges on the carrier which is an ionic exchanger. Immobilization is similar to the adsorption process and share similar pros and cons, although the resultant attachment is usually stronger than by adsorption. The carriers are classified into anion or cation exchangers respectively based on the properties of exchanging the anions ( Cl, OH etc ) of the carrier with anions of the solution ( enzyme ) and the cation ( H+, Na+ etc ) with cations of the solution. DEAE-cellulose, AE-cellulose, DEAE-Sephadex, amberlite, CM-cellulose, etc are used commonly for immobilization of enzymes by ionic adsorption.

Pencillin amidase on CM-cellulose ( Carleysmith et al., 1980 ), glucose oxidase on amberlite IRA ( Klei et al., 1978 ), glucoamylase on DEAE-cellulose ( Maeda et al., 1979 ) and urease on vermiculite ( Tarafdar and Chhonkar, 1982 ) have been immobilized by the ionic binding method.

2.3.4 Metal linking

This method is based on the chelating properties of the transition metals. Mainly titanium and zirconium ( Zr ) metal salts are used to activate the surface of a support. Glucoamylase on alumina ( Allen et al., 1979 ), controlled pore glass ( Cabral et al.,
Covalent binding

Covalent coupling for the immobilization of enzymes is based on the formation of a covalent bond between the enzyme molecules and carrier material. The protein functional groups that commonly take part in the covalent binding of the enzyme to the support (Paul and Kosaku, 1976) are as listed on Table 2.4 in the next page.

Table 2.4: Reactive residues of proteins (Mosbach, 1987)

<table>
<thead>
<tr>
<th>Residue</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>-NH₂</td>
<td>Amino groups of lysine and N-terminal amino acids</td>
</tr>
<tr>
<td>-SH</td>
<td>Sulphydryl group of cysteine</td>
</tr>
<tr>
<td>-COOH</td>
<td>Carboxyl groups of aspartate (Asp) and glutamate (Glu) and C-terminal carboxyl groups</td>
</tr>
<tr>
<td></td>
<td>Phenolic hydroxyl groups of tyrosine (Tyr)</td>
</tr>
<tr>
<td>-NH-C=NH NH₂</td>
<td>Guanidino groups of arginine (Arg)</td>
</tr>
<tr>
<td></td>
<td>Imidazole group of histidine (His)</td>
</tr>
<tr>
<td>-S-S-</td>
<td>Disulfide group of cysteine (Cys)</td>
</tr>
<tr>
<td></td>
<td>Indole group of tryptophan (Trp)</td>
</tr>
<tr>
<td>CH₃-S-</td>
<td>Thioether group of methionine (Met)</td>
</tr>
<tr>
<td>-CH₂OH</td>
<td>Hydroxyl group of serine (Ser) and threonine (Thr)</td>
</tr>
</tbody>
</table>
There are many established methods for covalent immobilization of enzymes (Rehm and Reed, 1987). Table 2.5 gives a summary of the variations for covalent bonding. The major classes of coupling reaction are listed in Table 2.6.

**Table 2.5: Variation in enzyme immobilization by covalent bonding** (Mosbach, 1987)

<table>
<thead>
<tr>
<th>Reactions of enzyme with:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Reactive polymer where no activation is involved (e.g. maleic anhydride polymers)</td>
</tr>
<tr>
<td>2</td>
<td>Polymer (where activation involves the chemical conversion of a functional group of the polymer followed by the addition of the enzyme)</td>
</tr>
<tr>
<td>3</td>
<td>Polymer (where activation involves the chemical conversion of a functional group of the polymer with a reagent in the presence of the enzyme, e.g. carbodiimide coupling with carboxyl-containing polymers)</td>
</tr>
<tr>
<td>4</td>
<td>Polymer (where activation involves the chemical conversion of a functional group of the polymer with a multifunctional, low molecular weight reagent followed by addition of the enzyme, e.g. glutaraldehyde modification of amino-containing polymers)</td>
</tr>
</tbody>
</table>
An immobilized enzyme may have properties different from that of the soluble free enzyme. Factors that may affect the properties of the immobilized enzyme can be support related or enzyme related as shown in Table 2.7.
Table 2.7: Factors that can affect the properties of immobilized enzymes (Mosbach, 1987)

<table>
<thead>
<tr>
<th>A : Support-related factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Diffusion layer around the immobilized enzyme particles</td>
</tr>
<tr>
<td>2. Steric repulsion of substrates</td>
</tr>
<tr>
<td>3. Molecular size of support</td>
</tr>
<tr>
<td>4. Flexibility of the support backbone</td>
</tr>
<tr>
<td>5. Degree of hydrophilicity or hydrophobicity</td>
</tr>
<tr>
<td>6. Macrostatic interactions</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B : Enzyme-related factors:</th>
</tr>
</thead>
<tbody>
<tr>
<td>7. Local and net charge</td>
</tr>
<tr>
<td>8. Conformational charges</td>
</tr>
<tr>
<td>9. Transformation of catalytically essential amino acid residues</td>
</tr>
</tbody>
</table>

2.3.6 Entrapment

The following gives an outline for the facilitation of enzyme entrapment.

Enzyme molecules are:

(i) entrapped within the interstitial space of cross-linked, insoluble polymer (gel entrapment),

(ii) entrapped within the microcavities of synthetic fibres (fibre entrapment) or

(iii) enclosed in a semi-permeable membrane (micro-encapsulation)
This method is attractive when the enzyme molecule is very large compared to the substrate, when the enzyme is susceptible to chemicals and deactivation and when strong interaction between the enzyme and the support is undesirable.

2.3.6.1 Gel entrapment

This method involves entrapping the enzyme within the interstitial spaces of a cross-linked water-insoluble gel. Polyacrylamide is the most commonly used polymer for immobilization of enzymes. The other polymers used are dimethyl acrylamide, polyvinyl alcohol, 2-hydroxy ethylacrylate, collagen, gelatin, agar, carrageenan and alginates.

Algin and alginates have been known for about 60 years in food and other industries because of their unique colloidal behavior and their ability to thicken, stabilize, emulsify, suspend, form films and produce gels (Cotrell and Kovocs, 1980). Alginate-type systems may be used as safe, non-toxic, readily available media or transporting agents which are suitable even for the administration of drugs. Several methods have been developed for microencapsulating biologically active molecules, tissue and cells so that they remain viable within a protected semi-permeable membrane which permits the passage the low molecular weight substances but not cells and high molecular proteins (Sefton and Broghton, 1982). A biocompatible and durable alginate–poly–L-Lysine (PLL) in the form of a capsule membrane was developed by Geraldine et al (1984) for the treatment of diabetes.
Alginates were probably the first polymers utilized for entrapment of biocatalysts by ionotropic gel formation or polymerization of polyelectrolytes by multivalent ions. Alginate came to be one of the most favored ones for immobilization of plant cells (Hulst and Tramper, 1989). This material became more and more frequently used also in enzyme immobilization. Immobilization in alginate gels is safe, fast, mild, simple, cheap and a versatile technique which may be applied with a wide range of biocatalysts, (Birnbaum et al., 1983; Bucke, 1987). Alginate gels are commercially advantageous because of its minimum cost of alginate, which is sold by weight (Bucke, 1987).

However, this mild simple and rapid technique has some disadvantages. Alginate gels are destroyed by high concentrations of K\(^+\) and Mg\(^{2+}\) ions, phosphate and chelating agents. The beads also have a tendency to swell in the presence of monovalent cations. Limitation of oxygen transfer within the gels may be a problem or an advantage depending on the type of biocatalyst entrapped.

Entrapment using alginate gel, however, is inapplicable to most enzymes since the pore size of the gel is so large that enzymes leak out the gel network (Tanaka et al., 1984 and Bucke, 1987).
2.3.7 Ultra filtration membrane and hollow fibre devices

These methods of immobilization usually allow the enzyme to operate in its native state continuously over a long period. The procedure is simple and best suited for high molecular weight substrates. Hollow fibre devices offer an extremely large surface area (Gerhartz, 1990). However, enzyme inactivation is possible due to vigorous agitation and high shear forces. Leakage can occur and fine control of residence time is required. Mass transfer resistance can also occur.

2.4 Effects of immobilization on the properties of enzymes

Immobilization may alter the properties of the immobilized enzymes by the following effects:

(1) Conformational

- Changes in conformation of the enzyme due to
  (i) modification of amino acid residues at the active sites
  (ii) changes in the protein conformation of the enzyme
  (iii) changes in the charges of the enzyme

(2) Steric

- Steric hindrance due to interaction between the enzyme and the substrate

(3) Partitioning

- Due to the chemical nature of the support matrix, the support may interact (e.g. hydrophilic or electrostatically) with low molecular weight species

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in solution and create a modified microenvironment different from the bulk.

(4) Mass transfer of diffusional - Substrates (or products) may encounter diffusional their transport to (from) the active sites of the immobilized enzymes from (to) the bulk solution.

Observed changes in the kinetics and properties of the immobilized enzyme will depend on the resultant effects from these interactions of these factors.

2.5 Studies on immobilization of laccase

Immobilization of laccase with various supports, the different coupling techniques and its effect of the immobilized enzymes compared to the free were studied and discussed.

Laccase from *Neurospora crassa* was immobilized by two different techniques by Froehner (1975). The first was by covalent attachment to sepharose 4B activated with cyanogen bromide. The second method was by adsorption to concanavalin A-sepharose via the carbohydrate moiety. Results showed that the immobilization procedure utilizing CNBr-activated sepharose yields about 60% of the initial laccase activity. By the concanavalin A-sepharose procedure an activity of nearly 100% were obtained. Both immobilized preparations could be stored for at least several months without detectable loss of activity. No activity could be detected in supernatant indicating that leakage of
the enzyme did not occur. The free enzyme and the immobilized enzymes were active over a wide pH range and no apparent differences.

In another interesting study by Leonowicz et al. (1988), laccase of the basidiomycete, *Trametes versicolor* was immobilized onto porous glass beads that were activated with 3-aminopropyltri-ethoxysilane and glutaraldehyde. Results showed that 90% of the enzyme activity was retained after immobilization. The immobilized enzyme was active in a wider pH and temperature range. At 80°C, the immobilized enzyme was highly active by maintaining 90% of original activity whereas the free enzyme showed only 35% of original activity. The resistance of the enzyme to high temperatures was greatly improved by immobilization. Weetall (1970), reported that enzymes immobilized on glass showed in general increased thermal stability compared to enzymes immobilized on organic carriers. Messing and Weetall (1970) found that porous glass was a superior support for enzyme immobilization because of its structural stability and large surface area.

The work by Davis and Burn (1990), involved the entrapment of laccase in alginate beads. This improved decolourization by a factor of 3.5 times for cotton mill effluent. However, this method was not suitable for continuous use because the enzymes were rapidly released into solution. Co-polymerization of laccase with L-tyrosine increased the efficiency of decolourization of pulp mill effluent by 28 times.
Laccase was covalently immobilized onto activated carbon by Davis and Burns in 1992. Their findings showed that the immobilized enzyme was stable at pH values from 4.0 to 9.0. The immobilized enzyme at 4°C lost 38% activity in the first 4 days and then a further 22% activity in 126 days. It showed increased stability to low pH although the pH optimum was not changed. Carbon immobilized laccase (Davis and Burns, 1992) removed colour from pulp mill effluent at 115 colour units per enzyme unit per hour and the removal rate increased with increasing effluent concentration. Activated carbon can be easily derivatised to produce many different functional groups like the amino, carboxyl and hydroxy group of phenol. It was also reported to have good mechanical strength, readily available and has been already used in the food and waste industries.

Laccase from *Coriolus hirsutus* was bound to threadlike DEAE-cellulose (Ghindilis *et al.*, 1992). It was clearly seen that the enzyme became more stable upon immobilization. The immobilized enzyme retained almost all of its activity for one month, whereas the activity of the free enzyme decreased by more than 70% after being stored for the same period.

In a different study by Brenna and Bianchi (1994), laccase from a mutant strain of *Trametes versicolor* was coupled with agarose based activated matrixes (Affi Gel-10 and 15). The matrixes were activated by reaction with 2% polyethylenemine and then treated with glutaraldehyde. NaBH₄ was also added to stabilize immobilized enzyme derivative. The immobilized enzyme was found to be stable with negligible loss in
activity for a period of six months. The immobilized enzyme showed a similar pH profile to that of the free enzyme. Affi-Gel 15 was also used by Reyes et al. (1999) to immobilize laccase from Coriolopsis gallica UAMH8260. This laccase immobilized on agarose is stable for several months when not in use. It retained 70% of initial activity after being used for a total of 21 hours for three months. This result is in contrast to the findings by Davis and Burns (1992) which showed on exponential decrease in activity by the laccase from Coriolus versicolor immobilized on activated carbon. However, enzyme immobilization did not change significantly the laccase activity profile at different temperatures.

Laccase from Trametes versicolor was immobilized by the adsorption method to various supports such as glass, glass powder, silica gel and Nylon 66 membrane (Ruiz et al., 2000). The immobilized enzyme activity and stability was determined in diethylether, ethylacetate and methylene chloride. The best results for the oxidation of syringaldazine was obtained with Nylon 66 membrane. Activity of immobilized laccase was stable for more than 72 hours in diethylether and ethylacetate, while exposure to methylene chloride resulted in a decrease of activity regardless of the support material.