DEVELOPMENT OF CROSS-LINKED ENZYME CRYSTALS AS ROBUST BIOCATALYSTS IN ORGANIC MEDIA

THESIS SUBMITTED TO THE
UNIVERSITY OF KERALA
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN BIOTECHNOLOGY UNDER THE FACULTY OF APPLIED SCIENCE

BY

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UNDER THE SUPERVISION OF

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DECEMBER 2004

DECLARATION

I, Mr.J.Jegan Roy do hereby declare that the matter embodied in this thesis entitled "**Development of Cross-linked Enzyme Crystals as robust biocatalysts in organic media**" is a bonafide record of the investigation carried out by me in the Chemical Science Division of Regional Research Laboratory, Trivandrum, under the guidance of Dr. T. Emilia Abraham and no part of this thesis has been submitted elsewhere for the award of any other degree or diploma.

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CERTIFICATE

Certified that the work embodied in this thesis entitled "**Development of Cross-linked Enzyme Crystals as robust biocatalysts in organic media**" is a record of bonafide research carried out by Mr.J.Jegan Roy under my supervision in fulfilment of the requirements for the degree of Doctor of Philosophy in Biotechnology of University of Kerala and the same has not been submitted elsewhere for any other degree or diploma

Dated
16-12-2004

Dr. T. Emilia Abraham
(Supervising Guide)

ACKNOWLEDGEMENTS

I express my gratitude to my research guide Dr (Mrs) T.Emilia Abraham for allowing me to choose my research problem cross-linked enzyme crystal technology and for giving me absolute freedom. Her excellent guidance and thoughtful suggestions were of inestimable value towards the over-all improvement of my research work.

I would like to acknowledge the present and former directors of RRL-T for providing all the necessary facilities for carrying out my doctoral programme.

I am extremely grateful to Dr.M.S.Thakur and Prof. N.G.Karanth of CFTRI, Mysore for allowing me to do the biosensor work in their Lab.

I am thankful to Dr.C.K.S.Pillai, for his advice during the period of my assessment.

I wish to acknowledge Prof.Peter Koshy and Dr. Prabhakara Rao, Scientist, RRL for the SEM photographs of the enzyme samples.

I wish to acknowledge Dr.Rukmini Sugumaran and Mr.Prakash, RRL for the surface area and porosity measurement of the crystal samples.

My heartfelt thanks are due to Dr.L.V.Bindhu, Postdoc Fellow, Nanyang Technical University Singapore for her help and support given to me during the period of my research.

I would like to thank my colleagues Mrs. Nisha, Miss. Sindhu, Mrs.Akhila Miss.sangeetha, Mrs.Meera and Mr.Venu for their help during my research work.

I would like to thank my dear friends Mr.Raja Singh, Mr.Prem, Mr.Ravi, Mr.Nithi, Mr.Prabhu and Dr.T.P.D.Rajan for their help during the period my research work and thesis writing.

I wish to express my appreciation to the staff members of RRL-T for the immense help and co-operation extended to me during my tenure at RRL.

Financial assistance from the Council of scientific and industrial research in the form of research fellowship is gratefully acknowledged.

I owe much to my parents and sisters who have supported and encouraged me throughout my academic carrier.

Above all I am extremely obliged to the Almighty for all his blessings

J.Jegan Roy

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ABBREVIATIONS

Å	Angstrom
aa	amino acid
ABTS	2,2'-Azinobis (3- ethylbenzthiazoline-sulfonic acid)
AOT	Aerosol OT
CD	Catalytic domain
CLEC	Cross-linked enzyme *crystals*
DMSO	Dimethyl sulfoxide
mg	milligram
ml	millilitre
mM	millimolar
nm	nanometre
o-	ortho
p-	para
PBR	Packed Bed Reactor
PEG	Polyethylene glycol
PVP	Polyvinyl propilidine
SBD	Starch binding domain
SEM	Scanning Electron Micrograph
THF	Tetrahydrofuran
v/v	volume/volume
w/v	weight/volume
μg	microgram
μl	microlitre

PREFACE

Cross-linked enzyme crystal (CLEC) technology is one of the most exciting developments in the area of biocatalysis. Cross-linking of enzyme crystals brings about both stabilization and immobilization of enzyme without dilution of the activity. CLECs have special advantages over soluble or conventionally immobilized enzymes for bioprocessing. They are heterogeneous catalysts and can be readily isolated, recycled and reused many times. CLECs can withstand the shear forces associated with processing equipment and pumps, all of which cause particle attrition and fragmentation. They have high stability at elevated temperature and in the presence of neat organic solvents. The present study explains, for the first time, the rational development of cross-linked enzyme crystals of laccase & glucoamylase and their applications.

The thesis consists of six chapters. The first Chapter presents a brief over view of the recent developments in the field of cross-linked enzyme crystals. The various strategies in crystallization and cross-linking and how to improve the enzyme stability in organic solvents have been explained.

The Second chapter deals with the preparation and properties of cross-linked crystals of laccase enzyme (EC 1.10.3.2), which belongs to the family of multi copper oxidases. An introduction to its general structure, catalytic mechanism, and its application is given. Standardization of conditions for crystallization & cross-linking of laccase, and the resultant crystal structure, crystal size, thermal stability, pH stability, solvent stability were explained in this chapter. The Cross-linked enzyme crystals of laccase was found to have 4 times improved thermal stability and a half-life of 123 minutes at 60°C as against 24 minute for the native enzyme.

Comparison of the reaction kinetics of CLEC laccase with native enzyme is explained in the chapter three. Substrates like ABTS, catechol, pyrogallol, guaiacol, catechin and syringaldazine were used for the kinetic studies. ABTS was found to be the best substrate for both CLEC and native laccase with low Km values of 0.8595 and 0.1412 respectively. The catalytic efficiency (K_{cat}/K_m) for ABTS oxidation of native and CLEC of laccase was found to be 1.673×10^5 and 3.73×10^3 respectively.

The application of CLEC laccase in a biosensor for the amperometric determination of phenolic compounds was explained in the chapter four. The

concentrations of Catechol, guaiacol, pyrogallol, catechin, and ferulic acid in aqueous solution were determined by using this amperometic biosensor. The biosensor could be used for the detection of antioxidants like ferulic acid & pyrogallol in food and beverages and the presence of polyphenols like catechol & catechins, which is a quality index of teal eaves. The sensor could also be used to monitor organic pollutants like 2-amino phenol, in wastewater at varying concentrations (50-1000 µmol)

The application of CLEC laccase for the production of Purpurogallin is explained in Chapter five. Purpurogallin originally isolated from *Quercus nutgall*. It has antioxidant activity and it is an inhibitor of epidermal growth factor receptor protein tyrosine kinase activity and inhibitor of xanthane oxidase. Pyrogallol was biotransformed into purpurogallin in a Packed Bed Reactor at 28°±2C, at different residence time and substrate concentrations. 76% conversion was obtained at the residence time of 7.1 sec, when 3 mM pyrogallol was used as the substrate. The highest Purpurogallin production had occurred at the residence time of 3.5 sec, when 5 mM pyrogallol was used as the substrate. The CLEC laccase had good reactor stability and maintained the same activity even after 650 min of continuous reactor operation.

The sixth chapter deals with the preparation and properties of CLEC glucoamylase (EC 3.2.1.3), an industrially useful starch-hydrolyzing enzyme. Crystallization and cross-linking conditions of glucoamylase were standardized and reaction kinetic was studied. CLEC glucoamylase had a better thermo stability above 60°C unlike the immobilized glucoamylase. Continuous production of glucose from 10% (v/v) starch and maltodextrin (12.5 DE) using CLEC glucoamylase in a Packed Bed Reactor 60°C gave a productivity of 110.58 g/L/h at a residence time of 7.6 min and 714.10 g/L/h at a residence time of 3.4 min, respectively. The CLEC glucoamylase had a half-life of 10 hours under the reactor conditions.

Development of a robust biocatalyst of an oxidoreductase enzyme like Laccase and a hydrolytic enzyme like glucoamylase, which has diverse industrial uses, has been accomplished.

CHAPTER I

INTRODUCTION

1 Introduction

Enzymes are recognized as useful tools for accomplishing industrially important chemical reactions in stereo-, regio- and chemoselective ways. Biocatalytic routes that have emerged over the last few decades offer the promise of radically altering chemical manufacturing processes. The use of enzymes as catalyst in industrial scale synthesis of specialty chemicals and pharmaceuticals has received much attention in the recent years (Aleksey Zaks, 2001). In these purposes, enzymes have to be used in organic solvent environments for extended periods.

1.1 Features of enzymes and their classification

- Enzymes catalyze the reactions under mild condition like neutral pH and temperature (20-40 °C).
- Enzymes increase the reactions up to 10^7 fold faster rates than a chemical catalyst.
- Enzymes are stereo, regio and chemo selective catalyst. So there is no unwanted side product formation.

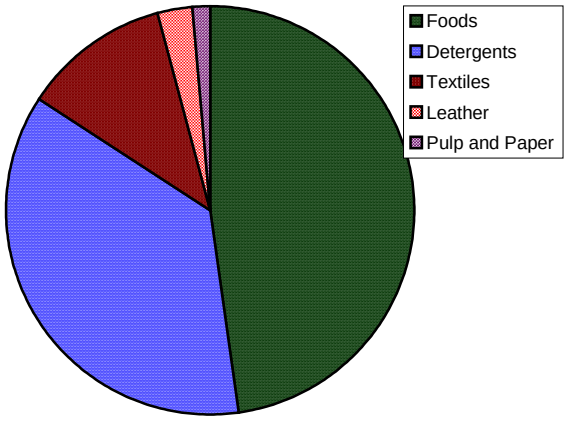
Enzymes are classified into six groups

1. Oxidoreductases : Enzymes of this group catalyze oxidation reactions involving oxygenations such as C- H to C- OH, or overall removal or addition of hydrogen atom equivalents as for CH (OH) to C=O .
2. Transferases : These enzymes mediate the transfer of groups like acyl, sugar, phosphoryl and aldehyde or ketone moieties from one molecule to another.
3. Hydrolases : The range of functional groups hydrolysed by this group includes glycosides, anhydrides and esters as well as amides, peptides and other C=N containing functions.
4. Lyases : These enzymes catalyse additions usually of HX to double bonds such as C=C, C=N and C=O and the reverse processes.
5. Isomerases : Various isomerisations, including C=C bond migration, cis/trans isomerisation, and racemisation can be effected.
6. Ligases : These are often synthetases and mediate the formation of C-O, C-S, C-N, C-C and phosphate –ester bonds.

1.1.2 Enzyme Market

Enzyme market is worth $2 billion per annum (www.biotechsupportindia.com), with annual growth rate of 3- 5 %. Stunning progress in the engineering of enzymes using advanced techniques of biotechnology continues to open new markets, with enzymes being increasingly tailored for specific applications. Properties such as stability, activity at high temperature, wide range of substrates, greater reaction speed and wider pH range are often at the top list of properties desired by industries Food market will continue to be largest for enzymes, followed by detergent enzymes.

Figure 1.1 World Enzyme Market



Novo Nordisk and Genencor share 44% and 22% of world enzyme market. The other important enzyme companies are DSM, Danisco Cultor, AB enzymes and Amano. Carbohydrases will remain the single largest type of enzymes due to their dominance in the large corn milling market. Lipases and proteases penetrate the detergent market and is receiving strong interest for chemical synthesis such as chiral resolution and peptide synthesis. Enzymes such as lipases are used in enzyme replacement therapies such as glucocerebrosidase. Prospects for the agriculture market vary, with demand for phytase and other animal feed enzymes. Enzymes like amylase are used in starch industries for the conversion of starch to glucose syrup. Laccase enzyme is widely used in textile industries for denim finishing.

Indian enzyme market is estimated to be Rs. 1600 million in the year 2003-04. The consumption of enzymes in India is predominantly in the detergents market (32%) followed

by starch market (15%). In comparison with the world market, the use of enzymes in India is rather limited. Moreover, 70% of domestic demand is imported. However, Indian enzyme market is growing, because of increased awareness of this eco-friendly processes. Indian players include Biocon India, EPIC enzymes, ABL limited etc.

1.1.3 Enzyme stabilization for Industrial use

Industry needs enzymes for extended periods of use in organic solvent environments for the production of fine chemicals like pharmaceuticals, agrochemicals, fragrances and flavors; food additives and consumer care products. One of the major challenges in the biocatalytic processes is to improve the activity and stability of the enzymes. Biomolecular engineering (Ryu and Nam, 2000) techniques are nowadays employed to enhance the useful properties of the enzymes like pH stability, thermal stability and increased activity etc. Direct evolution (Bornscheuer and Pohl, 2001) approach is favored for many industrial enzymes owing to the difficulties of relating the desired applications with required properties. The direct evolution, called molecular evolution through DNA shuffling, involves preparation of protein variants by recombining gene fragments by in vitro methods. Other techniques include changing a single or few amino acid residues (Cedrone et al., 2000), either by exchanging functional domain, or by introducing a small protein fragment scaffold enzyme. Even though the properties of the enzymes can be improved by any of these methods, the recycling of the enzyme is important for continuous process for a high productivity.

Immobilization circumvents this problem and, in addition, improves the economy of the processes by the reuse of the biocatalyst. However, enzyme immobilization is costly and requires an inert matrix on to which the enzyme can be immobilized and many a time the matrix should be chemically modified to couple the enzyme. The interaction between the enzyme and the matrix dilutes the effective concentration of the enzymes. The activity loading (Tisher and Kasche, 1998) of the enzyme on the support is usually 0.1% to 10% w/w. Hence, the immobilization has only partially solved the problem of low activity of enzymes for biotransformations in non-aqueous medium. Cross-linked enzyme technology (CLEC) provides a unique approach to solve the above disadvantages of immobilization. Table 1.1 provides the comparison of the properties of soluble enzyme, immobilized enzyme

and CLEC. Cross-linking of enzymes results in both stabilization and immobilization of the enzyme without dilution of activity (Haring and Schreier, 1999).

Table 1.1 Comparison of the characteristic features of soluble, immobilized enzymes and CLEC

Character	Soluble Enzyme	Immobilized Enzyme	Cross-linked Enzyme Crystal
Enzyme purity	Enzyme of any purity	Even crude enzyme can be immobilized	Only pure enzymes can be used
Stability	Can be stored at concentrated form with stabilizers at refrigerated temperature as a powder or solution	Store at refrigerated temperature in wet condition	Higher stability due to cross-linking. Can be stored at room temperature as a powder
Specific activity	High Specific activity	Dilutes the activity due to the interaction with the matrix.	High specific activity due to high volumetric activity
Reaction in Aq/Org media	Only in aqueous media, inactivated in organic media	React in both aqueous and less in organic media	React in both aqueous and organic media
Separation from the reaction mixture	Difficult to separate from reaction mixture	Can be separated by filtration or centrifugation	Easily separated by Filtration or centrifugation
pH and thermal stability	Not stable over a range of pH and temperature	Not stable over a range of pH and temperature	Stable over a range of pH and temperature.
Productivity	Low productivity	High productivity	Very high productivity

1.2 Features of cross-linked enzyme crystals for bioprocessing

CLECs are chemically cross-linked enzyme crystals having special advantages over soluble or conventionally immobilized enzymes for bioprocessing.

Compared to immobilized enzymes or soluble enzymes, CLECs have a higher activity per unit volume. The enzyme concentrations within a CLEC are close to theoretical limits. CLEC particles are uniform and microporus (Vilenchik et al., 1998), in contrast to soluble enzymes, and remain monodisperse on reconstitution, even in organic solvents. Owing to their insoluble nature in both organic and aqueous media, separation from product is easy by settling or filtration, thereby eliminating a chance for contamination.

CLECs can withstand the shear forces associated with processing equipment such as stirred tanks, cross-flow micro filters, and pumps, all of which cause particle attrition and fragmentation. CLECs are robust biocatalysts, and extended agitation in suspension at high mixing speeds using either a turbine (high shear) or a propeller blade (moderate shear) does not lead to particle breakage (Lee et al., 2002).

CLECs are heterogeneous catalysts and can readily be isolated, recycled, and reused many times. The high operational stability allows reactions at higher temperatures and in aqueous organic solvent mixtures or neat organics, thus increasing the substrate solubility. The ability to withstand proteolysis and autolysis makes possible the use of high concentrations of this modified enzyme in hydrolytic reactions. The intermolecular contacts and cross-links between enzymes in the crystal lattice of a CLEC stabilize the enzyme and prevent denaturation. CLECs can easily be freeze-dried or air-dried, and in that form can be stored indefinitely at room temperature (Margolin, 1996). Long shelf life solves storage problems and eases handling of enzymes as ordinary chemicals (St.Clair, and Navia, 1992). CLECs give improved yield under harsh conditions or in situations requiring high throughput, and enable process chemists to concentrate on maximizing yields.

CLEC catalysts are basically purified enzymes. Pure enzymes have maximal selectivity, but are costly and have low stability, so crude enzyme preparations are used commercially even though they normally contain cell debris, nucleic acids, inactive proteins, and pigments. Such catalyst cocktails may sometimes catalyze competing reactions, interfere with purification of the final product, and thus make the process difficult and expensive. Contamination of the reaction mixtures by proteins and the products of protein

self-digestion is a serious problem in the synthesis of peptides or other pharmaceuticals since such contaminants can cause anaphylactic shock. Thus in these applications, a thorough purification of the product is required. Cross-linked enzyme crystals eliminate these problems since they are pure, heterogeneous, and free of unwanted protein contaminants.

CLECs have high specific activity. The entire volume of a CLEC consists of an active catalyst with no inert carrier as opposed to the situation for immobilized enzymes. Enzyme concentrations within the crystal approach the theoretical packing limit (Timpson and Wasserthal, 1998) for molecules of a given size. Thus, the volumetric activity of CLECs is two to four orders of magnitude higher than that of both conventional and immobilized enzymes, which reduces both the reaction time and the volume of enzyme required, thereby maximizing the volumetric productivity.

CLECs are environmentally benign and easy to dispose of compared to traditional chemical catalysts (precious metals), resolving agents or coupling agents. The cost savings from simplified product work-up and catalyst disposal procedures alone can be very significant. In a batch configuration CLECs can be recycled and used may times, and in column configuration they can be used for a long time. Thus, this technology is cost-effective. The increased selectivity of CLECs as well as their high stability at elevated temperature and in the presence of organic solvents significantly broadens the synthetic potential of the enzyme catalysts. Many biotransformation processes that chemists could not even contemplate before are now possible with CLECs.

CLECs do not require expensive supports or complex immobilization procedures, and they exhibit the highest possible protein density. The high specific activity means a CLEC will generate the largest possible signal (Navia and St.Clair, 1999(b)) from even the smallest substrate (analyte) concentration. Proteins in crystal form are uniformly arranged, which produces a linear and predictable signal.

Soluble enzymes are vulnerable to proteolysis as well as contamination in many biosensor environments. CLECs are very stable towards degradation by protease since both the protein-protein interactions necessary for this process are hindered, and also access of the protease to the crystal lattice is limited.

1.3 History of the development of Cross-linked enzyme crystals

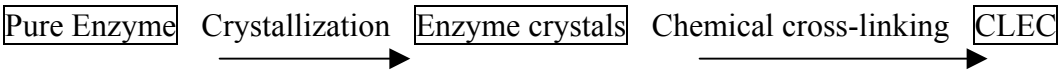
The first reported preparation of cross-linked enzyme crystals was by Quiocho and Richards in 1964. They prepared crystals of carboxypeptidase-A and cross-linked them with glutaraldehyde. However, the CLEC they prepared retained only 5% of the original activity and showed a measurable increase in mechanical stability. The authors quite correctly observed, "cross-linked enzyme crystals, particularly ones of small size where the diffusion problem is not serious, may be useful as reagents which can be removed by sedimentation and filtration. Two years later, the same authors reported that only the lysine residues of carboxypeptidase-A enzyme were modified by glutaraldehyde cross-linking.

The kinetic properties of CLECs of the protease substilin were reported by Tuchsen and Ottesen in 1977. They reported that CLECs of substilin were highly effective catalysts with increased thermal stability and increased stability toward acid compared to the soluble enzyme and showed essentially no auto digestion at 30° C. After 8 years 1985, a group at the Louis Pasteur University in Strasbourg, France, prepared cross-linked crystals of horse liver alcohol dehydrogenase (Lee et al, 1986). The CLEC could be used as redox catalysts with no addition of coenzyme and having increased stability towards organic solvents.

Up to this time, CLECs had been prepared largely for academic interest, until Navia and St.Clair (1992) reported for the first time the commercial development of CLECs having combined high activity with high stability. In 1992-93 the use of cross-linked enzyme crystals in biocatalysis started when Altus Biologics was formed to commercialize the technology developed by Navia et al. Altus has obtained very broad patent coverage for its technology. Today many CLEC forms exclusively from Altus Biologics under the brand names, ChiroCLEC^{TM}, PeptiCLEC^{TM} and Syntha CLEC^{TM}.

1.4 Preparation of Cross-Linked Enzyme Crystals

CLECs are prepared stepwise, with the first step being controlled precipitation of enzymes into micro crystals. The second step is cross-linking with bifunctional agents to form strong covalent intra- and intermolecular bonds between ε-amino group of lysine residues (Santis and Jones, 1999) or by cross-linking between carbohydrate moieties (Margolin et al., 2002) of the enzymes. Inexpensive chemical cross-linking locks the enzyme in the crystalline form, thus enhancing both thermal and proteolytic stability as well as insolubility in both aqueous and organic solvents.



1.4.1 Enzyme Crystallization

Crystallization is a powerful tool for the purification, isolation and long-term storage of enzymes (Dounce and Allen, 1988). The effectiveness of crystallization for purification is usually greater than that of any other method. In general, enzymes are much more stable in crystalline form than in soluble or amorphous form. In addition, crystallized enzymes are more concentrated and are purer. Crystallization of macromolecules requires the creation of a supersaturated state. This is a non-equilibrium condition in which some quantity above the solubility limit of the macromolecules should be present in solution. Crystallization strategies for enzymes have been extensively reviewed in research papers (Mcpherson, 1990 & Enrico et al., 1994 & Feher, 1986 & Wiencek, 1999 & Rosenberger, 1996 & Mcpherson et al., 1995) and books (Ducruix and Giege, 1992 & Mcpherson, 1999). Crystallization is an important step in CLEC preparation because the quality of crystal determines the stability of the cross-linked enzyme since the enzyme crystal itself acts as its own support (Margolin, 1998). Crystallization of enzymes is achieved by adjusting the rate of solvent evaporation, pH, and temperature, and by manipulation of protein and precipitant concentration. During the preparation of a CLEC, the crystallization conditions should be optimized to get CLECs of size appropriate to the intended applications. The size of the crystals (Tiller, 1986 and Johns, 1999) can be changed by controlling the kinetics of crystallization. Crystals of 50-150 μm size are preferable for biocatalysis applications since they offer a combination of good filtration properties and activity. Particle size of around 10μm is preferred for biosensor and cosmetic applications (Govarthan, 1999). In these cases, it is crucial to define a set of crystallization conditions that are amenable to scale up, can tolerate some variation of input, provide a high yield of crystals with uniform size and shape and have minimum loss of functional activity (Boistelle and Astier, 1988).

1.4.1.1 Standardization and screening of crystallization process

When attempting the crystallization of macromolecules, first find some set of initial conditions, which yield crystals, even if they are too small or misshapen. Then, using those conditions as a starting point, optimize crystal growth by varying all the parameters, such as precipitant type (inorganic salts organic solvents, or polymers such as PEG of different

molecular weight), pH, temperatures and buffer system. It is also important to determine the solubility properties, and to screen additives and other variables, which affect the crystallization process such as temperature (Christopher, 1998 & Durban and Feher, 1996), pH (Zeppezauer, 1971) and enzyme concentration (Bob et al., 1994 & Stoscheck, 1990). Mutant proteins may crystallize under very similar conditions with only minor changes in precipitant concentrations and pH, which is needed to compensate for changes in solubility and charge. There are several techniques for the screening process of crystallization experiments such as vapor diffusion, batch crystallization, microdialysis, and microbatch under oil. Once screening is completed, further optimization is required, which is amenable to the tried and true statistical method (Carter, 1997)

1.4.1.1.1 Protein precipitation

So many precipitating agents are currently used for enzyme crystallization. The commonly used precipitants are inorganic salts, organic solvents and polymers (PEG)

a) Inorganic salts

The commonly used salts are $(NH_4)_2SO_4$, Na_2SO_4, Ammonium formate, ammonium phosphate, lithium sulfate, magnesium chloride, calcium chloride, Sodium Citrate, Sodium chloride, etc (Dixon and Webb,1961 & Ries and Ducruix, 1989). These salts steals water molecule from the surface of the enzyme molecules. The distribution of hydrophilic and hydrophobic residues at the surface of the enzyme molecules is the feature that determines solubility in various solvents. Hydrophobic groups on the surface of the protein molecules are responsible for the “Salting out”of enzymes. Salting out which is a global dehydration effect, or lowering of the chemical activity of water, there are also specific protein-ion interactions that may have important consequences. Salting out never precipitates the entire enzyme, but just reduces the solubility. Neutral salts are always used for precipitation. 15-80 % of neutral salt with the increment of 5% is used for crystallization of enzymes.

b) Organic Solvents

The most popular volatile organic solvents ethanol, acetone, isopropanol, tert.butanol, 1, 3- propanediol, DMSO, methanol and non-volatile alcohols MPD (2 methyl 2, 4 pentane diol) and 1, 6 hexanediol are used in enzyme crystallization (Timasheff and Arakawa, 1988 & Davey, 1986). Generally MPD and 1,6 hexanediol are used. These organic solvents act as precipitant by lowering the chemical activity of water. This means

that these solvents steel water molecules from enzymes in solution through hydrogen bonding. This is turn reduces the dielectric constant of the solution. This reduction in dielectric constant causes enhancement in electrostatic interactions between protein molecules and an augmentation of the electric attraction between opposite charge on the surface of enzyme molecules and hence to a reduction in solubility. But the organic solvents should be used at low temperature (4°C or lower).

c) Polymers

The commonly used polymers (Patel and Mcpherson, 1995 & Mcpherson, 1985) for protein crystallization is poly (acrylic acid) poly ethylene glycol (PEG), poly propylene glycol, poly vinyl alcohol (Mol.wt 15,000), poly (vinyl pyrolidine) (Ducruix and Giege, 1992). PEG is the water soluble polymer and the distinct advantage of poly ethylene glycol over other reagents is that most of the proteins (but not all) crystallize within a fairly narrow range of poly ethylene glycol concentration, this being about 4-18% w/v. The time required for crystal growth with PEG as the precipitant is also generally shorter than with ammonium sulfate or MPD but occasionally longer than required by volatile organic solvents such as ethanol etc. All of the poly ethylene glycol having mol.wt from 400 to 20000 has provided crystals. PEGs of molecular weight 4000 to 6000 seems to be very effective for most protein systems (Mcpherson, 1990). Since larger molecular mass PEG probably does not even enter the crystals and therefore do not directly contact the interior molecules. PEG will also hydrate as well as alter the dielectric constant of the solvent. However, the volume exclusion effect must account for its effectiveness especially for the high molecular weight PEG. Poly ethylene glycol is useful over the entire pH range and over a broad temperature range and shows no anomalous effects. 3 to 18 % of PEG with increment of 3% is suitable for the screening of protein crystallization. PEG is also preferred to ammonium sulphate or other salts because certain heavy atoms are insoluble under high salt conditions (Johns, 1999).

1.4.1.1.2 Variables affecting enzyme crystal growth

In all the crystallization process, the production of protein crystals requires to bring the protein into a supersaturated liquid state. Crystallization requires the gradual creation of a supersaturated solution of macromolecule followed by spontaneous formation of crystal growth centers or nuclei. The degree of supersaturation determines the nucleation as well as the crystal growth rate. The variables affect the solubility of the enzyme, which can also be

used to control supersaturation in the system and thus indirectly influences the rates of nucleation and growth (Boistelle and Astier, 1988). The major variables affecting protein crystal growth are temperature, pH, and concentration of enzyme solution, concentration of precipitants.

a) Effect of temperature on Enzyme crystal growth

Temperature induced crystallization (Christopher et al., 1998 & Durban and Feher, 1996) is generally a useful technique which influences nucleation and crystal growth by changing the solubility and supersaturation of the sample. Controlled manipulation of temperature during the screening and optimization of production of crystals is a prerequisite for successful and reproducible crystal growth of the enzyme with temperature dependent solubility. Temperature is amenable to control, thereby allowing precise control approach to nucleation and post nucleation crystal growth. According to classical thermodynamics, the Van't Hoff's equation can be written for the solubility of protein in solution by varying temperature. If the heat of crystallization is $\Delta Hcry$, and solubility of protein (C_1^*) and temperature are known. The solubility of unknown protein (C^*) at temperature T can be determined by

$\ln(C^*/C_1^*) = (\Delta Hcry/R)\ (1/T - 1/T_1)$ where R=universal gas constant (Boistelle and Astier,1998)

Temperature provides precise, quick and reversible control of super saturation of enzymes. Temperatures also affect the quantity, size and quality of crystal as well as sample solubility and preliminary crystal growth (Dixon and Webb,1961). Crystallization screens and experiments are performed at room temperature or at 4°C. Most proteins will denature at temperature above 40°C. So reasonable range of temperature to screen and optimize crystallization is from 4°C to 40°C. When incubations are done at room temperature, the temperature control and stability are often minimal. In an open room, temperature fluctuations may be significant, especially over 24 hours period and on weekends when thermostatic control of the room environment can fluctuate 10°C or more. Incubation at 4°C and other temperature are often more stable since the incubation is performed in some type of incubator. Controlled temperature is important for consistent results. Temperature fluctuations can be useful in obtaining high quality crystals by screening a larger range of

crystallization conditions. The temperature dependent solubility changes can be used for screening and optimization of protein crystallization. However, increasing temperature increases the disorder of the reagent molecule. In the crystallization, experiment the temperature can be manipulated, to improve the sample-sample as well as sample reagent and reagent-reagent interactions (Ries and Ducruix, 1989).

b) Effect of pH on enzyme crystal growth

The next most important variable in enzyme crystal growth is pH. Protein solubility will change dramatically as pH is altered by even 0.5 units. However, some systems are sensitive to pH changes as small as 0.1 units (Timasheff and Arakawa, 1988). The enzyme of interest will often dictate acceptable pH ranges for crystallization. Only pH condition that maintain the folded structure of protein are acceptable condition for protein crystal growth. Not only the net charge on the protein change with pH, but also the distribution of those charges, the dipole moment of the protein, its conformation influences crystallization. The protein solubility is minimal at the isoelectric point (pI) where its net charge is zero (Enrico et al, 1994). Some proteins are not stable near their pI value and hence pH may not be a significant variable in these cases. If refining the pH for optimal growth, it is important that the difference between the amorphous precipitate, microcrystals and large single crystals might be only difference in pH of less than 0.5. The preferred pH range for most protein Crystallization is pH between 3.5 to 9.0 with the increment of 0.5. (Mcpherson, 1990)

c) Effect of precipitant and enzyme concentration on crystal growth

The separation of protein molecules from a solution according to the methods based on variation of precipitant concentration at constant pH and temperature based on alteration of pH and temperature or some other variables at constant precipitant concentration (Davey, 1986). Crystallization may also be accomplished by increasing the concentration of precipitating agent to a point just below the super saturation and then adjusting the pH or temperature to reduce the solubility of protein. Another way of achieving super saturation of protein solutions is by the slow increase in concentration of the precipitants such as salts or poly (ethylene glycol). The organic solvent competes to some extent like salt for water molecules, but it reduces the dielectric capacity of the intervening solvent.

In very concentrated solutions, protein molecules may aggregate as an amorphous precipitate. The aggregates may be random in nature and lead to linear and branched chain

oligomers, and eventually precipitates. Freely soluble proteins usually require high molecular concentration to crystallize. The preferred protein stock solution should be 5-30 mg/ml of protein for screening purposes. If the supply of the protein is limited, a conc. of 5-6 mg/ml may be sufficient. Generally faster crystallization processes yields smaller micro crystals. Diluted enzyme solutions can be used for crystallization, in turn it will take more time to produce the crystal and yields a better crystal. For physical studies of macromolecule crystal growth, concentration of macromolecule may be a parameter for regulating super saturation.

1.4.1.1.3 Sparse Matrix Crystallization

In the crystallization of enzymes, it is very difficult to do a complete screening of crystallization parameters by fixing the precipitant, pH, temperature, etc. The best method to arrive at crystallization conditions is sparse matrix (Jancarik and Kim, 1991) screens, in which samples selected randomly from a complete combinatorial matrix are used to narrow down the parameters for subsequent optimization experiments. In the process of optimization, the variables such as precipitant concentration, pH, temperature, etc., are expanded to a finely sampled multidimensional matrix with conditions centered on the initial condition that produces crystals.

1.4.1.1.4 Screening of crystallization process

There are several techniques for the screening process of crystallization experiments like vapour diffusion, batch, microbatch under oil, and microdialysis. Once screening is completed, further optimization is required, and such optimization is amenable to tried and true statistical method.

a) Vapor drop diffusion method

Vapor diffusion (Chayen, 1998) experiments rely on water (and other volatile species) evaporation from a small droplet containing the sample protein and precipitant to slowly increase both the protein and precipitant concentration within the droplet. Vapour diffusion method has proven to be the most popular technique for crystallization of proteins. This method allows one to simultaneously increase the protein concentration and decrease the protein solubility owing to the increased precipitant concentration (Christopher et al., 1998).

The advantage of the vapor diffusion method is speed and simplicity and needs only small amount of sample. Micro- Bridges, Glass Sitting Drop rods with VDX or Libro plates, Cryschem plate are the commercial plates available for vapor diffusion crystallization (Hampton Research).

b) Batch Crystallization

Bach crystallization techniques (Schall et al., 1996) are more suitable for optimizing protein crystal growth than vapor diffusion because the system variables are more easily controlled. In the batch technique, the protein sample and precipitant are mixed with appropriate additives creating a homogeneous crystallization medium requiring no equilibrium with a reservoir. Batch crystallization has become a popular technique for implementing a temperature controlled protein crystallization (Zeppezauer, 1971) and yields uniform particles with excellent filtration properties. Batch crystallization is quite attractive for its simplicity and can ultimately be scale up.

c) Micro dialysis crystallization

Dialysis crystallization (Zeppezauer et al, 1968) involves placing the sample in a dialysis button, which is sealed with membrane. Microdialysis chambers are manufactured from plastic cores to accommodate volumes ranging from 5 to 50µl. This is an excellent technique provided that the precipitant is a small molecule like salts, organic solvents, it can easily penetrate through the dialysis tubing, and the protein is slowly brought into equilibrium with the precipitant solutions. Dialysis experiment is used to take the sample from the presence of a high ionic strength solution to a lower ionic solution. Salts, ligands and compounds smaller than the pore size of the dialysis membrane will leave as long as their concentration is lower on the opposite side of the membrane. Water will transfer through the dialysis membrane until the precipitant activity is equalized on both the sides of the membrane. Dialysis is the best method for screening a variety of solution conditions at a fixed protein concentration (Bob et al., 1994).

d) Micro batch crystallization under oil

In this technique, (Arcy et al., 1996) a small drop of the sample combined with the precipitant kept under oil, usually paraffin oil. Nowadays mixture of silicone oil and paraffin oil (1:1) is used for this purpose. This oil allows water vapor to permeate from the drop. This is also like a vapor diffusion experiment where the drop volume shrinks resulting in a

gradual concentration of protein. When the paraffin oil was substituted with silicone fluid, a dramatic time of crystal appearance was observed. Low viscosity of oils results less time for crystallization. 1 to 5 cSt viscosity is always used for optimum results (Stoscheck, 1990).

1.4.1.2 Bulk crystallization of enzymes

Bulk crystallization of enzymes from crude protein mixtures is a highly efficient scalable process and reduces purification costs. The goal is to promote the crystallization of enzyme in large volumes, commonly reaching several thousands of liters, of rather impure solutions from culture broth (Barba and Mcpherson, 1999). In most industrial processes, the aim of crystallization is to generate a concentrated, highly purified stable form of enzyme in a single economical step within a short time, at a lower production cost (Jacobsen et al, 1996). Most of the laboratory purification methods like HPLC, gel permeation, affinity chromatography, etc., are not suitable for the large-scale purification of the enzymes, and the reagents used for these procedures are too expensive. In a well-developed process, the protein yield can be very high and also give the best purification (>99%) in a single step. Control of crystal size is important during the process, since crystalline products obtained from bulk protein crystallization are often of small average size with a wide crystal size distribution. Optimization of crystallization with respect to the target crystal size is based on a fundamental understanding of the process, and in particular, on the knowledge of phase equilibrium, and control of crystallization kinetics (Johns, 1999) and supersaturation (Chgernov, 1984). The number, size, and properties of the enzyme crystals formed depend on the location of the initial conditions on the solubility phase diagram (Barba and Mcpherson, 1999). The solution should be at a level of sufficient supersaturation at which nucleation occurs spontaneously and the crystals formed grow significantly larger. At lower supersaturation, crystals will not be formed at all. At higher supersaturations, the nucleation will be so prolific that the protein available for crystallization will be spread over the large number of crystals formed thus leading only to microcrystals. At very high supersaturations, protein will just precipitates as an amorphous powder. Crystallization of the enzymes is usually done using clarified fermentation broth that has been concentrated, normally by ultrafiltration. Before concentration, the solution should be filtered or centrifuged to perfect clarity to eliminate any amorphous material or debris, including materials like glycogen, starch, and other macromolecular contamination. The concentrated enzyme should have a

high amount of protein (>50mg/ml) and supersaturation is attained on addition of precipitant. The pH for crystallization is generally adjusted to values at or near the isoelectric point (Barba and Mcpherson, 1999) of the protein to take advantage of the lower solubility at these pH values.

Batch crystallization (in a batch stirred reactor) can be used for large-scale production of protein crystals because of its inherent simplicity and reproducibility. During isothermal batch crystallization, the supersaturation falls rapidly from an initial high value to much lower values, especially when supersaturation is achieved by the addition of a precipitant. In most large-scale crystallizations, halide salts or PEG is preferred for producing supersaturation. Bulk crystallization of some of the commercial enzymes such as insulin, glucose isomerase, asparginase, subtilisin, lipases, thermolysin, and penicillin acylase has been reported in patents (Barba, and Mcpherson,1999). A schematic representation of the bulk crystallization process is given in Figure 1.2.

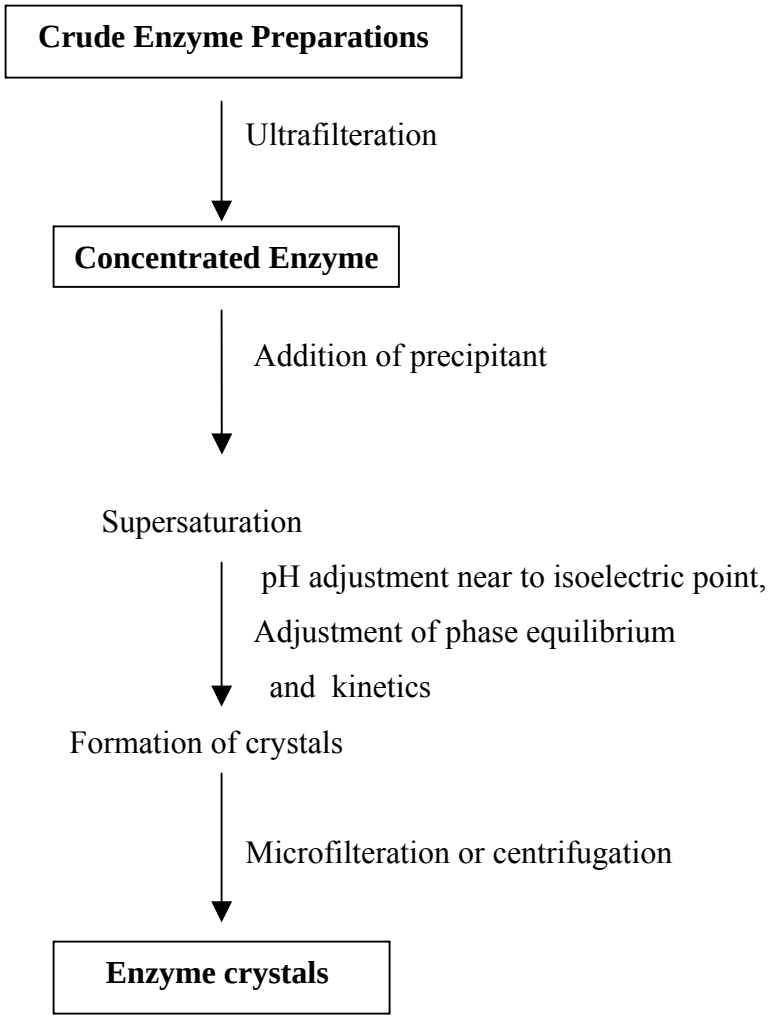


Figure 1.2 Schematic representation of bulk crystallization of enzymes

1.4.1.3 Limitations of crystallization

Some enzymes are difficult to purify and crystallize because their molecules can exist in several conformations (Durban and Feher, 1996), which prevent the formation of a highly ordered crystal. Enzymes have a large number of sites capable of intermolecular interactions, but only relatively few sites will produce the precise alignment of molecules necessary for crystal formation. The binding energies of protein-protein contacts are comparable to those between small molecules, but there are fewer contacts for protein crystals in proportion to their molecular weight, so this energy is apparently weak. Hence,

the supersaturation stage is also far higher for proteins when compared with small molecules. By varying the crystallization conditions and using different precipitants, one can produce crystals with a most favorable conformation. Some crystallization conditions can have specific detrimental effects, such as the loss of cofactor. In the crystallization, some crystal forms are functionally inactive or less active than other crystal forms of the same enzyme. This happens when the active centers are not exposed to the solution volume available for substrates, or the channels in a given crystal form are too narrow for the substrate to enter.

1.4.2 Cross-linking of enzyme crystals

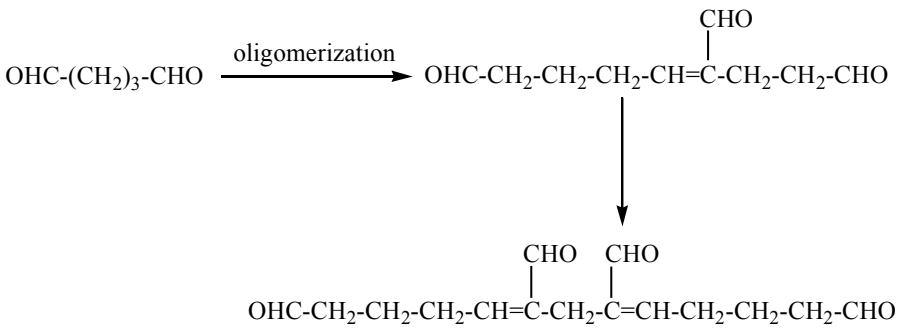
A chemical cross-link, which does not disturb the crystal lattice structure, provides additional stabilization to an enzyme. In an immobilized enzyme, the enzyme is linked by a point attachment to a two dimensional solid surface, but a protein in a cross-linked crystal is stabilized by links throughout its three dimensional structure (Lalonde, 1997). In cross-linked enzyme crystals the lattice interactions, when fixed by chemical cross-links, are particularly important in preventing denaturation, especially in organic solvents. The combination of the crystalline lattice contacts and the covalent cross-linking results in a 100 to 1000-fold increase in protein stability towards thermal deactivation and organic solvent denaturation. The inter- and intramolecular covalent cross-links provide an additional barrier to catalyst deactivation. The cross-linking interactions prevent the constituent enzyme molecules in the crystal from redissolving, thus effectively immobilizing the enzyme molecules into microcrystalline particles. Uniformity (Navia and St.Clair, 1997(a)) is also maintained by the intermolecular contacts and the chemical cross-links between the protein molecules constituting the crystal lattice. Intermolecular (Rose et al., 1995) cross-linking is necessary to maintain crystal structures in environments different from the crystallization liquor, and also increases stability to storage, even at elevated temperatures. The crystals are highly active, easy to handle, recyclable, possible to incorporate in fabrics, and indifferent to shear and foaming (Govarthan, 1999).

Chemical cross-linking of an enzyme crystal results in stabilization of the crystal lattice by introducing covalent links between the constituent enzyme molecules of the crystal. This makes it possible to transfer the enzyme into an alternative reaction environment that might otherwise be incompatible with the intact soluble protein. While

crystallization of a protein creates a precise spatial arrangement of the molecules, subsequent cross-linking inside the crystal locks the proteins in place. Many reagents and newer methods are now available for chemical cross-linking. Cross-linkers have been used to brace a protein and to connect it intermolecularly with another protein molecule. Two types of chemical cross-linkers are used for the cross-linking (Margolin and Navia, 2001) of biomacromolecules: homobifunctional and heterobifunctional cross-linkers. Homobifunctional reagents are commonly used for cross-linking of enzyme crystals, having similar reactive groups. These reagents couple functional groups like two aldehydes, two amines or two thiols. The commonly used homobifunctional cross-linking agents include dialdehyde cross-linkers (glyoxal, glutaraldehyde, succinaldehyde), diamine cross-linkers (ethylenediamine, hexamethylenediamine, octanediamine, etc.), bis(imido esters), and bis (succinimidyl esters). In heterobifunctional reagents, the reactive groups are different, allowing the formation of cross-links between unlike functional groups. They are used to cross-link two different molecules like enzyme with antibody, or nucleic acid with drug or peptide, etc. Increasing the chain length of the cross-linking agent increases the flexibility (Wong and Wong, 1992) of the cross-linked enzyme crystal. The cross-linking conditions such as pH, temperature, protein concentration, reagent concentration, cross-linking time, and reagent addition rate must be carefully optimized. The cross-linking can be done in an organic solvent such as acetone (David et al, 2001) or DMSO (Sobolov et al, 1996) to reduce reaction time, since only few minutes are needed for cross-linking in these solvents. Excessive cross-linking may lead to aggregation, protein precipitation, loss of activity, and distortion of the crystal lattice (Margolin, 1996).

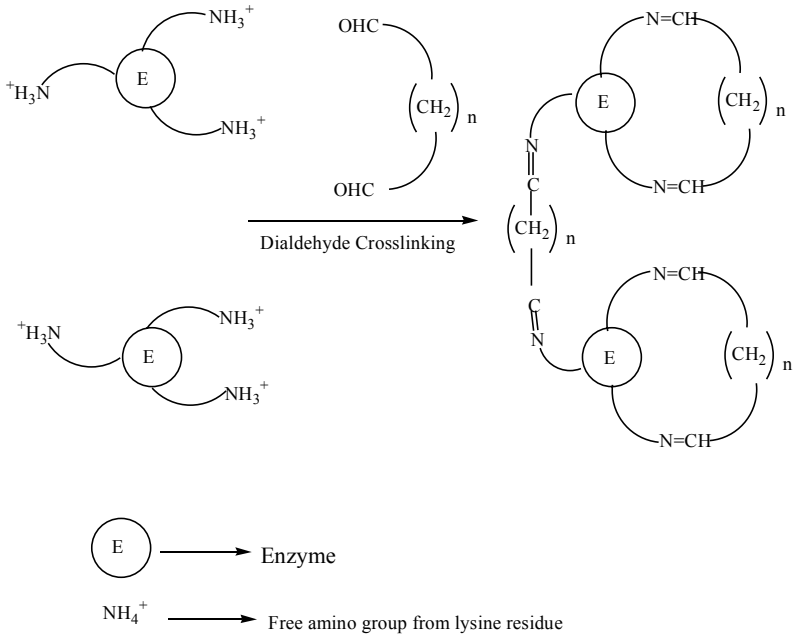
1.4.2.1Glutaraldehyde Cross-linking

Glutaraldehyde, a dialdehyde reagent, is the most popular cross-linking agent for enzyme crystals. Glutaraldehyde is safe, inexpensive, and easily handled. The ability of glutaraldehyde to form a mixture of oligomers (scheme 1.1) of different lengths and structures in aqueous solutions makes the use of this cross-linker somewhat unpredictable (Tashima et al,1991). The long chain of oligomers of glutaradehyde formed in aqueous solutions.



Scheme 1.1. Oligomerization of Glutaraldehyde

Cross-linking with glutaraldehyde forms strong covalent bonds between the ε-amino groups of lysine residues within and between the enzyme molecules (i,e., intra- and intermolecular imine cross-linking) in a crystal lattice (Santis and Jones,1999). Because many of the linkages are between adjacent macromolecules in the lattice, the crystals become cross-linked throughout. The cross-linking is also irreversible and therefore cannot be explained by simple imine or Schiffs base formation (Scheme 1.2), and the mechanism is not fully understood. Cross-linking with glutaraldehyde confers several advantages. The enzyme crystals, which are normally very fragile, become more sturdy and robust after cross-linking so that there is much less chance of damage in handling. They become completely insoluble under a variety of conditions but remain permeable to solute, and are insensitive to pH and temperature, which can be varied over a wide range without dissolution or deterioration of the cross-linked crystals (Klaus, 1976). The advantage of glutaraldehyde cross-linking is that it gives rise to a stable, three-dimensional crystal net that is resistant to denaturation by urea. The cross-linking conditions should be carefully optimized with different glutaraldehyde concentrations at different cross-linking times. 0.1 to 5% of glutaradehyde is reported to give better cross-linking. Crystals cross-linked with excessive glutaraldehyde are yellow-brown in color and mechanically hard. Analysis of the amino acid sequence after cross-linking gives the number of lysine residues that have been cross-linked. Rose *et al* (1995) have reported that in the case of thermolysin . CLEC, approximately eight of the eleven lysine residues are found to be modified. The other three lysine residues that are involved in the intra- and intermolecular interactions are not accessible to glutaraldehyde.



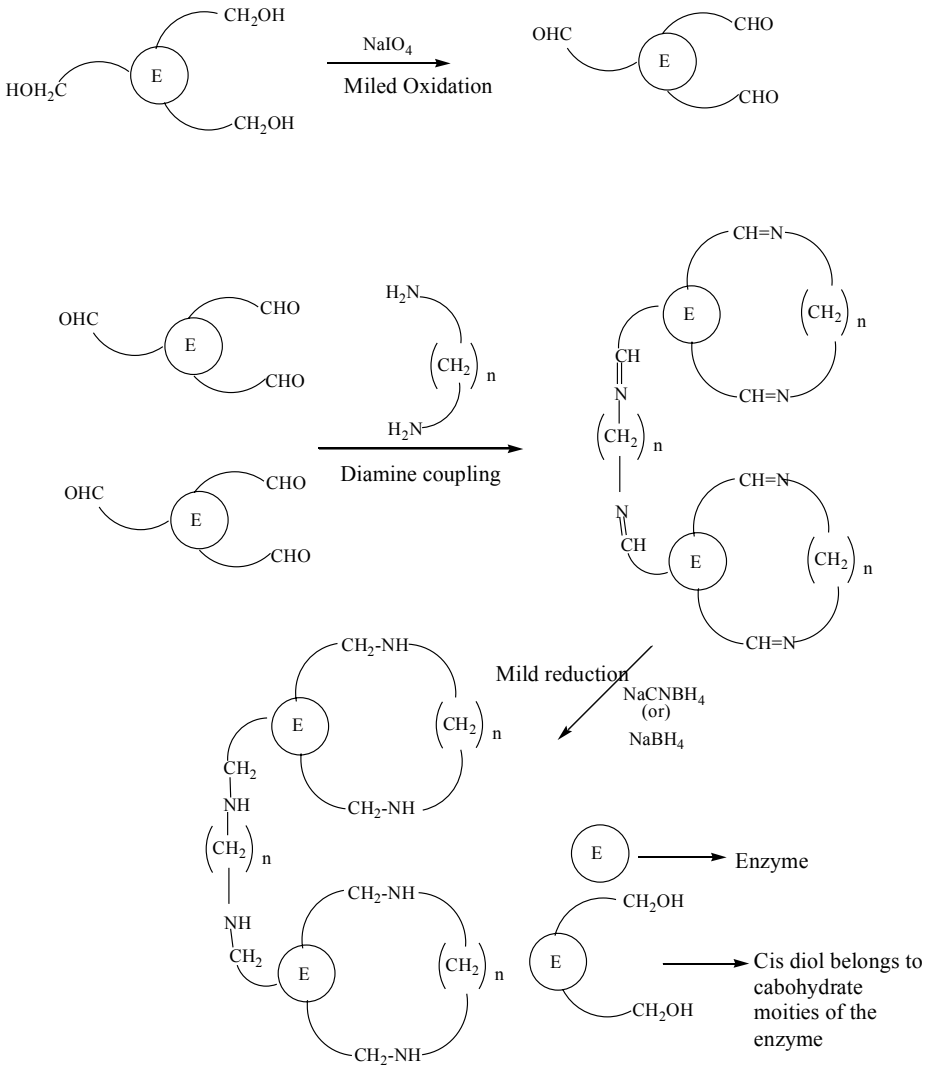
Scheme 1.2. Inter and intra molecular imine crosslinking of enzyme crystals through dialdehyde coupling

1.4.2.2 Carbohydrate Cross-linking

Another method for stabilization of the crystal lattice is exclusive cross-linking of the carbohydrate moieties of glycoproteins (Margolin et al., 2002) or a combination of carbohydrate and amino acid side chains. In such carbohydrate cross-linked glycoprotein crystals, the lattice interactions are fixed by chemical cross-links, which are particularly important in providing stability to storage under harsh environments and in preventing denaturation. In the case of enzymes having a lysine residue in their active sites, carbohydrate cross-linking is preferred, since glutaraldehyde has a great affinity for lysine, which may lead to enzyme inactivation by active site fixation. This method advantageously accomplishes the crystallization of glycoproteins in large-scale, without the need of the cumbersome and potentially denaturing effects of chemical deglycosilation.

Carbohydrate cross-linked crystals are produced by the initial oxidation of the carbohydrate moieties, which cleaves cis-diol groups to produce a di-aldehyde. This is followed by cross-linking with a bifunctional reagent such as a diamine thereby forming a schiffs base which can be reduced under mild conditions using $NaCNBH_4$ or $NaBH_4$ (Scheme 1.3). The diamine cross-linking agents can be ethylenediamine, hexamethylene-

diamine, diaminooctane, adipic acid dihydrazide (Zutsanna and Bence, 1999), etc. CLEC glucose oxidase and lipase have been prepared by this carbohydrate cross-linking method.



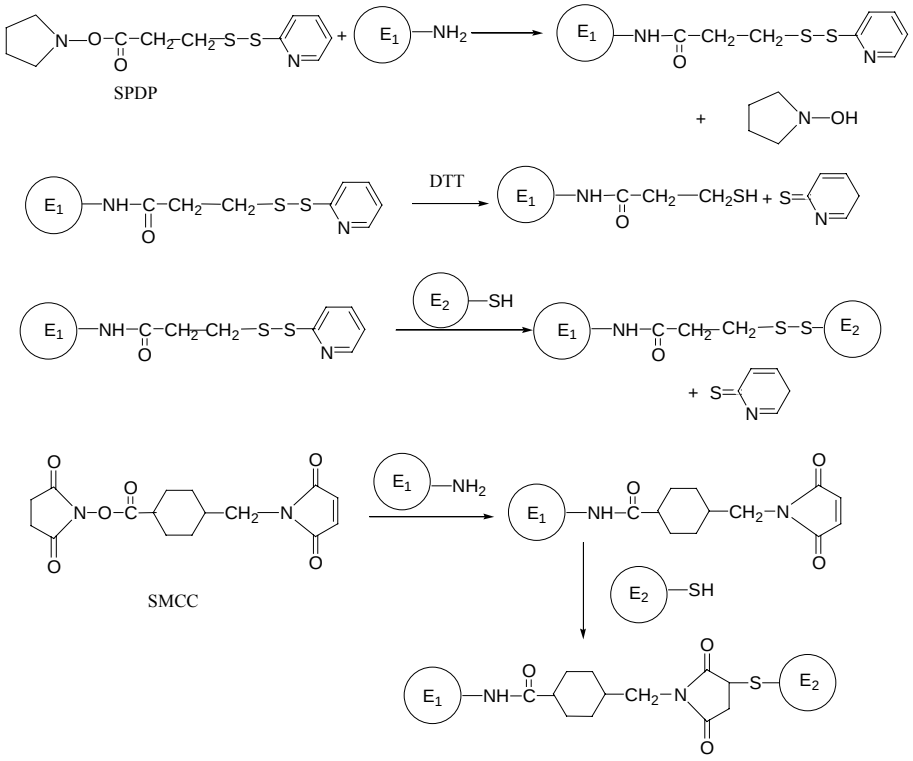
Scheme 1.3. Cabohydrate crosslinking of enzyme

1.4.2.3 Thiol Cross-linking

Another possible cross-linking method is through the thiol groups in the enzyme crystals. Here a heteroconjugate is formed, which involves indirect coupling of an amine group in one protein molecule to a thiol group of a second biomolecule, usually by two or three reaction steps. The first step is the introduction of thiol groups in the enzyme crystals. If the enzyme does not have enough free thiol groups, they can be introduced by selectively reducing cystine disulfides with reagents such as dithiothreitol (DTT)

(Mark et al, 2001) or β-mercaptoethanol or tris-(2-carboxyethyl) phosphine (TCEP) or tris-(2–cyanoethyl) phospine. Amines can be indirectly thiolated by reaction with succinimidyl 3-(2-pyridyldithio) propionate (SPDP)(Carlsson et al., 1978) (Scheme 1.4). Excess of these reagents must be removed by dialysis or gel filtration.

The second step involves the coupling of the thiol containing enzyme molecule with another enzyme molecule containing amine groups using heterobifunctional reagents like maleimides or iodoacetamides. Succinimidyl trans-4-(maleimidylmethyl) cyclohexane-1-carboxylate (SMCC)(Yoshitake et al,1979) is one of the maleimide heterobifunctional reagents used to introduce a thio-reactive group at an amine site of an enzyme followed by cross-linking with a thiol-containing enzyme molecule.



Scheme 1.4. Schematic representation of Amine-Thiol cross-linking of Enzyme crystals

1.4.2.4 Other Cross-linking methods

Other cross-linking methods, such as metal chelation, carbodiimide coupling and diazo coupling which are used for the modification of immobilization matrix, cannot be used for the cross-linking of protein crystals because the reagents may inhibit or inactivate the enzymes when added directly to the crystals.

1.4.2.5 Limitations of Cross-linking of enzyme crystals

When enzyme crystals are cross-linked in a three-dimensional lattice by a cross-linking agent, microscopic channels of about 20-55Å are formed between the two crystals. The diffusion limitations (Margolin, 1996) of CLECs are defined by size, length, and microenvironment (surface charge, etc.) of these channels. Cross-linkers of sufficient length could block the channels wall-to-wall resulting in particles with peculiar properties. In some cases, crystals remain under-cross-linked, which results in having the outer layers fixed, but without much cross-linking in the interior. This type of CLEC will look normal in the mother liquor but expand in low ionic strength buffers and shrink in concentrated ones, thereby losing storage and mechanical stability. The diffusion inside the expanded CLEC is less hindered, although it may be that the cross-linked "rind" on the surface determines the actual rate of entrance and departure of the substrate. The size of the substrate (Margolin and Navia, 2001) is also important, because substrates with high molecular weight may not be able to diffuse through the channels and hence have reduced reactivity.

Different lattice packing in enzyme crystals can dramatically change the cross-linking pattern of CLECs, presenting different numbers of reactive residues to other cross-linker molecules with different distances between them. Thus, the crystal structure, the crystal packing information, and the nature and length of cross-linking reagents are important factors in rationally engineering CLECs. Cross-linked enzyme crystals already reported in the literature is given in Table 1.2.

Table 1.2 Preparation of Cross-linked enzyme crystals

Enzyme Name	Crystallization Conditions	Cross-linking Conditions	Reference
Fructose Diphosphate Aldose	45% NH_4SO_4 Soln at 22°C for 1-2 weeks	1-16 mM conc. glutaraldehyde in 0.5mM triethanolamine buffer at 0° C for one hour	Sobolov et al (1994)
Glucose Isomerase	NH_4SO_4 as precipitant at pH 7.0 for 20 hrs in 16°C	12.5% glutaraldehyde (v/v) with 20% lysine soln for 3.5 hour at 100 rpm	Visuri (1995) Ossipastine et al (2000)
Hydroxynitrile lyase	46 mg/ml of protein 5% PEG 8000 and 25%MPD in citrate buffer at pH 5.4 for 5 days 23°C	0.1 to 1% glutaraldehyde in acetone at 300 rpm for 5 minutes	David et al (2001)
Glucose oxidase	18% PEG 6000, 32% 2-propanol in 0.2 M acetate buffer pH 5.0 at 6°C at 100 rpm for 24 hrs	$NaIO_4$ oxidation in dark. Cross-liked using 0.25 M 1,8 diamino-octane with 9% PEG 6000 and 16% 2-propanol in 0.1 M acetate buffer pH 5.0	Margolin et al (2002)
Lipase	1.93 to 4.63 mg/ml protein 47.5% v/v MPD and 0.4 mM $CaCl_2$ in MES buffer at pH 5.9 in 200 rpm	5% glutaraldehyde v/v in MES buffer pH 5.9 for 3 hours	Margolin et al(2002), Jeetendra et al (2000)

α- chymotripsin	2.4 M NH_4SO_4. 5.7 mg/ml protein Conc. in 0.1 M citrate buffer at pH 4.	5% glutaraldehyde v/v in 0.1 M citrate buffer at pH 4	Jeetendra et al (2000)
Yeast alcohol dehydrogenase	14 mg/ml protein conc. PEG 4000 16% v/v in 50mM Tris buffer at pH 8 with 2 mM β-nicotinamide adenine dinucleotide (β-NAD) in 100 rpm at room temperature for 3 days.	5% glutaraldehyde v/v in 50mM Tris-buffer at pH 8 for 2 hours.	Sobolov et al(1996) Lee et al (2000)
Lactate dehydrogenase	5 mg/ml protein conc.35% NH_4SO_4 in 0.1M phosphate buffer at pH 7.5 at 25ºC	15 mM glutaraldehyde in 0.5 M triethanolamine buffer pH 7.5 at 4ºC over night	Sobolov et al (1996)
Thermolysin	100mg/ml protein conc. in 1M Calcium acetate solution, 30% DMSO in 50mM Tris buffer pH7.0	12.5% glutaraldehyde in 50mM Tris buffer pH 6.5 for 1 hour	St.Clair et al (1992)
Chloroperoxidase	7 mg/ml of protein 14% PEG 8000 in 10mM phosphate buffer at pH 5.0 at 18°C	1000-6000 molar excess of glutaraldehyde at pH 6.5 in 0.1M sodium cacodylate buffer for 1 hour at room temperature.	Marcela et al (2002)
Subtilisin	Na_2SO_4 as precipitant, enzyme in 0.33M cacodylate buffer at pH 5.6 in 30ºC.	1.5% glutaraldehyde solution in 30mM cacodylate buffer at pH 7.5, with 13% Na_2SO_4	Bruggink et al (1998)

Horseradish peroxidase	30mg/ml protein in 10mM phosphate buffer at pH 7.2 with 1,5 pentanediol and (10%)NH_4SO_4	1% glutaraldehyde in acetone (v/v) for 5 min at 200 rpm in 4ºC	Bindhu et al
Glucoamylase	65% $(NH_4)_2SO_4$ with 20% 2-propanol in 0.5 M acetate buffer at pH 4.5 for 16 h at 4°C	2%glutaraldehyde (v/v) in 0.2 M phosphate buffer at pH 7.0 for 1h	Emilia et al (2004)

1.5 Structure of a CLEC

Cross-linked enzyme crystals are crystals, within the crystal lattice the concentration of protein approaches the theoretical limit. The crystallinity is absolutely required to achieve the stability exhibited by CLECs (Rose et al, 1995). Crystals of proteins (enzymes) are not solid structures. They typically contain 30-65%, by volume, open channels, which are usually filled with solvent (Matthews, 1968). CLECs are micro porous materials with limited pore size of (15-100 Å) and surface areas between 500 and 2000 m^2/mg and this are the same range as for inorganic zeolites (Michael, 2001). CLECs, however, have pore diameters in the range of 3-8 nm, as compared 0.2-1.0 nm for normal inorganic zeolites. Indeed, CLECs can be thought of as bioorganic zeolites. A Computer generated picture of a CLEC is given in Figure 1.3

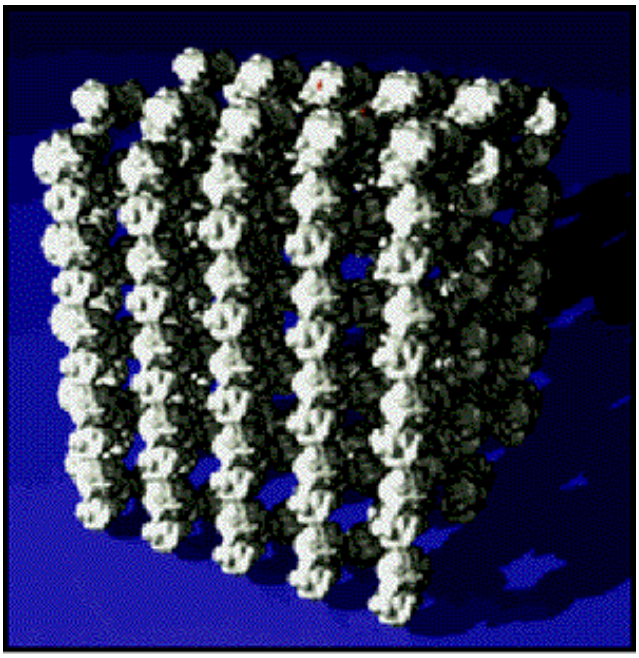


Figure 1.3 Computer generated Structure of a CLEC (taken from (www.altus.com))

1.6 Cross-linked enzyme aggregates (CLEA)

The physical aggregation (Brown and Glatz 1986). of enzyme molecules into super molecular structure can be induced, without perturbation of the original three-dimensional structure of the protein by the addition of salts, organic solvents, or nonionic polymers to protein solution. These solid aggregates are held together by noncovalent bonding and readily collapse and redissolve when dispersed in an aqueous medium. The chemical cross-linking of these physical aggregates would produce cross-linked enzyme aggregates in which the reorganized superstructure of the aggregates and their activity would be maintained. Cross-linked enzyme aggregates can also be prepared by a simple method using cross-linking agents. Cross-linked enzyme aggregates of Penicillin acylase (Linqiu et al, 2002) (E.C.3.5.1.11) and Lipase (Lopez-serrano et al 2002) have been prepared by glutaraldehyde cross-linking.

1.7 Stability of CLECs

1.7.1 Chemical Stability

Biocatalytic stability under chemical process conditions is critical for their large-scale commercial applications of CLECs. Stability towards organic substrates, solvents, heat, mechanical shear, and pressure are requirements for long catalyst lifetimes (Lalonde,

1997). The increased stability of a protein molecule in a crystal is due to additional ionic and hydrophobic contacts between the protein molecules. When a protein is transferred from a solution to crystalline form, an increase in the number of both polar (electrostatic) and hydrophobic interactions (Islam and Weaver,1990) among the protein molecules may significantly enhance protein stability against heat and other denaturants by preventing unfolding, aggregation, or dissociation. The crystalline enzyme maintains its native conformation at elevated temperature and has a lower tendency to aggregate. The chemical cross-linking of these protein crystals provide additional stabilization. Figure 1.4 is a schematic representation of the relative stability of a CLEC, a soluble enzyme, and an immobilized enzyme

Cross-linked soluble enzymes do not show increased stability. Tolerance of temperature, pH, and organic solvents probably arises from protein-protein interactions and the contacts that occur in the crystal lattice which are also maintained in CLECs. In a CLEC, the enzyme molecules are linked together in a three-dimensional lattice with ordered microscopic channels between them. This lattice provides structural strength and durability, since the total energy of crystallization as well as the energy needed to denature the cross-linked protein will be high. The microscopic channels allow the substrate to pass readily through the crystal lattice and access the active sites of the enzyme. The degree of stabilization (Mozhaev, 1993) depends on the number of contacts, both intermolecular and intramolecular, among the enzyme molecules. Cross-linking of protein crystals also provides additional stabilization against unfolding and thus leads to both chemical and mechanical stability.

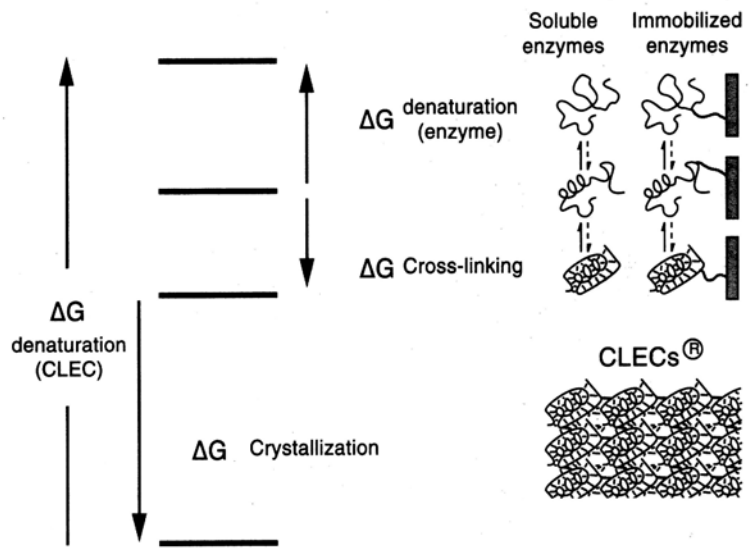


Fig 1.4 Comparison of Stability of a CLEC, immobilized and soluble enzyme taken from Ref (Lalonde,1999)

1.7.2 Mechanical Stability

For the industrial application of CLECs as biocatalysts, it is important to understand their mechanical properties in relation to process design and operation. A CLEC must withstand the shear forces associated with processing equipment such as stirring tanks and cross-flow micro filters, which may cause particle attrition and fragmentation. Stability of the cross-linked enzyme crystals has been reviewed by Margolin et al (2001). The enhanced stability of protein crystals by chemical cross-linking also extends to mechanical stability. Mechanical stability of a CLEC also depends on the shape of the crystals. Lee et al (2002) have studied the mechanical stability of both hexagonal and rod-shaped crystals of alcohol dehydrogenase (YADH). Hexagonal crystals exhibited some breakage during mechanical shearing at a disk rotational speed of 27000 rpm, which may be due to the shear-induced attrition, but no breakage was found in rod-shaped crystals. Both crystal forms showed no significant change in catalytic activity induced by shearing. The controlled exposure of protein crystals to lower concentration of cross-linkers, followed by treatments with butylamine that remove excess reagent, has produced more mechanically stable crystals (Mcpherson, 1999). The formation of heavy atom derivatives without crystal disruption showed a notable reduction in radiation damage. CLEAs are too soft and hence may exhibit

poor stability when used in stirred tanks or in packed bed reactors. For the production of a sturdy process biocatalyst, this can be overcome by encapsulation of CLEAs into a very rigid polyvinyl alcohol network or a sol gel matrix by a suitable immobilization technique.

1.7.3 Thermal stability

Soluble enzymes are typically easily inactivated by heat. It does not require much energy to disrupt the hydrogen bonds and other weak forces, which hold an enzyme in its active conformation. The thermal stability of CLECs can be two to three orders of magnitude greater than that of soluble protein (Michael, 2001). When an enzyme forms a crystal, a very large number of stabilizing contacts are formed between individual enzyme molecules. Energy must be put into the system in order to disrupt these new contacts. On top of that, additional energy is required to break the covalent cross-links before the CLEC begins to dissolve and then denature. The increased thermal stability offers several major benefits to the organic chemist using CLECs as catalyst. First, unlike soluble and immobilized forms, no special storage conditions are required for CLECs. Second, since enzyme catalyzed reactions are generally slower in organic solvents and with non-natural substrates, the ability to run such reactions at higher temperatures to increase the rate is a significant benefit to the chemist.

1.7.4 Stability in Organic Solvent

The enzyme activity in organic solvents is intimately related to the water content, size, and morphology of the catalyst particle, and to the enzyme microenvironment (Bell et al, 1995). Generally, the catalytic activity of enzymes in neat organic solvents (Klibanov, 1997) is far lower than that in water. The organic solvents lack the ability to engage in multiple hydrogen bonds with water molecules and also have lower dielectric constants, leading to stronger intraprotein electrostatic interactions, which leads to the loss of activity. The effect of different organic solvents on the catalyst is not always uniform and properties such as solvent hydrophobicity, hydrogen bonding capacity, and water miscibility have a profound influence on the structural integrity and catalytic activity of enzymes. Proteins in hydrophobic solvents (Mattos and Ringe, 2001) are thought to retain their native structure because of kinetic trapping, resulting from stronger hydrogen bonding between the protein atoms and a more rigid structure in the absence of water. Polar solvents can easily strip water from the surface of the protein by competing strongly for hydrogen bonds between

protein atoms. Solvents like DMSO and DMF usually denature the structure to a largely unfolded state (Knubovets et al, 1999). However, this denaturation will not happen to either crystalline or lyophilized enzymes (Griebenov and Klibanov, 1997).

Protein crystal structure in organic solvents suggested the idea that organic solvents cannot be used to map binding surfaces of proteins, since organic solvents bind only at few positions, primarily at the active site and in crystal contacts. In both cases, the anhydrous environment presumably locks the enzyme molecule kinetically in its prior conformation. The insolubility of CLEC catalysts in water and organic solvents allows recovery by simple filtration or use in a column reactor.CLECs need to be formulated as a free flowing dry powder with low moisture content (1-10%) to function in near-anhydrous organic solvents.

1.8 Characterization of a New CLEC

1.8.1 Catalytic properties

A new CLEC can be characterized as similar to an immobilized enzyme as proposed by Van Ginkel et al (1983). The catalytic activity and stability at different temperatures, pHs, in various organic solvents, and in aqueous-organic mixtures is measured by assaying the activity of the CLEC after a long-term exposure to the above conditions. Stability studies of the CLEC enable us to determine the robustness of the catalyst under different operational conditions. A study of the stability of the CLEC in the presence of proteases should be examined in order to apply to a crude preparation that may include even traces of microbial proteases. Kinetic properties of the CLEC such as catalytic efficiency, K_{cat}, K_m and substrate specificity have to be determined for comparison to both the soluble and the immobilized form.

1.8.2 Physical and mechanical properties

The crystal size, shape and density give the basic data related to the mechanical properties of the crystal. This may also indicate the existence of any diffusion limitation. The mechanical stability of the CLEC is also important under physical conditions that are found in the most frequently used reactor configuration i.e., a mechanically stirred vessel. The porosity of the CLEC should also be determined. A CLEC has well defined pores of limited size leading to the enzyme molecules within its body. As a result, a substrate larger than the available pore size will not be able to penetrate the body of the CLEC particle. Hence, a CLEC with limited pore size would be unsuitable for many enzymatic reactions of

commercial interest involving substrates larger than the pore size of the CLEC. Determination of the number of filtration cycles needed for product recovery allows prediction of the processing time. Figure 1.5 represents the protocol for the characterization of a CLEC, as proposed by Jeetendra et al (2000).

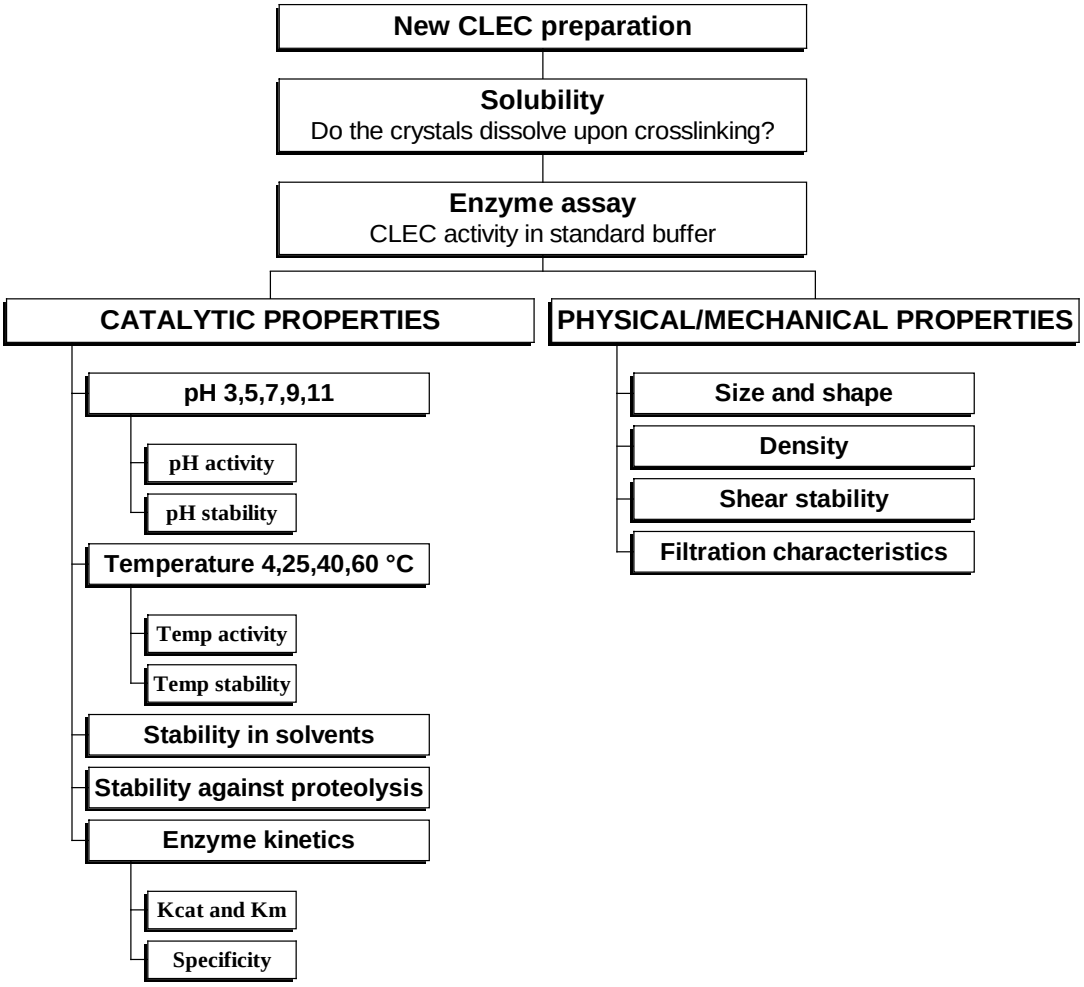


Fig 1.5 Protocol for the characterization of a New CLEC taken from ref (Jeetendra et al, 2000)

1.9 Activation of CLEC in organic solvents

This has been done by adding small amounts of surfactants (Feher, 1986) during the drying process. Surfactants often possess strong denaturing potential, which when used in small concentrations, neverthless can generate enzyme preparations with extraordinary properties. Surfactants are usually mixed with an aqueous solution of an enzyme, and the mixture is dewatered (Mattos and Ringe, 1996 & Lee and Dordick, 2002) by lyophilization,

vacuum drying, etc. The resulting enzyme preparation is used as catalyst having enhanced activity in organic solvents. Surfactants may help to maintain a better water balance and the native conformation of enzymes. Surfactants may also facilitate the transfer of hydrophobic substrate molecules through the layer of tightly bound water to the binding site of the enzyme. Enzymes can be extracted into hydrophobic organic phases in the presence of surfactants like Aerosol OT (AOT) (Paragkar and Dordick, 1994) or others (Okahata and Mori, 1997) in small concentration (1mM) to give highly active preparations more stable in organic solvents than the soluble enzyme preparations. Like surfactants, some additives may also improve the activity of the enzymes in organic solvents. Additives such as methyl β-cyclodextrin (Meana, 2002) (MβCD) dramatically activate the serine protease Subtilisin Carlsberg in THF and in 1,4-dioxane. The transesterification rate of sec-phenethyl alcohol with vinyl butyrate is reported to be increased 164 fold in THF by subtilisin Carlsberg (Griebenow, 1999) lyophilized with methyl β-cyclodextrin(MβCD) .

1.9.1 Crown ether activation of CLECs in organic solvents

Crown ethers are macrocyclic organic molecules that have been shown to activate enzymes following their co-lyophilization from an aqueous solution. The effects of crown ether (Dirk-Jan etal, 1998) treatment under various conditions give support to the hypothesis that removal of bound water molecules from the active enzyme during the drying process is the origin of the observed enzyme activation. Crown ethers might prevent the formation of deactivating inter- and intra-molecular salt bridges in organic solvents by complexation of the ammonium functions of lysine residues, or may contribute to an enhanced substrate binding and consequently to a higher enzymatic activity by facilitating the transport of water molecules from the active site into the bulk organic solvent (Sakurai et al, 1998). The capability of crown ethers to form complexes with ammonium groups (Gokel, 1991) (lysine residues) and water molecules by hydrogen bonding is well defined.

In aqueous solutions, the transfer of water molecules from an active site to the bulk solvent is entropically favorable due to the increase in transitional and rotational freedom. Increased K_m values and thus lowered enzymatic activity are found after the transfer of enzymes from an aqueous solution to organic solvents (Ryu and Dordick, 1989). Crown ethers, which are able to complex with water molecules in organic solvents, will facilitate the transport of water from the active site to the organic solvent during the process of drying

the enzyme crystal. Lyophilization of subtilisin Carlsberg crystals in the presence of 18-crown-6 (Dirk-Jan etal, 1998) had increased its activity by 8.5 times. There is a reduction of activity in the absence of crown ether during lyophilization of enzyme crystals might originate from a distortion of the enzyme conformation by crystallization of the water in the solvent, which fills the channels of the crystals. The presence of 18-crown-6 may act as a lyophilization-protecting agent.

In cross-linked enzyme crystals, most of the lysine residues react with the cross-linking agent and consequently get converted into imine functions. Rose et al (1995). has observed that in the case of thermolysin CLEC, amino acid analysis reveals that eight out of eleven lysine residues are modified. Moreover, unreacted free lysine residues in the CLEC may seem to be of minor importance for the activation effect of crown ether in organic solvent. However, in the case of enzyme crystals, crown ether activation is very important to prevent the loss of activity during lyophilization, since the properties of cross-linked enzyme crystals depend only on active enzyme crystals.

1.9.2 Lyophilized CLEC

Lyophilizing enzymes in the presence of structure preserving lyoprotectants (Klibanov, 2001) such as sugars and poly ethylene glycols, and some inorganic salts and crown ethers, minimize the denaturing effects of organic solvents. These lyoprotectants prevent protein aggregation in organic solvents. Similarly, CLECs when subjected to lyophilization with various surfactants or additives and inorganic salts or crown ethers produced lyophilized CLEC with minimum denaturation. Most of the CLECs (subtilisin, thermolysin, protease, lipase) retain their activity in organic solvents (Khalaf et al, 1996 & Khalaf, 2000 & Kui and Klibanov, 1996 & Noritomi et al, 1998). These cross-linked enzyme crystals in organic solvents may be used in a number of chemical processes such as organic synthesis of specialty chemicals and pharmaceuticals, synthesis of intermediates, and also for the chiral synthesis and resolution of optically pure pharmaceuticals. Enzymatic conversion processes such as oxidation, reduction, esterification, coupling reactions and asymmetric conversion including stereo selective, stereo specific and regioselective reactions can be done with CLECs for the production (Partride et al, 1996 & Rose et al, 1996 & Wang et al, 1997 & Zelinski and Waldmann, 1997) of organic molecules, peptides, carbohydrates, etc.

1.10 Productivity of CLEC

The productivity of a catalyst is the quantity of product per kilo of the catalyst. For a biocatalytic process, the catalyst cost should be less than 5-10% of the product value. The process development and scale-up are accelerated by recommendations on catalyst loading and depends on catalyst recovery and reusability. A biocatalyst which performs in organic solvents, aqueous-organic mixtures, at elevated temperatures, and under high shear conditions results in increased catalyst productivity (Lalonde, 1997). The final productivity of a CLEC catalyst is further increased by the number of times the catalyst can be recycled. The increased volumetric activity is essential for commercial scale up since it extends to an increased reactor productivity (Timpson and Wasserthal, 1998). The entire volume of the CLEC catalyst consists of active material, unlike an immobilized enzyme on an inert carrier. The CLEC catalysts are highly stable, active, and recyclable due to easy filtration or centrifugation properties, but routine immobilization methods do not guarantee enzyme stability and have low specific activity. The enzyme loading (Tisher and Kasche, 1998) on an inert matrix will be only 0.1% to 10% w/w of the total, and hence a large amount of immobilized enzyme is needed for high reaction rates. However, in CLEC catalysts, the enzyme is packed as crystals and hence the volumetric activity is orders of magnitude higher than that of conventional soluble or immobilized enzymes. For biotransformation reactions, small amounts of CLEC are required to produce large amounts of products, and the reaction will be completed within a shorter time.

The catalyst to product ratio (Lalonde, 1997) for CLEC reactions normally ranges from1: 100 to 1:5000 for a single reaction cycle. If the enzyme crystals can be reused 10–20 times, the final productivity will be in the 1: 1000 to 1:100,000 range. The cost of commercially available CLEC catalyst ranges from $12000 to $350000/kg (according to the enzyme used). Thus, for example, suppose, if the productivity is about 1:10,000 then 1 kg of product would be formed by using 100mg of CLEC costing $5. The resolution of 1-phenylethanol with vinyl acetate in toluene catalyzed by CLEC lipase (PS) is the best example for CLEC productivity. In this reaction, substrate to catalyst ratio is 4600. Thus, we see that CLEC catalysts have high productivity for biotransformation reactions when compared to soluble or immobilized enzymes. Commercially available CLECs are given in Table 1.3. These are the products of Altus Biologics, Cambridge, USA

Table 1.3 Commercially Available Cross-Linked Enzyme Crystals

Product Name	Enzyme/source	pH Stability	Organic solvent Compatibility	Reaction Catalyzed
Chiro CLEC-CR	Lipase/*Candida rugosa*	2 - 8.5	Hyrophobic solvents	Chiral resolution Ester synthesis and hydrolysis and Transesterification
Chiro CLEC-PC	Lipase/ *Pseudomonas cepasia*	4 - 8	MeOH, Toluene Isopropanol, 3º-butyl methyl ether	Chiral resolution Ester synthesis and hydrolysis and transesterification
Syntha CLEC-PA	Penicilline acylase / *E.coli*	5 – 9	EtOAc,Toluene	Ester amide cleavage and synthesis
Pepti CLEC-TR	Thermolysin/ *Bacillus thermoproteolytics*	4-9	DMF, Acetone, THF,Ethanol	Hydrolysis of peptides
Pepti CLEC-BL	Subtilisin *Bacillus Licheniforms*	5-10	Acetone,EtOH DMSO,3-butanol, DMF,Acetonitrile	Protein and peptide hydrolysis and synthesis

1.11 Application of CLEC enzymes

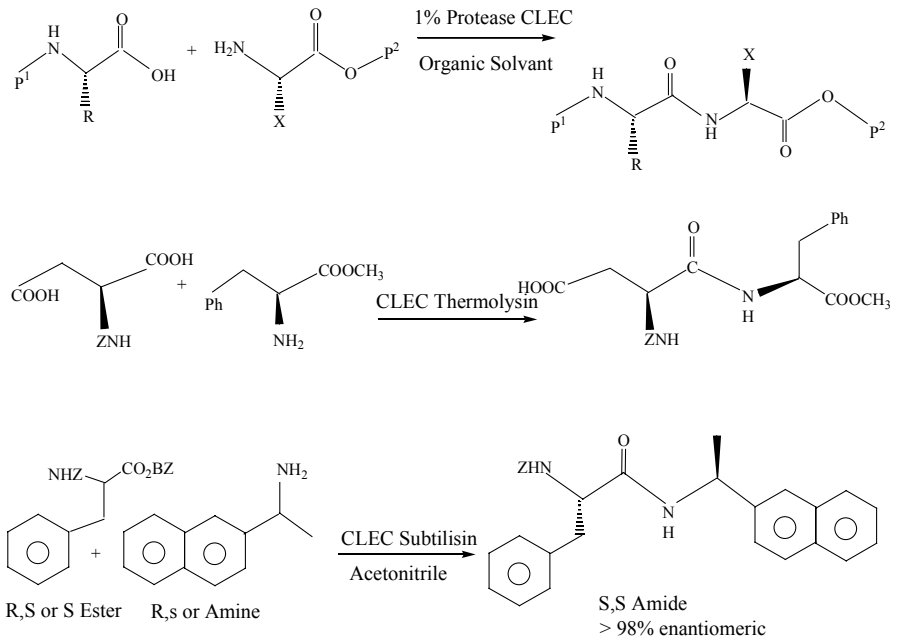
The major applications of CLEC catalysts involve high concentrations of organic solvents and substrates for which the soluble enzyme cannot be used. These occur mainly in the areas of synthetic organic chemistry, biomedical applications and environmental catalysis.

1.11.1 Synthetic Chemistry

Applications of CLECs in synthetic chemistry (Michael, 2001) arise from their enhanced stability without loss of specific activity in both aqueous and organic media. CLEC catalysts are more enantioselective than crude enzyme mixture. CLECs remain insoluble throughout the process and can be recycled many times, which increases their productivity.

1.11.1.1 Synthesis of peptides and peptidomimetics

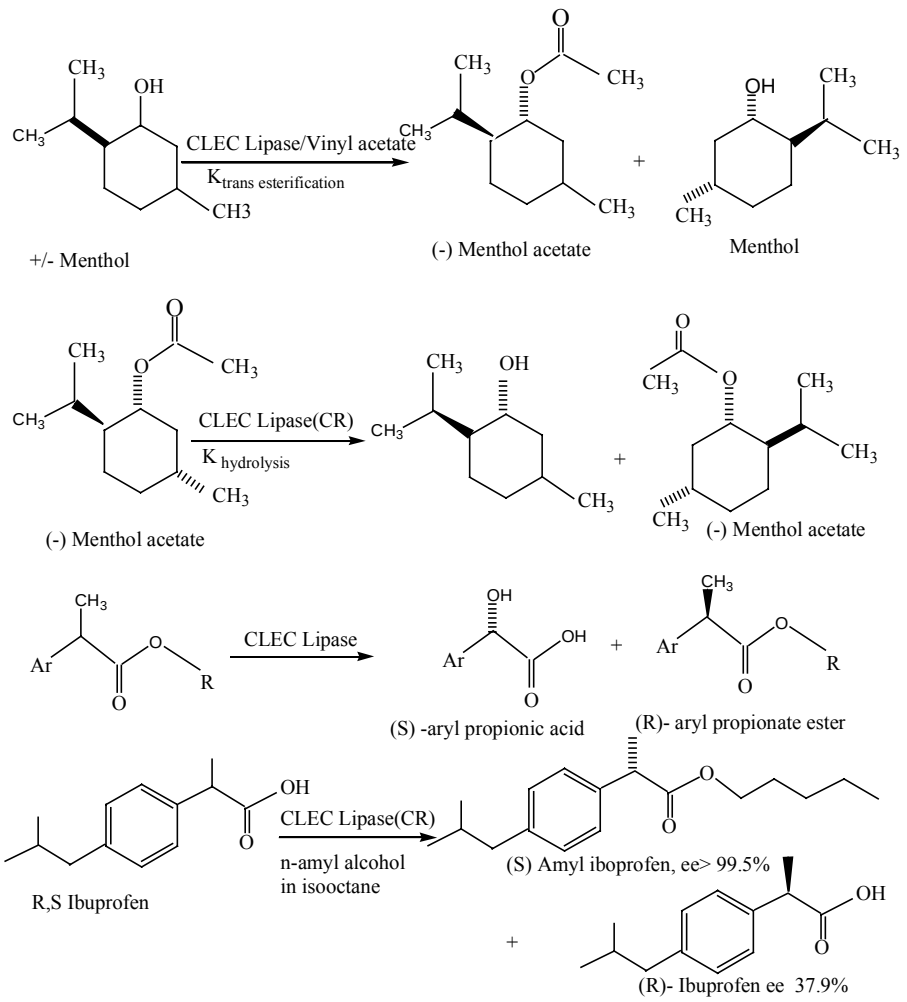
Peptides and peptidomimetics are an important group of modern drugs. Peptides are typically prepared either by the stepwise coupling of the individual amino acids or by the condensation of amino acids and/or peptide fragments in solutions. The proteases catalyzes peptide bond hydrolysis, but the reaction equilibrium can be reversed to peptide bond formation in the presence of organic solvents or by product precipitation. However, proteases are unstable in the presence of organic solvents, and the reaction mixture contains peptide and protein fragments, which are difficult to separate from the product. CLEC proteases circumvent these problems in peptide synthesis (Kullmann, 1987) and hence are better suited for the large-scale synthesis of peptides and peptidomimetics. Subtilisin-CLEC (Wang et al, 1997 & Wang et al, 1996) effectively catalyzes the synthesis of peptides giving > 90% yield by accepting both L and D amino acid amides as nucleophiles. Thermolysin-CLECs (Rose et al, 1995) were efficient in the synthesis of several peptides in 90% EtOH and were used in a multicycle preparation of aspartame in ethyl acetate for 18 cycles, but the soluble enzyme can hardly be used for 2 cycles (Scheme 1.5). The above two CLEC proteases are commercially available as pepti CLEC-BL (Subtilisin from *Bacillus licheniformis)* and pepti CLEC-TR (Thermolysin from *Bacillus thermoproteolyticus)*, respectively, from Altus Biologics,Cambridge, USA.



Scheme 1.5. Synthesis of peptides an d peptidomimetics

1.11.1.2 Chiral resolution applications

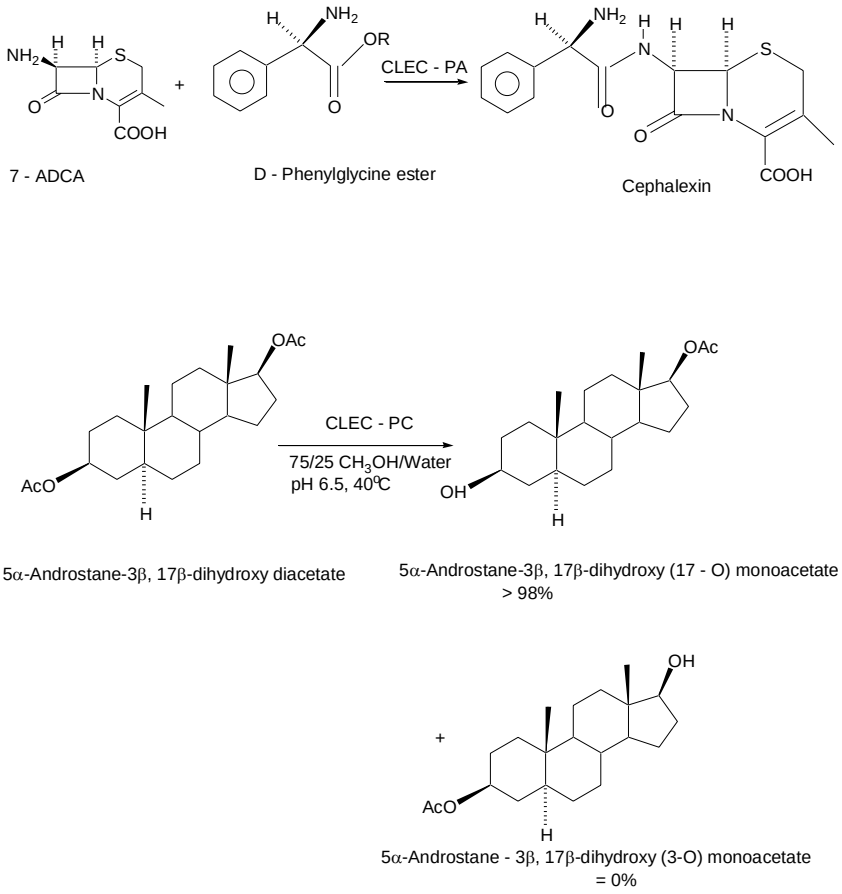
The major use of enzymes in biocatalysis is for the resolution of racemates to obtain enantiomerically pure compounds. The commercial CLEC form of lipases and esterases are important in kinetic resolution in organic synthesis (Kazlauskas and Bornscheuer, 1998 & Chen et al, 1982)(Scheme 1.6). These enzymes are used to catalyze the enantioselective hydrolysis or synthesis of esters. The chief advantage of the CLEC form is in the resolution of alcohols via the stereospecific transesterification in organic media, or the hydrolysis of racemic esters, where high concentration of organic solvent or substrate is required. The rate of organic phase transesterification can be two orders of magnitude greater than that of the crude enzyme. CLEC lipase (Persichetti et al, 1996 & Lalonde et al, 1997 & St.Clair et al, 2000) (*Candida rugosa*) catalyzes the acylation of menthol and hydrolyse the arylpropionate esters, with a 4 to 50 fold increase in enantioselectivity over the crude lipase (Lalonde,1998) preparation. CLEC-subtilisin catalyzed resolution of sec-phenethyl alcohol and trans-sobrerol was studied by Colombo et al (1998) in organic solvents. Large-scale resolution (Collins et al, 1998) of the racemic mixture (R)(S)-sec- phenethyl acetate to R-sec-phenethyl alcohol was studied by Collin *et al* using Chiro CLEC-Lipase (PS)(Altus).



Scheme 1.6. Chiral resolution Applications

1.11.1.3 Chemo selective and regioselective transformations

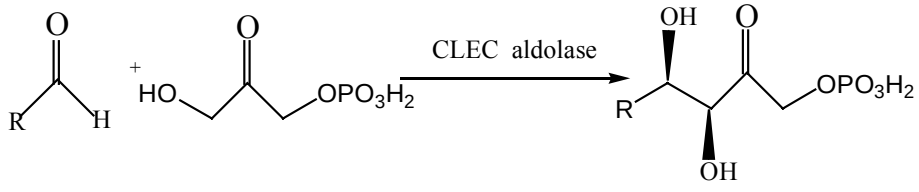
The chemical process for semisynthetic antibiotic manufacture requires extremely low temperatures and includes several steps for protection, activation, and deprotection that generate many times more waste than the product. The catalytic coupling of D-amino acids with 6-aminopenicillanic acid or 7-aminodesacetoxycephalosporanic acid (7-ADCA) derivatives exploits the chemoselectivity of the enzyme penicillin acylase (PA) (Collins et al, 1998). CLEC-PA catalyzes the direct coupling (Lalonde,1997) of D-phenylglycine ester or amine with 7-ADCA at room temperature in water at near neutral pH (Scheme 1.7). The CLEC form of PA is used at 1-2% by weight of enzyme and even after 1,000 batch reactions, the catalyst retained 70% of its original activity (Lanonde, 1998).



Scheme 1.7. Chemoselective and Regioselective transformations

1.11.1.4 Carbon-Carbon bond forming Reactions

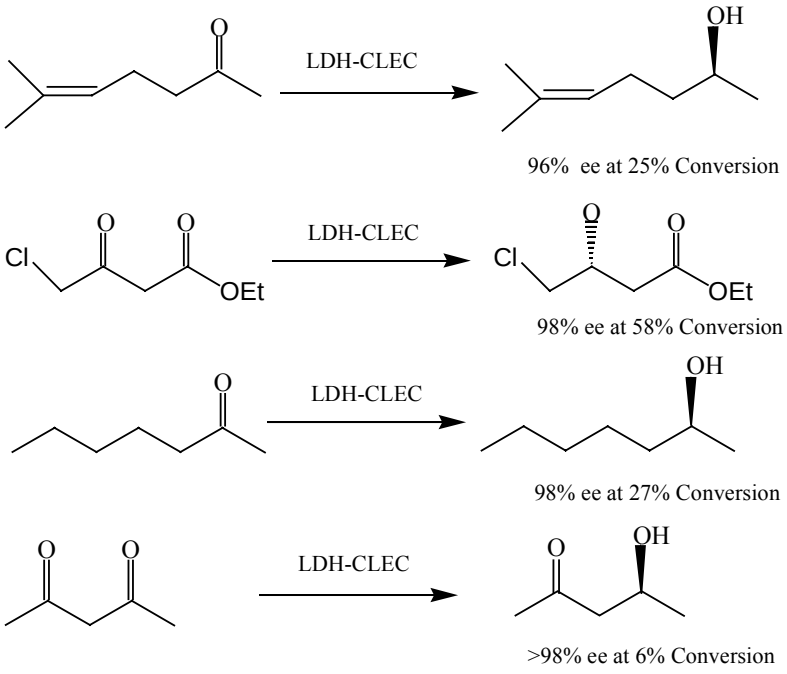
Carbon-Carbon bond forming Reactions are some of the most important transformations in organic chemistry. Sobolov et al (1994) reported that CLECs of fructose 1,6-diphosphate aldose from rabbit muscle are much more stable than the soluble enzyme. The synthetic potential of CLEC fructose 1,6-diphosphate aldolase was demonstrated by the preparation of a series of compounds by Carbon-Carbon bond forming.



Scheme 1.8. Carbon-Carbon bond forming using CLEC Aldolase

1.11.1.5 Reduction reactions using CLEC

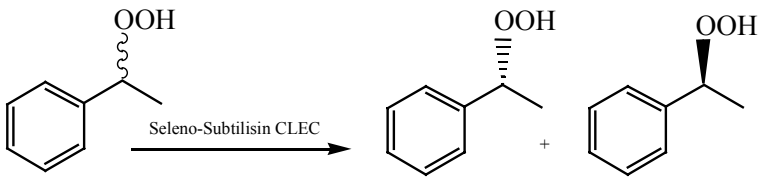
Reduction of achiral precursors is often used to produce chiral products. The advantage of this approach is that the theoretical yield of product is 100% compared to the 50% theoretical maximum for the resolution of racemates. CLECs of lactate dehydrogenase (LDH) have been used to prepare L-lactic acid from pyruvic acid in an electrolytic cell. The LDH CLECs maintained constant activity over 25 days and were much less sensitive to pH than the soluble enzyme. CLECs of horse liver alcohol dehydrogenase (Michael, 2001) used to reduce a number of ketones to chiral alcohols (Scheme 9). This CLEC exhibited the activity similar to the soluble enzyme and were more stable toward heat and the presence of alcohols.



Scheme 1.9. Reduction of ketones using LDH-CLEC

1.11.1.6 Resolution using semisynthetic seleno-subtilisin CLEC

Haring and Schreier have modified the active site of subtilisin cross-linked enzyme crystals by introducing selenium into it and thereby converting the enzyme into a peroxidase (Haring and Schreier, 1998). The kinetic resolution of racemic 2-hydroxy-1-phenylethyl hydroperoxide was done using the semisynthetic CLEC (Scheme 1.10). The reaction time was 25-30 minutes with an ee of 97%. The semisynthetic CLEC was reused for 10 times.



Sheme 1.10. Kinetic resolution using seleno-subtilisin CLEC

1.11.2 Biosensor Applications of CLEC

A biosensor is a device that detects, transmits, and records information regarding a biological change. Biosensors integrate a biological component with an electronic transducer, there by converting a biochemical signal into an electronic response. Generally, biosensors are immobilized molecules connected to some type of optical, electrical, electromagnetic, or chemical signal transducer that produces a signal in the presence of an analyte biomolecule. The function of a biosensor depends on the biochemical specificity of the bioactive material. CLECs exhibit the highest protein density and specific activity, and will produce clear signal even in the presence of small amounts of substrate. In the analysis of samples with a biosensor, it is particularly desirable to produce the largest possible detectable signal from the smallest possible quantity of substrate and catalyst. CLEC formulations (Navia and St.Clair, 1999(a)) may be used as the biosensor component, which detects an analyte of interest in a sample. CLECs allow improved contact between itself and analyte of interest in both aqueous and organic media. A CLEC-based Glucose oxidase (Maltos etal, 2001) biosensor can be used to measure the glucose level in blood. CLEC urease (Navia and St.Clair, 1999(a)) from Jack-bean can be used in clinical biosensor applications to measure urea levels in the circulatory fluid as an early indication of renal disease. In our laboratory, we have developed cross-linked enzyme crystals of Horseradish peroxidase, which can be used in a biosensor to monitor organic pollutants.

1.11.3 Biomedical Applications

Most of the drugs in research and development are peptides or peptide-like synthetic organic compounds. These peptide drugs are quickly broken down into smaller units in the gut, mostly by proteolytic enzymes in the stomach. The improved stability of CLECs towards proteolysis may make these compounds attractive alternatives to intravenously administered peptide and peptide-like drugs.

Protein/peptide crystalline drugs are carrier free, pure, stable, and storable at room temperature. Crystals are the most concentrated form of proteins and are useful as certain drugs, such as antibodies (St. Clair etal, 1999), which require high concentrations at the delivery stage. CLECs are ideally suited for diagnostic application because of their greatly enhanced stability and excellent bioactivity. The CLEC system functions as a self-delivery vehicle for drugs incorporating high concentration of therapeutic agents into a non-dissolvable or biodegradable crystal matrix.

Enzyme therapy such as lipase therapy (Navia and St.Clair, 1999(b)) can be performed by administering cross-linked lipase crystals orally. The digestive disorders linked with cystic fibrosis or pancreatitis can be traced to improper levels of lipase enzymes in the duodenum. However, CLEC formulations of such therapeutically beneficial enzymes and other proteins can be used in the gut lumen without being degraded by extremes of pH or endogenous protease action. CLEC glucose oxidase/peroxidase test strips can be used as a diagnostic reagent to detect the level of glucose in blood.

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CHAPTER II

PREPARATION AND CHARACTERIZATION OF CROSS-LINKED ENZYME CRYSTALS OF LACCASE

2.1 Introduction

Laccase (Xu, 1999) is a versatile enzyme belongs to the oxidases group. Laccase (benzenediol:oxygen oxidoreductase, (E.C 1.10.3.2) a polyphenol oxidase belongs to the family of blue multi copper-oxidases. It is one of the blue copper oxidases, which catalyze the four-electron reduction of oxygen to water. Laccase differ from peroxidase in that it does not require hydrogen peroxide to oxidize substrates, which makes it superior to peroxidases. Here the electrons are transferred to molecular oxygen (O_2) yielding water. Laccases are widely spread in fungi and higher plants and few reports are about bacterial laccase.

2.1.1 Laccase General Properties (Xu, 1999)

Most of the laccases are extracellular and some of them are intracellular found in fungi and Insects. The pI value is 3-7 for fungal laccases and 3-9 for Plant laccases. The Molecular Weight ranges from 60- 100 Kda. The redox potential was found to be 780mV. Laccase can oxidize organic compounds like anilines, thiols, phenols (diphenols and amino phenols) and strongly prefers O_2 as its oxidizing substrate

2.1.2 History and Occurrence of Laccase

a) Plant Laccase

The laccase enzyme was first discovered from the juice of the Japanese lacquer tree *Rhus Venicifera* by Yoshid etal (1883). The other plants (Table 2.1) producing significant amount of laccase (Mayer et al, 1966,1979,1987,2002 & Benfield et al., 1964) are *Azer pesudoplatanus, Nicotiana Tobaccum,* and *Pinus taeda*.

Table 2.1 Plant sources of Laccase

Plant Source	Presence of Laccase
Rhus Vernicifera	Latex of the Tree
Populus euramericana	Xylem tissue
Rhus succedanea	Latex of the tree
Azer pseudoplatanus	Cell Culture
Pinus taeda	Xylem Tissue
Nicotiana Tobaccum	Leaves
Aesculus parviflora	Leaves

Plant laccases (Solomon et al, 1996) are extracellular monomeric proteins with 22-45% glycosilation. Plant laccase has four Cu^{++} ions per molecules. *Rhus Vernicifera* is found to be not involved in lignin biosynthesis, since it cannot oxidize monolignols. Laccase catalyze the polymerization of Urushiol into lacquer, but the dominant products of the partial oxidation of urushiol by *Rhus Vernicifera* laccase are biphenyls and aryl side chain products. Aryl side chain products are thought to be formed by the electrophilic substitution of a heptatrienyl cation on the urushiol ring. The mechanism for the formation of biphenyl products are believed to be radical substitution of the semiquinone formed by the one-electron oxidation of Urushiol by laccase with another molecule of urushiol. Most of the plant laccases are capable of oxidative coupling monolignols to dimmer or trimer. Plant laccase activity was found in the xylem cell walls of a wide variety of plant species. The plant enzyme being intra-cellular may be expected to have its pH optima nearer to the physiological range. The difference in pH optima may point also to the difference in function. The plant enzyme may be involved in the synthetic process of lignin formation.

b) Fungal Laccases

The important source of fungal laccases (Xu, 1999) are *Trametes versicolor,Pleurotus ostreatus,Pholiota mutabilis, Pycnoporus cinnabarinus,Aspergillus spp.,Coriolus hirsutus* and *Russula delica* and *Russula foetens* (Mushroom)

c) Bacterial Laccase

Bacterial laccase has been extensively studied in *Azospirillum lipoferum* and its mutant strains (Grigorious et al, 2000 & Faure et al, 1994). These bacteria are widespread in soil and in the rhizosphere of a variety of grasses and cereals.

2.1.3 How to differentiate Laccase from other oxidases

Laccase catalyses the oxidation of various polyhydric phenols but does not oxidize p-cresol and tyrosine. In addition, that resorcinol is not oxidized by laccase. Laccase shows absorption peaks at 280 and 600 nm and a shoulder at 330 nm. The type 1 copper atom in laccase, which is responsible for the intense blue color of the enzyme, has a strong electronic absorption around 600 nm. The two type 3 copper atoms that form a binary complex, shown a strong absorption shoulder at 330 nm. Type 2 and Type 3 sites constructed a trinuclear copper cluster center.

Laccase and catechol oxidase are copper oxidases but can be differentiated by the use of cinnamic acid derivatives which inhibits selectively the catechol oxidase and cationic detergents such as CTAB which will inhibit selectively the Laccase. Tropolone acting as a Cu^{++} chelator is very effective in inhibiting catechol oxidase at micro molar concentration. However, laccase is not inhibited by Tropolone. Catechol oxidase catalyses the oxidation of monophenols and o-diphenols to the corresponding quinones, via the formation of a hydroxylated intermediate in the case of monophenols. However, laccase will oxidize only diphenols not monophenols.

2.1.4 Laccase substrates & inhibiters

Laccase has broad substrate specificity towards aromatic compounds containing electron-withdrawing groups like hydroxyl and amine. This enzyme reacts with many substrates under H-atom abstraction and formation of radicals, which may undergo further non-enzymatic reactions leading to the often-observed polymerized products. The characteristic substrates are ortho and para diphenols which are oxidized by the enzyme to ortho and para quinones respectively. Fungal laccase can oxidizes phenolic compounds that are not diphenolics as for example phenolic aldehydes, acids or alcohols in which laccase catalyze the break down of the C-aryl-C alkyl bond that explains it's activity in lignin metabolisum. Laccase oxidize a broad range of substrates (Table 2.2) such as phenols, methoxy-substitued phenols,diamines and some inorganic compounds like potassium ferrocyanide. Syringaldazine is the best substrate for the qualitative detection of laccase in fungi, because Syringaldazine is non autooxidizable (in the absence of H_2O_2) laccase specific, very sensitive substrate (ε_{max}= 65000 M^{-1} cm^{-1}). ABTS (non phenolic) is another good substrate for laccase and forms ABTS radical. Hence, ABTS is found suitable for screening its one electron oxidation product, which is soluble in water, stable and intensely green.

Table 2.2 The substrates and their products formed by the laccase

Substrate	**Product**	**Color development**	**λmax(nm)**	**$\varepsilon_{max}(M^{-1}cm^{-1})$**
Catechol	o-benzoquinone	Yellow	450	2,211
Hydroquinone	p-Benzoquinone	Yellow	248	17,252
Pyrogallol	Purpurogallin	Yellow	450	4,400
2,6dimethoxy phenol	3,5,3',5' tetramethoxy diphenoquinone	Yellow	468	35,645
Guiacol	Biphenoquinone	Yellow	470	26,600
ABTS	$ABTS^{\cdot+}$	Blue	420	36,000
Syringaldazine	Quinones	Purple	525	65,000
Catechin	o-quinone	Yellow	390	4019

Inhibiters

The inhibitors of laccase are small anions, halides (exclude Iodide), azide, cyanide, Metal ions (Hg^{2+}), fatty acids, sulfhydryl reagents, and quaternary ammonium detergents.

2.1.5 Structure of Laccase

Laccase is a glycoprotein constitutes 10-45% of carbohydrate moieties including hexosamine, glucose, manose, galactose, fucose and arabinose where mannose is a major component (Bligny et al, 1986). Laccase contains about 500 amino acid residues. The molecular weight of laccase from various sources varies and the major discrepancies may well relate to the carbohydrate moiety of the molecule. The laccase as an active holoenzyme form is a dimeric or tetrameric glycoprotein, which contains four copper atoms per monomer bound to three redox sites. Copper sites have historically divided into three classes, copper type 1(T1) or blue copper, type 2(T2) or normal copper, type3 (T3) or coupled binuclear copper center based on their spectroscopic features, which reflect the geometric and electron structure of the active site (Yaropolov et al, 1994). The four Copper atoms differ from each other in their characteristic electronic paramagnetic resonance (EPR) signals. The three types of Copper atoms differ in accessibility to solvents, in spectra, and in

the features of their surroundings. Copper of the first type (copper 1) is available to the action of solvents, including water. Copper of type 2 was proven to play an important role in structural nonspecific stabilization of anionic binding in the copper 3 active site (Thurston,1994).

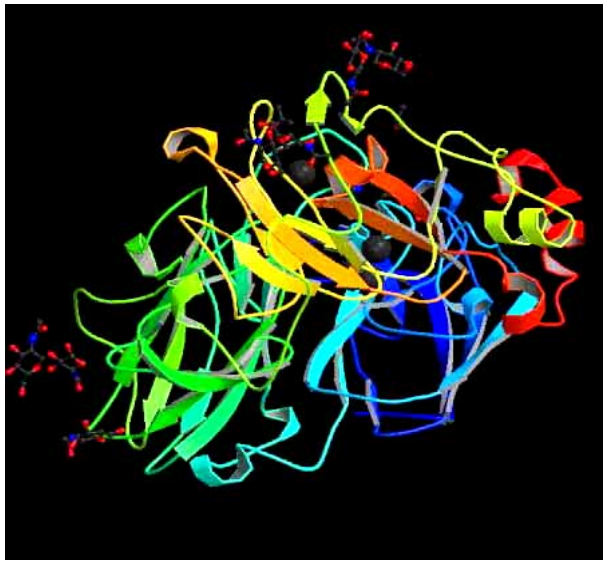
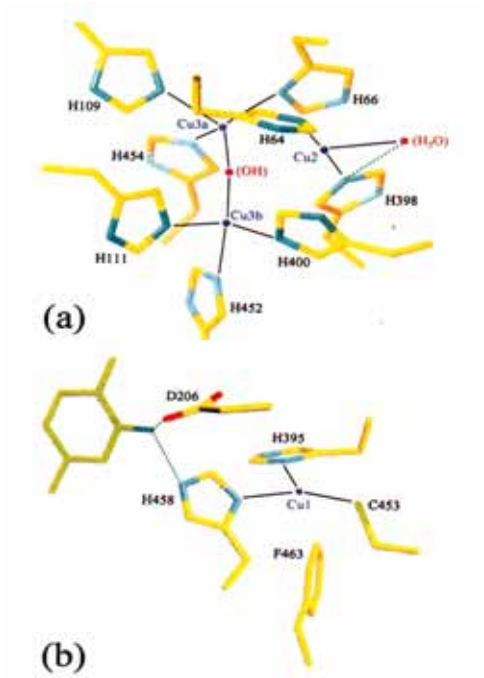


Fig 2.1 3D structure of laccase from *Trametes versicolor* (PDB code: 1GYC)

2.1.5.1 Active site of Laccase

The interaction of an enzyme with its substrate can be illustrated by the lock-and-key model. Only a substrate (the lock), which can dock to the active center (the key), will be converted at the catalytic center, which is a part of the active center. The active site of laccase consists of four Copper ions of three types involved in a coordinated oxygen reduction. The cavity of the active site having the dimensions (10 X 10 X 20 Å) approximately (Thomas et al, 2002). The T1 site contains the type 1 blue copper which is tightly coordinated to a cysteine or methionine (depending on the laccase source) responsible for an intense absorption band around 600 nm ($\varepsilon \approx 5000\ M^{-1}cm^{-1}$) and an electron paramagnetic resonance (EPR) spectra (A_{11}= 43-95 X 10^{-4} cm^{-1}) with narrow hyperfine splitting giving its blue color to the enzyme. The type 1 copper (T1) is coordinated with N-atom of His-458 and His-395 and sulphur atom of Cys-453. The type 2 copper (T2) atom is coordinated with the amino acid residues His 398 & His 64 and a water molecule (H_2O) has a charecteristic (EPR) signal (A_{11}= 158-201 X 10^{-4} cm^{-1}) (Solomon et al, 1996).

In the type 3 (T#) copper pair, each copper is coordinated with the N-atom of three Histidine residues. Cu 1 (T3) is coordinated with His 109, His 454 & His 64 and Cu 2 (T3) is coordinated with His 111, His 452 & His 400. The T3 copper pair has an absorption band around 330 nm (($\varepsilon \approx 5000$ $M^{-1}cm^{-1}$) but it is EPR silent, indicative of strongly antiferromagnetically coupled T3 copper pair bridged by a hydroxide. This enzyme couples the oxidation of the substrate, which takes place at type 1 copper site with the reduction of dioxygen to water, taking place at the type 2 and type 3 trinuclear copper cluster site. The reduction of enzyme by the substrate (no color) and oxidation by oxygen (return to blue color) can be followed visually and spectrophotometrically.



(a) The trinuclear T2/T3 site (b) T1 site

Fig 2.2 Active site of Laccase (Thomas et al, 2002)

2.1.5.2 Structural classification of Trametes versicolor laccase

1. Class: All beta proteins
2. Family: Multidomain cupredoxins
3. Superfamily:Cupredoxins contains copper-binding site
4. Fold:Cupredoxin-like consists of three domains of this fold sandwich; 7 strands in 2 sheets, greek-key variations: some members have additional 1-2 strands
5. Amino acids 499 and Lysine residues 39

2.1.6 Catalytic Mechanism of Laccase

Substrate oxidation by laccase is a one-electron reaction generating a free radical (Yaropolov et al, 1994). As one electron substrate oxidation is coupled to four-electron reduction of oxygen, Laccase must operate as a battery, storing electrons from individual oxidation reactions in order to reduce molecular oxygen. The initial step of catalysis by laccase was shown to involve a step of one electron transfer to a specific copper-containing site to form a free radical from an organic substrate. The initial step of oxygen reduction to water consists of the transfer of two electrons from the reduced dyad of type 3 copper ions to form O_2^{2-}. This is followed by one electron transfer from type 1 copper to oxygen intermediate to form water molecules and O-radical. A shift in redox state of the enzyme can stabilize O- radical. The fourth electron passes from copper 2 ion to O- radical to form the second water molecule.

$$Ph\text{-}OH + Cu^{2+} \xrightarrow{\text{Laccase}} Ph\text{-}O^{\cdot} + Cu^{+} + H^{+}$$

$$2\,Cu^{+} + \tfrac{1}{2}\,O_2 \longrightarrow 2\,H^{+} + 2\,Cu^{2+} + H_2O$$

The overall catalytic mechanism of laccase has given in Fig 2.3.

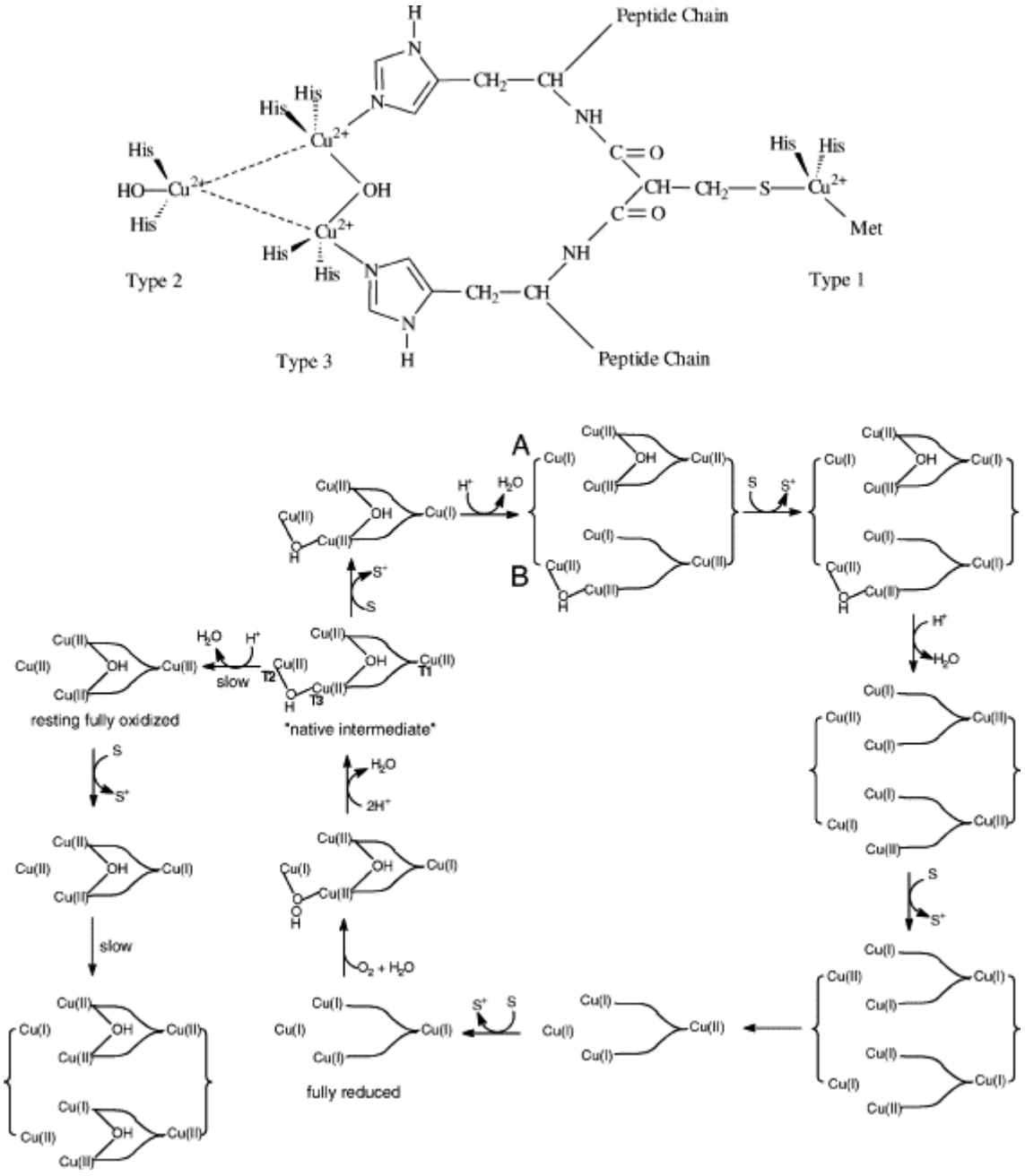


Fig 2.3 Catalytic mechanism of Laccase (Duran et al, 2002)

Role of mediators in laccase catalysis

A mediator could be a small molecule that acts as a sort of 'electron shuttle': once it is oxidized by the enzyme, it diffuses away from the enzymatic pocket and in turn oxidises any substrate that, due to its size, could not directly enter the enzymatic pocket (Banci et al, 1999). Alternatively, the oxidized mediator could rely on an oxidation mechanism not available to the enzyme, thereby extending the range of substrates accessible to it (Hildén et al, 2000). ABTS, TEMPO (tetra methyl pyppiridine N-oxide), HAA (Hydroxy anthranilic acid), VLA (violuric acid), HBT (1, hydroxy benzotriazole) were found to be the best mediators for laccase enzyme (Johannes and Majcherczyk, 2000).

2.1.7 Application of Laccase

a) Delignification

Conventional methods of delignification or decolorization of paper pulp involves either chlorine or oxygen based chemical oxidants (ClO_2 or O_3) and they are cost effective. These methods have serious drawbacks such as disposal of chlorinated byproducts and the loss of cellulose fiber strength. Microbial or enzymatic delignification systems that overcome these drawbacks can be easily adapted to current pulp production lines as attractive alternatives. Various laccasees have been shown capable of degrading either natural lignin (wood chips or kraft pulp) or synthetic lignin models (Mikhail et al, 2001). These laccases could directly either oxidize phenolic components exposed in lignin or in the presence of proper redox mediator, can indirectly oxidize heterogeneous phenolic and nonphenolic components (Sealey and Ragauskas, 1998 & Call and Mücke, 1997). As a result of laccase oxidation, radicals could be generated in lignin, resulting in subsequent aliphatic or aromatic C-C bond cleavage and depolymerization.

b) Production of fuel ethanol from renewable raw material

To improve the production of fuel ethanol from renewable raw materials, laccase from *Trametes versicolor*, was expressed under control of the PGK1 promoter in S.cerevisiae to increase its resistance to phenolic inhibitors in lignocellulose hydrolysates (Larsson et al., 2001). The results show that there was a definite advantage of using laccase expressing yeast strains for producing ethanol from lignocellulose.

c) Wine clarification

Many groups of phenolic compounds (Cantarillic et al, 1986) are found in wines; cinnamic acid derivatives and catechins are present in different quantities in all wines, while rose and red wines are characterized by anthocyanidins. Polyphenol removel should be selective to avoid any undesirable alteration in the wine's organoleptic characteristics. These polyphenolic would be oxidized by enzyme, polymerized and then removed by clarification. Laccase from *Trametes versicolor* (Minussi et al, 1998(b)) has great potential for phenolic compound removal from wines. More than 90% ferulic acid (Minussi et al, 1998(a)) was removed from a model solution and 34% removal of phenolic compounds from wines was obtained. Laccase immobilized on copper-chelate carrier that can be regenerated was used successfully to remove phenols from white grap must(Servili et al, 2000). The phenols especially epicatechin, ferulic acid and o-coumaric acids were partially removed from wine by the enzymatic treatment.

d) Bioremediation of organic pollutants

Laccase has been used to oxidatively detoxify or remove various aromatic xenobiotics and pollutants found in industrial waste and contaminated soil or water. The laccase catalysis could result in either direct degradation or polymerization. Bioremediation of pollutants include dechlorination of chlorophenols (Dec and Bollag, 1995) mineralization of polycyclic aromatic hydrocarbons (Bezalel et al, 1996), decolorization of pulp or cotton mill effluent (Davis and Burns, 1990), and bleaching of textile dyes. Laccase catalyzed textile dye-bleaching (Chivukula and Renganathan, 1995) may also be useful in finishing dyed cotton fabric. Replacing conventional chemical oxidants (e.g. hypochlorite), a laccase-based system has been shown capable of bleaching indigo dye in denim and achieving various bleached appearances in the fabric (Pedersen and Kierulff, 1996).

e) Dye decolourization

Even though azo dyes are not good substrates for laccase, it can be degraded by the enzyme. Wong and Yu (1999) reported that laccase could degrade azo dye through the mediation of small molecule metabolites. The mechanism of laccase catalyzed dye degradation is not yet clear (Rogaski et al, 1991). It catalyses the oxidation of organic pollutants by the reduction of molecular oxygen to water in the absence of hydrogen peroxide (Thurston, 1994). The activity of *Trametes versicolor ATCC 48424* in the form of

complex pellets showed high rate of decolorization of an azodye, Acid violet 7 (Zhang and Yu, 2000). *Trametes versicolor ATCC 20869*, which produces laccase and MnP was able to decolorize Amaranth dye (Swamy and Ramsay, 1999). The laccase secreted by *Pcynoporous sanginues* was used for the partial *decolorization* of Orange G and Amaranth (Pointing and Vrijmoed, 2000). In our work, a mixture of textile azo dyes were degraded in a Packed Bed Reactor (PBR) (Resmi et al, 2003) and Rotating Biological Contactor (RBC)(Emilia et al, 2003) using a microbial consortia immobilized on lattrite pebbles. The consortium was found to produce laccase enzyme, which could degrade the azo dyes.

f) Medical and personal care applications

Laccase has been shown to oxidize, polymerize and detoxify urushiol, thus reducing the effect of poison ivy dermatitis (Hendrix and Duhe, 1981). Laccase can oxidize iodide to produce iodine which is a reagent widely used as disinfectants (generation of iodine in situ) (Xu, 1996). Current hair dyeing or waving processes often involve oxidative or additive chemicals either have unpleasant odors, are irritant to tissues, or difficult to handle. A laccase-based system may overcome these drawbacks by replacing harsh chemicals and operating at milder conditions (in terms of pH and solvent). Laccase catalyzed oxidation, transformation and cross-linking of various precursors have been reported to result in satisfactory hair dyeing or waving. Laccase based system may also improve or complement the cosmetic effect (in terms of color type, shade, and compatibility with hair type) achieved by conventional chemical methods (Tsujino et al, 1991).

g) Biosensor and Biofuel cell Application

The oxidation catalyzed by Laccase could be useful in biosensor for detecting O_2 and wide variety of reducing substrates (especially phenols and anilines). Two types of laccase based O_2 sensor is widely used. One type monitors visible spectral changes (at 600 nm) of laccase resulting from the reoxidation of the type 1 copper (I) in laccase by O_2 (Gardiol et al, 1996). Another type monitors current or voltage change from a modified oxygen electrode on which O_2 reduction is enhanced under the electrocatalysis of immobilized laccase (Yaropolov, et al, 1994). For detecting phenols, anilines, or other reducing substrates, three types of laccase-based sensors have reported. One type detects the photometric change resulted from the oxidation of a chromogenic substrate, another type uses an electrode that replaces O_2 as the acceptor for the electron flown from the substrate (Yaropolov, et al,

1994). For these applications, laccase is either immobilized or as free solution, and the coupled to redox mediators and physical converter is either optic, amperometic, or piezo electric crystal or a field-effect transitor or thermister, etc.

Electrical contacting of redox enzymes with electrode supports attracts research efforts directed to the development of biofuelcell elements. Emerging application of biofuel cells are in powering miniaturized implanted medical devices and chemical syntheses of pharmaceuticals and fine chemicals (Wong and Schwaneberg, 2003). Laccase based miniature biofuel cell has been developed, which operates at a highest voltage of 0.78 V (Mano et al, 2003)

h) Synthesis of various organic compounds especially polymers.

Laccase catalysis can be used to synthesize various functional organic compounds, which include polymers with specific mechanical, electrical, or optical properties, textile dyes (Baker et al, 1996), cosmetic pigments (Junino and Martin, 1994), flavor agents (Falconnier et al, 1994), pesticides and heterocyclic compounds (Eggert et al, 1995). Laccase can be used to synthesis several complex medicinal agents, which include triazolo(benzo) thiadiazines (a group of anti-inflammatory, analgestic, central stimulant/depressant antisecretory and sedative depressant agent) (Bhalerao et al, 1994), vinblastine (a cytostatic, antitumor drug) (Yaropolov et al, 1994), penicillin X dimmer (Agematu et al,1993), cephalosporin antibiotics (Agematu et al,1993) and dimerized vinolidine (a plant alkaloid for treating neoplastic diseases) (Sariaslani et al,1983).

2.1.8 Need for the development of CLEC Laccase

Laccase has been already crystallized for the structural elucidation (Ducros et al, 1998 & Thomas et al, 2002). There is no report available on the cross-linked enzyme crystals of laccase. This is for the first time we have made cross-linked enzyme crystals of laccase. The aim of the work is develop CLEC of laccase that can be suitable for biosensor and biotransformation applications.

2.2 Material and Methods

2.2.1 Materials

Laccase (E.C.1.10.3.2) from *Trametes versicolor* was purchased from Fluka (0.8 U/mg,Catechol units) and Glutaraldehyde, ABTS (2,2'-Azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid)), Aerosol OT, β-cyclodextrin, 18-crown-6 ether were purchased from Sigma (St.Louis,USA). Ammonium sulphate and other organic solvents used were in analytical grade.

2.2.2 Methods

2.2.2.1 Preparation of Cross-linked laccase crystals

Crystallization of Laccase

Laccase was crystallized by batch method. 50 mg of crude laccase was dissolved in 3 ml of 0.1M Sodium Acetate buffer at pH 5.6. Ammonium sulphate (65-85% saturation) was added in small portions over 3 hours by stirring. Then the super saturated solution was kept at 5° C for 20 hours. The crystals were separated by centrifugation at 2000 rpm for 8 minutes.

Cross-linking of Laccase crystals

The laccase crystals were cross-linked using glutaraldehyde (0.5- 3.0%) (v/v) solution in isopropanol for 20 minutes at 25°C. After cross-linking, it was washed 3 times with 0.1 M sodium acetate buffer pH 5.6 and stored at same buffer at pH 5.6.

2.2.2.2 Laccase assay measurement

The laccase assay (Eggert et al, 1996) was done using ABTS (5 mM final concentration) as substrate in 0.1 M sodium acetate buffer pH 5.0. In 3ml assay volume, 2 ml of buffer and 750µl of 20mM ABTS and 250 µl of enzyme solution were taken, the optical density of the oxidation product was read at 420 nm (ε_{max} = 36000 $M^{-1}cm^{-1}$).

2.2.2.3 Estimation of Protein

The determination of protein was carried out by Lowry's method (Lowry et al, 1951) BSA was used as the standard, and the absorbance was read at 660 nm. The cross-linked enzyme crystals were washed thoroughly to remove excess reagents before estimation.

2.2.2.4 Assay of CLEC Laccae

Assay of CLEC laccase was done using ABTS (5mM final concentration) as substrate. Instead of directly taking measurement, the assay mixture (in 3ml assay volume, 2.25 ml of buffer and 750µl of 20mM ABTS and 1 mg of CLEC Laccase) containing the CLEC laccase was stirred continuously for 1 minute and then the increase in absorbance was monitored at definite time intervals at 420 nm. One unit of Laccase is the amount of enzyme needed for the conversion 1 µmol of substrate to its product.

$$\% \text{ of activity yield} = \frac{\text{CLEC activity (after crystallization and cross-linking)}}{\text{Total initial activity}} \times 100$$

2.2.2.5 Surface area

Surface area was measured by the BET (Brunauer, Emmett and Teller) technique using a Zetasizer (Melvern, UK) in which liquid nitrogen was the adsorbent.

2.2.2.6 Crystal structure

The crystal structure was observed under a scanning electron microscope (JEOL, Japan) at 10 kV accelerating voltage, after sputtering with gold.

2.2.2.7 pH profile of CLEC laccase

pH profile of CLEC laccase was determined by assaying it in different pH(3.0-8.0) of buffer.

2.2.2.8 Effect of additives on the activity of CLEC Laccase

1mM conc. of 18-crown-6 ether, AOT (Aerosol OT), β-cyclodextrin were mixed with CLEC Laccase in aqueous medium and lyophilized till dry.

2.2.2.9 Thermal Stability

Thermal stability of CLEC laccase and soluble Laccase was determined by incubating 1mg of CLEC/soluble enzyme in 2.0 ml of 0.1 M acetate buffer pH 5.0 at various temperatures ranging from 40 -70°C. The thermal inactivation constant was determined from the equation $K = A \exp(-E_D/RT)$

E_D = Inactivation constant

R = Gas constant (8.314 KJ mol^{-1} K^{-1})

A plot of lnK Vs 1/T was drawn. The slope of the straight line gives the value of E_D/R

2.2.2.10 Kinetics of thermal inactivation

Kinetics of thermal inactivation of CLEC and soluble Laccase were studied at different temperature between 40-70 ±0.1°C in a Shaking water bath (Julabo, Germany). 1mg of CLEC/ native enzyme was added to 2.0 ml of 0.1 M acetate buffer pH 5.0 and kept in a constant temperature bath at the desired temperature. After every half an hour interval, the assay of the biocatalyst was carried out by rapidly cooling the reaction mixture to room temperature.

The thermal inactivation constant was determined from the Arrhenius equation (Espenson, 1981).

$K = A \exp(-E_a/RT)$ Where, E_a = Energy of activation

R = universal Gas constant (8.314×10^{-3} KJ $Kmol^{-1}$ K^{-1})

A plot of Log % residual activity Vs time was drawn (Fig 11) at different temperatures, the slope gave the values of inactivation rate constant k_r (Gouda et al, 2003).

2.2.2.11 Activation energy calculation

The thermal stability of CLEC and soluble laccase was determined by the inactivation rate constant (k_r) as a function of temperature, in the range 40-70 °C. the temperature dependence of k_r was analyzed from Arrhenius plot (ln k_r Vs 1/T), The activation energy (Ea) was obtained from the slope of the plot.

Half-life of CLEC and soluble laccase was calculated from the quadratic equation for each temperature.

2.2.2.12 Organic Solvent Stability

Organic solvent stability of the CLEC laccase was done by incubating 1mg of CLEC in various organic solvents and organic solvent-water (50%,75%, and 100%) mixtures for 30 hours. The organic solvents were chosen according to the hydrophobicity, dielectric constants, and log P values. After the incubation period, the assay of CLEC laccase was done by the procedure mentioned earlier. The activity was compared with the original activity and the activity relation was calculated.

2.3. Result and discussions

2.3.1 Properties of the cross-linked enzyme crystals (CLEC) of Laccase

Laccase enzyme was crystallized by batch crystallization method and the crystals obtained were of 2-25 μM in size (Figure 2.4) and channels were formed in between the crystals (Figure 2.5). The general properties of CLEC laccase was given in Table 2.3.

Fig.2.4 Scanning Electron Microscopic view of Laccase crystals

Fig 2.5. Channels formed between the laccase crystals

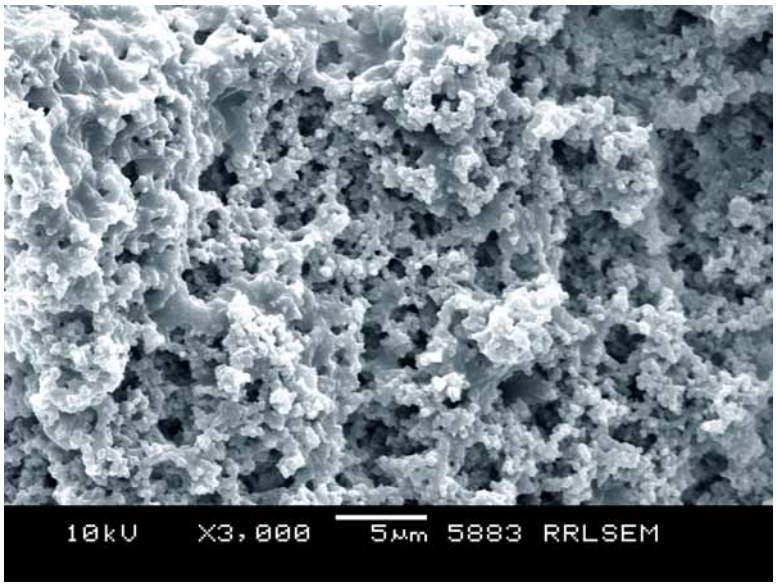


Table 2.3 General properties of CLEC Laccase

Property	CLEC Laccase
pH optimum	5.5
Thermal Stability	upto 60 °C
Specific activity	1612IU/mg crystal
K_m (ABTS)	0.8595 mM
V_{max} (ABTS)	2873.2 µmol/mg/min
K_{cat} (ABTS)	3.21 (S^{-1})
K_{cat}/K_m (ABTS)	3.73 x 10^3 $M^{-1}S^{-1}$
Crystal size	2–25 µm
Surface Area	2.456 m^2/g
Pore size	12-15 Å

Table 2.4 Enzyme activity in crystallization mother liquor

NH_4SO_4 cut off	Laccase activity (IU)
60%	186573
65%	32806
70%	14840
75%	**6790**
80%	13626
85%	13813

The maximum activity was retained at 75% ammonium sulphate saturation (Table 2.4) and the crystals formed in this condition were taken for cross-linking. The crystals were cross-linked with glutaraldehyde, the cross–links were formed between the ε-amino groups of the lysine residues of the enzyme and aldehyde group by forming a schiffs base. A glutaraldehyde concentration of 0.5 - 1.0 % (v/v) did not produce good cross-linked crystals and the crystals got dissolved in the assay mixture. The best glutaraldehyde concentration was found to be 1.5% for cross-linking and further increase in the concentration of

glutaraldehyde reduced the catalytic activity of the crystal (Figure 2.6). The total retention of enzyme activity after crystallization (75% NH_4SO_4) and cross-linking with 1.5% glutaraldehyde was 64%.

Fig 2.6 Cross-linked crystals activity of laccase at different Glutaraldehyde concentration

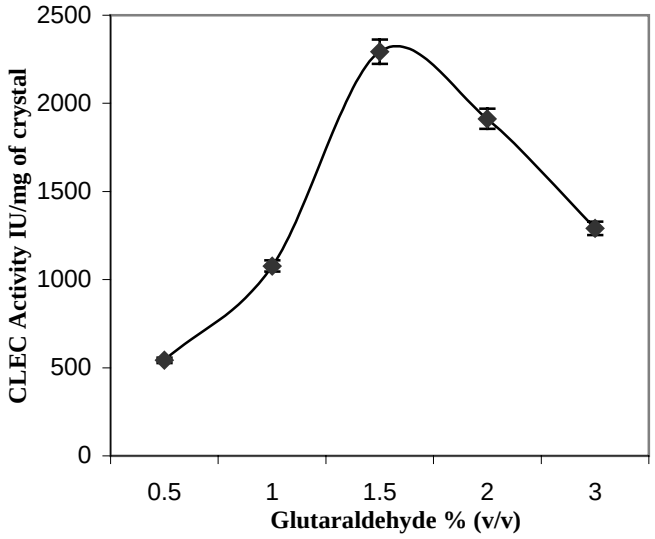


Fig 2.7 pH profile of CLEC Laccase

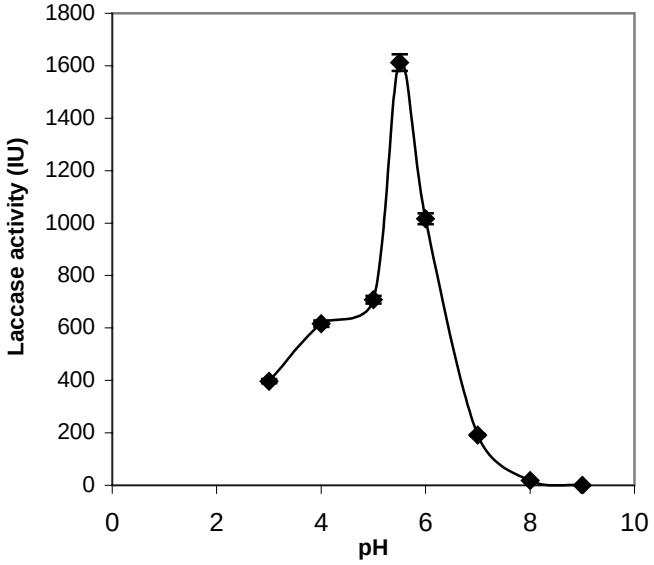
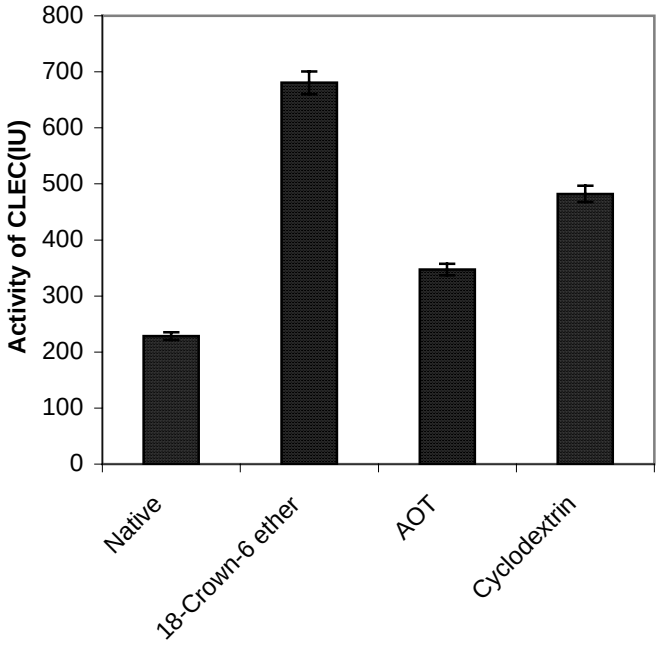


Fig 2.8 Effect of additives (1mM.Conc) on CLEC activity



CLEC Laccase showed a maximum activity at pH 5.6 (Figure 2.7) and the activity of CLEC was improved by addition of surfactant (1mM) and freeze-dried (Figure 2.8). Surfactants may help to maintain a better water balance and the native conformation of enzymes. Surfactants may also facilitate the transfer of hydrophobic substrate molecules through the layer of tightly bound water to the binding site of the enzyme (Lee and Dordick, 2002). 18-crown-6 ether was found to be the good additive, because it can complex with the ammonium functions of lysine residues and water molecules by hydrogen bonding (Gokel, 1991).

2.3.2 Thermal Stability of CLEC Laccase

Thermal stability of CLEC laccase was improved by 4 fold, after the cross-linking. According to earlier reports, the thermal stability of CLECs can be two or three order of magnitude greater than that of the soluble enzyme (Grim, 2001). The crystalline enzyme maintains its native conformation at elevated temperature and found to have lower tendency to aggregate. This is due to the fact that in the CLECs, the enzyme molecules are symmetrically arranged and hence their native conformation is stabilized. When an enzyme forms a crystal, a very large number of stabilizing contacts are formed between individual

enzyme molecules. Energy must be put into the system in order to disrupt these new contacts. Additional energy is required to break the covalent cross-links before the CLEC begins to dissolve and then denature. The relative activity of CLEC laccase increased more than 400% at 40°C (Fig 9) during the 1st 90 minutes of incubation and thereafter decreased slowly. After that, the activity was started decreasing and the half-life of CLEC laccase at 40°C was found to be 558 minutes. Compared to the CLEC laccase the relative activity of native enzyme was increased to 150% during the 1st hour of incubation at 40°C (Fig 10), thereafter it decreased rapidly.

Fig 2.9 Thermal Activation/Inactivation of CLEC Laccase at different temperatures

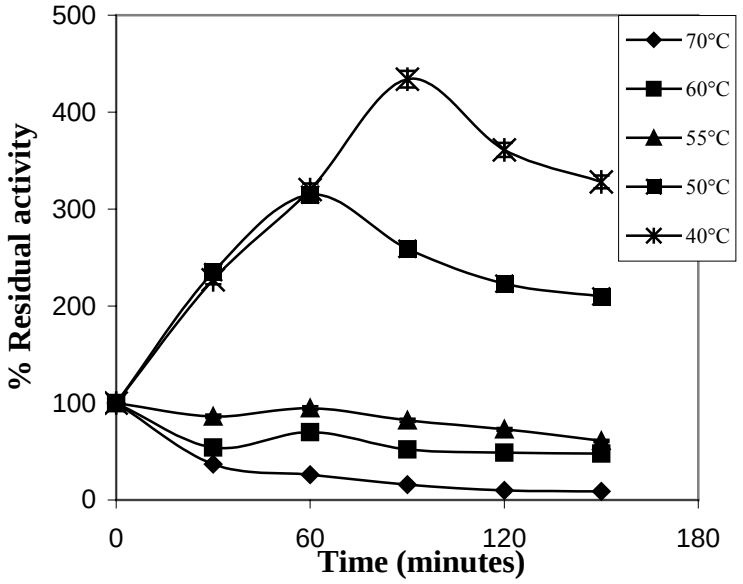
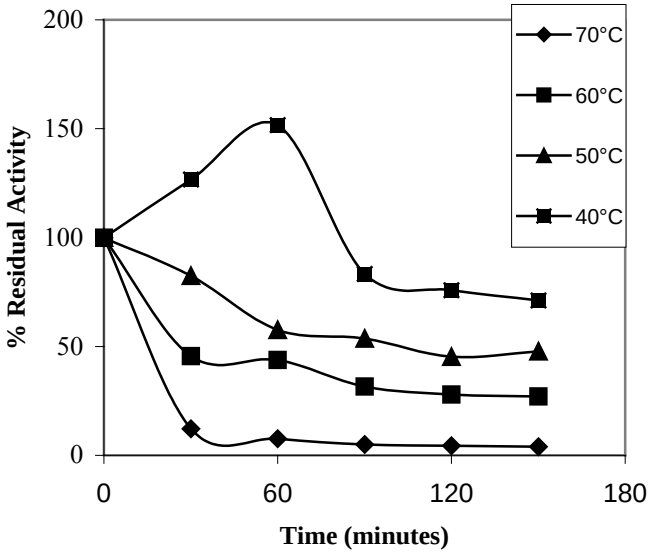


Fig 2.10.Thermal Activation/Inactivation of Soluble Laccase at different temperatures



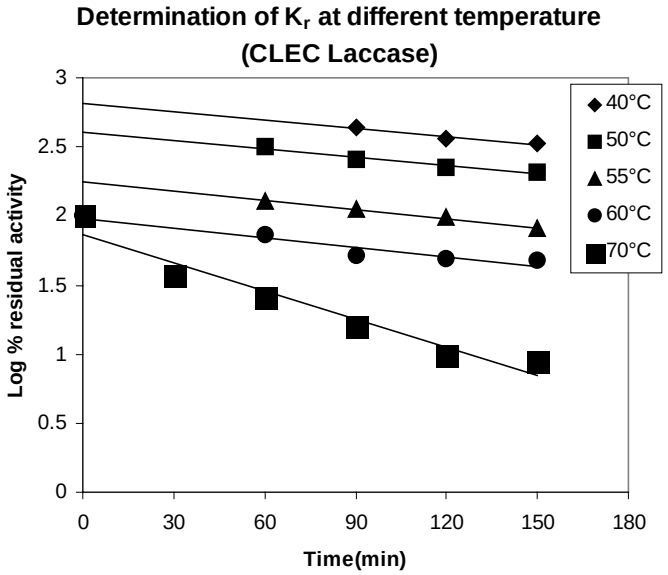
Above a temperature of 40°C, the activity started decreasing when the incubation time was increased. At 50°C, the activity increased to 300% during 1st 60 minutes of incubation, then it started decreasing. The half-life of CLEC laccase was found to be 453 minutes at 50°C and 229 minutes at 55°C. The thermal stability started decreasing above 55°C. The half-life of CLEC laccase was 123 minutes at 60°C and 25 minutes at 70°C, where as native laccase had a half-life of only 24 minutes at 60°C and 6 minutes at 70°C (Table 5).

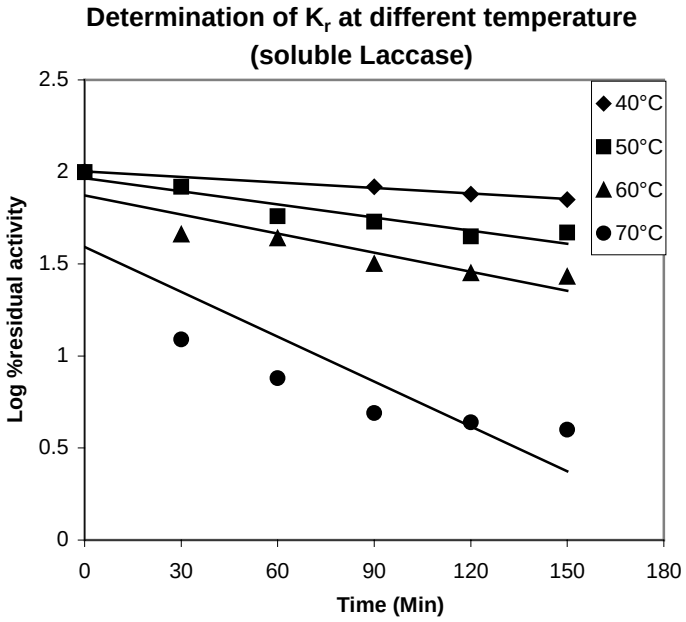
Table 2.5 Half-Life of Soluble and CLEC Laccase at various temperatures

Temperature	CLEC	Soluble Laccase
40°C	558 minutes	304 minutes
50°C	453 minutes	112 minutes
55°C	229minutes	55 minutes
60°C	123 minutes	24 minutes
70°C	25 minutes	6 minutes

` The activation energy (Ea) of CLEC and native laccase were found to be -31.59 KJ $Kmol^{-1}$. K^{-1}. and –55.04 KJ $Kmol^{-1}$. K^{-1}. CLEC laccase had activation energy of 14.45 KJ K mol^{-1}. K^{-1}. higher than that of native enzyme. A Plot of Log % residual activity Vs time was drawn (Fig 11) at different temperatures, the slope gave the values of inactivation rate constant k_r

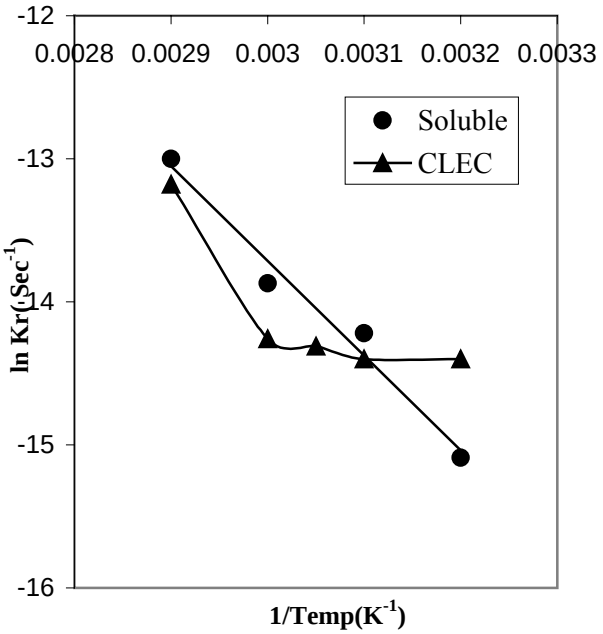
Fig 2.11 Determination of K_r at different temperatures





Arrhenius plot of thermal inactivation (Fig 12) showed that native enzyme had a steep inactivation curve from 70°C to 40°C. However, in CLEC laccase, there was a steep inactivation from 70°C to 60°C after that there is little decrease in thermal stability, and as such the CLEC laccase does not obey Arrhenius equation and the Ea obtained is not the true one (because the slope was not accurate).

Fig 2.12 Arrhenius plot for the thermal inactivation of soluble and CLEC laccase



Native laccase had thermal stability and half-life four times lower than that of CLEC. The increased thermal stability may be due to the pre-ordered arrangement of the molecules by inter- and intramolecular cross-links between the crystals, and hence the rigidity of the three-dimensional arrangement of molecules in the CLEC (Marcela et al, 2002). The increased thermal stability of CLEC laccase offers major advantages to the organic chemist, to perform the laccase catalyzed reactions at higher temperature thereby increasing the reaction rate.

2.3.3 Stability of CLEC laccase in organic solvent

Table 2.6 Stability of CLEC in Organic Solvents (Retention of Activity %)

Solvent	Dielectric Constant	log P	Polarity	100% solvent	50% solvent	75% solvent
Hexane	1.9	4.0	Non polar	29.6	83.6	37.5
Toluene	2.4	2.73	Non polar	50	95	58
Isoocatane	2.1	4.5	Non polar	42	90	83
Cyclohexane	2.0	3.44	Non polar	24	68.5	42.5
Ethyl acetate	6.0	0.73	Dipolar aprotic	2.3	52.6	30
Chloroform	4.8	1.97	Non polar	5.1	52.9	11.7
Isopropanol	19.9	0.05	Polar protic	5.0	40.5	9.5
Acetone	20.7	-0.24	Dipolar aprotic	1.6	50.5	3.5
Ethanol	24.6	-0.30	Polar protic	3.5	10.5	N.D
Methanol	32.7	-0.74	Polar protic	0	4.3	N.D
Acetonitrile	37.5	-0.34	Dipolar aprotic	2.14	10.2	N.D
water	80.2	-	polar aprotic	53		

N.D- Not determined

Generally, enzyme activity in different organic solvents is not always uniform and properties such as solvent hydrophobicity, hydrogen bonding capacity and water miscibility have profound influence on the structural integrity and catalytic activity of enzymes. Enzyme activation in organic solvents involves considerable conformational changes, which results in unfolding of protein molecules.

According to Laane et al (1986) high biocatalytic activities are favored in solvents having log P between 2 and 4. CLEC laccase had higher activity in non-polar organic solvents (Table 2.6) like hexane, toluene, isooctane and cyclohexane due to their lower dielectric constant (~ 2) and higher log P values (2.73- 4.0). CLEC had medium activity in organic solvents like ethyl acetate, chloroform, isopropanol and acetone. Their log P values are in between 0.73 to – 0.24 and the dielectric constant between 4.8 to 20.7. Solvents like ethanol, methanol and acetonitrile was found to have very low activity due to their higher dielectric constant. There was no activity detected for CLEC laccase in solvents like, DMSO, THF and 1,4 dioxane. The decrease in activity in these polar solvents is due to the stripping of water from the surface of the enzyme by competing through hydrogen bonds between the protein atoms (Dordick, 1989 & Klibanov, 1989& Gorman and Dordick 1992). The increase in stability of CLEC in organic solvents is due to the number of covalent bonds between enzyme molecules created by the glutaraldehyde cross-linking. Cross-linking increases the rigidity of the enzyme molecules and hence reduces the unfolding of the three dimensional structure of the protein by the organic solvents.

2.4 Conclusion

Cross-linked enzyme crystals of laccase were 2-25 μM in size after crystallization using 75% of ammonium sulphate saturation and cross-linked with 1.5% (v/v) solution of glutaraldehyde. The optimun pH of CLEC Laccase was found to be 5.6. CLEC laccase had four fold improved thermal stability over soluble laccase. CLEC laccase was more stable in non-polar solvents like hexane, toluene, isooctane and cyclohexane than polar solvents.

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CHAPTER III

KINETIC STUDIES OF CLEC LACCASE

3.1 Introduction

Living systems depends on chemical reactions, which, on their own, would occur at extremely slow rates. Enzymes are catalysts, which reduce the needed activation energy so these reactions proceed at rates that are useful to the cell. Kinetic information of enzymes is essential in the practical application of enzymes and kinetic parameters are useful when developing process based on a reaction studied in laboratory. It is necessary for the evaluation of an enzyme used in medical research clinical diagnosis, pharmaceutical research or drug development, because it provides essential information about how an enzyme will behave or respond in given situations (Murphy et al, 2002) and also be essential in the prediction of the toxic and metabolic effects of drugs.

Enzymes are specialized proteins, which are able to conduct chemical reactions under biological conditions. Most enzymes have very specific functions, and convert specific substrates to the corresponding products. Since amino acids are chiral, enzymes also show chirality, and will often act on only one enantiomer of a given substrate. Enzymes are extremely efficient, far more so than chemical catalysts which carry out similar reactions. As dynamic molecules, enzymes change their conformation in order to function properly.

An enzyme catalyses a chemical reaction by lowering the activation energy of the reaction and speeds up the rate at which the reaction reaches equilibrium but does not affect the equilibrium itself. Therefore, an enzyme can catalyze a reaction in either direction depending on the relative ratio of compounds present. The substrate is bound to the enzyme to form an enzyme-substrate complex (ES), which then undergoes reaction to form the enzyme-bound product (EP). The product is then released and the enzyme is free to bind another substrate molecules.

The active site of an enzyme is usually a hollow or cleft on the protein surface into which the substrate can fit and bind. The substrate is usually bound to amino acids present in the binding site by a variety of interactions, such as hydrogen bonding, ionic bonding, van der Waals interactions or dipole-dipole interactions (Figure 5.1). For example, a substrate must bind to a serine residue by H-bonding, to an aspartate residue by ionic binding and to a phenylalanine residue by van der Waals interactions. These binding interactions must be

strong enough to hold the substrate long enough for enzyme-catalyzed reaction to take place, but weak enough to allow the product to depart once it is formed.

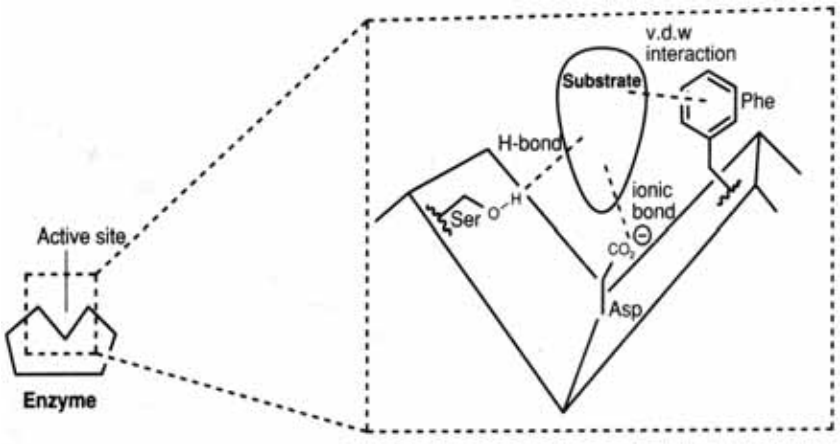


Fig 3.1 Substrate binding mechanism in enzyme active site

The active site also contains amino acids, which assist in the reaction mechanism. Nucleophilic amino acids, such as serine or cysteine are commonly involved in enzyme-catalyzed mechanisms and will form a temporary covalent bond with the substrate as part of the reaction mechanism. The amino acid histidine is commonly involved as an acid/base catalyst. This is because the imidazole ring of histidine residue can equilibrate between the ionized and nonionized forms, allowing the amino acid to act both as a source as a 'sink' for proteins.

The mechanism of an enzyme-catalyzed reaction is a composite description of all the events that take place at a molecular and atomic level from the initial binding of substrates to the release of the products (Fersht, 1984 and 1986).

1. Binding of substrates to the active site of enzyme
2. Conformational changes of enzyme (or) substrate (or) both as a result of binding.
3. Changes in chemical bonding by way of transition states and intermediates.
4. Further conformational changes on formation of products.
5. Release of product on the solvent water.

The term enzyme kinetics (Cornish-Bowden, 1995(a)) implies a study of the speed, rate or velocity of an enzyme-catalyzed reaction, and of the various factors, which may affect this. Enzyme kinetic parameters are derived from rate equations based on mathematical treatment

of data from enzyme-catalyzed reactions. That is, once the kinetic characteristics of an enzyme are known, a model, with its associated equations, can be developed for the number of substrates binding at the active site. Kinetics are typically determined using a steady-state approximation and by following initial rates measured at different substrate concentration (Cornish-Bowden, 1995(b)).

3.1.1 The Effects of Substrate Concentration on Reaction Rate

In an enzyme catalyzed reaction, measuring reaction velocity at various different concentrations, does not give a straight line like non-catalyzed chemical reaction but a curve. V_{max} is the reaction velocity at very high, saturating, concentrations of substrate and it is directly *proportional* to the concentration of enzyme.

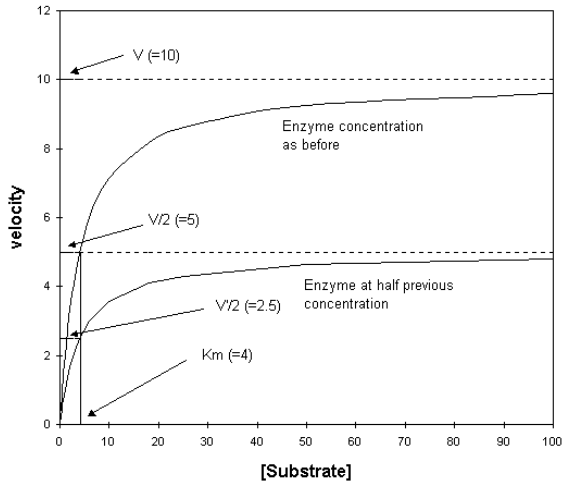


Fig 3.2 Graph of velocity/[substrate]

The maximal velocity is never actually achieved. The slope of the graph just keeps getting shallower and the velocity would only stop rising if you could reach infinite substrate concentration. This means that V_{max} can never be directly measured and the highest velocity measured is about 9.5 units while the true V_{max} is 10 (Figure 3.2).

3.1.2 Michaelis-Menten Equation

Consider a single substrate enzyme-catalyzed reaction where there is one substrate-binding site per enzyme. The simple general equation for such a reaction is

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + P \rightarrow (1)$$

In the initial period of reaction, the product concentration is negligible and the formation of ES from product can be ignored (Cornish-Bowden, 1976).

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \rightarrow (2)$$

The rate of formation of ES at any time t is k_1 [E][S]

Where [E] is the concentration of enzyme

[S] is the concentration of substrate

The rate of breakdown of ES back to E and S is k_{-1} [ES]

Where, [ES] is the concentration of enzyme substrate complex.

In 1913 Michaelis-Menten, assumption that there is an equilibrium between enzyme, substrate and enzyme-substrate complex was almost instantly set up and maintained, the break down of enzyme-substrate complex to products being slow to disturb this equilibrium. By his assumption

$$k_1[E][S] = k_{-1}[ES] \rightarrow (3)$$

In 1925 Briggs and Haldane modified Michaelis-Menten equation by a more valid assumption. By their assumption,

The rate of formation of [ES] at any time t is as same as k_1 [E][S], but the rate of break down of ES at time t is k_{-1} [ES] + k_2 [ES], because ES can break down to form products or reform reactant. By their assumption,

$$k_1 [E][S] = k_{-1} [ES] + k_2 [ES]$$

ie, $$k_1 [E][S] = [ES] (k_{-1} + k_2) \rightarrow (4)$$

$$\frac{[E][S]}{[ES]} = \frac{k_{-1} + k_2}{k_1}$$

Put $$\frac{k_{-1} + k_2}{k_1} = K_m$$

Therfore, $$K_m = \frac{[E][S]}{[ES]} \rightarrow (5)$$

Km in another constant, Substituting [E] = [E_0]-[ES] in eqn (5) where [E_0] is the total enzyme concentration, [E] is the concentration of free enzyme and [ES] is the concentration of bound enzyme.

$$Km = \frac{([E_o]-[ES])[S]}{[ES]}$$

From which $$[ES] = \frac{[E_o][S]}{[S]+K_m} \rightarrow (6)$$

Rate of formation of product $$v_o = k_2[ES] \rightarrow (7)$$

Therefore, $$[ES] = \frac{v_o}{k_2}$$ Substitute the value in eqn(6)

$$v_o = \frac{k_2[E_o][S]}{[S]+K_m} \rightarrow (8)$$

when the substrate concentration is very high, [ES] is negligible and all the enzyme present as the enzyme substrate complex and the limiting initial velocity V_{max} is reached

$$V_{max} = k_2[E_o] \rightarrow (9)$$

Therfore,

$$v_0 = \frac{V_{max}[S]}{[S]+K_m} \rightarrow (10)$$ at constant E_o

The substrate concentration is usually much greater than the enzyme concentration.

So, $[S] \approx [S_0]$ substitute in eqn (10)

$$v_0 = \frac{V_{max}[S_0]}{[S_0]+K_m} \rightarrow (11)$$ at constant E_o

Eqn (11) is Michaelis –Menten equation and K_m is called Michaelis constant

A graph of v_o against [S] will have the form of a rectangular hyperbola (Michaelis –Menten Plot)(Figure 3.3)

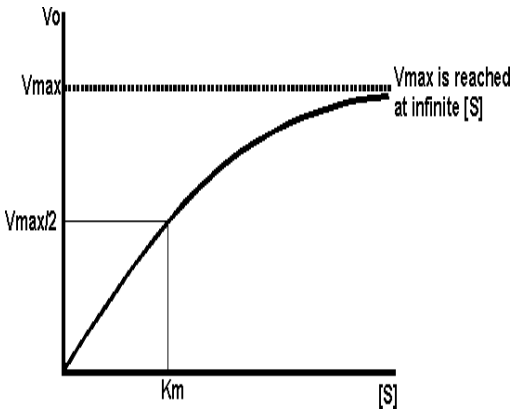


Fig 3.3 Michaelis –Menten Plot

V_{max} is the maximum initial velocity can be calculated from the graph. It will have the same unit of v_o. K_m can also be obtained from the above graph, by puttingn $v_o = ½ V_{max}$ in Michaelis –Menten equation

$$\frac{V_{max}}{2} = \frac{V_{max}[S_0]}{[S_0] + K_m}$$

$$ie, (V_{max})([S_0] + K_m) = 2V_{max}[S_0]$$

$$2[S_0] - [S_0] = K_m$$

$$[S_0] = K_m$$

Therefore , K_m is the value of $[S_0]$ which gives an initial velocity equal to $½ V_{max}$.

K_m also have the same units as $[S_0]$.

3.1.3 Lineweaver-Burk Plot

The graph of Michaelis-Menten equation v_0 against $[S_0]$ is unsatisfactory as a means of determining V_{max} and K_m. The value of v_0 approaches V_{max} in tangential fashion, only actually attaining it, according to Michaelis-Menten equation at infinite substrate concentration. Hence it isvery difficult to use a plot of v_0 against $[S_0]$ to obtain an accurate value of V_{max}, and hence of K_m. Lineweaver and Burk (1934) overcame this problem and they took the Michaelis-Menten equation and inverted it

$$\frac{1}{v_0} = \frac{[S_0] + K_m}{V_{max}[S_0]}$$

$$\frac{1}{v_0} = \frac{[S_0]}{V_{max}[S_0]} + \frac{K_m}{V_{max}[S_0]}$$

$$\frac{1}{v_0} = \frac{K_m}{V_{max}} . \frac{1}{[S_0]} + \frac{1}{V_{max}} \rightarrow (12)$$

(This is the Lineweaver-Burk equation)

This is the form of y= mx+c, which is the equation of a straight line graph, a plot of y against x has a slope m and intercept c on the y-axis.

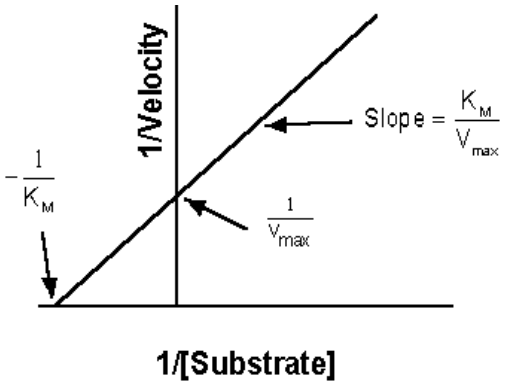


Fig 3.4 Lineweaver-Burk plot

A plot of 1/ [velocity] Vs 1/ [Substrate] (Lineweaver-Burk plot (Figure 3.4)) for systems obeying the Michaelis-Menten equation. The graph, being linear, can be extrapolated even if no experiment has been performed at anything approximating to a saturating substrate concentration, and from the extrapolated graph, the values of K_m and V_{max} can be determined. Departure from linearity for a particular enzyme-catalyzed reaction indicates that the assumptions inherent in the Michaelis-Menten equation are not valid in this instance.

3.1.4 Eadie-Hofstee plot

The Lineweaver-Burk plot has been criticized on several grounds. Firstly, and of the least importance, the extrapolation across the $1/ v_0$ axis to determine the value of $– 1/ K_m$ sometimes reaches the edge of the graph paper before reaching the $1/[S_0]$ axis, possibly resulting in the graph having to be redrawn with altered axes. Secondly, it is said to give undue weight to measurements made at low substrate concentrations. When results are

likely to be most inaccurate. Thirly, departures from linearity are less obvious than in some other plots, particularly the Eadie-Hofstee and Hanes plots.

The Eade-Hofstee plot takes as its starting point the Lineweaver-Burk equation, based in turn on the Michaelis-Menten equation. Both sides of the Michaelis-Menton equation multiply by v_0V_{max}

$$\frac{1}{v_0}(v_0V_{max}) = \frac{Km}{V_{max}}.\frac{1}{[S_0]}(v_0V_{max}) + \frac{1}{V_{max}}(v_0V_{max})$$

$$Therefore, v_0 = -K_m\frac{v_0}{[S_0]} + V_{max}$$

This is also in the form of y= mx+c, which is the equation of a straight-line graph. A plot of [velocity] vs [velocity/substrate] gives a straight line (Figure 3.5) , where the slope is $-K_m$ and the intercept is V_{max}.

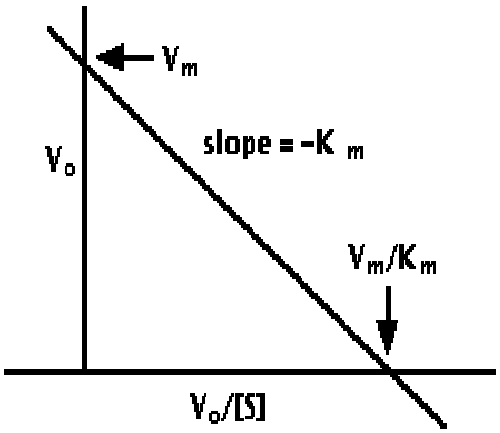


Fig 3.5 Eade-Hofstee plot

3.1.5 Hanes Plot

The Hanes plot similarly starts with the Lineweaver-Burk equation, which is further multiplied throughout by $[S_0]$.

$$\frac{1}{v_0}[S_0] = \frac{K_m}{V_{max}}\frac{1}{[S_0]}[S_0] + \frac{1}{V_{max}}[S_0]$$

$$Therefore, \frac{[S_0]}{v_0} = \frac{1}{V_{max}}[S_0] + \frac{K_m}{V_{max}}$$

A plot of [substrate] vs [substrate] /velocity gives straight line (Figure 3.6) in the form of y=mx+c

The slope gives the value of $1/V_{max}$ and the Y intercept gives the value of $-K_m$

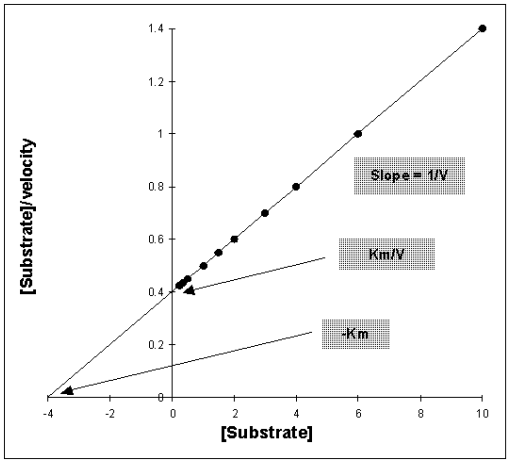


Fig 3.6 Hanes plot

The slope gives a direct readout of K_m. To avoid having to measure the slope it is probably best to calculate V_{max} from the K_m/V intercept.

The use of the direct linear plot for determining kinetic parameters

The Lineweaver-Burk, Eadie-Hofstee and Hanes plots are all rather similar to each other, in that they are based on an algebraic conversion of the Michaelis equation to give a

straight-line equation. The direct linear plot is a very different use of a straight-line technique.

The Constant V_{max} is the maximum velocity represents the amount of product formed per milligram of catalyst in a period.

The term K_m gives an indication of the affinity of the enzyme for a substrate. A lower Km value indicates a high affinity of an enzyme for substarate, whereas a higher K_m value indicates a lower affinity.

The constant K_{cat} is called turn over number, often applied to enzyme-catalyzed reaction. This is obtained from the general expression $V_{max} = K_{cat} [E_0]$, $K_{cat} = V_{max}/E_0$.
It represents the maximum number of substrate molecules, which can be converted to products per molecule of enzyme per unit time.

The term K_{cat}/ K_m is the catalytic efficiency of the enzyme. A higher value indicates that the limiting factor for the overall reaction is the frequency of collisions between enzyme and substrate molecules. A lower value would be more in keeping with the equilibrium assumption. The comparison of K_{cat}/ K_m for alternative substrate can also be used as a measure of the specificity of an enzyme.

3 Materials and Methods

3.1 Materials

Laccase from *Trametus versicolor* (E.C.1.10.3.2) (0.8 U/mg of powder, catechol units) was purchased from Fluka and Glutaraldehyde, ABTS (2,2'-Azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid)), Catechol, Catechin, Pyrogallol, Guaiacol, were purchased from Sigma (St.Louis, USA). Ammonium sulphate (Enzyme grade) was purchased from SISCO Laboratories, India. All other reagents used were of analytical grade.

3.2 Methods

3.2.1 Crystallization of Laccase

Laccase was crystallized by batch method. 50 mg of crude laccase was dissolved in 3 ml of 0.1M Sodium acetate buffer, at pH 5.6. Ammonium sulphate (75% saturation) was added in small portions over 3 hours by stirring at 5±1° C . Then the super saturated solution was kept at this temperature undisturbed for 20 hours. The crystals formed were separated by centrifugation at 2000 rpm for 8 minutes.

3.2.2 Cross-linking of Laccase crystals

The laccase crystals were cross-linked using 1.5 % glutaraldehyde (v/v) solution in isopropanol for 20 minutes at 25°C. After cross-linking, it was washed 3 times with 0.1 M sodium acetate buffer at pH 5.6 to remove excess glutaraldehyde and stored in the same buffer at pH 5.6.

3.2.3 Estimation of Protein

The determination of protein was carried out by Lowry's method (Lowry et al, 1951) BSA was used as the standard, and the absorbance was read at 660 nm. The cross-linked enzyme crystals were washed thoroughly to remove excess reagents before estimation.

3.2.4 Enzyme assay measurement

The Enzyme assay (Eggert et al, 1996 & Jimenez-Atienzar et al, 2004) was done with 0.1 mg of laccase in a reaction medium containing the substrate in 0.1 M sodium acetate buffer at pH 5.0 and the color of the oxidation products were read at the appropriate wavelength in a spectrophotometer (Shimadzu, UV 2100). The oxidation product of ABTS was read at 420 nm (ε_{max}= 36000 $M^{-1}cm^{-1}$), of Guaiacol at 470 nm ($\varepsilon_{max.}$ = 26600 $M^{-1}cm^{-1}$), of syringaldazine in 10% DMF was read at 530 nm ($\varepsilon_{max.}$ = 65000 $M^{-1}cm^{-1}$), of catechol was read at 450 nm ($\varepsilon_{max.}$ = 2211 $M^{-1}cm^{-1}$), of pyrogallol was read at 450 nm ($\varepsilon_{max.}$ = 4400 $M^{-1}cm^{-1}$) and that of catechin in 10% methanol was read at 390 nm ($\varepsilon_{max.}$ = 4019 $M^{-1}cm^{-1}$). The unit of enzyme activity was expressed in units /mg of enzyme protein.

3.2.5 Assay of CLEC Laccase

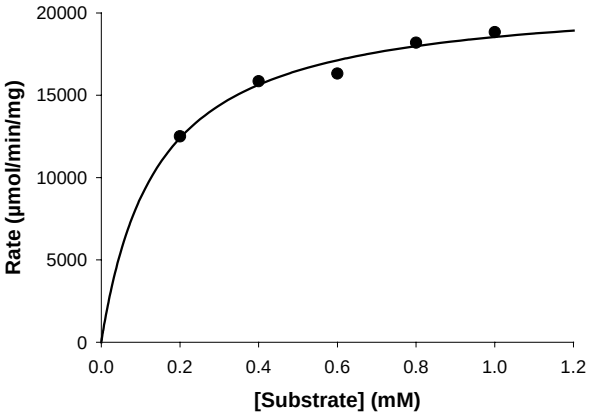
The Assay of CLEC laccase was done using ABTS (5mM final concentration) as substrate. Instead of directly taking measurement, the assay mixture (in 3ml assay volume, 2.25 ml of buffer and 750µl of 20mM ABTS and 1 mg of CLEC Laccase) containing the CLEC laccase was stirred continuously for 1 minute and then the increase in absorbance was monitored at definite time intervals at 420 nm in a spectrophotometer. One unit of Laccase is the amount of enzyme needed for the conversion of 1 µmol of substrate to its product.

3.2.6 Enzyme kinetics

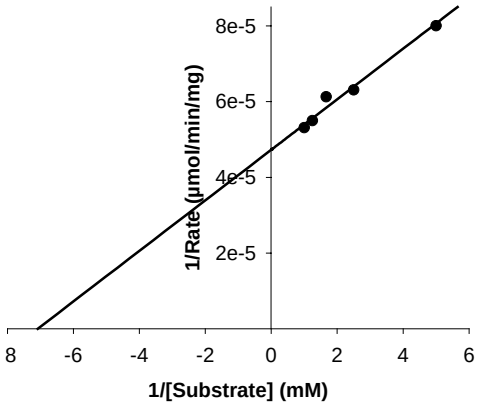
Kinetics of soluble enzyme as well as CLEC was done at 0.1 M sodium acetate buffer pH 5.0 by varying the substrate concentration, while keeping the concentration of biocatalyst constant (1612 IU/mg of CLEC laccase). Using Enzyme "Kinetics pro" (Sigma plot) software (www.systat.com/products/SigmaPlot/modules/enzyme_kinetics/), the Michaelis-Menten constant (K_m) and velocity maximum V_{max} were calculated from the mean value of Lineweaver-Burk plot (A plot of 1/ [V] Vs 1 / [S]), Eadie-Hofstee plot (A plot of [V] vs [V]/[S]) and Hanes plot (A plot of [S]/[V] Vs [S]). From those values, K_{cat} and catalytic efficiency K_{cat}/K_m values were calculated.

3.3 Results and Discussion

3.3.1 Results

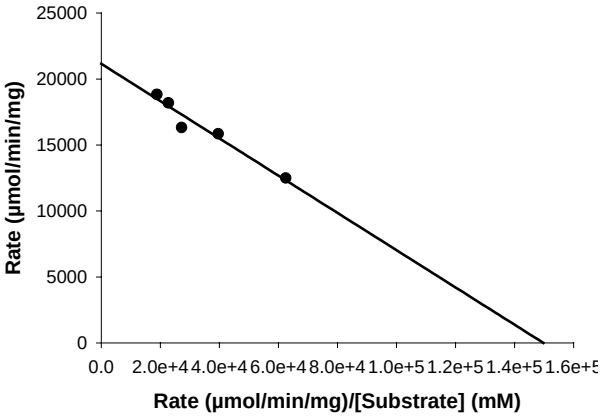


Michaelis-Menten Plot

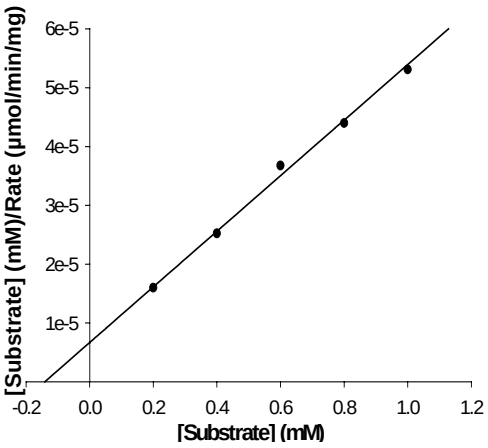


Lineweaver-Burk plot

Average Vmax = 2.116e+4
Km = 0.1412

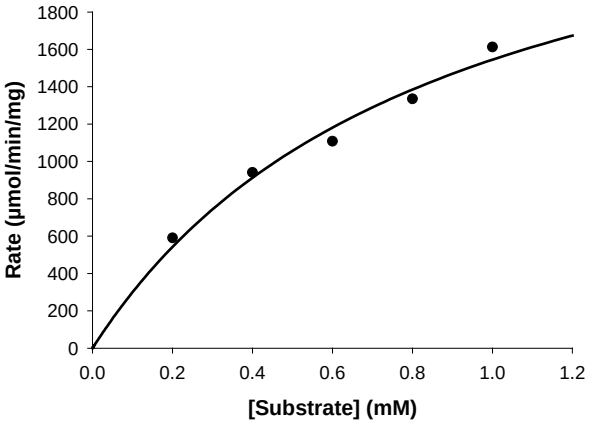


Eadie-Hofstee Plot

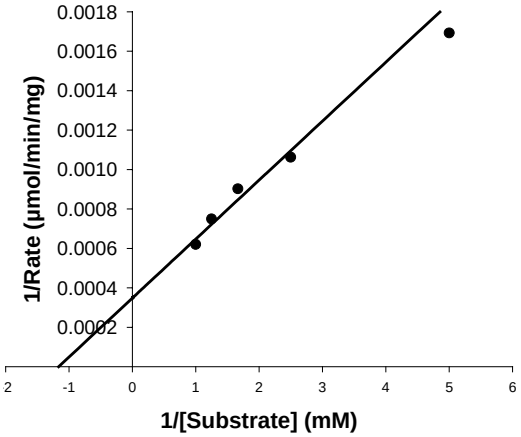


Hanes-Woolf plot

Fig 3.7 Kinetic behavior of soluble laccase ABTS as substrate

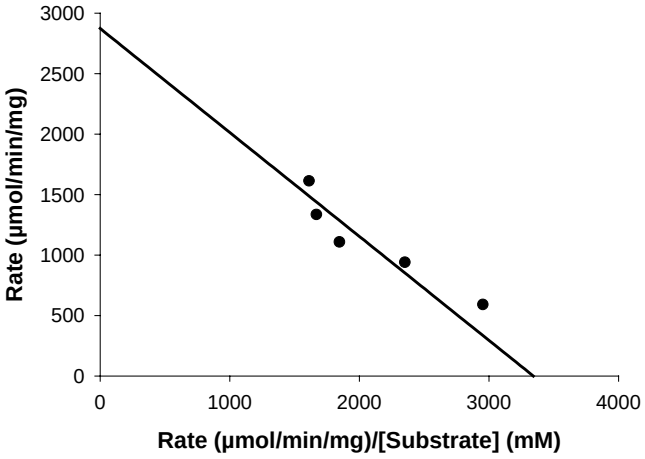


Michaelis-Menten Plot

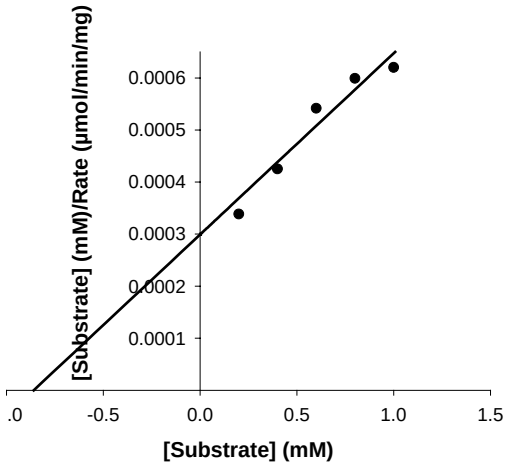


Lineweaver-Burk plot

Average Vmax = 2873.2
Km = 0.8595

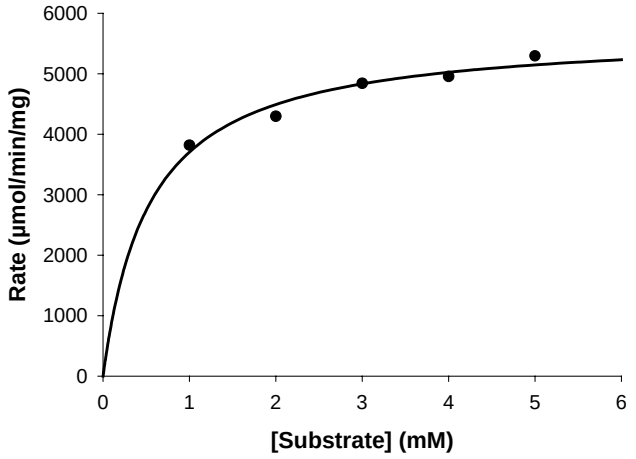


Eadie-Hofstee Plot

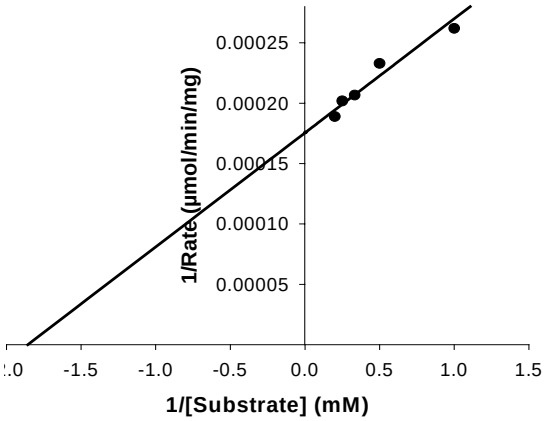


Hanes-Woolf plot

Fig 3.8 Kinetic behavior of CLEC laccase ABTS as substrate

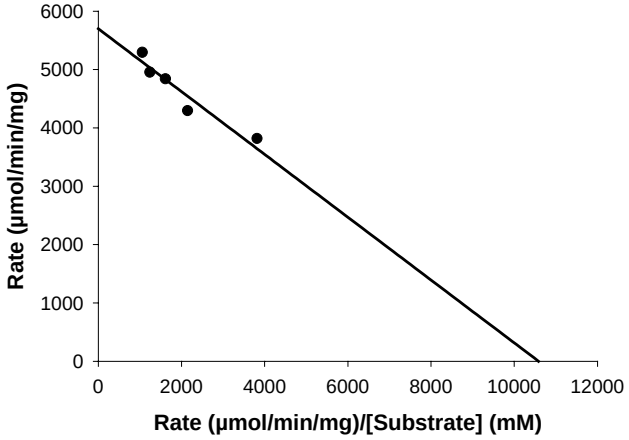


Michaelis-Menten Plot

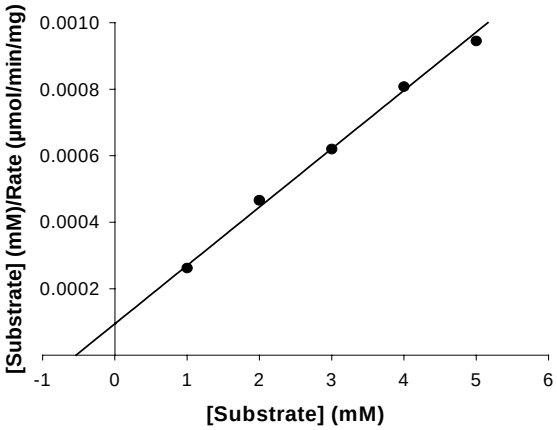


Lineweaver-Burk plot

Average Vmax = 5699.9
Km = 0.5385

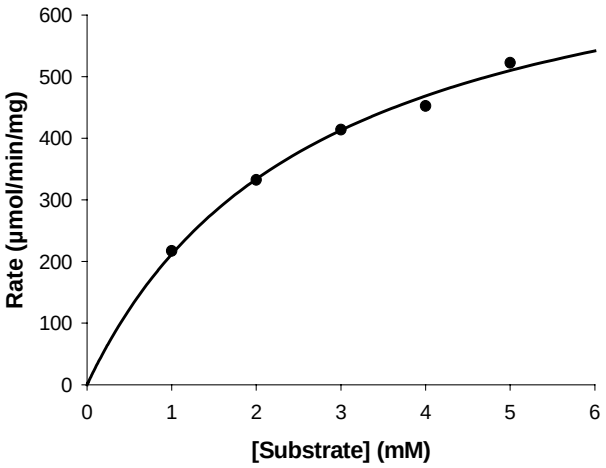


Eadie-Hofstee Plot

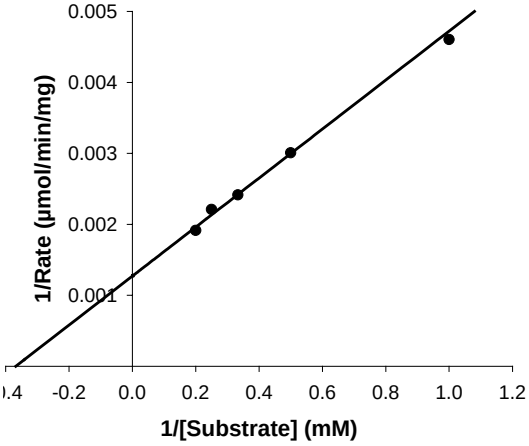


Hanes-Woolf plot

Fig 3.9 Kinetic behavior of soluble laccase catechol as substrate



Michaelis-Menten Plot

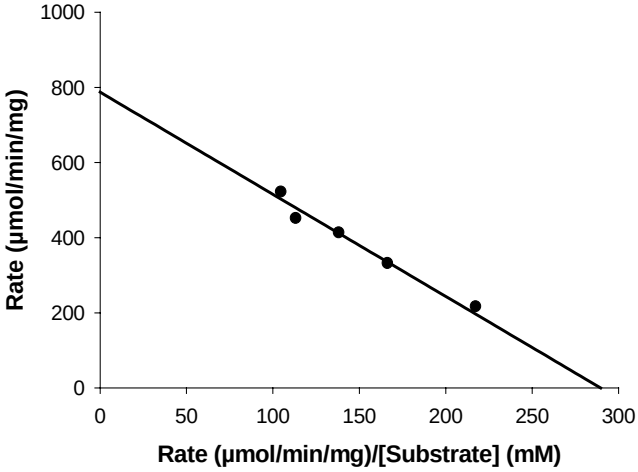


Lineweaver-Burk plot

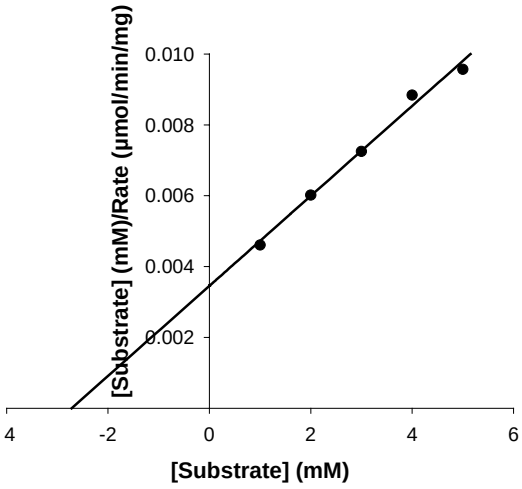
Average

Vmax = 787.2

Km = 2.7

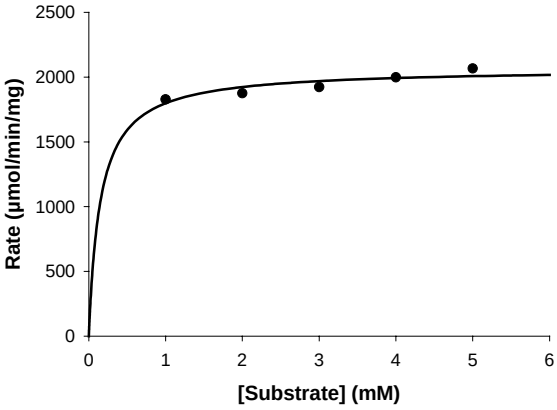


Eadie-Hofstee Plot

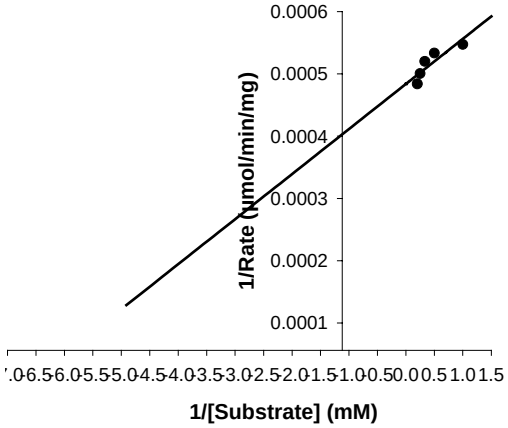


Hanes-Woolf plot

Fig 3.10 Kinetic behavior of CLEC laccase catechol as substrate

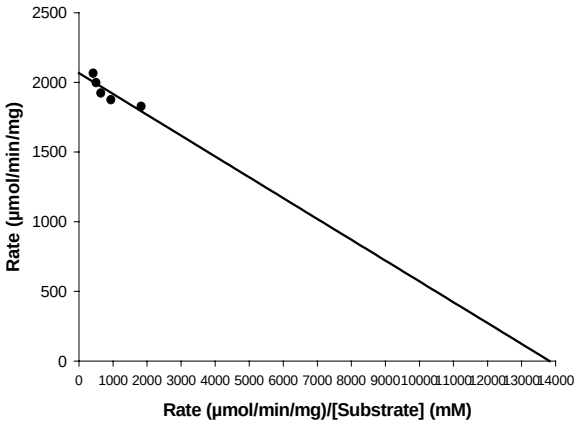


Michaelis-Menten Plot

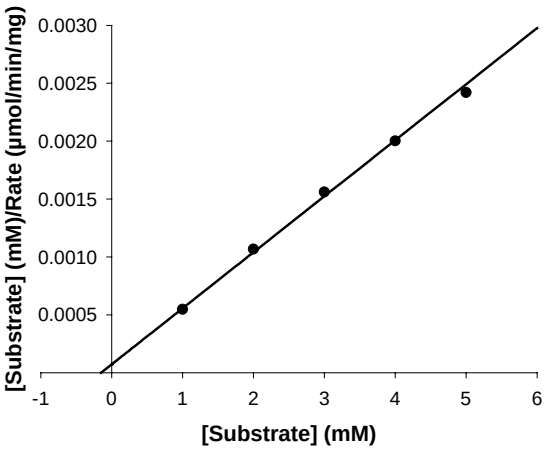


Lineweaver-Burk plot

Average Vmax = 2066.7
Km = 0.1495

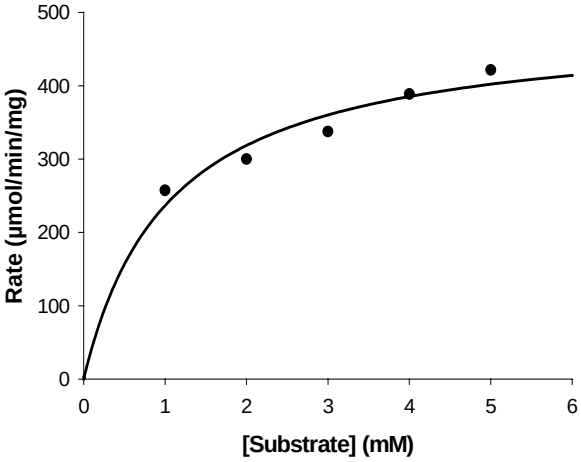


Eadie-Hofstee Plot



Hanes-Woolf plot

Fig 3.11 Kinetic behavior of soluble laccase pyrogallol as substrate



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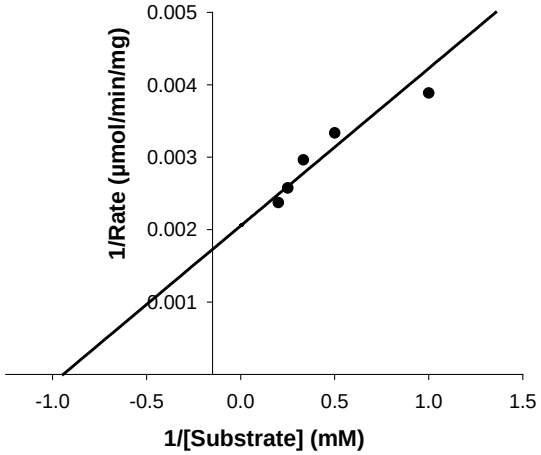
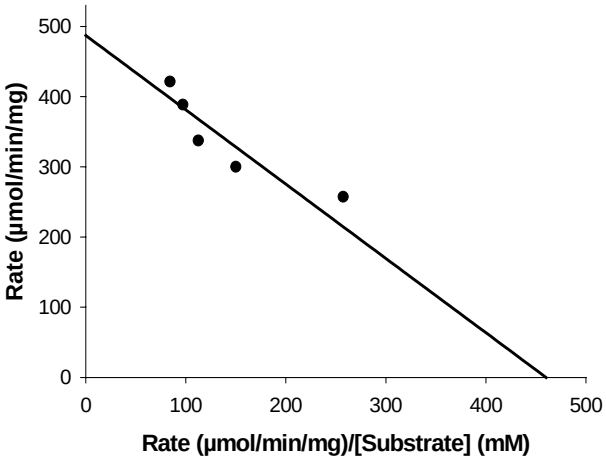


Lineweaver-Burk plot

Average Vmax = 487.2
Km = 1.1



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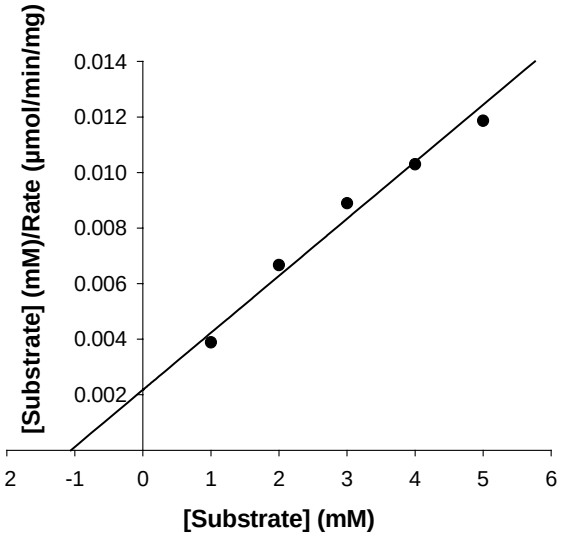
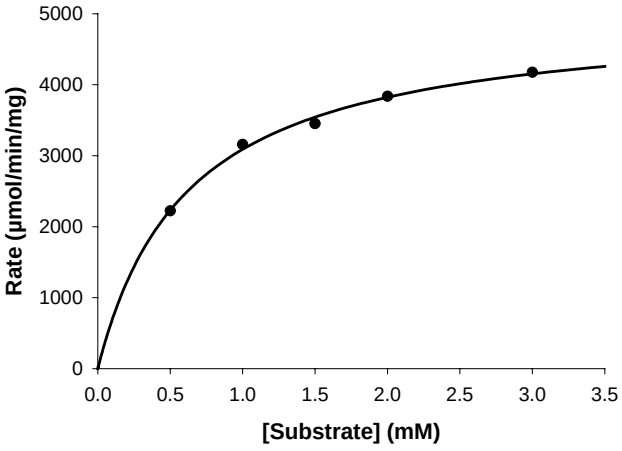
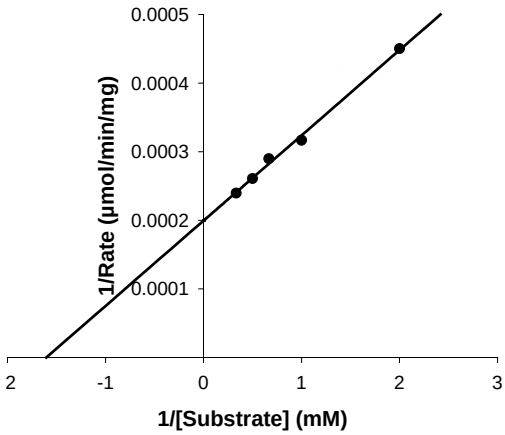


Hanes-Woolf plot

Fig 3.12 Kinetic behavior of CLEC laccase pyrogallol as substrate

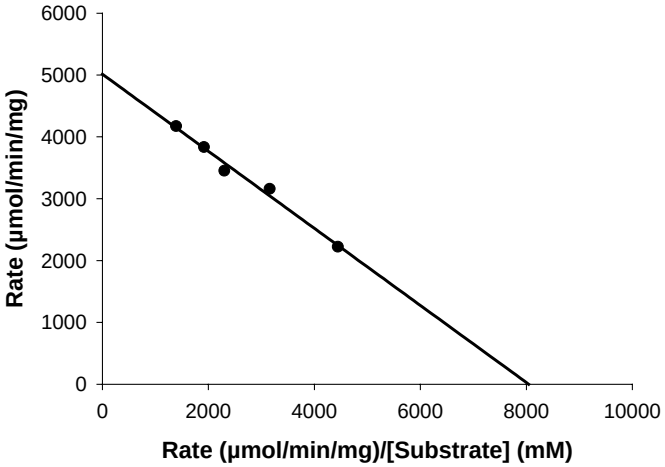


Michaelis-Menten Plot

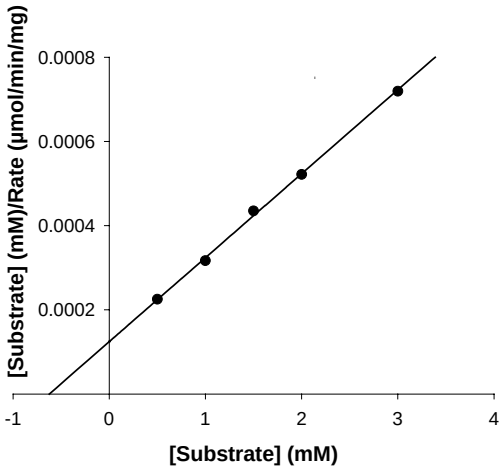


Lineweaver-Burk plot

Average Vmax = 5014.
Km = 0.6232

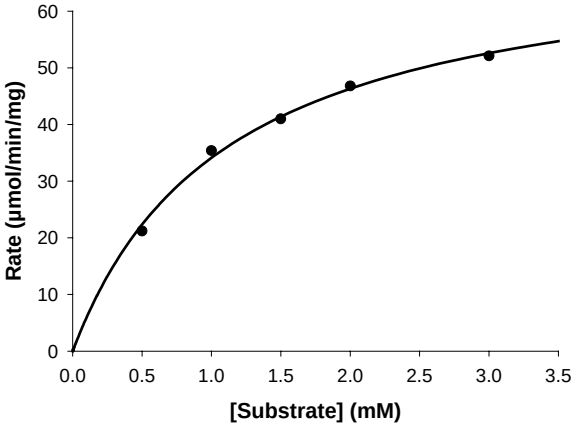


Eadie-Hofstee Plot



Hanes-Woolf plot

Fig 3.13 Kinetic behavior of soluble laccase guaiacol as substrate



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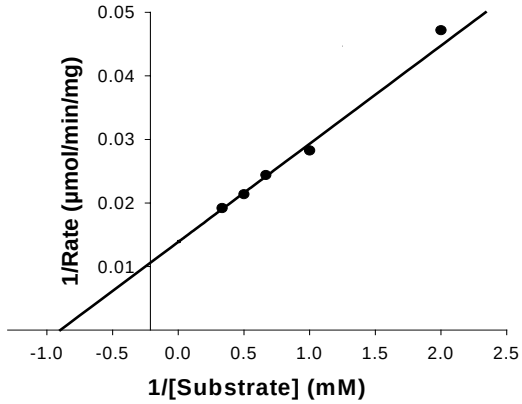

Lineweaver-Burk plot

Average Vmax = 72.1
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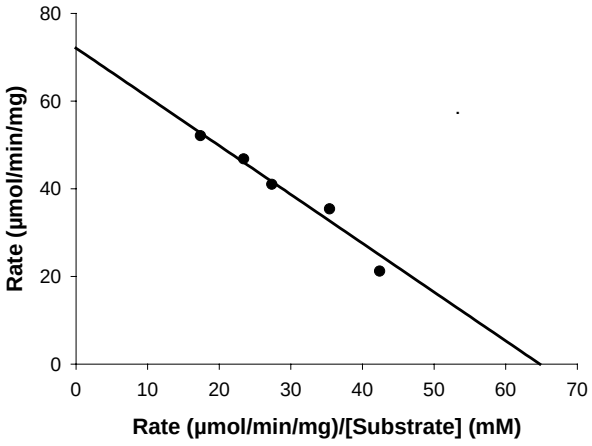
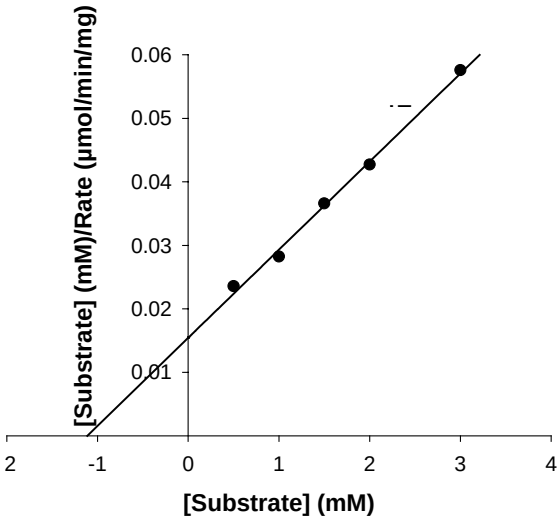
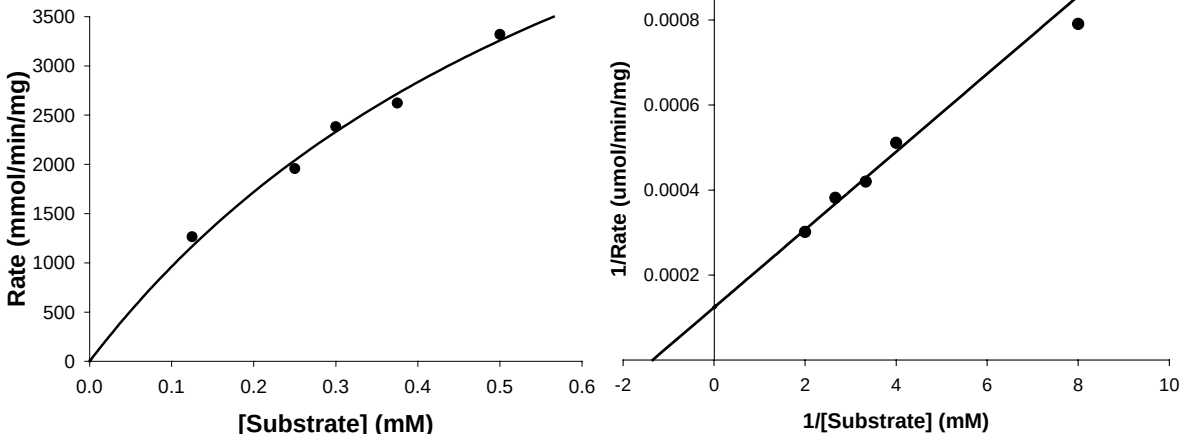


Eadie-Hofstee Plot



Hanes-Woolf plot

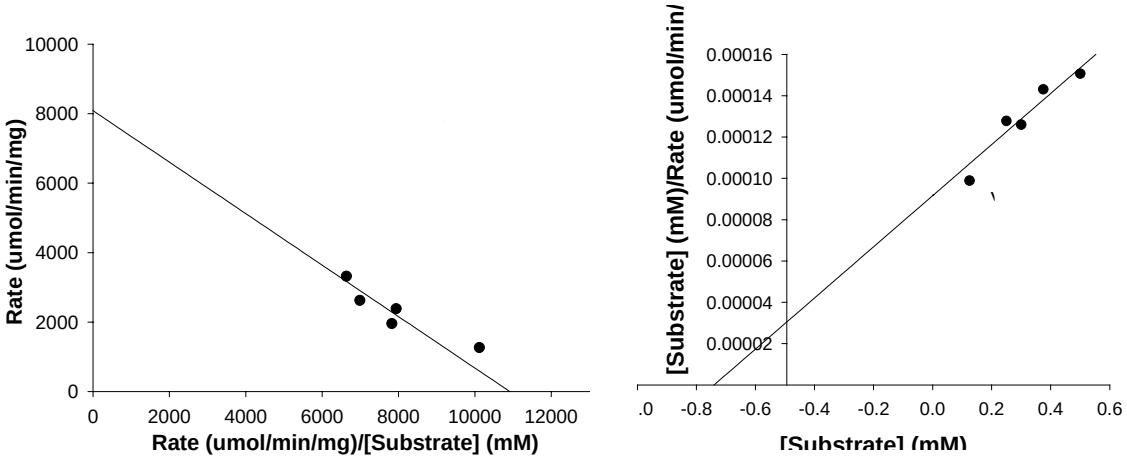
Fig 3.14 Kinetic behavior of CLEC laccase guaiacol as substrate



Michaelis-Menten Plot

Lineweaver-Burk plot

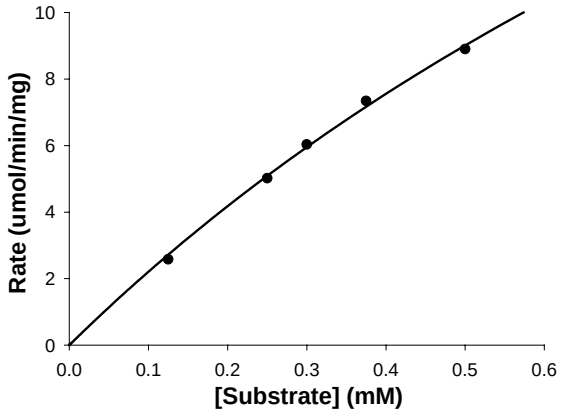
Average Vmax = 8084.6
Km = 0.7407



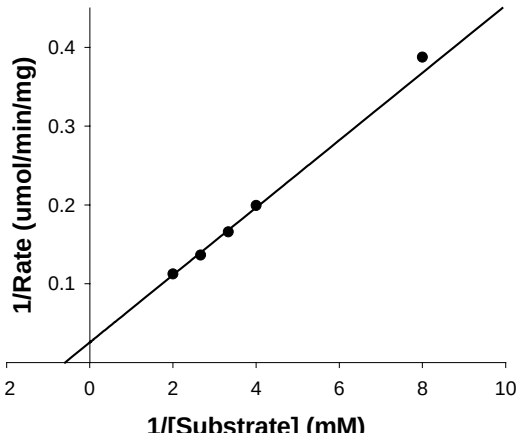
Eadie-Hofstee Plot

Hanes-Woolf plot

Fig 3.15 Kinetic behavior of soluble laccase syringaldazine as substrate

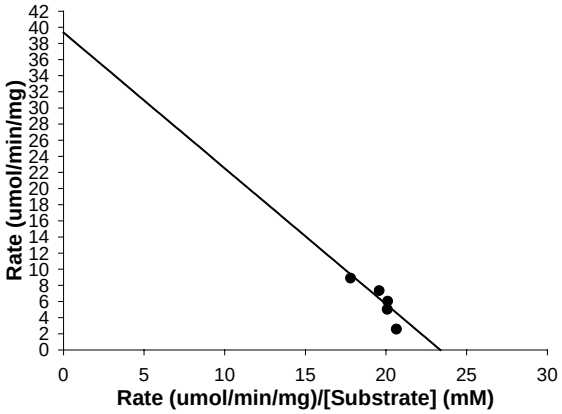


Michaelis-Menten Plot

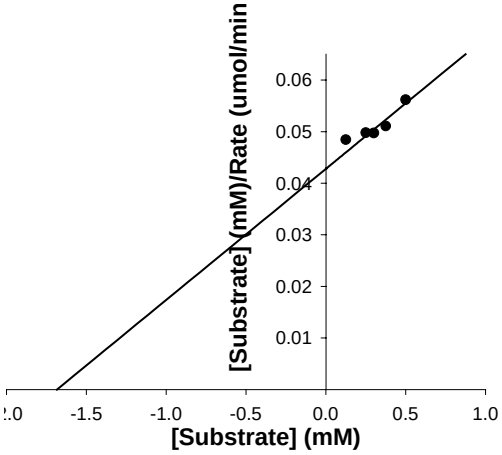


Lineweaver-Burk plot

Average Vmax = 39.4
Km = 1.7

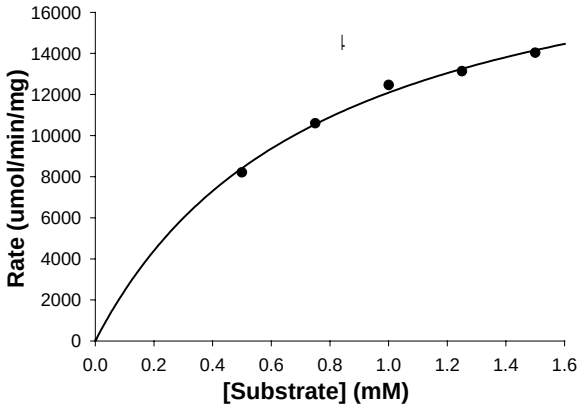


Eadie-Hofstee Plot

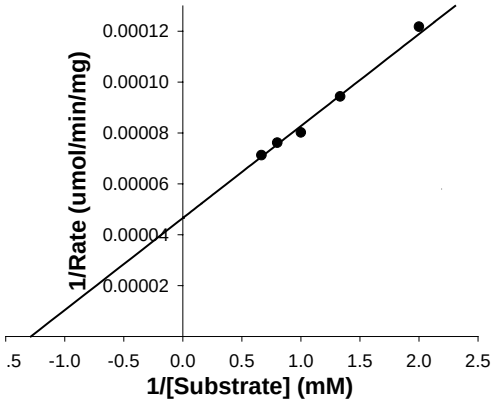


Hanes-Woolf plot

Fig 3.16 Kinetic behavior of CLEC laccase syringaldazine as substrate

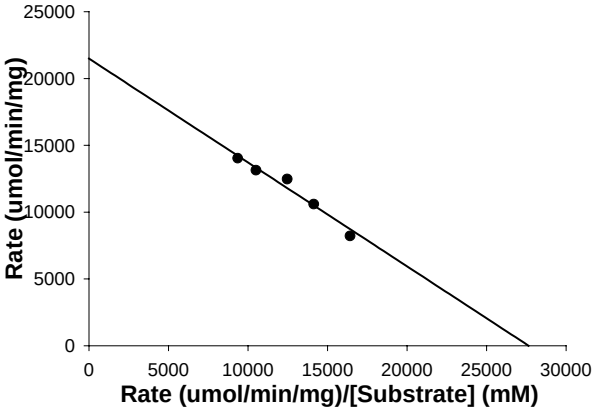


Michaelis-Menten Plot

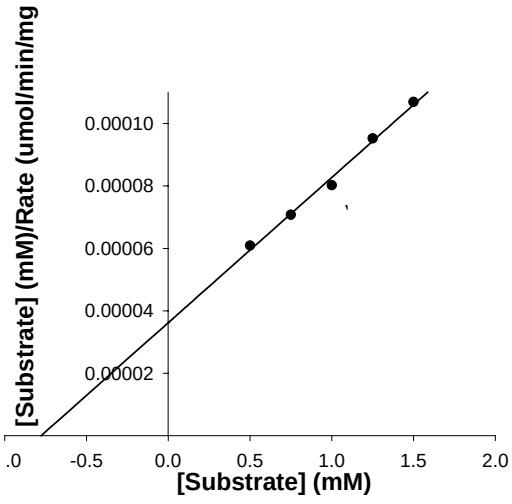


Lineweaver-Burk plot

Average Vmax = 2.149e+4
Km = 0.7775

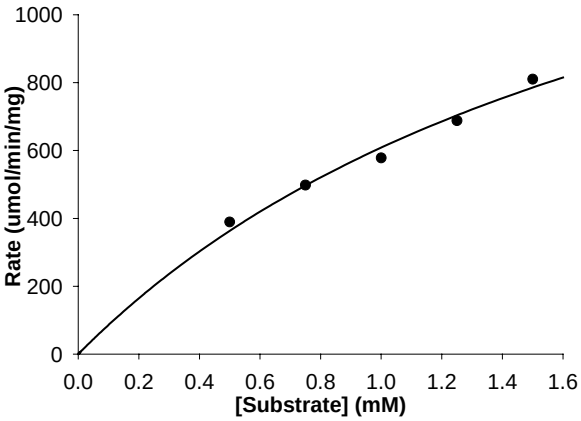


Eadie-Hofstee Plot

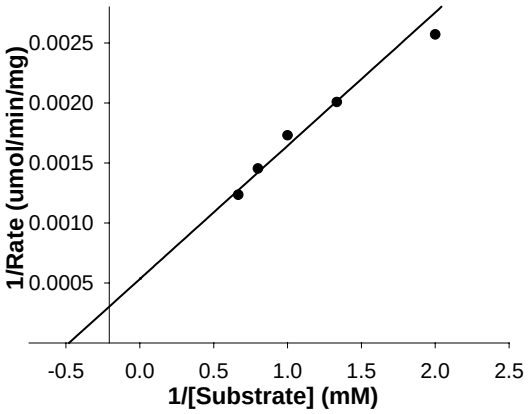


Hanes-Woolf plot

Fig 3.17 Kinetic behavior of soluble laccase catechin as substrate

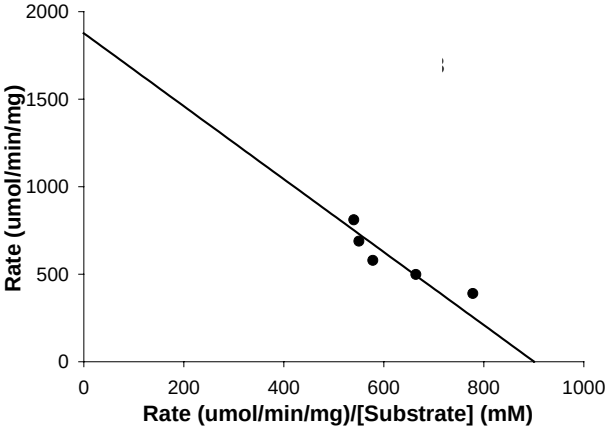


Michaelis-Menten Plot

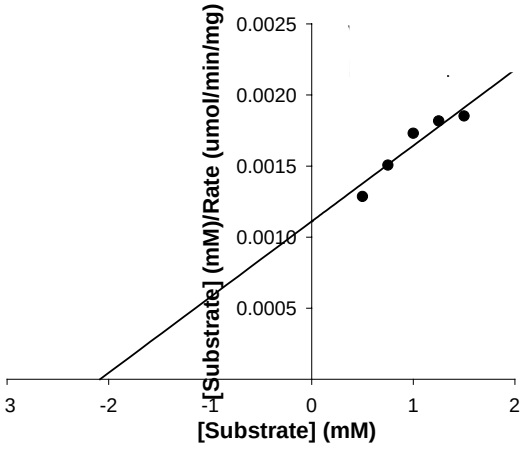


Lineweaver-Burk plot

Average Vmax = 1876.3
Km = 2.1



Eadie-Hofstee Plot



Hanes-Woolf plot

Fig 3.18 Kinetic behavior of CLEC laccase catechin as substrate

Table 3.1 Kinetic Constants for the oxidation of various Substrates by soluble Laccase

Substrate	V_{max} (µmol/min/mg)	K_{cat} (S^{-1})	K_m (mM)	K_{cat}/K_m ($S^{-1}M^{-1}$)
ABTS	21155.5	23.62	0.1412	1.673×10^5
Catechol	5699.9	6.36	0.5385	1.181×10^4
Pyrogallol	2067.0	2.31	0.1495	1.540×10^4
Guaiacol	5014.0	5.6	0.6232	0.898×10^4
Syringaldazine	8084.6	9.03	0.7407	1.220×10^4
Catechin	21490.8	23.99	0.7775	3.085×10^4

Table 3.2 Kinetic Constants for the oxidation of various Substrates by CLEC Laccase

Substrate	V_{max} (µmol/min/mg)	K_{cat} (S^{-1})	K_m (mM)	K_{cat}/K_m ($S^{-1}M^{-1}$)
ABTS	2873.2	3.210	0.8595	3.730×10^3
Catechol	787.1	0.879	2.717	0.323×10^3
Pyrogallol	487.2	0.544	1.059	0.514×10^3
Guaiacol	72.67	0.0811	1.113	0.072×10^3
Syringaldazine	39.36	0.044	1.684	0.026×10^3
Catechin	1876	2.094	2.084	1.005×10^3

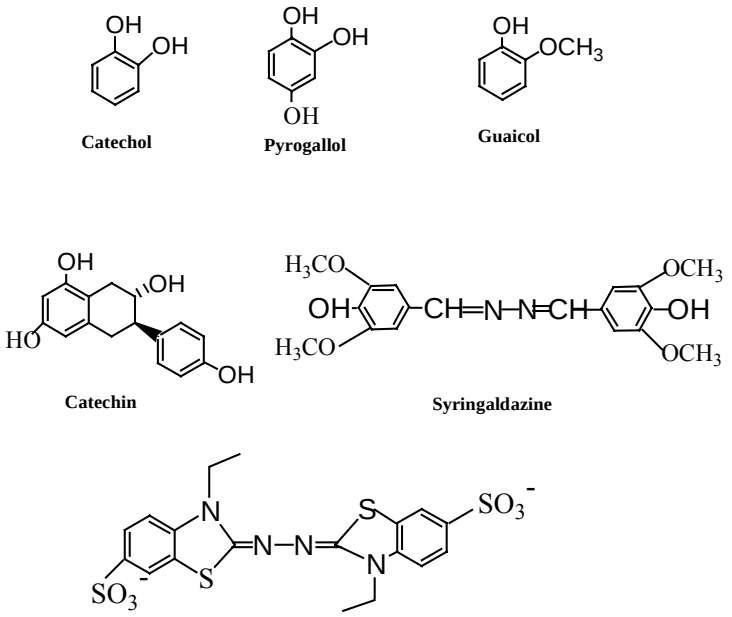
3.3.1 Discussion:

The catalysis of various substrates by laccase was carried out at pH 5.0 and the results are shown in Fig 3.7-3.18 & Table 3.1 & 3.2

Laccase catalysis involves the following events,

1. Reduction of type 1 copper by reducing substrate
2. Internal electron transfer from type 1 copper to the type 2 and type 3 copper
3. Reduction of O_2 to water at the type 2 and type 3 copper site.

The oxidation of reduced substrates by laccase typically involves the loss of a single electron and the formation of a free radical (Yaropolov et al, 1994). The radical is in general unstable and may undergo further laccase-catalyzed oxidation (e.g., to form quinone from phenol). The electron transfer from substrate to type 1 copper is probably controlled by redox potential difference (Xu, 1996) between the substrate & the Cu site. A lower oxidation potential of substrate or a higher redox potential of laccase (type 1 site) often results in a higher rate for substrate oxidation. It seems that the binding pocket of reducing substrate (or type 1 copper site) is quite shallow and has limited steric effect on simple phenol substrate (Xu, 1996 & Ducros et al, 1998). In contrast, the binding pocket (or type 2 and 3 copper site) appears to restrict the access of oxidizing agents other than O_2. Activation of O_2 likely involves chemical bond formation on the trinuclear copper cluster. Under turnover conditions, the rate-limiting step may be the oxidation of substrate, whereas under transient or anaerobic conditions, the internal electron transfer step may be rate-limiting step (Xu, 1999) (Scheme 3.1 Substrates used for enzyme kinetics).



Scheme 3.1 Substrates used for enzyme kinetics

The Michaelis-Menten constant (K_m) and velocity maximum $V_{max.}$ were calculated using Kinetics Pro software (Sigma plot 7.0). The kinetic constants of CLEC as well as native laccase was determined by the oxidation of various substrates such as ABTS, Syringaldazine, catechol, catechin, guaiacol and pyrogallol (Table 1 and 2). The catalytic efficiency of native enzyme is better for all the substrate studied.

The kinetic behavior of the immobilized biocatalyst will depends on the following parameters

1. Diffusion limitations
2. Rigidity of the immobilized biocatalyst
3. Conformational changes after immobilization
4. Partitioning of the product from active site environment to solvent phase.
5. Difference in redox potentials of substrates
6. Steric hindrance of bulky substituents.

The K_m value of the enzyme reaction is lower for the substrates like ABTS and pyrogallol for the soluble as well as CLEC laccase, indicates the high affinity of the laccase for the two substrates. Especially K_m value is lower for ABTS when compared to pyrogallol is due to the special interaction between the thiol and T1 copper (many sulfur-containing

groups have high affinity toward copper ion) (Xu, 1996) is involved in addtion of simple substrate docking to the T1 pocket. ABTS differ from phenolic substrate because its oxidation does not involve proton transfer, but only a single electron transfer. The affinity of ABTS to transfer the charge between Cu (II) (type 2 copper) to the S atom of ABTS (Michele and David, 1999). Asp 206 involved in the type 1 Cu site, allows different protonation sites for pH 3.0 & 5.0. At pH 5.0 (the experiments have been carried out) carboxylic function of Asp is dissociated, so negative charge should stabilize the partial positive charge of proton extracted from the reducing substrate by the enzyme. The K_m value for the substrates catechol, Guaiacol, syringaldazine and catechin were high for soluble enzyme indicates lower affinity of these substrates compared to ABTS and pyrogallol. The K_m value of guaiacol and syringaldazine for CLEC was higher than that of ABTS and pyrogallol indicates medium affinity. Substrates like catechin and catechol, the K_m value of CLEC laccase was 2.084 & 2.717 indicates lower affinity of these substrates for CLEC laccase. This may be due to the rigidity of the CLEC after cross-linking with glutaraldehyde preventing changes in its conformation according to the conformation of substrate, which binds to the active site of the enzyme. In general, CLEC laccase shows lower affinity for all the substrates.

The turn over number (K_{cat}) of the soluble enzyme was higher for the substrates ABTS & catechin that indicates the higher number of substrate molecules converted into their products per mole of the enzyme (or) the rate of the reaction was high. The K_{cat} value is lower with the substrate guaiacol for both soluble as well as CLEC laccase. The reason may be due to the partitioning of the product from the active site environment to the bulk phase (solvent). The turn over number of syringaldazine was medium for soluble laccase but was drastically reduced to a lower value in the case of CLEC laccase. This may be due to the diffusion limitations and the bulky substituent at the 4^{th} position of C-atom which will reduce the product partition from the active site of laccase in CLEC to the bulk phase.

The lower catalytic efficiency (K_{cat}/K_m) of CLEC laccase, compared to the native enzyme may be due to the partial loss of Type 2 (T2) copper, which is known to be labile, during crystallization process. In *Trametes versicolor* laccase, Type 3 Cu either 1 or 2 bound to three-histidine and oxygen atom, while Type 2 copper is coordinated to only two histidines and weakly to a water molecule (Thomas et al, 2002). It is reported in the previous

studies that during crystallization, the enzyme was trapped in a form devoid of the Type 2 (T2) copper mainly due to the enzymatic deglycosylation carried out prior to crystallization and this depletion of T2 Cu is known to render laccases with inactive/or reduces the activity, (Reinhammar and Oda, 1979).The another reason for the lower catalytic efficiency of CLEC laccase for all the substrates is also due to the rigidity of enzyme protein molecule and microenvironment changes occurring around the active site as a result of cross-linking. The rigidity of the enzyme molecules will not allow any changes in the conformation of active site in order to accommodate the substrate. ABTS and Catechin was found to have a favorable catalytic efficiency for both soluble (1.673 X 10^5 $S^{-1}M^{-1}$ & 3.085 X 10^4 $S^{-1}M^{-1}$) and CLEC laccase (3.73 X 10^3 $S^{-1}M^{-1}$ & 1.005 X 10^3 $S^{-1}M^{-1}$). The catalytic efficiency in between 1.1 X 10^4 – 1.5 X 10^4 $S^{-1}M^{-1}$ for soluble enzyme for the substrates like catechol, syringaldazine and pyrogallol. With substrates like catachol and pyrogallol, the CLEC laccase had a catalytic efficiency of 0.323X 10^3 $S^{-1}M^{-1}$ & 0.514 X 10^4 $S^{-1}M^{-1}$. With Guaiacol, both native and CLEC laccase has a lower catalytic efficiency.

Another possibility for the lower catalytic efficiency may be due to the inadequate exposure of the active centers of laccase for substrates, or the channels in the crystal form are too narrow where the substrates or products could not diffuse into it (diffusion limitations)(Margolin and Navia, 2001). Cross-linking could result in diffusion limitations, which may reduce the catalytic turn over of the CLEC. Even in the absence of diffusion limitations, as in the case of small substrates the enzyme active centers may be sterically hindered by the neighboring enzyme molecules and thus be inaccessible to the substrate (Klibanov, 1997). Differences in the redox potentials of the substrates along with the difference in their solubilities may also be responsible for the kinetic behavior of CLEC. Partitioning of the substrate and products will also change the rate of the reaction. ABTS was found to be the best substrate for CLEC and native laccase with low Km value 0.8595and 0.1412 respectively. The best catalytic efficiency (K_{cat} /K_m) of native and CLEC of laccase was found to be 1.673 x 10^5 and 3.73 x 10^3 respectively for ABTS oxidation.

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CHAPTER IV
BIOSENSOR APPLICATION OF CLEC LACCASE FOR THE DETERMINATION OF PHENOLIC COMPOUNDS

4.1 Introduction

Biosensors technology have emerged as an important discipline of biotechnology. A biosensor is an analytical device, which converts a biological response into an electrical signal. The term 'biosensor' is often used to cover sensor devices used in order to determine the concentration of substances and other parameters of biological interest even where they do not utilise a biological system directly (Lowe, 1984). Bio-molecules, utilized as biosensors, are selective in their interaction with other molecules and the reactions always follow the same kinetics. This property of molecular specificity is used as the basis in designing biosensors. A biosensor is a device that detects, transmits and records information regarding a physiological or biochemical change (D'souza, 2001). Biosensors make use of a variety of transducers such as electrochemical, optical, acoustic and electronic (Blum and Coulet, 1991). The biosensor produces discrete or continuous digital electronic signals, which are proportional to a single analyte or a related group of analytes (Turner et al, 1987). Boehringer Mannheim and Bayer have developed biosensors for diagnostic purpose and the combined sales dominate 85% of the world market.

4.1.1 Features of Biosensors

a)Specificity

The advantage of biological sensing elements is their remarkable ability to distinguish between the analyte of interest and similar substances. With biosensors, it is possible to measure specific analytes with great accuracy.

b) Speed

The analyte tracers or catalytic products can be directly and instantaneously measured.

c)Simplicity

The receptor and transducer are integrated into one single sensor by simply dipping the sensor in the sample.

d) Continues monitoring

In a biosensor, an immobilized enzyme or biomolecules can be used repeatedly and this feature allows these devices to be continuously used for multiple assays.

4.1.2 History of Biosensors

Table 4.1 History of the Biosensor Development

Year	Biosensor Development
1956	Invention of the oxygen electrode (by Leland Clark)
1962	First description of a biosensor: an amperometric enzyme electrode for glucose
1969	Potentiometric biosensor: urease immobilised on an ammonia electrode to detect urea
1970	Invention of the Ion-Selective Field-Effect Transistor (ISFET)
1972/1975	Commercial Biosensor: Yellow Springs Instruments glucose biosensor
1975	Microbe-based biosensor Immunosensor: ovalbumin on a platinum wire Invention of the pO_2 / pCO_2 optode
1986	Bedside artificial pancreas (Miles)
1980	Fiber optic pH sensor for in vivo blood gases
1982	Fiber optic-based biosensor for glucose
1983	Surface plasmon resonance (SPR) immunosensor
1984	Mediated amperometric biosensor: ferrocene used with glucose oxidase for the detection of glucose
1987	MediSense ExacTech blood glucose biosensor
1990	Pharmacia Biacore SPR-based biosensor system released

Within each permutation lies a myriad of alternative transduction strategies and each approach can be applied to numerous analytical problems in health care (Alcock and Turner, 1994), food and drink (Kress-Rogers, 1996), the process industries (White and Turner,

1997), environmental monitoring (Dennison and Turner, 1995), defence and security. The design of integrated systems approaches to patterning sensitive elements and methods to improve the sensitivity, stability and selectivity of biosensors are key areas.

4.1.3 Biomaterials Used in Biosensor

Enzymes, antibodies, DNA, receptors, organelles and microorganisms as well as animal and plant cells or tissues have been used as biological sensing elements. Some of the major attributes of a good biosensing system are its specificity, sensitivity, reliability, portability, (in most cases) ability to function even in optically opaque solutions, real-time analysis and simplicity of operation (Mulchandani and Rogers, 1998 & Rella et al, 1996).

A number of novel and stable enzymes is required for the analysis of a variety of complex chemicals contained in varied environmental conditions encountered in industrial processing as well as waste management. Highly thermostable enzymes that can withstand even autoclaving temperatures may be required for the *in situ* monitoring of fermentors and other high-temperature processes (Daniel, 1996 & Arnold, 1998). In addition to thermal stability, other important environmental conditions often required are the ability to act in highly acidic, alkaline, hydrophobic (organic solvent), or oxidizing environments. Currently, there is also an interest in cold active enzymes. These enzymes have low temperature optima, or they exhibit high activities even at low temperatures (Brenchly, 1996). The basic advantage of such enzymes is the possibility of carrying out process monitoring even under chilled storage conditions normally practiced in food and allied industries. In this direction a variety of extremophiles such as the thermophiles, alkalophiles, and halophiles have gained importance in the production of enzymes for use in biosensors and other applications.

4.1.3.1 Enzyme Sensor

Enzymes make excellent analytical reagents due to their specificity, selectivity and efficiency. They are often used to determine the concentration of their substrates (as analytes) by means of the resultant initial reaction rates. If the reaction conditions and enzyme concentrations are kept constant at low substrate concentrations, these rates of reaction (v) are proportional to the substrate concentrations ([S]). When $[S] < 0.1\ K_m$, simplifies to give $v = (V_{max}/K_m)[S]$.

Biosensors using enzymes in their biological function generate the signal by either product formation, the disappearance of substrate, or co-enzyme conversion. Sometimes these reactions are superimposed with another biochemical event such as use of inhibition kinetics or coupling with other reaction(s).

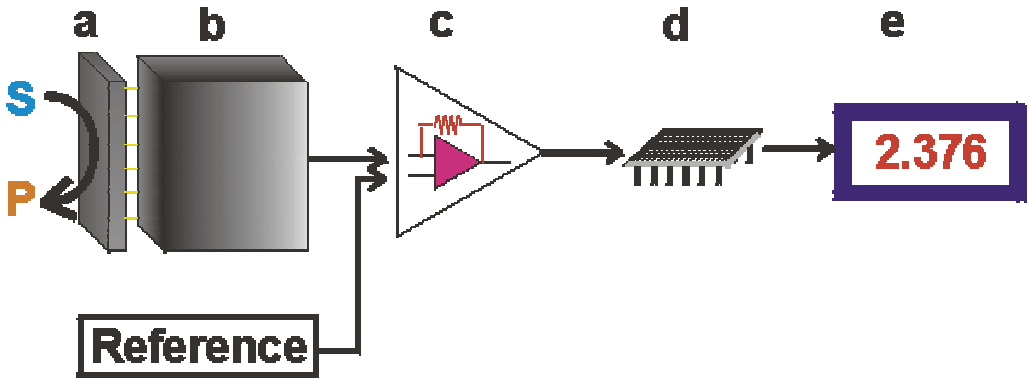


Fig 4.1 Schematic diagram of components of a biosensor

(a) The biocatalyst converts the substrate to product

(b) Transducer, which converts it to an electrical signal

(c), The output from the transducer is amplified

(d) Processer

(e) Display

The key part of a biosensor is the transducer, which makes use of a physical change accompanying the reaction (Figure 4.2). The following picture shows the different types of transducers used in a biosensor.

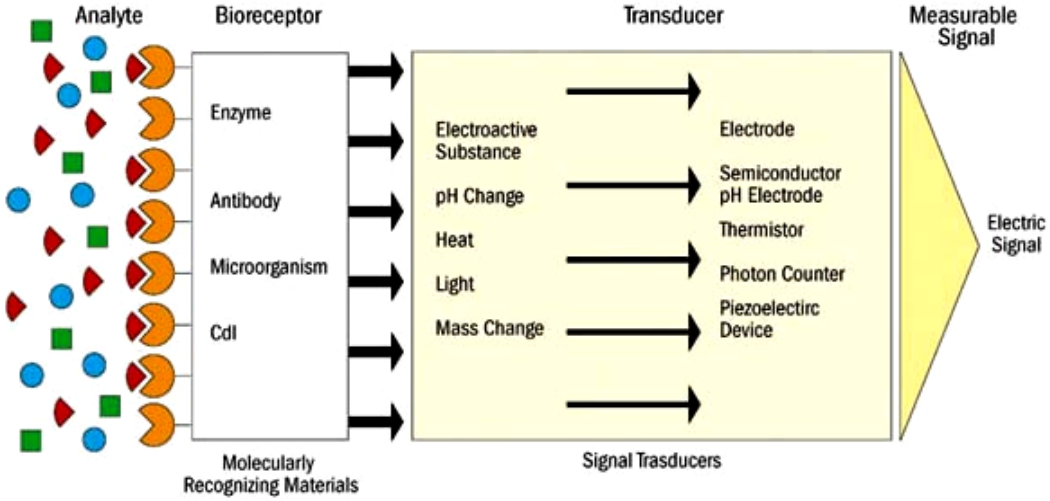


Fig 4.2 Schematic diagram of typical biosensor mechanisms

There are three so-called 'generations' of biosensors, First generation biosensors where the normal product of the reaction diffuses to the transducer and causes the electrical response, second generation biosensors which involve specific 'mediators' between the reaction and the transducer in order to generate improved response, and third generation biosensors where the reaction itself causes the response and no product or mediator diffusion is directly involved.

The electrical signal from the transducer is often low and superimposed upon a relatively high and noisy (i.e.,containing a high frequency signal component of an apparently random nature, due to electrical interference or generated within the electronic components of the transducer) baseline. The signal processing normally involves subtracting a 'reference' baseline signal, derived from a similar transducer without any biocatalytic membrane, from the sample signal, amplifying the resultant signal difference and electronically filtering (smoothing) out the unwanted signal noise. The relatively slow nature of the biosensor response considerably eases the problem of electrical noise filtration. The analogue signal produced at this stage may be output directly but is usually converted to a digital signal and passed to a microprocessor stage where the data is processed, converted to concentration units and output to a display device or data store.

A successful biosensor must possess at least some of the following beneficial features:

1. The biocatalyst must be highly specific for the purpose of the analyses, be stable under normal storage conditions and, show good stability over a large number of assays.
2. The reaction should be as independent of such physical parameters as stirring, pH and temperature as is manageable. This would allow the analysis of samples with minimal pre-treatment. If the reaction involves cofactors or coenzymes these should, preferably, also be co-immobilised with the enzyme
3. The response should be accurate, precise, reproducible and linear over the useful analytical range, without dilution or concentration. It should also be free from electrical noise.
4. If the biosensor is to be used for invasive monitoring in clinical situations, the probe must be tiny and biocompatible, having no toxic or antigenic effects. If it is to be used in fermenters, it should be sterilisable. This is preferably performed by autoclaving but no biosensor enzymes can presently withstand such drastic wet-heat treatment. In either case, the biosensor should not be prone to fouling or proteolysis.
5. The complete biosensor should be cheap, small, portable and capable of being used by semi-skilled operators.

4.1.4 Immobilization of enzymes for Biosensor applications

The basic requirement of a biosensor is that the biologic material brings the physicochemical changes in close proximity to a transducer. Immobilization not only helps in forming the required close proximity of the biomaterial with the transducer but also in stabilizing it for reuse (D'Souza, 2001). The biologic material has been immobilized directly on the transducer or, in most cases, in membranes, which can subsequently be mounted on the transducer (Mulchandani and Rogers, 1998).

Immobilization, in general, is known to stabilize the enzymes. Most of the stabilization efforts have involved either chemical cross-linking or protein engineering techniques. In this respect, useful strategies have been evolved for immobilization-stabilization of enzymes by multipoint covalent attachment to gels (Blanco et al., 1988).

Such approaches stabilize enzymes toward the conformational changes induced by heat, organic solvents, and so forth. Biomaterials can be immobilized either through adsorption, entrapment, covalent binding, cross-linking, or a combination of all these techniques (D'souza, 1989 & Bickerstaff, 1997 & D'souza, 1999). Selection of a technique and support depends on the nature of enzyme, nature of substrate, and configuration of the transducer used.

The choice of support and technique for the preparation of membranes often has been dictated by the low diffusional resistance of the membrane. The biological material has been immobilized directly on to the transducer or in most cases, in membranes, which can subsequently be mounted on to the transducer. This gives a catalytic redundancy, which is sufficient to ensure an increase in the apparent stabilisation of the immobilised enzyme. Even where there is some inactivation of the immobilised enzyme over a period of time, this inactivation is usually steady and predictable. Any activity decay is easily incorporated into an analytical scheme by regularly interpolating standards between the analyses of unknown samples. For these reasons, many such immobilised enzyme systems are re-usable over a period of several months. Clearly, this results in a considerable saving in terms of the enzymes' cost relative to the analytical usage of soluble enzymes.

When the reaction occurring at the enzyme-immobilised membrane of a biosensor, it is limited by the rate of external diffusion, the reaction process will possess a number of valuable analytical assets. It follows that the biocatalyst gives a proportional change in reaction rate in response to the reactant (substrate) concentration over a substantial linear range, several times the intrinsic K_m. This is very useful, When the analyte concentrations are 10 times more concentrated than that can be normally determined by use of the free enzyme in solution.

4.1.5 Types of Biosensors

a) Calorimetric biosensors

The basic principle of such biosensors is that all biochemical reactions involve a change in enthalpy, and this is detected by calorimetric biosensors. Many enzyme catalyzed reactions are exothermic, generating heat, which may be used as a basis for measuring the rate of reaction and, hence, the analyte concentration. The temperature changes are usually

determined by means of thermistors (Mosbach and Danielsson, 1981) at the entrance and exit of small packed bed columns containing immobilised enzymes within a constant temperature environment.

b) Potentiometric biosensors

Potentiometric biosensors make use of ion-selective electrodes in order to transduce the biological reaction into an electrical signal. In the simplest terms, this consists of an immobilised enzyme membrane surrounding the pH-meter probe where the catalysed reaction generates or absorbs hydrogen ions. The reaction occurring next to the thin sensing glass membrane causes a change in pH, which may be read directly from the pH-meter's display.

c) Conductometric biosensors

Conductometric biosensors measure the changes in the conductance of the biological component arising between a pair of metal electrodes. In 1994, Contractor et al. have constructed biosensors for estimation of glucose, urea neutral lipid/lipase and hemoglobin/pepsin by monitoring the change in the electronic conductivity arising as a change in redox potential and/or pH of the microenvironment in the polymer matrix.

d) Amperometric biosensors

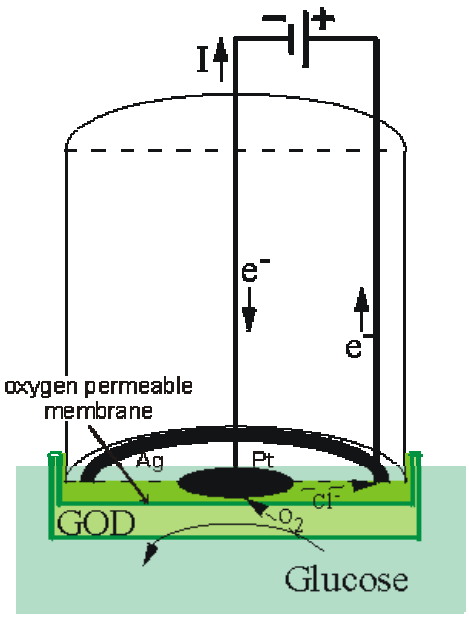
Amperometric biosensors measure the current produced during the oxidation or reduction of a product or reactant usually at a constant applied potential. The most important factor affecting the functioning of amperometric biosensors is the electron transfer between catalytic molecule, usually oxidase or dehydrogenase, and the electrode surface most often involving mediation or conducting polymer. They generally have response times, dynamic ranges and sensitivities similar to the potentiometric biosensors. The simplest amperometric biosensors in common usage involve the Clark oxygen electrode. This consists of a platinum cathode at which oxygen is reduced and a silver/silver chloride reference electrode. When a potential of -0.6 V, relative to the Ag/AgCl electrode is applied to the platinum cathode, a current proportional to the oxygen concentration is produced. Normally both electrodes are bathed in a solution of saturated potassium chloride and separated from the bulk solution by

an oxygen-permeable plastic membrane (e.g. Teflon, polytetrafluoroethylene). The following reactions occur:

Ag anode $4Ag + 4Cl^- \longrightarrow 4\ AgCl + 4e^-$

Pt cathode $O_2 + 4H^+ + 4e^- \longrightarrow 2\ H_2O$

The efficient reduction of oxygen at the surface of the cathode causes the oxygen concentration there to be effectively zero. The rate of this electrochemical reduction therefore depends on the rate of diffusion of the oxygen from the bulk solution, which is dependent on the concentration gradient and hence the bulk oxygen concentration. It is clear that a small, but significant, proportion of the oxygen present in the bulk is consumed by this process; the oxygen electrode measuring the rate of a process, which is far from equilibrium, whereas ion-selective, electrodes are used close to equilibrium conditions. This causes the oxygen electrode to be much more sensitive to changes in the temperature than potentiometric sensors. A typical application for this simple type of biosensor is the determination of glucose concentrations by the use of an immobilised glucose oxidase membrane.The reaction results in a reduction of the oxygen concentration as it diffuses through the biocatalytic membrane to the cathode, this being detected by a reduction in the current between the electrodes(Figure 4.3). Other oxidases may be used in a similar manner for the analysis of their substrates (e.g. alcohol oxidase, D- and L-amino acid oxidases, cholesterol oxidase, galactose oxidase, and urate oxidase)



4.3 Schematic diagram of a simple amperometric biosensor

A potential is applied between the central platinum cathode and the annular silver anode. This generates a current (I), which is carried between the electrodes by means of a saturated solution of KCl. This electrode compartment is separated from the biocatalyst (here shown glucose oxidase, GOD) by a thin plastic membrane, permeable only to oxygen. The analyte solution is separated from the biocatalyst by another membrane, permeable to the substrate(s) and product(s). This biosensor is normally about 1 cm in diameter but has been scaled down to 0.25 mm diameter using a Pt wire cathode within a silver plated steel needle anode and utilising dip-coated membranes.

The major problem with these biosensors is their dependence on the dissolved oxygen concentration. This may be overcome by the use of 'mediators', which transfer the electrons directly to the electrode bypassing the reduction of the oxygen co-substrate.

e) Optical biosensors

Optical biosensors are based on the measurement of light absorbed or emitted as consequence of a biochemical reaction. In such type of biosensors, light waves are guided by means of optical fibers to suitable detectors and are used for the detection of pH, O_2 and CO_2 etc. There are two main areas of development in optical biosensors. These involve

determining changes in light absorption between the reactants and products of a reaction, or measuring the light output by a luminescent process.

f) Piezo-electric biosensors

These biosensors operate on the principle of generation of electric dipoles on subjecting an anisotropic natural crystal to mechanical stress. Due to the adsorption of an analyte, the mass of the crystal is increased resulting in altered frequency of oscillation. This frequency change is easily detected by relatively unsophisticated electronic circuits. These types of biosensors have been utilized for the measurement of ammonia, hydrogen, methane, carbon monoxide, nitrous oxide and other organophosphorous compounds.

g) Immunosensors

Biosensors may be used in conjunction with enzyme-linked immunosorbent assays (ELISA). ELISA is used to detect and amplify an antigen-antibody reaction; the amount of enzyme-linked antigen bound to the immobilised antibody being determined by the relative concentration of the free and conjugated antigen and quantified by the rate of enzymic reaction. Enzymes with high turnover numbers are used in order to achieve rapid response. The sensitivity of such assays may be further enhanced by utilising enzyme-catalysed reactions, which give intrinsically greater response; for instance, those giving rise to highly coloured, fluorescent or bioluminescent products.

h) DNA Biosensors

DNA biosensors have an enormous application in clinical diagnostics of inherited diseases, rapid detection of pathogenic infections, and screening of cDNA colonies required in molecular biology. Present methods of genetic analysis require pre- and post-treatments to modify DNAs with probes as proteins. Recently, DNA integrated electroactive polymers (thin films or LB monolayers) have provided intelligent materials which possess superior intelligent material properties of self-assembly, self-multiplication, self-repair, self-degradation, redundancy, and self-diagnosis (Minehan and et al, 1994). Immobilization of

DNA on a conducting polymer matrix facilitates the detection of a signal (amperometric or potentiometric) generated because of interaction of proteins or drugs with DNA.

i) Nanobiosensor

Nanotechnology is playing an increasingly important role in the development of biosensors (Vo-Dinh et al., 2001 & Haruyama, 2003 & Jain, 2003). Nanotechnology involves the study, manipulation, creation and use of materials, devices and systems typically with dimensions smaller than 100 nm. Nanomaterials, or matrices with at least one of their dimensions ranging in scale from 1 to 100 nm, display unique physical and chemical features because of effects such as the quantum size effect, mini size effect, surface effect and macro-quantum tunnel effect.

Use of nanomaterials in biosensors allows the use of many new signal transduction technologies in their manufacture. Because of their submicron size, nanosensors, nanoprobes and other nanosystems are revolutionizing the fields of chemical and biological analysis, to enable rapid analysis of multiple substances in vivo. Various nanostructures include nanotubes, nanofibers, nanorods, nanoparticles and thin films have been investigated to determine their properties and possible applications in biosensors. Nanoparticles have numerous possible applications in biosensors. For example, functional nanoparticles (electronic, optical and magnetic) bound to biological molecules (e.g. peptides, proteins, nucleic acids) have been developed for use in biosensors to detect and amplify various signals. Some of the nanoparticle-based sensors include the acoustic wave biosensors, optical biosensors, magnetic and electrochemical biosensors.

4.1.6 Application of Biosensors

Biosensors provide a rapid and convenient alternative to conventional methods for monitoring chemical substances in fields as diverse as medicine, environment, fermentation, and food processing (Mulchandani and Rogers, 1995 & Ramsay, 1998 & Nikolelis et al, 1998). In medicine, biosensors can be used for the monitoring of blood glucose, urea, cholesterol, lactate, neurotransmitters, and so on. They are also gaining importance in monitoring the assimilable nutrients, intermediates, and end products of fermentation and in

food industry for shelf-life assessment (freshness) of fish, meat, and vegetables, microbial contamination,and olfaction (Mulchandani and Rogers,1995 & Ramsay,1998).Biosensors will play a major role in environmental monitoring (Nikolelis et al,1998, Rogers and Gerlach,1996) such as for estimation of BOD (Biological Oxygen Demand) (Marty et al ,1997) and for assay of explosives such as TNT (2,4,6-trinitrotoluene) and RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) (Bart et al, 1997) as well as other toxic and mutagenic environmental chemicals including pesticides (Trojanowicz and Hitchman, 1996) herbicides, and heavy metals (Nikolelis et al, 1998 & Rogers and Gerlach,1996). Biosensors come in different sizes and shapes and make use of a variety of transducers such as pH- or ion-selective electrodes, thermistors, optical fibers, and piezoelectric crystals (Mulchandani and Rogers, 1998 & Blum and Coulet, 1991). Advances in computational techniques now allow us to model both electron transfer reactions and receptor binding interactions with increasing accuracy.

4.1.7 Commercial Biosensors

Glucose biosensor is commercially available for the determination of glucose level in blood by glucose oxidase enzyme. Urease enzyme is used in biosensor to determine the level of urea. Lactate oxidase and lactate dehydrogenase are used in lactate biosensor. Cholestrol oxidase is used in the determination of cholestrol in the body.

4.1.8 Need for the CLEC Laccase based Biosensor

The importance of using biosensors for environmental surveillance becomes more prevalent in literature with the emphasis to phenol determination and control (Rogers, 1995). Phenols are byproducts of large-scale production and use of man-made organics such as drugs, dyes, antioxidants, paper pulp and pesticides and cause ecologically undesirable effects (Canofeni et al., 1994). Most phenols exhibit different toxicities, and their determination is very important for evaluating the total toxicity of an environmental sample. In general, phenolic compounds are subjected to chromatographic separation before detection. However, the separation takes time, and often requires pre- concentration. In addition, the equipment is expensive and is not generally portable. A device, which permits detection of phenols in aqueous solutions at concentration in the low micro molar range with minimal sample preparation, will be useful (Vianello et al., 2004)

Many biosensors have been developed in the past using the catalytic activity of the redox enzymes for phenol determination. Biosensors are developed with enzymes such as tyrosinase, peroxidase, and laccase, etc (Duran and Esposito, 2000) using different electrode materials, flow systems and sample pretreatment techniques. Laccase belongs to multi copper containing oxidase performing four-electron reduction of oxygen, with aromatic phenols and amines and with substituted phenols and amines used as electron donors (Yaropolov et al, 1994 & Xu, 1999). The active site of laccase consists of four copper atoms involved in the coordinated oxygen reduction. Laccase does not require H_2O_2 as co-substrate and any co-factors for its catalysis and hence the construction of the biosensor becomes simple. Phenolic substrate is subjected to a one-electron oxidation giving rise to an aryl radical. The active species can be converted to a quinone in the second stage of the oxidation (Marko-Varga et al, 1995). A recombinant fungal laccase was immobilized and used in biosensor for the detection of pyrocatechol and phenols under steady state and flow through regions (Kulys and Vidziunaite, 2003).

Purified enzymes have been most commonly used in the construction of biosensors due to their high specific activities as well as high analytical specificity. Purified enzymes are, however, expensive and unstable, thus limiting their applications in the field of biosensors. CLEC Technology avoids the lengthy and expensive operations of enzyme purification, as well as marix modified cost. CLECs have high specific activity and in purer form hence generate a clear signal when it is used in biosensor applications. (Navia and St. Clair, 1999) from the smallest possible quantity of substrate (analyte). Proteins in crystal form are uniformly arranged and this uniformity produces a linear and predictable signal. Hence, CLEC formulations may be used as the component of biosensor for detecting an analyte of interest in a sample.

This chapter deals with the use of CLEC laccase as a catalyst in a biosensor for the determination of phenolic compounds.

4.2 Materials and Methods

4.2.1 Materials

Laccase from *Trametus versicolor* (E.C.1.10.3.2) was purchased from Fluka and Glutaraldehyde, ABTS (2,2'-Azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid), Catechol, Catechin, Pyrogallol, Guaiacol, β-cyclodextrin were purchased from Sigma (St.Louis,USA). Ammonium sulphate and other reagents used are in analytical grade.

4.2.2 Methods

4.2.2.1 Preparation of Cross-linked enzyme crystals of Laccase

4.2.2.1.1 Crystallization of Laccase

Laccase was crystallized by batch method. 50 mg of crude laccase was dissolved in 3 ml of Sodium acetate buffer, 0.1M at pH 5.6. Ammonium sulphate (75% saturation) was added in small portions over 3 hours by stirring at 5±1° C . Then the super saturated solution was kept at this temperature undisturbed for 20 hours. The crystals formed were separated by centrifugation at 2000 rpm for 8 minutes.

4.2.2.1.2 Cross-linking of Laccase crystals

The laccase crystals were cross-linked using 1.5 % glutaraldehyde (v/v) solution in isopropanol for 20 minutes at 25°C. After cross-linking, it was washed 3 times with 0.1 M sodium acetate buffer at pH 5.6 to remove excess glutaraldehyde and stored in the same buffer at pH 5.6.

4.2.2.2 Enzyme assay

The laccase assay was done using ABTS (5 mM final concentration) as substrate in 0.1 M sodium acetate buffer pH 5.0. In 3ml assay volume, 2 ml of buffer and 750µl of 20mM ABTS and 250 µl of enzyme solution were taken, the optical density of the oxidation product was read at 420 nm (ε_{max} = 36000 $M^{-1}cm^{-1}$).

4.2.2.3 Assay of CLEC Laccase

Assay of CLEC laccase was done using ABTS (5mM final concentration) as substrate.Instead of directly taking measurement, the assay mixture (in 3ml assay volume, 2.25 ml of buffer and 750µl of 20mM ABTS and 1 mg of CLEC Laccase) containing the CLEC laccase was stirred continuously for 1 minute and then the increase in absorbance was monitored at definite time intervals at 420 nm.

4.2.2.4 Biosensor Electrode Development

A Clark's electrode with an amperometric detection system was used for detection of phenols. The Clarke-type electrode consists of a Gold (Au) cathode and a reference Ag/AgCl- electrode covered with saturated KCl electrolyte enclosed within a Teflon membrane. The electrode response was measured by an amperometric detector system, which was fabricated at Central Food Technological Research Institute, Mysore, India. During biochemical reactions, O_2 is consumed which was monitored as current decrease. This current decrease was converted into voltage using above system and response in voltage was displayed which is proportional to the concentration of substrate. Response curves were obtained for different substrate with varying concentrations. The standard solution of respective substrate was injected in sample cell and continuously stirred during the measurement. Response times were measured as the time required to achieve 95% of the final steady state response. Schematic representation of Clark's electrode that used for the determination of phenol has given below (Figure 4.4).

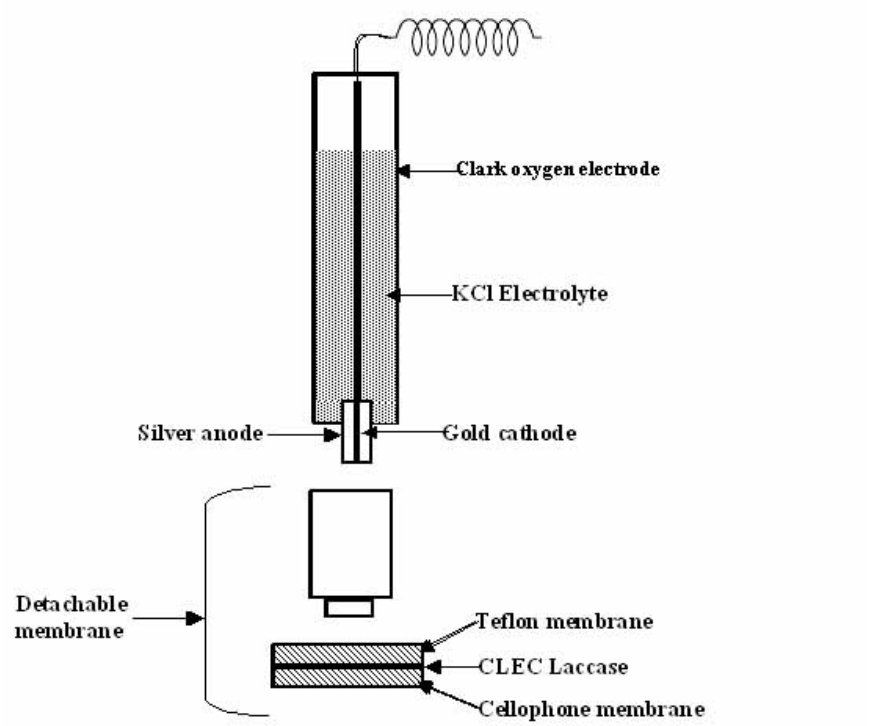


Fig 4.4 Schematic representation of Clark's electrode used in the Biosensor

Several key parameters such as concentration of phenols as substrates and pH have been investigated in order to optimize the response properties of this biosensor at 28 ± 2 °C.

4.2.2.5 Preparation of Membrane for biosensor application:

1 mg of Cross-linked enzyme crystals (114 IU) was mixed in 30% PVP (Poly Vinyl Propylidone) gel in a cellophane membrane and allowed to dry for 30 minutes. The enzyme containing membrane was mounted on to the Clark's electrode and electrode was connected to amperometric detector. An individual enzyme membrane was built by securing a micro porous dialysis membrane to the tip of the sensor body with an o-ring, and the redox reaction was monitored amperometrically.

4.2.2.6 Measurement of Linearity and Kinetics

Linearity and kinetics measurements were done with some phenolic substrates (Fig 4.6 & 4.11-4.16) having a concentration from 0.1mM to 0.5mM levels in 0.1M sodium acetate buffer at pH 5.5. The increase in voltage was read for 5 minutes at a time interval of 20 seconds at 28 ± 2 °C.

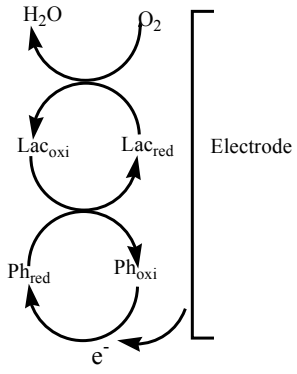
4.3 Results and discussion:

It is possible to design a specific biosensor choosing a versatile oxidoreductase enzyme like laccase, which catalyses a whole group of related phenolics of similar molecular structure. Free and immobilized laccase enzyme has been used for the detection of varity of analytes. Free enzyme can be used only once, where as immobilized enzyme can be reused many times but the activity loading is less. It was observed that cross-linked enzyme crystals exhibit the highest protein density and specific activity and hence is useful in the biosensor applications, as it improves the contact between itself and the analyte both in aqueous and/or organic media. CLEC laccase has been used for in biosensor for the first time.

The cross-linked enzyme crystals used for the experiment were of 2-20 μm in size. The CLEC laccase crystals were entrapped in PVP gel and mounted on a modified Clarks electrode, and used for the detection of phenols by amperometric method. The active site of laccase consists of four Copper ions involved in a coordinated oxygen reduction. Copper sites have historically divided into three classes copper type 1(T1) or blue copper, type 2(T2) or normal copper, type3 (T3) or coupled binuclear copper center based on their

spectroscopic features, which reflect the geometric and electron structure of the active site. This enzyme couples the oxidation of the substrate, which takes place at type 1 copper site with the reduction of dioxygen to water taking place at the type 2 and type 3 trinuclear copper cluster site (Yaropolov et al., 1994). The enzyme molecules, at the surface of the electrode are oxidized by oxygen (Figure 4.5) followed by their re-reduction to original state by phenolic compounds.

4.3.1 Biosensor response for various substrates



Lac_{oxi} Oxidised form of Laccase enzyme

Lac_{red} Reduced form of Laccase enzyme

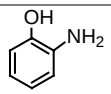
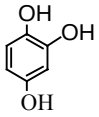
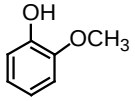
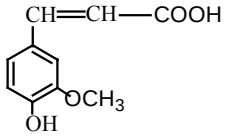
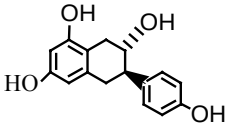
Ph_{oxi} Oxidised form of Phenolic Compound

Ph_{red} Reduced form of phenolic compound

Fig 4.5 Reaction mechanisam in Laccase Biosensor

The electrode response of these products was measured by the use of a modified Clark electrode (Table 4.2).

Table 4.2 Electrode response of CLEC laccase to various phenols and their products

Substrates Used (0.5mM)	**Structure of the substrates**	**Product formed**	**Response to oxidized phenols (V) in 180 Sec**	**Relative Response (%)**
2-amino phenol		Iminoquinone	0.42	100.0%
Catechol		o-Benzoquinone	0.46	109.5%
Pyrogallol		Purpurogallin	0.47	112.0%
Guaiacol		Biphenoquinone	0.29	69.0%
Ferulic Acid		Diferulic acid	0.20	47.6%
Catechin		o-Quinone	0.09	21.4%

The analysis of analyte in the sample in biosensor is particularly desirable to produce the largest possible detectable signal from the smallest possible quantity of substrate and catalyst. It was observed that as the molecular weight of the substrate increases the response

time also increases, and this may be due to diffusion limitations with in the cross-linked enzyme crystal lattice and in the gel matrix where in CLEC is embedded. The diffusion limitations of CLECs are defined by size, length and microenvironment (surface charge etc) of microscopic channels (20-55°A) (Michael, 2001), which are formed between the two individual crystals, when cross-linked to form a three-dimensional lattice. The size of the substrate (Margolin and Navia, 2001) is also important, because substrates with high molecular weight may not be able to diffuse through the channels and hence reduce the reactivity. In this biosensor, the CLEC is embedded in 30% PVP gel, which again leads to additional diffusion limitation for the larger substrates. Thus, to detect catechin and ABTS the sensor takes more time to start responding. Catechin start responding only after 60 seconds where as ABTS (non phenolic) responds only after 140 seconds (Table 4.3).

Table 4.3 Response time for various substrates using CLEC laccase

Substrate 0.5mM(Conc)	**Molecular weight**	**Response time (Sec)**	**Relative response time (%)**
2-amino phenol	109.11	120 ± 20	100%
Catechol	110.1	140 ± 20	85.7%
Pyrogallol	126.11	120 ± 20	100%
Guaiacol	124.1	120 ± 20	100%
Ferulic Acid	194.2	140 ± 20	85.7%
Catechin	290.3	300 ± 20	40%
ABTS (non phenolic)	548.7	400 ± 20	30%
Phenol		No response	
3-amino phenol		No response	
4-nitro phenol		No response	
2,4 - dinitro phenol		No response	

The higher concentration of the substrates above an optimum inhibits the enzyme, hence reduces the sensitivity of the device. Substrates like catechol, catechin, and guaicol form brown colored reaction products, which in turn deposits on the surface on the enzyme membrane, and subsequently inactivating the enzyme.

4.3.2 Calibration curve for phenolic compounds:

Among all the phenolic analytes tested 2-amino phenol gives the best response (R^2=0.9969). All the analytes showed a good correlation coefficient $R^2 > 0.97$. From the linearity graph (Figure 4.6.), the correlation coefficient R^2 and linearity line were calculated. From the graph it was observed that biosensor sensitivity is better for substrates like pyrogallol, catechol and 2-amino phenol with a higher slope of 0.913, 0.8274 and 0.82 respectively (Taken from the line, m value (refer Table 4.4)). However, the other three substrates used namely guaiacol, ferulic acid, catechin having a lower slope of 0.505, 0.354 and 0.247 respectively has lower sensitivity and hence, is not detected to the same extent. Pyrogallol, catechol and 2-amino phenol and guaiacol gave an increase in response upto 60 seconds after that the voltage reading remained constant.

Fig 4.6. Linearity graph of CLEC Laccase based Biosensor with different substrates

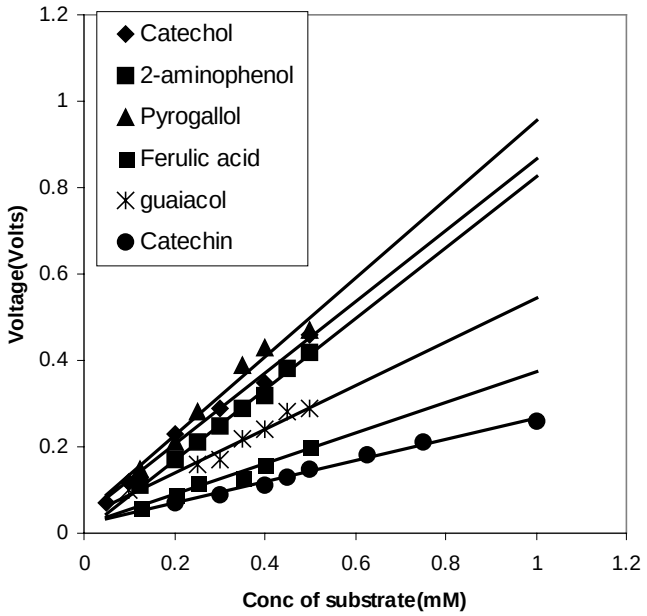
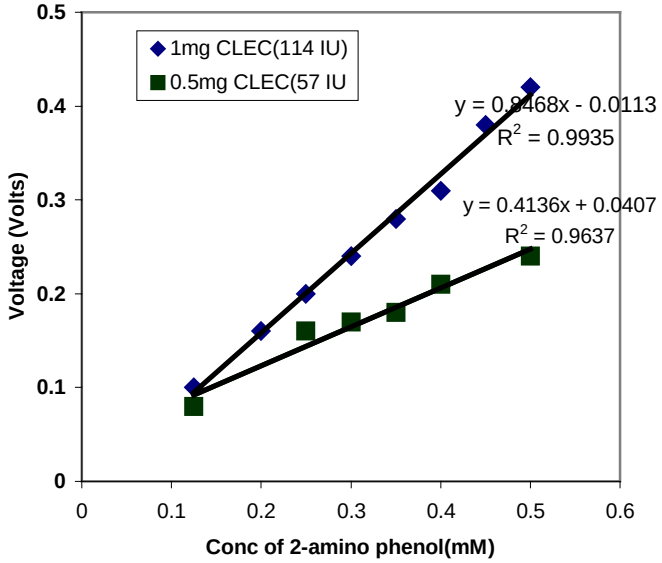


Table 4.4 Correlation coefficient R2 and linearity line of substrates

Substrate	Correlation Coefficient R^2	Linearity Line
2-amino phenol	0.997	Y= 0.820x + 0.0048
Catechol	0.988	Y= 0.827x + 0.0396
Pyrogallol	0.970	Y= 0.913x + 0.0440
Guaiacol	0.973	Y= 0.505x + 0.0389
Ferulic Acid	0.971	Y= 0.3542x + 0.0189
Catechin	0.992	Y= 0.242x + 0.0195

From the graph (Figure 4.6) it is observed that biosensor linearity of response is better for substrates like pyrogallol, catechol and 2-amino phenol with a higher slope of 0.913, 0.8274 and 0.82 respectively (Table 4.3) (Taken from the line, m value). However, the other three substrates used guaiacol, ferulic acid, catechin having a lower slope of 0.505, 0.354 and 0.247 respectively, is not obviously detected to the same extent. The linearity also increases with increase enzyme concentration (Figure 4.7).

Fig 4.7 Effect of CLEC Enzyme Concentration on linearity



The specificity of the laccase-modified electrode is demonstrated in Figure 4.8. It was found that the CLEC enzyme membrane responded well to pyrogallol, catachol and 2-amino phenol giving 0.47 V, 0.46 V, and 0.42 V respectively.

Fig 4.8 Biosensor response at different Enzyme Concentration with various substrates

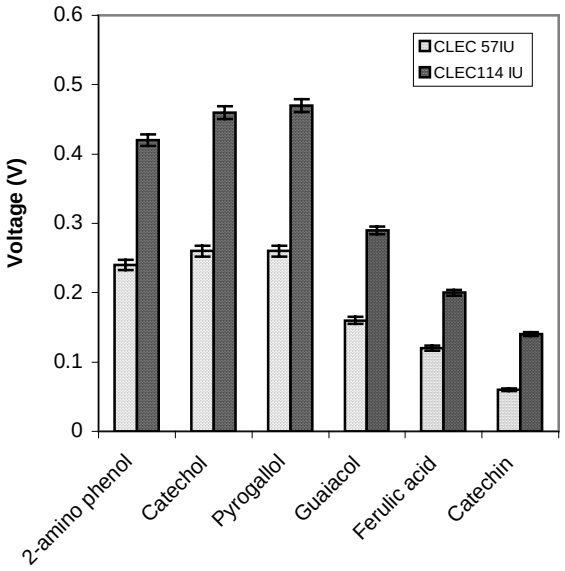


Fig 4.9 Linearity graph of CLEC Laccase at 60 second

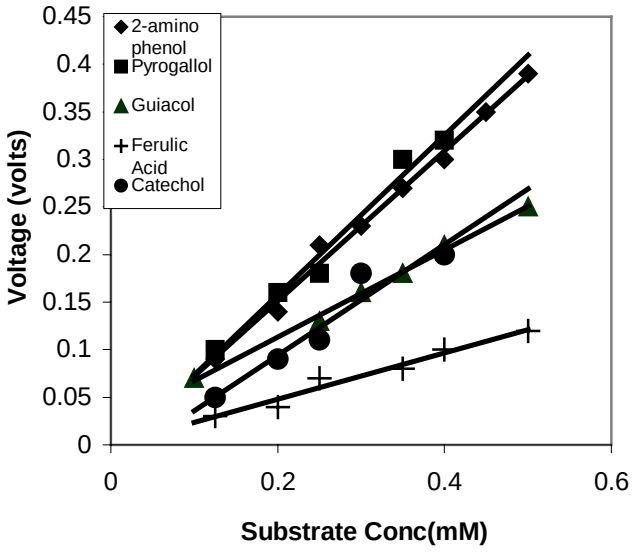
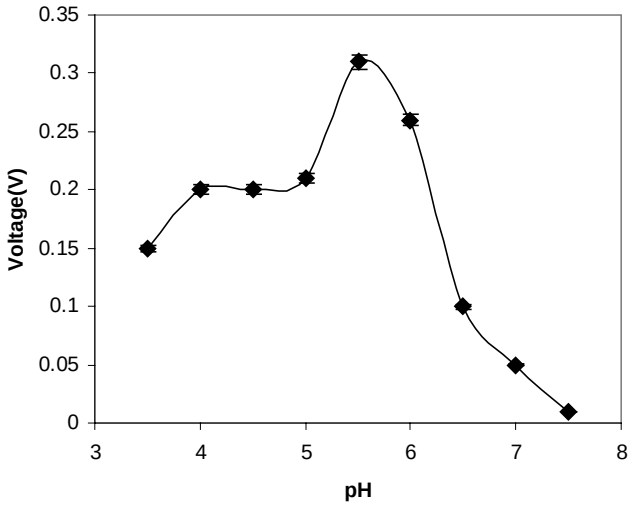


Figure 4.9 represents the linearity graph of CLEC laccase at 60 second for different substrate concentrations. Catechin started responding only after 60 seconds, so the linearity line could not be drawn for catechin.

The effect of pH has been determined by comparing the relative sensor response to the phenolic substrates over the pH range from 3.5 to 7.5. (Figure 4.10) Shows the pH profile obtained and the maximum sensor response was over the pH range of 5.5 - 6.0. Only 68% of the maximum response was obtained at pH 5.0 and only 16% of the maximum response was obtained at pH 7.0.

Fig 4.10 pH profile of CLEC Laccase (catechol as substrate)



The optimum response over the pH just below neutral pH is an advantage for this biosensor. The soluble laccase enzyme showed pH optima around pH 3-4. The cross-linking has shifted the useful pH range to 5.5 to 6.0, hence more useful in the biosensor detection. The lowest substrate concentration that could be determined using this biosensor was determined by the extrapolation of the response curve and hence it is possible to detect the phenolics in ppb levels.

The phenols like 3-amino phenol, phenol, 4-nitro phenol, 2,4 dinitro phenol, having a concentration range of 0.05 – 0.5 mM did not respond to biosensor. This is due to the fact that in 3-amino phenol the amino group is in meta position and hence laccase does not catalyze the reaction, hence the biosensor could not respond .The other three phenols are mono phenols and laccase does not catalyze the oxidation of monophenols, thus biosensor

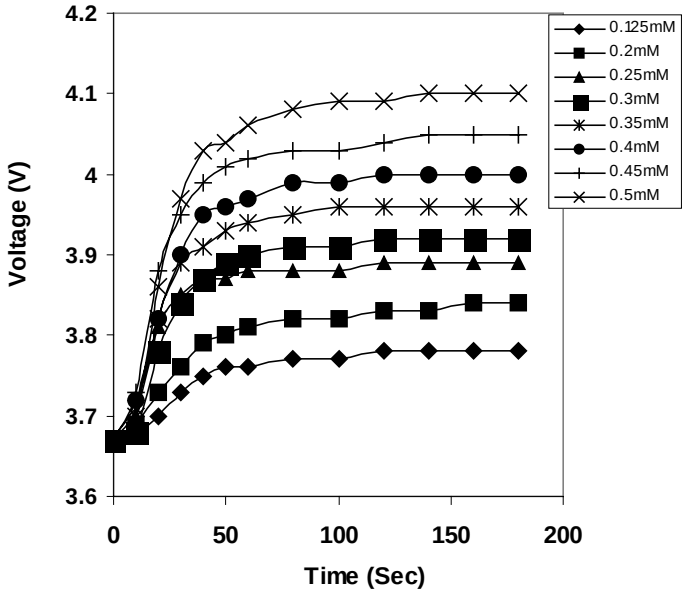
could not respond to these compounds. Cross-linking the laccase crystals does not alter the catalytic activity and specificity.

4.3.3 Kinetic Behavior of biosensor for various substrates

a) Response of the substrate (2-amono phenol):

From the Figure 4.11 it is observed that 2-amonophenol injected at varying concentration gave good response from 0.5mM to 0.125mM with good linearity (R^2 = 0.9969). Therefore, it is possible to monitor 2-aminophenol which is one of the pollutant in the environment.

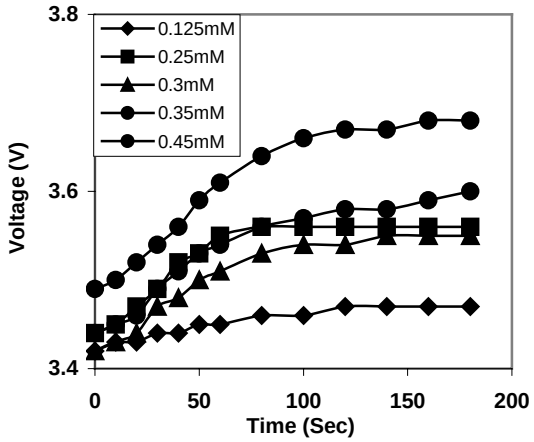
Fig 4.11 Kinetic behavier of CLEC laccase Biosensor using with 2-amino phenol as substrate



b) Response of the substrate (Catechol):

Catechol gave a good response from 0.125mM to 0.45 mM (Figure 4.12) upto 60 seconds, after that it got stabilized. It is possible to monitor catechol present in tea leaves.

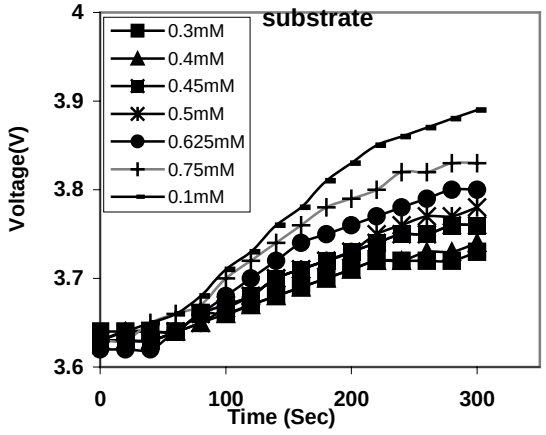
Fig 4.12 Kinetic behavier of CLEC laccase Biosensor using with catechol as substrate



c) Response of the substrate (Catechin):

Catechin gave a good response from 0.2mM to 0.1 mM upto 300 (Figure 4.13) seconds. It started responding only after 60 seconds because due to its high molecular weight so there was a diffusion limitation. It is possible to monitor catechin present in tea leaves.

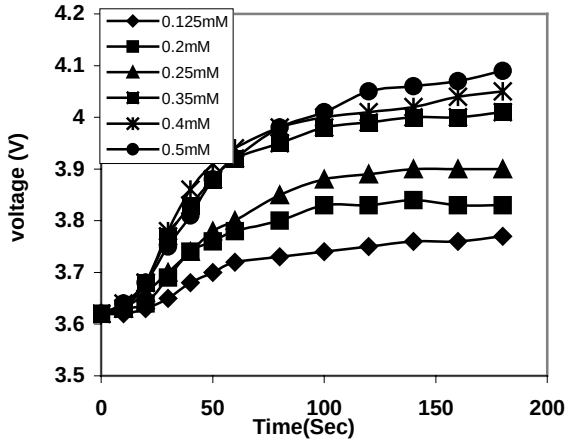
Fig 4.13 Kinetic behavier of CLEC laccase Biosensor using with catechin as substrate



d) Response of the substrate (Pyrogallol):

Pyrogallol gave a good response from 0.125mM to 0.5 mM upto 60 seconds (Figure 4.14). After that, it got stabilized. It is possible to monitor Pyrogallol, which is an antioxidant present food and beverages.

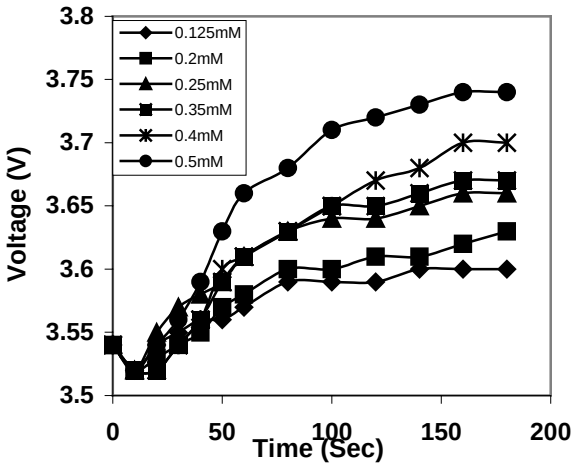
Fig 4.14 Kinetic behavier of CLEC laccase Biosensor using with pyrogallol as substrate



e) Response of the substrate (Ferulic Acid):

Ferulic acid gave a good response from 0.125mM to 0.5 mM upto 60 seconds (Figure 4.15). After that, it got stabilized. It is possible to monitor ferulic acid, which is an antioxidant present food and beverages.

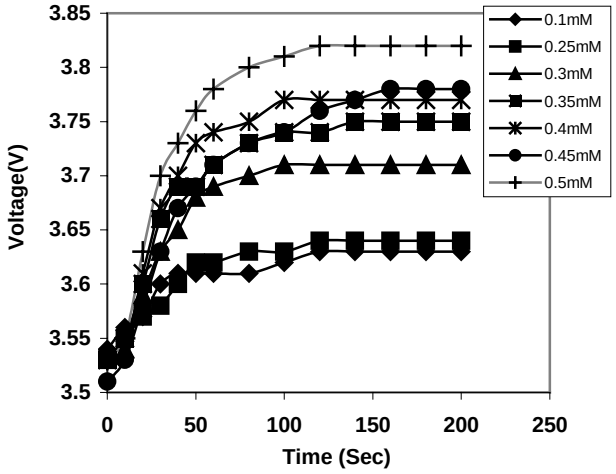
Fig 4.15 Kinetic behavier of CLEC laccase Biosensor using with ferulic acid as substrate



f) Response of the substrate (Guaiacol):

Guaiacol gave a good response from 0.1mM to 0.5 mM upto 60 seconds (Figure 4.16). After that, it got stabilized. It is possible to monitor the presence of guiacol.

Fig 4.16 Kinetic behavier of CLEC laccase Biosensor using with guaiacol as substrate



4.3.4 Operational Stability

The CLEC biosensor was reused for 30 cycles of reaction and detection. 40% of the activity was lost after 30 cycles and the loss is mainly be due to the accumulation of the reaction product formed on the CLEC during each reaction cycle. Most of the phenols that we have tested formed colored products over the crystals, and thus slowly inactivating it, by masking the active site.

4.3.5 Utility of the Biosensor

The present biosensor could be used for the detection of antioxidants like ferulic acid and pyrogallol in food and beverages and the presence of polyphenols like catechol and catechins present in tealeaves, which is an index of the quality of a particular tea. The sensor could also be used to monitor organic pollutants like 2-amino phenol, in wastewater.

4.3.6 Conclusions

Sensitive rapid and precise determination of phenols and its derivatives is important in environmental control and protection. Cross-linked enzyme crystals exhibit the highest protein density and specific activity. Thus, CLEC can be used in the biosensor applications, which detects an analyte of interest in sample and also improves the contact between itself and the analyte both in aqueous and organic media. The Cross-Linked enzyme crystals of laccase has an added advantage over the soluble enzyme in the biosensor application as its optimum pH range is in the range of 5.5 to 6, which is nearer to the neutral, compared to the soluble enzyme which has the optimum pH of 3-4. This biosensor could be used to detect the quantity of catechol and catechins in tea and the antioxidants like pyrogallol and ferulic acid in food and beverages and organic pollutants like 2-amino phenol in wastewater.

4.4 References

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CHAPTER V
BIOTRANSFORMATION OF PYROGALLOL TO PURPUROGALLIN USING CLEC LACCASE

5.1 Introduction

Biotransformations using enymes represents a useful tool for the production of various chemicals such as pharmaceuticals, agrochemicals, fragrances and flavors and consumer care products, because of the advantages of regio-stereo and chemo-selectivity. Commonly the word biotransformation is used to describe the transformation of a substrate or precursor into a certain product by a biocatalyst in only few steps. The major challenge in the biotransformation process is the stabilization of enzymes in organic solvents.

The major challenge in the biotransformation process is the stabilization of enzymes in organic solvents. Application of Cross-linked enzyme crystals in biotransformations is mainly because of the use of high conc. of organic solvents and substrates where the soluble enzyme cannot be used (Grim, 2001). Biotransformations using CLEC enzymes were done for peptide synthesis, chiral resolution, chemo- and regio-selective transformation, carbon-carbon bond formation & reduction reactions (Margolin and Navia, 2001).

Generally, laccase catalyses the oxidation of pyrogallol to purpurogallin which is also used for the assay of laccase enzyme. Chemical synthesis of purpurogallin was not available in literature. However, it is originally isolated from the plant Quercus nutgall (white oak). It has antioxidant activity (Wu et al, 1996), biological activity (Inamori et al, 1997) and it is an inhibitor of epidermal growth factor receptor protein tyrosine kinase activity (Abou-Karam and Shier, 1999) and inhibitor of xanthane oxidase (Sheu et al, 1998). Biotransformation of Pyrogallol to Purpurogallin using CLEC laccase is a single step eco-friendly method. The continuous production of purpurogallin using CLEC laccase is a novel process and the product recovery is simple, and is cost effective.

5.2 Materials and Methods

5.2.1 Materials

Laccase from *Trametus versicolor* (E.C.1.10.3.2) (0.8U/mg, catechol as substrate) was purchased from Fluka and Glutaraldehyde,ABTS(2,2'-Azino-bis(3-ethylbenz-thiazoline-6-sulfonicacid)), pyrogallol, purpurogallin, β-cyclodextrin were purchased from Sigma (St.Louis,USA). Ammonium sulphate and other reagents used were of analytical grade.

5.2.2 Methods

5.2.2.1 Crystallization of Laccase

Laccase was crystallized by batch method. 50 mg of crude laccase was dissolved in 3 ml of 0.1M Sodium Acetate buffer at pH 5.6. Ammonium sulphate (75% saturation) was added in small portions over 3 hours by stirring. Then the super saturated solution was kept at 5°C for 20 hours. The crystals were separated by centrifugation at 2000 rpm for 8 minutes.

5.2.2.2 Cross-linking of Laccase crystals

The laccase crystals were cross-linked using glutaraldehyde (1.5%) (v/v) solution in isopropanol for 20 minutes at 25°C. After cross-linking, it was washed 3 times with 0.1 M sodium acetate buffer at pH 5.6 and stored in the same buffer.

The cross-linked laccase crystals were mixed with 1mM β-cyclodextrin, and freeze-dried. This β-cyclodextrin coated CLEC laccase was used for the biotransformation of pyrogallol to purpurogallin.

5.2.2.3 Enzyme assay measurement

The laccase assay was done using ABTS (5 mM final concentration) as substrate in 0.1 M sodium acetate buffer pH 5.0. In 3ml assay volume, 2 ml of buffer and 750µl of 20mM ABTS and 250 µl of enzyme solution were taken, the optical density of the oxidation product was read at 420 nm (ε_{max} = 36000 $M^{-1}cm^{-1}$).

5.2.2.4 Estimation of Protein

Protein estimation was done by Lowry's method using BSA as standard.

5.2.2.5 Assay of CLEC

Assay of CLEC laccase was done using ABTS (5mM final concentration) as substrate.Instead of directly taking measurement, the assay mixture (in 3ml assay volume, 2.25 ml of buffer and 750µl of 20mM ABTS and 1 mg of CLEC Laccase) containing the CLEC laccase was stirred continuously for 1 minute and then the increase in absorbance was monitored at definite time intervals at 420 nm.

5.2.2.6 Continuous biotransformation of pyrogallol to Purpurogallin using CLEC Laccase as the catalyst in a Packed Bed Reactor (PBR)

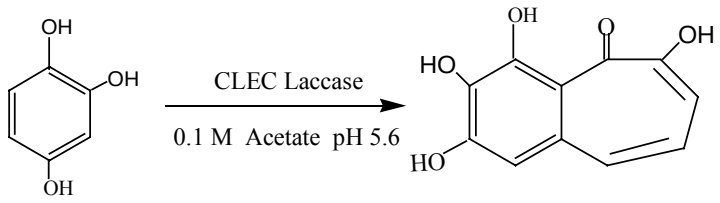
Continuous transformation of pyrogallol to purpurogallin was carried out in a packed-bed reactor (Fig 1). A glass column (12 ×1.6 cm and V_o = 0.188 mL) with 120mg of CLEC Laccase (4193 IU, ABTS units) was used, this catalyst was packed in between two layers of acid washed and purified high silica sand and the reaction was carried out at 28±2°C. Continuous production of purpurogallin from pyrogallol was carried out at by pumping the pyrogallol (pH 5.6) solution in the upward direction at concentrations of 3mM, 5mM and 7mM. The biotransformation was conducted at different residence times and product effluent was collected from the top of the packed column. The column was fully covered with aluminum foil to exclude light and to prevent any auto oxidation.

Dilution rate (h^{-1}) = Flow rate / Void volume (V_0)

Residence time (h) = 1/ Dilution rate

Productivity = Product formed/ L x Dilution rate

Reaction Rate = mg of product formed/ h / mg of CLEC used



Pyrogallol **Pupurogallin**

Scheme 5.1 Biotransformation of pyrogallol to purpurogallin using CLEC Laccase

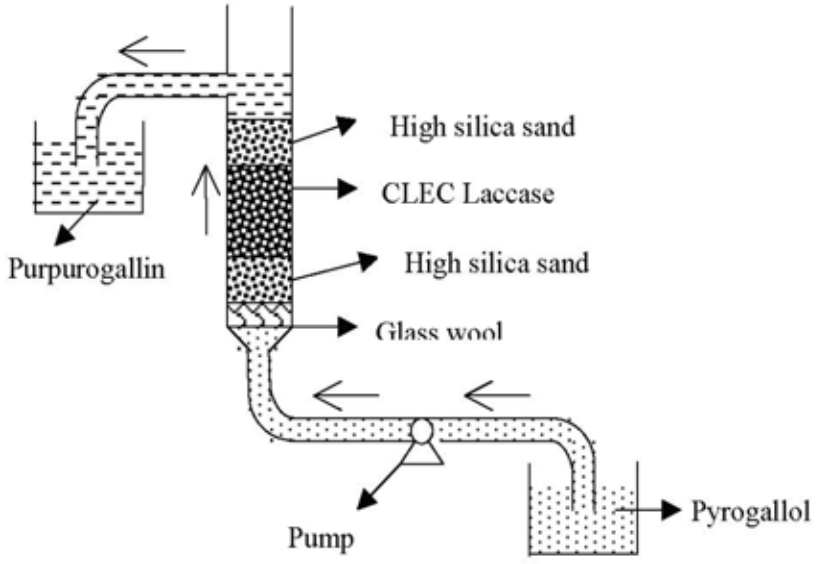


Fig 5.1 Schematic representation of Packed Bed Reactor for the biotransformation of pyrogallol to Purpurogallin using CLEC Laccase

5.2.2.7 Separation of Purpurogallin from unreacted pyrogallol

Using silica gel column with solvent combination of 95% chloroform and 5% methanol (v/v) was used for the separation. The product separated was conformed by IR spectrum and GC-MS. Productivity and the percentage of conversion were calculated by comparing the optical density of the product with the standard purpurogallin.

5.3 Results and Discussion

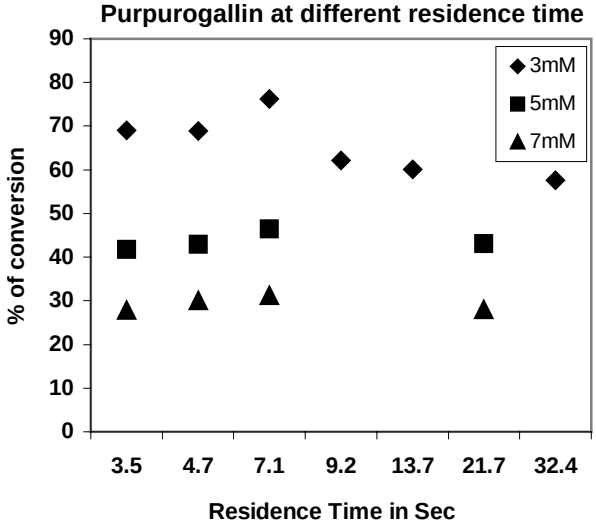
Cross-linked enzyme crystals of laccase were prepared by crystallizing laccase enzyme using $(NH_4)_2SO_4$ (75% saturation) followed by cross-linking the crystals with 1.5% glutaraldehyde. After crystallization and cross-linking, the enzyme activity retention was 64%. The crystals formed were in 2-20μm size, and it was further lyophilized with 1 mM β-cyclodextrin. The β-cyclodextrin coated CLEC was packed in the column and the pyrogallol was passed upwardly through the column using a peristaltic pump at 28±2°C. The converted product contained both purpurogallin and unreacted pyrogallol. Pupurogallin was separated from unreacted pyrogallol by passing through a silica column using 95% chloroform and 5% methanol (v/v) mixture as eluent.

Table 5.1 Reactor studies in the biotransformation of pyrogallol to purpurogallin

Conc. (mM)	Flow rate ml/h	Dilution Rate (h^{-1})	Residence Time(Sec)	Productivity g / l / h	Conversion %	Reaction rate (mg product /mg CLEC/h)
3	19.2	102.1	32.4	21.79	57.67	175 mg
	49.2	261.7	13.7	58.16	60.06	484 mg
	73.2	389.9	9.2	89.73	62.19	748 mg
	96.0	510.6	7.1	144.13	76.28	1201 mg
	144	765.9	4.7	195.08	68.83	1626 mg
	192	1021.3	3.5	261.14	69.11	2176 mg
5	31.2	165.9	21.7	47.98	43.15	399 mg
	96.0	510.6	7.1	149.64	46.52	1247 mg
	144	765.9	4.7	207.13	42.92	1726 mg
	192	1021.3	3.5	269.03	41.83	2242 mg
7	31.2	165.9	21.7	40.96	27.95	341 mg
	96.0	510.6	7.1	140.61	31.18	1172 mg
	144	765.9	4.7	203.38	30.07	1695 mg
	192	1021.3	3.5	251.09	27.84	2092 mg

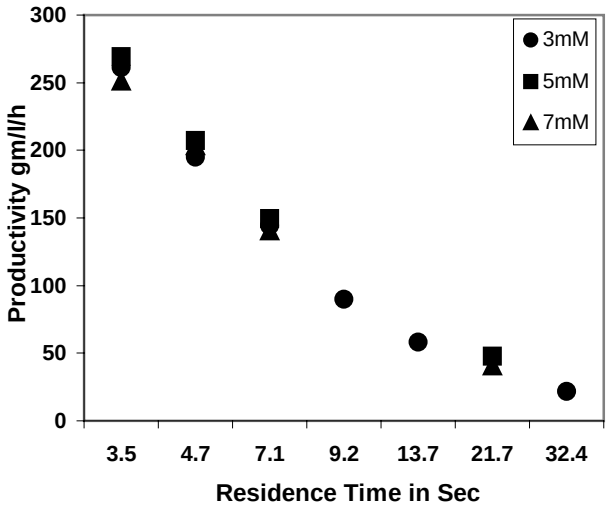
The percentage of conversion of pyrogallol to purpurogallin is given in Figure 5.2. The maximum percentage of conversion (76.28%) was obtained when 3 mM pyrogallol was used at a residence time of 7.1 sec and the % of conversion subsequently became steadily at around 60%. At higher pyrogallol concentration of 5mM and 7 mM, the percentage of conversion came down to 46.52% and 31.18% respectively at a residence time of 7.1 sec.

Fig 5.2 % of Conversion of pyrogallol to Purpurogallin at different residence time



The productivity of the PBR for purpurogallin production at various substrate concentrations and at different residence times is given in Figure 5.3. The optimum residence time in all the concentrations of pyrogallol was 3.5 sec. The maximum productivity (269.03 g/l/h) was obtained for 5mM pyrogallol and for 3 mM and 7 mM, it was 261.14 g/l/h and 251.1 g/l/h respectively.

Fig 5.3 Productivity of purpurogallin at different residence time



The reaction rate of CLEC laccase for purpurogallin synthesis was maximum (2241.94 mg purpurogallin/mg CLEC/h) at a residence time of 3.5 sec, when 5 mM conc. of pyrogallol (Figure 5.4& 5.5) was used as the substrate. The reaction rate was also maximum at a residence time of 3.5 sec for all the different pyrogallol concentrations. The catalyst to product ratio calculated in the present biotransformation is 1:2241. If the catalyst is used for at least 10 hours, the productivity ratio will also increase substantially to 1:22410, making the process much more economical.

Fig 5.4 Rate of the reaction for the production of purpurogallin at different flow rate

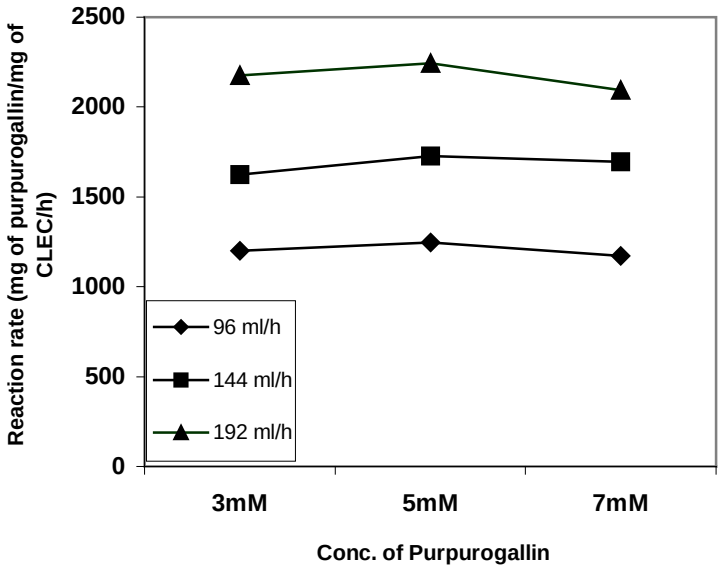
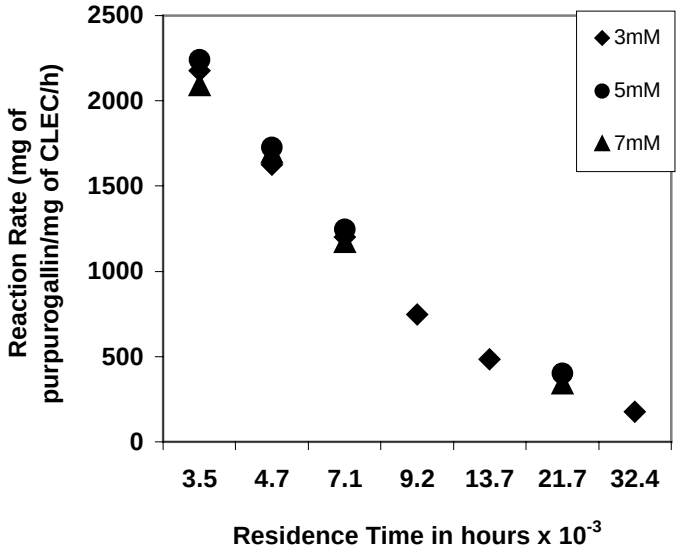
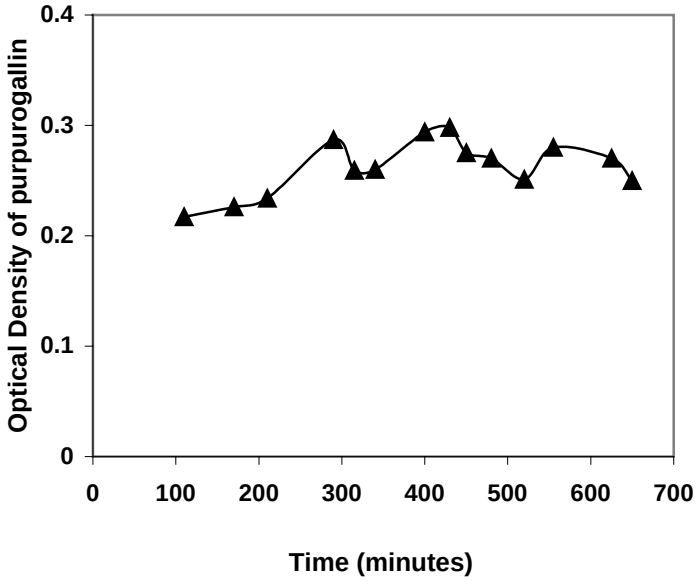


Fig 5.5 Reaction rate of purpurogallin production at different residence time



The reactor kinetics for the purpurogallin production was given in Figure 5.6. The CLEC laccase had very high reuse stability and even after 650 minutes (~ 11 hours) of continuous use, the reaction rate had not come down substantially and hence the half-life was not determined.

Fig 5.6 Reactor kinetics in Purpurogallin production



5.4 Cost effectiveness of CLEC Laccase as against Soluble Laccase

Cost of Laccase (T. versicolor) from Fluka = $ 44/gm

1 gm of soluble laccase produced 7.5 gm CLEC

Cost of crystallization and cross-linking

Cost of 1 gm Laccase	$ 44
$(NH_4)_2SO_4$ (60 gm)	$ 2
Glutaraldehyde	$ 1
Buffer & cosolvent	$ 1
β-cyclodextrin	$ 1
Other reagents	$ 1
	$ 50
Processing cost	$ 50
Total cost for 7.5 gm CLEC	$ 100

Biotransformation of pyrogallol to purpurogallin

1 mg of CLEC Laccase produces 2.2 gm purpurogallin/h

Therfore, 1 gm of CLEC Laccase produces 2.2 kg of purpurogallin/h

From 5.4 kg of pyrogallol (41% conversion)/ h at max. productivity

CLEC Laccase can be used minimum 10 hrs (we have used for 65 hrs)

54.0 kg pyrogallol + 1 gm CLEC ⟶ 22.0 kg purpurogallin

2 X [($ 9610) + ($ 14)] ⟶ $ 271480 ($ 61.7/ 5g)

(Processing cost is taken as the same as that of the material cost)

Value addition = $\frac{271480}{19248}$ = 14 times the processing cost.

5.5 Conclusions

The continuous production of purpurogallin using CLEC laccase is a novel process and reaction is very fast and the product recovery is simple, having no byproducts and hazardous chemicals to deal with and hence the overall process is very economical.The CLEC laccase when used in a packed bed configuration, catalyzed the conversion (76.28%) of 3 mM pyrogallol to purpurogallin within 7.1 sec. In all the concentrations of pyrogallol supplied, the maximum productivity (269.03 g/l/h) was obtained at a residence time of 3.5 sec. The productivity was found to be 261.14 g/l/h and 251.1 g/l/h when 3 mM and 7 mM concentration respectively of pyrogallol were used. The catalyst to product ratio was 1:2241 for this present biotransformation. The Purpurogallin was easily separated from the unreacted pyrogallol using a silica gel column and eluting with 95% chloroform and 5% methanol (V/V). The CLEC laccase had a very high reuse stability.

5.6 References

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CHAPTER VI

CROSS-LINKED ENZYME CRYSTALS OF GLUCOAMYLASE

6.1 Introduction

Glucoamylase (1,4-α-D-Glucan glucohydrolase, EC.3.2.1.3) is an exo-acting carbohydrase, which cleaves glucose units consecutively from the non-reducing end of starch and related poly and oligo saccharides in stepwise manner. Most glucoamylases are also able to hydrolyse the 1,6- α-linkages in the branching points of amylopectins, although at a slower rate than the 1,4- α linkages. Glucoamylase is also known as amyloglucosidase or gluc-amylase or γ amylase.

Fleming (1968) classified glucoamylases into two groups, one of which converts starch and β-limit dextrins completely into glucose and the other, which converts starch 80% and β-limit dextrins 40% into glucose. Panose and α-limit dextrins, however,are completely hydrolysed to glucose by the both groups.

6.1.1 Sources

a) Bacterial glucoamylase

Aerobic bacteria such as *Bacillus stearothermophillus*(Srivastava,1984), *Flavobacterium sp* (Bender,1981), *Halobacterium sodamense*(Oken,1983) have been reported to produce glucoamylase. A few anaerobic bacteria such as *Clostridium sp* (Tuchiya, 1988) produce glucoamylase.

b) Fungal Glucoamylase

Major fungal source (Pandey, 1995 & Saha and Zeikus, 1989) of glucoamylase is *Aspergillus sp* and *Rizopus sp* and *yeast* strains of *Endomycopsis capsularies*, *Schwanniomyces occidentalis* and *Saccharomyces diastaticus*. In the *Aspergillus specis*, *A.niger, A.awamori, A.candidus, A.oryzae* are important.

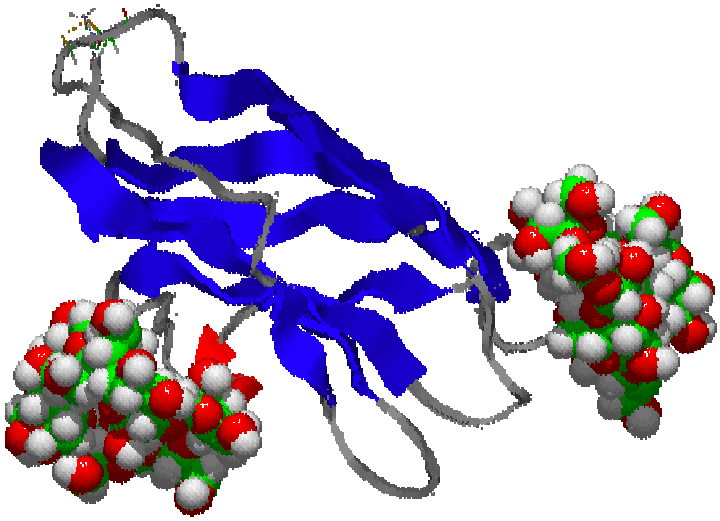
6.1.2. General Characteristics of Glucoamylase (Saha and Zeikus, 1989)

Molecular weight	50-112 Kda
Cabohydrate content	3.2 - 20%
Isoelectric Point	pH 3.4 -7.0
pH optimum	4.0-5.0
Temperature optimum	40- 60 °C
Thermal Stability	up to 60 °C
Metal ion requirement	None

Inhibitors	Amino alcohol with no stereochemistry (amino ethanol, 2- amino-2 methyl propane-1,3-diol, Tris (hydroxy methyl) amino-methane

6.1.3 Structure of Glucoamylase

Fig 6.1 Structure of glucoamylase



1. Class: All beta proteins [48724]
2. Fold: Prealbumin-like [49451]
   sandwich; 7 strands in 2 sheets, greek-key
   variations: some members have additional 1-2 strands to common fold
3. Superfamily: Starch-binding domain [49452]
4. Family: Starch-binding domain [49453]
5. Protein: Glucoamilase, granular starch-binding domain [49460]
6. Species: *Aspergillus niger* [49461]

6.1.3.1 Catalytic domain

The catalytic domain (CD) of GA from *A. awamori var.* 13 α-helixes of which 12 form an (α / α) 6-barrel. In this fold, six outer and six inner α -helixes surround the funnel-shaped active site, constituted by the six highly conserved α→ α segments (Coutinho and Reilly, 1994 &. Coutinho and Reilly, 1997) that connect the N-termini of the inner with the C-termini of the outer helixes (Aleshin et al, 1992 & Harris et al,1993 & Aleshin et al,1994(a)& Aleshin et al, 1994(b)) (Figure 6.2).

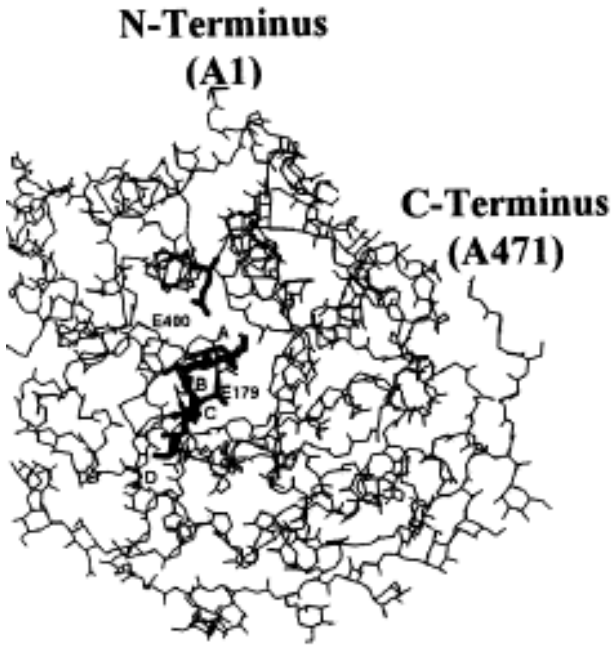


Fig 6.2 Catalytic domain (aa 1-471) of Aspergillus awamori var. Glucoamylase complexed with the pseudotetrasaccharide acarbose (rings A, B, C, D are indicated). The C- and N-termini are indicated together with the side chains of the two catalytic residues E179 and E400 (taken from Aleshin et al, 1994(b))

The catalytic site includes the general acid and base catalysts Glu179 and Glu400 situated at the bottom of a pocket (Sierks et al, 1990 & Frandsen et al, 1994). CDs of GAs from *A. awamori var.A. niger and S. fibuligera* share a very similar fold. The *S. fibuligera* GA contains 14 α -helices, 12 of which makes up the (α / α) 6-motif in an organization identical to that of A. *awamori var*. and *A. niger* CD (Sevcik et al , 1998). Two extra short helices protrude from the α -helix connecting loops in the first and the last pair of

antiparallel helices in the fold (Sevcik et al, 1998). The most pronounced difference between these GAs, however, is the lack of SBD in the *S. fibuligera* enzyme (Coutinho and Reilly, 1994(a) & Hostinova et al, 1991). A single Serine replacement between two very closely related *S. fibuligera* GAs is responsible for activity differences (Solovicova et,al, 1999).

6.1.3.2 Starch-binding domain

The C-terminal SBD of *A.niger* was prepared both by proteolysis and in recombinant form, and solution structures of the free and the β-cyclodextrin- complexed SBD were determined by NMR spectroscopy (Sorimachi et al, 1996 & Sorimachi et al, 1997). SBD consists of eight β -strands organized in two L-sheets forming a twisted β-barrel structure (Sorimachi et al, 1996 & Belshaw et al, 1991). Two starch-binding sites, seen to accommodate the starch mimic β -cyclodextrin (Belshaw and Williamson, 1993 & Sigurskjold et al,1994), are located on opposite sides of the `top' of the domain, i.e., away from the linker attachment point as seen in Figure.6.3 (Sorimachi et al, 1996 & Sorimachi et al,1997).

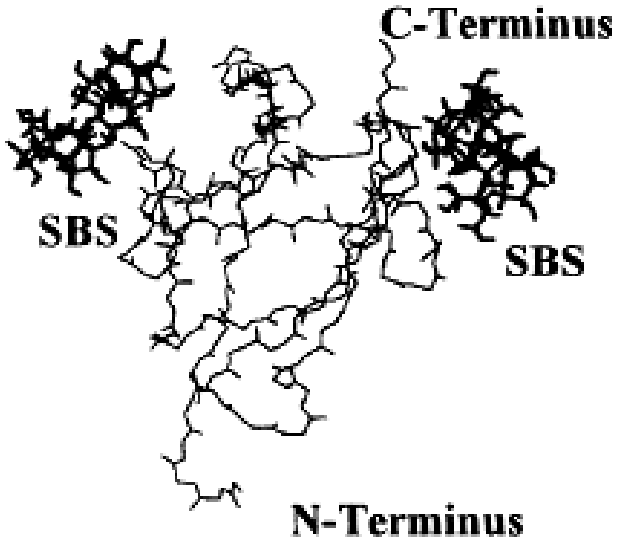


Fig 6.3 Starch binding domain (SBD) of. *niger* GA complexed with the starch mimic β-cyclodextrin at the two binding sites. The C- and N-termini are indicated (taken from Sorimachi et al,1997)

These sites display distinctly different structure and non-covalent interactions with β -cyclodextrin. Mutagenesis studies revealed, however, that they vary only little in affinity, Ka being 3.6 $x10^{4}$ and 1.6 x 10^{5} M^{-1}, respectively (Williamson et al, 1997). Also the enthalpies and entropies of binding are similar as analysis of the binding thermodynamics of β -cyclodextrin and SBD by isothermal titration calorimetry (ITC) did not resolve the two sites (Sigurskjold et al, 1998 & Sigurskjold et al, 1994).

6.1.3.3 Linker region

The serine- and threonine-rich O-glycosylated region of *A. niger* GA (Amino acids 440-508) contains a very highly O-glycosylated C-terminal segment of about 30 amino acids that connects with SBD (Svensson et al, 1983). This particular part of the linker has been attributed roles in stability, secretion, and digestion of raw starch [Libby et al, 1994]. Mass spectrometric analysis of the peptide Asn430-Phe519 shows a high degree of heterogeneity in the amount of attached sugars. Based on a calculated molecular mass of the peptide of 8562.28 Da and the experimentally determined mass of 18 991.3 Da for the glycoform of the lowest molecular mass, approximately 63 moles of hexose are attached to the peptide. The O-glycosidically linked units range from single mannosyl to branched mannotriosyl in wild-type *A. niger* GA (Gunnarsson et al, 1984). Heterologous expression of *A. niger* GA results in large host-dependent variation in the content of sugars ranging from hypermannosylation by *Saccharomyces cerevisiae* to modest over-glycosylation by both Pichia pastoris and a laboratory strain of A. niger (Fierobe et al, 1997). The first part (amino acid 440-471) of the O-glycosylated region carries, as seen in the structural model of GA, about 10 exposed single mannosyl residues,which together with the two N-glycosidically linked units at Asp171 and Asp395 form a belt of carbohydrate around the globular CD (Aleshin et al, 1992). The highly O-glycosylated part of the linker (aa 472-508) is not included in the structural model, but it has been speculated that this part surrounds CD in a continuation from residue 471, to place SBD with one of the two binding sites near the active site. This resembles the architecture of cyclodextrin glucanotransferase in which a homologous C-terminal SBD is situated relative to CD to direct the substrate chain into the active site via one binding site of SBD and to be bound onto soluble or insoluble polymeric substrate at the

other (Penninga et al, 1996). The full-length linker is anticipated to be conformationally flexible in accordance with the formation of 1:1 complexes between a bifunctional inhibitor and GA (Sigurskjold et al, 1998 & Payre et al, 1994).

6.1.3.4 Overall structure

SBD was earlier shown to be required for degradation of raw starch by GA, the natural G2 form (aa 1-512) without SBD having very low activity on raw starch (Svensson et al, 1983). Recently, isolated SBD acting on starch granules together with G2 showed a synergistic effect on the degradation of the insoluble substrate, suggesting that SBD binds onto starch as an individual entity and disrupts the compact structure of the starch granule facilitating the hydrolysis by CD (Southall et al, 1999). The complete three-dimensional structure of intact GA comprising CD, the linker region, and SBD, is not known. In an attempt to delineate the relative position of CD and SBD, scanning tunneling microscopy indicated that the two domains are around 90Å apart [Kramer et al, 1993]. Recently, a series of bifunctional inhibitors, in which the CD specific pseudotetrasaccharide inhibitor acarbose and the SBD specific ligand β-cyclodextrin were coupled via thioglycoside linkages, was used to further analyse binding to the different domains. The bifunctional molecules were synthesized without and with varying lengths of poly (ethyleneglycol) spacers connected to the reducing end of acarbose and C6 of a glucose ring in β-cyclodextrin shown in Figure 6.4 (Payre et al, 1999).

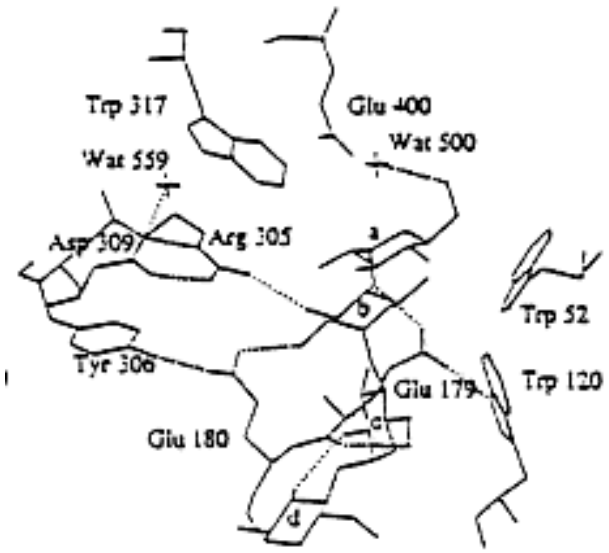


Fig. 6.4 The active site of glucoamylase from *A. awamori* var. *X100* with bound D-*gluco*-dihydroacarbose (Stoffer et al, 1995**) The four rings are marked a, b, c and d. Hydrogen bond interactions less than 3.0 Å are represented by dashed lines, that of Trp52 to Glu179 is 3.04 Å and therefore does not appear. The α-flank of the active site as that on the right side of the ligand and the β-flank as that on the left side is defined in this representation.**

Four different heterobifunctional inhibitors were demonstrated by ITC to bind simultaneously at the active site of CD and one of the binding sites on SBD (Sigurskjold et al, 1998). It was thus concluded that in solution the two domains of the GA molecule either are in, or can be brought into, close proximity. The sum of enthalpies in binding of acarbose and β -cyclodextrin gave essentially the same value as found for the enthalpy of the bifunctional ligands. The binding affinities, however, were reduced approximately 105 times compared to that of acarbose due to strong entropy penalties in binding of double-headed inhibitors (Sigurskjold et al,1998).Dynamic light-scattering measurements on the binding of the bifunctional inhibitors suggested co-operation between the domains (Payre et al, 1994). The inhibitors were concluded to bind in a bimolecular complex with occupation of one site at each of the CD and SBD.

6.1.4 Mechanism of action

6.1.4.1 Catalytic site

The widely accepted mechanism of hydrolysis involves proton transfer to the glycosidic oxygen of the scissile bond from a general acid catalyst; formation of an oxocarbenium ion; and a nucleophilic attack of water assisted by a general base catalyst (McCarter and Withers,1994 & Sinnott,1990 & Tanaka et al, 1994). Glu179 and Glu400 in GA from A. niger have been identified as the general acid and the general base catalyst, respectively, and pH-dependencies of steady-state kinetic parameters are in accordance with a rate determining hydrolysis step involving these two catalytic residues (Sierks et al, 1990). Also in accordance with this is the observation that mutation of Glu 400 to Gln results in a reduction of k_{cat} to 3% of wild-type, showing the marked influence of this residue on the rate determining step (Frandsen et al, 1994). The GA catalysis occurs with inversion of the anomeric configuration (Figure 6.5) in a single displacement mechanism and the gap between the catalytic acids is 9.2 Å as is typical for inverting glycoside hydrolases (McCarter and Withers, 1994 & Davies and Henrissat , 1995).

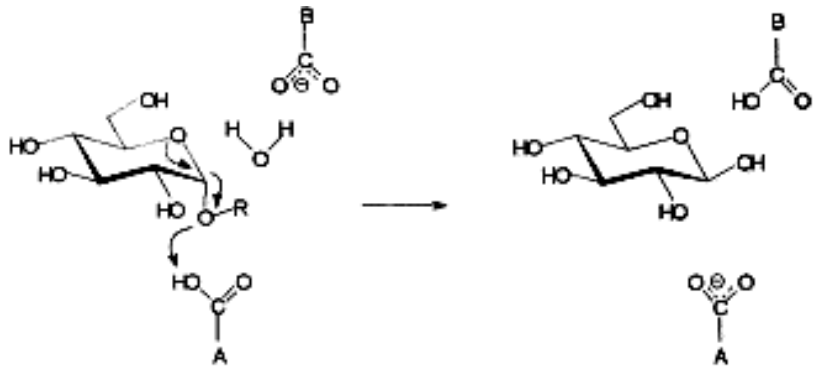


Fig 6.5 The generally accepted catalytic mechanism of GA illustrating the action of the catalytic base E400 (top) and acid E179 (bottom) in the water-assisted hydrolysis of substrate involving inversion of the configuration of the anomeric carbon.

In contrast, the distance between the catalytic acids in retaining glycoside hydrolases is only 4.8-5.5 Å and hydrolysis occurs in a double displacement mechanism that includes a covalent intermediate (McCarter and Withers, 1994 & Davies and Henrissat, 1995). In this mechanism the proposed covalent bond between substrate and protein has the consequence that high precision of the spatial positioning of the two catalytic groups is necessary for the nucleophilic attack on the glycosidic bond (Lawson et al, 1996). Such a strict geometrical requirement for the catalytic site seems not to apply for the inverting GA, as it was best illustrated by the elevated activity of GA from A. niger in which the catalytic base was replaced by cysteine which was subsequently oxidized to cysteinesulphinic acid (Fierobe et al, 1998).

6.1.4.2 Binding mechanism

Conserved tryptophan residues are involved in interactions of the GAs with substrates and inhibitors (Firsov et al, 1994 & Svensson and Sierks, 1992) and changes in intrinsic enzyme fluorescence result as binding occurs. These changes were earlier assumed to involve only a tryptophan in subsite +1 (Hiromi et al, 1983), but recent structure and function studies have shown that in addition to this tryptophan a number of other tryptophans are involved (Clarke and Svensson, 1984(a) & Clarke and Svensson, 1984(b)). Pre-steady-state kinetics analysis of the binding mechanism of wild-type and mutant *A. niger* GA has been based on the intrinsic protein fluorescence changes that occur when substrates and inhibitors bind (Olsen et al, 1982). Formation of complexes with single exponential kinetics was seen in all cases and analysis of their concentration dependencies all showed results in accordance with a three-step reaction mechanism (Model I) of catalysis involving two intermediates: ES, the initial association complex, and E*S, the Michaelis complex (i.e., the most stable enzyme-substrate intermediate) (Tanaka et al, 1982, Ohnishi and Hiromi, 1978).

6.1.5 Glucoamylase substrates

Substrates like starch or related poly and oligoscaaharides had been reported to be hydrolyzed by glucoamylase. Compounds like starch,glycogen,amylose,amylopectin dextrins,maltodextrins,maltose,isomaltose,dextran,panosan,etc.Pretreatment of these substrates is necessary for glucoamylase action. Pretreatment such as

liquefaction/gelatinization gives better hydrolysis rate and improved product yields. The hydrolysis of starch substrates by glucoamylase can be affected by other amylolytic enzymes such as α-amylase, β-amylase, pullulanase, isoamylase or α-glucosidase contaminations in a glucoamylase preparations. However, α-amylase contamination can be detected using an oxidized starch substrate.Pullulanase or isoamylase contamination can be easily avoided by using amylose as substrate(Marshall and Whelan, 1971).

α-glucosidase can be traced in the presence of glucoamylase by comparing the results of enzyme incubation with starch solution and with starch plus 0.05% maltose solution (Kujawski and Wergrzyn, 1980)

6.1.6 Applications of Glucoamylase

The most important use of glucoamylase is in the production of high glucose syrups (96-98% glucose) from liquefied starch. An aqueous slurry of starch (30 - 40% DS) is gelatinized and partially hydrolyzed using acid or thermo stable α-amylase to DE 10-20. Glucose is produced by saccharifying the liquefied starch with either glucoamylase alone or with glucoamylase and a debranching enzyme such as pullulanase or isoamylase. The saccharification process is much slower and takes 24-72 h depending upon the dose of glucoamylase. High glucose syrups are usually used either for the production of crystalline D-glucose or as a starting material for the production of high fructose syrups (Saha and Zeikus, 1989).

Another important use of glucoamylase is in alcohol fermentation. In the conventional alcoholic fermentation, the starchy materials are pre-cooked for liquefaction and then saccharified by glucoamylase before fermentation by yeast (Saha and Zeikus,1989). Ethanol can also be produced directly from raw starch in a single step process, which combines liquefacation, saccharification and yeast fermentation, by glucoamylase. The alcoholic fermentation of grains without cooking stage has been performed on an industrial scale.

6.1.7 Need for the Development of CLEC Glucoamylase

Industries need glucoamylase with high productivity and stability for repeated use over an extended period. Immobilization of glucoamylase provides many important advantages over the use of soluble enzymes namely, reusability, continuous operation,

controlled product formation, and simplified and efficient processing (Turecek, et al, 1990). Continuous reactors provide high productivities and minimize downtime, enzyme costs, and capital investment (Maugh,1984). Much work has been carried out to immobilize glucoamylase on various supports (Jun-ichi et al,2000), but these preparations had disadvantages that include low-temperature stability and enzyme activities, gradual enzyme inactivation over the period of storage, and low glucose yields. With immobilized glucoamylase the saccharification can be conducted continuously in a packed-bed reactor, thereby reducing the reaction time and production cost. Glucoamylase has been immobilized by ion-exchange processes, physical adsorption, covalent bonding, gel entrapment, etc. (Tramper, 1985 & Weetall, 1975 & Lee et al, 1980), but the drawback is the tendency of glucoamylase to get inactivated above 50 °C. This reduces the commercial feasibility of the process, since at lower temperatures the threat of microbial contamination is quite high. The company, Novozymes, has patented a process for the production of glucoamylase from a new strain of microbe, *Talaromyces Dupont*, which is thermostable up to 70 °C (Nielson, 2002).

Cross-linked enzyme crystals (CLECs) have several key characteristics that confer significant advantages over conventional enzyme immobilization methods (Margolin,1996 & Govardhan, 1999 & Margolin and Navia, 2001) like enhanced temperature stability, absence of an inert support, uniformity across crystal volume, catalysis under harsh conditions, such as elevated temperature, denaturing organic solvents, etc. These combined effects represent a major improvement in the reaction efficiency by maximizing the productivity of a given quantity of enzyme catalyst, which is generally the most expensive component of the reaction process (Kalaf et al, 1996). The insoluble nature of CLECs facilitates easy separation of the enzyme from the reaction medium that increases the reuse efficiency of the enzyme.

There has been no report about glucoamylase CLEC in the literature, and, hence, attempts have been made to provide a process for the preparation of a cross-linked glucoamylase composition with improved stability and activity.

6.2 Materials and Methods

6.2.1 Materials

Glucoamylase (amyloglucosidase, E.C: 3.2.1.3, α-(1→4)- glucan glucohydrolase) from *Aspergillus niger* (AMG 3L 3388 IU/mL, protein 609.8 mg/mL) was obtained from Novozymes (Denmark). Glutaraldehyde was from BDH (UK). Soluble starch and ammonium sulfate were purchased from E. Merck (India). Maltodextrin from obtained from Grain Processing Corporation (Iowa, USA). Hexane, dodecane, chloroform, Me_2SO, *n*-octane, 2-propanol, and Tween-80 were obtained from BDH, S.D fine Chemicals and E. Merck (India).

6.2.2 Methods

6.2.2.1 Purification of glucoamylase

The commercial glucoamylase obtained from Novozymes was further precipitated with ammonium sulfate (40–80% cutoff) and dialyzed against sodium acetate buffer at pH 4.5 or by passing through a column of Sephadex G-25.

6.2.2.2 Preparation of Cross-linked glucoamylase crystals

The purified glucoamylase enzyme was crystallized by the batch method using ammonium sulfate as precipitant (65% saturation) along with 20% 2-propanol as cosolvent in acetate buffer (0.5 M, pH 4.5). The solution was stirred at 4 °C for 30 min and then kept for 16 h at the same temperature. The crystals formed were separated by centrifugation, and the crystals were suspended in the minimum amount of acetate buffer (0.5 M, pH 4.5) containing starch (1%) and/or bovine serum albumin (BSA, 1mg/mL). The glucoamylase crystals were chemically cross-linked using glutaraldehyde (50%), and the final concentration was 2% (v/v) in 0.2 M phosphate buffer of pH 7.0. After 1 h, the cross-linked crystals formed were separated by centrifugation at 2000 rpm and washed several times with 0.2 M acetate buffer pH 4.5 until the crystals were free of glutaraldehyde. The preparation was then lyophilized with a nonionic surfactant and stored at room temperature (28 ± 2 °C).

6.2.2.3 Determination of physical characteristics

a) Density

Density of the glucoamylase CLEC was obtained using a helium Auto pycnometer (Micromeritics model 1320).

b) Surface area

Surface area was measured by the BET (Brunauer, Emmett and Teller) technique using a Zetasizer (Melvern, UK) in which liquid nitrogen was the adsorbent.

c) Crystal structure

The crystal structure was observed under a scanning electron microscope (JEOL, Japan) at 10 kV accelerating voltage, after sputtering with gold. A photograph of the material is shown in Figure 6.7.

6.2.2.4 Determination of enzyme activity

Enzyme solution (0.2 mL) is added to 1 mL of 4% starch and 4% maltodextrin solution in 0.2 M acetate buffer (pH 4.5), and the mixture is incubated at 60 °C for exactly 1 h. The enzyme reaction is then terminated by adding 0.8 mL of 4 N NaOH. The dextrose formed was determined using the Lane-Eynon method (Pazur and Ando, 1960). Activity of glucoamylase CLEC was determined by the same procedure using 454 mg of CLEC. The amount of enzyme that is capable of producing 1 micromole of dextrose per minute under experimental conditions is defined as 1 enzyme unit (1 IU).

6.2.2.5 Determination of protein

The determination of protein was carried out by Lowry's method (Lowry et al, 1951) BSA was used as the standard, and the absorbance was read at 660 nm. The cross-linked enzyme crystals were washed thoroughly to remove excess reagents before estimation.

6.2.2.6 Determination of reducing sugar and dextrose equivalent (DE)

The dextrose equivalent was determined by the Lane-Eynon titrimetric method (Bernetti, 2000), and reducing sugar was determined by the DNS method (Miller, 1959). Percentage conversion was calculated as glucose obtained × 0.9. DE [dextrose (glucose) equivalent] refers to the reducing sugar content of a material, calculated as glucose (dextrose) and expressed as percent of total solids.

6.2.2.7 Thermo stability of Glucoamylase CLEC

CLEC (454 mg, sp. activity 0.068 I.U/mg protein) was incubated for 0.5–1 h at temperatures ranging from 60–90 °C in the presence of 4% (w/v) starch and in the absence of any starch. After incubation, 1 mL of 4% starch solution at pH 4.5 was added and incubated at 60 °C for 1 h, and the glucose produced was monitored.

6.2.2.8 Effect of pH and substrate concentration

In order to study the effect of pH and substrate concentration on the activity of the cross-linked enzyme, 454 mg CLECs (Activity 5302.7 IU) was incubated with 1mL of 4% starch solution having a pH varying from 2.5 to 8.5, and soluble starch concentration ranges from 10 to 350 mg/mL at 60°C for 1 hour. The glucose produced by hydrolysis was estimated (Bernetti, 2000). For same procedure was repeated using 0.2 mL of the soluble enzyme (sp. activity 0.085 I.U/mg protein), and the activities were compared.

6.2.2.9 Kinetic studies of CLEC glucoamylase using starch as substrate

Kinetics of soluble enzyme as well as CLEC was done at 0.2 M sodium acetate buffer pH 4.5 by varying the substrate concentration (starch 0.04 mg/ml to 0.3 mg/ml), while keeping the concentration of biocatalyst constant. The Michaelis-Menten constant (K_m) and velocity maximum V_{max} were calculated from Lineweaver-Burk plot (A plot of (1/[S] Vs 1/[V]). From those values, K_{cat} and catalytic efficiency K_{cat}/K_m values were calculated (Table 1).

6.2.2.10 Effect of organic solvents on the activity of Cross-linked enzyme crystals (CLEC) of glucoamylase

To 10 mL of 2% (w/v) maltodextrin (DE 12.5) in different water–organic solvent mixtures (1:1) at pH 4.5 was added 20 mg of CLEC glucoamylase, and the mixtures were incubated at 60 °C for 1 h. The resultant reducing sugar was estimated by the DNS method.

6.2.2.11 Continuous hydrolysis of starch in a packed-bed reactor (PBR) using CLEC glucoamylase

Continuous hydrolysis of soluble starch to glucose was carried out in a packed-bed reactor (Figure 6.6). A jacketed glass column (114 × 8 mm and V_o = 3.88 mL) with 3.5 g of CLEC glucoamylase was used, and the hydrolysis was carried out at 60°C. Continuous saccharification was carried out by passing 4% and 10% (w/v) solution of starch (pH 4.5) and 10% maltodextrin (DE 12.5, pH 4.5) through the column in different residence times using a pump at a dilution rate of 7–17 bed volumes per h.

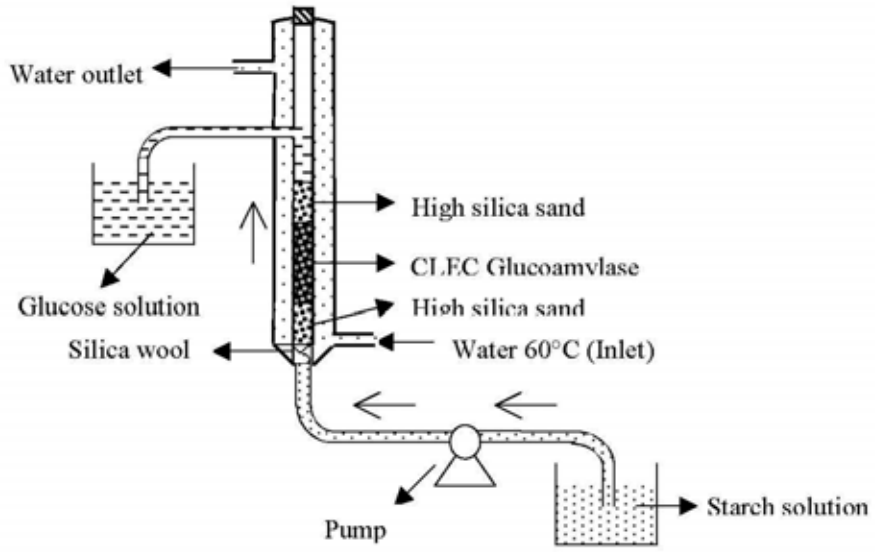


Fig. 6.6 Schematic representation of Packed Bed Reactor for the continuous production of glucose from starch using CLEC glucoamylase

6.3. Result and Discussion

6.3.1 Properties of the cross-linked enzyme crystals (CLEC) of glucoamylase

Glucoamylase catalyses the hydrolysis of glucosidic linkages with inversion of aromatic configuration (Sauer et al, 2000). *Aspergillus niger* glucoamylase occurs in two natural forms. The larger one, GA1, consists of two globular parts with separate functions. Amino acid residues 1–471 form the catalytic globular domain (CD), and AAs 509–616 form the globular starch-binding domain (SBD). The carbohydrates are attached exclusively to the peptide region having AAs 471–508 between the two domains. The smaller, natural proteolytic fragment GA II comprises residues 1–512, only, and thus neither binds to or hydrolyses raw starch (Pazur and Ando, 1960). We assume that in the *A. niger* glucoamylase only AA residues 1–471, which form the catalytic globular domain (CD), have crystallized. There is a lack of any report that the full length of glucoamylase has ever been crystallized (Sauer et al, 2000). The crystals formed were separated and cross-linked with glutaraldehyde (2% v/v) solution. The cross-linked crystals of 10–100 μm were obtained (Figure 6.7).

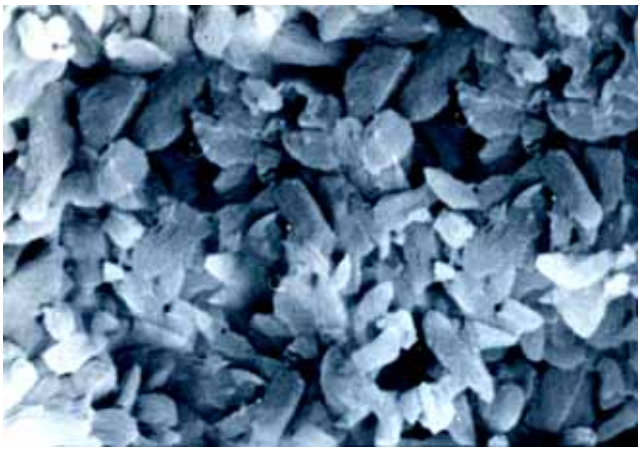


Fig 6.7 SEM photograph of CLEC glucoamylase

(Magnification X 1000)

The crystals were light brown in color and fluffy. The active site of the glucoamylase was blocked by soluble starch before cross-linking with glutaraldehyde to prevent any inactivation of the enzyme. Addition of a small quantity of a protein like bovine serum albumin (BSA) enhances the activity of the crystal. After crystallization and cross-linking, 63.15% of the enzyme protein was retained, and the activity yield was found to be 50.66%. The soluble enzyme had a specific activity of 5.56 IU/mg protein where as the CLEC had a specific activity of 11.68 IU/mg crystal protein. A general characteristic of CLEC and soluble glucoamylase has given in Table 6.1.

Table 6.1. General characteristics of CLEC and soluble glucoamylase

Property	CLEC glucoamylase	Soluble glucoamylase
pH optimum	4.5	4.5
Thermal Stability	upto 70 °C	upto 60 °C
Specific activity	11.68 IU/mg crystal	5.56 IU/mg Protein
K_m (starch)	0.0283 mM	0.0026mM
V_{max}	9.9 µmol/mg/min	0.963µmol/mg/min
K_{cat}	0.0174 (S^{-1})	0.00169(S^{-1})
K_{cat}/K_m	614.84 $M^{-1}S^{-1}$	650 $M^{-1}S^{-1}$
Crystal Shape	Rhombohedral	
Crystal size	10–100 µm	
Density	1.8926 g/cm^2	
Surface Area	0.7867 m^2/g	

6.3.2 Thermal Stability of CLEC glucoamylase

Immobilized glucoamylase previously produced in the laboratory (Abraham et al, 1991) has an inherent disadvantage of having a lower conversion rate and a thermal stability of less than 65°C when used in continuous operations. Cross-linking of glucoamylases via carbohydrate of linker regions is known to decrease thermal stability (Sasvari and Asboth, 1999). Hence cross-linking by glutaraldehyde via lysine residues was tried. The cross-linked glucoamylase crystals that we have developed have circumvented some of the above disadvantages and have exhibited better thermal stability. These CLECS are shown to retain 98.6% activity at 70 °C for 1 hour, 77% activity at 80 °C for 1 hour and 51.4% of the original activity at 90 °C for 0.5 hour in the presence of starch (Table 6.2).

Table 6.2 Thermal stability of CLEC glucoamylase in the presence and absence of 4% starch (w/v) at pH 4.5.

Temperature	Duration of incubation (h)	Soluble glucoamylase activity (%)	CLEC glucoamylase activity (%)
70 °C	1 (with starch)	83.46	98.6
80 ° C	1 (with starch)	62.62	77.0
90 ° C	0.5 (with starch)	44.89	51.4
70 ° C	1 (without starch)	negligible	82
80 ° C	0.5 (without starch)	negligible	30.82
90 ° C	0.5 (without starch)	negligible	negligible

The presence of starch has a protective effect on the enzyme toward denaturation at elevated temperatures. The inactivation coefficient calculated from the Arrhenius equation is $Ea = -58.67$ kJ mol^{-1}. Cross-linking of the enzyme by glutaraldehyde seems to prevent the unfolding of glucoamylase induced by heat. In the absence of starch, the thermal stability is moderate having retained 82% of activity at 70 °C for 1 h and 30.82% of activity at 80 °C for 0.5 hour. Hence, the glucoamylase CLEC can be used in biotransforamtion of other substrates at elevated temperatures, which makes it more versatile.

6.3.3 pH optima and Kinetic studies of CLEC glucoamylase

Fig 6.8. pH profile of CLEC and soluble glucoamylase

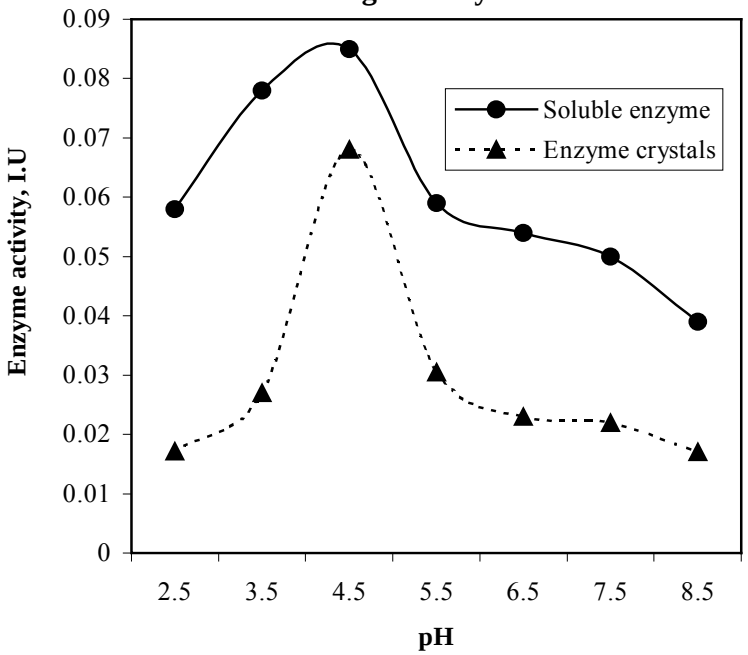
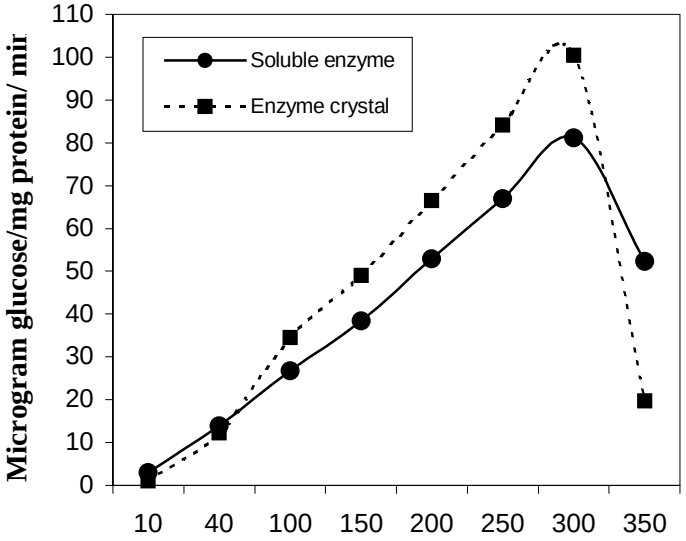


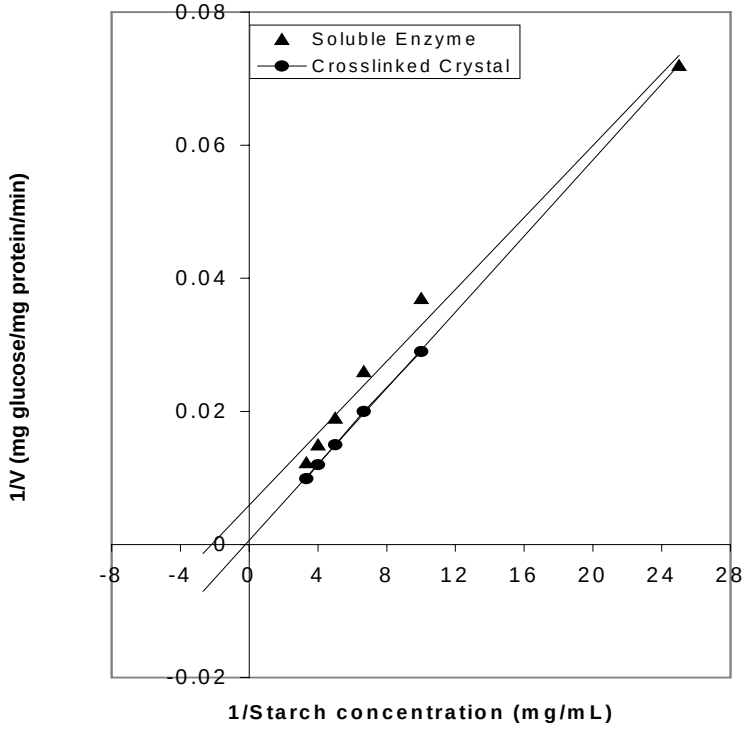
Figure 6.8 shows the relationship between the pH and the enzyme activity in the case of the cross-linked enzyme crystals and the soluble enzyme for the hydrolysis of soluble starch (4% w/v). Compared to the soluble enzyme, the activity of the CLEC is slightly lower because of the lower concentration of enzyme in the crystal, as well as due to the hindrance of substrate's access to the active site and the restricted flexibility of the catalytic sites. This CLEC has also a narrow pH optimum at pH 4.5, which in turn warrants strict pH control during hydrolysis.The effect of starch concentration on the activity of both the soluble enzyme and the CLEC is given in Figure 6.9.

Fig 6.9 Effect of substrate concentration on CLEC glucoamylase



CLEC glucoamylase has a better activity at higher substrate concentrations up to 300 mg/mL of soluble starch. The Michaelis constant, K_m for the cross-linked glucoamylase crystal is 0.0283 mM with a V_{max} of 9.9 μmol/mg /min, which is 10-fold higher than the soluble enzyme where K_m is only 0.0026 mM with a V_{max} of 0.963 μmol / mg /min with starch as substrate at pH 4.5 at 60 °C. The Michaelis constants K_m and V_{max} were obtained from the Lineweaver–Burk plot (Figure 6.10).

Fig. 6.10 Lineweaver--Burk plot for soluble and CLEC Glucoamylase



The affinity of the substrate is reduced due to the diffusion limitations of the starch polymer getting into the active site of the enzyme's crystal lattice.

6.3.4 Continuous hydrolysis of starch by CLEC.

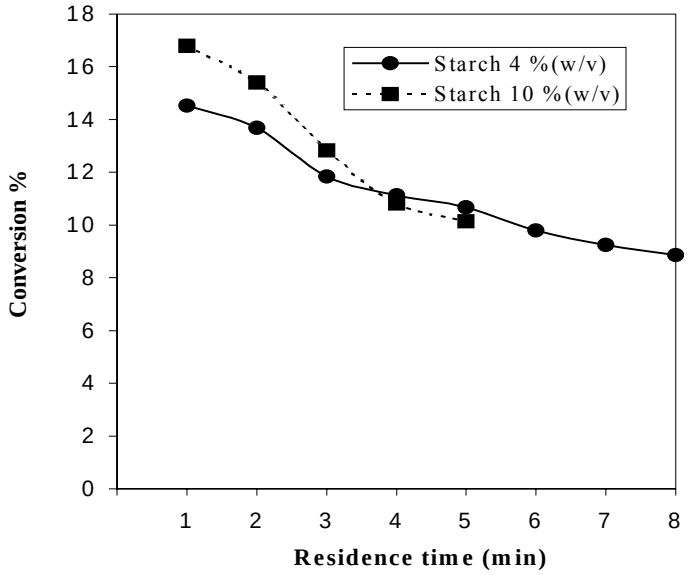
The hydrolysis of substrates using CLEC glucoamylase packed in a glass column was carried out at 60 ± 2 °C. The productivity of the reactor system at various substrate concentrations and dilutions is given in Table 6.3.

Table 6.3. Productivity of CLEC glucoamylase in a packed-bed reactor (PBR) for the hydrolysis of starch and maltodextrin (12.5 DE) at 60 ± 2 °C

Substrate (w/v)	Residence time (min)	Glucose produced (g/L)	Productivity g/L/h
Starch (4%)	5.4	4.972	55.13
Starch (10%)	7.6	14.07	110.58
Maltodextrin (10%)	5.65	43.73	463.69
Maltodextrin (10%)	3.39	40.42	714.10

At 4% (w/v) soluble starch feed, a productivity of 55.13 g/L/h was obtained at a residence time of 5.4 minutes. When the concentration of the soluble starch was increased to 10% (w/v), the productivity was also increased to 110.58 g/L/h, and the residence time was 7.6 minutes (Figure 6.11).

Fig 6.11. Hydrolysis of starch to glucose by CLEC glucoamylase in a packed-bed reactor (PBR) at 60 °C



When a more suitable substrate for glucoamylase, maltodextrin of DE 12.5, at a concentration of 10% (w/v) was used, the productivity markedly increased, and a value of 463.7 g/L/h was obtained at a residence time of 5.6 minutes. When the residence time was further reduced to 3.4 minutes, a higher productivity of 714.1 g/L/h was obtained. These results are mainly due to the small size of the substrate, which reduces the diffusion limitations of the hydrolysis.

After 10 h of continuous saccharification of a 4% (w/v) soluble starch solution, the activity of the CLEC declined to 2.3 IU/g from 5.1 IU/g of the crystal. Hence the half-life of the enzyme was about 10 h at 60 ± 2 °C.

6.3.5 Effect of organic solvents on CLEC activity.

When water and organic solvents were taken in ratios of 1:1, the CLEC exhibited high activity in water–hexane, water–*n*-dodecane and water–chloroform mixtures (27–28 mg of glucose produced per hour). The activity was less in 1:1 mixtures of water–2-propanol, water–1,4-dioxane and water–Me_2SO (Table 6.4).

Table 6.4. Effect of organic Solvents on CLEC glucoamylase on glucose-releasing activity

Water–organic solvent mixture (1:1)	Amount of glucose produced (mg/h)
2-propyl alcohol	13.55
***n*-dodecane**	**28.94**
1,4-dioxane	11.35
Me_2SO	14.05
hexane	**28.94**
chloroform	27.02
water	20.39

Comparatively, CLEC is found to be more active in water-immiscible solvents than in water-miscible solvents. CLEC in hexane and in *n*-dodecane medium hydrolyzed and produced 28.94 mg glucose/hour. The catalytic performance of CLEC glucoamylase was lower in 1,4-dioxane and 2-propanol with a glucose production of 11.35 and 13.55 mg glucose/hour, respectively, compared to that in aqueous medium, giving 20.39 mg

glucose/hour. The results show that careful selection of the solvent is necessary either for biotransformations or for the synthesis of oligosaccharides.

6.4 Conclusions

Crystallization followed by cross-linking is an efficient method for the production of a thermostable glucoamylase enzyme that also has good activity in organic media. Cross-linking of the enzyme crystals resulted in a significant change in the kinetic parameters as evident from the increase in K_m and V_{max} of the cross-linked glucoamylase crystal, during soluble starch hydrolysis. The CLEC must, however, be used at pH 4.5 to get the optimum activity, and it must also be able to withstand a temperature of 80 °C for 30 minutes, even without the protection of starch.

The CLEC can be used for the continuous production of glucose from soluble starch or maltodextrin at 60 °C in a packed-bed reactor within short reaction times. The crystals are robust and can also be used in solvent media for many biotransformations, including the synthesis of novel oligosaccharides. In addition, our experiments showed that CLECs made by this method when subjected to lyophilization, with or without surfactants, yield lyophilized a CLEC glucoamylase that can be stored at nonrefrigerated temperatures for extended periods of time. Further studies are needed to determine the effects of cross-linking on the CD, SBD and the linker regions of the glucoamylase enzyme.

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1. **J.Jegan Roy**. and T.Emilia Abraham. "Strategies in making Cross-linked enzyme crystals", **Chemical *Reviews***, 2004 , Vol 104(9), 3705-3721.
2. Tholath Emilia Abraham, **Jegan Roy Joseph**, Lekshmi Bai.V.Bindhu, Jayakumar. "Cross-linked Glucoamylase Crystals as potent catalyst for Biotransformations". ***Cabohydrate Research,*** 2004, Vol 339, 1099-1104.
3. **J. Jegan Roy,** T.Emilia Abraham, K.S. Abhijith, P.V. Sujith kumar, .and M.S.Thakur, " Biosensor for the determination of phenols based on Cross-linked enzyme crystals (CLEC) of Laccase". (in press) ***Biosens.Bioelectron***. (2004)
4. **J**.**Jegan Roy,** S.Sumi, K.Sangeetha, T. Emilia Abraham. "Chemical modification and Immobilization of Papain" (in press). ***J. Chemtechnol. Biotechnol.*** (2004)
5. **J**.**Jegan Roy,** and T.Emilia Abraham "Cross-linked enzyme crystals of Laccase: Thermal stability and kinetic studies". Communicated.
6. **J. Jegan Roy** and T.Emilia Abraham **"**Biotransformation of pyrogallol to purpurogallin in a Packed Bed Reactor using Cross-Linked Enzyme Crystals of Laccase". Communicated.
7. Resmi C.Senan, T.S.Shaffiqu, **J.Jegan Roy**, T.Emilia Abraham. "Aerobic degradation of a mixture of azo dyes in a packed bed reactor having bacteria coated laterite pebbles". ***Biotechnol. Progress***. 2003, Vol 19, 647-651.
8. T.Emilia Abraham, Reshmi Senan, T.S.shaffiqu, **Jegan J. Roy**, T.P.Poulose, and P.P.Thomas. "Bioremediation of textile azo dyes by an aerobic bacterial consortium using rotating biological contactor". ***Biotechnol.Progress***, 2003, Vol 19, 1372-1376.
9. Shaffique, T.S., **Jegan Roy,J.** , Aswathi Nair and T.Emilia Abraham, "Degradation of textile dyes by plant peroxidases" ***Applied Biochemistry and Biotechnology.*** 2002, Vol 102-103, 315-326

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11. Resmi.C. Senan, Shaffiqu,T.S., **Jegan Roy,J.**, Akhila Rajan, T.Emilia Abraham and P.Prema "Biodegradation of dyes from coir and textile effluents" ***Coir News*** 2002, Vol 31,21-27.

Paper/Poster presentations in Conference

1. Shaffique, T.S., **Jegan Roy,J.**, Aswathi Nair and T.Emilia Abraham, "Degradation of textile dyes by plant peroxidases" poster presented in International conference New horizons in Biotechnoloy held at Trivandrum April 2001.
2. **Jegan Roy,J.,** Sumi,S., Sangeetha,K., Emilia Abraham,T. "Chemical modification and Immobilization of Papain" poster presented in the National conference "Protein structure and function" held at IIT Mumbai in October 2002.
3. **Jegan Roy, J** and Emilia Abraham,T. "Development of cross-linked enzyme crystals of Laccase enzyme" Poster presented in the International conference 10th FAOBMB Congress held at IISc Bangalore December 2003.
4. **Jegan Roy,J. and** Emilia Abraham,T., Thakur,M.S. "Biosensor based on Cross-linked enzyme crystals (CLEC) of Laccase for the determination of phenols" poster presented in the International conference Macro 2004 held at Trivandrum December 2004.

Patent Filed/Applied

1. Emilia Abraham,T.; Nisha Rani,D.; Thomson,K.; **Jegan Roy,J**. "A process for the production of plant cell and callus cultures" NF 0468/2003.