CHARACTERIZATION OF ALKALI STABLE FUNGAL CELLULASES AND THEIR POTENTIAL INDUSTRIAL APPLICATIONS

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BY

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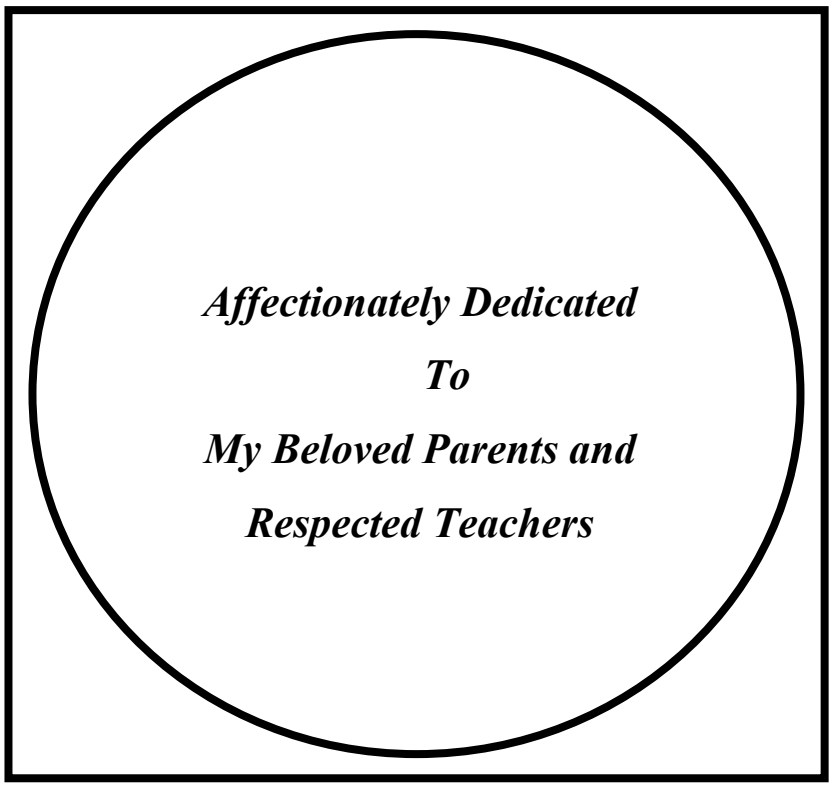
Affectionately Dedicated
To
My Beloved Parents and
Respected Teachers

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CHAPTER 6

DECLARATION

Certified that the work incorporated in the Ph.D. thesis **" CHARACTERIZATION OF ALKALI STABLE FUNGAL CELLULASES AND THEIR POTENTIAL INDUSTRIAL APPLICATIONS "** submitted by ***Mr. Santosh Vyas*** was carried out by the candidate under my guidance. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

Research Guide
Dr. Anil H. Lachke

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ABSTRACT

Alkaline active and/or alkali stable cellulases have gained commercial importance because of their potential applications in textile, paper/pulp, and detergent industries. These enzymes are useful in denim washing, 'Biopolishing' of textiles as well as in deinking operations during recycling of Mixed Office Waste Paper (MOW). However, fungi capable of growing at alkaline pH and secreting extracellular alkaline active cellulases are rare amongst microorganisms. The present investigation was undertaken to isolate alkaline active and alkali stable cellulases from the promising culture(s). Alkalophilic microorganisms growing in alkaline habitats in nature were screened for selection of promising cultures that can produce extracellular cellulases of desired properties. The best culture was selected and identified as alkalotolerent *Fusarium* sp. The fungus grows luxuriously at pH 10. It produces extra cellular cellulase preparation that showed activity in broad pH range from 4 to 10 and optimum temperature 60^{o}C. The enzyme was stable in alkaline pH range 8-10 and showed interesting short fibre forming activity. These properties suggested it has potential for industrial applications. For the successful industrial applications of the isolated cellulase components it is necessary to study their structural and functional relationship with different cellulosic substrates. In this respect the present study deals with biochemical characterization of cellulolytic enzyme and their applications in deinking of Mixed Office Waste paper.

The highlights of the present investigations are as follows.

1. Isolation of industrially important but rare alkaline active cellulases and its enhanced production by statistical optimization of submerged fermentation conditions.
2. Purification and characterization of Endoglucanase A
3. Biophysical characterization of cellulose hydrolysis by Endoglucanase A and its mode of action on different cellulosic substrates.
4. Immobilization of cellulase on novel nanoscale supports to protect them from denaturation in adverse physicochemical conditions and make them reusable.
5. Successful application of cellulases in enzymatic deinking of MOW Paper and deducing the probable mechanism of biodeinking.

The thesis is consisting of six Chapters.

Chapter 1 General Introduction

This Chapter presents general introduction to this thesis. The discussion comprises of literature survey with reference to fungal cellulases, their occurrence, properties, and mechanism of action, enzyme structure, protein engineering and industrial applications of cellulases.

Chapter 2 Enhanced Production of Alkaline Active Cellulases: The Statistical Optimization of Submerged Fermentation Conditions

This Chapter describes screening and identification of an alkalotolerent fungus *Fusarium* sp for the production of alkaline active cellulases.The fungus grows luxuriously in the pH range of 5 to 10 and at 30°C. Submerged fermentation was carried out in 250 ml shake flasks containing 50 ml of Reese basal medium at 30°C. The results showed that refined cellulosic substrate such as Cellulose 123 is favorable for production of cellulases. The enzyme can be produced on agricultural residues like bagasse pith, corncob, and wheat bran. Ammonium sulphate (0.14%), yeast extract (0.05%), urea (0.06%) were found to be the best inorganic, organic and complex nitrogen sources for cellulase production by the fungus. Tween 80 (0.1%) was necessary to release the cellulase complex in the medium.

The optimal concentration of these selected media components were determined using statistical experimental design. Two level fractional factorial designs in five selected variables mentioned above and their concentrations were constructed. The experimental measurements of activity were fitted to a polynomial model and solved by a regression method using singular value decomposition. The optimum concentrations of the constituents in the medium thus found are as follows (in g/l): cellulose powder, 25; ammonium sulphate 1.4 ; yeast extract, 1; urea, 1 and Tween 80, 0.05 %. The optimum starting pH for enzyme production was found 7.0. The results obtained through fractional factorial design were confirmed in 1l shake flasks. The maximum endoglucanase activity obtained on 8^{th} day was 28-30 IU/ml which is 4 fold higher as compared with enzyme yield about 7-8 IU /ml that was obtained in basal medium. The crude culture filtrate showed following extra-cellular cellulolytic and

xylanolytic activities: CMCase, Filter Paper degrading (FPase), β-D-glucosidase, β-D-xylanase and β-D-xylosidase.

Chapter 3 Enzyme Purification and Characterization

The enzyme Endoglucanase A (1,4-β-D glucan-4 glucanohydrolase (EC 3.2.1.4)) was purified to homogeneity from the culture filtrate of alkalotolerent *Fusarium* sp. The enzyme was purified 13 fold by ultrafiltration (PM-10), gel filtration chromatography on BioGel P-100 matrix and preparative isoelectric focusing. The molecular weight of the enzyme was determined as 37.8 KD by gel filtration and 45 KD on SDS PAGE respectively. The pI of enzyme was determined as 4.5. Further characterization studies on Endoglucanase A showed that the enzyme is active in broad pH range of 4 to 9 with pH and temperature optima at 5.0 and 60^{o}C respectively. The enzyme is stable in alkaline pH range (7-9) at 50-60^{0}C.The thermostability of enzyme was studied in the temperature range of 50-70^{0}C. Endoglucanase A shows half-life of 1.5h at 70^{0}C. Differential scanning calorimetric studies indicates that the enzyme unfolds in 2 different stages. The thermal stability of enzyme is improved in the presence of different sugar alcohols. Trehalose was found best in protecting enzyme from thermal denaturation. Km and Vmax values at optimum conditions were found as, 2.0 mg/ml and 641 μmoles/min/mg respectively. The enzyme action is unaffected in the presence of various metal ions. However Hg^{2+} at 1 mM concentration completely inhibited enzyme action.

Chapter 4 Substrate Specificity : Characterization of Cellulose Hydrolysis by Endoglucanase A and Its Mode of Action

The substrate specificity on Endoglucanase A from alkalotolerent *Fusarium* sp showed that the enzyme readily hydrolyzes carboxymethyl cellulose (CMC), Walseth cellulose and lichenan. It did not hydrolyze laminarin that consists of β-(1→3) and β-(1→6) linkages. CM pachyman with β -(1→3) and sophorose with β-(1→2) glycosidic linkages. Thus, the substrate specificity of Endoglucanase A is restricted to β 1-4 linkages only. The enzyme did not show β-D-xylanase, β-D-xylosidase and β- D-glucosidase activity. The enzyme samples were used for the determination of

randomness of their attack on CMC. The specific viscosity of 1% CMC solution was rapidly lowered as a function of time with subsequent slow release of reducing sugars indicating random cleavage of the substrate by the enzyme. Thus, the enzyme shown a typical endo type of action. The enzyme is inhibited by different concentrations (0.1%, 1% and 5%) of cellobiose and glucose. The product of enzymic hydrolysis were determined by high-pressure liquid chromatography (HPLC). The Endoglucanase A did not hydrolyzed cellobiose. However, it attacked cellotriose and higher chain length cello-oligosachharides, preferentially at the internal glycosidic bonds.

Cellulosic substrates with different crystallinity were examined for the bound water analysis by Differential Thermal Analysis (DTA) and Thermogravimetry (TG). The samples were heated in the range of 30-100°C at a rate of 3°C /min. DTA vaporization curves for different cellulose samples indicated that the bound water (W_b) was vaporized at higher temperature than free water (W_f) at the surface. The weight loss was observed in two stages, corresponding to W_f and W_b in TG curves. The bound water content was dependent on the degree of crystallinity of cellulose. Higher the crystallinity, lower is the bound water content. Among different cellulose substrates Walseth cellulose showed highest bound water content 0.920 g/g of the substrate at the same time it was found to be least crystalline (Crystallinity index = 31%). The possible application of bound water analysis in understanding the hydrolysis of cellulosic substrates of different crystallinity is discussed in this Chapter.

Chapter 5 Immobilization of Cellulases on Novel Supports

The enzyme Endoglucanase A from an alkalotolerant *Fusarium* sp has been immobilized in / on different novel supports such as fatty amine films and nanoscale curved surfaces using different strategies. The comparison of various biochemical properties of immobilized enzyme with free enzyme is presented in this Chapter.

A: *Immobilization in thermally evaporated fatty amine films*

The Endoglucanase A was electrostatically encapsulated in thermally evaporated fatty amine films and the enzymatic activity of the biocomposite film under different pH and temperature conditions were studied. The characterization of immobilized enzyme in film form was carried out by Fourier Transformed Infrared spectroscopy (FTIR). Substrate protection of the enzyme by carboxymethyl cellulose (CMC) was essential to stabilize the enzyme against inactivation in the lipid matrix.

The optimum temperature of the enzyme was increased by 10°C as compared to free enzyme in solution. The enhanced stability at high temperatures was coupled with improved catalytic activity of the enzyme-lipid biocomposite films at alkaline pH conditions.

B: *Enzyme bioconjugates with colloidal gold nanoparticles*

The utility of nanoscale-curved surfaces provided by colloidal gold particles was exploited for immobilization of Endoglucanase A. The high surface-to-volume ratio offered by colloidal particles resulted in higher loading of enzyme as compared to that afforded by other immobilization protocols. Covalent and other secondary interactions are responsible for this immobilization process. The formation of endoglucanase-colloidal gold bioconjugates was accomplished by simple mixing of the two solutions at pH 5. The bioconjugate formation process was monitored by UV-visible spectroscopic studies, while Transmission Electron Microscopy (TEM) measurements were used to characterize the particle size of the bioconjugates. The secondary and tertiary structure of the enzyme in the bioconjugate system was studied by FTIR and fluorescence spectroscopy, respectively. The enzyme activity in the bioconjugate system was comparable with that of free enzyme in solution. The enzyme in the bioconjugate system showed an increased half-life at 60°C and pH 9 in comparison with free enzyme in solution.

C: *Three dimensional assembly of gold nanoparticles on polyurethane microspheres to form 'Core-Shell' structures*

The separation of colloidal gold bioconjugates (Chapter 5 B) from the reaction mixture is difficult. Therefore, the reuse of the enzyme is not possible. To overcome this problem, a novel biocatalyst was synthesized by immobilizing enzyme on the surface of modified polyurethane microspheres by gold nanoparticles. The binding of the gold nanoparticles to the polyurethane polymer surface occurs through nitrogen groups in the polymer backbone. These surface modifications induce strong binding of enzyme Endoglucanase A on polyurethane microspheres. Scanning Electron Microscopic (SEM) studies revealed the binding of endoglucanases in 3-D core shell assembly. These microspheres were conveniently separated from reaction mixture by centrifugation. It is possible to reuse the Endoglucanase-nanogold-Pu assembly for at least five cycles. As the enzyme is present on the surface of the microspheres, it does not require any substrate protection. The Endoglucanase-nanogold-Pu assembly

showed comparable specific activity to free enzyme in solution and improved temperature stability.

Chapter 6 Enzymatic Deinking of Mixed Office Waste (MOW) Paper

The major difficulty in recycling of waste paper is removal of inks. In this work the extracellular cellulase preparation from alkalotolerent *Fusarium* sp was used for deinking of Mixed Office Waste (MOW) papers. The enzyme treatment resulted in the increase in brightness with the reduction in ink counts of the recycled paper. The objective of the present work is to understand the mechanism of action of alkaline active cellulase complex from alkalotolerant *Fusarium* sp during deinking of MOW-paper. The loosening and release of toner particles from the printed papers due to random acting endoglucanase components facilitate 'Biodeinking' of MOW-papers. The experimental results suggested a possible mechanism of enzymatic deinking of MOW-papers for recycling purpose.

The refined extracellular cellulase preparation was fractionated on Bio Gel P-100 gel filtration column. Two separate enzyme fractions namely Endo A and Endo B were obtained and they showed 2.3 and 4.6 fold increase in their specific activities, respectively. The viscometric analysis indicated the increase in fluidity ($1/\eta_{sp}$) of 1% carboxymethyl cellulose solution. The data revealed that Endo B was 1.9 fold more random as compared to Endo A. Both the endoglucanases showed comparable patterns of their ability to release short fibres in the reaction mixture when filter paper was used as the substrate. The average size of released short fibres was ranged between 20-100μm. Adsorption characteristics of the endoglucanases were examined using increasing concentrations of enzyme and their effect on rate of the hydrolytic reaction. The data indicated that the enzyme action is prominent at the surface of substrate rather than at their cracked edges. The X-ray diffractograms showed a marginal increase in the degree of crystallinity of the substrates in the initial stages of the enzymatic action. Amorphous regions of the cellulose substrates appeared to be readily attacked by the enzyme. The enzyme treatment resulted in the increase in brightness with the reduction in ink counts of the recycled paper. Based on the distinct properties of endoglucanases a probable mechanism of enzymatic deinking process is presented schematically. Also, the fabrication of the cost effective, laboratory scale flotation device that can be used for deinking of various grades of wastepaper is described as a separate annexure at the end of this Chapter.

ABBREVIATIONS

AU-PU	: Colloidal gold–Polyurethane Conjugates
BSA	: Bovine Serum Albumin
CBD	: Cellulose Binding Domains
CMC	: Carboxy Methyl Cellulose
EDAX	: Energy Dispersive Analysis of X-rays
FTIR	: Fourier transformed infrared spectroscopy
$HAuCl_4$	: Chloroauric acid
Km	: Michelis Menten Constant
MOW	: Mixed Office Waste
ODA	: Octadecylamine
PAGE	: Polyacrylamide Gel Electrophoresis
P NPG	: *P* Nitrophenyl -β- D- glucopyranoside
P NPX	: *P* Nitrophenyl -β- D- xylopyranoside
P HBAH	: *P* hydroxybenzoic acid hydrazide
PU	: Polyurethane
SEM	: Scanning Electron Microscope
SDS	: Sodium Dodecyl Sulphate
TEM	: Transmission Electron Microscopy
V/ W	: Volume/ Weight (concentration)
W/W	: Weight / Volume (concentration)

Chapter 1

General Introduction

Cellulose is the most abundant and renewable biopolymer on the earth. An estimated synthesis rate of cellulose is approximately 4 X 10^{10} tonnes per year. Although it is the most promising renewable energy source to overcome the problems of energy resource, chemicals and food in the future, its enormous potential was recognized only after the cellulose degrading enzymes or cellulases had been identified. The research activity on the cellulases started with the biodeterioration problem faced by US Army in South Pacific during World War II. A fungus was found to be a causative agent for spoilage of tents, sandbags, soldier uniforms and other clothing's. Several organizations within US Army had to set up laboratories to find an immediate solution to this problem (Reese,1976). Later the fungus was identified as *Trichoderma viridae* (Simmons,1977). It was quickly recognized that cellulases, a group of hydrolytic enzymes could certainly prove beneficial in converting cellulosic waste to glucose, soluble sugars and industrially important chemicals.

Till date, a variety of microorganisms that are capable of producing cellulases have been isolated (Lunjungdahl and Eriksson, 1985; Beguin and Aubert, 1993). Attempts are going on to increase cellulase yields by mutation, protoplast fusion, and genetic engineering techniques (Lachke et al., 1986; Brown et al., 1986). The significant progress is seen in purification, characterization of different cellulase components and in understanding their substrate specificities and mode of action (Berghem and Pettersson, 1973; Berghem et al., 1976; Hurst et al., 1977; Hong et al., 1986; Lachke et al., 1987; Sahasrabudhe et al., 1987, Bhat et al., 1989,1990; Claeyssens and Aerts, 1992; Schülein, 1997). Recently, 3D structures of the different cellulolytic enzymes have been solved (Davies et al., 1993,1995; Devine et al., 1994; Sulzenbacher et al., 1996,1997). This has advanced our understanding regarding structure - function relationships of enzyme with their substrates. Most of these studies were driven by potential industrial applications of cellulases. This chapter includes discussion on fundamental and applied aspects of fungal cellulases.

1.1. The Structure of Cellulose

The chemical composition of cellulose is simple. However, its physical structure and morphology is heterogeneous and complex (Teeri, 1997). The polymeric chain of cellulose consists of over 10,000 D-glucose residues linked with β-1,4 glycosidic bonds (Fig.1.1).

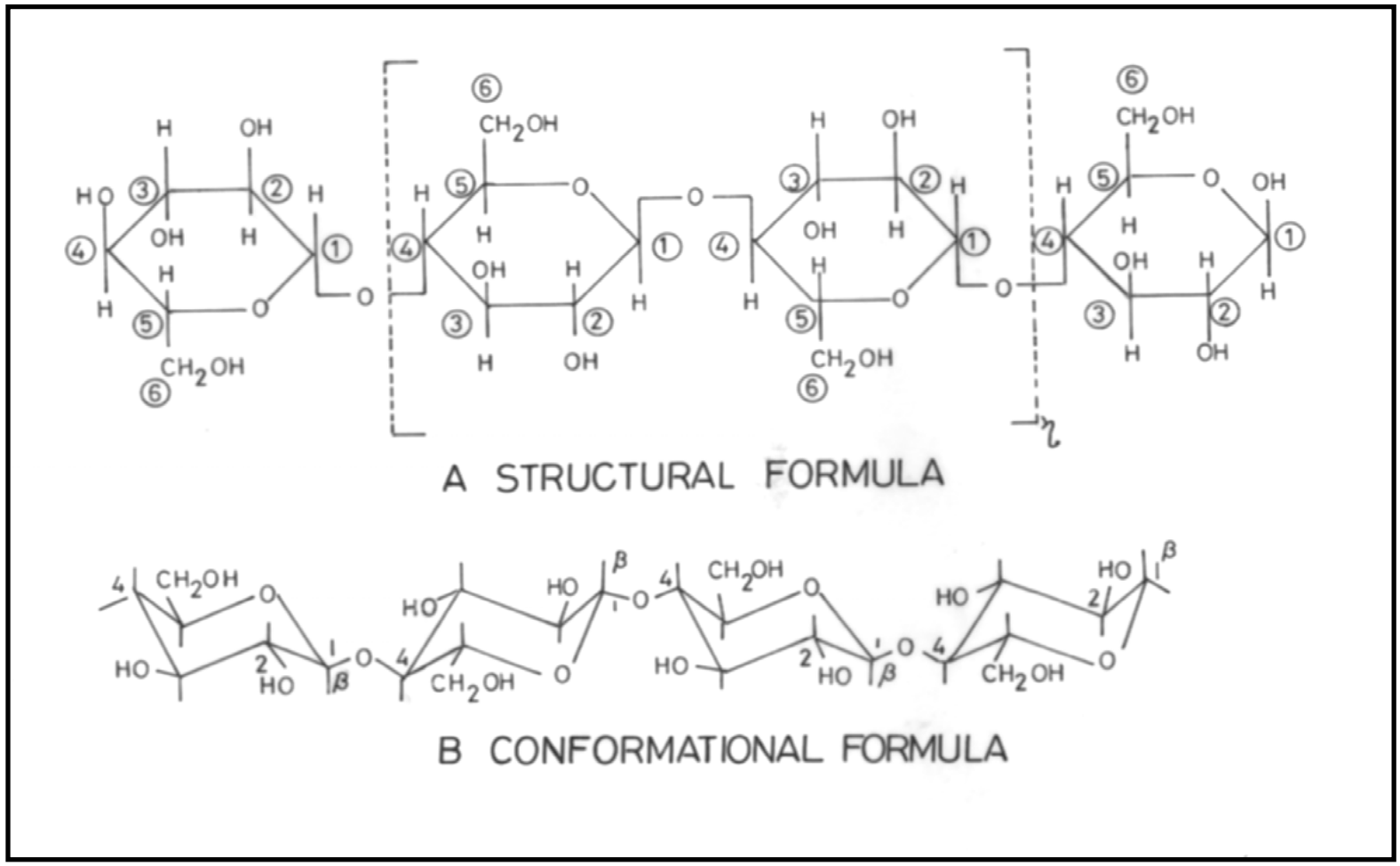


Fig. 1.1. The structure of cellulose

The resulting chains are insoluble. They adhere to each other in parallel fashion to form crystalline microfibrills. The individual crystal of cellulose contains tens of glucan chains in parallel orientation with their reducing ends at one terminus and non-reducing chain end at other. Although highly crystalline, the structure of cellulose is not uniform. The native cellulose contains both highly crystalline and less ordered amorphous regions (Atalla, 1993). Further, complexity in the structure of native cellulose also arises due to hemicellulose, lignin and pectin present along with cellulose as plant cell wall components. Due to such a complex structure of cellulosic substrates, its efficient solubilization requires presence of different enzymes in a typical cellulolytic enzyme complex.

1.2. Typical Cellulolytic System

Although all cellulolytic enzymes share the same chemical specificity for β-1,4-glycosidic bonds they show difference in their specificities towards macroscopic properties of substrate. Generally, a typical cellulolytic complex includes a variety of hydrolytic and oxidative enzymes. Hydrolytic enzymes such as endoglucanase [1,4-β-D glucan glucanohydrolase; (EC 3.2.1.4)], cellobiohydrolase (CBH) [1,4-β-D glucan cellobiohydrolase; (EC 3.2.1.91)], 1,4-β-D-glucan glucohydrolase (EC 3.2.1.74) and β-glucosidase [1,4-β-D glucoside glucohydrolase, (EC 3.2.1.21)] are involved in degradation of crystalline cellulose to glucose. The enzyme action is generally initiated by random acting endoglucanases at amorphous regions within cellulose chain to produce cello-oligosaccharides. The cellobiohydrolases then act sequentially by removing cellobiose units from non-reducing as well as reducing ends of the cellulose chains. Finally, β glucosidase completes the hydrolysis by degrading cellobiose to glucose (Eveleigh, 1987). The organization of native cellulose and its hydrolysis by different endoglucanases and cellobiohydolases is demonstrated schematically in Fig.1.2. Oxidative enzymes, such as cellobiose dehydrogenase (EC 1.1.5.1) (Westermark and Eriksson, 1974), cellobiose oxidase (EC 1.1.99.18) (Morpeth, 1985) has been characterized from cellulolytic fungus *Phanerochaete chrysosporium*. Oxidative enzymes are found to induce hydrolytic components of cellulase complex. Lactonases [(D-glucano-1, 5, lactonohydrolase (EC 3.1.1.17)] are responsible for hydrolysis of inhibitors of cellulase system (Bruchmann et al., 1987).

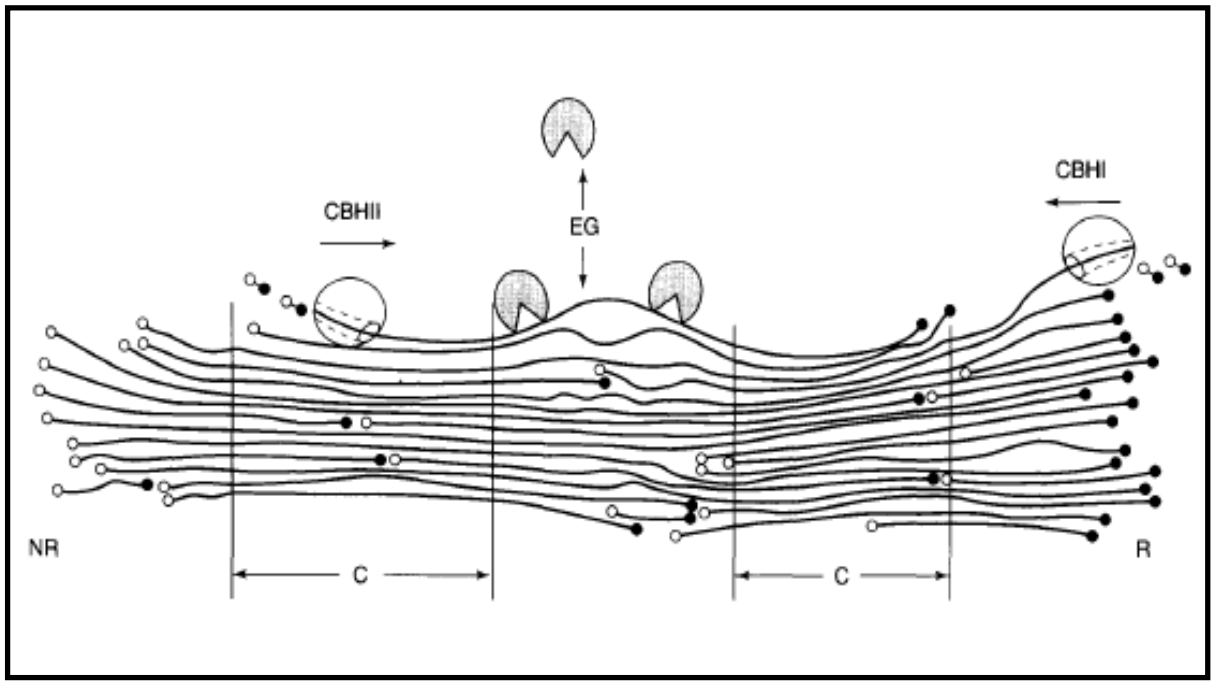


Fig.1.2.Schematic representation of the organization of crystalline cellulose and its hydrolysis with cellulolytic enzymes.

The native structure of cellulose is composed of crystalline and amorphous regions. The less organized amorphous region at the centre of cellulose chain is attacked by endoglucanases. This is followed by attack of cellobiohydrolases (CBHs) at reducing or non reducing ends of cellulose chain. Symbols: • R reducing ends, ○ NR nonreducing ends, C crystalline regions, CBH I: Cellobiohydrolase I, CBH II: Cellobiohydrolase II, EG: Endoglucanase. *(Adopted from Teeri ,1997)*

1.3. Fungal Cellulases

1.3.1. Occurrence

Cellulases are produced by wide variety of bacteria, fungi, actinomycetes, aerobes and anaerobes, mesophiles and thermophiles. However, fungi are the most studied organisms because of their higher enzyme yields and capacities to produce complete cellulase complex. Our knowledge about fungal cellulases is mainly due to studies on hypersecretory fungi e.g. *Trichoderma reesei* (Kubicek, 1992; Kubicek et al. 1993), *Penicillium pinophilum* (Lachke et al.,1986; Bhat et al., 1989), *Penicillium funiculosum* (Wood and McCrae, 1982; Lachke et al., 1983), *Fusarium oxysporum* (Kumar et al., 1991;Christakopoulos et al., 1995a,b), *Aspergillus niger* (Okada, 1985; Hurst et al., 1977), *Sclerotium rolfsii* (Lachke and Deshpande, 1988) and *Humicola sp.* (Hayashida et al., 1988; Schülein, 1997). However, other cellulolytic systems from *Phanerochaete chrysosporium* (*S. pulverulentum, Eriksson*, 1978), *Taleromyces emersonii* (McHale and Coughlan, 1980), *Melanocarpus albomyces* (Arja et al., 2004) and from anaerobic fungi belonging to genera *Neocallimastix*, *Cacomyces, Oprinomyces* (Wood, 1992) have also been well characterized. The cellulases have also been isolated from thermophillic fungi such as *Sporotrichum thermophile* (Coutts and Smith, 1976), *Thermoascus auranticus* (Khandke et al., 1989), *Chaetomium thermophile* (Erikssen and Goksoyr, 1977), *Humicola griseus* (Hayashida and Mo, 1986) and *Myceliopthora thermophila* (Roy et al., 1990). These fungi are of particular interest because of their capacities to produce thermostable cellulases. These enzymes have shown to be stable at highly acidic or alkaline pHs as well as temperatures up to 90°C. Recently, enzymes that are active and stable in neutral to alkaline conditions have gained significant commercial importance due to their ecofriendly applications in detergent, textile and paper industries. However, unlike thermophiles, alkalophillic fungi are very rare. Till date such enzymes have been reported from very few fungi such as *Humicola* sp. (Hayashida et al., 1988; Schülein, 1997), *Cephalosporium* sp (Kang and Rhee, 1995) and from a unknown desert basidiomycetes sp. (Sreenath et al., 1996). In the present investigation alkaline active, alkali stable cellulase preparation was obtained from an alkalotolerent *Fusarium* sp. The four fold enhanced production of the enzyme, with the help of statistical optimization studies is described in Chapter 2. The other industrially exploited,

extracellular cellulase systems from *Humicola insolens* and *Trichoderma reesei* are shortly described in the text below.

1.3.2. Physiological role of fungal cellulases

The cellulases from fungi hydrolyzes cellulose to simple soluble sugars that are utilized by fungus as a carbon source in their metabolism. Some specialized group of fungal species e.g. *Pythium, Leptomitus* synthesize cellulases to help in extension of their cellulosic mycelial cell walls. As the cellulose is a major component of the plant cell wall, cellulases play key role in establishing host pathogen interactions in case of plant pathogens (Osagie and Obuekwe,1991). Different anaerobic fungi belonging to genera *Neocallimastix*, *Cacomyces, Oprinomyces* have been isolated from ruminants and other herbivorous animals (Wood et al., 1988). These fungi produce extracellular cellulases that are involved in breakdown of lignocellulose in the rumen. In order to overcome complexity of cellulosic substrates, fungi often produce a variety of cellulase components that differ in their molecular characteristics such as molecular weight, amino acid composition and sequence, isoelectric point and carbohydrate content etc. They also differ in their capacities to adsorb on the cellulose, substrate specificity and even in their catalytic activities.

1.3.3.. Multiplicity of fungal cellulase components

In fungi, cellulase components occur in multiple forms (Labudova and Farcas, 1983). Molecular cloning studies revealed that diversity of cellulolytic components is far less at genetic level. The multiplicity in cellulases can be attributed to the events at transcriptional / translational level or may be due to post-translational events such as glycosylation or proteolysis. Fungal cellulases are generally glycosylated at asparagine (N-linked) or serine and threonine (O-linked) residues. The degree of glycosylation could be responsible for multiplicity of fungal cellulase components (Gum and Brown 1977; Van Ardsell et al., 1987). The principle sugar in glycosylated residues is mannose. The other commonly occurring sugars are identified as glucose, xylose, galactose, N-acetyl glucosamine and galactosamine (Salovuori et al., 1987). This glycosylation of cellulase plays pivotal role in substrate binding (Darnell et al., 1986). The glycosylation also protects enzyme from proteolysis and helps in secretion of

proteins. In *T. reesei* 'N' linked glycosylation appears to stabilize cellulase structure by protecting them from protease attacks during secretion (Sanchez et al., 1982; Merivuori et al.,1985). Proteolysis is another important phenomenon for multiplicity of cellulase complex (Gong and Tsao, 1979; Eriksson and Pettersson, 1982; Hayashida and Mo, 1986). A cellobiohydrolase component from *T. reesei* was partially digested by proteases into two components; a catalytic core of molecular weight 55 kDa was unable to bind crystalline cellulose but it was still active towards soluble substrates and a 10 kDa binding domain containing binding site (Van Tibeurgh et al., 1986).

1.3.4. *Some Industrially important fungal cellulase systems*

1.3.4.1. Humicola insolens cellulases

The thermophilic, micromycetes fungus *Humicola insolens* produces at least seven different cellulase components. These cellulases are active in neutral to alkaline pH range (7-10) and have gained considerable commercial importance. They can be utilized for total biomass conversion as well as in other specialized commercial applications such as detergents, textile industry, and paper and pulp industries (Schülein et al., 1993). Out of seven different cellulases of *H. insolens*, five are endoglucanases while two belong to cellobiohydrolase class (Schülein, 1997). Based on the catalytic core sequence, they are classified under different families (Henrissat and Bairoch, 1993). The molecular properties of these different enzymes are compared in Table 1.1. These cellulases are different in their catalytic activities on different substrates. It was found that presence of cellulose binding domains (CBD) in cellulase lowers apparent Km. This was attributed to dispersing action of cellulose binding domain. Presence of CBD also reduces apparent Kcat values (Schülein, 1997).

1.3.4.2. Trichoderma reesei cellulases

The fungus *Trichoderma reesei* is traditionally used by various enzyme manufacturers to produce fungal cellulases. It is one of the best-characterized fungal cellulase systems (Montenecourt, 1983; Kubicek, 1992; Kubicek et al., 1993). The fungus produces large amount of different cellulases that act synergistically for complete hydrolysis of crystalline cellulose to glucose. The fungus produces at least

three different types of endoglucanases (EG I, EGII and EG III), two different cellobiohydrolases (CBH I, CBH II) and β glucosidase (Shoemaker et al., 1983; Teeri et al., 1987). Some of their molecular properties are highlighted in Table 1.2.

Table 1.1. Cellulases from *Humicola insolens* and their properties.

Enzyme	Family	MW KDa	pI	Presence of CBD
EGI	7	50	5.5	Absent
EG II	5	50	7.0	Present
EG III	12	26	5.2	Absent
EG V	45	43	5.2	Present
EG VI	6	43	5.0	Present
CBH I	7	72	4.5	Present
CBH II	6	65	Isozymes in the range of 4.6-5.2	Present

Table 1.2. Cellulases from *Trichoderma reesei* and their properties.

Enzyme	MW KDa	pI	Degree of Glycosylation %	References
EGI	54	4.7	4	Shoemaker et al.,1983 Bhikhabhai et al., 1984
EG II	49.8	5.5	15	Saloheimo et al., 1988
EG III	20-23.5	7.0-7.7	12-15	Beldman et al., 1985
CBH I	42-72	3.5-4.2	1.4-10.4	Bhikhabhai et al., 1984 Bhikhabhai et al., 1985
CBH II	50-58	5.0-6.3	8-18	Fägerstam & Petterson, 1980.

MW - molecular weight **pI** - isoelectric pH **CBD -** Cellulose Binding Domain

1.4. Factors Affecting Cellulose Hydrolysis

As described previously, cellulose does not occur alone in free thread like chain. It is usually present in bundles of fibrillar units with supra molecular structure consisting of crystalline and amorphous regions. Thus it is important to know which cellulase specificities are affected by differences in physiochemical properties of the cellulose surfaces. Ultimately, stereochemistry of cellulose chains may prove to be a key factor for both the existence of multiple isozymes of cellulase components and the synergistic effect exhibited by these components in saccharification of cellulosic substrates. Both factors such as substrate characteristics and enzyme properties can limit the rate and degree of cellulose hydrolysis.

1.4.1. *Substrate characteristics that affects cellulose hydrolysis*

Anatomical and ultra structural characteristics of cellulose fibres that can potentially limit hydrolysis of cellulose are summarized in the Table 1.3. Each of these characteristics is briefly discussed below.

1.4.1.1. *Degree of polymerization* (D.P.)

The degradation of insoluble cellulosic substrates finally results in generating soluble cello-oligosaccharides, i.e. cellulose molecules with D.P.< 6 units. However, there is mixed opinion about degree of polymerization affecting the rate of cellulose hydrolysis. A number of researchers have demonstrated that the molecular weight or D.P. of residual cellulosic material following hydrolysis was similar to that of original substrate (Walseth, 1952; Phillip et al., 1981). On the contatrary other researchers have shown that recombinant cellulases effectively reduced D.P. of different cellulose substrates by various mechanisms (Kleman-Leyer, 1994; Stälbrand et al., 1998). The most accepted opinion suggests that the extent of enzymatic degradation is affected by D.P. of cellulosic substrate. Beyond a definite molecular weight range cellulose is recalcitrant to hydrolysis and its degradation is limited (Chang et al., 1991).

Table 1.3. Anatomical and ultrastructural characteristics of cellulosic fibres that limits its enzymatic hydrolysis

Structural level	Characteristic	Description	References
Microfibril	Molecular orientation	Parrallel Vs Antiparrallel cellulose	Chanzy and Henrissat, 1985
	Crystallinity (CrI)	Ratio of crystalline to amorphous cellulose	Fan et al., 1980,1981; Nazhad et al., 1995 Mansfield et al., 1997
	Degree of polymerization	Molecular chain length of cellulose	Lee and Kim, 1983; Hoshino et al., 1993; Kleman-Leyer et al., 1994
Fibril	Lattice structure	Cellulose lattice (I, II, III, V or X)	Lenz et al., 1988
	Composition	Structural moieties (Carbohydrate and lignin)	Grethlein et al., 1984; Mooney et al., 1998
	Particle size	Fibril dimensions	Sinitsyn, 1991; Nazhad et al., 1995 ; Gama et al., 1997
Fibre	Intrinsic strength	Tensile strength	Pere et al., 1995; Mansfield et al., 1997
	Dimensions	Fibre length, cell wall thickness, coarseness	Converse, 1993; Ramos et al., 1993

Fibre (contd.)	Weight loss	Carbohydrate solubilization	Fan et al., 1980, Gikes et al., 1993 Gama et al., 1997; Mansfield et al., 1997
	Surface area	Total surface area	Stone et al., 1969; Coughlan, 1985a ; Converse et al., 1990
	Water retention	Fibre swelling	Stork et al., 1995
	Pore structure	Pore distribution on fibre surface	Bendzalova et al.,1996 ; Henrissat et al., 1988.
	Surface characteristics	Microscopic determination (SEM, TEM, AFM,CLSM)	White and Brown, 1981; Gikes et al., 1993 ; Daniel, 1994.

Modified from Mansfield et al., 1999

1.4.1.2. Crystallinity

Overall, it is accepted that the amorphous part of cellulose is hydrolyzed first by the enzyme leaving recalcitrant crystalline part unhydrolysed (Ghose and Das, 1971). This observation suggested that the crystallinity of cellulose play major role in limiting rate of its hydrolysis (Fan et al., 1980,1981). However latter studies on lignocellulosic substrates ruled out direct correlation between crystallinity of cellulosic substrates and its hydrolysis (Puri,1984). Now it is established that hydrolysis of crystalline cellulose is brought about by synergistic action of tightly binding cellobiohydrolases (Teeri, 1997). Thus it depends mainly on properties of enzyme rather than the nature of substrate.

1.4.1.3 Accessible surface area

The area available in the form of pores, cavities and spaces between microfibrils in the form of the cell lumen, pit apertures and pit membranes are the actual surface area accessible to the enzyme molecule in the solution (Stone et al., 1969; Grethlein et al., 1985). Different studies found that the relative digestibility of cellulosic substrates was directly proportional to the accessibility of enzyme molecule (Grethlein et al., 1984). It was also observed that the removal of hemicellulose and the redistribution of lignin during the pretreatment of *Pinus radiata* increases the surface area present in the form of pores, thus increasing the accessibility to enzymes (Wong et al., 1988).

1.4.1.4 Particle size

Adsorption of cellulases on cellulose surface is the key step in efficient hydrolysis of cellulosic substrates. Smaller particle size of cellulosic substrates offers larger surface area for enzyme adsorption and hence preferred for improved degradation of lignocellulosics. It has been shown that the smaller sized fractions within pulp are hydrolyzed preferentially in the initial stages of the hydrolysis reaction (Jackson et al., 1993; Mansfield et al., 1996). The studies demonstrated that pretreatment of cellulosic substrates results in decreased particle size and improved hydrolysis yields (Sawada et al., 1995).

1.4.1.5. Lignin distribution

Lignin content in lignocellulosic substrates negatively influences the hydrolysis reaction. Cellulases are irreversibly adsorbed on lignin and that prevents their action on cellulose (Converse et al., 1990; Lee et al., 1994a). The removal of lignin improves hydrolysis of cellulosic material as it creats additional surface area for enzyme adsorption on the cellulosic substrates (Grethlein et al., 1984, Stone et al., 1969).

1.4.2. *Enzyme characteristics important in cellulose hydrolysis*

Various characteristics of cellulases such as their adsorption capacities, inhibition by end products, stability under different environmental conditions (pH, temperature etc.) and synergism between different enzyme components have shown to influence enzymatic hydrolysis of cellulose.

1.4.2.1. Adsorption of cellulases on cellulose

The capacity of cellulases to adsorb on the cellulose surfaces is an important characteristic because the hydrolysis of cellulose requires effective interaction between the substrate and enzyme. This adsorption of cellulase is facilitated by cellulose binding domain (CBD) of the enzyme (Wilson et al.,1995). These CBDs are non-catalytic regions found in tightly binding cellulases and are rich in hydroxy amino acids (Linder and Teeri, 1997). It is thought that the aromatic amino acid residues such as tryptophan, tyrosine packs sugar rings giving extra stability and specificity to enzyme substrate complex (Macarron et al.,1995). Binding of cellulases on cellulose with CBD improves rate of enzymatic cellulose hydrolysis. The binding of enzyme on cellulose surface results in increased concentration of enzyme at the surface. Further, it liberates cellulose chains from the surface of crystalline cellulose by non-hydrolytic mechanism (Din et al., 1991, Teeri et al., 1992). The removal of CBD from enzyme reduces hydrolytic efficiency of enzyme on crystalline cellulose but not on the amorphous cellulose (Tomme et al.,1988). The adsorption of cellulases on cellulose is also influenced by substrate characterization such as crystallinity, D.P, surface area (Mansfield et al., 1997).

1.4.2.2. End product inhibition

One of the key factors that affect cellulose hydrolysis includes inhibition of cellulolytic enzymes by their end products. Both cellobiose and glucose have shown to inhibit cellulose hydrolysis either competitively (Sternberg,1976) and/or noncompetitively (Howell and Mangat, 1978). According to Ghose and Das (1971), inhibition due to glucose is weaker than cellobiose. Reese (1963), reported that hydrolysis of cellulose can be inhibited by cellobiose. Halliwell and Griffin (1973) has demonstrated that cellobiose inhibited *T. koningii cellobiohydrolase* competitively. Other workers suggested that family 7 cellulases from *Trichoderma reesei* (Biely et al., 1991) and EG I from *Humicola* sp (Schülein, 1997) shows such characteristic inhibition of endoglucanases by different cellobiose concentrations. In the present studies it is observed that Endoglucanase A was inhibited by different concentrations of cellobiose and glucose.

1.4.2.3. Stability under different conditions

It is observed that under industrial processing conditions such as high temperatures, pHs, enzymes get rapidly inactivated. Hence, the stability and activity of proteins under industrial operational conditions is the most important characteristics of enzyme that affect cellulose hydrolysis. Thermostable cellulases that are active and stable at temperatures 60°C and above are preferred for enzymatic cellulose hydrolysis (Klyosov, 1988). Similarly cellulases that are stable and active under alkaline conditions are preferred for their applications in detergents, textile and paper industries (Vyas et al., 2003). Various extremophilic microorganisms are being explored to obtain such stable enzymes. The cellulase stabilities can also be improved by their immobilization and by protein engineering of cellulases (Mosbach, 1976; Schülein, 2000; Sastry, 2002). During the present investigations it was demonstrated that immobilization of Endoglucanse A in/on different supports improved their temperature and pH stabilities (Chapter 5). Other environmental factors such as the presence of heavy metals, surfactants can also affect stability and activity of cellulolytic enzymes.

1.4.2.4. Synergism between components of the cellulolytic system

Extensive investigations by various researchers indicated that synergistic action between the different cellulase components is required for efficient cellulose hydrolysis (Wood and McCrae, 1978; Wood et al.,1989). Different types of synergism shown by multiple forms of individual components are as follows: (a) between endoglucanase and CBH (Wood et al., 1980); (b) exo-exo synergism between two distinct CBHs (Fägerstam and Pettersson LG.1980; Henrissat et al., 1985); (c) endo-endo-type interactions between endoglucanases (Mansfield et al., 1998) and (d) between β -glucosidase and either endoglucanase or CBH (Eriksson and Wood, 1985). Although different models have been proposed to explain synergism in these systems, the degree of synergism has shown to vary with the nature of cellulosic substrate used. It was demonstrated that with *Trichoderma* CBHs synergism was more when crystalline substrates were used (Nidetzky and Clayesson, 1994). At the same time synergism between CBH and EG1 was highest when semi crystalline substrates were used and almost zero with crystalline cellulose substrate such as valonia cellulose (Reidel, 1997). Thus, it can be concluded that, both the substrate quality and enzyme properties are the key factors that affect cellulose hydrolysis by cellulases. Some of these properties and their role in cellulose hydrolysis are presented in Chapter 4.

1.5. Classification of Cellulases

The sequence-based classification for cellulases has been originated from its early sequence- based alignments (Henrissat et al., 1989). Over 80 different families of the glycoside hydrolases have been identified on the basis of amino acid sequence similarities (Henrissat, 1991). The latest up date on all known sequence based families of glycoside hydrolases is available at URL http://afmb.cnrs-mrs.fr/vpedro/ CAZY/db.html. A large number of cloned cellulase genes fall under ten of the sequence- derived families (5-9, 12, 26, 44, 45,48). The three-dimensional structures are available for cellulases from families 5 to 9, 12, 45 and 48 (Henrissat and Davies, 1997; Davies, 1998). Despite the fact that they all cleave β-1,4-glycosidic bonds, they display a variety of topologies ranging from all β-sheet proteins, through β/α barrels to all α-helical proteins. The first 3-D structure of a cellulase, the *Trichoderma reesei*

Cel6A (CBH II) catalytic core, was published in 1990 (Rouvinen et al., 1990). Since then, more than 20 different cellulase structures have been solved covering eight different family folds. This includes the structure of three different family representatives of `cellobiohydrolases'. A notable feature of cellulases and many glycoside hydrolases is their modularity (Cutinho and Henrissat, 1999). Proteins are rarely found as single catalytic domain entities. They display a modular structure with one (occasionally more) catalytic domain linked to one or several non-catalytic modules. The non-catalytic modules are frequently involved in protein-carbohydrate and protein-protein interactions. All the non-catalytic modules, including carbohydrate-binding domains, have also been classified into sequence-related families (Tomme et al., 1995; Cutinho and Henrissat, 1999). Based on the catalytic core sequence, cellulases have been classified under different families (Henrissat and Bairoch, 1993).

1.6. Enzyme Structure

Small angle X-ray diffraction studies on different enzymes have been utilized to predict 3D structures of different cellulases (Abuja et al., 1988; Rouvinen et al., 1990; Esterbauer et al., 1991; Davies et al., 1993; Divne et al., 1994; Sulzenbacher et al., 1996,1997). The catalytic domain of *T. reesei* Cellobiohydrolase (CBH II) show large α / β protein with several folds. The active site of CBH II is located at carboxy-terminal end of the parallel β barrel in an in an enclosed tunnel, through which cellulose thread can be entrapped. Two aspartic acid groups appeared at center of tunnel as catalytic residues (Rouvinen et al., 1990). Four glucose residues binds at subsite A B C D. The catalytic domain of the CBH I is also a large single domain protein with two large antiparallel β sheets that face each other to form β sandwich. The active site tunnel of CBH I is longer than CBH II. It possess 7 glycosidic binding sites. The catalytic residues are two glutamic acids (Henrissat, 1991). The difference in active site is reflected through its mode of action. It has been suggested that CBH I cleaves cellulose chain from reducing end and releases cellobiose units, whereas CBH II hydrolyses the same substrates from non reducing end. 3D structure of Endoglucanase E2 from fungus *T. fusca* is similar to catalytic core of CBH II of *T.reesei* (Spezio et al., 1993). However one of the exoglucanase active site loops is missing and it adopts conformation lending to an open active site of endoglucanase E2.

Similarly, sequence based comparison and determination of 3D structures of other cellulases suggested that mode of action on polymeric substrates are dictated by shape of their active sites. Endoglucanases with open active sites can bind and act in the middle of the glucan chains, while exoglucanases with tunnel shaped active site are confined to chain ends for their action. A comparison of homologous exoglucanase-endoglucanase pair to illustrate their differences in active site topologies is demonstrated in Fig. 1.3.

1.7. Mechanism of Action of Cellulases

The fundamental studies on catalytic mechanisms of cellulases have shown that enzyme cleaves β 1-4 glycosidic bonds using acid-base catalysis (Sinnott, 1990; Davies and Henrissat, 1995; Zechel et al., 2000). Catalysis may be performed with either inversion or net retention of the anomeric configuration of the substrate (Fig.1.4). Inversion is a simple single displacement reaction. A catalytic acid gives protonic assistance to leaving group departure, whilst a catalytic base is required to deprotonate water for nucleophilic substitution at the anomeric centre. The acid and base are typically located some 7-13 Å apart in order to accommodate the nucleophilic water `below' the pyranoside ring. On many cellulase systems, the identification, indeed the existence, of the catalytic base remains controversial. The retention mechanism is a double displacement essentially as described by Koshland (1953). A covalent glycosyl enzyme intermediate is formed and subsequently hydrolysed, via oxacarbenium ion-like transition states. This requires two essential residues, an enzymatic nucleophile and a catalytic acid/base. It first serves as a classical Bronsted acid, protonating the leaving group to assist departure and then functions as a base, deprotonating the incoming water nucleophile for the second step. The nucleophile and acid/base are always found some 5-6 Å apart on all systems studied thus far. It has been demonstrated that, since catalytic mechanism is dictated by the location of functional groups on the protein, the stereochemistry of catalysis is conserved within each family (Schülein, 2000).

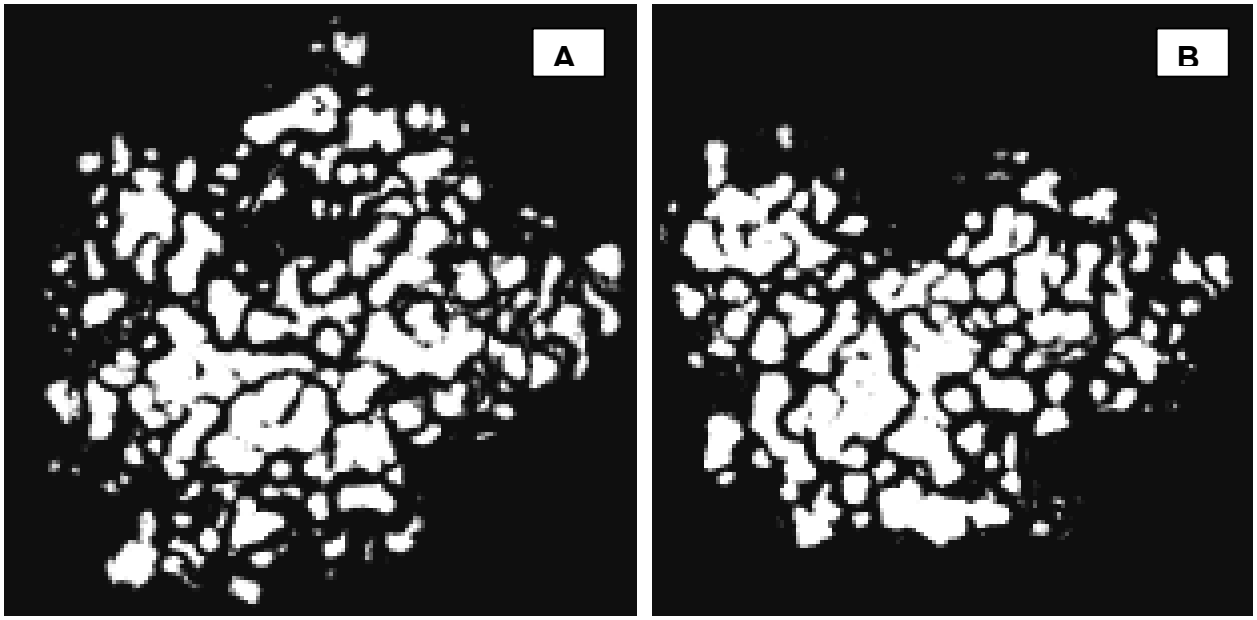


Fig.1.3 Comparison of homologous exoglucanase-endoglucanase pair to illustrate their differences in active site topologies.

A: *Trichoderma reesei* Cellobiohydrolase (CBH II) has a tunnel shaped active site due to presence of two surface loops.
B: *Thermomonospora fusca Endoglucanase* (E2) has tunnel shaped active site, as the surface loops are absent. This open conformation of active site in endoglucanase allows easy access to internal glycosidic bonds. *(Adopted from Teeri, 1997)*

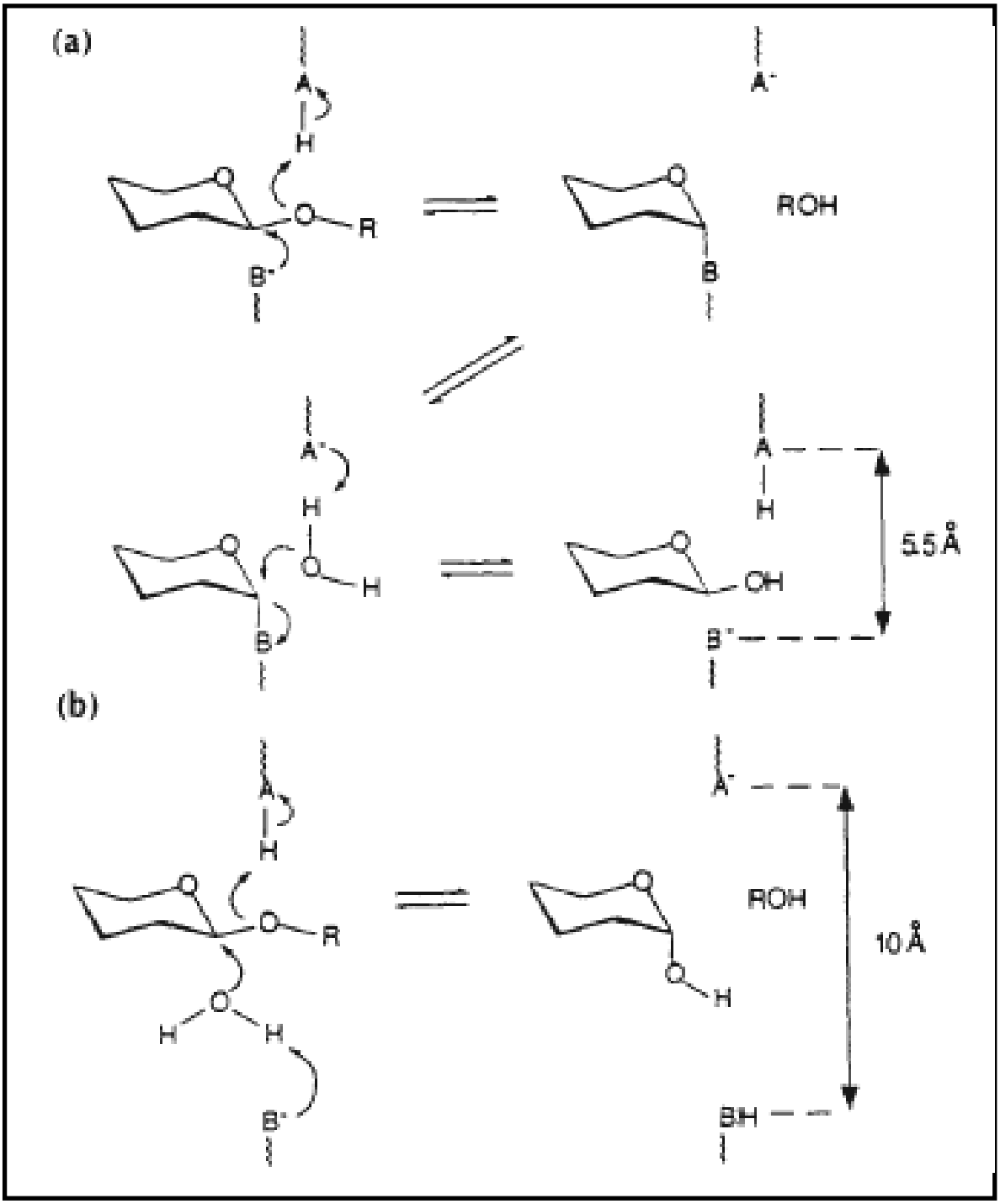


Fig.1.4 The two major mechanisms of enzymatic hydrolysis of glycosidic bonds.

(a) The retaining mechanism, in which the glycosidic oxygen is protonated by the acid catalyst (AH) and nucleophilic assistance to aglycon departure is provided by base (B^-).The resulting glycosyl enzyme is hydrolysed by water molecules and this second nucleophilic substitution at anomeric carbon generates a product with the same stereochemistry.
(b) The inverting mechanism, in which protonation of glycosidic oxygen and aglycon departure are accompanied by concomitant attack of a water molecule that is activated by the base residue (B^-). This single nucleophilic substitution yields a product with opposite stereochemistry to the substrate. (Adopted *from Davies and Henrissat,1995*)

1.8. Protein Engineering of Cellulases

Protein engineering of cellulases has predominantly been used to improve biochemical and catalytic activities of cellulases. It is also used as an efficient tool in the study of their catalytic mechanisms (Schülein, 2000). The sequence-based family classification of cellulolytic enzymes is helpful in the identification of potential active centre residues. These residues have been mutated and mutants are used as vehicles in understanding of protein-ligand complexes at three-dimensional level. The substrate specificity studies with this approach has led to the engineering of enzymes with modified specificities towards crystalline cellulose substrates (Meinke et al., 1995). The construction of hydrolytically inactive glycosyl hydrolase mutants that can deliver `designer' oligosaccharide in high yield is another successful example of protein engineering of cellulases (Fort et al., 2000; Mayer et al., 2000). Some properties of industrial enzymes, such as the detergent compatibility of the *Humicola insolens* Cel45A have also been enhanced with their protein engineering (Otzen et al., 1999). A number of examples of successful protein engineering of cellulases are summarized in Table.1.4

1.9. Applications of Cellulases

A cellulase complex containing all three major cellulolytic components are involved in the process of saccharification where agricultural waste residues can be converted to glucose, single cell proteins and biofuels like ethanol. Despite of last 30 years of research, this application has not yet been economically feasible due to cost of enzymes and lack of efficiency of cellulases. Recently, cellulases gained significant commercial importance due to their potential applications in food, animal feed, detergents, paper and pulp, and textile Industries.

1.9.1. *Fungal cellulases in food industry*

Cellulases are used for various applications in food industries (Coughlan,1985b, 1985c; Mandels, 1985; Beguin and Aubert,1993). These includes; (1) for the improvement of the extraction process of fruit juices and oils from seeds,

Table.1.4. Protein Engineering of Cellulases

System	Objective	Statergy	Reference
Bacillus sp (NK_1) Cellulase, Family 5 Endoglucanase	Alteration in pH optima Alkaline – Neutral	Mutation at Ser.287- Asn Ala 296-Ser	Nakamura et al., 1991
T.fusca E 2 Family 6 Endocellulase	To study entry of substrate molecules in active site and to improve enzyme activity	Arg.237-Ala Show increased activity on CMC Lys-259-His near active site yields higher activity on CMC and acid swollen cellulose.	Zang et al. ,1997 Zang et al., 2000
C.fimi Cellobiohydrolase Family 6 CelB	Alteration of substrate specificity Crystalline ⟶ Amorphous	Deletion of C terminal proximal loop resulted in structure similar to Endoglucanase that showed improved catalytic activity on CMC.	Meinke et al., 1995
Humicola insolense Endoglucanase Cel 7B	Alteration in subsite to bind longer chain substrate	Mutation at Ser 37-Trp and Pro–39 Trp improved binding to longer chain substrates shows lowered Km on such substrates	Davies et al.,1997
H. insolense Endoglucanase Cel 45A	Improved stability in the presence of detergents	Mutation at Argr-158-Glu reduced affinity towards detergents and improved enzyme stability in the presence of detergents	Otzen et al., 1999
H. insolense Endoglucanase Cel 7B	Glycosyl Hydrolases ⟶ Glycosynthases	Replacement of carboxylate nucleophile of retaining β glycosidases Glu--- Ser	Fort et al., 2000 Mayer et al., 2000

(2) in the clarification of fruit juices, (3) for better soaking efficiencies and homogeneous water absorption of cereals, (4) in removal of external soybean coat in production of fermented soybean foods such as Soya sauce, (5) in the isolation of proteins from soyabean and coconut, (6) for efficient isolation of starch from corn and sweet potato, (7) for the gelatinization of seaweeds to digest ball-milled lignocellulose which can be utilized as food additives. Cellulases can also be used for (a) improving the nutritive quality of fermented foods, (b) improving rehydrability of dried vegetables and soup mixtures, (c) the production of cello-oligosaccharides, glucose and other soluble sugars from cellulosic waste and (d) for removal of cell wall which helps in releasing flavour enzymes, polysaccharides and proteins. In brewery and wine industries, cellulases are used to hydrolyze β -1,3 and β-1,4 glucans, which are present in low-grade barley to help in filtration of beer. Cellulases are also used to increase the aroma in wines.

1.9.2. *Fungal cellulases in detergents*

The enzymes used in detergents constitute major portion of industrial enzymes. Until now the use of enzymes in detergents was mainly limited to application of proteases, amylases, lipases as detergent additives. The incorporation of cellulases as detergent components improves washing performance of detergents (Barbesgaard et al., 1984; Hoshino and Susumo, 1997). It is known that washing of cotton containing fabrics by normal detergents generally causes a pronounced unpleasant harshness of the fabric. Along with dirt removal and colour clarification cellulase treatment also helps as fabric softener by reducing harshness of the cotton containing fabrics. Several bacterial cellulases are available in the market that can be used for this purpose, but as the enzyme yields from bacteria are very low, their costs go up. Fungal cellulases can be more important from commercial point of view. Many types of cellulases derived from fungi have pH optimum around 5. As the pH increases above 7, their activity is greatly reduced. Usually the pH of the detergent solution is 7.0-10.5. Fortunately, certain fungal cellulases produced from strains of *Humicola* sp, *Fusarium* sp, *Myceliophthora* sp and *Cephalosporium* sp (Kang and Rhee, 1995) are active at a pH range mentioned earlier. The monocomponent enzyme formulations of fungal endoglucanases are preferred, as they are required in low quantities and do not show action over crystalline regions of cotton fibres (Schülein et al.,1997).

1.9.3. *Fungal cellulases in textile applications*

Cellulase offers an environment friendly alternative to the harsh chemicals being used for fabric finishing in textile industry. The successful application of cellulases in textile started with the process called ' Biostoning '. In this process, the cellulase enzyme is used in place of pumice stone to produce characteristic abraded, faded appearance of jeans. The enzyme cuts bond between cotton fibres and insoluble, surface adhering particles of indigo pigment to produce the necessary faded effect. The enzyme process is more attractive as it overcomes several limitations of traditional process. Even small amount of enzyme dose replaces several kilogrammes of stones in the washing machines that indirectly helps in higher processing capacities as well as high productivity. The enzyme treatment is fast and also avoids damage due to mechanical action of stones both on the garments and washing machines. According to Cavaco Paulo (1998), cellulases rich in endoglucanase components are important in the process of ' Biostoning.' Avicel adsorbable endoglucanases are suitable for this application. It was found that cellulases with hydrophobic amino acid domains on the surface binds indigo dye and act as emulsifier helping them to separate from cellulose fibres (Gusakov et al., 2000). The back staining problems such as loss of contrast, reddening of the original blue shade can be avoided if the process is carried around neutral pH. Hence, cellulases that are operating best in neutral to alkaline pH range are in demands for ' Biostoning '.

Cellulases are useful in polishing of jute and its different blends. ' Biopolishing' is the registered trademark of the Novo-Nordisk Industries for the process of cellulase treatments to cotton fibres and their blends. A hairy ball of fuzz protruding out of yarn is called as pill in textile trade. These pills present serious quality problem because they result in unattractive knotty fabric appearance. During the biopolishing process, multicomponent or monocomponent cellulase treatments cleaves the fibre ends protruding out from the fabric. The process is generally carried out in the presence of anionic or cationic detergents and different fabric softeners are also used during the process. The susceptibility of enzymes to these various agents is important criterion for selection of enzymes for the process.

1.9.4. *Cellulases in Paper and Pulp Industries*

Fungal cellulases are proven useful in various ecofriendly applications in paper and pulp industry (Kirk and Jeffries, 1996). The mechanical process to improve paper strength by enhancing fibrillation and inter fibre bonding is called as beating or refining of pulp. Cellulases can be used for 'Biobeating' or Pulp refining. The first process of refining paper fibrils with cellulases from white rot fungus *Trametes suaveolens* was patented in 1968 by Yerkes, W.D. The process was successful in reducing beating time. Enhancement of drainage rates with cellulase enzymes has been another success story of cellulases in paper industry. Drainage rate determines the loss of water during paper production. Low drainage rate severely affects machine speed that ultimately results in high-energy consumption with low-productivity. This problem is more critical when secondary fibres are used for paper production. Secondary fibre pulp with the commercial cellulase preparation can dramatically enhance drainage rates of recycled fibres (Pommier et al., 1990).

During recycling of mixed office waste (MOW) papers, the deinking with cellulases play a key role in releasing thermally fused toners from fibre surfaces (Jeffries et al., 1994, Elgir et al., 2000, Vyas and Lachke, 2003). Alkaline active cellulases that are effective between pH 8-10 are preferred for this process. The exact role of cellulases in the deinking is still not clear. In the present studies it is observed that the alkaline active cellulases from alkalotolerant *Fusarium* sp. are useful for deinking of MOW paper. The low molecular weight endoglucanase from alkalotolerant *Fusarium* sp. is more random in its action and has more access to the surfaces of cellulose fibres. The randomly acting endoglucanases are responsible for overall deinking process. The probable mechanism of enzymatic deinking is described in Chapter 6 of this thesis.

Cellulases are also useful in other various applications. These miscellaneous applications of cellulases are described in Table 1.5

Table 1.5 Miscellaneous applications of cellulase enzymes

Area	Applications
Animal Feed	Improving digestibility of usual forage. Processing of wood and wood product as feed
Pharmaceuticals	Digestive aids Elimination of unwanted fibres (phytobezoars)
Pollution control	Cleaning of solid waste in municipal dumps. Digestion of excreta in septic tanks and drainage
Fermentation	Providing substrate for the synthesis of methane, ethanol, glycerol, citric acid, lactic acid, vitamins, antibiotics, and single cell proteins and in breweries.
Flavours	Cellulases treatments reduces maturation period of tobacco, tea leaves.
Research	To dissolve plant cell walls and to obtain protoplasts from plant cells.

1.10. Present Investigation

Alkaline active and/or alkali stable cellulases have gained commercial importance because of their potential applications in textile, paper/pulp, and detergent industries. However, fungi capable of growing at alkaline pH and secreting extracellular alkaline active cellulases are rare amongst microorganisms. The present investigation was undertaken to isolate alkaline active and alkali stable cellulases from the promising culture(s). Alkalophilic microorganisms growing in alkaline habitats in nature were screened for selection of promising cultures that can produce extracellular cellulases of desired properties.

In this regard the best culture was selected and identified as an alkalotolerent strain of *Fusarium* sp. The fungus grows luxuriously in the pH range of 5-10. It produces extracellular cellulase preparation that showed activity in broad pH range from 4 to 10 and optimum temperature 60^{o}C. The enzyme was stable in alkaline pH conditions and showed interesting short fibre forming activity. These properties suggested that the enzyme has potential for industrial applications. In this respect the biochemical characterization of cellulolytic enzyme and their applications in deinking of MOW paper are studied in detail.

The highlights of the present investigations are as follows.

(1) Enhanced production of alkaline active cellulases by statistical optimization of submerged fermentation conditions.
(2) Purification and characterization of Endoglucanase A
(3) Biophysical characterization of cellulose hydrolysis by Endoglucanase A and its mode of action.
(4) Immobilization of cellulase on novel supports to protect them from denaturation in adverse physicochemical conditions and make them reusable.
(5) Successful application of cellulases in enzymatic deinking of MOW Paper and deducing the probable mechanism of biodeinking.

Chapter 2

Enhanced Production of Alkaline Active Cellulases: The Statistical Optimization of Submerged Fermentation Conditions

Summary

This Chapter describes screening and identification of an alkalotolerent fungus *Fusarium* sp for the production of alkaline active cellulases. The fungus grows luxuriously in the pH range of 5 to 10 and at 30°C. Submerged fermentation was carried out in 250 ml shake flasks containing 50 ml of Reese basal medium at 30°C. The results showed that refined cellulosic substrate such as Cellulose 123 is favorable for production of cellulases. The enzyme can be produced on agricultural residues like bagasse pith, corncob, and wheat bran. Ammonium sulphate (0.14%), yeast extract (0.05%), urea (0.06%) were found to be the best inorganic, organic and complex nitrogen sources for cellulase production by the fungus. Tween 80 (0.1%) was necessary to release the cellulase complex in the medium.

The optimal concentration of these selected media components were determined using statistical experimental design. Two level fractional factorial designs in five selected variables mentioned above and their concentrations were constructed. The experimental measurements of activity were fitted to a polynomial model and solved by a regression method using singular value decomposition. The optimum concentrations of the constituents in the medium thus found are as follows (in g/l): cellulose powder, 25; ammonium sulphate 1.4 ; yeast extract, 1; urea, 1 and Tween 80, 0.05 %. The optimum starting pH for enzyme production was found 7.0. The results obtained through fractional factorial design were confirmed in 1l shake flasks. The maximum endoglucanase activity obtained on 8^{th} day was 28-30 IU/ml which is 4 fold higher as compared with enzyme yield about 7-8 IU /ml that was obtained in basal medium. The crude culture filtrate showed following extracellular cellulolytic and xylanolytic activities: CMCase, Filter Paper degrading (FPase), β-D-glucosidase, β-D-xylanase and β-D-xylosidase.

2.1. Introduction

Cellulase is a multicomponent enzyme system that is well studied because of its potential industrial applications (Vyas et al., 2003). Cellulolytic enzymes are produced by variety of bacteria, fungi, aerobes and anaerobes. They are detected in mesophiles and thermophiles (Bhat and Bhat, 1997). Each of these microorganisms can produce different kinds of cellulases that differ in their mode of action as well as properties like activity towards crystalline cellulose, activity and stability in acidic or alkaline pH. The most favoured sources for research and production of cellulases are fungi such as *Aspergillus niger* (Okada, 1985)*, Trichoderma reesi* (Kubicek, 1992), *Penicillium* sp (Bhat et al., 1989 Jørgensen et al., 2004)*, Sclerotium rolfsii* (Lachke and Deshpande, 1988), *Fusarium* sp (Christakopoulos et al., 1995) *and Humicola insolens* (Schülein,1997). Conventionally cellulases obtained from these fungi have been utilized in the bioconversion of lignocellulosic materials into biofuels like ethanol and single cell proteins (Wheals et al., 1999). Cellulases that are active and stable in an alkaline pH range are in demand because of their potential ecofriendly applications in textile (Cavaco-Paulo et al., 1998; Gusakov et al., 2000), detergents (Hoshino and Susumo, 1997; Obendorf et al., 2002) and pulp and paper industries (Kirk and Jeffries, 1996; Bajpai, 1999; Vyas and Lachke, 2004). However, fungal alkaline active cellulases are rare (Goyal et al., 1991). In this work, industrially important alkaline active cellulase preparation is obtained from an alkalotolerant fungus *Fusarium* sp. The refined enzyme preparation is active in a broad pH range of 4 to 10 with pH optima at 5.0 at 60°C. The enzyme is found useful in biodeinking of mixed office waste paper (Vyas and Lachke, 2003). However, enzyme yields obtained are low as compared to conventional acid cellulases obtained from hypercellulolytic fungi such as *Trichoderma* sp (Kubicek et al., 1993). The objective of the present work is to improve the yields of industrially important, but rare alkaline active fungal cellulases by optimization of the fermentation parameters. Higher enzyme yields of cellulases have been achieved by strain improvement through cloning techniques (Arja and Rajamaki, 2002), mutation studies (Lachke et al., 1986), and optimization of fermentation conditions (Tuncer et al., 1999). The conventional method of medium optimization involves varying one parameter at a time and keeping the other parameters constant. However, the process is time consuming, expensive and inaccurate especially when interactions between

different components are present (Haaland,1989). Fractional factorial experimental design is a useful model for studying the effect of several factors by varying them simultaneously (Box et al., 1978). The schematic explanation of how statistical approach is useful in process optimization is presented in Fig.2.1.The technique is useful in limiting the number of experiments. Various research workers have applied this technique for the optimization of culture conditions for prokaryotic as well as eukaryotic cells. (Chen, 1996; Chao et al., 2003), the optimization of processing parameters such as pH, temperature, aeration (Harris et al., 1990; Rao et al., 1993). This technique has also been employed for optimizing enzyme production by microorganisms (Gawande and Patkar, 1999; Varela et al., 2000).

The enhanced production of commercially important alkaline active cellulases from an alkalotolerant *Fusarium* sp is described in this Chapter. In order to improve the enzyme yields by media optimization two level fractional factorial designs in five variables (Cellulose 123, ammonium sulphate, yeast extract, urea and Tween 80) and their concentrations were constructed. The experimental measurements of activity showed that the enzyme yields were 4 fold higher than in non-optimized medium.

2.2 Materials and Methods

2.2.1. Chemicals

Following materials were purchased from the suppliers indicated: Avicel PH 101, 4-Hydroxybenzoicacid hydrazide (Fluka AG, Switzerland); Cellulose-123 (Carl Schleicher & Schüll Company, Germany); Solka floc SW 40 (Pure fibrous spruce wood pulp) (Brown Company. Germany); Absorbent cotton (Bengal Chemicals and Pharmaceuticals Work Ltd. India); sodium salt of carboxy methylcellulose (medium viscosity) (CMC), oat spelt xylan, *p*-nitrophenyl-β-D glucopyranoside (*p*-NPG), *p*-nitrophenyl-β-D-xylopyranoside, (*p*-NPX), 3,5 dinitrosalicylic acid (DNSA) (Sigma Chemical Co., U.S.A.). Tween 80 (Polyoxyethylene sorbitanmonolaurate), Tween 40 (Polyoxyethylene sorbitanmonopalmitate)(Atlas Chemical Industries,USA),Triton X-100 (alkylphenylpolyethyleneglycol)(Koch-lite,laboratories,UK), Tween20 (Polyoxyethylene sorbitanmonolaurete (Sigma Chemical Co., USA). Wheat bran, rice bran, corncob and bagasse pith were obtained locally. All the buffer salts and microbial media components were procured from the standard commercial sources and of highest quality available.

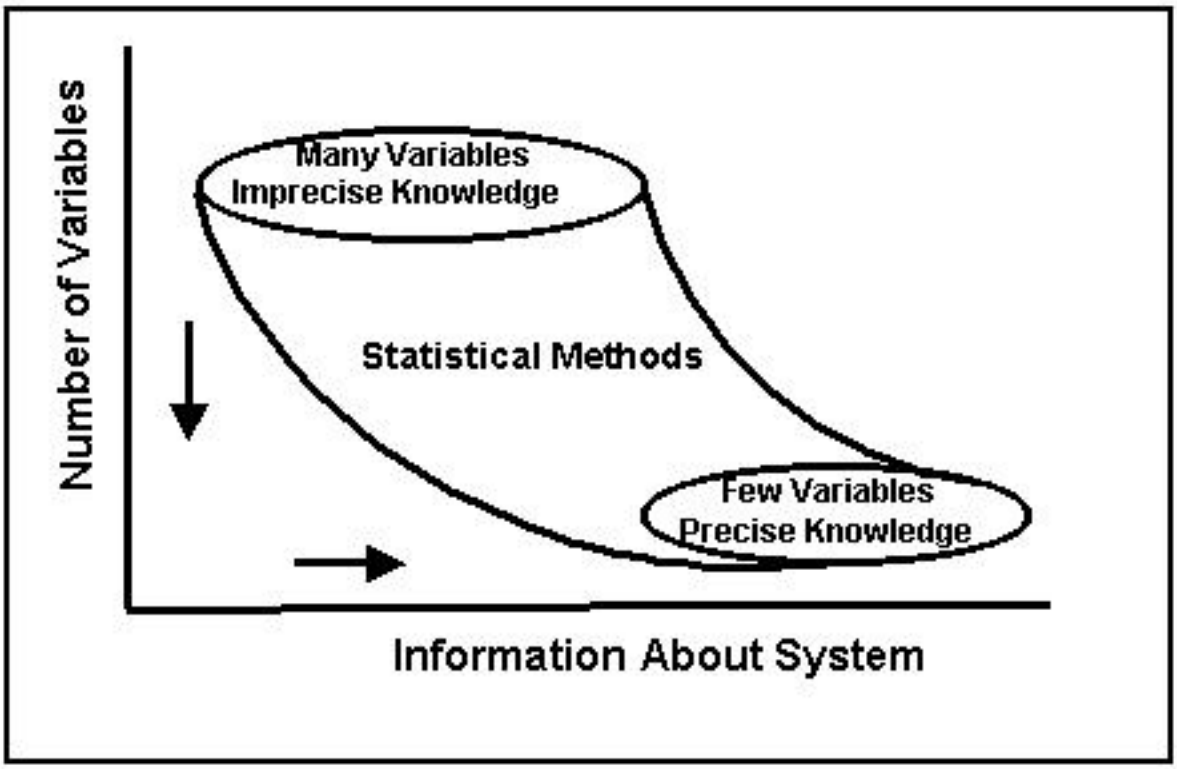


Fig. 2.1. Schematic demonstrating how stastistical optimization approach is useful in optimization process. (Adopted from Haaland, 1989)

2.2.2. *Microorganism, culture conditions and enzyme production*

The alkalotolerant fungal strain was screened and identified as *Fusarium* sp. on the basis of microscopic observations. It is maintained by periodic transfer on potato dextrose agar (0.5% dextrose), slants at 30°C. The cellulolytic nature of the fungus was confirmed by cultivation on CMC agar culture plate and by subsequent staining using Congo red staining method (Teather and Wood, 1982).The fungus was grown in M-1 medium (Reese and Mandels, 1963). The basal M-1 medium contained (in g/l of distilled water) KH_2PO_4, 2.0; $(NH_4)_2SO_4$, 1.4; $CaCl_2$. $2H_2O$, 0.3; $MgSO_4.7H_2O$, 0.3; urea, 0.3; Proteose peptone, 0.25; Yeast extract, 0.2; Cellulose-123, 10.0 and trace metal solution, 1ml [$FeSO_4.7H_2O$, 5 mg/ l ; $MnSO_4.7H_2O$, 5.6 mg/ l ; $ZnSO_4.7H_2O$, 3.34 mg/l; $CoCl_2.2H_2O$, 2 mg/ l], Tween 80, 1ml. The pH of the medium was adjusted to 7.0 after autoclaving by separately sterilized 1M Na_2CO_3.

The M-1 medium supplemented with 1% cellulose powder was inoculated with vegetative mycelia from 7-day-old sporulating slant on PDA. The culture was grown for 3 days and used as the inoculum. Enzyme production was carried out in 250 ml Erlenmeyer flask containing 50 ml M-1 medium with cellulose powder (1%) as the sole carbon source. The culture was incubated at 30°C on a rotary shaker at 200 rpm. The samples were withdrawn at regular intervals. The mycelium was removed by centrifugation at 7000 rpm at 4°C to obtain a clear crude broth. This preparation was used for measurement of enzyme activities. Results given here are the mean of at least duplicate experiments.

2.2.3. *Effect of culture conditions on enzyme production*

The fungus was grown in M-1 basal medium supplemented with different cellulosic substrate at 30°C. The enzyme production and change in pH was monitored for 10 days. The effect of inorganic nitrogen source was studied similarly by replacing ammonium sulphate from the medium by different inorganic nitrogen sources. Effect of initial pH on production was also studied by growing culture at different pH (4-10) at 30°C. To study the time course for enzyme production the fungus was grown in 250 ml flask with 50 ml M1 medium supplemented with 1 % cellulose powder under

submerged conditions. These flasks were incubated on rotary shaker at 200 rpm at 30°C up to 10 days. The samples were removed on alternate days and assayed for CMCase and FPase Activity.

2.2.4. *Enzyme assays*

The activity of endoglucanase (1,4-β-D-glucan-glucanohydrolase, EC 3.2.1.4), xylanase (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8), Filter paper degrading activity (FPase) were measured in terms of release of reducing sugars by 3,5 dinitrosalicylic acid (DNSA) method (Miller et al., 1960). An aliquot (0.5 ml) of appropriately diluted enzyme sample was mixed with 0.5 ml of 1% CMC solution prepared in 0.05 M sodium phosphate buffer, pH 7.0. The reaction mixture was incubated for 30 min at 60°C. The release of reducing sugars was estimated by DNSA reagent. For FPase, 50 mg (1X 6 cm) of Whatman No.1 paper was added to 1 ml of phosphate buffer, pH 7.0 and 1 ml diluted enzyme sample. The reaction mixture was incubated for 1 h and the released reducing sugars were estimated. The β-glucosidase (1,4-β-D-glucan glucohydrolase, EC 3.2.1.21), and β-xylosidase (1,4-β-D-xylan xylohydrolase, EC 3.2.1.37) activities were determined using synthetic substrates *P*-NPG, *P*-NPX, respectively at pH- 6.0 and 50°C (Lachke,1988). The various buffers used for observing effect of pH on the enzyme activity were 50 mM citrate buffer (pH 3-6), sodium phosphate buffer (pH 7- 8) and glycine-NaOH buffer (pH 8.5 to 10). For the determination of pH stability of the enzyme, it was pretreated in buffers of different pH for 10 h at 50°C and assayed at 60°C at pH 7.0.

1 IU of enzyme activity is defined as the amount of enzyme that liberates 1μmole glucose/min (for endoglucanase and FPase) or xylose/min (for xylanases) under the standard assay conditions. 1 IU of β -glucosidase and β-xylosidase has been defined as the amount of enzyme, that liberates 1μmole of *P* -nitrophenol/min under the standard assay conditions.

2.2.5. Factorial design and analysis of results

The optimal concentration of selected media components from above experiments were determined using statistical experimental design. Two level fractional

factorial designs in five variables mentioned above and their concentrations were constructed. The experimental data obtained from a two-level factorial design were fitted to equation (1) that follows polynomial expression for activity *A*.

$$A = \alpha_0 + \alpha_1 x_1 + \alpha_2 x_2 + \alpha_3 x_3 + \alpha_4 x_4 + \alpha_5 x_5 + \alpha_{12} x_1 x_2 + \alpha_{13} x_1 x_3 + \alpha_{14} x_1 x_4 + \alpha_{15} x_1 x_5 + \alpha_{23} x_2 x_3 + \alpha_{24} x_2 x_4 + \alpha_{25} x_2 x_5 + \alpha_{34} x_3 x_4 + \alpha_{35} x_3 x_5 + \alpha_{45} x_4 x_5 \quad (1)$$

Here the α_{ij}'s are fitted constants and x_1, x_2, x_3, x_4 and x_5 are the concentrations of cellulose powder, ammonium sulphate, yeast extract, urea and Tween 80 which are defined as

$$x_i = \frac{2C_i - C_i^h - C_i^l}{C_i^h - C_i^l}, \qquad i = 1,2,\mathrm{K},5, \quad (2)$$

where C^l and C^h are the low and high concentrations of the variables. The activities *A* were experimentally obtained for 16 different combinations of the variables taking either high (*h*) or low (*l*) values as shown in Table 2.6. After transforming the variables by Eq. (2) and substituting in Eq. (1) we solve for the polynomial coefficients α_{ij}. representing the activities in matrix notation A = F(x) a , where F(X) is the polynomial in x_i and a is the vector of polynomial coefficients. The polynomial coefficients are obtained by a = A F(X)$^{-1}$ where the matrix inverse can be computed by Singular Value Decomposition (SVD) (Press et al., 1992). Let, F (X) = U W V^T be SVD of matrix F(X) and its inverse is given by F(X)$^{-1}$ = V [diag (1/W$_i$)] U^T. Using the polynomial coefficients in Eq. (1) and by varying any two variables at a time while keeping others fixed at either their low or high values contour plots indicating regions of activity were obtained. Theoretically predicted activities are in good agreement with the

experimental observations. The combination of the variables leading to optimum (maximum) yield could be systematically inferred by carrying out planned experiments.

2.3. Results and Discussion

2.3.1. Characteristics of the fungal isolate

On the basis of morphology and microscopic observations the fungus was identified as an alkalotolerent *Fusarium* sp. (Fig. 2.2. A, B). The mycelium is septate, and extensively cottony with some tinge of purple colour. Conidiophore is variable and short branched. Conidia are hyaline, variable and principally of two types. Microconidia are slightly curved and ovoid shaped. Macroconidia are large, several celled and typically canoe shaped. The cultural studies showed that the fungus grows best at 30°C. However, the optimum growth was observed around pH 7.0. The culture grows luxuriously at pH 10, indicating its alkalotolerant nature. The cellulolytic nature of the culture was also confirmed by clearance zone formation on CMC agar plate (Fig.2.2C).

2.3.2. Effect of different cultural conditions on enzyme production

The first step in the process optimization is to identify the input variables that have the greatest influence on the response. Such variables were identified by varying one factor each time under shake flask conditions. The optimum temperature and pH for growth of the fungus was 30°C and 7.0 respectively. The effect of initial pH on the production medium showed that there is a difference in the growth and yield of enzyme (Table 2.1). The fungus grew well around pH 7 and produced high amounts of enzyme in neutral to alkaline medium confirming alkalotolerant nature of the fungal isolate. The production of cellulase was compared when the fungus was grown on various cellulosic substrates (Table 2.2). The highest amount of cellulase activities were induced on refined cellulosic substrates such as Cellulose 123, Avicel PH 101, Solka floc SW40. These substrates were superior to cotton, CM cellulose for production of cellulases. The enzyme can also be produced on agricultural residues like bagasse pith, corncob, and wheat bran. However yields obtained were moderate as compared to refined cellulosic substrates (Table 2.2).

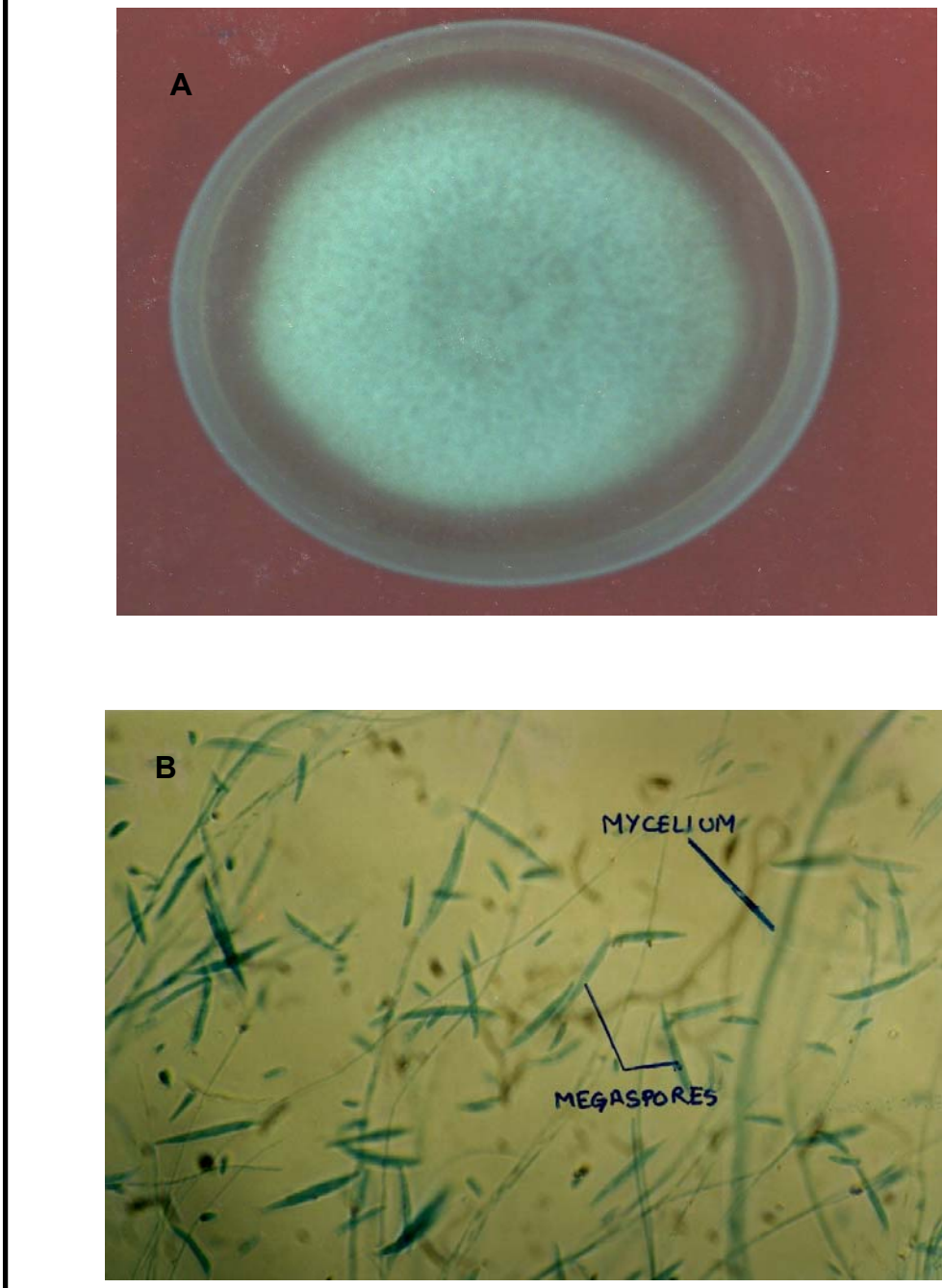


Fig. 2.2.
A : The alkalotolerent fungus *Fusarium* sp growing at alkaline pH

B : Microscopic view of the alkalotolerent *Fusarium* sp
Two different types of conidia (Macroconidia, Microconidia) are

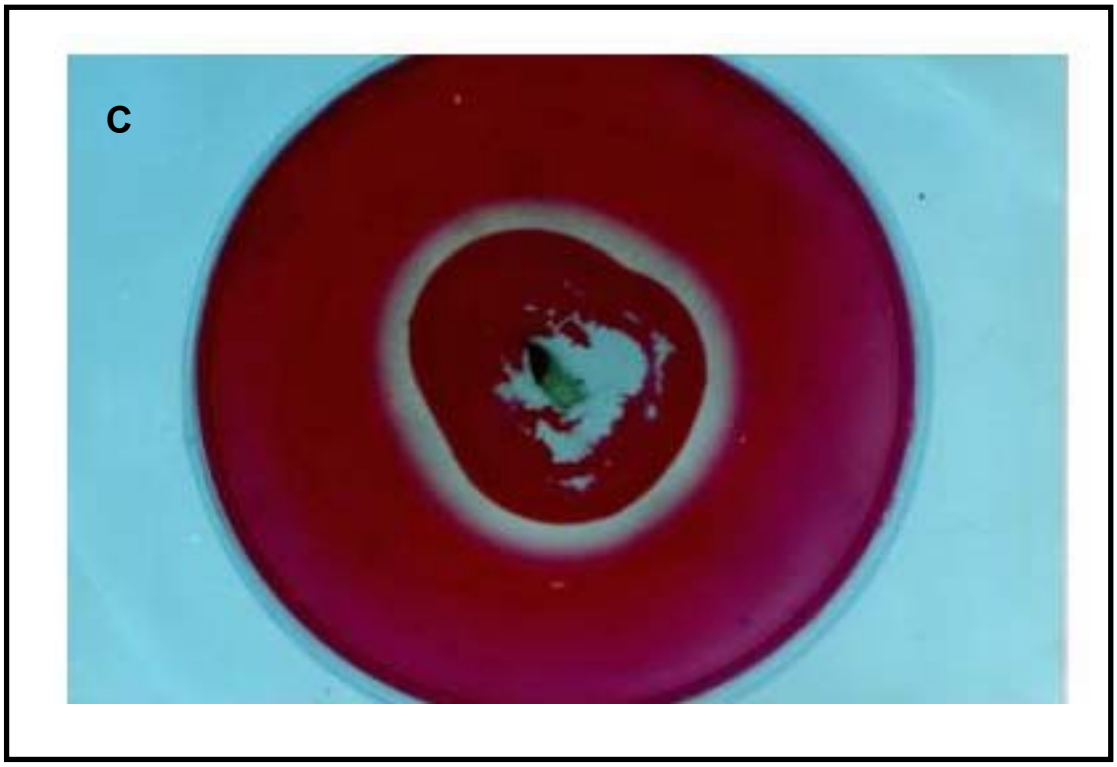


Fig.2.2.C. The zone of clearance produced by alkalotolerent *Fusarium* sp

The fungus was grown on CMC agar plate for 4 days. The plates were flooded with 0.1% Congo red solution for 15-20 min. The plates were washed 2 times with 5M NaCl solutions. Congo red forms red coloured complex with CMC.The zone of clearance appeared as CMC was hydrolysed with cellulases produced during fungal growth.

Table 2.1.Effect of initial pH on enzyme production by alkalotolerent *Fusarium* sp

Initial pH	Growth	Final pH	CMCase (IU/ml)	Fpase (FPU/ml)
4.0	+	4.0	4.1	0.18
5.0	+	5.0	5.8	0.28
6.0	++	5.0	5.8	0.28
7.0	+++	5.0	6.7	0.42
8.0	++	5.0	7.2	0.47
9.0	++	5.5	7.0	0.42
10.0	++	6.0	4.9	0.19

Table 2.2. Effect of different cellulosic substrates on production of cellulases by alkalotolerent *Fusarium* sp

Cellulose Substrates (1 %)	Final pH	CMCase (IU/ml)	FPase (FPU/ml)
Cellulose powder	5.0	8.4	0.59
Avicel	5.5	7.5	0.51
Solka floc (SW 40)	5.0	7.0	0.48
Filter paper	5.0	6.5	0.38
Roll milled cotton	5.0	5.9	0.39
CMC	6.5	5.3	0.49
Wheat bran	8.0	6.9	0.32
Rice bran	6.5	5.9	0.32
Corn cob	7.0	6.4	0.41
Bagasse pith	6.5	7.0	0.32
Jute powder	7.0	5.2	0.32

Samples were withdrawn on 8th day and assayed at pH 7.0 at 60 $^{\circ}$C

Previous workers reported that cellulases are inducible enzymes (Kubicek, 1992,1993). They can be induced with different inducers such as cellobiose, lactose and sophorose (Mandels and Reese, 1960; Mandels et al., 1962). However, most of the microorganisms have produced highest level of cellulases when grown on cellulose (Ryu and Mandels, 1980). Other researchers reported that refined cellulosic substrates such as Solka floc (Hayward et al., 2000), Avicel (Aiello et al., 1996) are better substrates for cellulase production than agricultural residues. This may be attributed to higher lignin content in agricultural residues affecting the cellulase production. (Aiello et al., 1996; Bigelow and Wyman, 2002). Various researchers have shown that different organic and inorganic nitrogen sources such as yeast extract (Ganguly and Mukherjee, 1995), Soyameal (Gomes et al., 2000) and corn steep liquor (Hayward et al., 2000) influence the cellulase production. Experiments regarding effect of various nitrogen sources on enzyme production demonstrated that there is substantial increase in the enzyme activity when the medium is supplemented with complex nitrogen sources like yeast extract and urea (Table 2.3).

Table 2.3.Effect of inorganic and organic nitrogen sources on enzyme production by alkalotolerent *Fusarium* sp

Nitrogen source (g/l)	Final pH	CMCase (lU/ml)	FPase (FPU/ml)
$(NH_4)_2 SO_4$ (1.4)	4.0	7.1	0.45
$(NH_4)_2 HPO_4$ (1.4)	5.0	7.0	0.41
KNO_3 (2.3)	8.5	4.6	0.17
$NaNO_3$ (2.8)	8.5	4.2	0.18
$(NH_4) NO_3$ (1.8)	5.0	5.0	0.23
NH_4Cl (2.0)	5.0	4.8	0.21
Protease peptone (0.25)	5.0	7.2	0.47
Yeast extract (0.25)	5.0	8.3	0.47
Soya meal (0.25)	5.5	6.2	0.36
Urea (0.30)	8.0	8.0	0.52

Samples were withdrawn on 8th day and assayed at pH 7.0 at 60°C

Amongst inorganic nitrogen sources, $(NH_4)_2SO_4$ and $(NH_4)_2HPO_4$ were suitable inorganic nitrogen source for enzyme production (Table 2.3). According to study carried out by Domingues et al. 2000, Tween 80 influences the morphology of *Trichoderma reesei Rut* C-30 as well as the enzyme production. Sukan et al., 1989 demonstrated that emulsification with Tween 80 led to higher cellulase activities presumably by causing increased permeability of cell membranes and/or by promoting the release of cell-bound enzymes. The effect of the addition of surfactant to alkalotolerent *Fusarium* sp is presented in Table 2.4.The results suggested that addition of surfactant (0.1%, v/v) was essential in order to facilitate the release of cellulases in the medium. Tween 80 was found to be the best surfactant in this regard (Table 2.4).

Table 2.4. Effect of various surfactants on cellulase production by alkalotolerent *Fusarium* sp

Surfactant (% V/V)	Final pH	CMCase (IU/ml)	FPase (FPU /ml)
Tween 20 (0.1)	5.3	3.32	0.26
Tween 40 (0.1)	5.0	6.10	0.41
Tween 80 (0.1)	4.8	8.04	0.45
Triton X- 100 (0.1)	6.2	4.25	0.18
None	5.5	0.17	0.07

2.3.3. **Production profile for cellulase production**

Production profiles for cellulases production by alkalotolerent *Fusarium* sp. reveals that onset of activity in the fermentation broth starts on 2nd day onwards. The maximum enzyme production obtained on 8th day while it remains constant thereafter with slight decrease after 10th day (Fig.2.3). The enzyme production is associated with drop in pH as observed previously by different workers in other cellulolytic microorganism. The crude culture filtrate shows different cellulolytic and xylanolytic activities (Table 2.5). However, the yields of these activities vary according to media composition.

Table 2.5. Enzyme activities in the culture filtrate

ACTIVITY	IU / ml
CMCase [Endo (1--4)β-D-glucan-glucano hydrolase (EC 3.2.1.4)]	8.80
FPase (Filter paper degrading activity)	0.52
β Glucosidase [β- D-Glucan glucohydrolase (EC 3.2.1.21)]	1.31
β- D-Xylanase [β-D-Xylan xylanohydrolase, (EC 3.2.1.8)]	0.72
β- D-Xylosidase [β-1,4-D-Xylan xylohydrolase (EC3.2.1.37)]	0.32
Protease	N.D.

The fungus was grown in M-1 medium as described in the text. Samples were withdrawn on 8th day and assayed for different activities.

N.D. = Not detected.

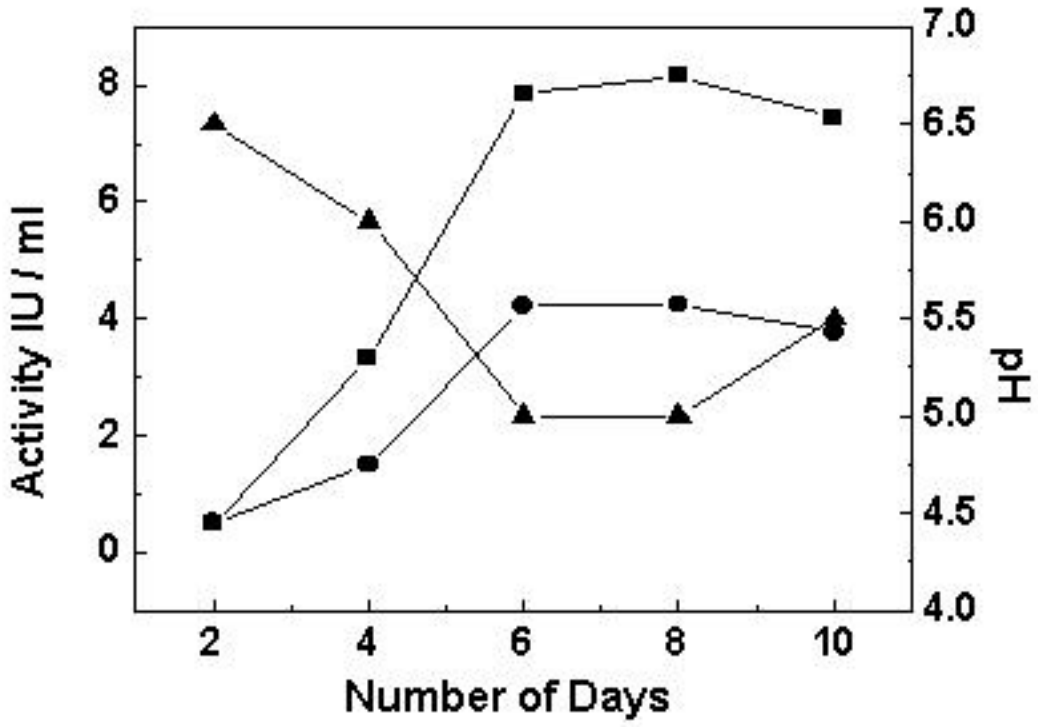


Fig.2.3. Cellulase production by alkalotolerent *Fusarium* sp

Symbols: CMCase activity (■), FPase activity [(●) X 10], pH (▲)

2.3.3. Enzyme properties

The refined enzyme preparation was active in a broad pH range of 4 to 10 with pH optima at 5.0 at 60°C. The enzyme is stable in an alkaline pH range of 8 -10 at 60°C (Fig.2.4). The half-life of the enzyme at pH 8.5 at 50°C was found to be 10 h. The enzyme showed maximum activity at 60°C and retained 80% of the maximum enzyme activity at 70°C (Fig.2.5). Microbial cellulases are generally active in acidic to neutral pH range (Durand et al., 1984). Kang and Rhee (1995), reported alkaline active carboxymethyl cellulases from a *Cephalosporium* strain. *Humicola* sp has been commercially exploited for the production of alkaline cellulases. These endoglucanases are optimally active in a broad pH range of 6-10 but their thermal stability in the alkaline pH range is low (Schülein, 1997). Although certain strains of *Trichoderma* sp and *Penicillium* sp produce high amounts of cellulases, their activities at alkaline pH are negligible (Beldman et al., 1985). The extracellular enzyme produced by alkalotolerant *Fusarium* strain reported in this work was active and stable under alkaline conditions indicating their potential for commercial use.

2.3.4. Factorial design

Fractional factorial experiment was designed with five significant variables namely (1) cellulose 123 (2) ammonium sulphate (3) yeast extract (4) urea and (5) Tween 80 obtained from initial optimization studies. The concentration levels of these variables were taken in a broad range as described in (Table 2.6). The results obtained from first factorial design showed that all variables had significant effects on enzyme production with cellulose powder and yeast extract concentrations having maximum effect (Table 2.7). Increasing the cellulose powder in the basal medium towards higher concentration level (2.5%) increased enzyme yields. However, the lower concentration levels of yeast extract, urea, Tween 80 were preferred for higher enzyme production. Fig.2.6 demonstrates the contour plots for cellulase production (level 1). Each display consists of lines of equal value of the response in terms of CMCase production. The pattern of contour shows how response changes as a function of two media components. Two different media components varied along with X and Y axis at the same time the other variables are kept constant to their minimal or maximum concentrations (for variable codes and media concentrations refer Table 2.6)

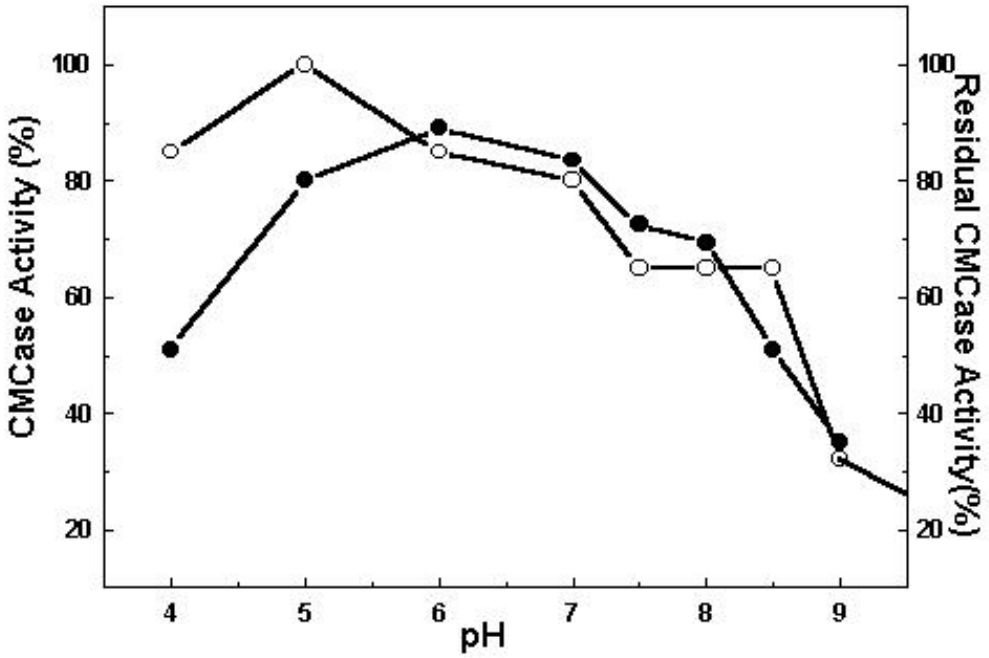


Fig.2.4. Effect of pH on activity (○) and stability (●) of CMCase from alkalotolerent *Fusarium* sp at 60 °C

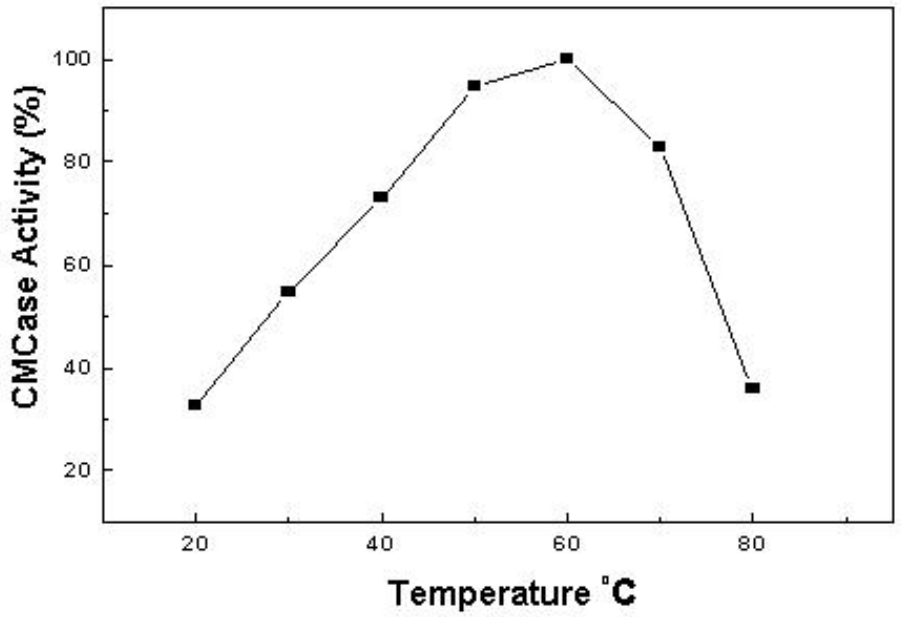


Fig.2.5.Effect of temperature on CMCase activity from alkalotolerent *Fusarium* sp

Table 2.6. Different variable codes and their concentration levels used in fractional factorial designs

Variables	Level 1		Level 2	
	Low (-)	High (+)	Low (-)	High (+)
X_1: Cellulose	0.25	2.5	2.5	5.0
X_2 : Ammonium sulphate	0.1	1.0	0.01	0.1
X_3 : Yeast Extract	0.1	1.0	0.01	0.1
X_4 : Urea	0.1	1.0	0.01	0.1
X_5 : Tween 80	0.05	0.25	0.01	0.05

All the values are expressed in % W/V, However for X_5.values are given in V / V %.

When experiments were conducted in the direction of steepest ascent obtained from 1st factorial data, there was further increase in the activity. Hence the same experimental design was followed by varying the concentration levels of each variable (Table 2.6). The trends for optimum concentration of variables for maximum cellulase activity were reversed. For example, when the concentration of cellulose powder decreased towards lower level values i.e. (2.5 %) and concentrations of other variables such as yeast extract, urea, and Tween 80 were increased in the second design, higher enzyme yields were obtained. The predicted activity through mathematical model is presented by response surface plot in the Fig.2.7. The surface plot display, three-dimensional representation of the relationship between the response i.e. cellulase production and the experimental media variables X_3 –X_4. In this plot Response (CMCase Activity in IU/ml) is plotted at vertical axis and the concentrations of the 2 different variables each time are at horizontal X and Y axis. Other variable are kept constant to their low (-) values. The response surface is generated by

Table 2.7. Experimental results of first fractional factorial design for cellulase production (level 1)

Flask no.	X_1	X_2	X_3	X_4	X_5	CMCase Activity (IU/ml)
1	-	-	-	-	-	1.02
2	-	-	-	+	+	0.22
3	-	-	+	-	+	0.22
4	-	-	+	+	-	0.29
5	-	+	-	-	+	1.57
6	-	+	-	+	-	0.92
7	-	+	+	-	-	0.46
8	-	+	+	+	+	0.44
9	+	-	-	-	-	18.52
10	+	-	-	+	+	0.29
11	+	-	+	-	+	0.09
12	+	-	+	+	-	0.31
13	+	+	-	-	+	18.14
14	+	+	-	+	-	0.44
15	+	+	+	-	-	0.15
16	+	+	+	+	+	0.18

Samples were withdrawn on 8th day and assayed in 0.05M Na phosphate buffer at pH 7.0 at 60°C.

mathematical model and that estimates true functional relationship between response and experimental factors. The highest enzyme activity is represented by lighter shade whereas negative effect of variable concentration on activity is denoted by darker shade. Similar plots can be generated by taking different media variables and their response in terms of enzyme production.

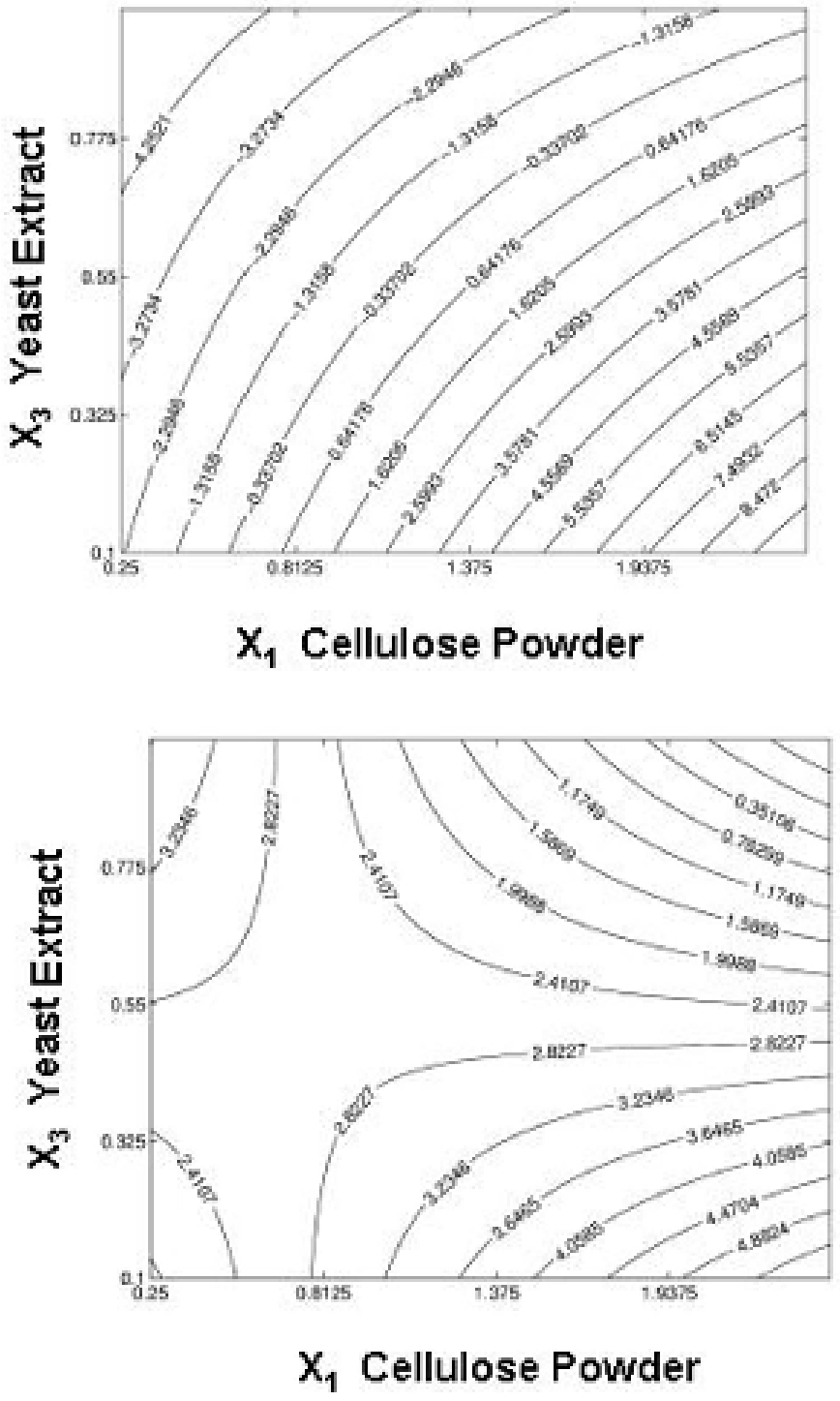


Fig.2.6. Contour plots

Response of cellulase production to variation in cellulose (X_1) and yeast extract (X_3) concentrations when the coded variables for ammonium sulphate (X_2), urea (X_4) and Tween 80 (X_5) were kept constant to low (A) and high (B) concentrations. The display consists of lines of equal value of the response in terms of CMCase production. The contour plot shows how the response changes as a function of two media components.

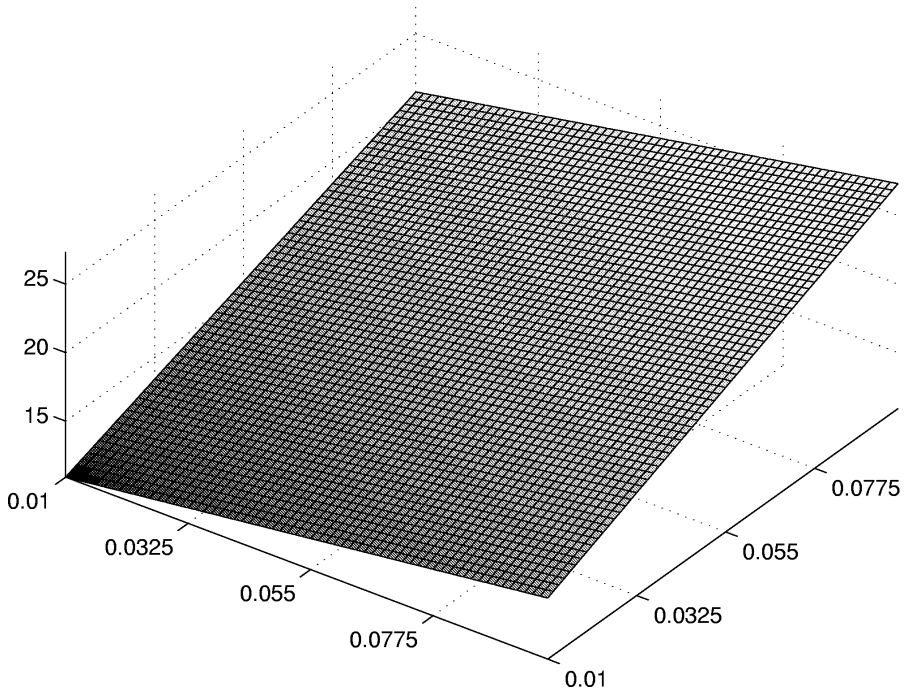


Figure 2.7. Response surface plot for prediction of enzyme yields for level II.

Other variables, Cellulose powder (X_1), Ammonium sulphate (X_2) and Tween 80 (X_5) were kept constant at their low concentrations values.

Table 2.8. Results of second fractional factorial design for cellulase production (level 2)

Flask no.	X_1	X_2	X_3	X_4	X_5	Predicted CMCase Activity (IU/ml)	Actual CMCase Activity (IU/ml)
1	-	-	-	-	-	2.40	2.22
2	-	-	-	+	+	23.2	24.07
3	-	-	+	-	+	6.31	4.62
4	-	-	+	+	+	26.63	27.77
5	-	+	-	-	+	0.71	1.85
6	-	+	-	+	-	9.46	7.77
7	-	+	+	-	-	0.55	1.29
8	-	+	+	+	+	18.69	18.51
9	+	-	-	-	-	1.10	1.29
10	+	-	-	+	+	3.70	2.96
11	+	-	+	-	+	-0.76	0.92
12	+	-	+	+	-	7.79	6.66
13	+	+	-	-	+	1.96	0.83
14	+	+	-	+	-	-1.04	0.65
15	+	+	+	-	-	2.59	1.85
16	+	+	+	+	+	2.40	2.59

Samples were withdrawn on 8th day and assayed in 0.05M Sodium phosphate buffer at pH 7.0 and at temperature 60°C.

Thus the concentrations of the key variables in the optimized production medium obtained from the factorial design were as follows (g/l): cellulose powder, 25; ammonium sulphate,0.1;yeast extract, 1.0; urea 1.0 and Tween 80 0.05 % v/v. Theoretically predicted activities were calculated as described in material methods above. The results obtained for level 2 are in good agreement with the experimentally obtained enzyme yields (Table 2.8). The results obtained through fractional factorial design were confirmed in 1l-shake flasks. The maximum CMCase activity obtained on 8^{th} day was 28-30 IU/ml that is four fold higher as compared with enzyme yields obtained in basal medium (Table 2.9).

Table 2.9. Comparison of basal medium Vs. optimized medium composition for production of alkaline active fungal cellulases by alkalotolerent *Fusarium* sp

Media components	**Reese basal medium (g / l)**	**Optimized production medium (g / l)**
KH_2PO_4	2.0	2.0
$(NH_4)_2SO_4$	1.4	0.1
$CaCl_2.2H_2O$	0.3	0.3
$MgSO_4.7H_2O$	0.3	0.3
Urea	0.3	1.0
Peptone	0.25	0.25
Yeast extract	0.2	1.0
Tween 80	1.0	0.05
Mineral Metal Solution	1.0 ml	1.0 ml
Cellulose-123 Powder	1%	2.5 %
CMCase Activity	**7- 8 IU/ml**	**28 - 30 IU/ml**

2.4. Conclusion

Microbial cellulases are generally active in acidic to neutral pH range. The enzyme preparation obtained in the present studies was found to be alkaline active and alkali stable indicating their potential for different industrial process. Different approaches such as cloning and over expression of cellulase genes, mutagenesis, solid state cultivation can be followed in order to increase the enzyme yields. However these techniques suffers from limitation such as complexity, use of hazardous chemical agents and scale up difficulties respectively. The systematic optimization of media components with the help of statistical factorial design is demonstrated in this Chapter. The optimally designed media resulted in 4 fold enhanced production of industrially important cellulases under submerged fermentation conditions.

Chapter 3

Enzyme Purification and Characterization

Summary

The enzyme Endoglucanase A (1,4-β-D glucan-4glucanohydrolase (EC 3.2.1.4) was purified to homogeneity from the culture filtrate of alkalotolerent *Fusarium* sp. The enzyme was purified 13 fold by ultrafiltration (PM-10), gel filtration chromatography on BioGel P-100 matrix and preparative isoelectric focusing. The molecular weight of the enzyme was determined as 37.8 KD by gel filtration and 45 KD on SDS PAGE respectively. The pI of enzyme was determined as 4.5.Endoglucanase A is active in a broad pH range of 4-9 with pH and temperature optima at 5.0 and 60°C, respectively. The enzyme is stable in alkaline pH range (7-9) at 50-60°C.The thermostability of enzyme was studied in the temperature range of 50-70°C. Endoglucanase A shows half-life of 1.5 h at 70°C. Differential scanning calorimetric studies indicated that the enzyme unfolds in 2 different stages. The thermostability of enzyme is improved in the presence of different sugar alcohols.Trehalose was found to be best in protecting enzyme from thermal denaturation. Km and Vmax values for Endoglucanase A at optimum conditions were found to be 2.0 mg/ml and 641 μmoles/min/mg, respectively. The enzyme action is unaffected in the presence of various metal ions. However, Hg^{2+} at 10 mM concentration completely inhibited enzyme action.

3.1. Introduction

Due to complex structure of cellulosic substrate, its efficient degradation requires presence of different enzymes in a typical cellulolytic enzyme complex. Hydrolytic enzymes such as endoglucanase [1,4-β-D glucan glucanohydrolase; (EC 3.2.1.4)], cellobiohydrolase (CBH) [1,4-β-D glucan cellobiohydrolase; (EC 3.2.1.91)], 1,4-β-D-glucan glucohydrolase (EC 3.2.1.74) and β-glucosidase [1,4-β-D glucoside glucohydrolase, (EC 3.2.1.21)] are involved in degradation of crystalline cellulose to glucose. Endoglucanases are important components of the system, as the enzyme action is generally initiated by random acting endoglucanases at amorphous regions within cellulose chain to produce cellooligosaccharides. Till date, significant progress is seen in purification, characterization of different cellulase components and in understanding their substrate specificities and mode of action (Berghem and Pettersson, 1973; Berghem et al., 1976; Hurst et al., 1977; Hong et al., 1986; Lachke et al., 1987; Sahasrabudhe et al., 1987, Bhat et al., 1989,1990; Claeyssens and Aerts, 1992; Schülein, 1997).

Fungal cellulase preparations that are rich in alkaline active and /or alkali stable endoglucanase components are desired for their applications in detergents and fabric finishing (Barbesgaard et al., 1984; Schülein et al., 1997). Work described in the previous Chapter demonstrated that the alkalotolerent *Fusarium* strain secrets high amounts of cellulases under submerged fermentation conditions. The preliminary studies suggested that the enzyme preparation possess desired properties for their industrial applications. The major endoglucanase component (Endoglucanase A) is purified 13 fold by ultrafiltration (PM-10), gel filtration chromatography on BioGel P-100 matrix and preparative isoelectric focusing. The purified endoglucanase A is homogeneous as revealed by single protein band on 7.5 % polyacrylamide gel. The purified enzyme showed a specific activity of 23.3 IU/mg of protein and is free from β glucosidase activity. The purpose of the work was to understand of the optimal conditions of Endoglucanase A from alkalotolerent *Fusarium* sp. The physicochemical characteristics of Endoglucanase A such as its molecular weight, pI, optimum pH, optimum temperature, Km and Vmax and their stabilities at different pH and temperatures are presented in this Chapter.

3.2. Materials and Methods

3.2.1. Chemicals

Following materials were purchased from the suppliers indicated: Hydroxyparabenzoic acid hydrazide (Fluka AG, Switzerland); BioGel P-100, Biolytes (electrolyte carrier pH 3-10) Bio-Rad Laboratories, Richmond, CA, U.S.A., sodium salt of carboxymethyl cellulose (medium viscosity), bovine serum albumin, molecular weight standards for gel filtration; alcohol dehydrogenase, bovine serum albumin, ovalbumin, carbonic anhydrase, cytochrome C, Comassie brilliant blue R-250,Sugar alcohols such as sorbitol, mannitol, xylitol and trehalose (Sigma Chemical Co. USA). Molecular weight markers for SDS PAGE (Low molecular weight kit no.17-0446-01, Amersham Biosciences). Acrylamide and N, N, N', N' Tetramethylethylenediamine (Eastman Kodak Co., USA) and N, N'-methylene bis acrylamide (Eastman Organic Chemicals, USA). All the buffer salts and microbial media components were procured from the standard commercial sources and of highest quality available.

3.2.2. Enzyme production

The enzyme production was carried out in 1000 ml Erlenmeyer flask as described in Chapter 2.The mycelium was removed by centrifugation at 7000 rpm at 4°C to obtain a clear crude broth. This preparation was used for measurement of enzyme activities.

3.2.3. Enzyme assays

The endoglucanase was measured in terms of release of reducing sugars by 3,5 dinitrosalicylic acid (DNSA) method (Miller et al., 1960). An aliquot (0.5 ml) of appropriately diluted enzyme sample was mixed with 0.5 ml of 1% CMC solution prepared in 0.05 M sodium phosphate buffer, pH 7.0. The reaction mixture was incubated for 30 min at 60°C. The release of reducing sugars was estimated by DNSA reagent. The β-glucosidase activity was determined using synthetic substrate *P*-NPG, at pH 6.0 and 50°C (Sadana et al., 1988).

1 IU of enzyme activity is defined as the amount of enzyme that liberates 1μmole glucose/min (for endoglucanase) under the standard assay conditions.

3.2.4. Protein content

Protein concentration was measured using bovine serum albumin standard as described by Lowry et al. (1951).

3.2.5. Purification of Endoglucanase A

3.2.5.1. Gel filtration on BioGel P-100

After harvesting mycelia by centrifugation at 7000 rpm for 10 min, the clear supernatant was used as crude cellulase extract. This crude extract was concentrated in Amicon ultrafiltration unit using PM 10 membrane (molecular weight cut off 10,000D). The enzyme concentrate was stored at 4°C until further use.

A column (160 X 1cm) of BioGel P-100 was equilibrated at 4°C with 0.05M sodium phosphate buffer pH 7.0 The hydrated gels were routinely equilibrated with the same buffer and degassed under vacuum before using. The eluted fractions, each of 2.5 ml, were collected at a flow rate of 10 ml h^{-1} and assayed for protein and /or enzymatic activity.

3.2.5.2. Preparative isoelectric focusing

The Endo A fraction obtained from gel filtration column was concentrated using an Amicon ultrafitration membrane (PM 10) unit. Finally a sample of the concentrated enzyme was purified by using miniscale isoelectric focusing (IEF) unit as described by Sathivel et al. (1995). This unit was previously fabricated in our laboratory and has various advantages such as rapid resolution of proteins, easy collection of protein fractions etc (Sathivel et al., 1994).This assembly of isoelectric focusing unit is demonstrated schematically in Fig.3.1.The IEF was carried out using Biolyte, carrier electrolytes in the pH range of (3-10) at 400 V and for 6-8 h. After electrofocousing is completed each fraction of 0.5 ml was removed from the column and the samples were analyzed for CMCase activity, pH and protein content.

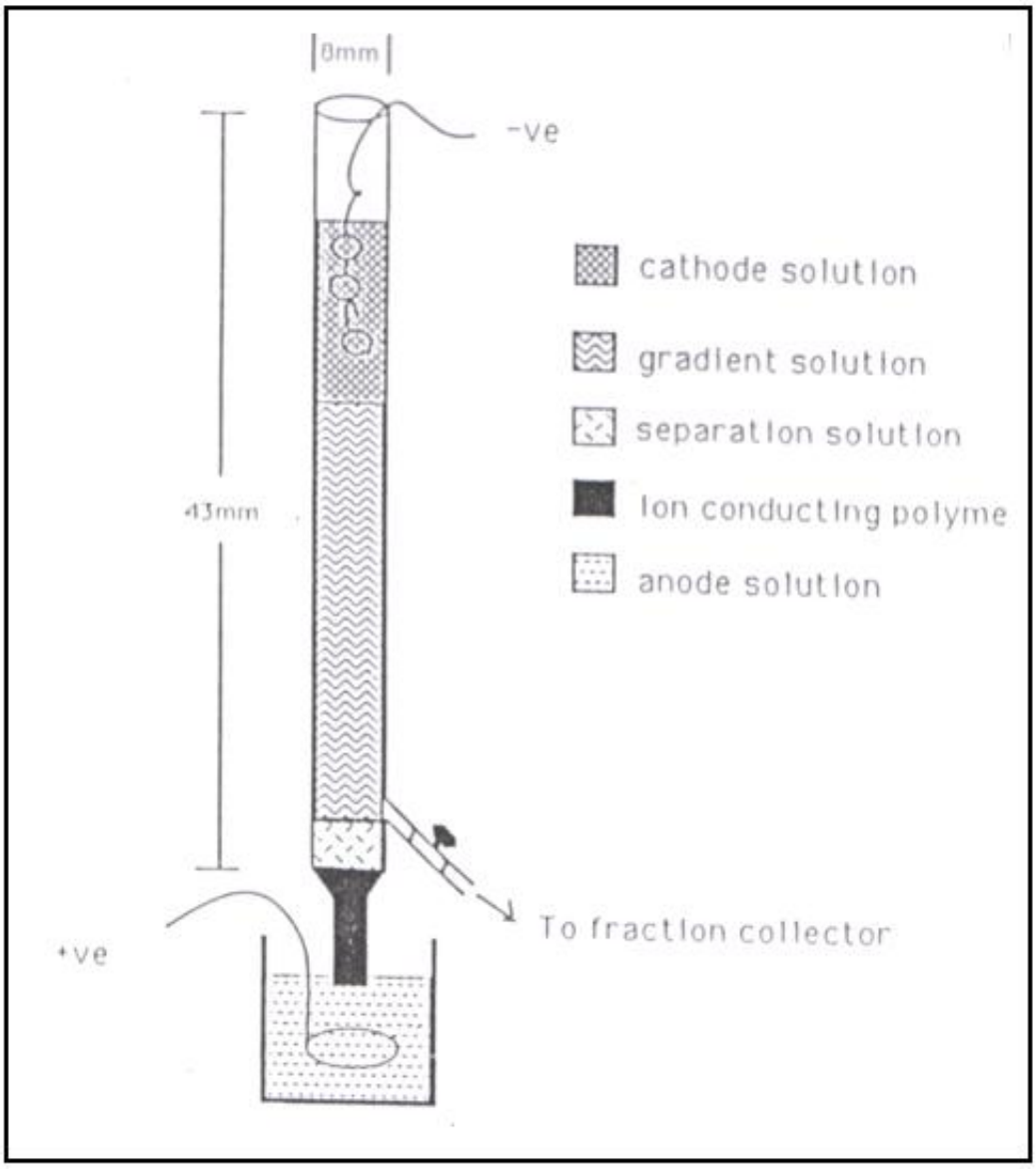


Fig. 3.1. Miniscale Isoelectric Focusing Unit

Light density solution :
5.0 ml Water + 0.2ml Ampholytes (pH 3.0 – 10.0) + 0.2ml Enzyme sample

High density solution :
3.0 ml Glycerol + 2.0 ml Water + 0.2ml Ampholytes (pH 3.0–10.0) + 0.2ml Enzyme sample

Separation solution :
3.0 ml Glycerol + 2.0 ml Water

Electrolyte solutions :
Cathode Solution: 0.1M NaOH
Anode Solution: 0.1M Phosphoric acid

Polyacrylamide gel electrophoresis under non-denaturating conditions was performed on 7.5 % (W/V) acrylamide gel using Tris–glycine buffer pH 8.3 (Weber and Osborn, 1969; Shapiro et al., 1967) and bands were visualized by silver staining (Blum et al., 1987)

3.2.6. *Characterization of Endoglucanase A*

3.2.6.1. Molecular weight determination by gel filtration chromatography

Molecular weight of purified enzyme Endoglucanase A was determined by the molecular sieve chromatography method of Andrews (1964). A column (160 X 1 cm) of BioGel P-100 (50-100 mesh) was equilibrated at 4°C with 0.05M sodium phosphate buffer pH 7.0.The void volume (V_0) of the column was estimated with Blue Dextran (molecular weight approx 2,000,000). The column was calibrated with 5 different marker proteins viz. alcohol dehydrogenase (1,50,000), bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (29,000), cytochrome C (12,400). One milligram each of marker proteins and 500μg of purified Endoglucanase A enzyme were dissolved in 1ml of 0.05M sodium phosphate buffer pH 7.0, and applied to the column. The elution of these proteins was performed with the same buffer at a flow rate of 12 ml/h. Fractions of 3.0 ml each were collected and the proteins were detected in the effluent by measuring the absorbance at 280 nm. The elution volume (Ve) for each protein was calculated from mid point of peaks. The purified Endoglucanase A was detected by assaying CMCase activity as described under materials and methods.

3.2.6.2. Molecular weight determination on SDS –PAGE

Molecular weight of purified Endoglucanase A and its possible subunit structure was estimated by their migration in SDS polyacrylamide gel as described by (Laemmli, 1970). The protein bands were stained by 0.25% Comassie brilliant blue R-250 in methanol: acetic acid: water (40:10:50) and destined with the same mixture without dye. Low molecular weight markers for SDS PAGE were was used as reference proteins. A plot of log molecular weight versus relative mobility of standard proteins was used to estimate molecular weight of Endoglucanase A.

3.2.6.3. Effect of pH on enzyme activity and stability

The optimum pH of the Endoglucanase A was determined by measuring its activity by incubating it at different pH values of the reaction mixtures for 30 min. The various buffers used for observing effect of pH on the enzyme activity were 50 mM citrate buffer (pH 3-6), sodium phosphate buffer (pH 7-8) and glycine-NaOH buffer (pH 8.5 to 10). For the determination of pH stability of the enzyme, it was pretreated in buffers of different pH at 50°C, 60°C for 2 h and assayed at 60°C at pH 7.0.

3.2.6.4. Effect of temperature on enzyme activity and stability

For determining the effect of temperature on Endoglucanase A, enzyme activities were estimated in a temperature range of 30-80°C. The energy of activiation for Endoglucanase A was calculated from Arrhenius plot. The temperature stability of the enzyme was detected by incubating 100μg enzyme at 50-70°C for different time intervals. The samples were removed periodically and assayed for residual CMCase activity under standard assay conditions. The effect of sugar alcohols on thermal stability of Endoglucanase A was monitored by incubating enzyme in the presence of 0.1M sugar alcohols such as sorbitol, mannitol, xylitol and trehalose at 60°C and pH 9.0. The samples were removed at different time intervals and assayed for residual activity at pH 7.0 and 60°C. A computer controlled Differential Scanning Calorimeter (Seiko,Japan)was used to study thermal denaturationof enzyme.100μg Endoglucanase A was slowly heated in the range of 30-90°C at a rate of 1°C.The course of protein unfolding was compared from thermograms obtained in the presence and absence of trehalose.

3.2.6.5. Kinetic constants

Kinetic constants such as Km, Vmax were determined under steady state conditions using various concentration of CMC as a substrates. The enzyme assay was carried out at different pH values (5,7,9) and at 60°C. The formation of reducing sugars in the reaction mixtures was estimated by *p* HBAH method (Hurst et al.,1977). It

is possible to determine the small quantity of the reducing sugars present in the substrate blank due to high sensitivity of the method.

3.2.6.6. Effect of metal ions on enzyme activity

For determining the effect of different metal ions on the CMCase activity of Endoglucanase A, the enzyme (10μg) was incubated with the respective metal ions at pH 7.0 and 50°C. The final concentration was 1mM and/or 10 mM for each metal ion. An aliquot (500μl) was taken after 30 min of incubation for the determination of CMCase activity under standard assay conditions.

3.3. Results and Discussion

***3.3.1**. Purification of Endoglucanase A*

3.3.1.1. Ultrafiltration and Gel filtration on BioGel P-100

After harvesting mycelia by centrifugation at 7000 rpm for 10 min, 100ml clear culture filtrate was concentrated in Amicon ultrafiltration unit with PM 10 membrane. Some of the low molecular weight proteins were separated from crude culture filtrate in this step. The enzyme concentrate showed 1.2 fold specific activity.

The enzyme concentrate from ultrafiltration step was fractionated on a BioGel P-100 column and the elution profile is presented in Fig. 3.2.The data indicated presence of 2 different endoglucanases in the concentrated crude broth. The peak Endo A (fractions 22-31) contained the major, high molecular weight Endoglucanase A. The other low molecular weight Endoglucanase B appears in later fractions (70-76) in Endo B peak and can be conveniently separated from major Endoglucanase A. The fractions from Endo A peak showing higher specific endoglucanase activity were pooled and concentrated in an Amicon ultrafiltration unit. In this step the specific activity of Endoglucanase A increased to 10.3 IU/mg of protein.

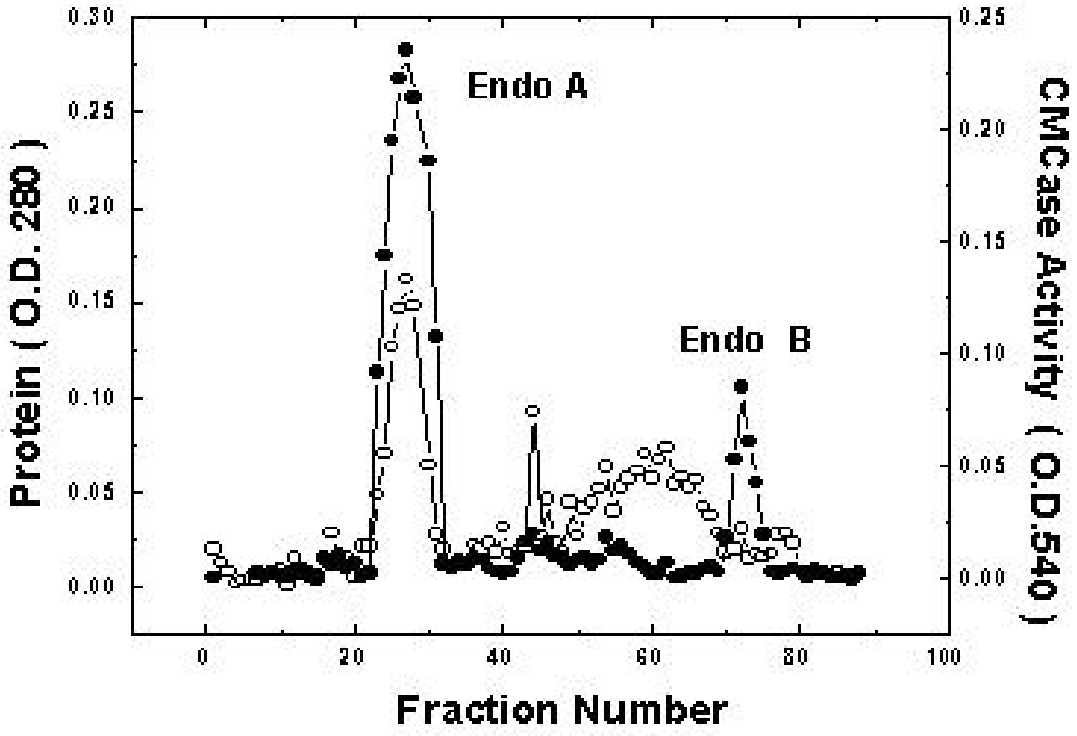


Fig.3.2.Gel filtration on BioGel P-100

Fractionantion of extracellular proteins from alkalotolerant *Fusarium* sp on BioGel P-100 column chromatography. Column dimensions (160 x 1 cm). Buffer 0.05 M sodium-phosphate buffer pH 7.0. Symbols Protein (O.D.280 nm) (o), CMCase activity (•).

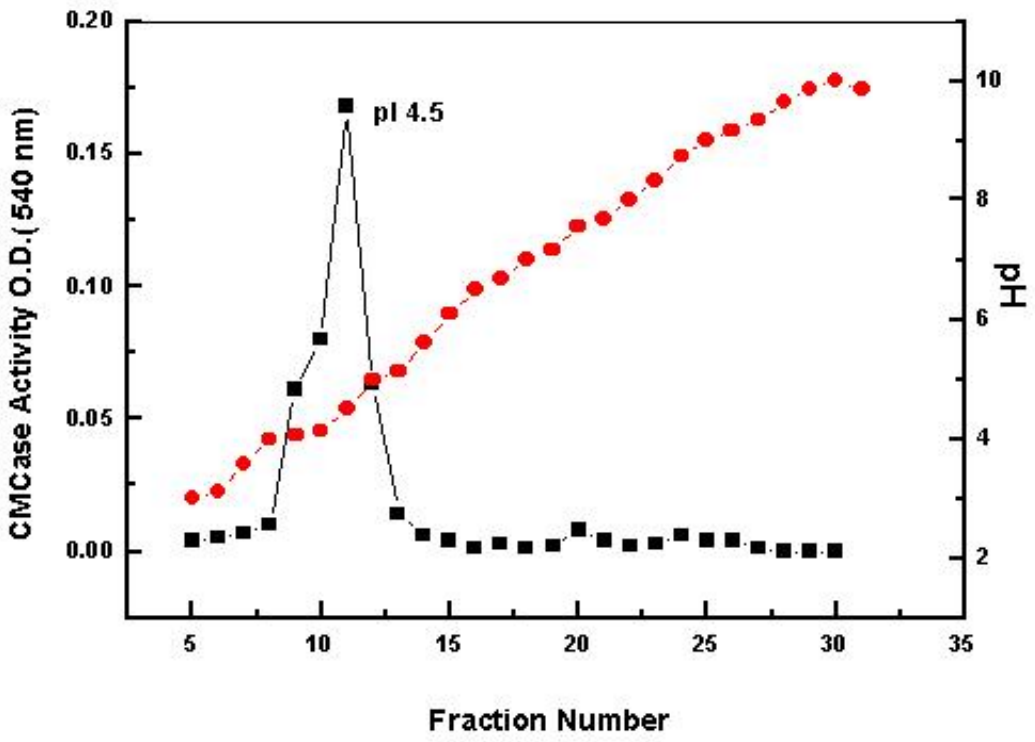


Fig.3.3. Purification of Endoglucanase A by isoelectric focusing

The electro focusing was carried by using ampholyte solution in the range of pH 3-10 at 1% final concentration. Endo A fraction obtained from previous step was dialyzed against 1% glycine for 10 h and concentrated in Amicon ultrafiltration unit .The sample was distributed throught the miniscale isoelectric focusing column.
Symbols: for pH (●) and for CMCase activity (O.D. 540) (■)

3.3.1.2. Preparative isoelectric focusing (IEF)

The pooled Endoglucanase A fraction obtained from gel filtration column was concentrated in an Amicon ultrafitration (PM 10) unit. Finally the concentrated enzyme was purified futher by using miniscale isoelectric focusing (IEF) unit. The glycerol density gradient was used as the stabilizing medium. Isoelectric focusing was carried out for 6 h at 400 volts giving a current of 2 mA. The voltage at the end of the run was 400 V and current was zero. The fraction of 0.5 ml were collected from the side arm of the unit and immediately processed for the determination pH, endoglucanase activity and protein. A single sharp peak around pH 4.5 was observed after isoelectric focusing. The fractions of high specific activity were pooled and dialyzed in a colloidon bag (Sortorius) to make enzyme free from electrolytes and glycerol. The pH and activity profile is presented in Fig.3.3. The purified enzyme thus obtained has the specific activity of 23.3 IU / mg.

The summary of the purification scheme for Endoglucanase A is presented in Table 3.1. The data indicated that the purified Endoglucanase A shows 13 fold higher specific activity with 12 % overall yields. The literature survey on purification of cellulases indicated multiple forms of endoglucanases are produced by many microorganisms (Gum and Brown, 1977; Beldman et al., 1985; Lachke and Deshpande, 1988; Bhat et al., 1989;Schülein M.1997). Its purification to homogeneity requires various steps such as gel filtration chromatography (Christakopoulos et al., 1995, Bhat et al., 1989), ion exchange chromatography (Wood and McCrae, 1972, Beldman et al., 1985), Affinity chromatography on Concanvalin A sepharose (Gong et al., 1979) and on different cellulosic substrates (Shoemaker and Brown, 1978; Schülein M.1997). Other research workers demonstrated application of techniques such as hydrophobic interaction chromatography (Mansfield et al., 1998; Tomaz and Queiroz .2004), preparative gel electrophoresis (Wilson DB, 1988) and Isoelectric focusing in purification of cellulases (Sadana et al, 1984). Recently few unconventional techniques such as fast protein liquid chromatography (Medve et al., 1998), immunoaffinity chromatography (Koivula et al., 1996) have been utilized for purification of cellulases. The most of the purification protocols resulted in purification of homogenous endoglucanase preparation, however yield obtained were low. This is because; multiple steps are needed in order to get rid of contaminating isozymes of cellulases.

3.3.1.3. *The criterion of purity*

The purified enzyme preparation obtained after isoelectric focusing was free of contaminating β glucosidase, and or xylanase activity. The analytical slab gel electrophoresis at pH 8.3, in 7.5 % (W/V) acrylamide gel revealed only one protein band (Fig 3.4).

Table 3.1. Summary of Purification of Endoglucanase A from alkalotolerent *Fusarium* sp

Step	***Total Activity (IU)***	***Total Proteins* (mg)**	***Specific Activity* (IU/mg)**	***Fold* Purification**	***Enzyme* Yield (%)**
Culture filtrate	700	380	1.8	---------	100
PM 10	680	309	2.2	1.2	97
BioGel P-100	440	40.7	10.8	6.0	62.8
IEF	84	3.60	23.3	13	12

3.3.2. *Characterization of Endoglucanase A*

3.3.2.1. Molecular weight determination by gel filtration chromatography

Molecular weight of purified enzyme Endoglucanase A was determined by the molecular sieve chromatography method on BioGel P-100 column. The void volume (V_0) and the elution volume (Ve) for each protein were estimated as described in materials and methods. The plot of Ve/V_0 against logarithm of molecular weight gave a straight line (Fig.3.5). The molecular weight of Endoglucanase A was found to be 37.5 KD by this method.

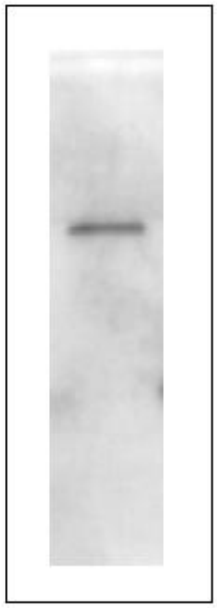


Fig.3.4. Analytical polyacrylamide gel electrophoresis of Endoglucanase A.

(Native PAGE: 7.5% (w/v) polyacrylamide gel, Tris–glycine buffer at pH 8.3, Current 16mA, protein loaded 5μg)

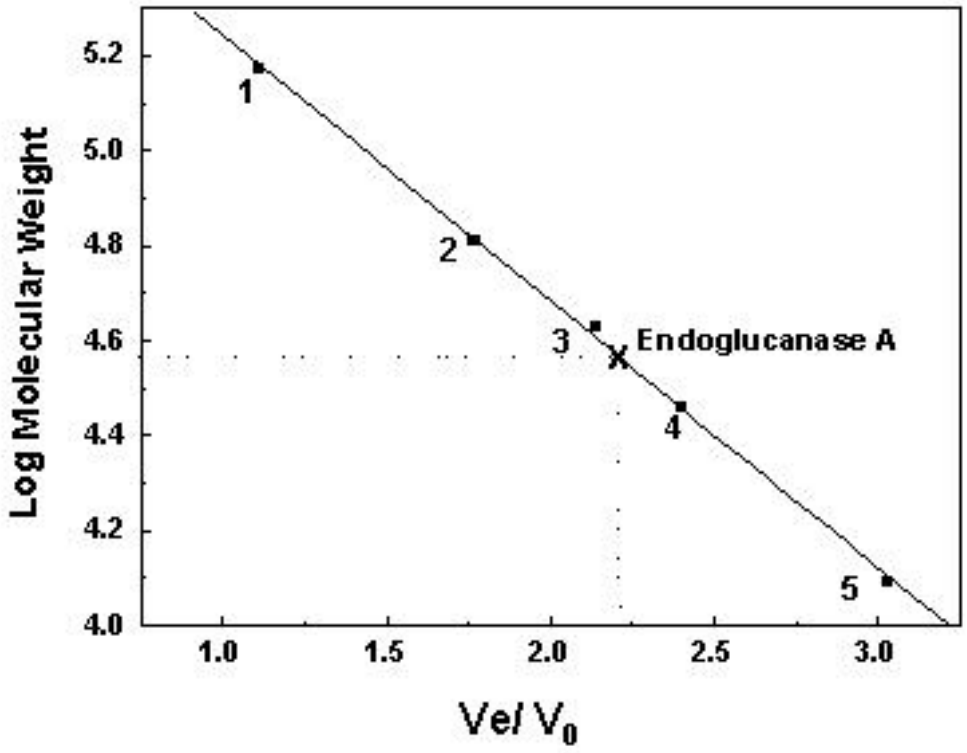


Fig. 3.5 Molecular weight determination of Endoglucanase A by Gel filtration

BioGel P-100 column (160 x 1 cm) was calibrated with 1. Alcohol Dehydrogenase (1,50000), 2.Bovine Serum Albumin (68,000), 3.Ovalbumin (43,000), 4. Carbonic anhydrase (29,000), 5.Cytochrome C (12,400). The column was equilibrated with 0.05M Na phosphate buffer pH 7.0 V_0 ,void volume, V_e, eluted volume

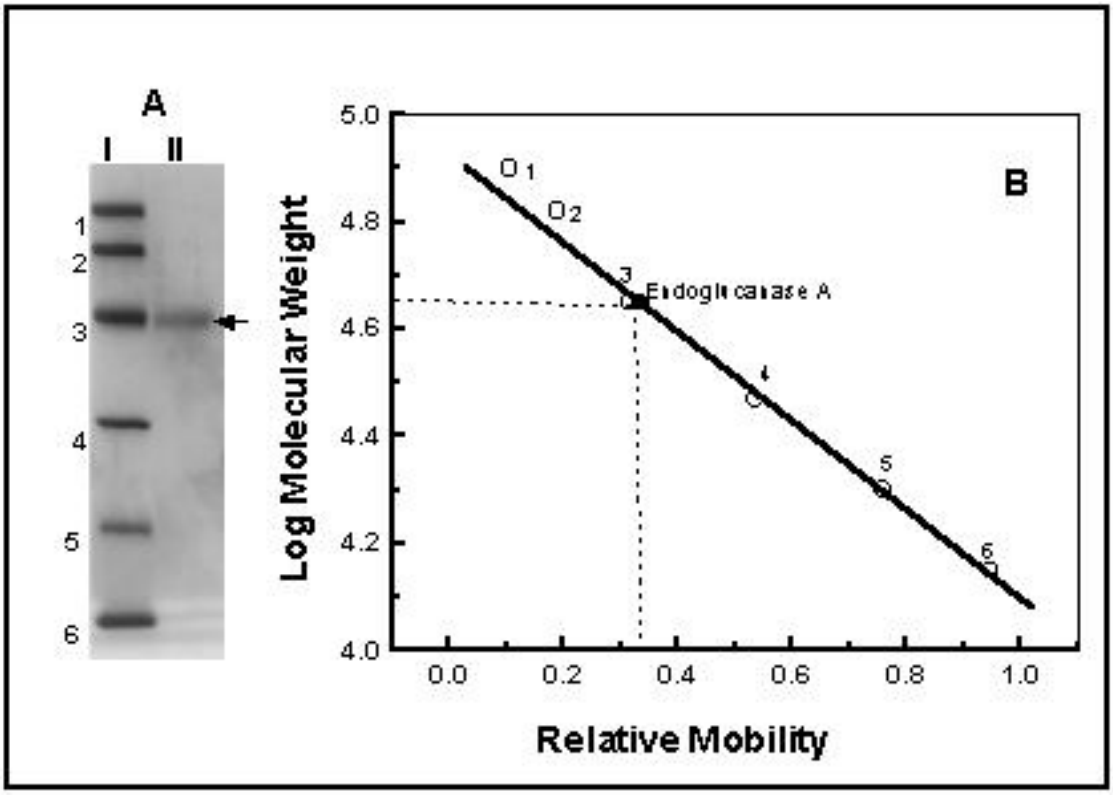


Fig.3.6A. SDS PAGE of Endoglucanase A

(7.5%(w/v) Polyacrylamide gel, Tris –glycine buffer at pH 8.3, Current 16mA, and protein loaded 5μg) Lane I Standard mixture, Lane II Purified Endoglucanase (5μg)

1.Phosphorylase b (97,000) 2. Albumin (66,000)
3.Ovalbumin (45,000) 4. Carbonic anhydrase (30,000)
5. Trypsin inhibitor (20,100) 6. α Lactalbumin (14,400)

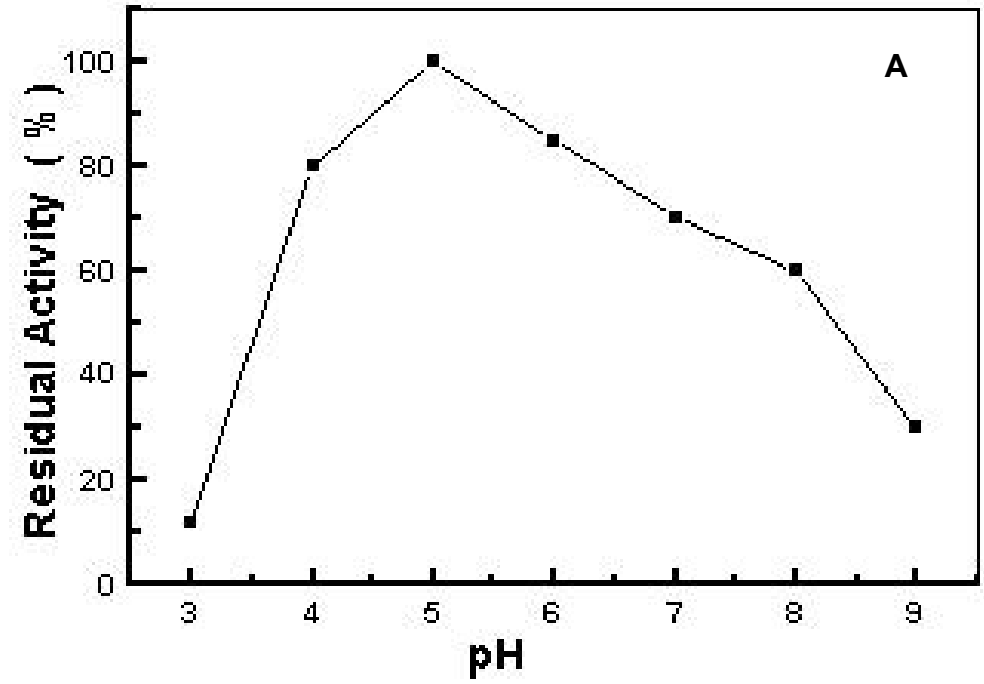
Fig. 3.6B Molecular weight determination by SDS PAGE

3.3.2.2. Molecular weight determination on SDS–PAGE

Molecular weight of purified Endoglucanase A and its possible subunit structure was estimated by their migration in SDS polyacrylamide gel as described by (Laemmli, 1970). The enzyme appeared as single band after treatment with 1% SDS in combination with 1% 2-mercaptoethanol and/or iodoacetamide (Fig.3.6). This indicated that the enzyme is composed of only one polypeptide chain. The plot of log molecular weight versus relative mobilities (Rf) of standard proteins and Endoglucanase A is presented in Fig.3.6.The data indicated that molecular weight of Endoglucanase A is 45KD.

3.3.2.3. Effect of pH on enzyme activity and stability

The optimum pH of the Endoglucanase A was determined by measuring its activity at different pH values. The purified enzyme Endoglucanase A was active in a broad pH range of 4 to 9 with pH optima 5.0 at 60°C (Fig. 3.7A). The enzyme showed 60% of its maximum activity at pH 8.0. The pH stabilities of the enzyme at operational temperatures (50-60°C) was determined by incubating 100μg of enzyme in respective buffer of different pH for 2 h. The enzyme was stable in alkaline pH range of 7-9 at 50°C (Fig.3.7). The Endoglucanase A retains around 75% activity after incubation for 2h at pH 8.0 while 50% residual activity was detected at pH 9.0. The half-life of the enzyme at pH 8.0 was 4h and 2h at 50°C and 60°C, respectively. Most microbial cellulases are active in acidic to neutral pH range (Durand et al., 1984). Kang and Rhee (1995), reported alkaline active carboxymethyl cellulases from a *Cephalosporium* strain. *Humicola* sp has been commercially exploited for the production of alkaline cellulases. These endoglucanases are optimally active in a broad pH range of 6-10 but their thermal stability in the alkaline pH range is low (Schülein, 1997). Although certain strains of *Trichoderma* sp and *Penicillium* sp produce high amounts of cellulases, their activities at alkaline pH are negligible (Beldman et al., 1985; Schmoll and Kubicek 2003). The enzyme produced by alkalotolerant *Fusarium* strain reported in this work was active and stable under alkaline conditions indicating their potential for commercial use.



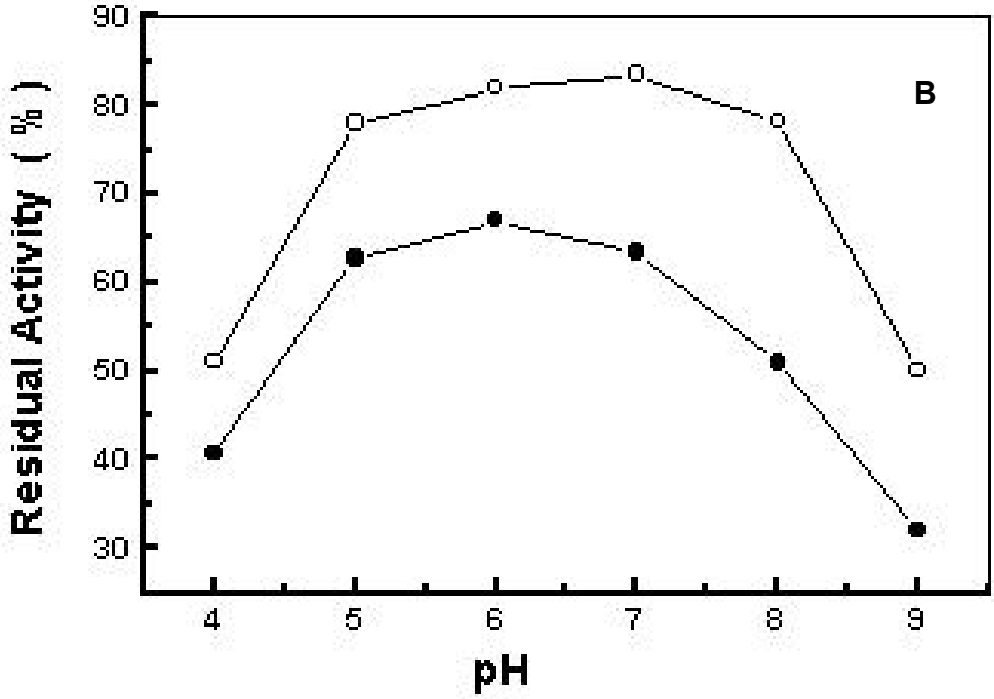


Fig.3.7A. Optimum pH of Endoglucanase A

Fig.3.7B.The effect of pH on stability of Endoglucanase A at 50°C (○) and 60°C (•).

3.3.2.4 Effect of temperature on enzyme activity and stability

For determining the effect of temperature on Endoglucanase A, enzyme activities were estimated in the temperature range of 30-80°C. The enzyme showed maximum activity at 60°C and retained 80% of the maximum activity at 70°C (Fig.3.8A). The energy of activation (Ea) was determined from Arrhenius plot (Fig.3.8B) and it was found to be 22.88 KJ/mole for Endoglucanase A. The temperature stability of the enzyme was detected by incubating 100 μg enzyme at 50-70°C and pH 7.0 for different time intervals. The heat stability curves of Endoglucanase A are presented in Fig.3.9. The enzyme retained 80 % of its maximum activity after 4h of incubation at 50° C.The enzymes was rapidly inactivated at 70°C and shows half-life of 1.5 h at this temperature. Thermostable cellulases that are active and stable at temperatures 60°C and above have been reported from thermophiic fungi such as *T.auranticus, Myceliopthora* sp These enzymes are preferred for enzymatic cellulose hydrolysis (Klyosov, 1988).

The Endoglucanase A gets rapidly inactivated when incubated under alkaline conditions (pH 9.0) and at higher temperatures such as 60°C simultaneously. It is observed that the enzyme stabilities can be improved with the addition of additives such as sugar alcohols. Endoglucanase A was incubated in the presence of 0.1M sugar alcohols such as sorbitol, mannitol, xylitol and trehalose at 60°C and pH 9.0. The samples were removed at different time intervals and assayed for residual activity at pH 7.0 and 60°C. 0.1M trehalose was found to be most suitable in stabilizing enzyme against thermal inactivation (Fig 3.10). Differential Scanning Calorimetry studies in the range of 30-90°C at heating rate of 1°C/min indicated the nature of unfolding of the protein on heating. The thermogram showed the endothermic nature of the unfolding of the enzyme. The protein appears to unfolding in two steps; at 50° C and 80°C. The similar thermogram obtained in the presence of 0.1M trehalose demonstrated that the unfolding is delayed in the presence of trehalose and occurred at 86°C (Fig. 3.11). Various workers previously reported that sugar alcohols improve thermal stability of enzyme (Back et al., 1979; Kelkar and Deshpande, 1991;Goerge et al., 2001). It is concluded that polyalcohols interact with water molecules in microenvironment of

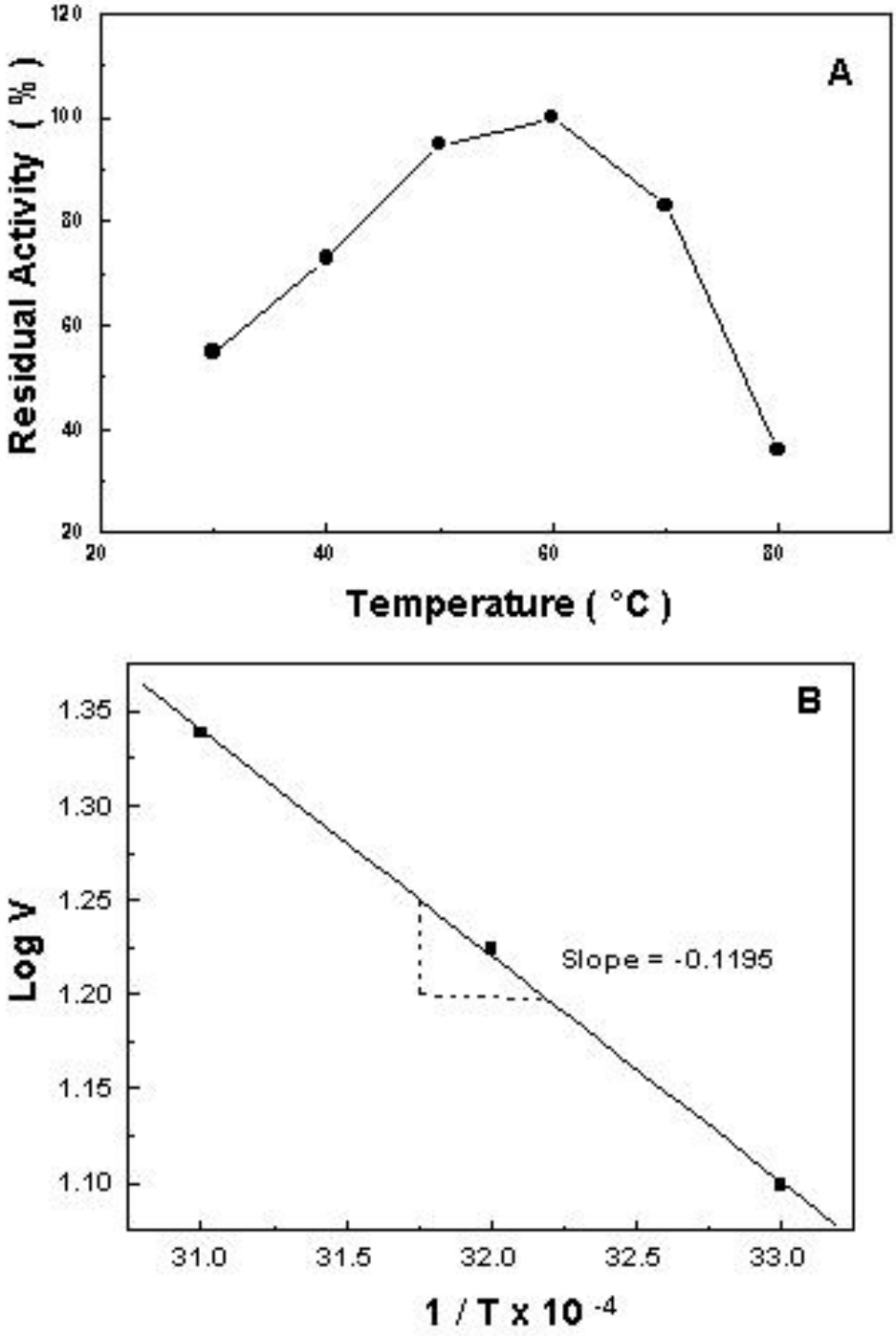


Fig. 3.8 Effect of temperature on rate of hydrolysis of CMC.

A: Optimum temperature for Endoglucanase A

B : Arrhenius plot for Endoglucanase A

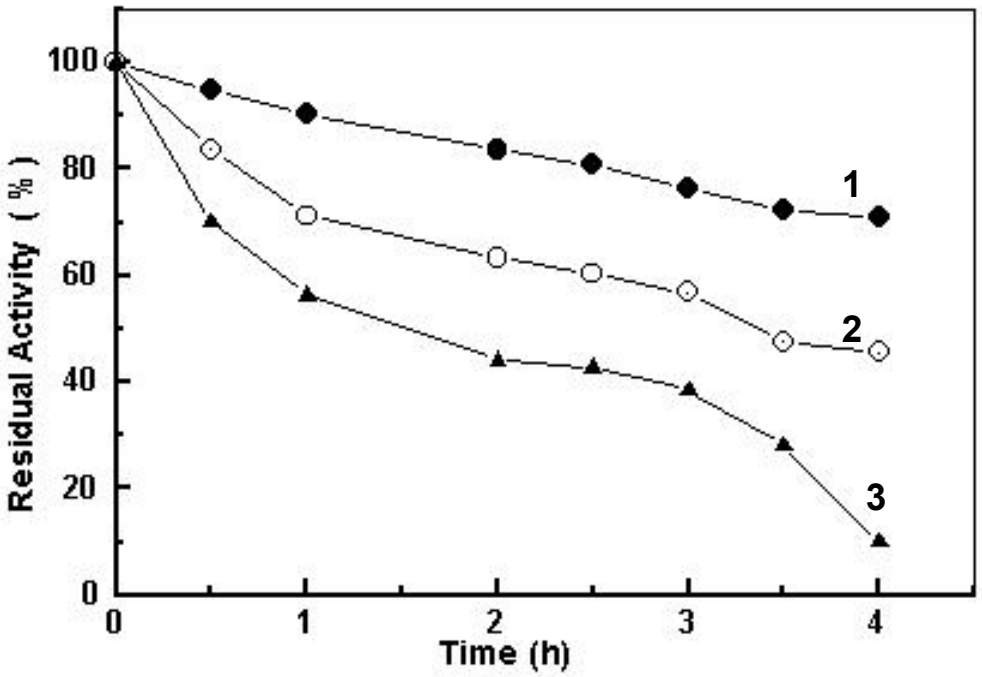


Fig. 3.9. Thermal stability of Endoglucanase A at different temperatures
Symbols **1**: 50°C, **2**: 60°C, and **3**: 70°C at pH 7. 0

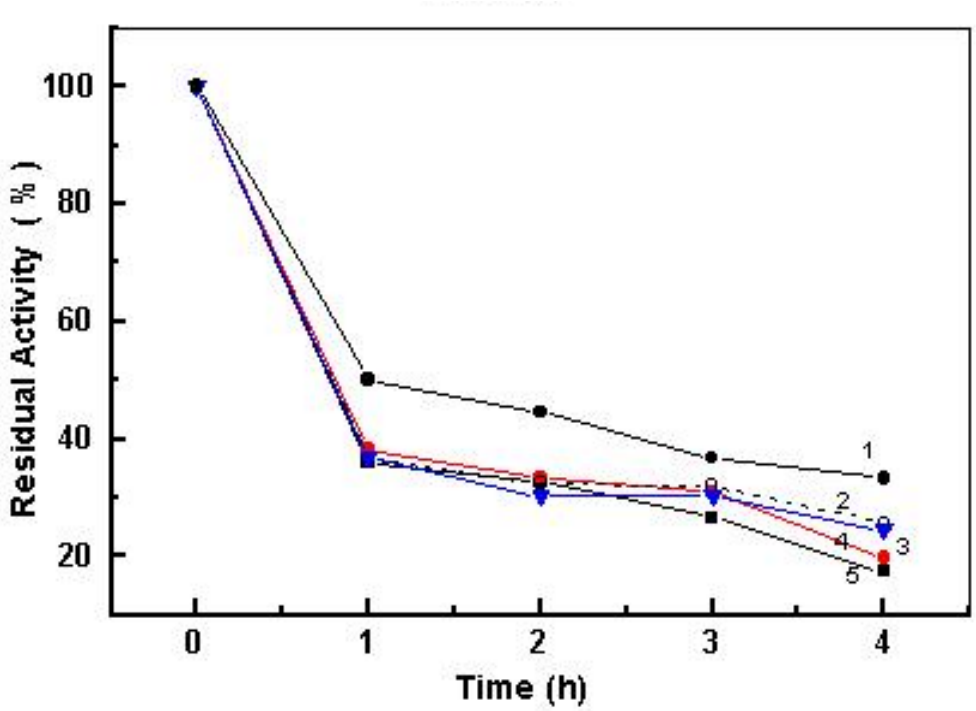


Fig. 3.10. Thermal stability of Endoglucanase A at pH 9 and 60° C in the presence of different additives
Symbols **1**: Trehalose, **2**: Xylitol , **3** :Sorbitol, **4**: Mannitol, **5:** Control without additives .

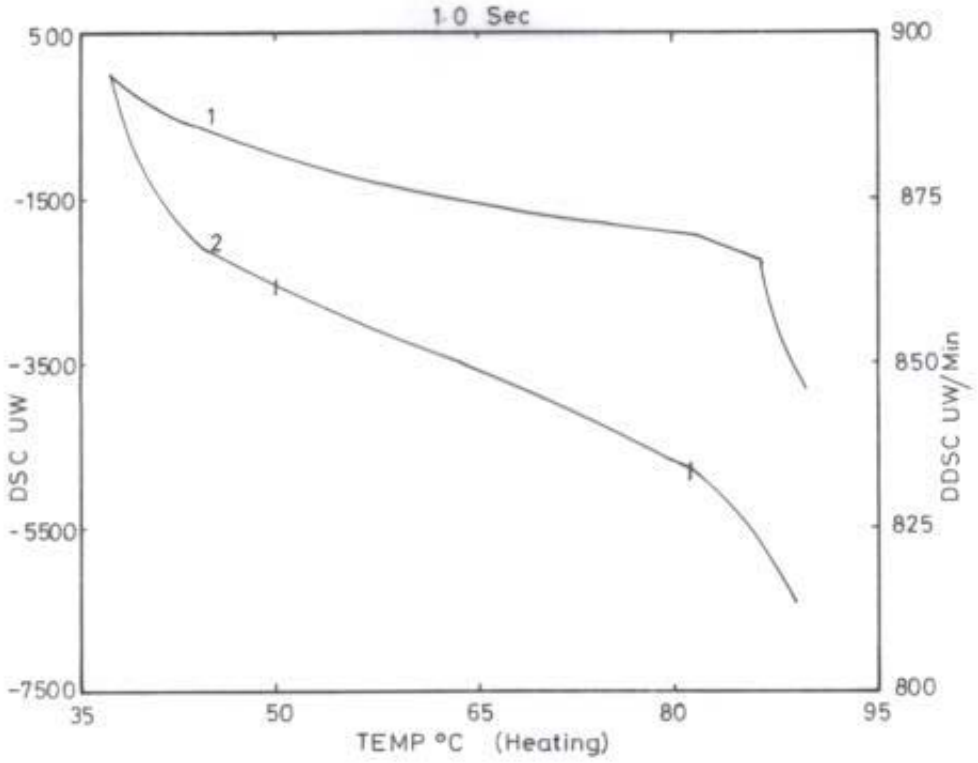


Fig. 3.11. Differential Scanning Calorimetric characterization for thermal denaturation of Endoglucanase A

1 : Thermogram of Endoglucanase A in the presence of 0.1M Trehalose

2 : Thermogram for native Endoglucanase A protein in the absence of additives.

Temperature range (30-90°C); Heating rate (1°C) ; Protein (100μg)

protein for their hydration. Trehalose can retain two water molecules. This alters the microenvironment of the protein and arrest molecular movement of enzyme responsible for its unfolding (Klibanov, 1983).

3.3.2.5 Kinetic constants

The measurement of initial rate of reducing sugars production from CMC at different substrate concentration yielded linear Lineweaver-Burk plots at all pH values. (Fig 3.12 A-C). Km, Vmax values obtained from these plots at different pH values are presented in Table 3.2.

Table 3.2 Kinetic constants for Endoglucanase A at different pH conditions

pH	Km (mg/ml)	Vmax (μmoles/min/mg)
5	2.0	641
7	4.42	250
9	10.10	176

High Km and subsequent low Vmax values at alkaline pH for Endoglucanase A indicate pH 9.0 is not optimal for the enzyme. Schulein et al., 1998 compared apparent kinetic constants for different endoglucanase at pH 5.5 and 8.5. According to their studies Km values for different fungal endoglucanases varied between 0.7-5.6mg /ml at pH 5.5.The kinetic constants obtained for Endoglucanase A in the present studies were in the range of values previously reported for different endoglucanases (Chapter 1).

3.3.2.6. Effect of metal ions on enzyme activity

The effect of different metal ions on the CMCase activity of Endoglucanase A is presented in Table 3.3. The activity of Endoglucanase A was not markedly inhibited by most metal ions such as Mo^{2+}, Zn^{2+}, $Fe2^{+}$, Li^{+}. The Endoglucanase activity was found completely inhibited at 10 mM concentrations of Hg^{2+}. The CMCase activity of alkali stable endoglucanase from *Cephalosporium* sp was inhibited 70-80% with 1mM concentrations of Hg^{2+}. The metal ions such as Cu^{2+}, As^{3+}, W^{2+} inhibited enzyme

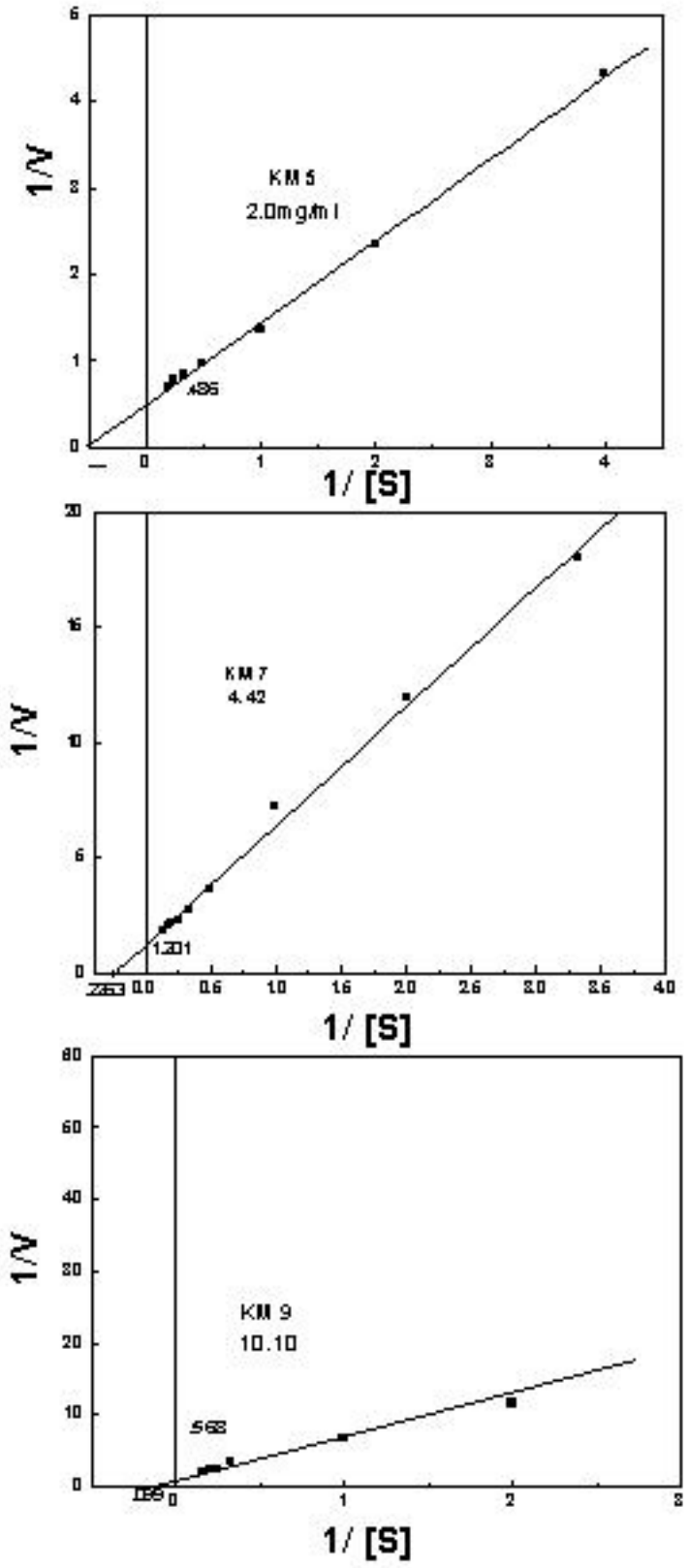


Fig.3.12 Lineweaver–Burk plots for the determination of Km of Endoglucanase A at different pH conditions at 60°C.
Substrate used was CMC, Na-salt (medium consistency)

Table 3.3. Effect of heavy metal ions on catalytic activity of Endoglucanase A

Metal ion	Concentration	Activity (%)
None	-	100
$MnCl_2$,, $2H_2O$	10mM	116
$MgCl_2$,, $4H_2O$	1mM 10mM	104 97
$CoCl_2$, , $6H_2O$	10mM	115
$CaCl_2$, $2H_2O$	10 mM	106
$HgCl_2$,	1mM 10 mM	12 N.D.
NaCl	10 mM	104.
KCl	10 mM	84
$FeSO_4$, $7H_2O$	10 mM	102
$ZnSO_4$, $7H_2O$	10 mM	101
$CuSO_4$,, $5H_2O$	10 mM	88
Li_2,SO_4,, H_2O	10 mM	105
Na_2,HA_SO_4,, $7H_2O$	1 mM 10 mM	108 81
Na_2WO_4, $2H_2O$	10 mM	86
Na_2MoO_4, $2H_2O$	10 mM	100
EDTA	10 mM	78

The enzyme Endoglucanase A (10μg) was incubated with the respective metal ions at pH 7.0 and 50°C. An aliquot (500μl) was taken after 30 min of incubation for the determination of CMCase activity under standard assay conditions.

activity up to 5-20%. Some stimulation in CMCase activity of Endoglucanase A (15%) was observed with Co^{2+}, Mn^{2+}. Similar stimulating effect of Mn^{2+} on endoglucanase from *Sclerotium rolfsii* was reported by Lachke, 1982. Inhibition of enzyme activity on addition of EDTA (10mM) indicated metal ions are needed for enzyme activity. Hoshino and Ito (1997) demonstrated that Ca^{2+}, Mg^{2+}, Co^{2+}, $Mn^{2+}$are essential for thermostability of alkaline cellulases from *Bacillus* sp.

3.4. Conclusion

Multiplicity and presence of different isozymes of cellulase components are the major difficulties in purification of these enzymes. Two distinct endoglucanases were seen in crude culture filtrates of Alkalotolerent *Fusarium* sp. However, surprisingly no isozymes were detected in purified enzyme fraction. The protocol described in the present study resulted in rapid 13 fold purification of Endoglucanase A in minimum steps. The purified enzyme Endoglucanase A is a single polypeptide chain. It shows molecular weight of 45 KD on SDS PAGE and 37.8 KD on gel filtration using BioGel P-100.The slow movement of enzyme in gel filtration matrix can be attributed to specific glycoprotein interaction of enzyme with matrix or irregular shape of the enzyme molecules. Different endoglucanases characterized previously show molecular weight in the range of 10-70KD.Thus the molecular weight value determined for Endoglucanase A fall in the range of previously reported values for endoglucanases. The enzyme Endoglucanase A shows minimum Km and maximum Vmax values at pH 5.0 indicating this pH is optimum for enzyme action. The high pH stability at alkaline conditions and high temperature stability at operational temperatures such as (50-70°C) indicates the possibility of enzyme applications in industrial processes.

Chapter 4

Substrate Specificity:

Characterization of Cellulose Hydrolysis by Endoglucanase A and Its Mode of Action

Summary

The substrate specificity on Endoglucanase A from alkalotolerent *Fusarium* sp showed that the enzyme readily hydrolyzes carboxymethyl cellulose (CMC), Walseth cellulose and lichenan. It did not hydrolyze laminarin that consists of β-(1→3) and β-(1→6) linkages. CM pachyman with β-(1→3) and sophorose with β-(1→2) glycosidic linkages. Thus, the substrate specificity of Endoglucanase A is restricted to β 1-4 linkages only. The enzyme did not show β-D-xylanase, β-D-xylosidase and β- D-glucosidase activity. The enzyme samples were used for the determination of randomness of their attack on CMC. The specific viscosity of 1% CMC solution was rapidly lowered as a function of time with subsequent slow release of reducing sugars indicating random cleavage of the substrate by the enzyme. Thus, the enzyme shown a typical endo type of action. The enzyme is inhibited by different concentrations (0.1%, 1% and 5%) of cellobiose and glucose. The product of enzymic hydrolysis were determined by high-pressure liquid chromatography (HPLC). The Endoglucanase A did not hydrolyzed cellobiose. However, it attacked cellotriose and higher chain length cello-oligosachharides, preferentially at the internal glycosidic bonds.

Cellulosic substrates with different crystallinity were examined for the bound water analysis by Differential Thermal Analysis (DTA) and Thermogravimetry (TG). The samples were heated in the range of 30-100°C at a rate of 3°C /min. DTA vaporization curves for different cellulose samples indicated that the bound water (W_b) was vaporized at higher temperature than free water (W_f) at the surface. The weight loss was observed in two stages, corresponding to W_f and W_b in TG curves. The bound water content was dependent on the degree of crystallinity of cellulose. Higher the crystallinity, lower is the bound water content. Among different cellulose substrates Walseth cellulose showed highest bound water content 0.920 g/g of the substrate at the same time it was found to be least crystalline (Crystallinity index = 31%). The possible application of bound water analysis in understanding the hydrolysis of cellulosic substrates of different crystallinity is discussed in this Chapter.

4.1. Introduction

Cellulose is the biopolymer on the earth that can be hydrolysed to soluble sugars either chemically or enzymatically. The hydrolysis of cellulose depends on composition of cellulolytic complex, properties of cellulases and complexity of cellulosic substrates (Mansfield et al., 1999). The chemical composition of cellulose is simple but its physical structure and morphology is heterogeneous and complex (Teeri, 1997). The polymeric chain of cellulose consists of over 10,000 D-glucose residues linked with β -1,4-glycosidic bonds. The resulting chains are insoluble and adhere to each other in parallel fashion to form crystalline microfibrils. The physical structure of cellulose is not always uniform. The native cellulose contains both highly crystalline and less ordered amorphous regions (Atalla, 1993). Due to complex structure of cellulosic substrates, its efficient solubilization requires presence of different enzymes of a typical cellulolytic complex.

In general, celluolytic enzymes share the same chemical specificity for β-1,4-glycosidic bonds. These are classified depending on their mode of action as endoglucanases [1,4-β-D glucan glucanohydrolase; (EC 3.2.1.4)] and exoglucanases [1,4-β-D glucan cellobiohydrolase; (EC 3.2.1.91)]. A considerable amount of research has been done to elucidate and expand on the mechanisms of cellulose hydrolysis (Ghose and Das, 1971; Coughlan, 1985a,1992; Enari and Niku-Paavola, 1987; Tomme et al., 1988; Wood et al., 1989; Klyosov, 1990; Woodward, 1991; Wood, 1992). Various models suggested the involvement of endo-endo (Mansfield et al., 1998) and exo-exo (Fägerstam and Petterson, 1980) synergism in hydrolysis of cellulose. However, the most accepted model on the mechanism of cellulose hydrolysis is based on the involvement of three different types of enzymes. These include a β-1-4 endoglucanase, β-1-4-exoglucanase and a β-glucosidase (cellobiase, EC 3.2.1.21). (Please refer Chapter 1 for detailed discussion).

The endoglucanases (EGs) act randomly to hydrolyze amorphous cellulose and soluble derivatives of cellulose (Eriksson and Wood, 1985). This involves the cleavage of β-1-4-glycosidic bonds with little release of reducing sugars. In contrast, cellobiohydrolases (CBHs) attack the cellulose from both the reducing and the

nonreducing ends of the chain (Henrissat et al., 1985; Warren, 1996). Various studies have indicated that the substrate specificities of the endoglucanases are considerably more complicated than predicted (Claeyssens et al., 1990; Claeyssens and Aerts, 1992). The process of cellulose hydrolysis to glucose with the help of complete cellulase complex is complicated. However, the application of specialized monocomponent cellulases in various industrial processes such as textile finishing, biodeinking of waste paper and detergent ingredient is the recent trend. For the successful application of cellulases in these processes understanding of structural and functional relationship of individual enzyme with its substrate becomes important. The studies in this Chapter are aimed at understanding the substrate specificity and characterization of cellulose hydrolysis by Endoglucanase A from alkalotolerent *Fusarium* sp. The linkage specificity studies using glucan substrates containing different glucosidic linkages revealed that the substrate specificity of Endoglucanase A is restricted to β 1-4 linkages only. The radom nature of enzyme attack on CMC is demonstrated by viscometric study. Viscometric study also suggested that the enzyme is partially inhibited by cellobiose and glucose at low concentrations. The mode of action of Endoglucanase A on cellulosic substrates is demonstrated with the help of end product analysis using different cello-oligosaccharides with varying chain length. This Chapter highlights the application of bound water analysis in confirming hydrolysis of amorphous cellulose by Endoglucanase A. Finally, a short account on short fibre formation is also presented in this Chapter.

4.2. Materials and Methods

4.2.1. Chemicals

Following materials were purchased from the suppliers indicated: Avicel PH 101, *P*-hydroxybenzoic acid hydrazide (*P*-HBAH) (Fluka AG, Switzerland); Cellulose-123 (Carl Schleicher & Schüll Company, Germany); Solka floc BW 100 (Pure fibrous spruce wood pulp) (Brown Company, Germany); Absorbent cotton (Bengal Chemicals and Pharmaceuticals Work Ltd, India); lichenin, dextran, sodium salt of carboxy methyl cellulose, (CMC, medium viscosity) (Sigma Chemical Co, U.S.A.); laminarin (Koch-Light Laboratories Ltd, England); HPLC grade glucose (G), cellobiose (G_2), cellotriose (G_3), cellotetraose (G_4), cellopentaose (G_5), cellohexaose (G_6) and the mixture of cellooligosacharides were obtained from E-Merck, Darmstadt Germany, CM-

pachyman and sophorose was a kind gift from Prof., B.A. Stone (Biochemistry Department, La Trobe University, Victoria, Australia) and Prof. E.T. Reese (U.S. Army Natick Laboratories, U.S.A.). Phosphoric acid-swollen cellulose (Walseth cellulose) was prepared as described by Wood (1988). Mixed Office Wastepaper (MOW) pulp was prepared as described in Chapter 6. All the buffer salts and microbial media components were procured from the standard commercial sources and of highest quality available.

4.2.2. *Enzyme production and purification of Endoglucanase A*

The alkaline active alkali stable cellulase preparation was obtained from alkalotolerant *Fusarium* sp. as described previously in Chapter 2. The enzyme Endoglucanase A was purified to homogeneity by Gel filtration on (BioGel P-100) and isoelectric focusing as described in Chapter 3.

4.2.3. *Enzyme assays and substrate specificity studies*

The activity of purified Endoglucanase-A on 1% CMC solution, Filter paper (FPase), and other cellulosic substrates containing β 1-4 linkages were measured in terms of release of reducing sugars by 3,5 dinitrosalicylic acid (DNSA) method (Miller et al., 1960) and *P*-HBAH Method (Hurst et al., 1977). Xylanase (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8), activity was tested using 1% solution of oat spelt xylan. The β-glucosidase (1,4-β-D-glucan glucohydrolase, EC 3.2.1.21), and β-xylosidase (1,4-β-D-xylan xylohydrolase, EC 3.2.1.37) activities were determined using synthetic substrates *P* - NPG, *P* -NPX, respectively at pH- 6.0 and 50°C.

The various buffers used for enzyme activity were 50 mM citrate buffer (pH 3-6) and sodium phosphate buffer (pH 7-8). One IU of enzyme activity was defined as the amount of enzyme that liberates 1μmole of reducing groups in terms of glucose/min (for cellulase) or xylose/min (for xylanases) under the standard assay conditions. One IU of β-glucosidase and β-xylosidase are defined as the amount of enzyme that liberates 1μmole of ρ-nitrophenol/min under the standard assay conditions (Lachke,1988).

4.2.4. Viscometric analysis

The relationship between the change in specific viscosity (η_{sp}) and reducing sugars released from CMC solution by Endoglucanase A was determined by viscometric analysis. An Ubbelöhde viscometer with a flow time of 50.3 $\pm$ 0.1 sec for 0.05 M Na-phosphate buffer pH 7.0 at 50°C was used. The viscometer was mounted in a water bath at 50°C and 10 ml of 1% CMC solution was pipetted into it. After 10 min, preincubated 3 ml diluted enzyme (3 μg) was introduced in the viscometer. The reaction mixture was gently mixed by blowing the air through capillary arm of the viscometer. A stop watch was started at the time of enzyme addition. The decrease in flow time was recorded at every 3-min interval for 30 min and simultaneously aliquots were removed and analyzed for reducing sugars by *P*-HBAH method (Hurst et al., 1977). The viscosity of the substrate at zero incubation time was determined by adding 3 ml buffer solution instead of the enzyme to 10 ml of the substrate solution. For enzyme inhibition studies, the experiments were carried out in the presence different concentrations (0.1%, 1%, 5%) of glucose and cellobiose. The initial flow at each concentration of cellobiose and glucose was separately determined.

4.2.5. Mode of action of Endoglucanase A on cellooligosaccharides

The mode of action of Endoglucanase A was studied by following the hydrolysis of different cello-oligosaccharides (G_2-G_6) and CMC. The pattern of cellooligosaccharides hydrolysis was followed by HPLC by analyzing the reaction products at different time intervals. The reaction mixtures (1.0 ml) consisting of 1mg substrate in 0.05M Na-phosphate buffer, pH 7.0 and the enzyme (3 μg) were incubated at 60°C for 2 hr. The samples 50μl were removed at different time intervals and analyzed on Waters sugar-pak-1 column (6.5x300mm) with deionised water (70°C) as eluant and at a flow rate of 0.4ml/min. Glucose and other oligosaccharides were detected with Waters 2410 Refractive index detector. Similarly standards for each oligosacharides were injected separately on the column.

4.2.6. *Sample preparation for X ray diffraction and TG / DTA studies*

All cellulose samples were purified by extracting contaminants using toluene and *n*-hexane successively. In order to avoid effect of particle size, the cellulose powder samples were passed through sieve of 200-mesh size. Cotton, Filter paper and MOW pulp samples were cut to fine powder to eliminate effect of fibre orientation. All cellulose samples under the investigation soaked for 4 h in deionised distilled water in order to facilitate binding of water molecules to cellulose samples. The samples were dried in vacuum desiccators for 3 days in order to remove excess of surface water. One part of these samples was dried and used for X-ray diffraction studies as described detail in *4.2.7* section. The other portion of each sample was used for vaporization studies as described in section *4.2.8*.

4.2.7. *Determination of the degree of crystallinity of cellulose samples*

The samples were prepared as described above. The X-ray diffraction of each set of samples was recorded using Philips Analytical X-ray diffractometer (PW 1710). The wavelength of Cu radiation source was 0.1540 (nm) and spectra were obtained at 30 mA with an accelerating voltage of 40 kV. Samples were scanned on the automated diffractometer from 9-40° of 2θ (Bragg angle) with data acquisition taken at intervals of 0.400 s. A peak resolution program (PC-APD diffraction software) was used to calculate crystallinity index. The crystallinity index (CrI) of cellulose was also calculated by empirical method described by Segal et al.(1959) using the equation:

$$\mathbf{CrI\ (\%) = I_{002} - I_{AM} / I_{002} \times 100}$$

Where I_{002} is the maximum intensity of the 002-lattice diffraction (reflection attributed to crystalline regions of the sample) and I_{AM} is the intensity of diffraction at Bragg angle 2θ =18° (reflection attributed to amorphous regions of the sample). The results obtained using both the methods were in good agreement.

4.2.8. *Vaporization measurements by TG / DTA*

A Seiko TG/ DTA 32 computer controlled unit was used for bound water analysis of different cellulosic samples. The small amount of distilled water was added with micro syringe to each sample and then total weight of the sample was directly recorded by microbalance fitted in the instrument with the accuracy 0.001 mg. The cellulose samples were heated in the range of 30-100°C at a rate of 3°C / min. The amount of bound water was calculated from thermograms by subtracting the weight loss due to vaporization of free water from total weight loss due to water content of the sample. Bound water (W_b) in samples = Total weight loss due to water content of the sample (W_t) - Weight loss due to free water in the sample (W_f)

$$W_b = W_t - W_f$$

In order to evaluate effect of endoglucanse action on the bound water content of cellulose samples, 50 mg of Walseth cellulose incubated along with 10 μg of purified Endoglucanase A. The reaction was carried out at different time intervals. The unhydrolysed part of Walseth cellulose was separated by centrifugation. The samples were washed to remove residual enzyme, dried and used for bound water analysis. In order to avoid experimental error the samples were run in triplicates and data are given as mean of all three sets. The values obtained are expressed as amount of bound water in g / g of the cellulosic substrates.

4.2.9. *Short fibre forming activity*

Short fibre formation was determined according to Halliwell and Riaz (1970) with a slight modification. The assay mixture contained 50 mg of Whatman no.1 filter paper and appropriately diluted enzyme (3μg) fraction in 0.05 M sodium phosphate buffer, pH 7.0 in a final volume of 5 ml. The residual filter paper was carefully removed by forceps leaving a suspension of insoluble short fibres. The short fibres were measured turbidometrically at 600 nm. (A control was run under identical conditions in the absence of enzyme). The suspension of insoluble short fibres was centrifuged at 10,000-x g for 5 min and supernatant was used for determination of reducing sugars by *P*-HBAH method.

Scanning electron microscopic studies

The sediment of short fibres after centrifugation was washed twice with deionised water and resuspended in 1.0 ml of distilled water. Out of that an aliquot of 0.1 ml was dried on aluminum foil and coated with thin gold layer. The length of the short fibres formed by action of Endoglucanase A was determined using a Leica Stereoscan 440 scanning electron microscope.

4.3. Results and Discussion

4.3.1. *Linkage specificity of Endoglucanase A*

Knowledge of the specificity requirement of endoglucanases has been considerably widened by the use of glucan substrates containing more than one type of glycosidic linkages. The purified Endoglucanase A was used to study their substrate specificity on different cellulosic substrates and D-glucans of various linkages. Table 4.1. summarizes the formation of reducing sugars from different substrates. Endoglucanase A did not hydrolyze laminarin that consists of β-(1→3) and β-(1→6) glycosidic linkages. CM pachyman with β-(1→3) and sophorose with β-(1→2) linkages also remained unaffected in the presence of enzyme. However, CMC, Walseth cellulose and lichenin were hydrolyzed readily by Endoglucanase A. This indicated that the substrate specificity of Endoglucanase A is restricted to β 1-4 linkages only. Amongst cellulosic substrates the enzyme rapidly hydrolyzed amorphous cellulose such as carboxymethyl cellulose than crystalline substrate such as filter paper. According to Hurst et al. (1978) endocellulase from *Aspergillus niger* hydrolyzed variety of substrates including barley glucan, lichenin and CM pachyman along with activities on cellulosic substrates. Few of the previously studied endoglucanases also show inherent xylanase activity (Kanda et al., 1976; Hurst et al., 1978; Sadana et al., 1984). However, Endoglucanase A described in present study did not show β-D-xylanase, β-D-xylosidase and β - glucosidase activity.

Table 4.1. Substrate specificity of Endoglucanase A

Substrate	Components	Linkage of D-Glycosyl group	Reducing Sugars (μg)
CMC	D-Glc →D-Glc	β-(1→ 4)	280
Walseth cellulose	D-Glc →D-Glc	β-(1→ 4)	16.4
Filter paper	D-Glc →D-Glc	β-(1→ 4)	9.6
Lichenan	D-Glc →D-Glc	β-(1→ 3)&β-(1→ 4)	22.5
Laminarin	D-Glc →D-Glc	β-(1→ 3)& β-(1→ 6)	-N.D.-
Sophorose	D-Glc →D-Glc	β-(1→ 2)	-N.D.-
CM pachyman	D-Glc →D-Glc	β-(1→ 3)	-N.D.-
Xylan	D-Xyl →D-Xyl	β-(1→ 4)	-N.D.-
Dextran	D-Glc →D-Glc	α-(1→ 6)	-N.D.-

N.D. = Not detected

4.3.2. Viscometric analysis

The decrease in specific viscosity (η_{sp}) of CMC solution due to hydrolytic action of enzyme is shown in Fig. 4.1. Suitably diluted enzyme samples were used for the determination of randomness of their attack on CMC. The specific viscosity of 1% CMC solution was rapidly reduced as a function of time with subsequent slow release of reducing sugars indicating random cleavage of above substrate. Thus the enzyme appeared to be a typical endoglucanase similar to some of the other endoglucanases reported from other microorganisms (Beldman et al., 1985; Hong et al., 1986; Bhat et al., 1989;Christakopoulos et al., 1995b).

One of the key factors that affect cellulose hydrolysis includes the end product inhibition of cellulolytic enzymes (Coughlan, 1985c). Both cellobiose and glucose have shown to inhibit cellulose hydrolysis (Lachke and Deshpande, 1988). The inhibition of cellulase due to glucose is weaker than cellobiose (Ghose and Das, 1971). Reese (1963) reported that hydrolysis of cellulose can be inhibited by cellobiose. Other workers suggested that this inhibition of endoglucanases by cellobiose could be competitive (Sternberg, 1976) as well as noncompetitive (Howell and Mangat, 1978). Halliwell and Griffin, 1973 have demonstrated that cellobiose inhibited *T. koningii* cellobiohydrolase competitively. The degree of inhibition by cellobiose depends on the relative concentration of substrate and inhibitor. Recently, Marju et al. (2004) showed that the inhibition of cellulases by cellobiose depends on the nature of the substrate. The hydrolysis of amorphous cellulose by endoglucanases from *T. reesei* was less affected by cellobiose inhibition as compared to that of crystalline substrates.

Wood and McCrae, (1978) studied the effect of various concentration of cellobiose on the action of endoglucanase E1, E3a, E3b, and E4 from *T. koningii.* Their studies indicated that E3a, E3b and E4 were inhibited to the extent of 40-84% at 29 mM concentration of cellobiose. In contrast, E1 component was stimulated by 115%, 190%, and 310% by 0.29 mM, 2.9 mM and 29 mM cellobiose respectively. Earlier these authors reported that *T. koningii* CMCase (Cx1) was inhibited 47% and 14% at 0.05% and 0.01% concentration of cellobiose, respectively. In the present studies Inhibition of Endoglucanase A by cellobiose and glucose was examined by viscometric method. The plots of fluidity of 1% CMC solution as a function of incubation time for Endoglucanase A in the absence and presence of cellobiose (0.1%, 1%, 5% W/V) or glucose (0.1%, 1%, 5%) are shown in Fig.4.2 A and B respectively. The difference in slopes refer to the extent of inhibition (Lachke, 1982). Cellobiose at 5 % concentration inhibited Endoglucanase A activity by 82% while 60% inhibition of Endoglucanase A activity was observed by glucose at 5% concentration. The percent inhibition of CMC liquefying activity of Endoglucanase A at different concentration of cellobiose and glucose is presented in Table 4.2.The family 7 cellulases from *Trichoderma reesei* (Biely et al., 1991) and EG I from *Humicola* sp (Schülein, 1997) showed such characteristic inhibition of endoglucanases by different cellobiose concentrations.

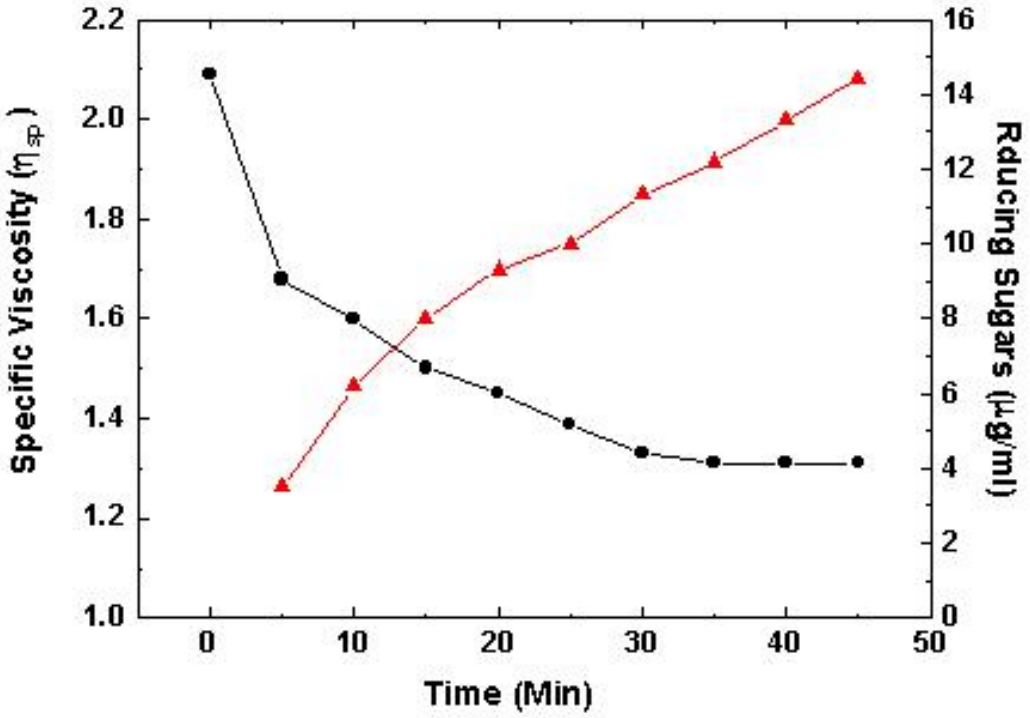


Fig. 4.1. Randomness of CMC hydrolysis by Endoglucanases - A

The time course of CMC (1.0 %) hydrolysis by (3 µg) of Endoglucanase-A at pH 7.0 at 50°C shows the lowering of its specific viscosity (ηsp) (●) with subsequent release of reducing sugars (▲) due to random action of Endoglucanase A.

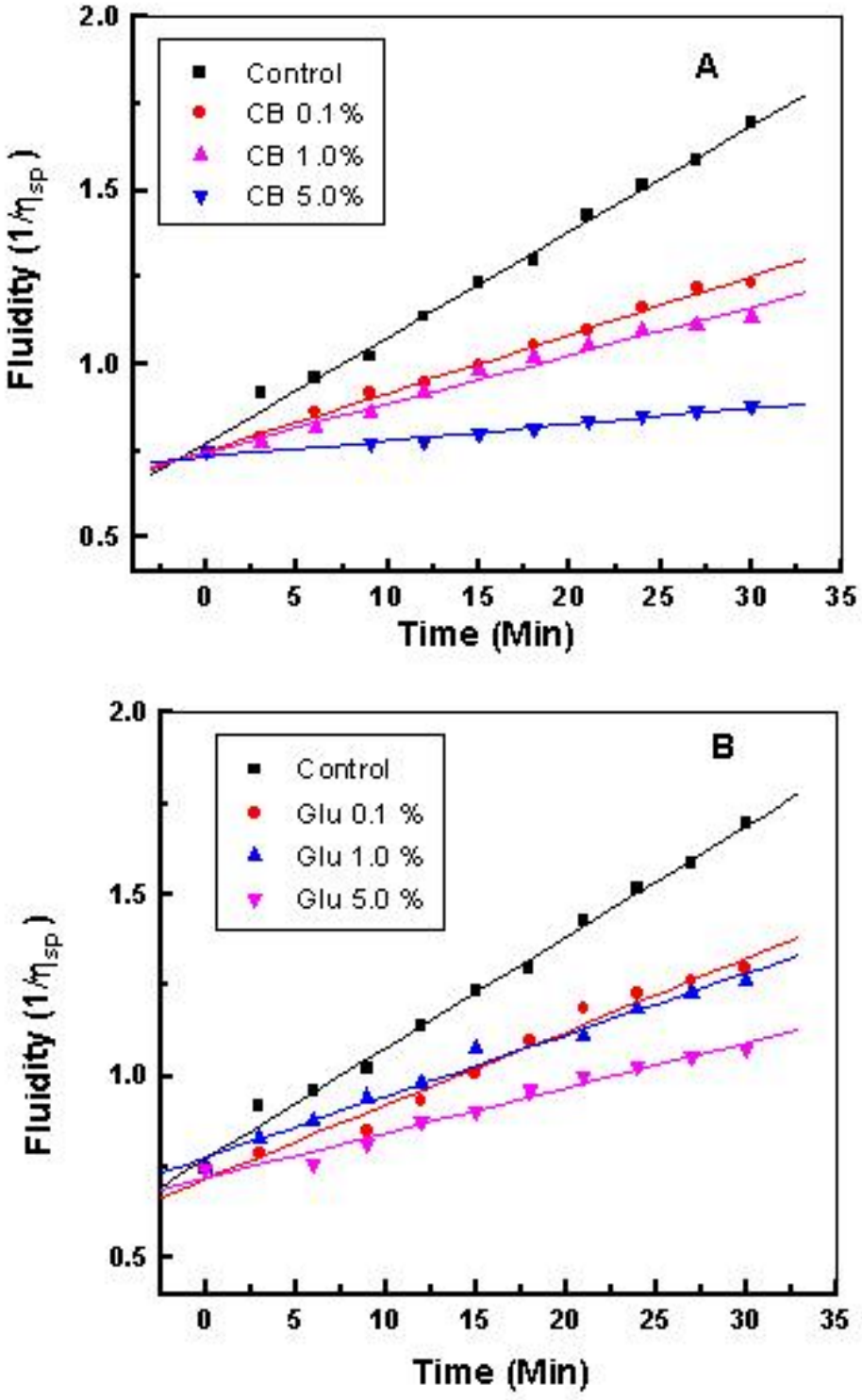


Fig.4.2. The relation between fluidity and incubation time during hydrolysis of CMC by Endoglucanase A in the presence of different concentrations of cellobiose (A) and glucose (B).

The percent inhibition of the CMC liquefying activity was calculated by comparing slopes of the lines in the presence and absence of cellobiose and glucose. (CB =Cellobiose; Glu =Glucose)

Table 4.2. Effect of cellobiose and glucose on activities of Endoglucanase A (Viscometric method)

Sugar	Concentration (%)	Endoglucanase A inhibition (%)
Cellobiose	0.1	52
	1.0	54
	5.0	82
Glucose	0.1	34
	1.0	45
	5.0	60

4.3.3. Mode of action of Endoglucanase A

The mode of action of Endoglucanase A on cellooligosacaharides (cellobiose-cellohexaose) was studied by monitoring the reducing sugars liberated on HPLC. The separation of different oligosacharides from standard mixture is presented in Fig 4.3A. The retention times (min.) for different oligosacharides were as follows: glucose (G, 12.5); cellobiose (G_2, 9.9), cellotriose (G_3, 8.4), cellotetraose (G_4, 7.7), cellopentaose (G_5, 7.2) and cellohexaose (G_6, 7.0).

4.3.3.1. Action of Endoglucanase A on cellobiose and cellotriose

No peak other than the substrate could be detected up to 24 h incubation with Endoglucanase A from cellobiose. However, from cellotriose after 1 h incubation small peaks corresponding to cellobiose and glucose were seen in HPLC chromatogram (Fig.4.3B). The results suggested that enzyme was capable of hydrolyzing cellotriose. The absence of any product formation from cellobiose confirmed that the enzyme was free from β glucosidase activity. Various workers previously, reported feeble activity of endoglucanases on cellotriose (Okada, 1976; Hurst et al., 1977). Christakopoulos et al. (1995b) reported low molecular weight endoglucanase from *Fusarium oxysporium* was

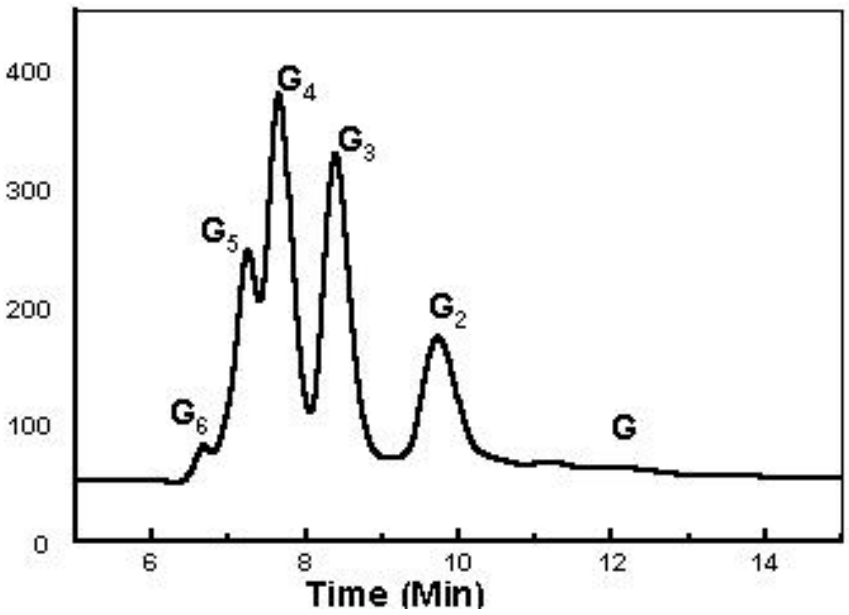


Fig.4.3A.Separation of standard oligosaccharides from standard mixture and their retention times.

Glucose (G_1, 12.5), cellobiose (G_2, 9.9), cellotriose (G_3, 8.4), cellotetraose (G_4, 7.7), cellopentaose (G_5, 7.2) and cellohexaose (G_6, 7.0).

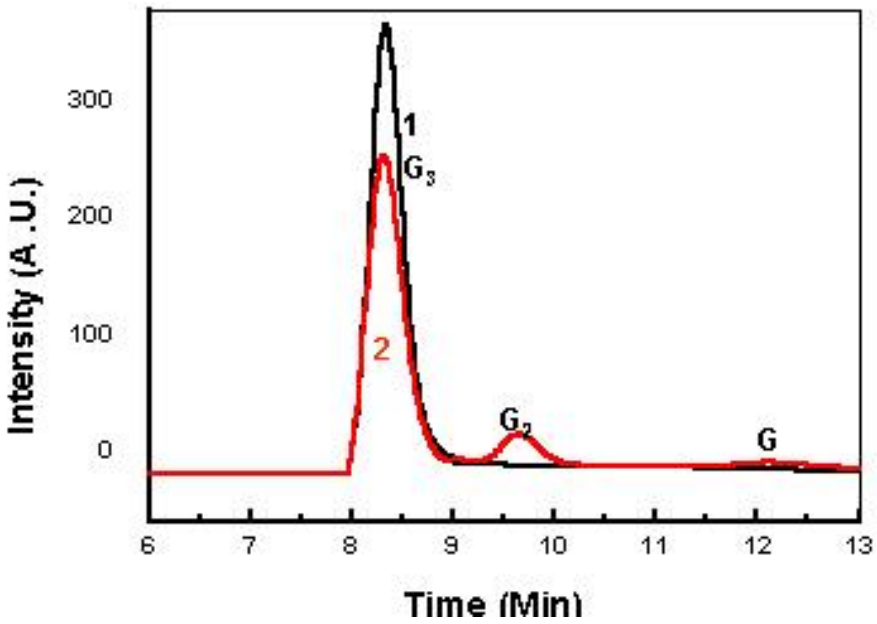


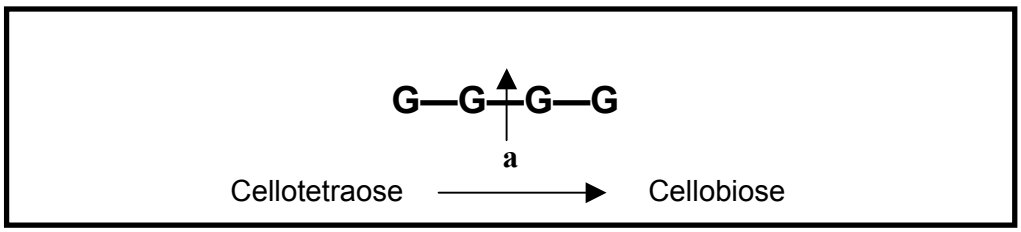
Fig.4.3B. Action of Endoglucanase A on cellotriose (G_3)

The reaction mixtures (1.0 ml) consisting of substrate (G_3,1mg) in 0.05M Na-phosphate pH 7.0 buffer and enzyme (3μg) were incubated at 60°C for 1h.The samples 50ml were removed at different time intervals and analyzed by (HPLC). 1: at 0 min; 2: at 1 h interval. After 1 h incubation clear peaks corresponding to cellobiose (G_2) and glucose (G) are seen.

capable of hydrolyzing 63% cellotriose in 1h. Schülein (1997) concluded endoglucanases belonging to family 7 can hydrolyze cellotriose efficiently due to their smaller substrate binding site.

4.3.3.2. *Cellotetraose*

With cellotetraose as a substrate, Endoglucanase A rapidly produced cellobiose as the sole product. Even after longer incubation period (1h), absence of peak corresponding to glucose and/or cellotriose in the chromatogram suggested that the enzyme cleaves central bond of cellotetraose (Fig 4.3C).



Bhat et al. (1990) demonstrated that endoglucanase III from *Penicillium pinophillum* attack at side linkage or internal linkage to yield cellobiose, cellotriose and glucose as the end products of cellotetraose hydrolysis. Sadana et al., (1984) observed that endo A from *Sclerotium rolfsii* feebly attacks cellotetraose to give cellobiose as a sole products.

Schoü et al., (1993) observed that Endoglucanase from *Fusarium oxysporium* liberated cellobiose as the major product on hydrolysis of soluble cellodextrins. The endoglucanase I from *Fusarium oxysporium* possesses 4 subsites for sugar binding (Sulzenbacher,1996,1997; Mackenzie, 1997). The kinetic studies and crystal structure studies showed that enzyme binds oligosaccharides between -2, -1, +1 and +2 subsites and cleaves substrate at internal -1, +1 region.

4.3.3.3 *Cellopentaose*

The hydrolysis of cellopentaose by Endoglucanase A in the initial stages of hydrolysis gave peaks corresponding to cellobiose and cellotriose. These were

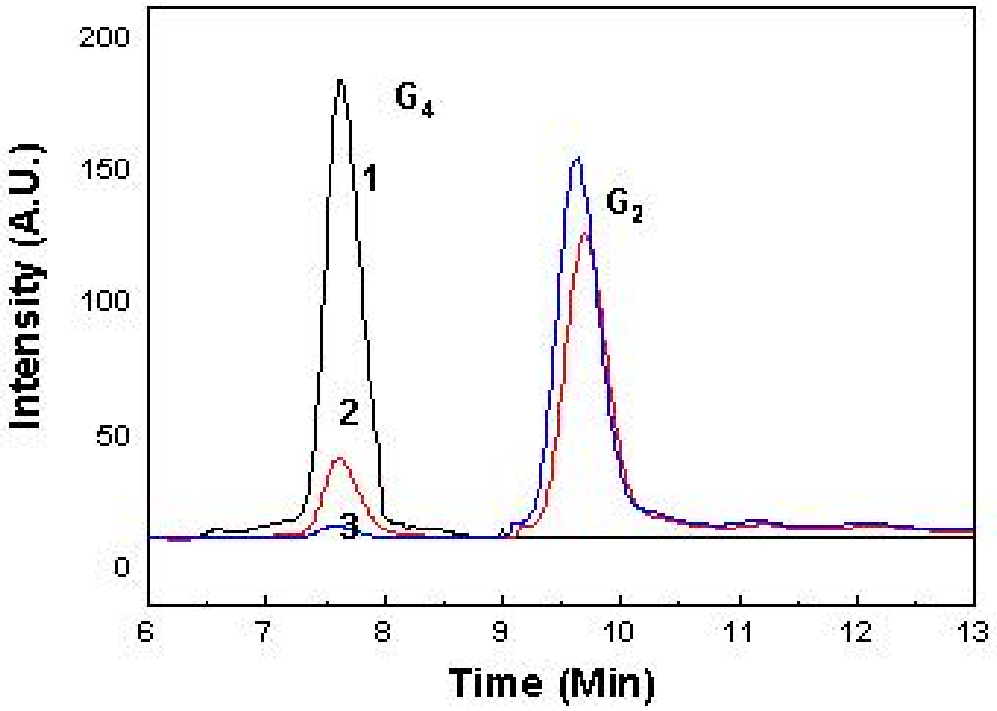


Fig.4.3C. Action of Endoglucanase A on cellotetraose (G_4)

The reaction mixtures (1.0 ml) consisting of substrate (G_4, 1mg) in 0.05M Na-phosphate pH 7.0 buffer and enzyme (3 μg) were incubated at 60°C for 1h. The samples 50 μl were removed at different time intervals and analyzed by (HPLC) 1: at 0 min; 2: at 30min and 3: at 1 h. interval. Cellobiose (G_2) appeared as major product of hydrolysis at all stages.

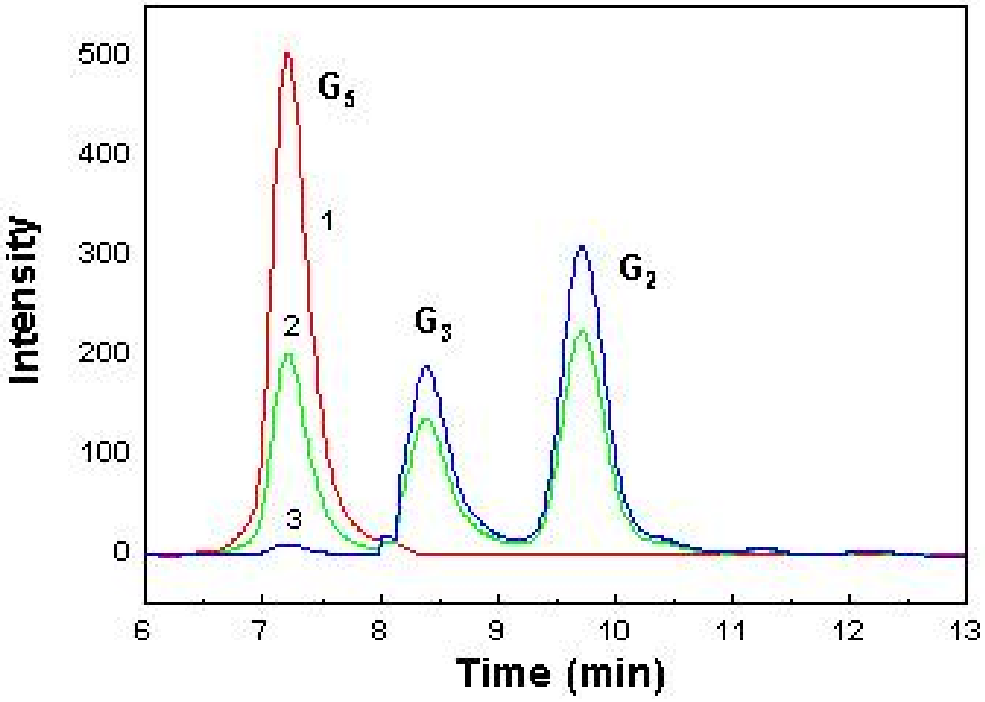
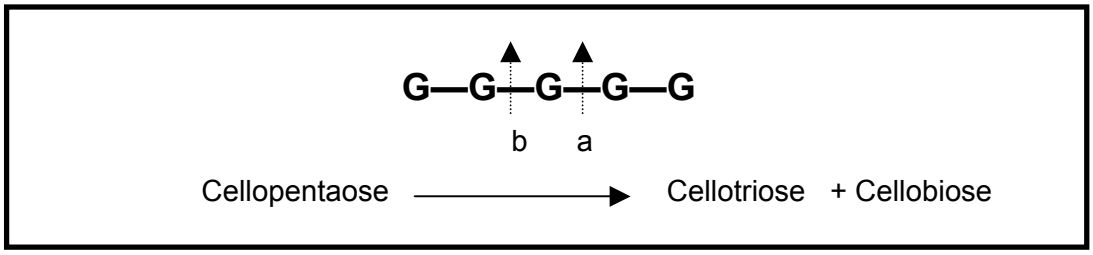


Fig.4.3D. Action of Endoglucanase A on cellopentaose (G_5)

The reaction mixtures (1.0 ml) consisting of substrate (G_5, 1mg) in 0.05M Na-phosphate pH 7.0 buffer and enzyme (3μg) were incubated at 60°C for 1hr. The samples 50μl were removed at different time intervals and analyzed by (HPLC) 1: at 0 min; 2: at 30 min and 3: at 1 h interval. Cellobiose (G_2) and cellotriose (G_3) appeared as the major products of hydrolysis at all stages

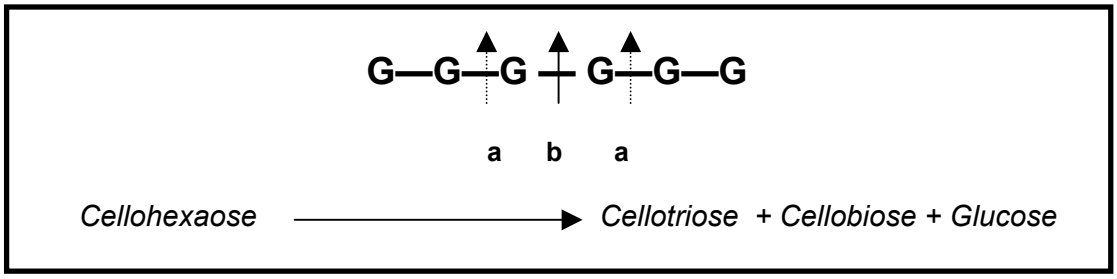
apparently produced in equal amounts in first 30 min of hydrolysis. The quantity of cellobiose and cellotriose increased in parallel with time. It is to be noted that neither glucose nor cellotetraose was detected during initial incubation period (Fig.4.3D). Absence of any glucose formed in the reaction mixture suggests that enzyme act preferentially in the internal linkages.



Similar, observation was recorded, by other researchers (Hurst et al., 1978; Sadana et al., 1984; Bhat et al.,1990).

4.3.3.3. *Cellohexaose*

The end products obtained from hydrolysis of cellohexaose by Endoglucanase A are shown in Fig.4.3E. The results indicated the rapid breakdown of cellohexaose in to cellotriose and cellobiose at 30 min interval. The enzyme rapidly splits cellohexaose at a and b conviently to yield cellobiose, cellotetraose and cellotriose respectively.



However, absence of cellotetraose from reaction products suggests that cellotetraose formed is quickly degraded into two molecules of cellobiose. Hence, higher amount of cellobiose is observed in reaction product. However, on prolonged incubation appearance of traces of glucose suggested further degradation of cellotriose into glucose and cellobiose.

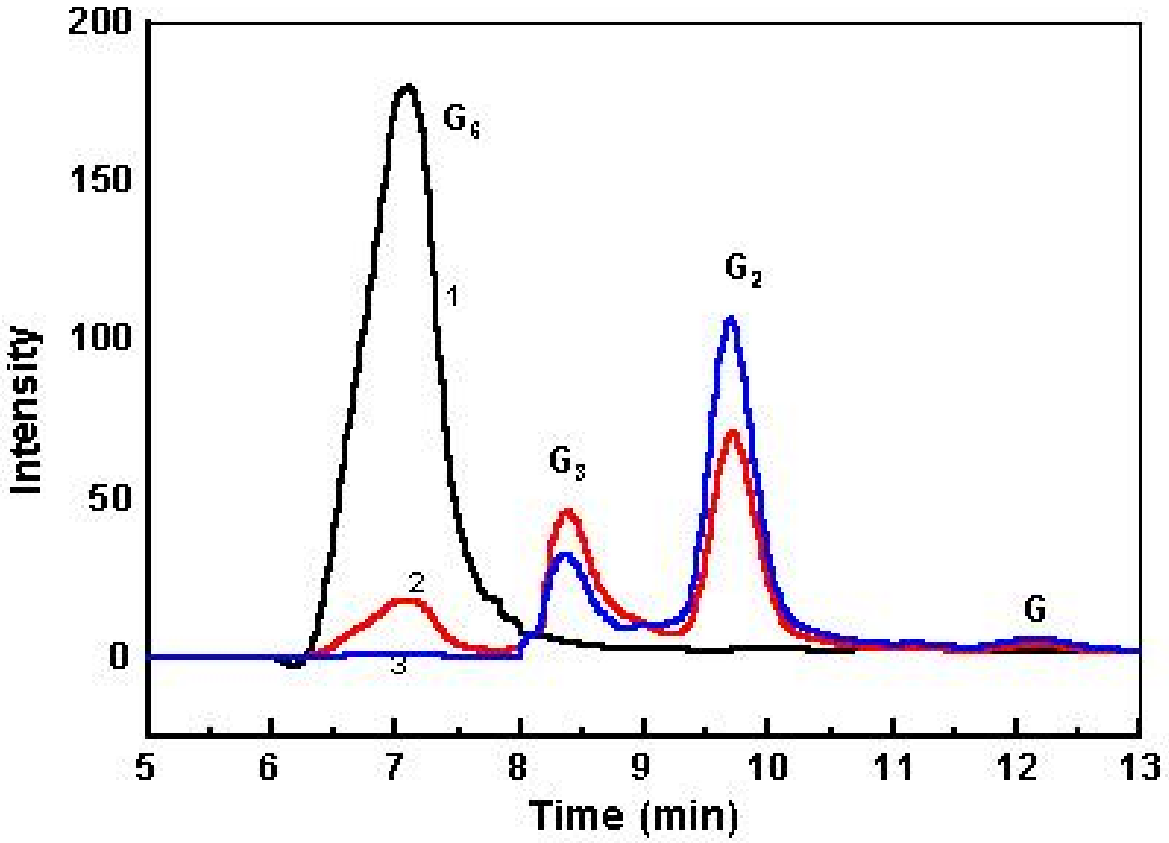


Fig.4.3E. Action of Endoglucanase A on cellohexaose (G_6).

The reaction mixtures (1.0 ml) consisting of substrate (G_6,1mg) in 0.05M Na- phosphate pH 7.0 buffer and enzyme (3μg) were incubated at 60°C for 1h. The samples 50μl were removed at different time intervals and analyzed by (HPL.C) .1: at 0 min ; 2: at 30 min and 3: at 1 h. interval. Cellobiose (G_2) and cellotriose (G_3) appeared as the major products of hydrolysis at all stages. Glucose was detected on prolonged incubation period

4.3.4. The crystallinity of cellulose samples

X-ray diffraction patterns of different cellulose samples are presented in Fig.4.4 In case of microcrystalline substrates, filter paper and Avicel P.H. 101, the diffraction peak of (101), ($1\overline{0}1$) and (002) appeared at 2θ of 14°, 16°, and 23° respectively. In case of selected cellulose samples the highest intensity peak occurred at (002) plane that appeared in the range of 22-24° of 2θ. However, in case of Walseth cellulose, cotton, MOW pulp, solka floc and cellulose powder there was shift of 2θ values at (001) plane. The crystallinity index values for Avicel P.H.-101, Walseth cellulose, Cellulose powder 123, Solka floc BW 100, Filter paper (Whatman No. 1), dewaxed cotton linters and MOW pulp were found to be 84, 31, 80, 83, 91, 75 and 83 % respectively.

4.3.5. Analysis for bound water content using TG / DTA studies

The interaction of water with the cellulose polymer markedly influences physicochemical properties of both cellulose and water (Hatakeyama et al., 1987). In polymers such as cellulose, melting and crystallization temperatures of bound water are higher and lower, respectively than that of free water (Nakamura et al., 1981; Hoffman and Hatakeyama,1995;Hatakeyama and Liu, 1998). As the heat of vaporization of water molecules is large, it can be used to determine traces of water in the samples. DSC studies on cotton, and regenerated cellulose samples suggested that the vaporization of bound water occurs at higher temperature than that of free water (Hatakeyama et al., 2000). In the present work thermogravimetric method is used to evaluate bound water content of different cellulosic samples (Fig.4.5). Walseth cellulose was heated at the rate of 3°C /min in the temperature range of 30-110°C. In this case the weight loss was observed in two stages I, II, corresponding to W_f and W_b, respectively. The evaporation of free water (W_f) at the surface occurs up to 60°C, while the same occurs at around 80°C for tightly bound water. This observation was confirmed as Differential thermogravimetric (DTG) curve in Fig.4.5.The values are expressed in g/g of cellulose substrates in Table 4.3.The amount of bound water varied in different samples. This is because the bound water content depends on molecular architecture of cellulose chains and its capacity to form hydrogen bonds with water molecules, DTA curves for Walseth cellulose and dewaxed cotton linters suggested

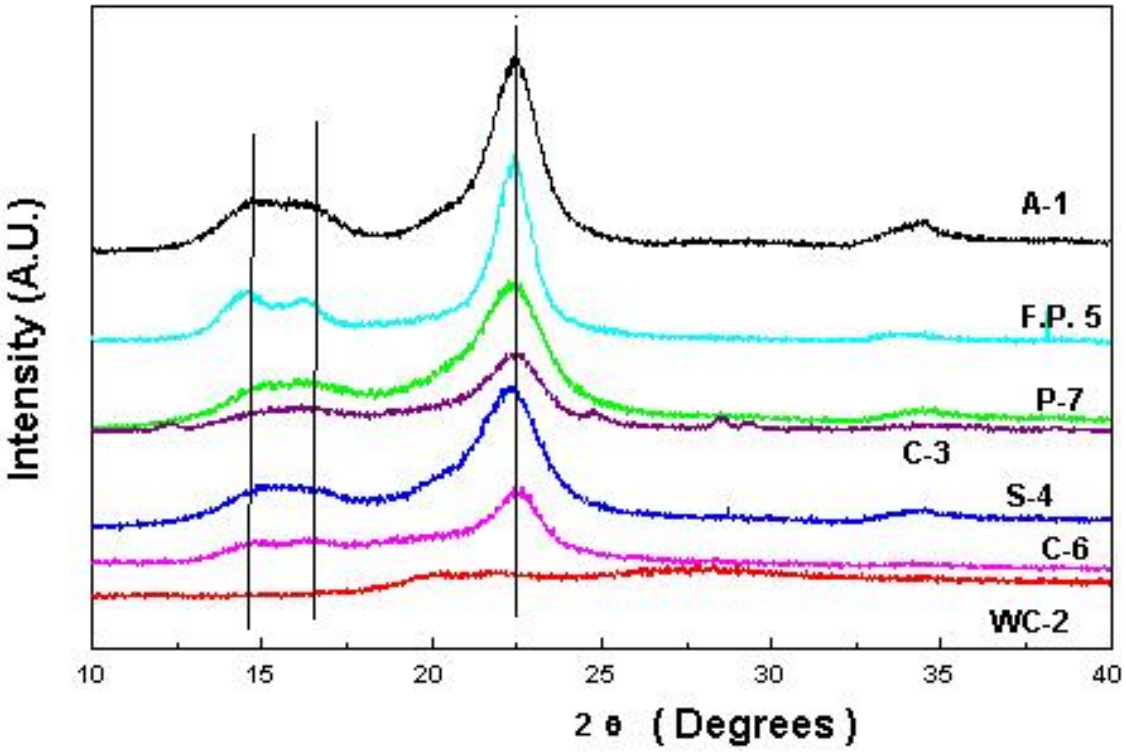


Fig.4.4. X ray diffraction patterns of different cellulose samples.

A-1= Avicel PH 101
C-3= Cellulose 123
F.P.-5 = Filter paper (Whatman No.1.)
P-7= Mixed Office Waste paper pulp
WC-2 = Walseth cellulose
S-4 = Solka floc (BW 100)
C-6= Dewaxed cotton linters

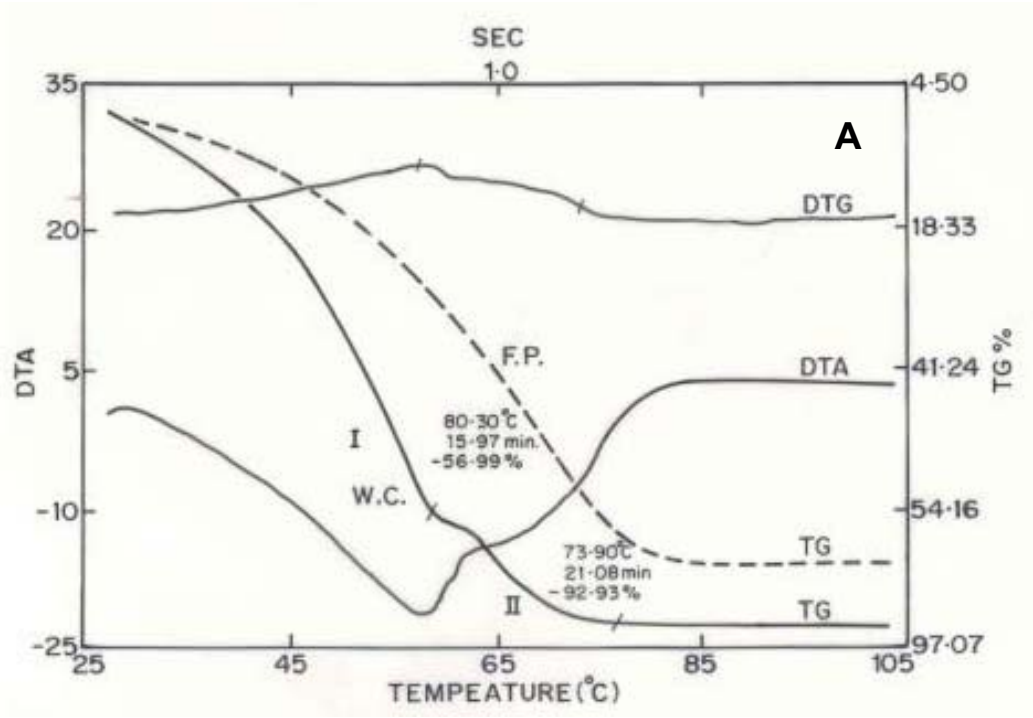


Fig.4.5. : TG/ DTA/ DTG thermograms for vaporization of water in the cellulose Samples.

TG : Curves for thermogravimetric data

DTA: Curve for differential thermal analysis of the same sample.
DTG: Curve for differential thermogravimetric analysis of the same sample.

I and II represents weight loss occurred due to vaporization of free water and bound water respectively.

A : Comparison of bound water content in WC (____) for walseth cellulose and F.P. (- - - - --) for filter paper.

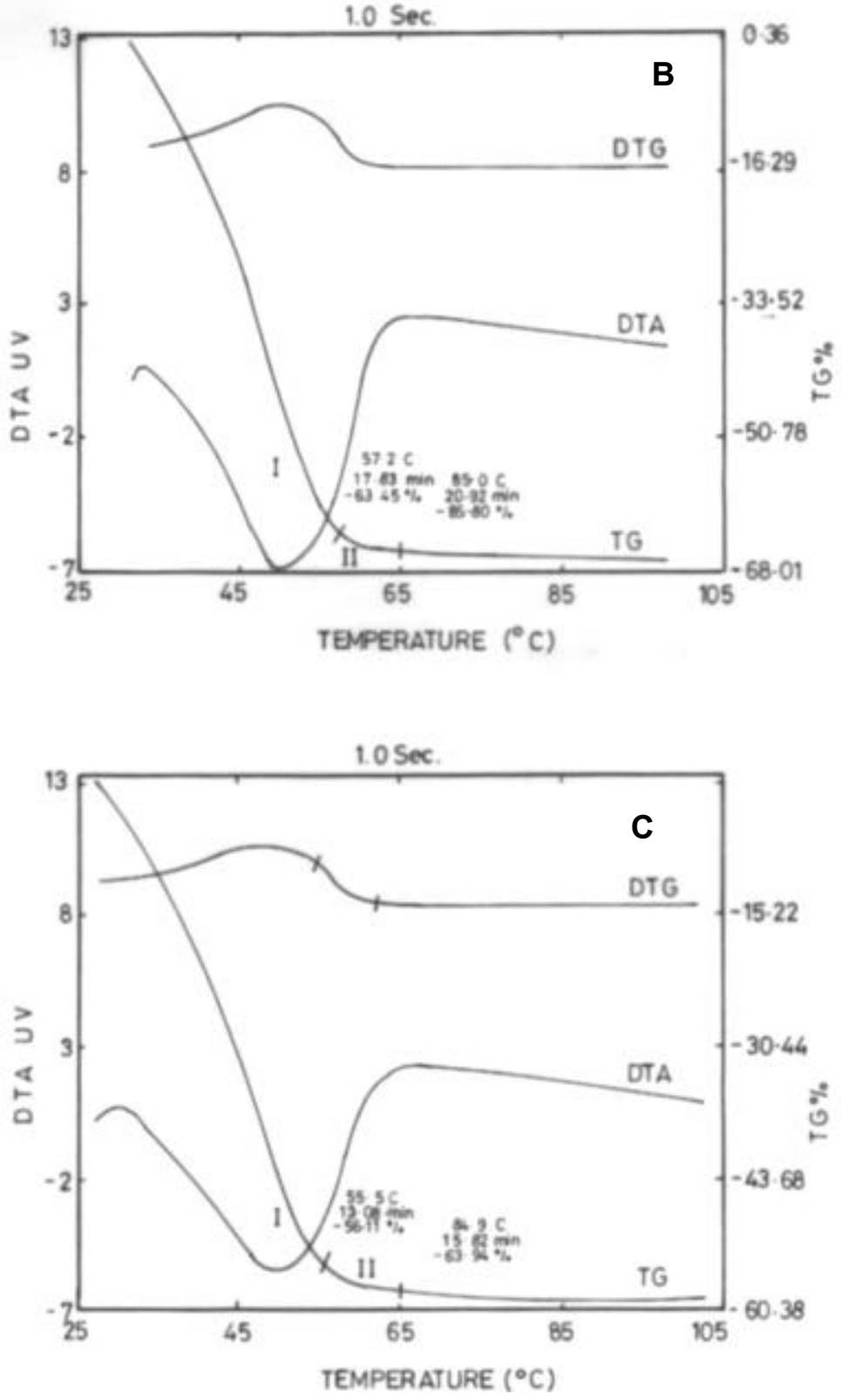


Fig.4.5 B : Bound water content in Avicel (A_1)

C : Bound water content in Cellulose powder (C_3)

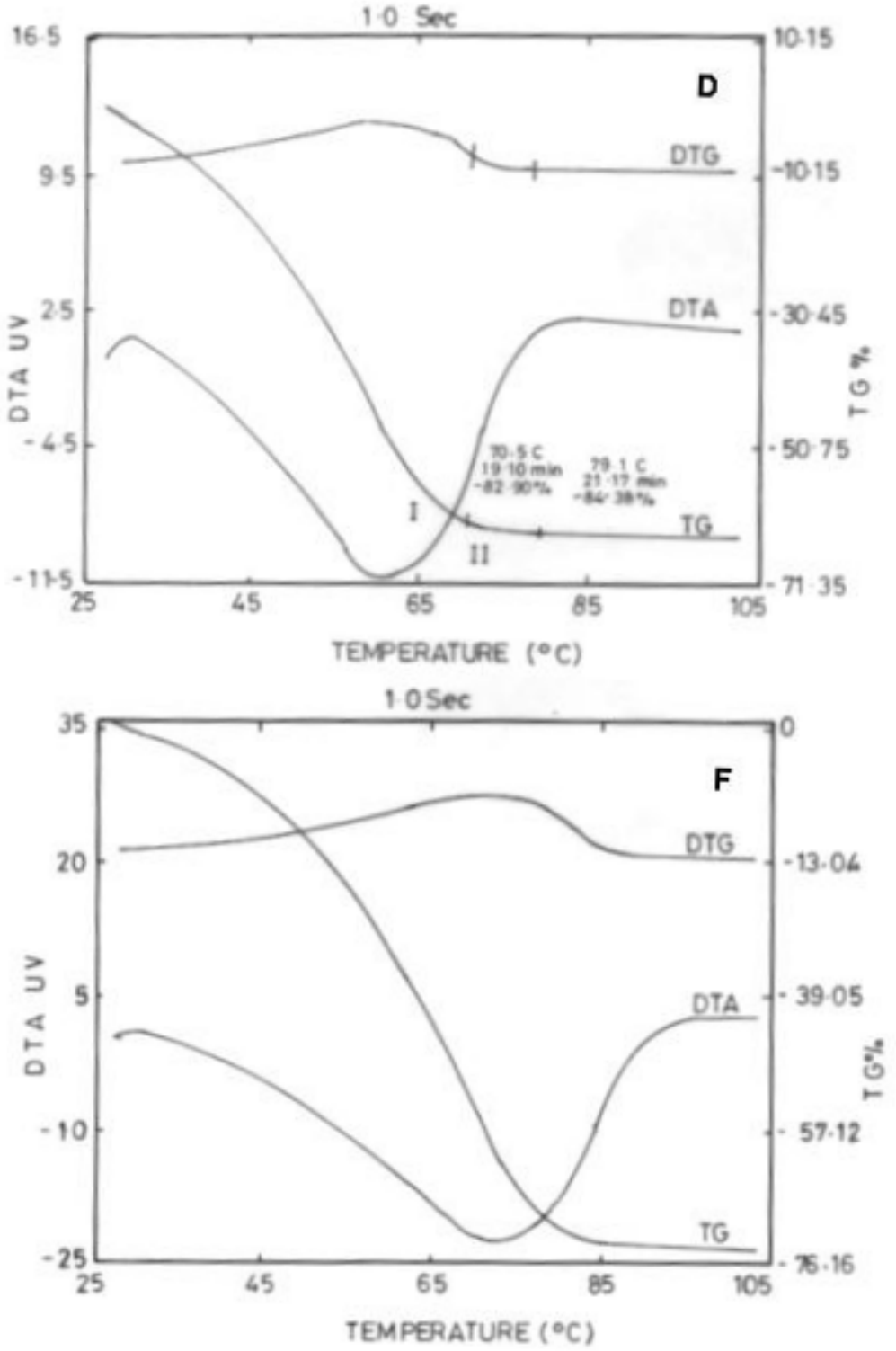


Fig.4.5 D : Bound water content in Solka Floc (S4)

E : Bound water content in Filter Paper (FP 5)

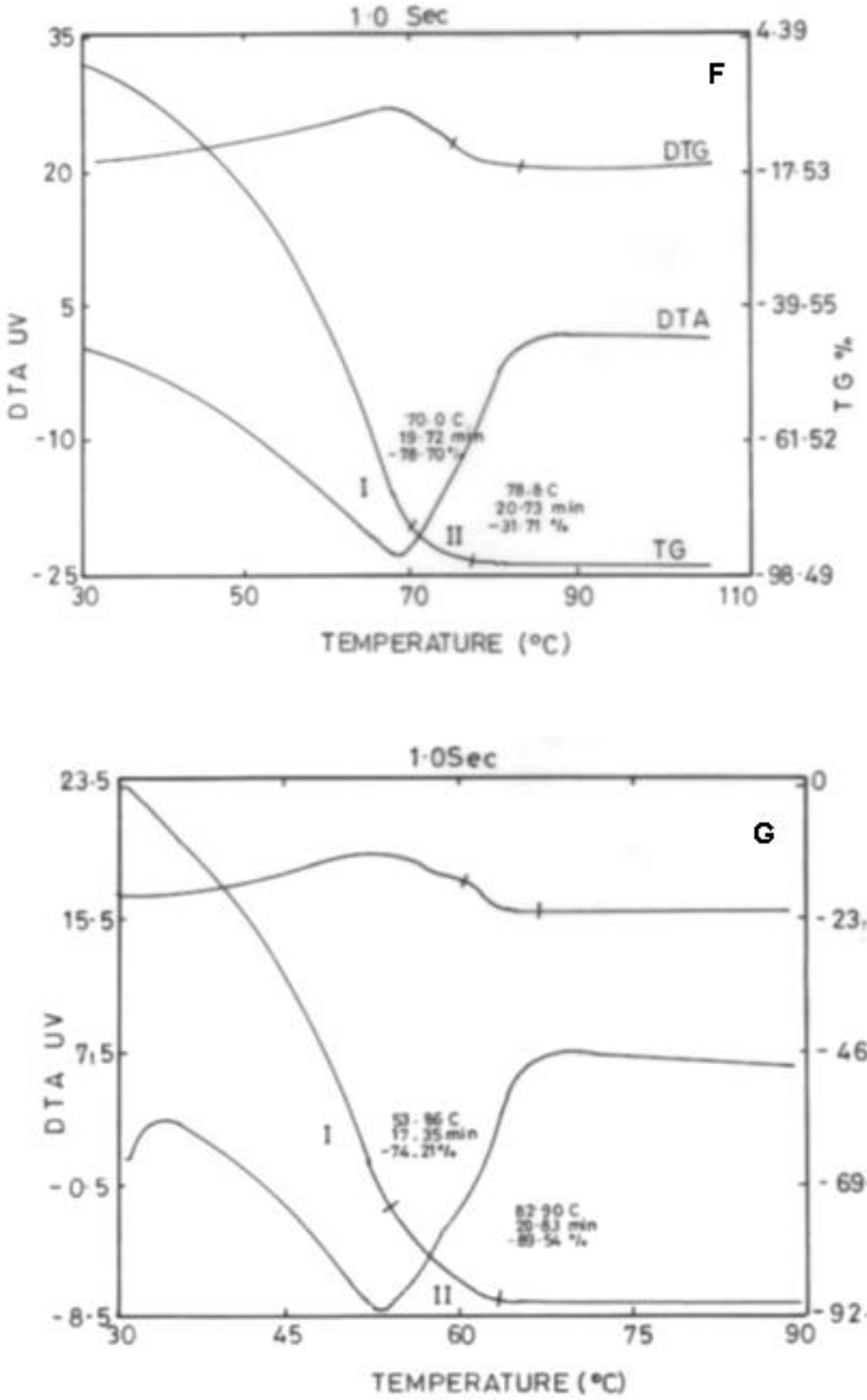


Fig.4.5 F : Bound water content in Cotton (C_6)

G : Bound water content in MOW Pulp (P_7)

vaporization of W_b occurs around 80°C that is the highest temperature in all the samples examined in the present study. This indicated that the W_b *is* tightly bound to cellulose chains in these substrates as compared to other substrates. The highest amount of bound water was observed in Walseth cellulose (phosphoric acid swollen cellulolse) and it was 0.92 g/g of substrate. In case of Whatman filter paper (Fig.4.5A) bound water was not detected. The bound water content was dependent on the degree of crystallinity of cellulose. Higher the crystallinity, lower is the bound water content (Table.4.3).

Table 4. 3. Effect of crystallinity index on bound water content of different cellulosic substrates

Sample	Substrate	Crystallinity Index (%)	Bound water g/g
A-1	Avicel P.H.101	84	0.068
W.C.- 2	Walseth Cellulose	31	0.920
C-3	Cellulose Powder (123)	80	0.096
S-4	Solka Floc	83	0.045
F.P.-5	Filter paper (No.1)	91	N.D.
C-6	Cotton	75	0.276
P-7	MOW Pulp	84	1.466

N.D.- Not detected

The moisture content of cellulose depends on the molecular structure of amorphous region of cellulose (Magane et al., 1947; Froix and Nelson, 1975). The water molecules can only diffuse through amorphous regions of cellulose samples (Long and Richman, 1960*)*. The amorphous regions in cellulose molecule can form

hydrogen bonds with water molecules and can be regarded as the site for adsorption of water molecules (Nakamura et al., 1981). Walseth cellulose is regenerated cellulose and *o* phosphoric acid treatment for its preparation resulted in decrease in the crystallinity of cellulose therefore, it showed the high amount of bound water content. Much higher amount of bound water was observed in MOW pulp despite high crystallinity of the samples. Different fillers such as calcium carbonates, starch are used for improving brightness and strength properties of high quality writing grade paper. MOW pulp is produced by repulping of such papers. Thus, the higher amounts of bound water content in these samples may be attributed to the capacity of these fillers to bind water molecules. The thermograms for vaporization of water in the different cellulose samples are presented in Fig.4.5.(A-G). The amorphous structure of natural cellulose is not completely random, but molecular chains are arranged unidirectional along the fibre axis of cellulose. The molecular rearrangement in the amorphous region of natural cellulose occurs reversibly by absorption and desorption of water (Hatakeyama and Hatakeyama, 1998). The molecular chains in these regions take an ordered form when a small amount of water is added. Molecular chains in the intermediate region reversibly change from regular structure to random molecular arrangement in the presence of a trace amount of water. Further, the higher order structure of amorphous cellulose chains convert to a more random form by loosing a small amount of bound water (Hatakeyama et al., 2000). Cellulose hydrolysis by microbial cellulases is often limited by degree of crystallinity of the substrates. Hydrogen bonds between cellulose microfibrils in crystalline regions are so strong that no further hydrogen bonds with water molecules can be formed. However, the amorphous regions of the cellulose can form hydrogen bonds with water molecules. As a result the water molecules in these regions remain as bound water. The Endoglucanase A is an endo acting cellulase that preferentially acts at these amorphous regions. The changes in amount of bound water during hydrolysis were monitored to determine the alteration in the amorphous regions. Data presented in Table 4.4 indicated that, the W_b content of Walseth cellulose was lowered during the enzymatic hydrolysis as a function of time. Similar studies using DSC to estimate the bound water content in cotton cloths are useful in establishing new mechanisms of detergency. The bacterial endoglucanase component added to detergents, acts at extensively soiled amorphous regions of cotton clothes and improves brightness of the soiled clothes (Murata et al., 1993). Thus, Differential Thermal Analysis and

Thermogravimetric methods to assess bound water in cellulose can be valuable in understanding the enzymatic hydrolysis of cellulosic substrates of different crystallinity.

Table 4.4.Time dependent hydrolysis of walseth cellulose and its effect on bound water content

Sample	Time (min)	Bound water g/g	Reducing sugars (μg)
1	0	1.930	2.3
2	30	1.698	20
3	60	1.559	50
4	120	1.186	82
5	240	1.032	110

4.3.6. *Short fibre formation mechanism and its significance in cellulose hydrolysis*

Various researchers suggested that the first step in enzymatic degradation of the cellulose microfibrills was their stratification along the longitudinal axis leading to the formation of even more thin subfibrills (White and Brown 1981; Chanzy and Henrissat 1983; Chanzy et al., 1983). Halliwell and Riaz, 1970 demonstrated that the low molecular weight endoglucanase from *T.koningii* is responsible for the formation of short fibres from native cellulose substrate. In the present studies enzymatic action of Endoglucanase A resulted in the release of short fibres from filter paper. The short fibre formation was also accompanied by reducing groups in the reaction mixture. The release of short fibres can be observed within 15-30 min. The fragmentation of filter paper increased initially with increase in time (Fig.4.6). However, there is decrease in short fibre formation after 6h.of interval without decrease in reducing sugar formation. This may be attributed to preferential hydrolysis of short fibres formed by Endoglucanase action. The SEM studies confirmed the short fibre formation by Endoglucanase A. The average size of the short fibre formed after 6h of incubation with Endoglucanase A varied between 20-100 μm as compared to 300-500μm in the control experiment without enzyme (Fig.4.7).

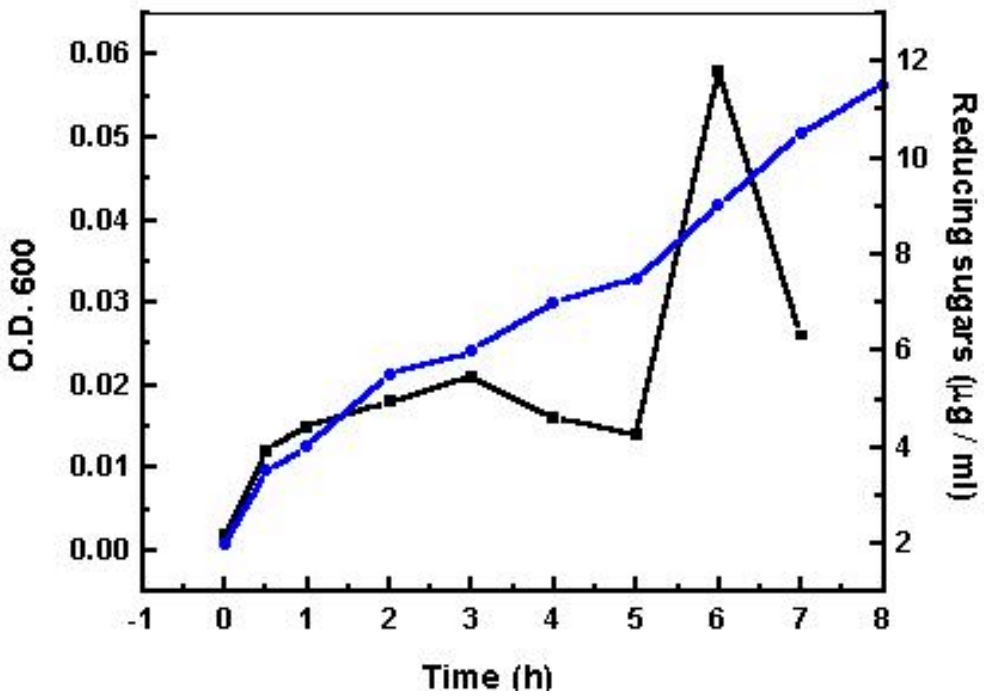


Fig.4.6. Short fibres formed from filter paper due to action of Endoglucanase A from alkalotolerant *Fusarium* sp

The rate of release of fibres in the reaction mixture was recorded from absorbance at 600 nm. The plotted values are corrected for the absorbance of the control.
Symbols short fibres (■), reducing sugars (•)

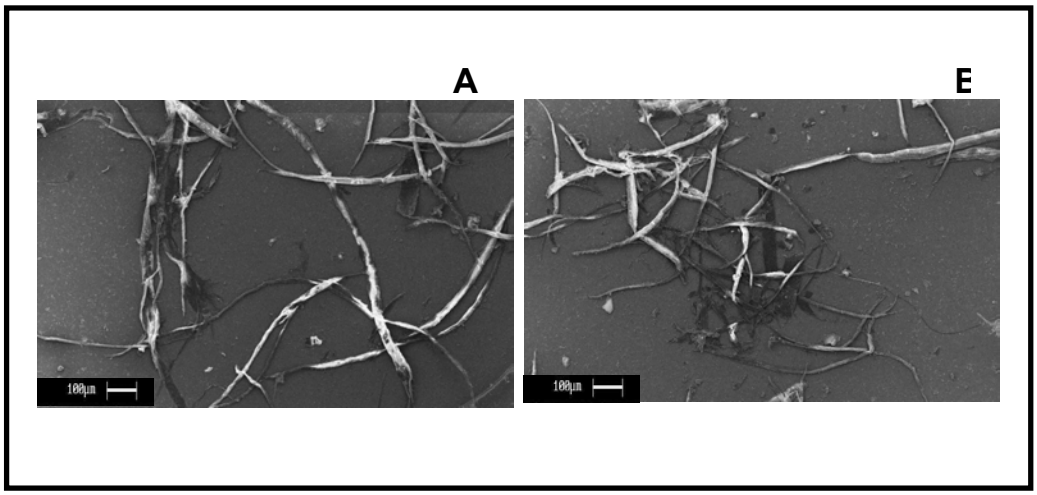


Fig. 4.7. The average size of cellulose fibres

A : Average size of cellulose fibres varied between 300-500mm in control (without enzyme) sample.

B : Short fibres formed after 6 h incubation with enzyme fraction Endo A size varied between 20- 100mm.

Many researchers attributed the short fibre formation activity as a property of endoglucanases (Halliwell and Riaz, 1970; Berghem and Petersson, 1973,1976). However, the exact mechanism of short fibre formation is still not clear. Acoording to White and Brown (1981) endoglucanases are responsible for short fibre formation, while Chanzy et al. (1983) suggested that it is the property of cellobiohydrolase. Vaheri (1982) suggested that oxidation of cellulose is an important step and –onic acids precedes the generation of short fibre by endoglucanases. The 1,4-β-D-glucan cellobiohydrolase from *T. viride* was completely devoid of short fibre forming activity. However, two purified endoglucanases from the same culture showed short fibre forming activity (Berghem and Petersson, 1973,1976). On the contrary, endo type of mode of action of (1--4)-β -D glucan cellobiohydrolase from *S. rolfsii* was demonstrated by Sadana and Patil (1985). Wood et al. (1989) suggested that addition of traces of endoglucanase to cellobiohydrolases I and II from *P. pinophilum* was necessary for the release of the short fibre. According to Klyosov (1990) the short fibre formation results due to tight adsorption of cellulases on cellulosic substrate. Both endoglucanases and cellobiohydrolases capable of tightly binding on cellulose can bring about mechanical dispersion of cellulose. Rabinowich et al. (1982) suggested that the mechanical dispersion is induced by adsorption of enzyme on cellulose defects followed by their penetration into interfibrilar space. This in turn concentrates the enzyme in cellulose defects, resulting in an increase in mechanical pressure on the walls of pores cavities and micro cracks of cellulose. The water in the defects in turn penetrates further and further inside the capillary spaces forcing them apart. This is followed by the hydrolytic action of enzyme at amorphous regions that cleaves the microfibrils apart.

4.4. Conclusion

The Endoglucanase A from alkalotolerent *Fusarium* sp has a typical endo type of action on the cellulose substrates. The substrate specificity of the enzyme was restricted to β -1-4 glycosyl linkages only. The enzyme was inhibited by low concentrations (0.1%, 1 % and 5%) of cellobiose and glucose. The product analysis of reaction mixtures with HPLC revealed that the Endoglucanase A did not hydrolyzed cellobiose. However, it attacked cellotriose and higher chain length cello-oligosachharide preferentially at the internal glycosidic bonds. The results indicated that the active site of the enzyme may be 4-residue long. It appears that the enzyme

belongs to family 7 cellulases because (i) it can hydrolyze cellotriose (ii) it is inhibited by low concentrations of glucose and cellobiose (iii) the major product of hydrolysis is cellobiose (iv) and its characteristic ability to form short fibres from cellulose substrate. These endoglucanases are efficient detergent additives and are useful in preventing back staining problem in denim washing process. The importance of short fibre forming activity of Endoglucanase A from alkalotolerent *Fusarium* sp in biodeinking of mixed office waste paper is demonstrated in Chpater 6. Thus the characterization studies on substrate specificity of enzyme in this Chapter marks the enzyme as a 'soft' cellulase having potential for industrial applications.

Chapter 5

Immobilization of Cellulases on Novel Supports

A: Immobilization of Endoglucanase in Fatty Amine Films

B: Enzyme Bioconjugates with Colloidal Gold Nanoparticles

C: Three Dimensional Assembly of Gold Nanoparticles on Polyurethane Microspheres to form 'Core-Shell' structures

Summary

The enzyme Endoglucanase A from an alkalotolerant *Fusarium* sp has been immobilized in / on different novel supports such as fatty amine films and nanoscale curved surfaces using different strategies. The comparison of various biochemical properties of immobilized enzyme with free enzyme is presented in this Chapter.

A: *Immobilization in thermally evaporated fatty amine films*

The Endoglucanase A was electrostatically encapsulated in thermally evaporated fatty amine films and the enzymatic activity of the biocomposite film under different pH and temperature conditions were studied. The characterization of immobilized enzyme in film form was carried out by Fourier Transformed Infrared spectroscopy (FTIR). Substrate protection of the enzyme by carboxymethyl cellulose (CMC) was essential to stabilize the enzyme against inactivation in the lipid matrix. The optimum temperature of the enzyme was increased by 10^{o}C as compared to free enzyme in solution. The enhanced stability at high temperatures was coupled with improved catalytic activity of the enzyme-lipid biocomposite films at alkaline pH conditions.

B: *Enzyme bioconjugates with colloidal gold nanoparticles*

The utility of nanoscale-curved surfaces provided by colloidal gold particles was exploited for immobilization of Endoglucanase A. The high surface-to-volume ratio offered by colloidal particles resulted in higher loading of enzyme as compared to that afforded by other immobilization protocols. Covalent and other secondary interactions are responsible for this immobilization process. The formation of endoglucanase-colloidal gold bioconjugates was accomplished by simple mixing of the two solutions at pH 5. The bioconjugate formation process was monitored by UV-visible spectroscopic studies, while Transmission Electron Microscopy (TEM) measurements were used to characterize the particle size of the bioconjugates. The secondary and tertiary structure of the enzyme in the bioconjugate system was studied by FTIR and fluorescence spectroscopy, respectively. The enzyme activity in

the bioconjugate system was comparable with that of free enzyme in solution. The enzyme in the bioconjugate system showed an increased half-life at 60^{o}C and pH 9 in comparison with free enzyme in solution.

C: Three dimensional assembly of gold nanoparticles on polyurethane microspheres to form 'Core-Shell' structures

The separation of colloidal gold bioconjugates (Chapter 5 B) from the reaction mixture is difficult. Therefore, the reuse of the enzyme is not possible. To overcome this problem, a novel biocatalyst was synthesized by immobilizing enzyme on the surface of modified polyurethane microspheres by gold nanoparticles. The binding of the gold nanoparticles to the polyurethane polymer surface occurs through nitrogen groups in the polymer backbone. These surface modifications induce strong binding of enzyme Endoglucanase A on polyurethane microspheres. Scanning Electron Microscopic (SEM) studies revealed the binding of endoglucanases in 3-D core shell assembly. These microspheres were conveniently separated from reaction mixture by centrifugation. It is possible to reuse the Endoglucanase-nanogold-Pu assembly for at least five cycles. As the enzyme is present on the surface of the microspheres, it does not require any substrate protection. The Endoglucanase-nanogold-Pu assembly showed comparable specific activity to free enzyme in solution and improved temperature stability.

Introduction

Cellulases are commercially important enzymes that have applications in various ecofriendly processes. Generally the stable enzyme preparations are preferred for industrial applications. This is because the high temperature, pH and other harsh processing conditions affect the enzymes activities. These problems can be partially overcome using immobilized enzymes over the free enzymes (Sastry, 2002). The immobilized enzymes have following advantages: (i) they are convenient for handling (ii) they can be easily separated from the product; thus lesser investments on downstream processing (iii) they can be reused for many reaction cycles that in turn reduces the cost of the process and (iv) they show improved thermal and pH stabilities and can be used in harsh operational conditions (Zaborsky, 1973).

A number of different interactions between the protein and the matrix can be utilized for its immobilization. This can be achieved by either modifying the protein (so as to suit the matrix) or by modifying the matrix for ease of immobilization. A variety of methods can be used to immobilize the enzymes. The general methods used in immobilization of cellulases are as follows: (i) physical adsorption to a solid phase (Fadda et al., 1989; Suvajittanont et al., 2000), (ii) covalent bonding to a solid phase (Rogalski, et al., 1985; Onyezilli, 1987; Garcia et al., 1989) (iii) covalent bonding to soluble polymers (Mishra et al., 1983; Taniguchi et al., 1989, 1992; Sardar et al., 2000) (iv) cross linking with bifunctional reagents (Flachner et al., 1999), (v) inclusion in the gel phase (Parikh, et al., 1974 ; Kumakura and Kaetsu, 1982, 1984 a,b, 1985) and (vi) encapsulation in different matrixes (Busto et al.,1998; Gole et al.,2001a). Although, physical adsorption is the simplest method for immobilizing an enzyme on a solid support, it is not suitable for cellulases. Much of the cellulase activity would be lost from the support during operation through desorption followed by preferential readsorption on the substrate (Ghose and Bisaria, 1979). Inclusion of enzymes in gel phase or encapsulation into matrix would require that the substrate be completely solubilized and allowed to diffuse freely. Covalent attachment of cellulases to a support would prevent loss caused by adsorption as well as mass transport problem seen in case of gel entrapment. However, selection of improper support and /or cross-linking agents may result in modification of enzyme surface and making it

inactive. Thus, for successful immobilization of any enzyme the selection of proper immobilization protocol is the key step (Mosbach, 1976; Kolot, 1981).

A variety of materials can be utilized for immobilization of cellulases based on different immobilization protocols. However, the choice of appropriate matrix depends on the ultimate application of immobilized enzyme. In general, following points needs to be considered before choosing the matrix for enzyme immobilization.

1. The matrix should be robust, stable and inert; it must be biocompatible and should not interfere with enzymes native structure.
2. The matrix should protect enzyme against microbial deterioration and should render the enzyme accessible to cofactor, metal ions etc.
3. The matrix must permit substrate accessibility to immobilized enzyme and thus avoid 'mass transport problem'.
4. The immobilization matrix should have high loading factor for the enzyme.
5. The immobilization process must be quick, inexpensive and should be ecofriendly.

Till date cellulases have been immobilized on variety of supports for their better performance in sacccharification processes (Ghose and Kostick, 1970; Mandels et al., 1971, Rao and Mishra, 1984; Chakrabarti and Storey, 1989; Taniguchi et al., 1992). However, the utility of the cellulases in other industrial applications such as in detergents, biodeinking may be greatly enhanced if it could be immobilized onto/within suitable solid supports. The immobilized enzyme that could be operated efficiently under alkaline conditions and at elevated temperatures is often desired. Most of the traditional supports used for immobilization of cellulases suffer from disadvantages such as loss of enzyme activity due to leaching out of the enzymes (Ghose and Kostick, 1970), poor substrate diffusion, Inactivation of enzyme due to modification of enzyme native structure etc. Recent developments in nanotechnology offer various kinds of nano materials that can be used as the templates for immobilization of biomolecules.

The studies in this Chapter are focused on immobilization of cellulases on novel supports. The specific objectives were: (i) to evaluate various nanoscale supports for immobilization of Endoglucanase A from alkalotolerent *Fusarium* sp

(ii) to determine optimum conditions for such immobilization process (iii) to characterize such biocomposits by biophysical techniques and (iv) to determine the optimum pH, temperature, and thermal stabilities of immobilized enzyme and compare them to those of free enzymes.

5A: Immobilization of Endoglucanase in Fatty Amine Films

5.A.1. Introduction

The studies on protein-lipid interactions have fundamental as well as technological importance. It is extremely difficult, if not impossible, to study the protein-lipid interactions *in-vivo* in membranes. Hence micelles, microemulsions, monolayers, bilayers, vesicles, host-guest systems, and polyions have been used as membrane mimetic agents (Fendler, 1982). Earlier investigators have demonstrated that lipids in the form of monolayers and bilayers can be used as a versatile matrix for intercalation of proteins/enzymes with negligible distortion to their three-dimensional (tertiary) structure. This enables them to perform their biological functions without hindrance (Hamachi et al., 1994; Mirksich et al., 1995; Sastry, 2002). Lipid membranes are advantageous due to their membrane mimicking capability, bio-friendliness, flexibility and inertness.

The entrapment of the enzyme, Endoglucanase A, in thermally evaporated fatty amine films such as octadecylamine is described here. The entrapment being driven to a large extent by electrostatic interactions between the protonated amine groups in the film and negative charges on the enzyme surface (Fig.5A.1). Substrate protection of the Endoglucanase by pre-complexing the enzyme with carboxymethyl cellulose (CMC) before immobilization was essential for retaining activity of the Endoglucanase enzyme in the encapsulated form. The immobilization of the enzyme in the lipid matrix resulted in a shift of the optimum temperature of an enzyme from 60°C to 70°C.The immobilized Endoglucanase A showed activity up to pH 9 under conditions where the activity of the free enzyme in solution falls rapidly.

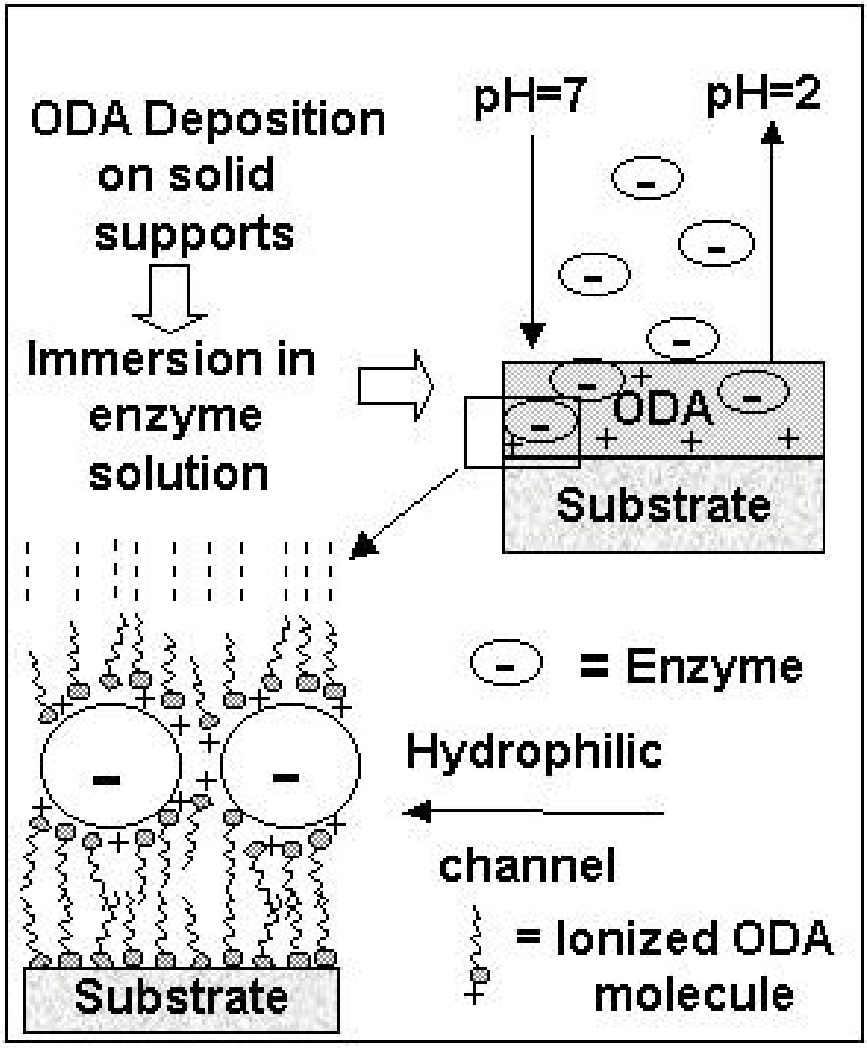


Fig.5A.1. Schematic diagram showing various steps involved in the lectrostatic immobilization of Endoglucanase in ODA films and leaching out of the enzyme by varying pH of the solution.

5.A.2. Materials and Methods

5.A.2.1. Chemicals

Octadecylamine (ODA) and carboxymethyl cellulose (CMC) were obtained from Aldrich and Sigma Chemicals, respectively and used without further purification. All buffer salts, and reagents were from standard commercial sources and of highest quality available.

5.A.2.2. Production and purification of Endoglucanase A

The alkalotolerant *Fusarium* sp was grown in Reese's media (Mandels et al., 1962) containing 1% cellulose powder and 0.05% Tween 80. The enzyme was produced as described in Chapter 2. Endoglucanase A was purified to homogeneity from the culture filtrate by ultrafiltration using Amicon filtration unit (filter PM 10), gel filtration on BioGel P-100 and isoelectric focusing as described in Chapter 3

5.A.2.3. Deposition of ODA thin films.

250 Å thick ODA films were deposited on gold-coated AT cut quartz crystals (for QCM measurements) and Si (111) substrates (for FTIR, SEM and enzyme activity measurements) by thermal evaporation in an Edwards E308 chamber at a pressure of 1×10^{-7} Torr. The thickness of the film was monitored *in-situ* during deposition with an Edwards quartz crystal thickness monitor.

5.A.2.4. Substrate protection of Endoglucanase A molecules

A 2 mg/ml solution of Endoglucanase A in 0.05M phosphate buffer, pH 7 was mixed with CMC in different proportions and the mixture were preincubated at 4°C for 30 minutes. An enzyme / CMC molar ratio of 1:2 was found to be ideal for encapsulation studies and used for all experimentation at 4°C.

5.A.2.5. *Encapsulation of the enzyme in ODA thin films.*

A 2 mg/ml solution of substrate-protected Endoglucanase A (1:2 molar ratio of enzyme to substrate) was prepared in different buffer solutions held at different pH values (pH 5, 0.05 M sodium citrate buffer; pH 7, 0.05 M sodium phosphate buffer; pH 9, 0.05 M Glycine-NaOH buffer). The enzyme diffusion into the thermally evaporated ODA films was monitored by immersion of separate 250 Å thick ODA covered gold coated AT cut quartz crystals for different time intervals in the different enzyme solutions at 4°C and measuring the frequency change of the crystals *ex-situ* after thorough washing in deionized water and drying over flowing nitrogen) of the crystals on an Edwards FTM 5 instrument operating at a frequency stability and resolution of $\pm$ 1 Hz. The frequency changes were converted to mass loading using the Sauerbrey formula (Sauerbrey, 1959).

5.A.2.6. *Contact angle measurements.*

Contact angle measurements of a sessile water drop (1μl) on bare Si (111) substrates, 250 Å thick ODA films on Si (111) substrates before and after encapsulation of the substrate protected enzyme at pH 7, as well as a drop-dried film of the substrate protected enzyme (pH 7) on the surface of a 250 Å thick ODA film were carried out on a Rame Hart 100 Goniometer.

5.A.2.7. *Scanning Electron Microscopy (SEM)*

SEM measurements on 250 Å thick ODA films on Si (111) wafers before and after incorporation of substrate protected enzyme together with films of the substrate protected enzyme drop-dried onto a 250 Å thick ODA film on Si (111) were performed on a Leica Stereoscan-440 scanning electron microscope.

5.A.2.8. *Secondary structure of enzyme entrapped in ODA films.*

FTIR measurements of the 250 Å thick ODA films on Si (111) substrates before and after entrapment of the substrate protected endoglucanase molecules at pH 7 were performed on a Shimadzu FTIR-8201 PC instrument operated in the diffuse

reflectance mode at a resolution of 4 cm^{-1}. To obtain good signal to noise ratio, 256 scans of the bio-conjugate film were taken in the range of 400 – 4000 cm^{-1}.

5.A.2.9. *Enzyme assays of the immobilized Endoglucanase A molecules.*

The 250 Å thick substrate protected Endoglucanase-ODA composite films were formed by encapsulation of mixtures of different molar ratios of Endoglucanase/ CMC at the three different pH values. The activity of these films was detected by using aqueous solution of CMC (10 mg/ml in sodium-phosphate buffer, pH 7) and incubating the mixture at 60°C for 1 h. The reducing sugars released were determined by the Nelson-Somogyi method (Nelson, 1944;Somogyi, 1952). For comparison, the activity of an identical amount of different molar ratios of Endoglucanase/CMC molecules (held at the three different pH values mentioned) in solution was determined as described above. As shown subsequently, the molar ratio of 1:2 (enzyme/CMC) yielded optimum enzyme activity in the film and was chosen for all further experimentation. Five 250 Å thick similarly loaded endoglucanase-CMC (1:2 molar ratio) films (film dimensions = 4 cm^2) were formed at the three different pH values, were tested for activity, to determine the standard deviation to the data. A control experiment was performed to estimate the enzyme activity of an as-deposited 250 Å thick ODA film under similar assay conditions (film dimensions = 4 cm^2). Another control experiment was performed in which a 250 Å thick substrate protected enzyme-ODA molecule composite film (4cm^2 film dimensions) was assayed at a temperature of 4°C, pH 7 (0.05 M sodium phosphate buffer). This was done in order to estimate reducing sugars released, if any, during the "protection phase".

5.A.2.10. *Temperature profile for activity of the Endoglucanase-ODA composite films.*

The activity of the substrate protected Endoglucanase-ODA (1:2 molar ratio) composite films formed at pH 7 was tested in a temperature range of 40-90°C and compared with that of the substrate protected enzyme (1: 2 molar ratio of enzyme: substrate; pH 7) in solution. These measurements were carried out for five Endoglucanase-ODA composite films at each one of the temperatures.

5.A.3. Results and Discussion

5.A.3.1. *Reversible immobilization of Endoglucanase in fatty amine matrix*

Three 250 Å thick octadecylamine (ODA) coated quartz crystals were immersed for different time intervals into substrate-protected Endoglucanase solutions (1:2 molar ratio of enzyme: substrate) held at different pH values of 5, 7 and 9 respectively at 4°C. As shown in Fig.5A.1. the enzyme molecules diffuse into the ODA matrix and are expected to be immobilized within the hydrophilic regions of the lipid bilayers of the fatty lipid matrix. The QCM mass uptake recorded from the above three films as a function of time of immersion in the enzyme solution is shown in Fig.5A.2.

Maximum incorporation of the enzyme takes place at pH 5 instead of pH 7 which is rather unexpected if one considers only electrostatic interactions controlling the diffusion process of the enzymes into the ODA matrix (pKb of ODA 10.5; pI of Endoglucanase 4.5). However, the incorporation of the substrate-protected enzyme at pH 9 is less than at both pH 7 and pH 5, indicating that electrostatic interactions do determine to a large extent the entrapment of the enzymes in the lipid matrix. The discrepancies observed could be attributed to the role of secondary interactions such as hydrophobic and hydrogen bonding that would also play a major role in the encapsulation process. One of the advantages of using electrostatic interactions based on functional groups, whose degree of ionization may be controlled by variation in solution pH is that the entrapped enzyme molecules may be 'leached out' of the biocomposite material. The inset of Fig.5A.2 shows the release of the substrate-protected Endoglucanase molecules by immersion of the fully loaded ODA film formed at pH 7 during immersion in the enzyme solution maintained at pH 2 and at pH 7. At pH 2, nearly 50 % of the entrapped enzyme molecules are released from the film, the residual molecules within the lipid matrix on complete neutralization of the electrostatic interactions further attesting to the role played by interactions such as hydrophobic, hydrogen bonding etc. in the enzyme immobilization energetic. As can be seen from the inset of Fig.5A.2, the release of enzyme molecules at pH 7 is negligible. At this pH, electrostatic interactions dominate and do not allow the removal of the enzyme molecules from the loaded enzyme-ODA composite film. This also supports the fact that the enzyme activity measured (as will be explained subsequently) from the biocomposite film is due to the enzyme entrapped within

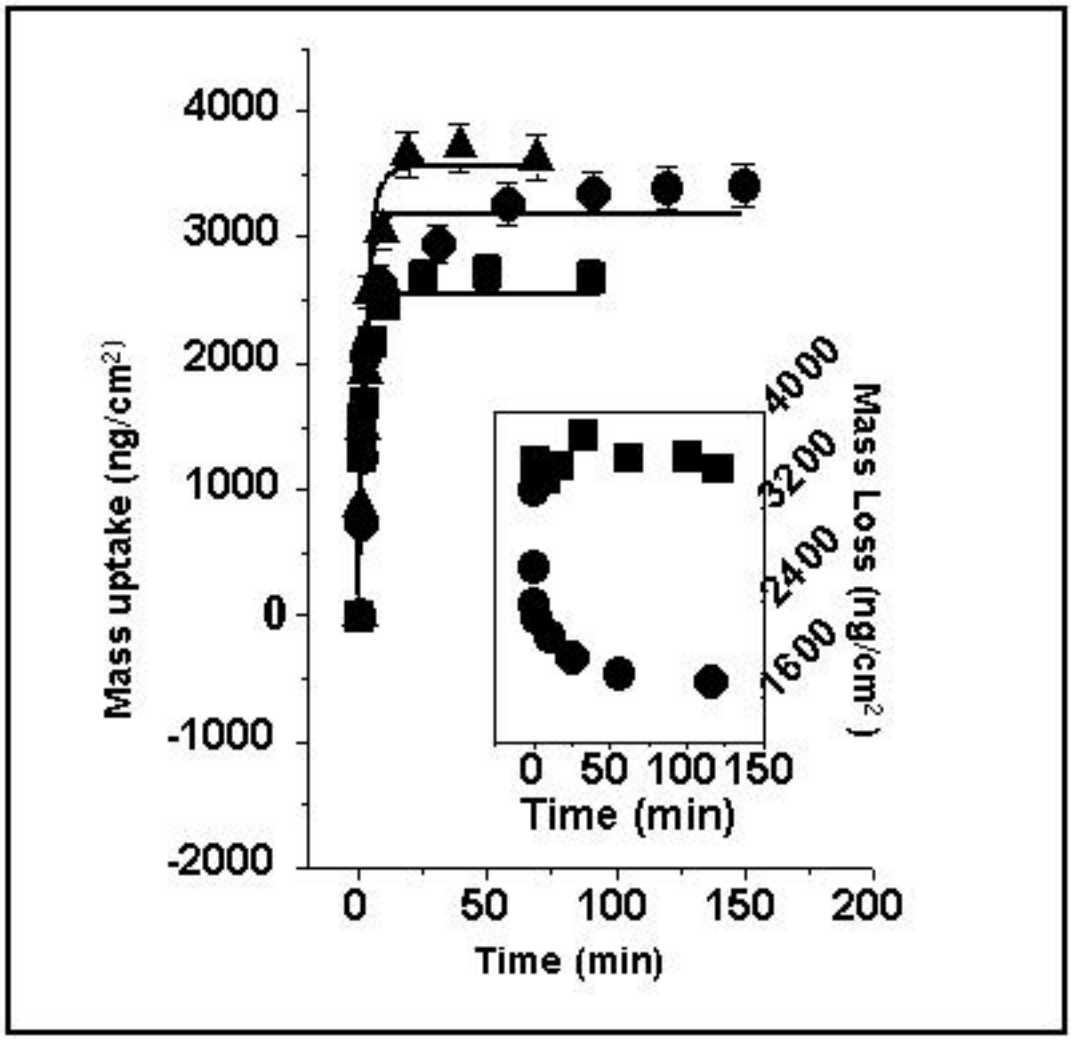


Fig.5A.2.QCM kinetics of entrapment of substrate protected Endoglucanase A in the 250 Å thick ODA films.

The fims were immersed in 2mg/ml substrate protected enzyme solution at different pH values. Symbols: pH 5 (▲); pH 7 (●); pH 9 (■). The solid lines are an aid to the eye and have no physical significance. The inset shows the QCM mass loss recorded during removal of enzyme at pH 2 (●) and pH 7(■) from the 250 Å thick enzyme-ODA composite film formed at pH 7.

and not due to enzyme in solution.

5.A.3.2. *Surface analysis of the enzyme-lipid biocomposite film.*

In order to rule out surface adsorption of the enzyme, a sessile water drop on a 250 Å thick ODA film as-deposited on Si (111) substrates before and after incorporation of substrate protected endoglucanase yielded contact angles of 90° and 78°, respectively. The small reduction in contact angle indicates some degree of distortion to the lipid bilayers after enzyme incorporation. Contact angle measurements of a plain Si (111) substrate, covered by drop-dried substrate protected enzyme film as well as an ODA film (250 Å thickness) on which a substrate protected enzyme film was deposited by drop-drying, yielded values of 15°, 18°and 20° respectively. The above results clearly indicate that the Endoglucanase molecules are entrapped within the lipid matrix and that surface adsorption of the enzyme molecules may be ruled out.

5.A.3.3. *Analysis of the enzyme-lipid biocomposite film by SEM*

SEM analysis was performed on a 250 Å thick ODA film on Si (111) substrate before and after the incorporation of substrate protected enzyme at pH 7. The SEM image of the substrate protected Endoglucanase-ODA composite film is shown in Fig.5A.3.The surface of this film is relatively smooth with little indication of the presence of the enzyme. Taken together with the contact angle measurements presented earlier, this suggests that the enzyme molecules are entrapped below the surface of the lipid film. For comparison, a film prepared by drop-drying the substrate protected enzyme solution (pH 7) on a 250 Å thick ODA film was analyzed by SEM (Fig.5A.3). The SEM image of this film clearly shows the presence of enzyme aggregates on the surface of the ODA film.

5.A.3.4. *Secondary structure of the encapsulated Endoglucanase A in ODA films.*

The position of the amide I and II bands in the FTIR spectra of proteins is a sensitive indicator of conformational changes in the protein secondary structure(Dong et al., 1992; Kumar and Mclendon, 1997). Fig.5A.4 shows the FTIR spectra recorded

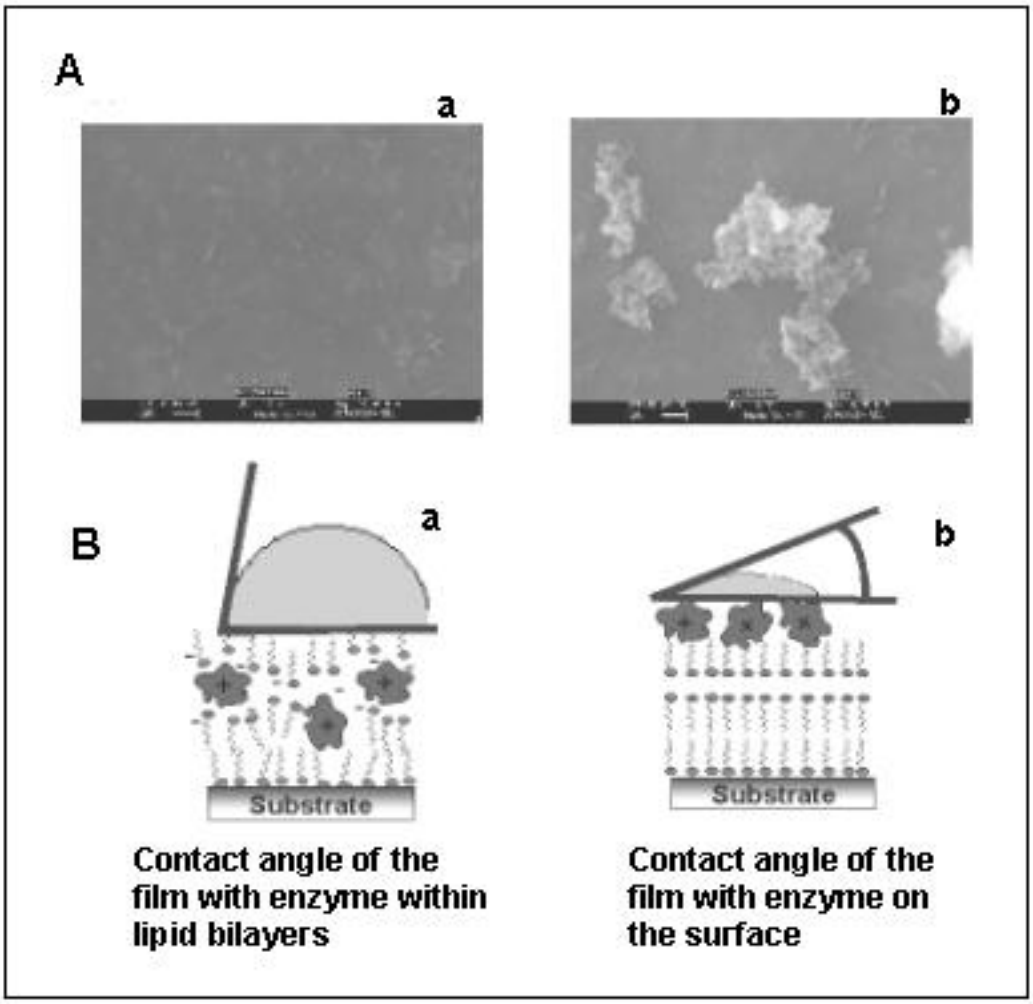


Fig.5A.3. (A) SEM micrographs recorded from a 250 Å thick ODA films.

a: Endoglucanase A immobilized within 250 Å thick ODA composite film.
b: A drop-dried enzyme film deposited on an as-deposited 250 Å thick ODA film.

Fig.5A.3. (B) The contact angle measurements of the biocomposite films.

a: The larger contact angle on biocomposit film clearly rules out surface adsorption.
b: The surface adsorbed enzyme has a low contact angle.

from a 250 Å thick as-deposited ODA film and the substrate-protected Endoglucanase-ODA composite film formed at pH 7. The band at 1646 cm^{-1} (feature a) is due to carbonyl stretch vibrations of the amide I band in the enzyme-ODA composite film and compares well with the amide I band observed by other researchers for encapsulated fungal protease in lipid films (Gole et al.,2000). While a small feature at this wavenumber does occur in the as-deposited ODA film (Fig.5A.4, curve 1), the intensity of this band increases in curve 2 clearly showing that it originates from the enzyme molecules in the composite film. The position of this band is close to that reported for native proteins by other workers (Phadtare et al., 2002). This indicates that the secondary structure of the protein in the ODA environment is relatively unperturbed. The N-H stretch vibration in amide linkages (the amide II band) is observed at 1546 cm^{-1} in the composite film (curve 2, feature c) that is clearly missing in the as-deposited ODA film (curve 1) and compares well with the other native proteins (Gole et al.,2000 , Phadtare et al., 2002). A feature at about 1568 cm^{-1} is observed in the as-prepared ODA film (curve 1, feature b) and is assigned to the N-H deformation vibration mode in the ODA matrix. The presence of well-defined amide bands indicates intactness of the secondary structure of the enzyme.

5.A.3.5. *Assays of the endoglucanase in the ODA films.*

As explained in the experimental section, the activity of the immobilized enzyme was determined by incubating the biocomposite film with CMC in pH 7.0 buffer at 60°C for 1h and estimating the sugars released in the reaction mixture. Initial experiments indicated that Endoglucanase molecules encapsulated in the ODA matrix without substrate protection showed a 58% loss in the activity relative to that of the free enzyme in solution (Table 5A.1). This result indicated inaccessibility of the active site of the entrapped enzyme molecules to substrate molecules in solution; possibly due to blockage of the active sites by electrostatic complexation with the lipid molecules. This necessitated substrate protection of the active site of enzyme by CMC. Studies on protection of the enzyme active site by precomplexing the enzyme with CMC in different ratios before immobilization into 250 Å thick ODA films was carried out at pH 7. Protection of the active site of enzyme was accomplished at an enzyme: substrate

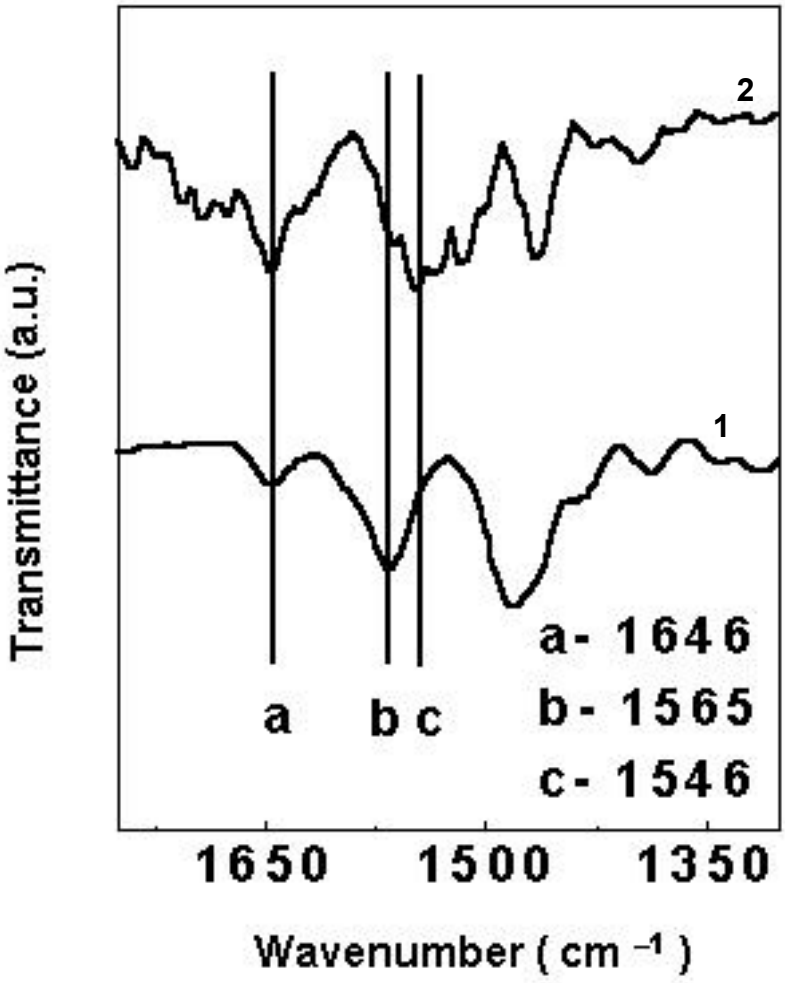


Fig.5A.4. FTIR studies for secondary structure of immobilized Endoglucanase A

Curve 1: 250 Å thick as-deposited ODA film.
Curve 2: 250 Å thick Endoglucanase-ODA biocomposit film.

Three bands labeled a,b,c were identified at different positions these are described in the text.

molar ratio of 1:2 that yielded optimum activity (Table 5A.2.). At this ratio of enzyme: substrate, the activity of the encapsulated enzyme relative to that of the free enzyme in solution is close to 91 % (Tables 5A.1.and 5A.2).

Table 5A.1. Comparison of Endoglucanase activity in solution and after the encapsulation in ODA films.

System	Relative specific activity (%)
Endoglucanase in solution	100
Endoglucanase-ODA composite film	42
Substrate protected Endoglucanase-ODA composite film, enzyme pre-complexed with CMC in the ratio 1:2.	91
ODA film (blank)	N.D.
Substrate protected Endoglucanase-ODA composite film, assayed at 4°C in pH 7 buffer	N.D.

N.D. Not detected

Relative specific activity (%) is the specific activity of the Endoglucanase in the encapsulated form relative to that of the free enzyme in solution at pH 7.0 and 60°C.

All enzyme activities were obtained from 5 separate experiments and were reproducible; the experimental data falls within range of < ± 10 % standard deviation.

Table 5A.2. Substrate protection of Endoglucanase A by different concentrations of carboxy methyl cellulose (CMC)

Enzyme: Substrate (CMC) molar ratios	**Relative specific activity (%)**
1:1	71
1:2	91
1:4	63

Relative specific activity (%) is the specific activity of the Endoglucanase in the encapsulated form relative to that of the free enzyme in solution at pH 7.0 and 60°C.

All enzyme activities were obtained from 5 separate experiments were reproducible; the experimental data falls within range of < ± 10 % standard deviation.

Hence, all further experiments were carried out by pre-complexing the substrate (CMC), with Endoglucanase prior to immobilization of the enzyme in the thermally evaporated ODA films. In order to check the reproducibility of the activity measurements, five similarly loaded films of the substrate-protected enzyme molecules were tested under identical assay conditions and yielded an activity of 18.1± 1.0 units/ mg. The small variance in the enzyme activity suggested that the protocol for the fabrication of the enzyme-lipid composite films is reproducible. The activity of the substrate-protected encapsulated enzyme (18.1± 1.0) compares favorably with the activity of the free enzyme in solution (20.0 ± 0.8 Units) indicating little compromise to the Endoglucanase activity on entrapment in the lipid matrix.

As discussed above, substrate protection is important in the case of Endoglucanase molecules entrapped in ODA films and a possible explanation is as follows. It is expected that the enzyme molecules are encapsulated in the hydrophillic regions of the lipid bilayers as illustrated in the schematic of Fig.5A.1. The location of the negatively charged acid sites on the enzyme surface would determine the electrostatic coordination of the enzyme with the protonated amine groups in the film and hence, the orientation of the enzyme molecules within the ODA bilayers. It is conceivable that if these negatively charged sites are located close to the active site of the enzyme, then the orientation of the enzyme in the lipid bilayers would not favour

easy access of substrate molecules from solution to the “blocked” active sites of the enzyme. It is observed that complexing flexible polycations with lactate and glycolate oxidases before immobilization into silicate sol gels dramatically stabilizes the enzyme by reducing the electrostatic interaction of the silica anions and the cationic arginine groups in the active sites (Heller and Heller, 1998). Similar protection mechanism might be possible in our case as well but in the absence of crystallographic information on the Endoglucanase-CMC system, it is difficult to make a definite statement on this aspect. Experimentally, however, an enzyme: substrate ratio of 1: 2 was determined to be ideal in optimising the activity of the encapsulated enzyme in the lipid matrix and is an important result of this investigation.

A control experiment was performed wherein a bare as-deposited 250 Å thick ODA film (film dimensions = 4cm^2) was analysed for enzyme activity under similar assay conditions. As shown in Table 5A.1, this film failed to show any enzymatic activity as would be expected. The possibility of reducing sugars released by possible enzyme-induced substrate degradation during the co-immobilization process at 4°C has also been investigated. The 250 Å thick substrate protected enzyme-ODA composite film formed at pH 7 and 4 °C (4 cm^2 film dimensions) was assayed in pH 7 buffer at 4°C without the substrate. This film failed to show any enzyme activity (Table 5A.1.) indicating that the enzyme does not degrade the substrate at 4°C.

5.A.3.6. *Shift in optimum temperature for the encapsulated endoglucanase in film.*

The temperature dependence of the activity of substrate-protected endoglucanase molecules in solution at pH 7 (Fig.5A.5.) was compared with that of the substrate-protected endoglucanase molecules encapsulated in 250 Å thick ODA films at pH 7. While the free enzyme in solution shows maximum activity at 60°C, the endoglucanase-ODA composite film shows optimum activity at 70°C. This 10°C shift in the optimum temperature was found to be reproducible for 5 separate endoglucanase–ODA films. Furthermore, the fact that the entrapped enzyme still show significant activity even above 70°C under conditions where the free enzyme molecules in solution loose activity rapidly. Significant stabilization of the enzyme molecules by the

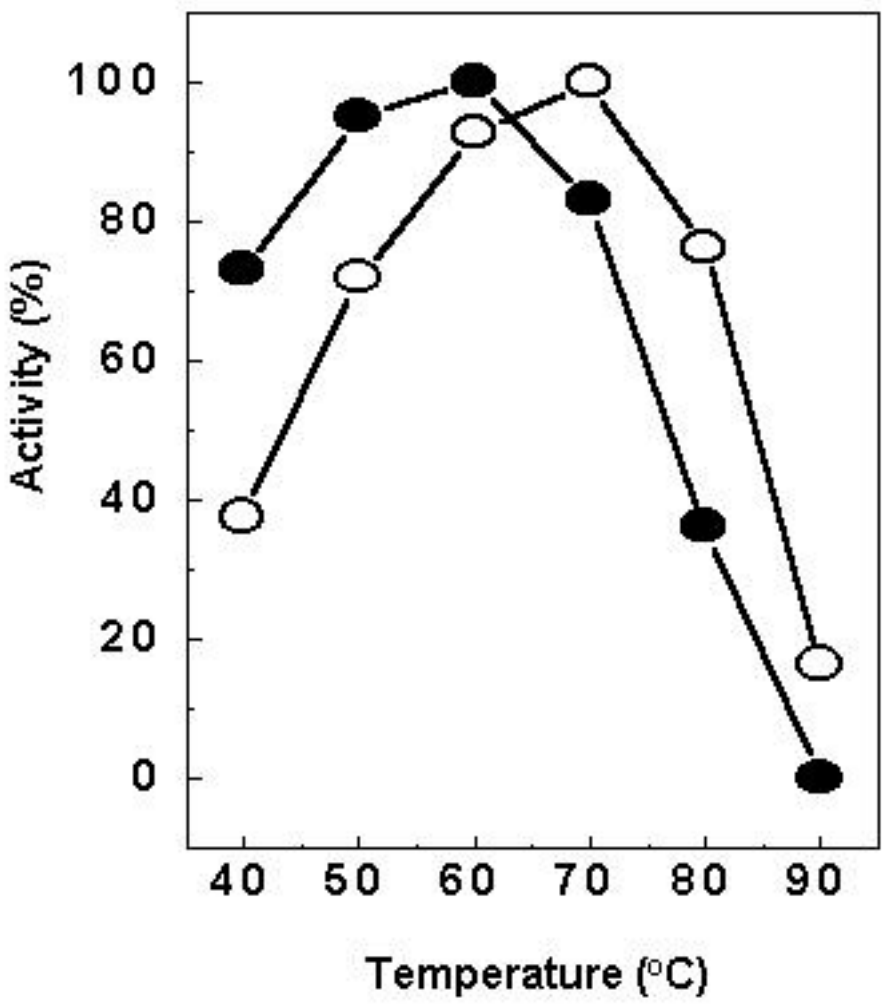


Fig. 5A.5. Effect of temperature on free (●) and immobilized (o) Endoglucanase A

the enzyme in the ODA matrix is expected to be responsible for this increased temperature stability of the immobilised Endoglucanase A. Similar broadening of temperature profiles was also reported previously in other immobilized cellulase enzymes (Rogalsky et al., 1985;Garcia et al., 1989).

5.A.3.7. *The pH dependence of immobilized enzyme*

The pH dependence of the activity of the substrate-protected Endoglucanase molecules in solution as well as in the encapsulated form is measured (Table 5A.3).

Table 5A.3. Comparison of the pH dependent Endoglucanase activity in solution and immobilized form

pH	Relative specific activity (%)	
	Solution form	**Immobilized form**
5	100	100
7	80	86
9	30	72

Relative specific activity (%) is the specific activity of the Endoglucanase in the encapsulated form relative to that of the free enzyme in solution under ambient assay conditions. The maximum specific activity in solution at pH 5 is taken to be 100 %.

In both cases the optimum activity of the enzyme occurs at pH 5. The decay in enzyme activity of the free substrate-protected enzyme molecules with increasing pH of the solution is much more rapid than that observed for the encapsulated Endoglucanase molecules. At pH 9, the activity of free substrate-protected enzyme in solution drops to 30 % of its original activity (at pH 5), while the encapsulated enzyme retains most of its original activity. This significant stabilization of the enzyme at high pH again points to the role of the ODA matrix in stabilizing the enzyme at higher pH values. The immobilized enzyme molecules have restricted mobility that prevents any possible structural changes, thereby 'locking' the enzyme in certain preferred orientation. The enhanced thermal stability of the substrate-protected Endoglucanase

molecules in the ODA films may be due to the hindered mobility of the entrapped enzyme molecules (Weetal H, 1969; Hernaiz and Crout, 2000). Secondly, the stability of the substrate protected encapsulated enzyme at high pH values as compared to that of substrate protected enzyme in solution indicates the role of local pH experienced by the enzyme in the lipid matrix. It has been observed that the surface acidity of a carboxylic acid functional group on a 2-D self assembled monolayer (SAM) and 3-D SAM is different than that for the free monomeric functional group in solution (Lee et al., 1994b; Gole et al., 1999). The titration curves for ionization of carboxylic acid terminated SAMs not only show a shift in the actual pK_a values but also that the width of these titration curves is found to increase (Lee et al., 1994b). Such a behavior is expected to be true for the amine functional groups in the ODA matrix as well. The combined effect of the broadening of the titration curves of both the ODA molecules in the lipid matrix and carboxylic acid groups on the enzyme surface would lead to considerable insensitivity of the local pH within the micro-environment of the lipid bilayers to variations in the bulk solution pH. This would satisfactorily explain the retention of the enzyme activity of the entrapped endoglucanase molecules with varying pH of the solution as observed in this study. The high temperature-high pH stability of the entrapped enzyme molecules in ODA matrixes highlights the utility of our technique.

5.A.4. Conclusion

The encapsulation of Endoglucanase in thermally evaporated fatty amine films by a simple beaker-based immersion process is demonstrated in this part of the Chapter. The enzyme molecules were entrapped in the lipid matrix by primarily electrostatic interactions. The use of electrostatic interactions enables 'leaching-out' of the entrapped enzyme molecules into solution according to variation of pH of the solution. Substrate-protection of the enzyme with the CMC is necessary to obtain significant activity of the entrapped enzyme. A salient feature of the work is the enhanced temperature stability of the enacapsulated enzyme relative to that of the free enzyme in solution. The immobilized enzyme also showed significant catalytic activity at elevated pH conditions where the free enzyme molecules in solution were relatively inactive. The retention of significant activity of the encapsulated Endoglucanase-ODA composite films under high temperature-high pH conditions marks them for potential industrial applications.

5B: Enzyme Bioconjugates with Colloidal Gold Nanoparticles

5.B.1. Introduction

The enzyme Endoglucanase A was successfully immobilized in thermally evaporated fatty amine films (part 5A, this Chapter). However, because of 2D nature of the system the protocol suffers with few notable disadvantages. These includes mass transport problem; need for substrate protection, and inability to reuse. Hence, the immobilization of biomolecules on to solid 3D surfaces is important. The utility of nanoscale-curved surfaces such as those provided by colloidal particles in immobilizing biomolecules has been recognized in the early 1980's (Rembaum and Dreyer, 1980). The high surface-to-volume ratio offered by colloidal particles results in the concentration of the immobilized entity being considerably higher than that afforded by protocols based on immobilization on planar, 2-D surfaces. Polymer colloidal particles have largely been used as templates for the immobilization of proteins/enzymes/DNA (Caruso and Mohwald,1999). Covalent and other secondary interactions are responsible for such an immobilization process. On the other hand, metal colloidal particles such as gold, silver and CdS have also been used as templates for the immobilization of biomolecules (Neiyemer et al., 2001). Studies on the formation of Endoglucase-colloidal gold bioconjugates and their catalytic activity are presented in this part of the Chapter. The formation of endoglucanase-colloidal gold biocomposites is accomplished by simple mixing of the two solutions at pH 5, centrifugation, washing and further resuspension. The bioconjugate formation process was monitored by UV-vis studies, while Transmission Electron Microscopy (TEM) measurements were used to characterize the particle size of the bioconjugates. The secondary and tertiary structure of the enzyme in the bioconjugate system was studied by Fourier Transform Infrared spectroscopy (FTIR) and Fluorescence spectroscopy. The activity of the enzyme in the bioconjugate system was comparable with that of free enzyme in solution. The enzyme in the bioconjugate system showed an increased half-life at 60°C and at pH 9 as compared to free enzyme in solution.

5.B.2. Materials and Methods

5.B.2.1. Colloidal gold synthesis

A 100 ml sample of a 1.25 x 10^{-4} M concentrated aqueous solution of chloroauric acid ($HAuCl_4$) (Aldrich Chemicals) was reduced by 0.01 g of sodium borohydride ($NaBH_4$) at room temperature to yield a ruby-red (surface plasmon absorption maximum at 512 nm) solution containing 35 $\pm$ 7 Å diameter gold nanoparticles (Patil et al., 1999).

5.B.2.2. Formation of Endoglucanase: colloidal gold bioconjugates

10^{-6} M Endoglucanase solution was, prepared in 0.05 M citrate buffer pH 5 was added to 10 ml of colloidal gold solution to yield a final enzyme concentration of 10^{-7} M in the colloidal solution. The pH of the colloidal gold solution was adjusted to 5 (by addition of dilute HCl) prior to the addition of the enzyme. The solution was stored for a period of 18 hours at 4°C and then centrifuged at 6000 rpm at 4°C to remove uncoordinated enzyme in the solution. The pellet so obtained was rinsed several times with buffer, centrifuged once again to remove free uncoordinated enzyme (if any) and then re-suspended in pH 5 buffer solution. The bioconjugates obtained were stored at 4°C for further experimentation. Initial experiments indicated that there is considerable loss in the amount of bioconjugate material during centrifugation. In order to overcome this loss, 0.07 % NaCl was added to the colloidal gold along with the enzyme. This improves the quality and quantity of the pellet formed upon centrifugation and the losses are considerably reduced.

5.B.2.3. UV-vis spectroscopy studies

The formation of endoglucanase: colloidal gold bioconjugates was monitored by UV-vis spectroscopy using a Shimadzu dual-beam spectrophotometer (model UV-1601 PC) operated at a resolution of 1 nm. The surface plasmon resonance due to colloidal gold (512 nm) (Patil et al., 1999) and the π-π^* transitions due to the tryptophan and tyrosine residues in the enzyme (Stoscheck, 1990) were monitored (280 nm)

immediately after addition of Endoglucanase to the colloidal gold solution and after centrifugation/re-suspension of the bioconjugate in appropriate buffers.

5.B.2.4. *Transmission Electron Microscopy (TEM)*

TEM measurements were performed on a JEOL Model 1200EX instrument operated at an accelerating voltage of 120 kV. Samples for TEM analysis were prepared by placing a drop of centrifuged/re-suspended endoglucanase: colloidal gold solution on a carbon-coated TEM copper grid. The film was allowed to dry for 1 min and the extra solution was removed using a blotting paper.

5.B.2.5. *Fourier transformed infrared spectroscopy (FTIR)*

FTIR was used to study the secondary structure of the enzyme in the bioconjugate. An Endoglucanase: colloidal gold film on a Si (111) substrate was prepared by drop-drying the bioconjugate solution. FTIR spectra of the films were recorded on a Shimadzu FTIR-8201 PC instrument operated in the diffuse reflectance mode at a resolution of 4 cm^{-1}. To obtain good signal to noise ratio, 256 scans of the bio-conjugate film were taken in the range 400 – 4000 cm^{-1}. For comparison, an FTIR spectrum of a film formed by drop-drying Endoglucanase solution onto Si (111) substrate was also recorded.

5.B.2.6. *Tertiary structure studies*

Fluorescence spectroscopy is a powerful tool for studying the tertiary structure of proteins and enzymes (Eftink and Ghiron, 1981). The fluorescence spectrum of the bioconjugate solution was studied using a Perkin-Elmer Luminescence Spectrophotometer (model LS 50B). The tryptophan residues in the enzyme were excited at 295 nm and the emission band was monitored in the range 300 to 500 nm. The shape and position of the maximum in the emission band was compared with free endoglucanse in solution. Fluorescence spectroscopy was also used to quantify the amount of enzyme present in the bioconjugate system. Since quenching of the fluorescence spectrum upon bioconjugate formation was observed, the quantification

was effected by monitoring the loss in intensity of the emission band in the supernatant after centrifugation of the bioconjugate solution.

5.B.2.7. *The enzyme assays for bioconjugates*

The activity of endoglucanase: colloidal gold bioconjugates in solution was determined by using CMC as a substrate (10 mg/ml in 0.05 M sodium-phosphate buffer, pH 7) and incubating the mixture at 60°C for 1 h. The reducing sugars released were determined by the DNSA method (Miller et al., 1959). Five separate measurements of the bioconjugate solutions were performed to check the reproducibility of the data. The concentration of Endoglucanase in the bioconjugate system was estimated via fluorescence measurements and by Lowry's method (1951). For comparison, enzyme activity of an identical amount of endoglucanase molecules in solution was determined as described above. The pH dependent variation of the Endoglucanase activity in the bioconjugates was studied in the pH range of 4-9.The buffers used were (pH 4-5, 0.05 M sodium citrate buffer; pH 6-7, 0.05 M sodium phosphate buffer; pH 8-9, 0.05 M glycine-NaOH buffer). Reproducibility of the data was tested in three similar experiments. In addition, the endoglucanase activity of the bioconjugates at pH 7 was tested in the temperature range 40-80°C and compared with that of identical amount of free enzyme molecules in solution under similar conditions. Three separate measurements at each temperature were performed to check the reproducibility of the assay. The stability of the bioconjugates was determined by incubating the bioconjugates at 60°C and at pH 9 for different time intervals and the residual enzyme activity was calculated and compared with same amount of free enzyme molecules in solution aged under similar conditions.

5.B.3. Results and Discussion

5.B.3.1. *Preparation of Endoglucanase: colloidal gold bioconjugates*

The pH of the as-prepared colloidal gold solution was around 9. The pH of colloidal gold was lowered prior to addition of the enzyme. A 10^{-6} M standard solution of Endoglucanase A was prepared in citrate buffer (0.05 M, pH = 5) and added to

colloidal gold solution at pH 5, to yield final concentration of enzyme to be 10^{-7} M. 0.07 % NaCl was added along with the enzyme in order to obtain a good pellet after centrifugation. The coordination of enzyme molecules with colloidal gold occurs possibly via the thiolate linkages through the cysteine residues (Sasaki et al., 1997) or by amine functional groups from primary amines through weak covalent interactions (Sastry et al., 2001).

5.B.3.2. *UV-vis studies*

Fig.5B.1a shows the UV-vis spectra of the as-prepared colloidal gold solution, Endoglucanase: colloidal gold bioconjugate immediately after addition of the enzyme and 0.07 % NaCl at pH 5 and after centrifugation and resuspension of the bioconjugate in pH 5 buffer solution. A broad peak at 512 nm in curve 1 is due to the surface plasmon resonance of colloidal gold particles. After addition of the enzyme, the surface plasmon band broadens and red shifts due to the interaction of the enzyme with colloidal gold particles. A feature at about 280 nm in the bioconjugate system arises due to the π-π^* electronic transitions from the tryptophan and tyrosine residues in the enzyme. After centrifugation-resuspension of the bioconjugate solution, the intensity of the plasmon band decreases due to some loss in the gold colloids. The intensity of π-π^* band at 280 nm diminishes indicating removal of excess uncoordinated enzyme molecules in solution.

5.B.3.3 *TEM measurements*

Fig.5B.1B shows the TEM micrograph of Endoglucanase: colloidal gold bioconjugate system formed on a carbon-coated copper grid as explained in the experimental section. At higher magnification, the film was observed to melt under the electron beam, due to possible electron-induced degradation of the enzyme molecules in the film. It is observed from Fig.5B.1B that the gold particles are ordered without the particles being in direct contact as is normally observed in TEM images of un-protected gold colloids. Furthermore, the separation between the particles appears to be fairly uniform indicating the presence of stabilizing Endoglucanase molecules between the gold particles. Thus, the gold particles stabilize the ensemble of enzyme molecules while preventing their aggregation. This is important in retaining the enzyme activity

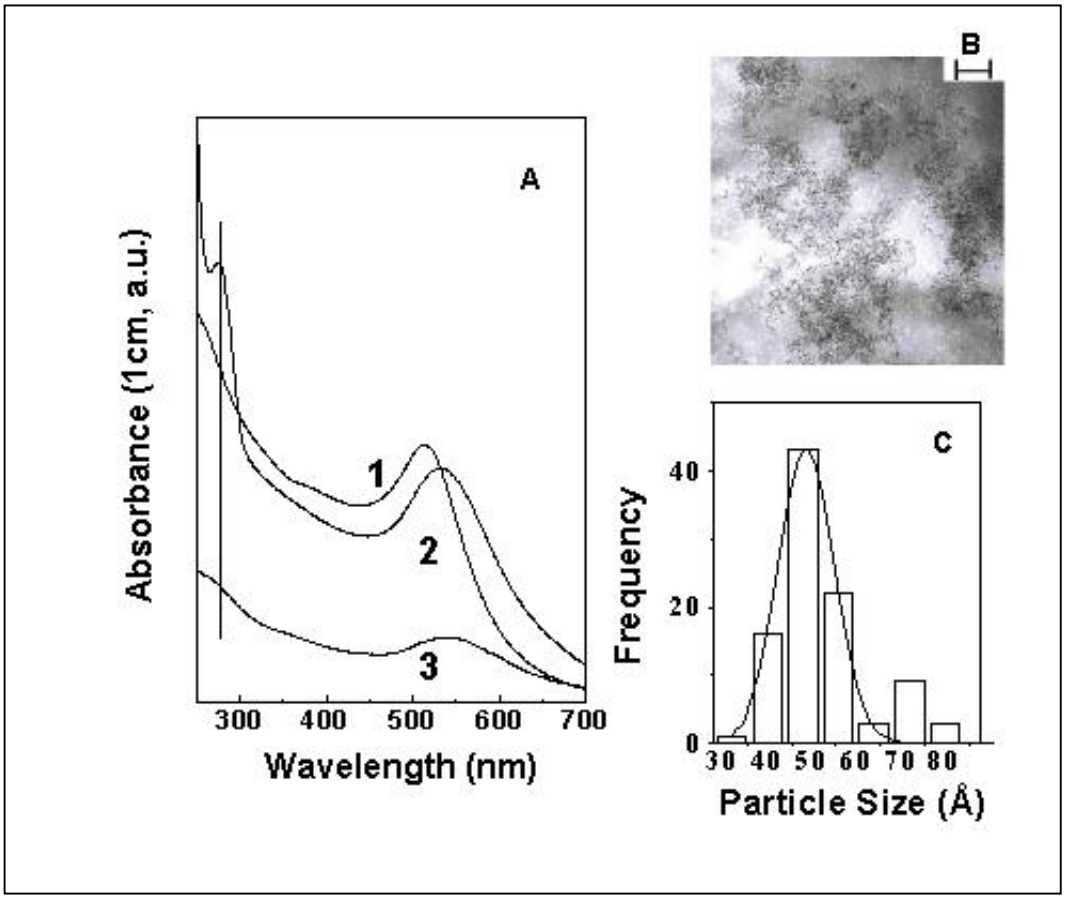


Fig.5B.1A UV-vis spectra

Curve 1: As-prepared colloidal gold solution.
Curve 2: Endoglucanase: gold bioconjugate solutions immediately after addition of endoglucanase to colloidal gold at pH 5.
Curve 3: The bioconjugate in buffer solution after centrifugation and resuspension.

Fig.5B.1B. TEM micrograph of a drop-dried endoglucanase: colloidal gold bioconjugate film. (⟷ 100nm)

FiG.5B.1C. Particle size distribution histogram estimated from the micrograph shown in Fig.5A.1B.

The solid line is a Gaussian fit to the data and yields a gold particle size of 49 Å $\pm$ 6 Å.

and is a desirable feature in enzyme immobilization protocols. Fig. 5B.1C is the particle size distribution of the particles shown in Fig.5B.1B and shows a mean particle diameter of 49 Å with a standard deviation of 6 Å. This result is in fairly good agreement with the expected size of the as-prepared colloidal gold of 35 $\pm$ 5 Å (Patil et al., 1999).

5.B.3.4. *FTIR studies*

A drop dried film of Endoglucanase*:* colloidal gold was prepared on Si (111) substrate and analyzed by FTIR measurements. Fig.5B.2A and B show the FTIR spectra of the Endoglucanase molecules in a film of the pure enzyme and that of the Endoglucanase*:* colloidal gold bioconjugate system in the spectral window 3500 – 2200 cm^{-1} and 1750 – 1200 cm^{-1}, respectively. A number of features can be seen in both the curves. The feature at 3216 cm^{-1} in curve 1 (feature a, Fig. 5B.2A) arises from the N-H stretching vibration mode in the endoglucanase molecules. This band shifts and appears as a broad shoulder at 3000 cm^{-1} in the Endoglucanase*:* colloidal gold system (curve 2, Fig.5B.2A). This shift in the band can be attributed to the binding of amine groups on the enzyme surface to colloidal gold. The methylene antisymmetric and symmetric vibrations at 2916 cm^{-1} and 2850 cm^{-1} arising in the enzyme molecules can be seen in curve 1 (Fig.5B.2A) respectively. These bands cannot be clearly seen in the bioconjugate system (curve 2, Fig.5B.2A) due to the broad shoulder at 3000 cm^{-1}. Another feature at about 2520 cm^{-1} can be clearly seen in the bioconjugate system (feature b, curve 2, Fig.5B.2A) and is tentatively assigned to the –SH stretch vibration bands in the cysteine residues in the enzyme (Templeton et al., 1999). The point to be noted is the fact that this band is absent in the enzyme. This can be explained as follows. It is well known that under reducing conditions the S-S bond breaks to form –SH bonds (White et al., 1978). We believe that in our case the excess borohydride present in the colloidal gold solution may reduce the S-S bond to form –SH bonds. This would be expected to lead to changes in the natural conformation of the enzyme. However, the enzyme in the bioconjugate shows enzyme activity comparable to that of the free enzyme in solution and indicates the intactness of the tertiary structure of the enzyme. The spectral window 1750 - 1200 cm^{-1} (Fig.5B.2B) provides information about the amide bands, which are sensitive to the secondary structure of the enzyme (Dong et al., 1992). The bands at 1650 cm^{-1}

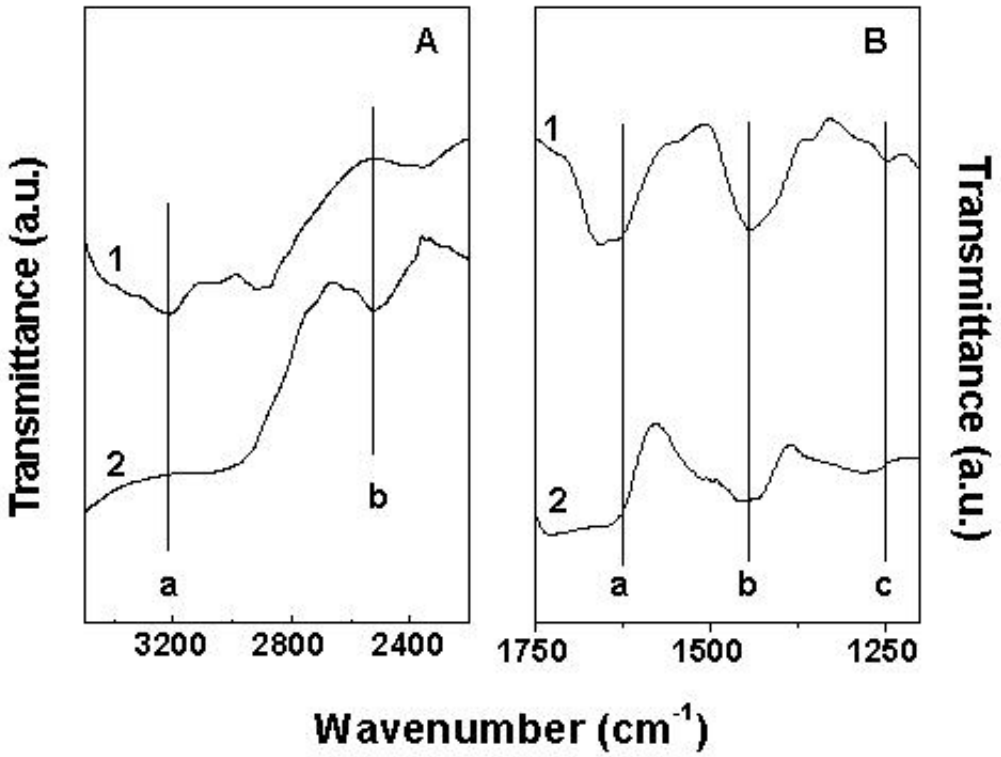


Fig.5B.2A. FTIR spectra recorded in the 3500 to 2200 cm^{-1} spectral region.

Fig.5B.2B. FTIR spectra recorded in the range 1750 cm^{-1} to 1200 cm^{-1}.

Curve 1: from a drop-dried Endoglucanase film on a Si (111) substrate.
Curve 2 : Endoglucanase : colloidal Au bioconjugate film on a Si (111) substrate.
(see text for assignments a,b,c).

and 1240 cm^{-1} in the drop-dried enzyme film (curve1, Fig.5B.2B) are assigned to the amide I and III bands. The amide I band originates from the C=O stretching vibrations of the peptide bonds (Dong et al., 1992) and amide III band arises from the C-N stretching modes of the polypeptide chains (Gole et al., 2000). The amide I band in the bioconjugate system (feature a, curve 2, Fig.5B.2B) shows some amount of broadening indicating structural changes in the enzyme due to bioconjugate formation. The amide III band in the bioconjugate system (feature c, curve 2, Fig.5B.2B) shows a small shift to lower wavenumbers along with some amount of broadening. This also might be due to coordination of enzyme molecules with gold particles. A broad feature at about 1440 cm^{-1} in the enzyme (feature b, curves 1and 2, Fig.5B.2B) is temporarily assigned to a combination of symmetric stretching modes of carboxylate groups and the methylene scissoring band. This band coincides with that observed by Caruso et al. (1988) for immobilized immunoglobulin.

5.B.3.5. *Tertiary structure studies*

Covalent immobilization of the enzyme often leads to modification of enzyme 's native structure and hence loss of activity. The enzyme activity depends upon the tertiary structure of the enzymes remaining unperturbed on formation of conjugates with colloidal gold. The tertiary structure of an enzyme can be studied by fluorescence measurements by exciting the sample at a particular wavelength and monitoring the fluorescence emission from the tryptophan or tyrosine residues in the enzyme. The standard procedure was followed to check the tertiary structure of Endoglucanase co-ordinated to colloidal gold particles by excitation of the π - π^* transition in the tryptophan residues at 295 nm. Fig.5B.3A shows the fluorescence spectrum of 10^{-9} M concentrated free enzyme molecules in 0.05 M citrate buffer, pH 5. The sample was excited at 295 nm, and the emission was monitored in the range 300 to 500 nm. A broad band at 340 nm is observed and indicates intactness of the tertiary structure of Endoglucanase in solution. Curve 2 shows the fluorescence spectrum of the endoglucanase*:* colloidal gold bioconjugate system recorded under similar excitation conditions. The nature of the curve and peak positions is quite similar to the free enzyme in solution indicating intactness of the tertiary structure of the enzyme after bioconjugate formation.

5.B.3.6. *Quantification of enzyme in the bioconjugate system*

Fluorescence spectroscopy was used to quantify the amount of endoglucanase in the bioconjugate solution (Gole et al., 2001b). This was done by comparing the tryptophan emission intensities of the bioconjugate versus that of free enzyme molecules in solution. However for the system in this study, a significant amount of fluorescence quenching of the tryptophan residues in the enzyme was observed after bioconjugate formation. Hence, an indirect method was used to calculate the amount of enzyme in the bioconjugate system. Fig.5B.3B shows the fluorescence emission intensity of the supernatant obtained after centrifugation and separation of the bioconjugates. For comparison, two different concentrations of free enzyme in buffers were also recorded (Fig.5B.3B, curve 1- 4 x 10^{-7}M; curve 3 -- 2 x 10^{-7} M). Knowing the initial concentration of enzyme added to the colloidal gold solution and subtracting the concentration of enzyme molecules in supernatant, it is possible to calculate the amount of enzyme left in the bioconjugate system. The amount of enzyme calculated by such a process was found to be 130 μg/ml. Lowry's method (1951) was also used to estimate the amount of endoglucanase in the supernatant obtained after the centrifugation of bioconjugate system. The amount of enzyme estimated by this protocol is 100 μg/ml. This compares favourably with the amount of enzyme estimated via fluorescence measurements.

5.B.3.7. *Enzyme assays for bioconjugates.*

The most significant aspect in protein-colloid bioconjugate system is the activity of the enzyme after binding onto the nanoparticle surface. The activity measurements of the bioconjugates were performed as explained in the experimental section and compared with that of the free enzymes in solution. The enzyme activity of the conjugates was performed at six different pH values. The enzyme activities obtained were comparable to that of free enzyme in solution (Table 5B.1). The reproducibility of the data was confirmed by three separate experiments performed under similar conditions. It was observed that the Endoglucanase activity both in solution and in the bioconjugates were comparable at all pH values (Table 5B.1). The temperature variation in enzyme activity of the bioconjugates was studied at pH 7 by

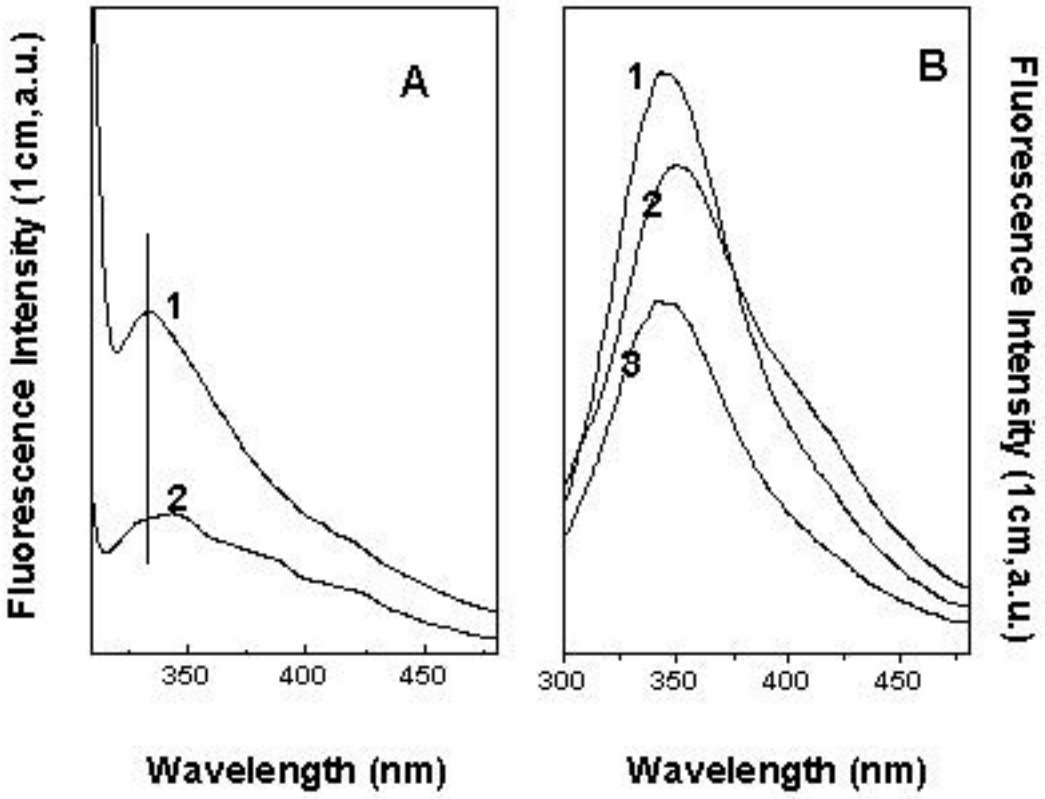


Fig.5B.3A. Fluorescence spectra of Endoglucanases A in free and immobilized form.

Curve 1: 10^{-9} M Endoglucanase A in buffer at pH 5

Curve 2: Endoglucanase: colloidal gold bioconjugate solution in buffer at pH 5

Fig.5B.3B: Quantification of enzyme in the bioconjugate system.

Curve 1: Fluorescence spectra of 4 x 10^{-7} M Endoglucanase A in buffer at pH 5.

Curve 2: Supernatant of the bioconjugate solution after centrifugation

Curve 3: 2 x 10^{-7} M concentrated Endoglucanase A in pH 5 buffer. (see text for details)

incubating separate solutions of the bioconjugates in the temperature range 40-80°C and further assaying for activity. The enzyme activity of the bioconjugate was compared with that of identical amount of free enzyme molecules in solution under similar conditions (Table 5B.2).

Table 5B.1Comparison of Endoglucanase activity in solution and in bioconjugate system at different pH

pH	Free enzyme **activity (%)**	**Bioconjugate system activity (%)**
4	85	85
5	100	100
6	85	95
7	80	81
8	60	63
9	30	35

Table 5B.2.Comparison of Endoglucanase activity in solution and in bioconjugate system at different temperatures

Temperature (°C)	Free enzyme **activity (%)**	**Bioconjugate system activity (%)**
40	73	66
50	95	90
60	100	100
70	83	70
80	36	42

Three separate measurements at each temperature were performed to check the reproducibility of the data. It is seen that the variation in activity of Endoglucanase in the bioconjugates with temperature closely follows the trend observed for the free enzyme molecules in solution.

The thermal stability of the bio-conjugate was monitored by incubating the bio-conjugates at 60°C and pH 9 for different time intervals and measuring the residual activity. This was compared with an identical amount of free enzyme in solution and the data obtained is shown in Fig.5B.4. A slow initial loss in the activity is seen with a further steady decrease to stabilize at a residual activity of 47% within 150 minutes. Free enzyme molecules in solution under similar conditions show a rapid initial decrease in the activity followed by a steady decrease until stabilization of the residual activity at 42% after 150 min. The half-life of the enzyme under these conditions was found to be 120 and 150 min for the free enzyme and bioconjugate, respectively

It is clear from the two curves that the bioconjugate system is more stable to temperature effects than free enzyme molecules in solution and shows a higher residual activity at all times at the temperature of 60°C. This does indicate that the colloidal gold particles in the bioconjugates play an important role in stabilizing the enzyme molecules from harsh temperature conditions.

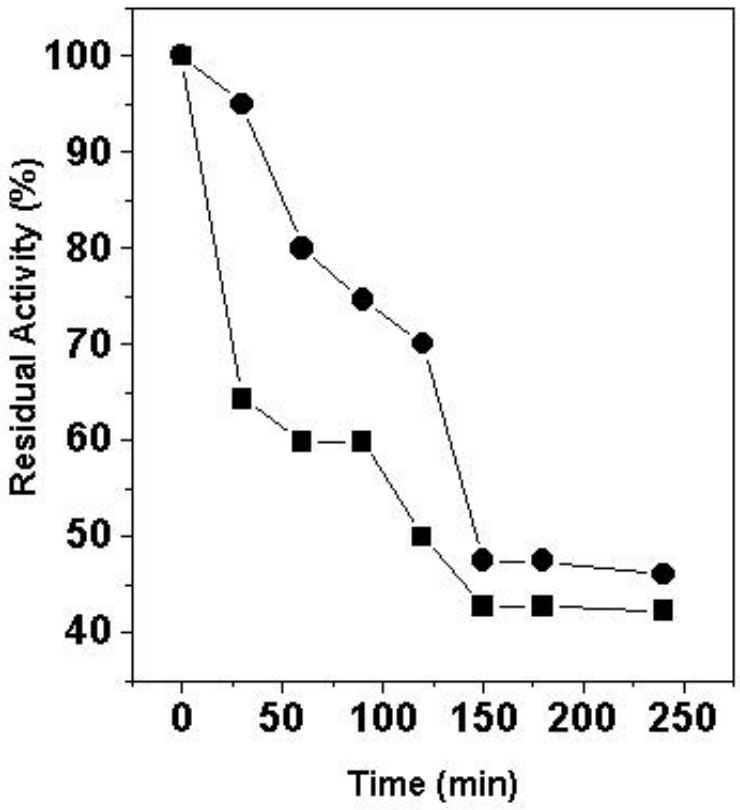


Fig.5B.4. Comparison of enzyme stability of Endoglucanase A free and bioconjugate forms at pH 9.0 and 60°C.

Symbols: free Endoglucanase A (■) and bioconjugates (●)

5.B.4. Conclusions

Endoglucanase: colloidal gold bio-conjugates have been prepared by simple addition of the enzyme to the colloidal gold solution. It is believed that the amine groups in the amino acids present on the surface of the enzyme bind covalently with the gold particle surface leading to the formation of the bioconjugate. TEM measurements show well-defined individual colloidal gold particles with definite particle-particle spacing indicating the role of enzyme molecules in stabilizing the colloidal gold particles. FTIR measurements indicate changes in the secondary structure of the enzyme in the biocomposite material. The enzyme activity of the Endoglucanase molecules in the bioconjugate both as a function of pH and temperature of the solution was comparable to free enzyme in solution indicating that the enzyme in the bioconjugate system is present in its natural conformation. The enzyme molecules in the bioconjugate exhibited a small increase in thermal stability relative to that of the free enzyme in solution at elevated temperatures and suggests possible industrial application for the bioconjugates.

5C: Three Dimensional Assembly of Gold Nanoparticles on Polyurethane Microspheres to form 'Core-Shell' Structures

(Enhancing the reusability of Endoglucanase-gold nanoparticle bioconjugates by tethering to polyurethane microspheres)

5.C.1. Introduction

The bioconjugates of Endoglucanase with the gold nanoparticles showed improved temperature stability (part 5B, same Chapter). However, the major drawback in this immobilization protocol was the low recovery and hence poor reusability of the bioconjugates. To overcome this problem, we assembled the gold nanoparticles on the surface of the bulky Polyurethane (PU) microspheres (Fig.5C.1). The binding of the gold nanoparticles to the polymer surface occurs through nitrogen groups in the polymer backbone. This obviates the need for surface-modification of the polymer spheres (Elgersma et al., 1990). The nanogold -labeled PU microspheres were used for immobilizing enzyme Endoglucanase A (Fig.5C.1). Endoglucanase-nanogold-PU micro spheres showed comparable activity and were reused over 5 cycles. As observed in earlier work (Chapter 5A), during immobilization of Endoglucanase enzyme in thermally evaporated ODA lipid films, the active sites of the enzyme were blocked due to electrostatic complexation with the lipid molecules. The substrate protection of enzyme active site was necessary in order to retain activity. Such substrate protection was not required in the present protocol, since the enzyme is on the surface of nanogold-PU composite materials.

5.C.2. Materials and Methods

5.C.2.1. Chemicals

The polyurethane microspheres of 2 μm mean diameter were synthesized as described by Ramnathan et al., 1998 and were supplied as a gift from Dr. S. Sivaram, Director, NCL, Pune. The enzyme Endoglucanase A was obtained from alkalotolerant *Fusarium* sp and purified as described previously in 5 A. The colloidal gold nanoparticles (diameter 35 ± 7 Å) was synthesized as described in part 5B of this Chapter.

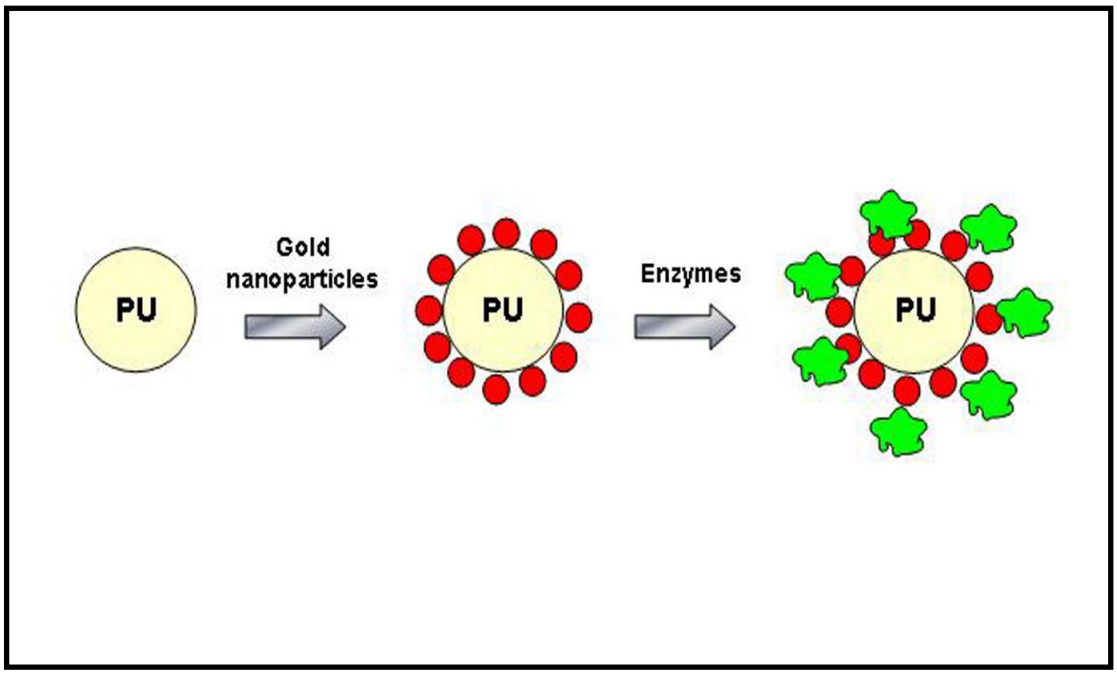


Fig.5C.1: Schematic of immobilization of Endoglucanase A on gold nanoparticles assembled on polyurethane microspheres.

Symbols: Polyurethane microspheres (PU); Gold nanoparticles ( ); Enzyme ( )

5.C.2.2. *Formation of nanogold-polyurethane composites*

10 mg of the PU microsphere powder was dispersed in 10 ml of hexane following which the dispersion was taken in a separating funnel along with 10 ml of the colloidal gold solution. Vigorous shaking of the biphasic mixture for 10 minutes yielded an emulsion-like phase that rapidly phase separated upon cessation of shaking. The originally ruby-red colloidal gold solution was now colourless (like hexane phase) and the PU powder had attained a purple colour and had accumulated at the hexane-water interface. The nanogold-coated PU spheres were separated by filtration, washed with double distilled water and dried in air

5.C.2.3. *Formation of Endoglucanase-nanogold-PU conjugates*

10 mg of the nanogold-PU powder was dispersed in 2 ml of 0.05 M, citrate buffer, pH 5 containing endoglucanase (250 μg/ml) under stirring at 4°C. After 4 h of stirring the endoglucanase-nanogold-PU spheres were separated by centrifugation. The powder so obtained was rinsed with 0.05 M citrate buffer, pH 5 solution and re-suspended in the same buffer solution and stored at 4°C prior to further experimentation.

5.C.2.4. *UV-vis spectroscopy studies*

The binding of colloidal gold to the polyurethane spheres was monitored by UV-vis spectroscopy on a Shimadzu dual beam spectrometer (model UV-1601 PC) operated as a resolution of 1 nm. The decrease in intensity of the surface plasmon resonance in the aqueous colloidal gold solution arising from binding of the gold nanoparticles to the PU microspheres (Patil et al., 1999) (resonance at 520 nm) was used to estimate the percentage coverage of the PU spheres by the gold nanoparticles. The probable structure of the endoglucanase-nanogold-PU biomaterial is illustrated in Fig. 5C.1.

5.C.2.5. *SEM and energy dispersive analysis of X-rays (EDAX) measurements*

Samples for SEM and EDAX measurement were prepared by drop-coating a film of the nanogold-PU and Endoglucanase-nanogold-PU solutions on a Si (111) wafers. These

measurements were performed on a Leica Stereoscan-440 scanning electron microscopy (SEM) equipped with a Phoenix EDAX attachment.

5.C.2.6 .*Quantitative analysis of enzyme in bioconjugates*

The concentration of Endoglucanase A in the bioconjugate system was estimated via fluorescence measurements on Perkin-Elmer Luminescence Spectrophotometer (model LS 50B). The tryptophan and tyrosine residues in the enzyme were excited at 280 nm and the emission band was monitored in the range 300 to 500 nm. The loss in fluorescent intensity (arising from π-π^* transitions in tryptophan and tyrosine residues in proteins) was used to quantify the amount of endoglucanase bound to the nanogold-PU spheres. The intensity in the emission band was compared with free endoglucanse in solution. The decrease in the intensity of supernatant after centrifugation was proportional to the enzyme bound to the nanogold-polyurethane material. Lowry's method (1951) was also used for the estimation of amount of protein present in the supernatant. Thus, amount of Endoglucanase enzyme in the bioconjugate system was estimated indirectly.

5.C.2.7. *Enzyme assays in immobilized system*

The activity of Endoglucanase-nanogold-PU in solution was determined using CMC as a substrate (10 mg/ml; 0.05 M sodium-phosphate buffer, pH 7.0) and incubating the mixture at 60°C for 1 h. The reducing sugars released were determined by Nelsons-Somogyi method (Nelson, 1944; Somogyi, 1952*)*. For comparison, the catalytic activity of an identical amount of free Endoglucanase molecules in solution was determined as described above. The pH dependent variation in the enzyme activity in the bioconjugates was studied at different pH values. The reproducibility of the data was tested in three similar experiments. The activity of the bioconjugates was detected in the temperature range 40-80°C at pH 7. The results were compared with that of identical amount of free enzyme molecules in solution under similar conditions.

5.C.3. Results and Discussion

In the earlier studies on bioconjugates with colloidal gold (Gole et al., 2002), the reuse of bioconjugates is not possible. In this protocol the enzyme is bound to massive nanogold-PU microspheres. This facilitated convenient separation of immobilized enzyme from the reaction mixtures and hence they were reused. Also during immobilization of Endoglucanase in thermally evaporated lipid films, substrate protection was essential (Gole et al., 2001a). Hence to overcome the problem we have immobilized Endoglucanase enzyme on gold nanoparticles bound to surface of polyurethane microspheres through nitrogen atoms in the urethane group (Fig.5C.1). In this protocol enzyme molecules are on the surface of the nanogold-PU composite material and, hence the substrate protection was not needed.

5.C.3.1. UV-vis studies.

Fig.5C.2 shows UV-vis spectra of the as-prepared colloidal gold solution and the gold solution after addition of PU spheres and filtration. The surface plasmon resonance in the as-prepared colloidal gold solution can be clearly seen at 520 nm (Patil et al., 1999*)*. After stirring the colloidal gold solution with the PU microspheres dispersed in hexane, there is loss in intensity of the surface plasmon resonance due to a decrease in the concentration of gold nanoparticles in the aqueous solution (curve 2). This indicates binding of colloidal gold particles to the PU microspheres through nitrogen atoms in polyurethane. It is well known that pyridine and primary amines bind to colloidal gold through nitrogen atoms (Blatchford et al., 1982; Leff et al., 1996). It is possible that a similar mechanism involving nitrogen atoms in PU in the entrapment of gold nanoparticles on the microspheres is operative in this study. The purple-coloured polyurethane spheres capped with gold nanoparticles were observed to assemble at the interface between the two liquids and could be separated and cast in the form of a film.

5.C.3.2. Enzyme quantitative studies.

Fluorescence spectroscopy was used to quantify the amount of enzyme in the bioconjugate. The tryptophan and tyrosine residues in the enzyme were excited at

280 nm and the emission band was monitored in the range 300 to 500 nm. The loss in the fluorescent intensity of the supernatant after centrifugation (arising from π-π^* transitions in tryptophan and tyrosine residues in proteins) was used to quantify the amount of endoglucanase bound to the nanogold-PU spheres. Fig.5C.3.shows the fluorescence emission intensity of initial concentration (250 μg/mL) of free endoglucanase in buffer and supernatant after centrifugation of bioconjugates. The concentration of enzyme in the bioconjugates was determined from the calibration curve. From the decrease in the intensity of the supernatant the amount of enzyme bound to polyurethane nanogold was found to be 240 μg/10 mg of nanogold-PU. Lowry's method, (Lowry et al., 1951) was also used for the estimation of protein in the supernatant after centrifugation of the bioconjugates. The amount of the enzyme was found to be 220 μg/10 mg of nanogold-PU. This compares favorably with the amount of enzyme estimated via fluorescence measurements.

5.C.3.3. *Scanning electron microscopy*

Fig.5C.4. shows representative SEM images of drop-cast films of the nanogold-PU core-shell material (A) and the Endoglucanase-nanogold-PU bioconjugate material (B) on Si (111) substrates. While the surface texture of the PU spheres capped with gold nanoparticles is quite smooth (Fig.5C.4A, the gold nanoparticles are not clearly visible at the resolution of the SEM measurement), the presence of the protein in the bioconjugate material is clearly observed as a thin coating surrounding the spheres. Fig. 5C.4B, indicates that the Endoglucanase molecules in the bioconjugate material are highly concentrated in the form of sheets, this was also observed during immobilization of pepsin on Polyurethane-gold composit templates (Phadtare et al., 2003). As will be seen below, the specific activity of the Endoglucanase molecules in the bioconjugate is not reduced by the immobilization process, possibly due to the presence of gold nanoparticles on the PU sphere surface. Spot profile EDAX measurements of the sheets confirmed that they were composed of only the enzyme (through the strong sulfur signal from cysteine residues of Endoglucanase). The sulfer signal was absent in the nanogold-PU core-shell material as expected.

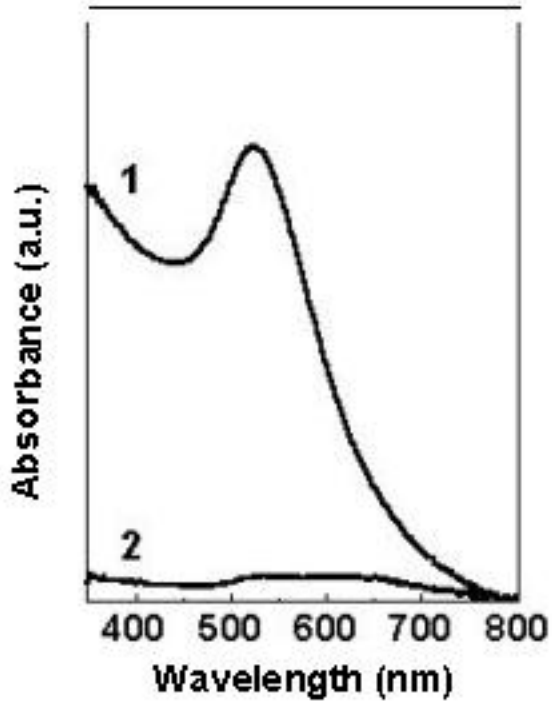


Fig. 5C.2. UV-Visible studies on the assembly of colloidal gold nanoparticles on polyurethane (PU) microspheres.
Curve 1: As-prepared colloidal gold solution
Curve 2: The gold solution after addition of PU spheres and filtration (text for details).

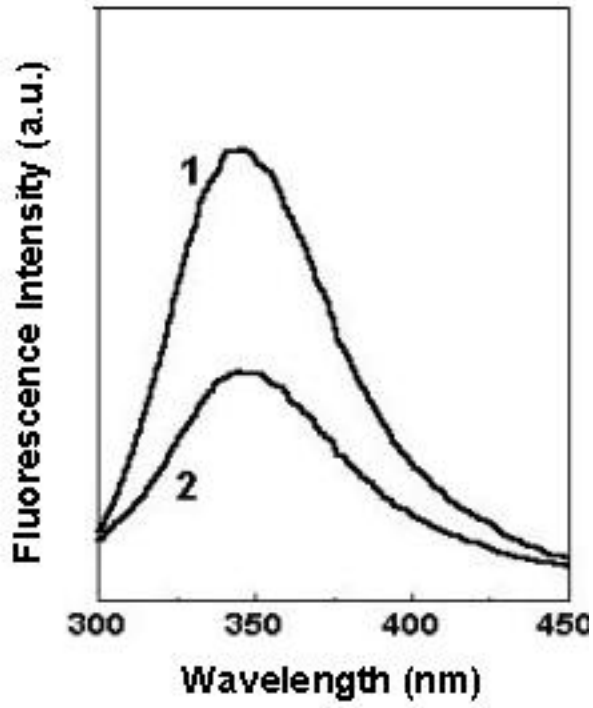


Fig. 5C.3. Fluorescence spectra for quantification of Endoglucanase A in bioconjugates
. Curve 1: Initial Endoglucanase A solution (250 μg/ml in buffer pH 5.0).
Curve 2: Supernatant after centrifugation of bioconjugates (text for details).

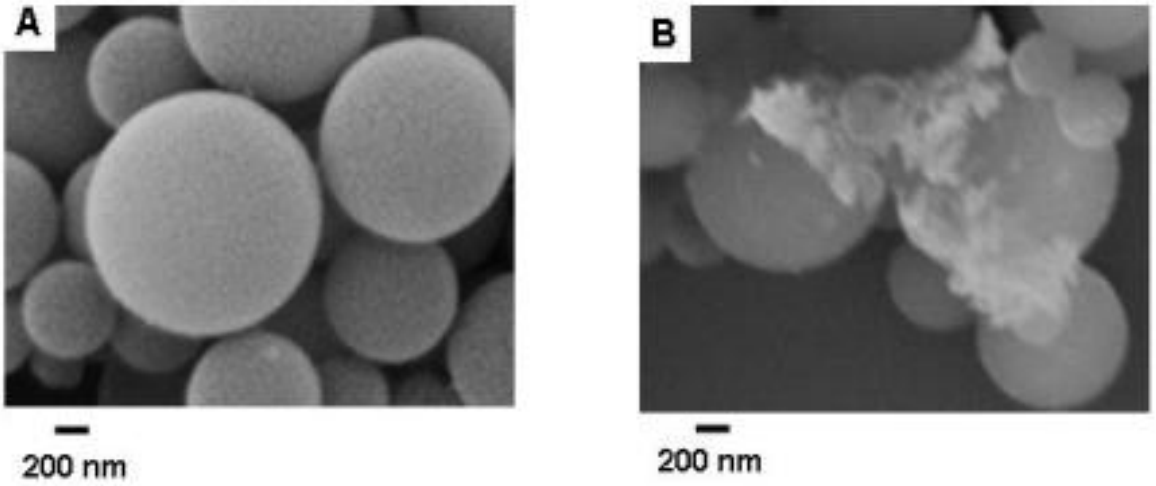


Fig.5C.4. SEM images of nanogold- PU 'core–shell '-like assembly

A: Nanogold-PU material (without enzyme)
B: Nanogold-PU-Endoglucanase A Bioconjugates (see text for details)

5.C.3.4. Enzyme activity in bioconjugates

The most significant aspect in enzyme-nanoparticle bioconjugate systems is retention of the enzyme activity after adsorption onto the nanoparticle surface. The enzyme activity in the bioconjugates was compared with that of the identical amount of free enzyme in the solution under similar assay conditions. Table 5C.1.shows the activity of the Endoglucanase-nanogold-PU bioconjugate for five cycles of reuses. The specific activity of free Endoglucanase in solution was determined to be 20 units/mg and that of the Endoglucanase-nanogold-PU bioconjugate was 18 units/mg. A marginal difference in the activity of the immobilized enzyme relative to the free Endoglucanase is seen and is attributed to experimental uncertainties. Thus, the Endoglucanase activity in the bioconjugate material is not compromised further to immobilization.

Table 5C.1. Endoglucanase activity in bioconjugates upon reuse

Experimental run	Endoglucanase gold PU spheres activity (%)	Endoglucanase-PU spheres activity (%)
1	**100**	**61**
2	**85**	**25**
3	**83**	**16**
4	**61**	**N.D.**
5	**44**	**N.D.**

N.D. Not detected

Activity (%) is the specific activity of the Endoglucanase in the immobilized form at pH 7.0 and 60°C. The maximum specific activity of Endoglucanase gold PU spheres is taken to be 100 %.

All enzyme activities were obtained from 5 separate experiments and were reproducible. The experimental data falls within range of < ± 10 % standard deviation.

The principle aim in immobilization protocols is enhancing the reusability of the enzyme. Since the Endoglucanase nanogold-PU bioconjugate material is easily separated from the reaction mixture by mild centrifugation, they were subsequently reused. Table 5C.1. shows the results of five cycles of reuse of the Endoglucanase-

nanogold-PU- bioconjugates. Care was taken to wash the bioconjugate material with the copious amounts of 0.05 M sodium phosphate buffer; pH 7 prior to each reuse. It is seen that there is a small decrease in the enzyme activity upon reuse. However, even after the third reuse, the Endoglucanase-nanogold-PU bioconjugate shows 83 % of the initial enzyme activity while it falls to 44 % after the fifth reuse. The decrease in the enzyme activity is likely to be due to the leaching of some amount of enzyme that is weakly bound to the surface of nanogold-PU microspheres. We believe the weakly bound enzyme could be present in the form of thin aggregated sheets as seen in the SEM picture of Fig.5C.4B.In the enzyme immobilization protocol described here, substrate molecules are easily accessible to the Endoglucanase immobilized on the surface of polyurethane-nanogold microspheres, thus rendering ineffective the so called 'mass transport problem' associated with the other enzyme immobilization methods. Thus, Endoglucanase in the bioconjugates behaves almost as if it is free yet retaining the advantages of immobilization such as ease of separation from the reaction medium etc.

In the previously described work on the immobilization of Endoglucanase in thermally evaporated lipid films, substrate protection of the enzyme was observed to be important in retaining activity of the immobilized enzyme. This step was important since otherwise, the active sites in the immobilized enzyme were blocked by the electrostatically complexed lipid molecules (Gole et al., 2001a). That such enzyme modification is not required in the protocol involving the use of gold nanoparticles tethered to PU microspheres is an important advance in the use of such scaffolds for enzyme immobilization. Clearly, the active site in Endoglucanase is not blocked when the enzyme is immobilized on the surface of gold nanoparticles.

In enzyme immobilization protocols, the operation of the biocatalyst at pH and temperature conditions far from form optimum temperature conditions becomes important and of industrial relevance. The enzyme activity of the Endoglucanase-nanogold-PU bioconjugate material was estimated at six different pH values (pH = 4, 5, 0.05 M sodium citrate buffer; pH = 6, 7, 0.05 M sodium phosphate buffer; pH = 8, 9, 0.05 M glycine-NaOH buffer). The activity of an identical amount of free endoglucanase in solution was measured under similar conditions. The reproducibility of the data was confirmed by three separate experiments performed under similar

conditions. It is observed from the Table 5C.2. that the enzyme activities of Endoglucanase both in solution as free enzyme and in the bioconjugate are almost identical at all pH values.

Table 5C.2. Comparison of Endoglucanase activity in solution and in Endoglucanase-nanogold- PU bioconjugates at different pH

pH	Free enzyme activity (%)	Bioconjugates activity (%)
4	85	87
5	100	100
6	85	87
7	80	65
8	60	44
9	30	18

The temperature variation in biocatalytic activity of the endoglucanase-nanogold-PU bioconjugate material was studied at pH 7 in the temperature range 40-80 $^{\circ}$C and compared with that of identical amount of free enzyme in solution under similar conditions (Table 5C.3). Three separate measurements at each temperature were performed to check the reproducibility of the data. It is seen that the endoglucanase-nanogold-PU bioconjugate shows enhanced stability at higher temperature compared to free enzyme in solution. At 70°C, the bioconjugate does not show any drop in activity while free enzyme in solution shows 83% of the maximum activity (Table 5C.1.). Similarly at 80°C, the bioconjugate is more thermally stable than the free enzyme molecules in solution (Table 5C.3). The increase in the thermal stability of the enzyme in the bioconjugates is due to the conformational integrity of the enzyme structures after binding to the gold nanoparticles through the amine groups and cysteine residues present in the enzymes.

Table 5C.3.Comparison of Endoglucanase activity in solution and in Endoglucanase - nanogold-PU bioconjugates at different temperatures.

Temperature (^{o}C)	Free enzyme activity (%)	Bioconjugates activity (%)
40	73	67
50	95	75
60	100	96
70	83	100
80	36	41

Such an increase in the thermal stability was also observed by Li et al., 2003 for pepsin immobilized on the surface of alumina nanoparticles. Thermal stability of the enzymes has also been observed after immobilization of various enzymes on the different solid supports (Arica et al., 1995; Akgol et al., 2002).

Control experiments were done where only polyurethane microspheres were used for direct immobilization of Endoglucanase. In such experiments, it was observed that the loading of the enzyme was considerably smaller that obtained by complexation with nanogold-decorated PU microspheres. The specific biocatalytic activity of endogolucanase directly bound to polyurethane microspheres was found to be 11 units/mg, which is lower than that for the endoglucanase-nanogold-PU bioconjugate system. This may be due to weak binding of the enzyme to polyurethane microspheres through electrostatic and/or hydrogen bonding interactions. We also observed a steep drop in activity during the first reuse cycle (50 % of the activity of the as-prepared Endoglucanase-PU bioconjugate) of the Endoglucanase-PU bioconjugate suggesting leaching of the enzyme during reuse. The presence of gold on the surface of the PU microspheres is thus crucial in retention of the enzyme during reuse.

5.C.4. Conclusion

The immobilization of Endoglucanase A on nanogold-labelled PU microspheres is described in this part of the thesis. Substrate protection of the enzyme prior to immobilization, which was a prerequisite in earlier studies on immobilization of Endoglucanse in thermally evaporated lipid films, is not essential in nanogold-PU bioconjugates. Since the enzyme is present on the surface of the nanogold-PU microspheres, substrates are easily accessible to the enzymes and consequently, the biocatalytic activity of the Endoglucanase-nanogold-PU material was comparable to the free enzyme in solution. The endoglucanase-nanogold-PU bioconjugate is easily separable from the reaction mixture by mild centrifugation and exhibited excellent reuse characteristics. The role of the underlying nanogold-polyurethane microspheres in stabilizing the enzyme under variable pH and temperature conditions has also been studied.

Finally to summarize, this Chapter describes studies on immobilization of cellulases on novel nanosupports. The encapsulation of endocellulase in thermally evaporated fatty amine films by a simple beaker-based immersion process and its enzymatic activity under different pH and temperature conditions were demonstrated. The optimum operation temperature shifted to higher values relative to that of the free enzyme in solution. Immobilization of cellulases on other novel supports such as colloidal gold nanoscale surfaces and three dimensional shell like assemblies using polyurethane microspheres has resulted in improved operational stability of enzyme as well as making them reusable. However, further research in this area demands studies on the kinetic parameters of immobilized systems, scale up of immobilization protocols and their applications in various industrial processes. One of the important limitations in the success of immobilized enzyme is characterization of enzymes in immobilized form. However, selection of nanoscale supports in the present studies, enanabled us to study molecular structure of immobilized enzyme and its interaction with the support. The methodology can be extended to variety of diagnostic enzymes mainly used for development of biosensors. The reasonably fast time-scales for the immobilization and the enzyme-friendly intercalation conditions are the other important features of immobilization protocols described in this Chapter.

Chapter 6

Enzymatic Deinking of Mixed Office Waste (MOW) Papers

Summary

The major difficulty in recycling of waste paper is removal of inks. In this work the extracellular cellulase preparation from alkalotolerent *Fusarium* sp was used for deinking of Mixed Office Waste (MOW) papers. The enzyme treatment resulted in the increase in brightness with the reduction in ink counts of the recycled paper. The objective of the present work is to understand the mechanism of action of alkaline active cellulase complex from alkalotolerant *Fusarium* sp during deinking of MOW-paper. The loosening and release of toner particles from the printed papers due to random acting endoglucanase components facilitate 'Biodeinking' of MOW-papers. The experimental results suggested a possible mechanism of enzymatic deinking of MOW-papers for recycling purpose.

The refined extracellular cellulase preparation was fractionated on BioGel P-100 gel filtration column. Two separate enzyme fractions namely Endo A and Endo B were obtained and they showed 2.3 and 4.6 fold increase in their specific activities, respectively. The viscometric analysis indicated the increase in fluidity ($1/\eta_{sp}$) of 1% carboxymethyl cellulose solution. The data revealed that Endo B was 1.9 fold more random as compared to Endo A. Both the endoglucanases showed comparable patterns of their ability to release short fibres in the reaction mixture when filter paper was used as the substrate. The average size of released short fibres was ranged between 20-100μm. Adsorption characteristics of the endoglucanases were examined using increasing concentrations of enzyme and their effect on rate of the hydrolytic reaction. The data indicated that the enzyme action is prominent at the surface of substrate rather than at their cracked edges. The X-ray diffractograms showed a marginal increase in the degree of crystallinity of the substrates in the initial stages of the enzymatic action. Amorphous regions of the cellulose substrates appeared to be readily attacked by the enzyme. The enzyme treatment resulted in the increase in brightness with the reduction in ink counts of the recycled paper. Based on the distinct properties of endoglucanases a probable mechanism of enzymatic deinking process is presented schematically. Also, the fabrication of the cost effective, laboratory scale flotation device that can be used for deinking of various grades of wastepaper is described as a separate annexure at the end of this Chapter.

6.1. Introduction

The demand for paper is rising at a fast rate. The global market for paper was about 80 million metric tons per annum at the beginning of 1980s, that is reached to 350 million metric tonnes by the year 2003 (Prasad, 2002). The paper industry is manufacturing above $ 200 billion worth paper annually and this industry is considered as one of the most polluting industries of the world. Most of the paper manufacturing steps utilize hazardous chemicals like alkali, chlorinated bleaching agents, reducing agents, surfactants, defoamers, deinking agents, sizing agents, binders, colorants and different additives. These chemicals have harmful impact on the environment. Also pulp and paper industry is heavily exploiting natural resources like timber, water and energy. For manufacturing one tonne of paper it requires 3.3 tonne wood and 0.4 tonne petroleum. Secondly, about 7 million metric tonnes of waste papers generated annually are occupying space in the form of land fillings. Obviously, recycling of wastepaper is desirable in order to save trees and energy. This in turn will reduce the effluents, pollutants and also the size of landfills. As a result the technologists are looking forward to develop the novel, ecofriendly way of recycling the waste papers.

6.1.1. Enzymes for pulp and paper industry

It is well known fact that microbial systems manage their chemistry rather more efficiently than man-made chemical plants, and that their wastes tend to be recyclable and biodegradable. As pulp is composed of cellulose, hemicellulose and lignin it can be modified with microbial enzymes. The enzymes are specific, safe and easy to control. They can accelerate the reaction at moderate conditions by replacing the harsh chemicals and are biodegradable. Thus, the selected enzymes are more advantageous than chemicals for the desired modification of the pulp. Several microbial enzymes are useful for developing ecofriendly technologies for paper and pulp manufacturing. These enzymes includes xylanases (Viikari et al., 1994, 1996), mannanases (Oksanen et al., 2000) for biobleaching of paper and pulp, cellulases for enzymatic deinking (Jeffries et al., 1994; Gubitz et al., 1998b ; Vyas and Lachke, 2003) and enhanced drainage rates (Stork et al., 1995), laccases and ligninases for delignification of pulp (Call, 1993; Call and Mücke, 1997); lipases, esterases for pith removal in the pulp and in deinking of waste papers (Ire et al., 1989; Fischer and Messner, 1992; Morbak et al.,1999) and

pectinases for various fibre handling operations and enzymatic retting of flax fibres (Reid and Ricard, 2000; Akin et al.,2001;Evans et al.,2002). Fungi are also useful in biomechanical pulping (Messner and Srebotnik, 1994; Akhtar et al., 1999). Several of these processes are commercialized and some of them are emerging rapidly (Table 6.1).

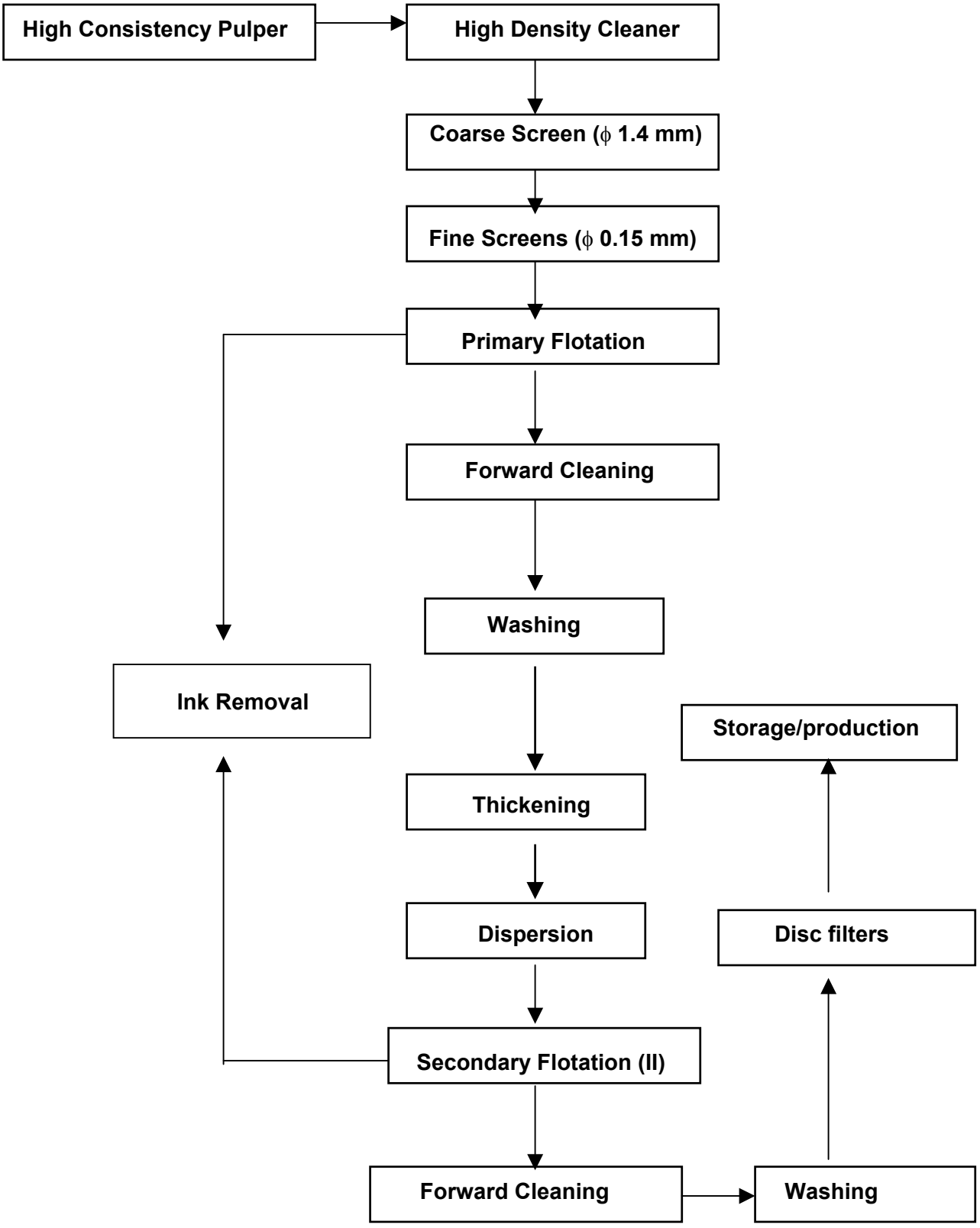
Table 6.1. Role of biotechnology in paper and pulp industry

Process	Mechanism	Microorganisms used
Fibre handling	Pectin removal using pectinanses	*Aspergillus* sp.
Biopulping	Using fungi for lignin breakdown (ligninase)	*Ceriporiopsis subvermispora*, white rot fungus *Lentus edodes*
Enzymatic pitch control	Sticky lipid removal by lipases	*Candida cylindracea*
Biobleaching	Xylan breakdown by xylanases	*Trichoderma* sp., *Trametous versicolor*
Sizing	Starch modification with amylases	*Bacillus stearothermophillus*
Refining	Removal fine particles with cellulases	*Trichoderma* sp.
Slime removal	Levan hydrolysis	--
Enzymatic deinking	Removal of ink particles from recycled fibre with alkaline active cellulases	*Humicola insolens*
Effluent treatment	Removal of lignosulfonates using microbial cultures	*Trametous versicolor, Aspergillus* sp., Sulfate reducing bacteria.

6.1.2. The deinking process

The process of removing ink and other contaminants from recycled fibre is termed as deinking. Although industry is familiar to the art of deinking for last 50 years, recycling of wastepaper into high-grade writing paper is comparatively new trend. Varieties of ink are used for different printing processes. These are considered as contact or non-contact ink types. Each of them requires different kind of strategies for deinking. Normally the ink contains non-polar pigments or dyes and an agent that carries the ink to paper and facilitates in binding. These agents are typically water insoluble oils or polymers as in case of toners. Thus, their removal is not an easy process. Washing and flotation are two most commonly used methods of deinking. Other methods like heat decolourization (Fujioka et al., 1999), irradiation (Macdonald and Nohr, 2000), organic solvent-based deinking (Fujioka et al., 2000), and magnetic deinking (Gubitz et al., 1998a) are still under experimental conditions. The choice of method depends on type of the ink to be removed and the desired quality of pulp. Black newspaper inks are generally removed by washing while coloured magazines are deinked by flotation. In both methods toxic chemicals are used. The schematic drawing of a typical deinking process used presently in the recycling industry is presented in Fig.6.1. Microbial enzymes can prove valuable alternative in order to reduce the use of toxic chemicals. Microbial cellulases (Jeffries et al., 1994; Vyas and Lachke, 2003) hemicellulases (Morkbak and Zimmermann, 1998), amylases (Zollner et al., 1998, Elegir et al., 2000), lipases (Morkbak et al., 1999) have shown promising results in increasing brightness of the fibres when used in combination with flotation deinking. The choice of enzymes to be used depends on the type of the ink to be removed as well as the type of the pulp. For example if the furnished pulp stock includes paper with oil based inks, (as generally in case of coloured magazine prints) then lipases are found useful in deinking of such pulp stock. Hemicellulases have proven useful in deinking of newsprint wastepaper. Deinking with hemicellulases has benefit of improved brightness of pulp due to its bio-bleaching effect. Amylases are also important in bleaching and brightening processes.

Fig. 6.1. Flow chart of Ecofriendly Deinking Process



6.1.3. Deinking of Mixed Office Waste (MOW) paper with cellulases

MOW paper includes laser printed and photocopier waste papers coated with toners. These toners are copolymers of styrene and acrylate that get thermally fused with cellulosic fibres of the paper during printing. Even costly dewatering, dispersion, additional flotation and washing process are not successful in separating inks from the fibres (Fig.6.1). Here is the real challenge for paper technologists because none of the conventional deinking methods can remove these inks from the paper. Moreover, it is the most valuable grade paper available in the market. Several researchers have proposed the application of cellulases to facilitate ecofriendly deinking process (Jeffries et al., 1994; Sreenath et al., 1996; Morkbak and Zimmermann, 1998b; Gubitz et al., 1998a,b; Qin et al., 1998; Elegir et al., 2000; Sanciolo et al., 2000).

As described previously in Chapter 2, different cellulase preparations that differ in their structural and functional properties can be obtained from variety of microbial sources. However, neutral to alkaline active cellulases are found more useful in deinking of waste papers. Acid cellulases that hydrolyze cellulose in acidic pH range of 3-6 can be used in acid sized (sulphite paper) paper. But alkaline sized papers are more acceptable because of their high fibre flexibility. Calcium carbonate is often used in the paper as a filler and brightner. It dissociates from fibres in alkaline environments and leaves fibres with alkaline pH (Sreenath et al., 1996). Hence alkaline active and alkali stable cellulases are necessary to process them for deinking. This automatically saves additional acid treatment required for acid cellulases to work. However, microbial alkaline cellulases are rare. A few *Bacillus* sp. produce cellulases that are active in alkaline pH. Unfortunately bacterial cellulases lack the complete cellulase complex. Thermal stability of enzyme is an additional important prerequisite for enzyme complex to be used for development of a commercial process. These all requirements motivated us for screening of alkalophilic fungi, producing alkaline active cellulases and applying them in biodeinking of MOW paper.

In most of the earlier studies mentioned above the focus was mainly laid on the evaluation of various enzymes and their effects on different paper and inks. However, the exact role of enzymes during deinking of waste papers is still poorly

understood. It is observed that the treatment with cellulase facilitates the removal of residual fibres from toner surfaces, which in turn enhances the flotation efficiencies (Jeffries et al., 1994, Sanciolo et al., 2000). The objective of the present work is to understand the mechanism of action of alkaline active cellulase complex from an alkalotolerant *Fusarium* sp. during deinking of MOW-paper. The loosening and release of toner particles from the printed papers due to random acting endoglucanase components facilitate biodeinking of MOW-papers. Based on the experimental results a possible mechanism of enzymatic deinking of MOW papers for recycling purpose is presented in this Chapter.

6.2. Materials and Methods

6.2.1. Enzyme production

The crude fungal cellulase preparation was obtained from the fungus, alkalotolerant *Fusarium* sp as described in Chapter 2.

6.2.2. Separation of Endo A and Endo B fractions

After harvesting mycelia by centrifugation at 10,000 X g for 10 min, the supernatant was used as crude cellulase extract. This crude extract was concentrated using freeze drier and fractionated on Bio-Gel P-100 gel filtration column as described in Chapter 3. Two active fractions Endo A and Endo B were pooled and concentrated using an Amicon ultrafitration membrane (PM 10).

6.2.3. Enzyme assays

The activity of endoglucanase, xylanase and filter paper degrading activity (FPase) were measured as described previously in Chapter 2

One international Unit (IU) of enzyme activity was defined as the amount of enzyme that liberates 1μmole glucose/min (for endoglucanase and FPase) or xylose/min (for xylanases) under the standard assay conditions. One IU of β-

glucosidase and β-xylosidase has been defined as the amount of enzyme, which liberates 1μmole of *P*-nitrophenol/min under the standard assay conditions.

6.2.4. Viscometric analysis

The relationship between the change in specific viscosity and reducing sugars released from CMC solution by endoglucanase was determined as follows (Sadana et al., 1984). A 13 ml reaction mixture containing 1% CMC (w/v) and enzyme in 0.05 M phosphate buffer pH 7.0 was incubated at 50°C for 30 min. The decrease in flow time was recorded at every 3-min interval for 30 min and simultaneously aliquots were removed, and reducing sugars were estimated by *P*-HBAH (*P*-hydroxybenzoic acid hydrazide) method (Hurst et al., 1977).

6.2.5. Short fibre forming activity

Short fibre formation was determined according to Halliwell and Riaz (1970) with a slight modification. The assay mixture contained 50 mg of Whatman no.1 filter paper and appropriately diluted enzyme fraction in 0.05 M sodium phosphate pH 7.0 buffers in a final volume of 5 ml. The residual filter paper was carefully removed by forceps leaving a suspension of insoluble short fibres. The short fibres were measured turbidometrically at 600 nm. (A control was run under identical conditions in the absence of enzyme). The suspension of insoluble short fibres was centrifuged at 10,000 X g for 5 min and supernatant was used for determination of reducing sugars by *P*-HBAH method.

6.2.6. Scanning electron microscopic studies

The sediment of short fibres after centrifugation was washed twice with deionised water and resuspended in 1.0 ml of distilled water. Out of that an aliquot of 0.1 ml was dried on aluminum foil and coated with thin gold layer. The length of the short fibres formed by action of endoglucanase was determined using a Leica Stereoscan 440 Scanning electron microscope.

6.2.7. Adsorption characteristics of cellulases on insoluble substrates

Enzymatic hydrolysis on various cellulosic substrates, namely filter paper, MOW pulp, Avicel PH-101 and bagasse pith was carried out to compare n values according to McLaren (1963). For this purpose 0.5 g of each substrate was incubated at varying enzyme concentrations 10, 20,30,40 IU/ml at pH 7.0 and 60°C for 4 h. The rate of reaction was determined from the amount of reducing sugars produced. The n values were calculated by plotting Log V against Log [E].

6.2.8. Determination of the degree of crystallinity of cellulose

Whatman filter paper No.1 (200 mg) was incubated with culture filtrate at 60° C for different time intervals (0, 6, 12, 18, 24 and 48 h). The filter paper was removed from reaction mixture at selected incubation time and washed with deionied distilled water to remove most of the non-adsorbed enzymes, soluble sugars and buffer salts. The sample was immediately lyophilized to avoid possible recrystallization of cellulose fibres.

The X ray diffraction of each set of samples was recorded using Philips Analytical X- ray diffractometer (PW 1710). The wavelength of Cu radiation source was 0.1540 (nm) and spectra were obtained at 30 mA with an accelerating voltage of 40 kV. Samples were scanned on the automated diffractometer from 9-40° of 2 θ (Bragg angle) with data acquisition taken at intervals of 0.400 s. A peak resolution program (PC-APD diffraction software) was used to calculate crystallinity index. The crystallinity index (CrI) of cellulose was also calculated by empirical method described by Segal et al. (1959) using following equation:

$$CrI\ (\%) = I_{002} - I_{AM} / I_{002} \times 100$$

Where I_{002} is the maximum intensity of the 002 lattice diffraction (reflection attributed to crystalline regions of the sample) and I_{AM} is the intensity of diffraction at Bragg angle $2\theta = 18°$ (reflection attributed to amorphous regions of the sample). The results obtained using both the methods were in good agreement. The apparent dimension of the cellulose crystallite were obtained by applying the Scherrer equation

to the data collected by peak resolution program. The average thickness of the crystallite at 002 plane of diffraction was calculated using following equation:

$$t(hkl) = K.\lambda / \beta.\cos\theta$$

Where t is thickness of crystal at (h k l) plane of diffraction, λ is the wavelength of X- ray source, K is the Scherrer constant (for cellulose K= 0.9) and β is the peak full width at half of the maximum height.

6.2.9. *Deinking trials*

Deinking trials for MOW papers were carried out at Pudumjee Pulp & Paper Mills Ltd. situated at Chinchwad Pune. The waste paper used in this study was a mixture of photocopier (Xerox) papers that were coated with toner and laser printouts. Each of them was cut in to small pieces and immersed in warm water at 50°C for at least 2 h. This paper was fiberized in medium consistency water-jacketed pulper for 10-15 min in the presence of 0.1% (v/v) nonionic surfactant. The temperature during pulping was 55°C and pH was adjusted to 8.5 with 1N NaOH. The suspension was mixed until paper particles were no longer visible. The enzyme treatments were carried out at 10 % final consistency at pH 8.0-8.5 at 55°C for 20 min. The enzyme dose was selected as 50 IU CMCase /100g of oven dried pulp. Prior to flotation the pulp samples were disintegrated in universal laboratory desintegrator at 5000 rpm for 5 min. The pulp samples from each trial run were taken after disintegration. Each sample was immediately filtered and the filtrate was frozen. Later these samples were thawed and centrifuged at 7000 rpm for 10 min. Supernatant was used to measure reducing sugars produced during enzyme action by *P*-HBAH method. The pulp was diluted to 1% consistency with distilled water. To separate toner particles from the fibres all the enzyme treatments were followed by 10 min flotation run in a 10 l capacity laboratory flotation unit at room temperature. This laboratory scale unit was fabricated in our laboratory (Vyas et al., 2003b). The foam containing floated ink particles were removed mechanically by the outflow provided as a slope from the top of one side of flotation unit. At the end of flotation the deinked fibres were recovered on a laboratory mesh from the drain valve of the flotation cell. Control runs were taken under identical

conditions replacing active enzyme by heat denatured enzyme preparation. After flotation, pulp consistency was determined for each sample. TAPPI Method T 205 om-88 was used to prepare handsheets from enzyme treated as well as control pulp. Five handsheets per run (weight 50 g/m^2) were made on Universal semiautomatic handsheet maker unit. The handsheets were placed on dry metal plates and covered with filter paper. Each metal plate was placed in to drying ring and handsheets were dried at room temperature. Handsheets from enzyme treated pulp, heat denatured control runs and handsheets from the pulp without flotation were compared for different optical and strength properties. The ink specks were counted by an optical scanner in visible (220μm to 80μm) to sub visible range (10-80μm). Brightness was measured at different places on hand sheet and values are expressed as the average % value (TAPPI T 452 om 92). Similarly, other strength properties like breaking length, burst index and tear index were determined using TAPPI standard tests. The ink specks in visible (220μm to 80μm) range were counted according to TAPPI standard method (TAPPI T 213). Brightness was measured at different places on hand sheet according to (TAPPI T 452 om 92) and values are expressed as the average % value.

6.3. Results and Discussion

6.3.1. Enzyme properties

The crude culture filtrate of alkalotolerant *Fusarium* sp showed following extra cellular cellulolytic and xylanolytic activities (IU/ml): CMCase, 8.80; FPase, 0.52; β-glucosidase, 1.31; β-xylanase, 0.72 and β-xylosidase, 0.32. The refined enzyme preparation is active in a broad pH range of 4 to 10 with a pH optima at 5.0 at 60°C. The enzyme is stable in an alkaline pH range of 8 -10 at 50°C. The half-life of the enzyme at pH 8.5 at 50°C was found to be 10 h. The enzyme showed maximum activity at 60°C and retained 80% of the maximum enzyme activity at 70°C (For details please refer Chapter 2). The alkalotolerant *Fusarium* strain reported in this work secretes high amount extracellular cellulolytic enzymes when cultivated under submerged fermentation. The enzyme preparation was found active and stable under alkaline conditions suggesting it's potential for deinking process.

6.3.2. Enzyme purification

The crude cellulase extract from culture filtrates of alkalotolerant *Fusarium* sp was concentrated by lyophilization. The concentrated protein was fractionated on Bio-Gel P 100. The elution profile is presented Chapter 3. The fractions Endo A and Endo B showed 2.3 and 4.6 fold increase in their specific activities, respectively. The elution profile indicated that the molecular weight of Endo B is lower as compared to Endo A. The separated fractions Endo A and Endo B were free from β-D-xylanase, β-D-xylosidase and β-D-glucosidase activity.

6.3.3. Viscometric analysis

The increase in fluidity ($1/\eta_{sp}$) of CM-cellulose solution due to hydrolytic action of Endo A and Endo B fractions is shown in Fig.6.2. Equal amounts of CMCase units from isolated fractions were used for the determination of randomness of their attack on 1% solution of CMC. The action of Endo B on CMC was more rapid as compared to that of Endo A. Earlier Investigators have interpreted that it is the magnitude of the slopes that determines the degree of randomness of hydrolytic action of enzymes; steeper the slope more random the enzymatic attack (Kanda et al., 1980; Sadana et al., 1984). From the ratio of the slopes it was found that Endo B fraction is 1.9 times more random in its hydrolytic action than Endo A.

6.3.4. Short fibre forming activity

Release of significant amount of short fibres as well as reducing groups from filter paper due to enzymatic action were observed on prolonged incubation (10 h) of reaction mixture. The release of short fibres can be observed within 15-30 min. The fragmentation of filter paper increased initially with increase in time (Fig.6.3). However, there is decrease in short fibre formation after 6 h of interval without decrease in reducing sugar formation. This may be attributed to preferential hydrolysis of short fibres formed by endoglucanase components. Both enzyme fractions showed similar patterns of short fibre formation. The Endo B fraction with a low molecular weight endoglucanase showed more short fibres forming activity than fraction Endo A. Earlier investigators have demonstrated that the low molecular weight endoglucanase from

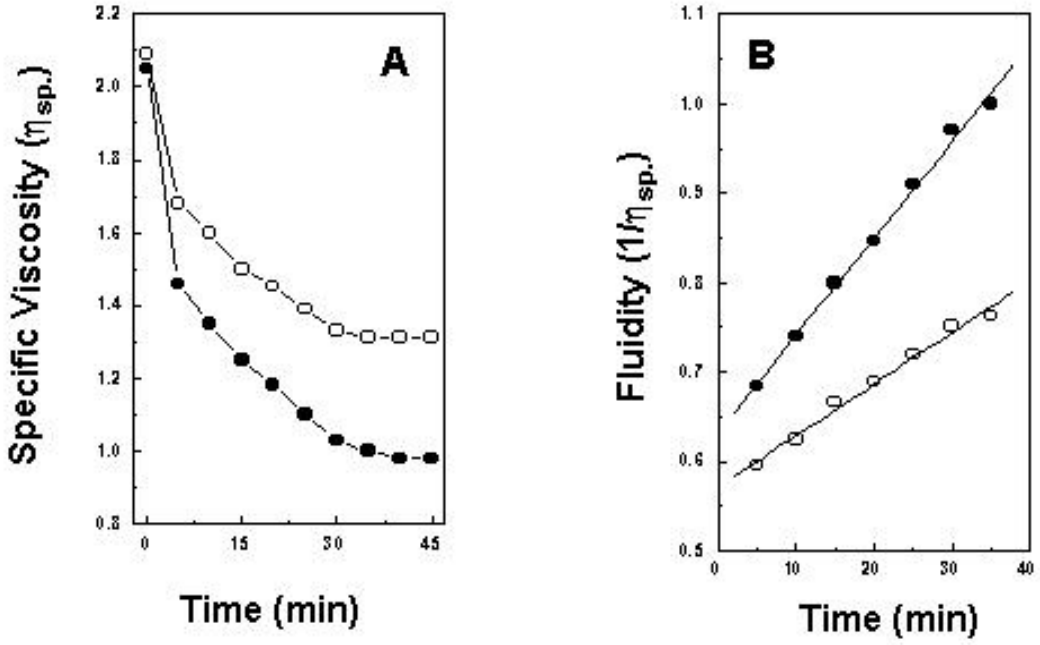


Fig. 6.2. Randomness of CMC hydrolysis by endoglucanases : The time course of CMC (1.0%) hydrolysis by isolated cellulolytic fractions Endo A and Endo B .

The figure shows that fraction Endo B is more random in action than fraction Endo A.

Symbols: Endo A (o), Endo B (●).

A. Lowering of specific viscosity (η_{sp}) of 1% CMC solution due to endoglucanases.
B. Increase in fluidity ($1/\eta_{sp}$) of 1% CMC solution due to endoglucanases.

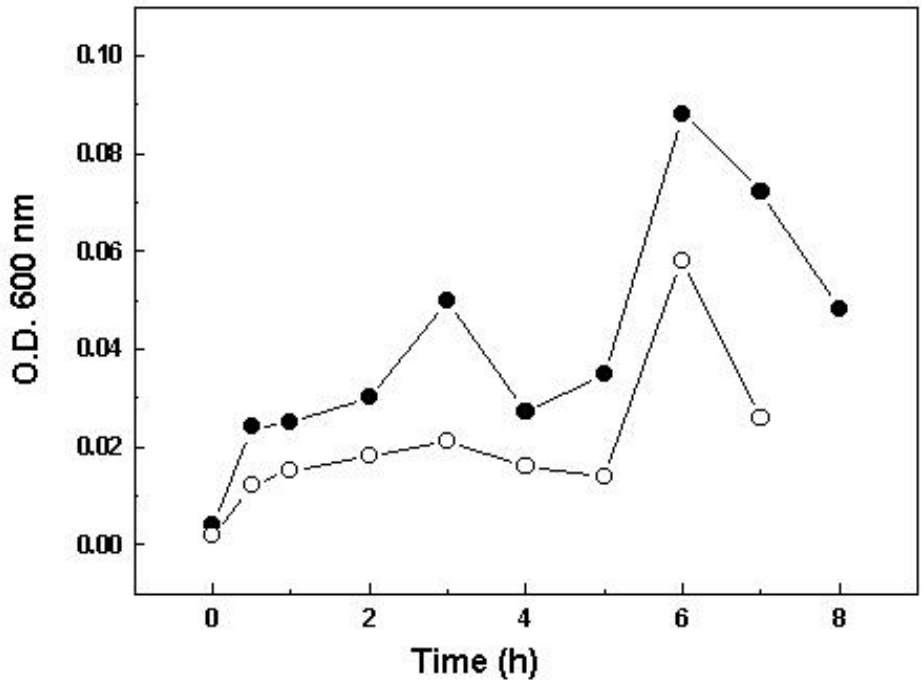


Fig. 6.3. Release of short fibres from filter paper due to action of endoglucanases from alkalotolerant *Fusarium* sp

The rate of release of fibres in the reaction mixture was recorded from absorbance at 600 nm. The plotted values are corrected for the absorbance of the control.

Symbols Endo A (o), Endo B (●)

T.koningii is responsible for the formation of short fibres from native cellulose substrate (Halliwell and Riaz, 1970). The 1,4-β-D-glucan cellobiohydrolase from *T. viride* was completely devoid of short fibre forming activity. However, two purified endoglucanases from the same culture showed short fibre forming activity (Berghem and Petersson, 1973,1976). On the contrary, endo type of mode of action of (1--4)-β-D glucan cellobiohydrolase from *S. rolfsii* was demonstrated by Sadana and Patil (1985). The scanning electron microscopic studies confirmed the short fibre formation by the endoglucanase from fraction Endo B. The average size of short fibre formed after 6 h of incubation with enzyme fraction varied between 20-100μm as compared to 300-500μm with control without enzyme (Please refer Fig.4.7).

6.3.5. *Adsorption characteristics of cellulases on insoluble substrates*

For insoluble substrate systems the initial rate of hydrolysis is not directly proportional to the total amount of enzyme in the system. When the effect of enzyme concentration on the rate of hydrolysis of filter paper, MOW pulp, Avicel and bagasse pulp was studied the data conformed to the equation developed by McLaren (1963) describing the hydrolytic rate of enzymatic reaction in heterogeneous system (Fig.6.4). The n values for filter paper, MOW pulp, Avicel and bagasse pith were calculated to be 0.31, 0.44, 0.28, and 0.50, respectively. The comparable n values were also observed in case of extracellular cellulases produced by *S. rolfsii* (Shewale and Sadana, 1979).

The n value describes the adsorption characteristics of enzyme on the surface of substrate. Adsorption of cellulase on cracks or edges of the surface gives n =1/3 and its adsorption on the surface of the gives n= 2/3. According to the theory, the low n value could be interpreted as indicating that the hydrolysis of microcrystalline cellulose takes place primarily on the cracked edges of the surface of particle. The n value for MOW pulp is 0.44. This suggested that during enzymatic deinking process the enzyme action take place at the surface.

6.3.6. *The degree of crystallinity of cellulose (CrI)*

The effect of enzymatic hydrolysis on the degree of crystallinity of the substrate obtained from X-ray diffractograms is shown in Fig.6.5.There is a slight increase in the degree of crystallinity of the substrate in the initial stages of enzymatic action.

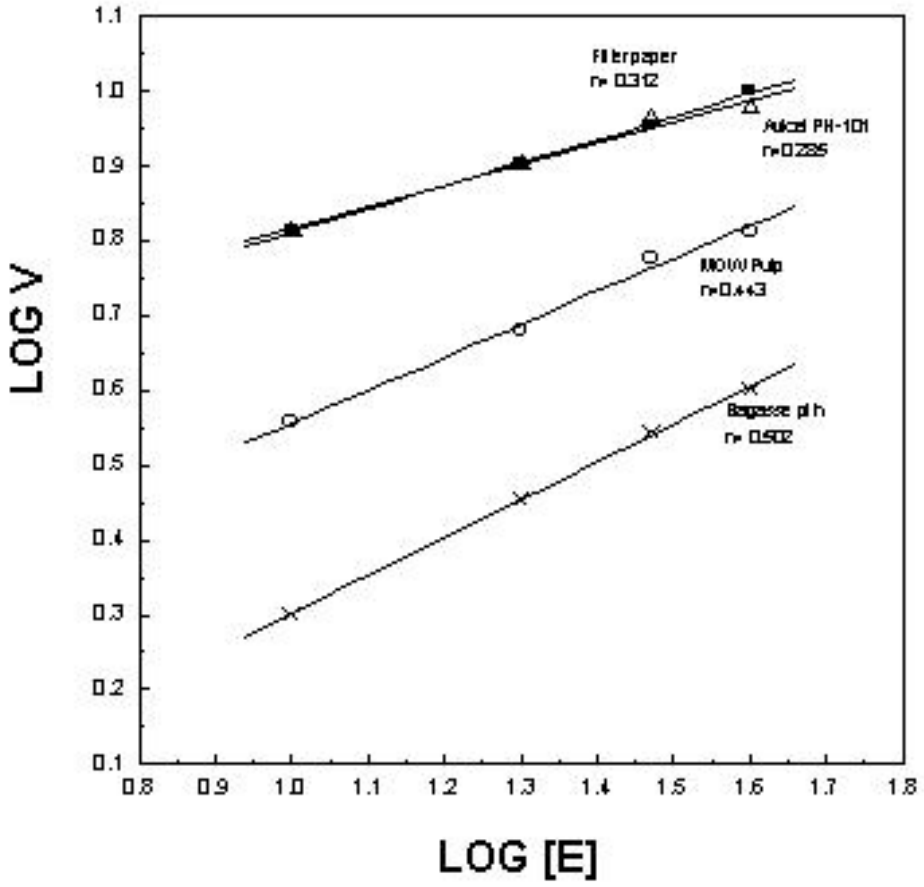


Fig.6.4. Determination of n value for different cellulosic substrates.

However, apparent change in crystallite size at 002 plane is negligible during initial period of hydrolysis (Table 6.2).

Table 6.2 Influence of enzymatic hydrolysis on the crystallinity index (CrI) of the cellulolytic substrate (Whatman no.1 filter paper)

	Hydrolysis Time (h)				
	0	6	12	24	48
Crystallinity Index (%)	92.04	93.10	93.57	91.30	92.38
Crystallite dimensions (nm) (002 plane)	5.48	5.48	5.50	5.07	5.69

Enzymatic hydrolysis was carried out at a substrate concentration 2% (w/v) and enzyme loading of 10 IU g^{-1} cellulose.

Crystallinity Index was calculated as described by Segal et al., (1959).

This observation is convincing evidence that the amorphous portion of the cellulose is more susceptible to the hydrolytic action of the endoglucanases than the crystalline portion of the substrate. Similar increase in crystallinity was also reported by earlier investigators (Lee et al., 1983;Nazhad et al., 1995). In the present case it is evident that more randomly acting endoglucanases act on amorphous regions of cellulose in the initial stages of enzyme action during deinking operation. This is followed by release of short fibres. The data obtained from X-ray diffraction very well supports the proposed enzymatic mechanism of deinking by us.

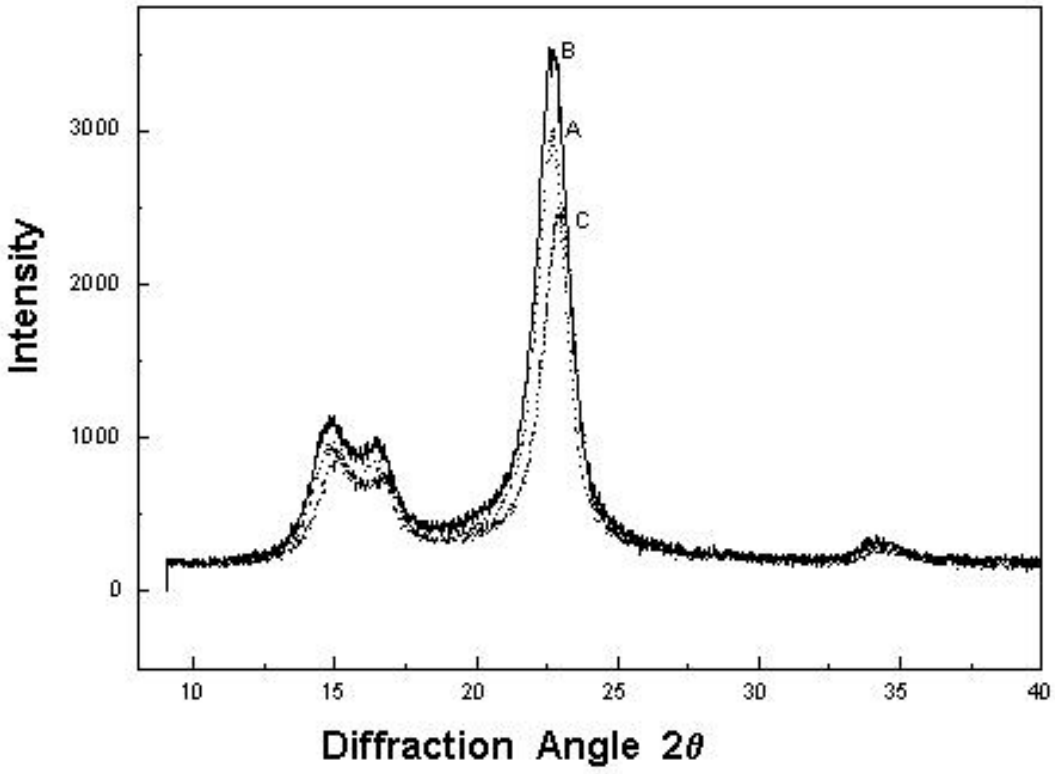


Fig. 6.5. X-ray diffractograms of Whatman No.1 filter paper during hydrolysis by alkaline active cellulase preparation from alkalotolerant *Fusarium* sp

(A) time = 0 h, (B) time= 12h, (C) time = 24h.

6.3.7. Deinking trials

The handsheets made from treated pulp and control pulp samples were compared for optical as well as mechanical strength properties in order to evaulate the influence of enzyme on deinking (Fig.6.6). The deinking process resulted in increase in brightness as compared to blank pulp samples that were not processed by flotation. Similarly, improved brightness by 3-4 points was shown by enzyme treated pulp over heat denatured control pulp samples. Clearly, the increase in brightness points can be attributed to enzymatic action that showed reduction in residual ink specks. The effect of ink particles on the brightness of paper has been evaluated by McKinney (1987). Simultaneously xylanolytic activities in the enzyme preparation can also aid in improved brightness of the enzyme treated pulp (Jeffries et al.,1994; Gubitz et al., 1998a). The enzyme treatment of MOW paper increased removal of ink in the presence of nonionic surfactant. Enzyme treatment resulted in reduction of ink specks after flotation (Table 6.3).

The smaller ink particles of the size < 200 μm were removed more effectively than larger ink particles (>220 μm). However, the reduction in larger size particles is also observed. It can be possible that the larger ink particles might have been broken down in to small particles due to enzyme action and small particles are effectively removed by flotation. Welt and Dinus (1998) have also recorded similar observation. The effect of enzyme treatment on physical and mechanical strength properties of the recycled paper is presented in Table 6.3.There is marginal improvement in burst index and breaking length with reduction in tear index values in case of enzyme treated handsheets. The primary fines in the recycled pulp have high affinity for water but does not contribute significantly to interfibre hydrogen bonding. Several studies have found that during enzymatic deinking, the cellulases act preferentially on the fines and microfibrils protruding out from the surfaces (Jeffries et al., 1994; Gliese et al., 1996; Gubitz et al., 1998b; Lee and Eom, 1999). This enzymatic action increases the freeness of the pulp (Jeffries et al., 1994; Lee and Eom, 1999). Moreover, preferential removal of fines improves the interfibrillar bonding that enhances strength properties like burst factor and tensile length. However, the decrease in tear strength could be due to localized debrillation of cellulose fibre.

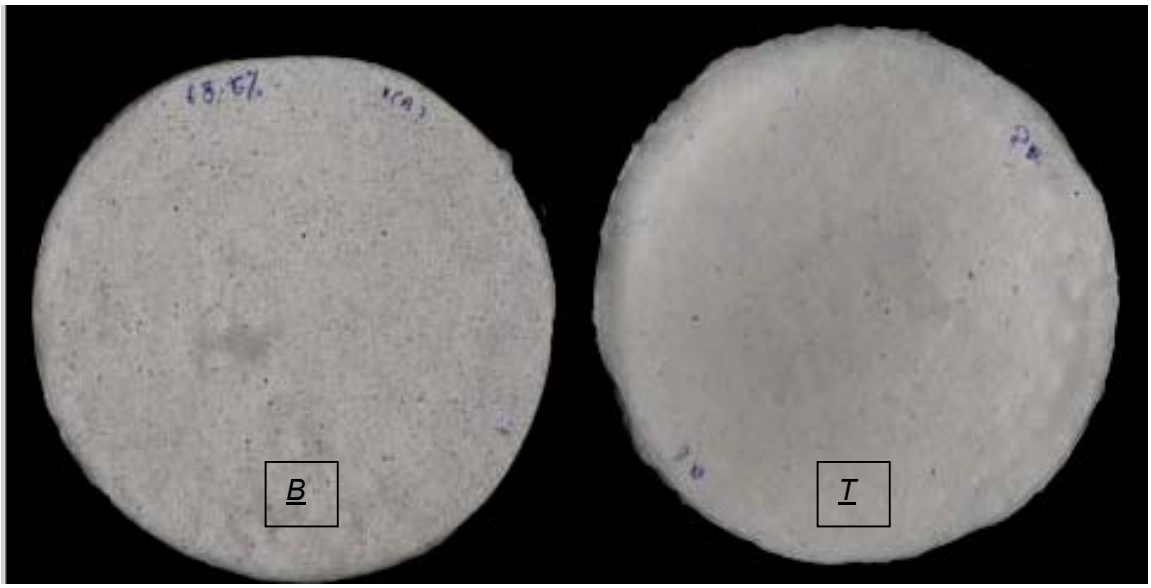


Fig.6.6. Comparison of hand sheets made from enzyme-deinked pulp (T) and from control pulp samples (B)

Hand sheets made from deinked pulp shows reduced of ink specs and improved brightness.

Table 6.3. Quality control tests for recycled paper handsheets made from enzyme deinked pulp

Q.C. Test	Blank	Control	Test Run
Brightness (%)	68.6	70.6	74.0
Opacity (%)	83.1	86.6	84.2
Ink Specks/m^2			
>220 μm	412	262	173
160μm	576	464	180
80-160 μm	472	178	56
10-80 μm	584	298	63
Breaking length (meters)	2295	3228	3300
Burst index (gscm /gsm)	17.14	21.4	22.8
Tear index	72.52	73.0	69.2

Handsheets were made after enzymatic treatment to the MOW paper pulp. The treatment was carried out in the presence of surfactant (0.1%) concentration at 10% consistency in the pulper for 30 min. The enzyme dose of 50 IU was selected for treating 100 g of pulp sample.

Each treatment was followed by 10 min floatation run at 1% consistency.

Control runs were taken under similar conditions replacing active enzyme by same volume of heat denatured enzyme preparation.

Blank samples were the pulp sample not treated with enzyme and also not processed by flotation.

The localized debrillation improves inter fibrilar bonding but tear strength goes down as it is been measured in cross direction to machine direction. Similar loss of tear index has also been observed by other researchers (Gliese et al., 1996; Lee and Eom, 1999). The estimation of reducing sugars from the enzyme treated pulp, shows that only 140 μg of sugar liberated per g of oven dried MOW pulp, while, no reducing

sugars could be detected in control pulp samples. This low value of the reducing sugars produced due to complete hydrolysis of fibers is also reflected in the strength maintenance. These observations supported the probable mechanism of deinking proposed in this study and it is depicted in Fig.6.7.

6.3.8. *The mechanism of enzymatic deinking*

Understanding enzymic action with the fibre is useful to minimise the possible negative effects on the strength of the paper and its quality. The role of cellulases in the deinking is still not clear. It has been suggested that the enzymes having higher filter paper degrading activities are effective in deinking (Jeffries et al., 1994). On the contrary other co-workers proved that that the filter paper degrading activity of the cellulase complex from *Aspergillus* L22 and *Trichoderma pseudokoningii* S28 had detrimental effect on paper strength (Qin et al.,1998,2000).Using different cellobiohydrolases and endoglucanases they showed different effects on the fibre properties. During deinking process individual endoglucanase treatment does not result in the decrease of average fibre length or coarseness, which is considered necessary for modification of the pulp fibre. The synergetic effect of the endoglucanases and cellobiohydrolases could lead to the cross breakage, and this is probably the major cause for the decrease in the paper strength. Gubitz et al. (1998 a,b) treated laser-printed wastepaper individually and with combinations of purified endoglucanases from *Gloeophyllum sepiarium* and *Gloeophyllum trabeum* and found that pure endoglucanases were responsible for most of the success in deinking. Although all enzyme treatments increased freeness and slightly decreased intrinsic fibre strength, the handsheet's strength was remained unaffected or even marginally improved.The characteristics of the individual endoglucanase plays an important role. According to their observations different deinking efficiencies and strength properties were measured with both the endoglucanases, that are closely related enzymes in terms of molecular properties and substrate specificities.

The present work describes the use of alkaline active cellulases from alkalotolerant *Fusarium* sp. for deinking of MOW paper. Based on the experimental results presented in this Chapter a schematic diagram indicating the probable mechanism of the enzymatic deinking process by cellulases is presented (Fig.6.7.). During photocopying, the toner particles of ink get entangled in microfibrils that are

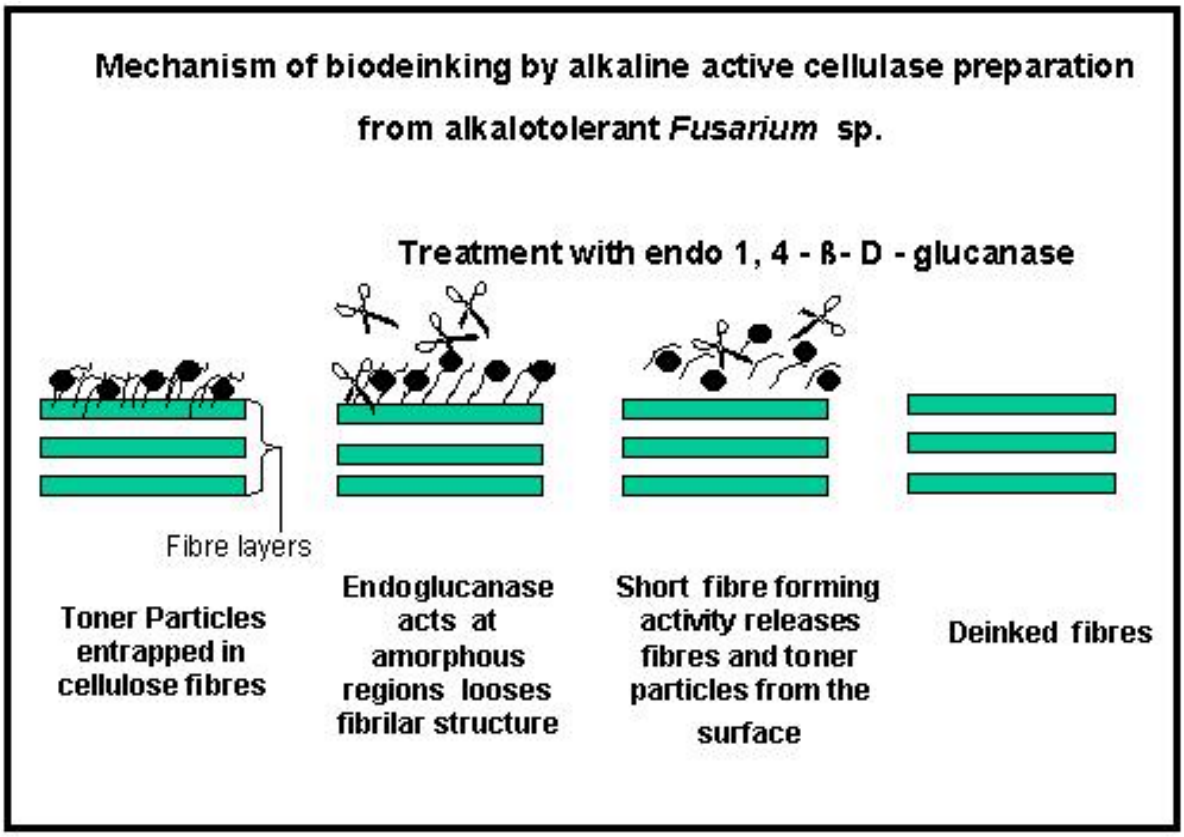


Fig.6.7. Schematic diagram showing probable mechanism of biodeinking by alkaline active endo 1,4 β-D glucanase preparation from alkalotolerent *Fusarium* sp

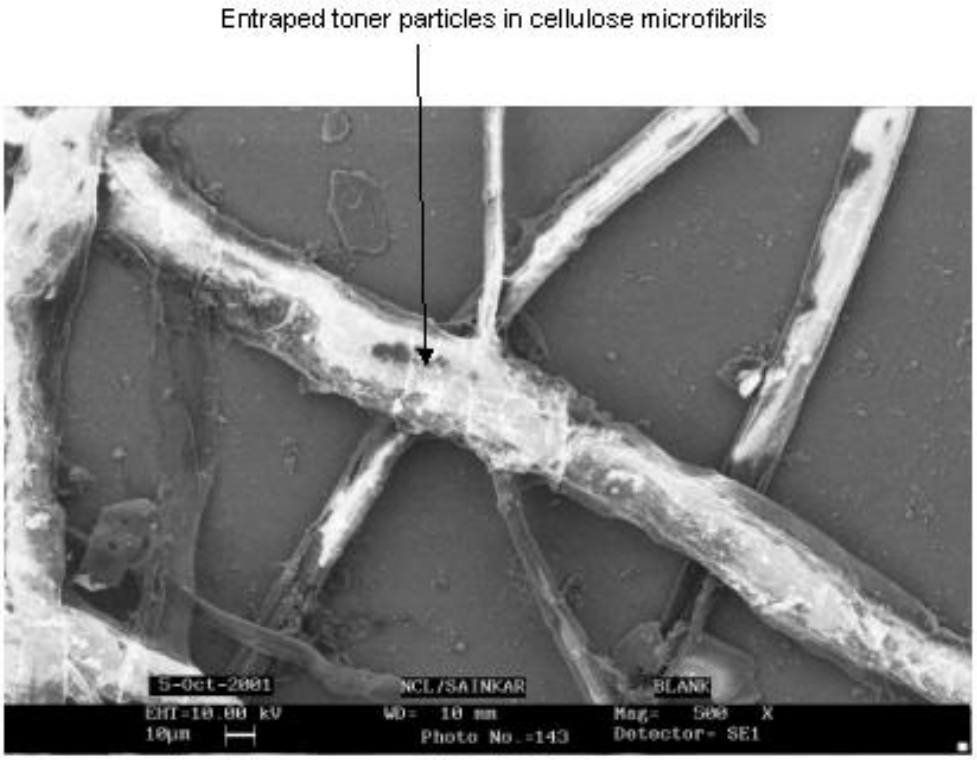


Fig.6.8. Scanning electron micrograph demonstrating the toner particles of ink entangled in microfibrils that are at the surface of the fibres

present at the surface of the fibre. This can be observed from a scanning electron microscopic image and EDAX analysis (Fig.6.8). During biodeinking process,cellulases act in different ways. It is well understood that endoglucanase initiate action on cellulose. The role of endoglucanase in overall process of cellulose degradation is to split the cellulose fibres at several amorphous sites and generates innumerous non-reducing ends of the chain. This action supports loosening of fibres which in turn helps in releasing ink particles from MOW paper during flotation deinking process in the presence of surfactants. Secondly, cellulases act at the frazzled surfaces of cellulose fibres and release short fibres from it. This capability of releasing short fibres can be assigned to mechano-chemical property of endoglucanase components. It can be interpreted that this short fibres forming activity also removes residual fibres from the toner surfaces. Finally endoglucanases release fibres from surface of hairy toners that in turn enhances the flotation efficiencies by increasing hydrophobicity of ink particles. From the data obtained during this work it seems that the randomly acting endoglucanases are useful for overall deinking process. The viscometric analysis revealed that Endo B from alkalotolerant *Fusarium* sp. is more random in its action and has more access to the surfaces of cellulose fibres.

The peeling effect of cellulases during their action on pulp fibres have been studied by several workers (Fan et al., 1980; Ramos et al., 1993; Jain et al., 2001). In the present work, the random attack of endoglucanases on the cellulose substrate as well as their fibre forming activity in the initial stages of action has been demonstrated. These distinct properties of endoglucanases facilitate the biodeinking of MOW paper.

6.4. Conclusion

The work described in this Chapter demonstrated that microbial cellulases can be useful in deinking of waste papers when used in combination with flotation deinking during recycling of wastepaper. The proposed mechanism of deinking suggested that the random attack of alkalistable endoglucanases on the cellulose substrate as well as their fibre forming activity in the initial stages of action of endoglucanases facilitate the biodeinking of MOW paper.

ANNEXURE

Fabrication of A Laboratory Scale Flotation Cell Device for Enzymatic Deinking of Waste Papers

During the recycling of wastepapers flotation deinking is the most widely used technique for deinking of variety of waste papers. The availability of a suitable laboratory scale flotation device is one of the limiting factors in evaluating performance of different enzyme preparations for deinking. The fabrication of the cost effective, laboratory scale flotation device that can be used for deinking of various grades of wastepaper is described here. The unit consists of aeration device, sparger, baffles for high air to stock ratios and high shear mixing. The sparger was designed in such a way that it gives microturbulent airflow necessary for removal of smaller ink particles. The deinking experiments were performed using cellulases from an alkalotolerant *Fusarium* sp. The highest efficiency of flotation was found to be around 80 %. The results indicated that the highest efficiency of deinking was possible when enzyme trials were followed by flotation run.

Experimental Section

All chemicals, MOW pulp samples and enzyme preparations were obtained as described previously in Chapter 6 .

Fabrication of flotation cell device

The efficiency of flotation depends on physical interaction between ink particles, air bubbles and hydrodynamic characteristics of the suspension (Herbert,1993). Considering these prerequisites a simplified flotation cell was designed (Figs 1 & 2). The working volume of the flotation cell was 10 l, with actual dimensions as L=260 mm., B= 260 mm. and H=220 mm. The cell was constructed with mild steel with standard thickness of 2.5 mm. Joints welded with an efficiency of 0.85. A hollow cylinder pipe of diameter 62.5 mm is fitted on the cylindrical sparger having diameter 120 mm. The shaft is introduced with impeller blade diameter of 30 mm in hollow cylinder. This shaft is rotated at 1500 rpm. using 1.5 Hp motor. The sparger hole has the diameter of 5 mm. For the removal of the foam containing floated ink particles the outflow is provided as a slope from the top of one side of flotation unit. The flotation efficiencies were tested during enzymatic deinking trials with cellulases, on MOW papers. The enzyme treatments were followed by flotation run at 1% consistencies in the same cell.

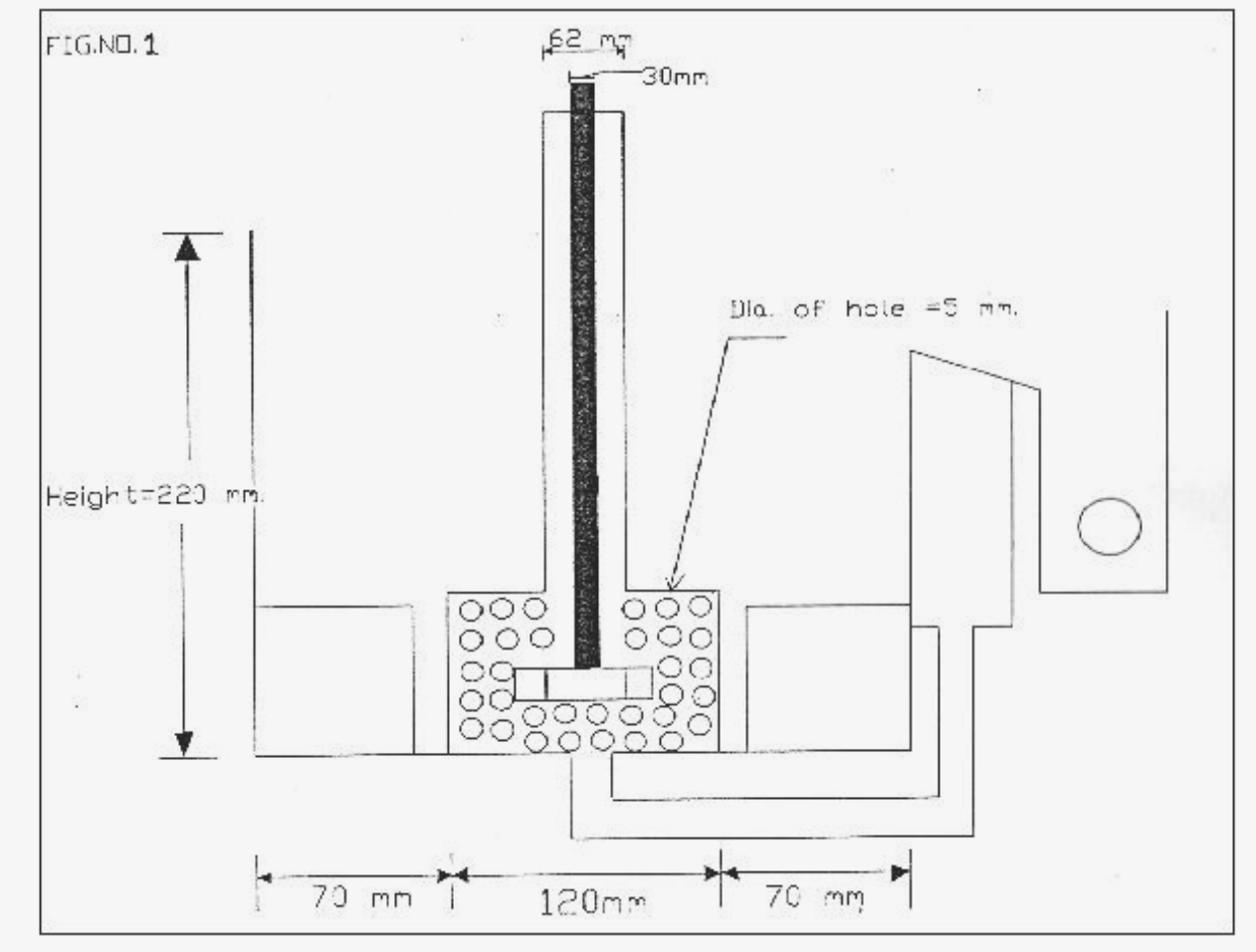


Fig. 1. Laboratory scale flotation cell device - Front view

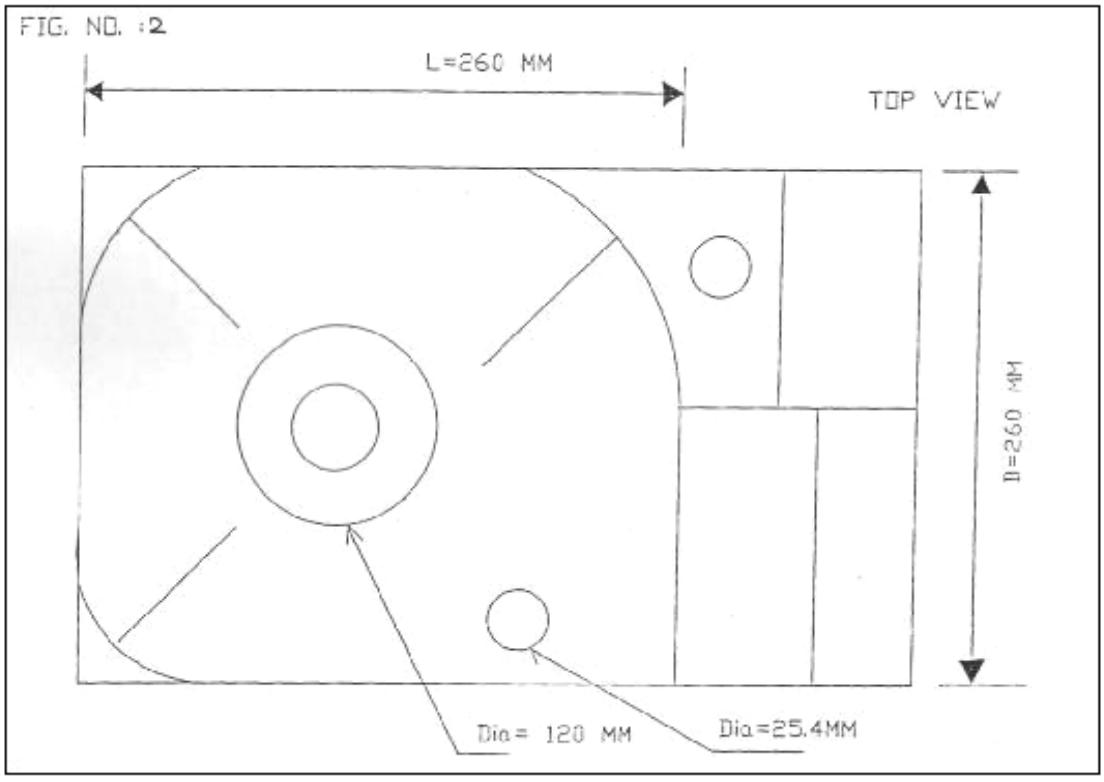


Fig. 2. Laboratory scale flotation cell device - Top view

Deinking trials

The MOW pulp used in this study was prepared from the mixture of photocopier (Xerox) papers and laser printouts waste paper. Enzyme treatments were carried out at 10 % final consistency in medium consistency water-jacketed pulper as described in previous Chapter. The pulp samples were disintegrated in Universal laboratory desintegrator at 5000 rpm for 5 min prior to flotation. The pulp was diluted to 1% consistency with distilled water. All the enzyme treated and control pulp samples were followed by 10 min flotation run in a newly fabricated laboratory flotation unit at room temperature. The foam containing floated ink particles were removed mechanically and the deinked fibres were recovered on a laboratory mesh from the drain valve of the flotation cell. For control, runs were taken under identical conditions replacing active enzyme by heat denatured enzyme preparation. After flotation, pulp consistency was determined for each sample. 5 handsheets per run (weight 50 g/m^2) from enzyme treated as well as control pulp were made on Universal semiautomatic handsheet maker unit as described in TAPPI Method T 205 om-88. The handsheets were placed on dry metal plates and covered with filter paper. Each metal plate was placed in to drying ring and handsheets were dried at room temperature. Handsheets from enzyme treated pulp and from control pulp, heat denatured control runs and handsheets from the pulp without flotation were compared for different optical properties. The ink specks were counted by an optical scanner in visible (220μm - 80μm) to sub visible range (10-80μm). Brightness was measured at different places on hand sheet and values are expressed as the average % value (TAPPI T 452 om 92). Similarly, other strength properties like breaking length, burst index and tear index were determined using TAPPI standard tests. The ink specks in visible (220μm to 80μm) range were counted according to TAPPI standard method (TAPPI T 213). The efficiency of deinking was calculated using following formula.

$$\text{Deinking efficiency \%} = \frac{\text{(No. of Ink specks in blank sample -- No. of ink specks in Test sample)}}{\text{No. of Ink specks in blank sample}} \times 100$$

The increasing varieties in the characteristics of printing inks make new demands on the function of flotation cell. The flotation cell must be capable of removing ink particles that lie under the visible as well as sub visible particle limits. Collectively they determine the brightness levels and the optical cleanliness (McKinney, 1987). The flotation cell must provide an environment where ink particles have a high probability of colliding with air bubbles. This helps ink particles in attaching to an air bubble that float to cell's top surface and finally leaving the fibres in the pulp suspension. The basic conditions required for this high probability of collision include high air to stock ratios, high shear mixing rates. This was achieved in the fabricated flotation device. During the flotation process the high vacuum is created in hollow cylindrical pipe. High speed of impeller blade ensures in high air generation. Impeller blades produce high shear mixing.

High air to stock addition rates ensure that there is large population of air bubbles to allow the ink particles to have a high probability of colliding with air bubble and floating to cell's surface. Generally, the air introduced is up to 10 times to stock volume (Gilkey, 1993). High shear mixing results in dispersion of large quantity of air into stock. Also, high shear increases the probability that ink particle to come in contact with air bubbles. This prevents ink particle from being trapped in the fibres (Herbert, 1993). Finally high shear mixing ensures that fibres stay suspended. In order to separate the ink particles from the fibres the ink particles must be hydrophobic so that they can adhere to the air bubble. This was achieved by using non-cationic surfactant.

Small ink particles ($< 200\ \mu m$) require a high relative velocity in order to break out if the streamline around the bubble comes into contact with bubble itself. Thus, the flotation of small particles requires a micro turbulent flow that is high enough to supply the necessary contact energy. At the same time, the size of the bubble is determined by the turbulent shear field. Smaller the bubble size, higher the degree of turbulence (Gikey,1993). The attachment probability increases as the diameter of air bubble decreases, since the particle can approach the air bubbles more easily. In the present flotation cell the micro turbulent airflow, was achieved by selecting sparger hole diameter of 5 mm. The data on ink counts and size of ink particles suggested that smaller ink particles were removed preferentially than the larger ink particles (Table 1).

Table 1 Quality control tests for recycled paper handsheets made from enzyme deinked pulp.

Q.C. Test	Blank	Control	Test Run
Brightness (%)	66.8	69.6	72.8
Ink specks/m^2			
>220 μm	492	242	157
160 μm	564	413	137
80-160 μm	432	144	58
10-80 μm	628	278	63

Handsheets were made after enzymatic treatment to MOW paper pulp. The treatment was carried out in the presence of surfactant (0.1%) at 10% consistency in the pulper for 30 min. Enzyme dose: 50 IU was selected for treating 100 g of pulp sample. Each treatment was followed by 10 min floatation run at 1% consistency.
Control run was taken under similar conditions replacing active enzyme by same volume of heat denatured enzyme preparation.
Blank: Pulp samples were neither treated with enzyme nor processed by flotation.

This also confirms the generation of micro turbulent airflow that resulted in preferential removal of smaller ink particles. This was observed during flotation of enzyme treated samples. The effect of ink particles on the brightness of paper has been evaluated by McKinney (1987). Generally enzyme action breakdown larger fibrils into smaller microfibrils, which are easily removed by flotation process. This results in further increase in brightness as well as reduction in ink counts Table 1. The blockage of air suction device by foam and stock deposit can become a serious problem during flotation process. While fabricating the flotation cell, this was avoided as there are no individual air hoses and the entry of compressed air takes place via a hollow cylindrical pipe that is installed outside the cell tank. The simplified air input device is introduced instead of costly air compressors used in commercial flotation cells. The novel sparger unit design to generate microturbulent airflow and absence of individual air hoses that can result in blockage of air device are the key highlights of fabricated flotation cell over commercially available flotation cells.

The deinking efficiency of fabricated flotation cell was determined as explained under experimental section. The highest efficiency of flotation was found to be around 80 %. The results indicated that the highest efficiency of deinking was possible when enzyme trials were followed by flotation run. During flotation the air bubble rises to the top carrying hydrophobic toner particles along with them. The large toner particles possess cellulose fibrils entrapped within them. These hairy toner particles affect flotation efficiencies severely by generating hydrodynamic drag on air bubble that is rising to the top. Cellulases separate toner particles from entrapped fibres. Now these non-hairy particles are hydrophobic and that can be easily separated by flotation. Thus, the efficiency of flotation increases dramatically by application of cellulases.

Fungal cellulase preparation from alkalotolerant *Fusarium* sp used in this study showed promising results when used in combination with flotation deinking during recycling of wastepapers. The laboratory scale flotation device described herein is cost effective, and it is found to be efficient in biodeinking of MOW papers. This unit can be used for studies on deinking of various grades of wastepaper and also for the evaluation of different enzyme preparations at laboratory scale level.

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GENERAL DISCUSSION

Cellulose is the most promising renewable energy source that can be utilized to overcome our problems of energy resource, chemicals and food in the future. However, its enormous potential can be exploited only after the clear understanding of structure function relationship of enzymatic cellulose hydrolysis . Till date, a variety of microorganisms that are capable of producing cellulases have been isolated .The significant progress is seen in purification, characterization of different cellulase components and in understanding their substrate specificities and mode of action. Recently, 3D structures of the different cellulolytic enzymes have been solved. This has advanced our understanding regarding structure - function relationships of enzyme with their substrates. Most of these studies were driven by potential industrial applications of cellulases.

Microbial cellulases are generally active in acidic to neutral pH range. Recently, enzymes that are active and stable in neutral to alkaline conditions have gained significant commercial importance due to their ecofriendly applications in detergent, textile and paper industries. However, alkalophillic fungi are very rare. Till date such enzymes have been reported from very few fungi such as *Humicola insolense, Cephalosporium* sp and from an unknown desert basidiomycetes sp. The objectives of the present work includes; isolation, production, purification and characterization of alkali stable fungal cellulases for their potential industrial applications such as biodeinking of waste papers.

Harnessing Biodiversity for Ecofriendly Biocatalysts

During the initial period of this research work attempts were made to explore microbial biodiversity for obtaining novel extremophilic microorganisms, which presumably holds a great wealth of biocatalytic potential. These studies resulted in isolation of alkalotolerant fungus, identified as alkalotolerant *Fusarium* sp. The fungus grows luxuriously in the pH range of 5-10 and produces extracellular alkaline active cellulases that have their potential ecofriendly applications in textile, detergents and paper and pulp industries. Different approaches such as cloning and over expression

of cellulase genes, mutagenesis, solid state cultivation can be followed in order to increase the enzyme yields. However these techniques suffers from limitation such as complexity, use of hazardous chemical agents and scale up difficulties respectively. In this respect the systematic optimization of media components with the help of statistical factorial design is demonstrated in the Chapter 2. The optimally designed media resulted in 4 fold-enhanced production of industrially important cellulases under submerged fermentation conditions.

Endoglucanase A : Understanding Its Ambience

Endoglucanases are important components of the cellulolytic system, as the enzyme action is generally initiated by random acting endoglucanases at amorphous regions within cellulose chain to produce cellooligosaccharides. The purification and physicochemical characterization of a major Endoglucanase (Endoglucanase A, 1,4-β-D glucan-4 glucanohydrolase (EC 3.2.1.4) is described in the Chapter 3 of the thesis. The purpose of the work was to understand of the optimal operational conditions of Endoglucanase A from alkalotolerent *Fusarium* sp. The enzyme Endoglucanase A was purified to homogeneity from the crude culture filtrate. The enzyme is purified 13 fold by ultrafiltration (PM-10), gel filtration chromatography on BioGel P-100 matrix and preparative isoelectric focusing. The molecular weight of the enzyme was determined as 37.8 KD by gel filtration and 45 KD on SDS PAGE respectively. The pI of enzyme was determined as 4.5.Endoglucanase A is active in a broad pH range of 4 to 9 with pH and temperature optima at 5.0 and 60°C, respectively. The enzyme is stable in alkaline pH range (7-9) at 50-60°C.The thermostability of enzyme was studied in the temperature range of 50-70°C. Endoglucanase A shows half-life of 1.5 h at 70°C. Differential scanning calorimetric studies indicated that the enzyme unfolds in 2 different stages. The thermostability of enzyme is improved in the presence of different sugar alcohols. Trehalose was found to be best in protecting enzyme from thermal denaturation. Km and Vmax values for Endoglucanase A at optimum conditions were found to be 2.0 mg/ml and 641 μmoles/min/mg, respectively. The enzyme action is unaffected in the presence of various metal ions however, Hg^{2+} at 1 mM concentration completely inhibited enzyme action.

How does Endoglucanase A act …… ?

The hydrolysis of cellulose depends on composition of cellulolytic complex, properties of cellulases and complexity of cellulosic substrates. However, the application of specialized monocomponent cellulases in various industrial processes such as textile finishing, biodeinking of waste paper and detergent ingredient is the recent trend. Various anatomical and ultra structural characteristics of cellulose fibres can potentially limit hydrolysis of cellulose. Various enzyme characteristics such as their adsorption capacities, inhibition by end products, stability under different environmental conditions (pH, temperature etc.) and synergism between different enzyme components have shown to influence enzymatic hydrolysis of cellulose. The studies in Chapter 4 describe the substrate specificity and characterization of cellulose hydrolysis by Endoglucanase A from alkalotolerent *Fusarium* sp. The thermogravimetric studies demonstrated the application of bound water analysis in confirming hydrolysis of amorphous cellulose by Endoglucanase A.

The Endoglucanase A from alkalotolerent *Fusarium* sp has a typical endo type of action on the cellulosic substrates. The substrate specificity of the enzyme was restricted to β 1-4 glycosyl linkages only. The enzyme was inhibited by low concentrations (0.1%, 1 % and 5%) of cellobiose and glucose. The mode of action of Endoglucanase A on cellulosic substrates is demonstrated with the help of end product analysis using different cello-oligosaccharides with varying chain length. The product analysis of reaction mixtures with HPLC revealed that the Endoglucanase A did not hydrolyzed cellobiose. However, it attacked cellotriose and higher chain length cello-oligosachharide preferentially at the internal glycosidic bonds. The results indicated that the active site of the enzyme may be 4-residue long. It appears that the enzyme belongs to family 7 cellulases because (i) it can hydrolyze cellotriose (ii) it is inhibited by low concentrations of glucose and cellobiose (iii) the major product of hydrolysis is cellobiose (iv) and its characteristic ability to form short fibres from cellulose substrate. These endoglucanases are efficient detergent additives and are useful in preventing back staining problem in denim washing process. Thus the characterization studies on substrate specificity of enzyme in this Chapter marks the enzyme as a 'soft' cellulase having potential for industrial applications.

Making Biocatalysts More Viable

The development of novel catalysts focuses on three approaches: 1.Search in natural habitats, such as geothermal sites, alkaline soda lakes for biocatalysts that can function at high temperatures and in industrial environments. 2. Modification of existing biocatalysts by physico-chemical methods. 3.Design of catalysts from organic or inorganic molecules. In the present work, the fragility of endoglucanase was minimized by developing various new immobilization protocols. In the Chapter 5 the studies on immobilization of cellulases on novel nanosupports are described. The encapsulation of Endoglucase A in thermally evaporated fatty amine films by a simple beaker-based immersion process and its enzymatic activity under different pH and temperature conditions were demonstrated. The optimum operation temperature shifted to higher values relative to that of the free enzyme in solution. Immobilization of cellulases on other novel supports such as colloidal gold nanoscale surfaces and three dimensional shell like assemblies using polyurethane microspheres has resulted in improved operational stability of enzyme as well as making them reusable. However, further research in this area demands studies on the kinetic parameters of immobilized systems, scale up of immobilization protocols and their applications in various industrial processes. One of the important limitations in the success of immobilized enzyme is characterization of enzymes in immobilized form. However, selection of nanoscale supports in the present studies, enanabled to study molecular structure of immobilized enzyme and its interaction with the support. The methodology can be extended to variety of diagnostic enzymes mainly used for development of biosensors. The reasonably fast time-scales for the immobilization and the enzyme-friendly intercalation conditions are the other important features of immobilization protocols described in this Chapter.

Towards environmentally clean process for recycling of high grade paper

Recycling of waste paper is the best way to protect natural resources such as water, timber, petroleum and energy. Removal of toners from toner coated xerox paper is presently one of the bottlenecks in recycling of mixed office waste (MOW) paper to writing grade paper. In the present work (Chapter 6) the extracellular cellulase preparation from alkalotolerent *Fusarium* sp was used for deinking of (MOW) papers.

The enzyme treatment resulted in the increase in brightness with the reduction in ink counts of the recycled paper. The objective of the present work is to understand the mechanism of action of alkaline active cellulase complex from alkalotolerant *Fusarium* sp during deinking of MOW-paper. The loosening and release of toner particles from the printed papers due to random acting endoglucanase components facilitate 'Biodeinking' of MOW-papers. The experimental results suggested a possible mechanism of enzymatic deinking of MOW-papers for recycling purpose.

Two separate enzyme fractions namely Endo A and Endo B were obtained gel filtration chromatography and they showed 2.3 and 4.6 fold increase in their specific activities, respectively.The viscometric analysis indicated the increase in fluidity ($1/\eta_{sp}$) of 1% carboxymethyl cellulose solution. The data revealed that Endo B was 1.9 fold more random as compared to Endo A. Both the endoglucanases showed comparable patterns of their ability to release short fibres in the reaction mixture when filter paper was used as the substrate. The average size of released short fibres was ranged between 20-100μm. Adsorption studies indicated that the enzyme action is prominent at the surface of substrate rather than at their cracked edges. The X-ray diffractograms showed a marginal increase in the degree of crystallinity of the substrates in the initial stages of the enzymatic action. Amorphous regions of the cellulose substrates appeared to be readily attacked by the enzyme. The enzyme treatment resulted in the increase in brightness with the reduction in ink counts of the recycled paper. Based on the distinct properties of endoglucanases a probable mechanism of enzymatic deinking process is presented schematically. Also, the fabrication of the cost effective, laboratory scale flotation device that can be used for deinking of various grades of wastepaper is described as a separate annexure at the end.

A jigsaw puzzle…. !

Understanding the process of cellulose hydrolysis with cellulases is similar to amalgamating pieces in a *jigsaw puzzle* wherein some fit and some don't. " *Whether there are a finite number of pieces, and whether there is even a border to the puzzle, is of course unknown. It will never be known until we finish the picture, if ever."* However isolation of novel enzymes from extremophilic microorganisms and their applications in developing ecofriendly processes are going to be major important drives for the cellulase research in future.

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