MOLECULAR CHARACTERIZATION AND APPLICATION OF GLYCOSIDASES FROM *THERMOMONOSPORA* SP.

Thesis submitted to University of Pune

For the degree of

DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY

By

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………Dedicated to my beloved father

“I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale.”

…Marie Curie

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T. R. Anish

CERTIFICATE

Certified that the work incorporated in the thesis entitled:

"**Molecular characterization and application of glycosidases from *Thermomonospora* sp.**", submitted by Mr. T. R. Anish, for the Degree of ***Doctor of Philosophy***, was carried out by the candidate under my supervision at Division of Biochemical Sciences, National Chemical Laboratory, Pune - 411 008, Maharashtra, India. Material obtained from other sources is duly acknowledged in the thesis.

Dr. Mala Rao
(Research Supervisor)

DECLARATION BY RESEARCH SCHOLAR

I hereby declare that the thesis entitled "**Molecular characterization and application of glycosidases from *Thermomonospora* sp.**", submitted for the Degree of ***Doctor of Philosophy*** to the University of Pune, has been carried out by me at Division of Biochemical Sciences, National Chemical Laboratory, Pune - 411 008, Maharashtra, India, under the supervision of Dr. Mala Rao. The work is original and has not been submitted in part or full by me for any other degree or diploma to any other University.

T. R. Anish
(Research Scholar)

ABBREVIATIONS

°C	Degree Celsius
µl	Micro liter
Å	Angstrom
APS	Ammonium persulphate
BLAST	Basic Local Alignment Search Tool
CD	Circular dichroism
CMC	Carboxy methyl cellulose
CP powder	Cellulose paper powder
$CsCl_2$	Cesium Chloride
Da	Dalton
ddNTP	Dideoxy nucleotide triphosphate
DEAE	Diethylaminoethyl
DNA	Deoxyribonucleic acid
DNSA	Dinitrosalicylic acid
dNTP	Deoxy nucleotide triphosphate
DTNB	5, 5'-dithiobis 2-nitrobenzoic acid
EDTA	Ethylene diamine tetra acetic acid
g	Gram
h	hours
HPLC	high performance liquid chromatography
IAA	Isoamyl alcohol
kDa	Kilo Dalton
KI	Potassium iodide
K_m	Michaelis Menton Constant
L	liter
LB	Luria Bertani
M	Molar
min	Minutes
ml	Milliliter
mM	Milli molar
NBS	*N*-Bromosuccinimide
NCBI	National Center for Biotechnology Information
nm	Nanometers
OD	Optical density
OPTA	*o*-phthalaldehyde
PAGE	Polyacrylamide gel electrophoresis

PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
pI	Isoelectric point
PNPG	*p*-Nitrophenyl b-D-glucopyranoside
PNPX	*p*-Nitrophenyl b-D-xylopyranoside
ppm	Parts per million
rev	Revolutions
RNAase	Ribonuclease
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBE	Tris Borate EDTA
TE	Tris-Ethylene diamine tetra acetic acid
T-EG	*Thermomonospora* endoglucanase
TEMED	N,N,N'N'- Tetramethyl ethylene diamine
TNBS	2,4,6-trinitrobenzenesulphonic acid
TSC	*Thermomonospora* sp. cellulase / 1,4 – β – D – Glucan Glucanohydrolase
V_{max}	Maximum velocity

ABSTRACT

Introduction

1,3-1,4-β-Glucans are polysaccharide components of the cell walls of the higher plant family Poaceae, apparently restricted to members of the Graminiae, and particularly abundant in the endosperm cell walls of cereals with commercial value such as barley, rye, sorghum, rice, and wheat. Structurally, they are linear glucans of up to 1200 β -D-glucosyl residues linked through β -1,3 and β -1,4 glycosidic bonds, with variations in the proportion of both types of linkages (25-30% L-1,3) and in the length of the mixed-linked segments, 1,3-1,4- β -D-glucan 4-glucanohydrolase (1,3-1,4- β -glucanase or lichenase) which exhibits a strict substrate specificity for cleavage of β -1,4 glycosidic bonds in 3-O-substituted glucopyranose units. The bacterial 1, 3-1, 4- β -glucanases are important biotechnological aids in the brewing and animal feedstuff industries. During malting, endogenous 1,3-1,4- β -glucanases are heat inactivated, and large contents of remaining high molecular weight L-glucans may cause severe problems such as reduced yields of extracts and lower filtration rates, as well as the appearance of gelatinous precipitates in the finished beer. Thus the level of activity of glucan hydrolases achieved during germination and the amount of their substrates are important factors for good quality brewers malt, and thermostable bacterial 1,3-1,4- β - glucanases are often added to reduce viscosity during mashing. In animal feedstuff, especially for broiler chickens and piglets, addition of enzymatic preparations containing bacterial β -glucanases improves digestibility of barley-based diets, and reduces sanitary problems (sticky droppings).

Cellulose is one of the most abundantly available renewable resources and its annual production is estimated to be about 100 billion tons (Coughman et al, 1985). Cellulose is a homopolymer of β-1,4 linked glucose units. Its potential as a renewable source of energy was exploited by many researchers. Cellulases comprise of three components: endo-glucanases, exo-glucanases and β-glucosidases, the synergistic action of all the three enzymes is required for complete degradation of cellulose. Cellulases and hemicellulases are used in clearing fruit juices and beer, extraction of juice and oil from fruit and seed pulp. Cellulases are also useful in the deinking process in paper industry and in animal feed as additive for monogastric animals. Recently, endoglucanases have

been successfully introduced in to laundry reagents along with alkaline proteases. Recently due to environmental pressures imposed on the textile industry and the need to minimize water contamination cellulases are gaining importance. The traditional use of pumice stone in a water-loaded tumbling machine caused severe wear and loss of tensile strength when used to achieve high degree of indigo fading. Introduction of cellulase enzyme to create the required effect without the use of stones and increasing their compatibility with other chemical processes is one of the thrust areas of research in textile industry.

The present work has been organized under the following headings

1. General introduction
2. Production of lichenase and xylanase from *Thermomonospora* sp.
3. Purification and molecular characterization of β-1, 3-1, 4-glucan-4-glucanohydrolase.
4. Molecular cloning and expression of a low molecular weight 1, 4-β- D glucan glucanohydrolase.
5. Industrial application of cellulase
   A) Entrapment of cellulase in a matrix for its controlled release.
   B) Evaluation of the cellulase activity in Textile industry on bio-finishing of Denims

Chapter 1

General Introduction

This chapter presents the literature survey of the research carried out on glycosidases mainly involved in plant biomass degradation, namely lichenases, with special reference to their occurrence, properties and industrial applications.

Chapter 2

Production of lichenase and xylanase from *Thermomonospora* sp.

The isolate secreting lichenase was identified to be a new species of *Thermomonospora* by 16S rDNA sequencing. It showed 98% homology toward *Thermomonospora mesouviformis*. The medium was optimized for the production of

lichenase from *Thermomonospora* sp. by surface response method. The optimized medium produced 2.5 to 3.0 times lichenase activity as compared to the basal medium. Cellulase and xylanase enzyme were co produced during the fermentation. A proportional increase in activities of cellulase and xylanase were observed during media optimization for lichenase. The optimum medium composition per 100ml obtained from factorial was Tween 80, 0.025μl; yeast extract, 2g; Ammonium sulphate, 0.5g; Urea, 0.05g and CPP, 2g. The methodology as a whole proved to be quite adequate for the design and optimization of a bioprocess. Using this method of experimental factorial design and response surface analysis it was possible to determine optimal cultivation medium to obtain high lichenase production. This method not only helps in establishing optimal fermentation media for operation but also predicts the possible yield and productivity which the system is disturbed in some way making it useful too to understand the potential for press control.

Chapter 3

Purification and molecular characterization of β-1, 3 -1, 4-glucan-4-glucanohydrolase

Section A

Purification and molecular characterization of β-1, 3 -1, 4-glucan-4-glucanohydrolase

A bifunctional high molecular weight (Mr, 64,500 Da) β-1-3, 1-4 glucan 4-glucanohydrolase was purified to homogeneity from *Thermomonospora* sp., exhibiting activity towards lichenan and xylan. A kinetic method was used to analyze the active site that hydrolyzes lichenan and xylan. The experimental data was in agreement with the theoretical values calculated for a single active site. Probing the conformation and microenvironment at active site of the enzyme by fluorescent chemo-affinity label, OPTA resulted in the formation of an isoindole derivative with complete inactivation of the enzyme to hydrolyse both lichenan and xylan confirmed the results of kinetic method. OPTA forms an isoindole derivative by cross-linking the proximal thiol and amino groups. The modification of cysteine and lysine residues by DTNB and TNBS

respectively abolished the ability of the enzyme to form an isoindole derivative with OPTA, indicating the participation of cysteine and lysine in the formation of isoindole complex.

Section B

Thermal inactivation of β-1, 3- 1, 4 D -glucan 4 glucanohydrolase from an alkalothermophilic *Thermomonospora* sp.

An insight in to the deactivation of the enzyme at 80°C was obtained using chemoaffinity labeling, intrinsic fluorescence and CD studies. OPTA binds to the active site of the enzyme when exposed to a temperature in the range of 30-80°C OPTA did not bind to the active site to yield an isoindole derivative on an exposure at 80°C for 10 minutes. Intrinsic fluorescence of the enzyme at different temperatures (30 – 80°C) showed quenching of fluorescence without any shift in the emission maxima indicating conservation of the tertiary structure of the enzyme. Similarly the CD spectra of the enzyme at different temperatures (30 – 80°C) showed no structural changes in the secondary structure. These results indicate that the inactivation of the enzyme at 80°C after exposure for 10min was due to a slight distortion in the active site and not due to any major structural changes in the enzyme.

Chapter 4

Molecular cloning and expression of a low molecular weight 1,4-β- D glucan glucanohydrolase

In the present study cloning of the gene encoding a low molecular weight 1, 4-β-D-glucan glucanohydrolase (TSC) from Thermomonospora sp. was attempted. The peptide sequences of the tryptic digest of TSC was used to design primers for amplification of the gene from the genome of Thermomonospora sp. The gene was partially amplified by PCR using the forward and reverse primers to yield an amplicon of 400bp. The predicted amino acid sequence of the 400bp amplicon revealed the presence of all the peptides from the tryptic digest indicating that the amplified fragment is of TSC. A characteristic 14bp inverted repeat sequence that lies in the 5' upstream region of the cellulase genes from *Thermomonospora fusca* has been reported by Spiridonov and

Wilson (1999). The genomic DNA was amplified using the forward primer to obtain an amplicon of 700bp containing the complete ORF of TSC gene. This was subsequently cloned in to pGEMT$^+$ vector and transformed in *E.coli*DH5α. The ORF was amplified from pGEMT$^+$ vector and was subcloned into an expression vector pET28(a)$^+$ and transformed in to *E.coli*BL21. The gene was expressed as a recombinant protein with an N-terminal His-tag for the ease of purification and a thrombin cleavage site for the separation of pure protein. The soluble recombinant protein was purified by Ni-NTA affinity chromatography. The purified protein has a molecular weight of 21kDa in contrast to 14.2kDa of TSC. The discrepancy in molecular weight may be due to post translational modification. The expressed protein did not exhibit any detectable endoglucanase activity probably due to misfolding of the protein due to its expression in a heterologous host.

Chapter 5

Industrial application of cellulase

Section A

Entrapment of cellulase in a matrix for its controlled release

Gum arabic, a biodegradable natural polymer was used as a matrix to encapsulate endoglucanase from *Thermomonospora* sp. The modified enzyme was found to retain complete biocatalytic activity. The modified enzyme exhibited a shift in the optimum temperature [50 - 55°C] and considerable increase in the pH and temperature stabilities as compared to the free enzyme. Encapsulation of the enzyme also protected the activity in presence of detergents and enhanced the shelf life. A 3-fold decrease in the initial rate of reaction indicated a controlled release of the enzyme conferring properties preferred for its potential application in the manufacture of detergents.

Section B

Evaluation of the cellulase activity in Textile industry on bio-finishing of Denims

Use of cellulase for denim washing is a standard eco-friendly technique to achieve desirable appearance and softness for cotton fabrics and denims. But enzymatic washing

of denim till date involved acid cellulase (*Trichoderma reesie*) and neutral cellulase (*Humicola isolens*) the use of which has a drawback of back staining of the indigo dye on to the fabric. Though it has been suggested that pH is a major factor in controlling back staining there are no reports on use of cellulase under alkaline conditions for denim washing. In this paper for the first time an alkali stable endoglucanase from alkalothermophilic *Thermomonospora* sp. (T-EG) has been used for denim biofinishing under alkaline conditions. T-EG is effective in removing hairiness with negligible weight loss and imparting softness to the fabric. Higher abrasive activity with lower backstaining was a preferred property for denim biofinishing exhibited by T-EG. The activities were comparable to acid and neutral cellulases that are being regularly used. The enzyme was also effective under non-buffering conditions which is and added advantage for use in textile industry. A probable mechanism of enzymatic finishing of cotton fabric has been represented based on the unique properties of T-EG.

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- **Anish R.**, Rao M. 2006 Biochemical characterization of a novel β- 1-3, 1- 4 glucan 4-glucanohydrolase from *Thermomonospora* sp. having a single active site for lichenan and xylan. Communicated
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- Attended National Seminar on Crystallography, at NCL, Pune from January 8-11, 2003.
- Attended workshop on "Macromolecular Crystallography" on January 12, 2003, at NCL, Pune.
- **Anish Ramakrishnan**, Md. S. Rahman, Mala Rao. Poster entitled "Biochemical characterization of a high molecular weight endoglucanase from an alkalothermophilic *Thermomonospora* sp.: Biotechnological applications of endoglucanases" presented at the International Conference and workshop on Innovations and Challenges in Biotechnology organized by Department of Industrial Biotechnology, BIHER Deemed University, Chennai during January 24-27, 2006.
  - *This poster was adjudged the 2nd best by the reviewing committee at the conference*
- Mala Rao, **Anish Ramakrishnan**, Jagtap Sharmili. Abstract entitled "Biochemical characterization of endoglucanase from cellulose utilizing *Thermomonospora* sp. and their application in the national conference on Forest Biodiversity Resources" Exploitation, conservation and management organized by Centre for Biodiversity and Forest Studies, Madurai Kamaraj University, Madurai during March 21-22, 2005

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- **Anish R.,** Rao M. Hemicellulose to ethanol: Hydrolysis and Fermentation. 2006. Editor: Ashok Pandey. Haworth press. Communicated.

CHAPTER I

GENERAL INTRODUCTION

INTRODUCTION – LICHENASE

The polysaccharides 1, 3-1, 4-β-Glucans are found in the cell walls of the plants from the family Poaceae, apparently restricted to members of the sub family Graminiae. They are abundant in the endosperm cell walls of cereals such as barley, rye, sorghum, rice, and wheat (Stone and Clarke, 1992). Structurally, they are linear glucans of up to 1200 β-D-glucosyl residues linked through β-1, 3 and β-1, 4 glycosidic bonds. Up to 90% of the water-soluble barley β-glucan consists of cellotriosyl and cellotetraosyl residues linked by a single β-1,3 glycosidic bond (Stone and Clarke, 1992; Parrish et al., 1960; Woodward et al., 1983) (Figure1).

The 1, 3-1,4-β-D-glucan 4-glucanohydrolases (1,3-1,4-β-glucanase or lichenase) are enzymes involved in the depolymerization of lichenan with a strict substrate specificity for cleavage of β-1,4 glycosidic bonds in 3-*O*-substituted glucopyranose units. The final hydrolysis products from barley β-glucan are the trisaccharide and the tetrasaccharide (Figure1). Few microorganisms have also been reported to secrete lichenases. The plant and microbial enzymes have neither amino acid sequence similarity nor related three dimensional structures.

Occurrence

Most of the known lichenase are of bacterial origin and belongs to family 16 of glycoside hydrolases. The most studied lichenases are produced by *Bacillus* species. Plant enzyme with similar specificity are grouped into family 17. Recently lichenases have been reported from fungi also (Chen et al., 1997).

Biochemical properties of lichenases

Lichenase have been mainly studied from *Bacillus* sp. The optimum temperature for lichenases from the genus *Bacillus* ranges from 45°C to 65°C. The pH optimum for lichenases from *Bacillus* reported is around neutrality (6–7.5) except for the enzymes of *B. brevis* which has a optimum pH 9. The alkalophilic *Bacillus* sp N137 exhibits activity in a broad pH range of 7–12. A range of pI 7.5 – 9.1 usually in the basic range has been

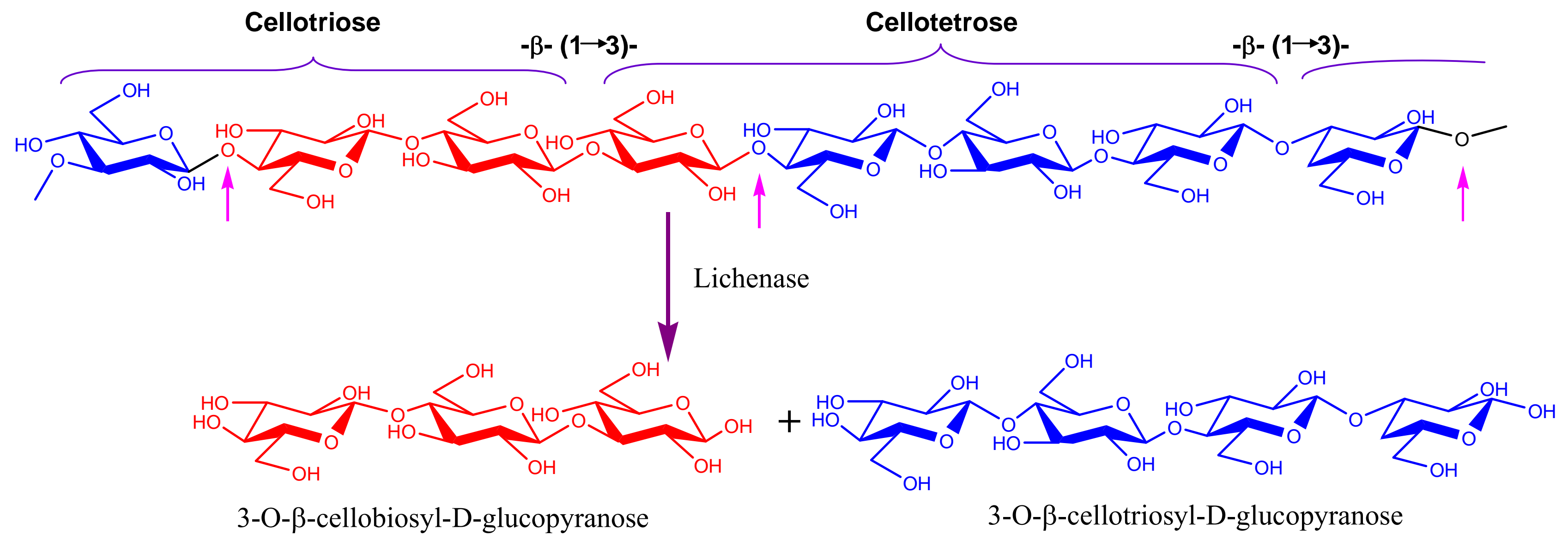


Figure 1: Enzymatic depolymerization of barley β-glucan by lichenase

reported in the *Bacillus*. Specific activities are in the range of 1200 to 4500 µmol min^{-1} mg^{-1} and K_m values of 1.2–1.5 mg ml^{-1} for barley β-glucan and 0.8–2 mg ml^{-1} for lichenan. *Bacillus* lichenases are monodomain proteins with molecular masses of 25–30 kDa. The biochemical properties of non-*Bacillus* lichenases are usually similar to *Bacillus* lichenase. *Clostridium thermocellum* had higher temperature optima of 80°C (Schimming et al., 1991) with 50°C optimum temperature. *Orpinomyces* and *Talaromyces emersonii*, exhibited a pH optima of 6 and 4.8 respectively with a temperature optima of 45°C and 80°C respectively (Chen et al., 1997).

Table 1: Biochemical properties of few lichenases

Organism	Optimum		Reference
	pH	Temperature (°C)	
Bacillus polymyxa	7	45	Borriss and Zemek 1981
Bacillus subtilis	6.5	55	Olsen et al., 1991
Bacillus macerans	7	65	Olsen et al.,1991
Bacillus brevis	9	65	Louw and Watson 1993
Bacillus sp. N137	6	65	Tabernero et al., 1994
Clostridium thermocellum	6.6–10	80	Schimming and Staudenbauer 1991
Enterococcus faecium	6.5	40	Beckmann et al., 2000
Fibrobacter succinogenes	6.0	50	Chen et al., 2001
Orpinomyces sp.	6.0	45	Chen and Ljungdahl 1997
Talaromyces emersonii	4.8	80	Chen and Ljungdahl 1997
Streptomyces bovis	6.5	50	Ekinci et al., 1997

Three dimensional structure of lichenases

The structures of two wild-type enzymes *B. licheniformis* (Hahn et al., 1995a) at 1.8 A^o resolution and *B. macerans* (Hahn et al., 1995b) at 1.6A^o resolution) as well as the hybrid H(A16-M) at 1.6A^o resolution (Hahn et al., 1995c) and different circularly permuted enzymes derived from the hybrid H(A16-M) or from the *B. macerans* wild-type have been solved by X-ray crystallography (Heinemann et al., 1996). The first solved

three-dimensional structure of a family 16 1,3-1,4-β-glucanase was that of the hybrid H(A16-M), which contains amino acid residues 1–16 of the mature *B. amyloliquefaciens* enzyme and residues 17–214 derived from the *B. macerans* protein (Keitel et al., 1993). The plant and microbial enzymes have neither amino acid sequence similarity nor related three-dimensional structure, being an example of convergent evolution towards the same substrate specificity. Whereas the plant enzymes belong to the glycosyl hydrolase family 17 (Henrissat, 1991; Henrissat and Bairoch, 1993; 1996) with an $(\beta/\alpha)_8$ barrel three dimensional structure, the microbial enzymes are classified as members of Family 16 with a jellyroll β-sandwich structure. They show nearly identical all anti-parallel β-sheet jellyroll architecture (Figure 2), a fold that is present in an increasingly large number of proteins and was first described for the plant lectins such as concanavalin A (Edelman et al., 1972). The core of the protein is formed by two β-sheets stacking atop each other in a sandwich like manner, which consist of seven anti-parallel strands each that are bent and create a cleft crossing one side of the protein where the substrate is bound. Loops between the β-strands are mostly stabilized by β-turns, and only one turn with α-helical geometry is observed. A major surface loop (residues 20–36 in *B. macerans* numbering) covers partially the binding site cleft created by the bending of the β-sheets, where the disulphide bond between Cys30 and Cys59 links this loop to the β-strand 56–64 of the protein core. On the convex side of the molecule, remote from the active site, a calcium ion is bound which plays a role in stabilizing the native protein structure. Cation binding has been analysed by comparing the crystal structures and stabilities of the hybrid H(A16-M) with bound Ca^{2+} or Na^{+} ions (Keitel et al., 1994). Calcium is bound with nearly perfect octahedral geometry coordinating to the backbone carbonyl oxygens of Pro7, Gly43 and Asp205, a carboxylate oxygen of Asp205 (*B. macerans* numbering), and two water molecules, whereas sodium is trigonal-bipyramidally coordinated and provides lower thermal stability to the folded protein.

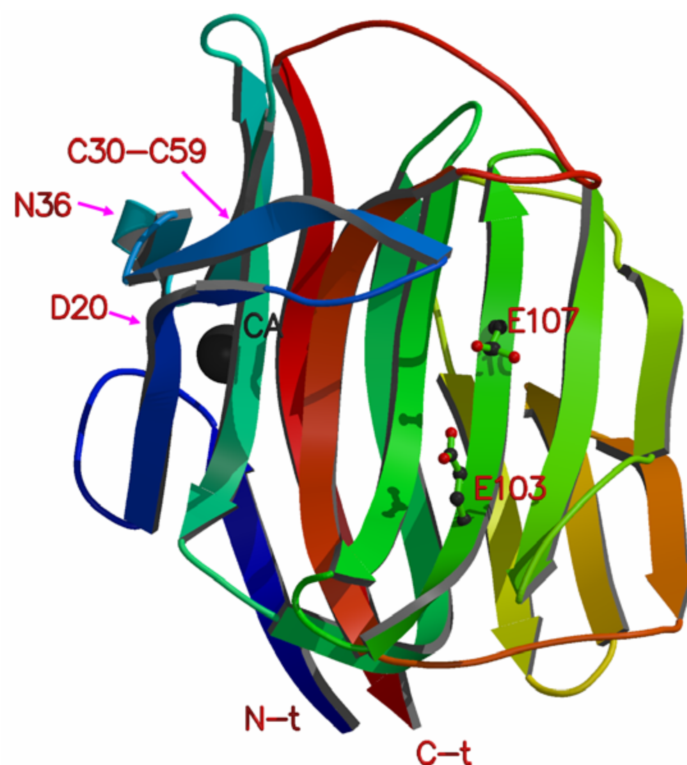


Figure 2: Three-dimensional structure of Bacillus 1,3-1,4-L-glucanases (*B. macerans* numbering). Backbone representation (with MOLSCRIPT (182)); the side chains of the catalytic residues E103 and E107 (equivalent to E134 and E138 in *B. licheniformis* numbering), and the disulphide bond Cys30-Cys59 are shown.

The cleft on the concave side of the molecule defines the oligosaccharide substrate binding site. It is lined with mainly aromatic residues on its walls and with acidic residues at the bottom. The catalytic residues are located in the same β-strand where there is a strict alternation of polar (acidic) and non-polar side chains, the first pointing toward the surface of the protein where they are able to interact with the substrate, the latter towards the hydrophobic interior. This pattern is disrupted in the 1,3-β-glucanases of family 16 by insertion of a methionine residue between Ile106 and the general acid/base Glu107 (in *B. macerans* numbering) (Hahn et al., 1995b).

Mechanism of enzyme action

Family 16 lichenases from bacterial origin are retaining enzymes as first shown for the *B. licheniformis* enzyme (Malet et al., 1993, Malet and Planas, 1997). The basic mechanism for a retaining glycosyl hydrolase involves a double-displacement mechanism as first proposed by Koshland in the early 1950s (Koshland, 1953) with participation of

two essential residues, the general acid/base catalyst and the nucleophile (Figure 3). The catalytic residues of *Bacillus* 1,3-1,4-β-glucanases have been identified by mutational analysis, affinity labelling and X-ray crystallography and thereafter the assignment extended to the rest of family 16 glycosyl hydrolases based on sequence similarities. According to this hypothesis, Glu134 in the *B. licheniformis* enzyme was substituted by Gln to yield a mutant protein with nearly abolished activity suggesting its essential role in catalysis (Planas et al., 1992). The T4 lysozyme model, however, did not hold because mutation of the other putative essential residue (Asp143→Asn) gave an active enzyme (Juncosa, 1994) and the viral lysozyme is an inverting enzyme whereas the bacterial lichenases were then proved to be retaining enzymes. Affinity labelling experiments on the highly homologous *B. amyloliquefaciens* 1,3-1,4-β-glucanase showed that Glu105 (the equivalent residue to Glu134 in *B. licheniformis*) was covalently modified by mechanism based inhibitors (epoxyalkyl β-oligosaccharides), proposing that residue as the likely catalytic nucleophile (Hoj et al., 1991). Likewise, the three-dimensional structure of a covalent protein–inhibitor complex of a hybrid 1,3-1,4-β-glucanase (*B. amyloliquefaciens–B. macerans*) with 3,4-epoxybutyl β-cellobioside showed that the inhibitor was covalently bound to Glu105 (Keitel et al., 1993), and that mutations on this glutamate residue also yielded inactive enzymes. A third residue (Asp136) was also found to be important, possibly acting as an auxiliary acidic group that may exert its effect by modulating the pK_a of the general acid. Recently, functional proof of Glu134 as the nucleophile and Glu138 as the general acid/base residue in the *B. licheniformis* enzyme has been provided by chemical rescue experiments (Viladot et al., 1998). As retaining glycosidases, the mechanism of bacterial 1,3-1,4-β-glucanases involves a double-displacement reaction assisted by general acid-base catalysis (Sinnott, 1990; Davies et al., 1998a). In *glycosylation* - the first step, the amino acid residue acting as a nucleophile displaces the aglycon in an inverting reaction assisted by proton transfer to the glycosidic oxygen from the general acid residue with formation of a covalent glycosyl–enzyme intermediate. The covalent nature of the intermediate may be general for most of the retaining glycosidases, except for lysozyme (Strynadka and James, 1991 and Matsumura and Kirsch, 1996) and glycosidases acting by substrate-assisted catalysis such as chitinases (Scheltinga et. al., 1995, Tews et al., 1997 and Brameld and Goddard.,

1998). This is supported by a substantial body of evidence including kinetic isotope effects (Sinnott, 1990), trapping experiments using substrate analogues, mainly 2-deoxy-2-fluoro glycosides (Wither et al., 1990; Adam et al., 1993 and Wither, 1995) for which the covalent intermediate is detected by mass spectrometry and X-ray crystallography (Street et al., 1992, Miao et al., 1994 and Davies et al., 1998b) and trapping of the intermediate between mutant enzymes and unmodified substrates (Notenboom et al., 1998; Mosi et al., 1997 and Uitdehaag et al., 1999). Nucleophilic assistance in the cleavage of the glycosidic bond in β-glycosidases would imply some distortion of the pyranose ring, like that observed in the crystal structure of a 1,4-β-glucanase with a non-hydrolysable substrate analogue (Sulzenbacher et al., 1996). The second step involved a deglycosylation mechanism in which the attack of a water molecule assisted by the conjugate base of the general acid to render the free sugar with overall retention of the anomeric configuration. Both steps proceed via transition states with considerable oxocarbonium-like character. For the same residue to act as a general acid in the first step and general base in the second, a significant shift in the pK_a of the carboxyl side chain must occur during the enzyme cycle (Mcintosh et al., 1996) Most of the kinetics with low molecular mass oligosaccharides addressed to establish the mechanistic details of bacterial 1,3-1,4-β-glucanases have been conducted on the enzyme from *Bacillus licheniformis* for which Glu_{138} and Glu_{134} are the general acid/base residue and catalytic nucleophile, respectively, as previously discussed. Except for the *B. brevis* enzyme, all *Bacillus* 1,3-1,4-β-glucanases have a single disulphide bond Cys61–Cys90 (*B. licheniformis* numbering) that connects the loop involved in substrate binding with a β-strand of the protein core. Its reduction had no effect on the catalytic parameters and it was initially deduced that the contribution of the disulphide bond to protein stability was low (Pons et al., 1995). Since 1,3-1,4-β-glucanases and 1,3-β-glucanases share some degree of sequence similarity but notably different substrate specificities, protein engineering has been attempted to modify specificity from one activity to the other. The amino acid sequence around the catalytic residues is highly conserved in bacterial lichenases. 1,3-β-glucanases have three extra amino acids before the glutamate residue acting as catalytic nucleophile and an inserted methionine between the nucleophile and general acid/base residues.

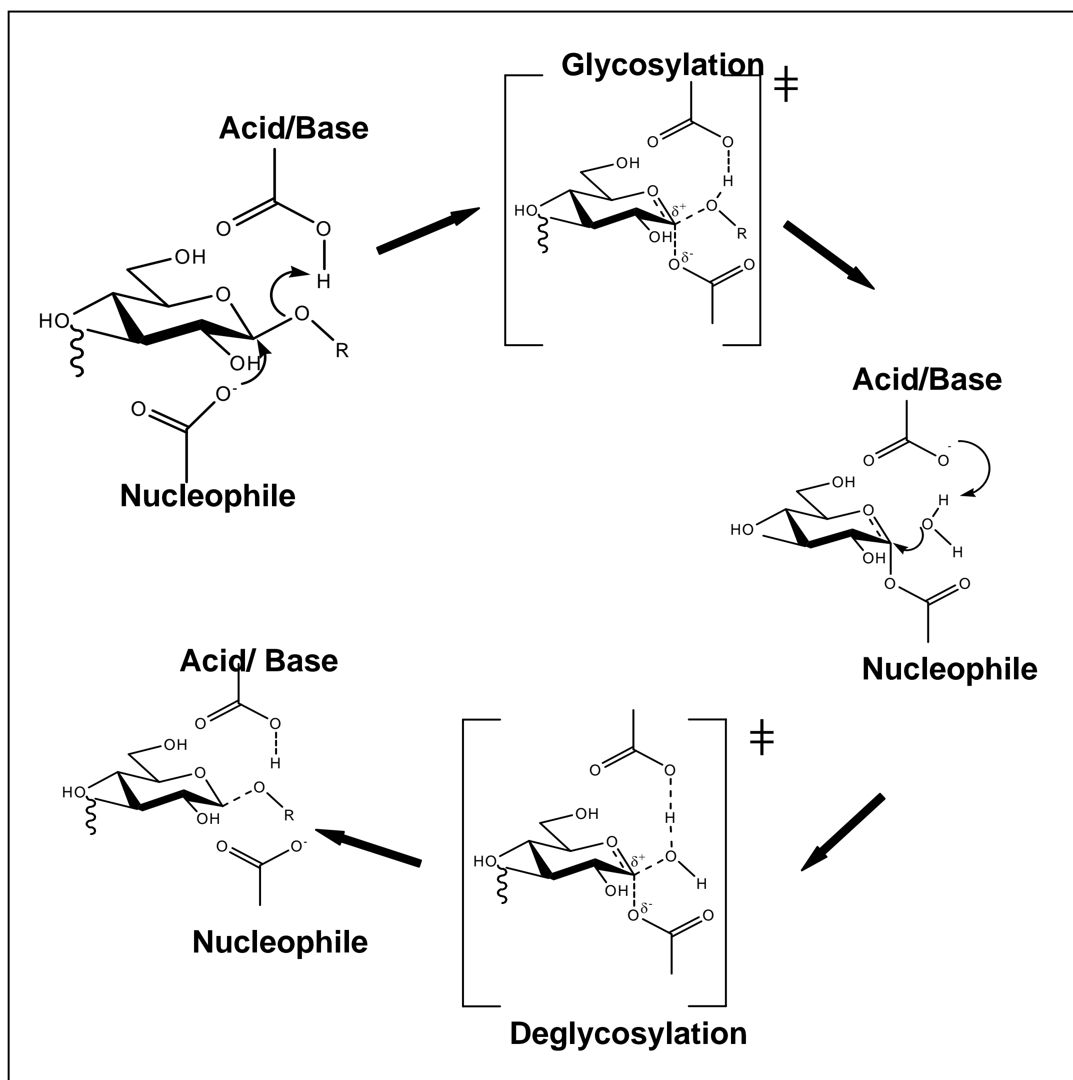


Figure 3: Retaining glycosidase mechanism involving a double displacement reaction

In *B. macerans* 1,3-1,4-β-glucanase mutant carrying an inserted methionine in this active site region was found to be enzymatically inactive (Hahn, 1995b). Likewise, deletion of the methionine in 1,3-β-glucanases from *P. furiosus* (Gueguen et al., 1997) and from *R. marinus* (Krah, 1998) did not change the substrate specificity but it did cause a severe reduction in its catalytic activity, suggesting a structural role of this residue in constituting the active site. Similarly, deletion of the extra three amino acid residues in the *R. marinus* laminarinase also failed to change specificity, resulting in a highly inactive enzyme (Krah et al., 1998).

Cloning and Expression of lichenase in heterologous host

Bacterial 1,3-1,4-β-glucanases have been expressed in a number of different host organisms including *Escherichia coli*, *Bacillus* strains, *Saccharomyces cerevisiae*, and transgenic barley and tobacco plants. Expression in *E. coli* (as the usual host after cloning of bacterial genes) was obtained under the control of its own *Bacillus* promoter and signal peptide (SP) sequence (Hinchliffe, 1984, Borriss et al., 1986, Gormley et al., 1988, Hecker et al., 1988, Tezuka et al., 1989, Riethdorf et al., 1990 and Planas et al., 1992). Several *Bacillus* strains have been used as host cells since *Bacillus* species offer considerable industrial utility for expression in bioreactors. Expression levels depend on the plasmid copy number and the strain (Borriss and Hofemeister, 1985 and Borris et al., 1982). Industrial 1,3-1,4-β-glucanases for brewing and animal feedstuff applications are being currently obtained from hyperproducing selected strains of *B. subtilis* and *B. amyloliquefaciens*. In *B. subtilis* strain deficient in lichenase, cellulase and xylanase has been constructed by deletions in the coding regions of the corresponding genes, thus producing a host useful to study expression of genes from other Gram-positives (Wolf et al., 1995). Other bacteria have been occasionally used in heterologous expression experiments of cloned lichenases, such as *Acetobacter methanolicus* (Miksch et al., 1997) and *Klebsiella planticola* (Miksch et al., 1999) or *Lactococcus lactis* and *Enterococcus faecalis* (Ekinci et al., 1997). Because of the use of thermostable *Bacillus* 1,3-1,4-β-glucanases in brewing, heterologous expression in yeast strains has been studied with the aim of producing a recombinant brewers yeast able to degrade malt β-glucans during fermentation. Expression in *Saccharomyces cerevisiae* of the *B. subtilis* gene including the bacterial promoter produced very low levels of lichenase activity (Hinchliffe and Box, 1984), whereas expression under the control of the yeast CYC1 (iso-1-cytochrome *c*) and ADH1 (alcohol dehydrogenase) promoters (Cantwell et al., 1986), the phosphoglycerate kinase promoter (Olsen and Thomsen, 1991), the yeast vacuolar endoprotease B promoter (Meldgaard et al., 1995), or the alcohol dehydrogenase II promoter (van Rensburg et al., 1997) gave high expression levels. *N*-Glycosylation when secreted from yeast increases enzyme thermostability as compared with the nonglycosylated forms expressed in *E. coli* (Oleson and Thomsen, 1991; Meldgaard and Svendsen, 1994), and the glycoforms produced depend**s** on strain and culture conditions

(Meldgaard et al., 1995). Transgenic plants have also been used as expression hosts of thermostable bacterial lichenases. A hybrid 1,3-1,4-β-glucanase (from *B. amyloliquefaciens* and *B. macerans*) have been cloned and expressed in transgenic barley as part of a breeding project to produce brewers barley plants expressing the enzyme during germination that survives the high temperature used in kiln drying of green malt (Jensen et al., 1996 and Hovarth et al., 2000). The enzyme from *Clostridium thermocellum* (*licB*) has been used as reporter gene for analysis of heterologous gene expression in transformed tobacco plants (Piruzian et al., 1998). Among fungi, 1,3 (4)-β-glucanase have been recently cloned from *Cochliobolus carbonum* (Gorlach, et al., 1998), *Phaffia rhodozyma* (Bang et al., 1999).

Gene expression and regulation

Enzyme expression by the *lic* operon is regulated by different overlapping mechanisms: catabolite repression via a CcpA-dependent and independent mechanisms (Kruger et al., 1993 and Tobishch et al., 1999), and temporal activation mediated by a drop in the intracellular GTP concentration after logarithmic growth at the onset of the stationary phase in response to nutrient limitation (Stulke et al., 1993). Recent studies have analysed the structure and regulation of the *lic* operon (Tobisch et al., 1999). It is transcribed from a sigma (A) dependent promoter and is inducible by the substrate**s:** lichenan, lichenan hydrolysate and cellobiose. Expression is positively controlled by the *LicR* regulator protein, the activity of which is stimulated by modification (phosphorylation) of two regulatory domains by the general phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS), and negatively regulated by modification of a third regulatory domain in the absence of oligomeric β-glucoside inducers. Upstream of the licS gene encoding the 1,3-1,4-β-glucanase, the licT gene codes for a transcriptional antiterminator (Schnetz et al., 1996). By mapping chromosomes of *B. subtilis* mutants, the gene organisation has been analysed (Borriss et al., 1986).

Characterization of lichenase gene

A number of *Bacillus* species secrete lichenases. The lichenase genes have been cloned and characterized from *Bacillus subtilis* (Cantwell and McConnell, 1983, Murphy et al., 1984), *Bacillus amyloliquefaciens* (Hofemeister et al., 1986), *Bacillus macerans* (Borriss et al., 1988 and Borriss et al., 1990), *Bacillus circulans* (Bueno et al., 1990a; b), *Bacillus polymyxa* (Gosalbes et al., 1991), *Bacillus licheniformis* (Lloberas et al., 1991), *Bacillus brevis* (Louw et al., 1993) and alkalophilic *Bacillus* sp. N137 (Tabernero, et al., 1994). The enzyme 1,3-1,4-β-glucanases from all bacilli share similarity on both the nucleotide and amino acid sequences (Planas, 2000).

Exceptionally *B. circulans* enzyme is also able to hydrolyse Carboxy Methyl Cellulose (CMC) thus not being a true lichenase as defined above. The *Bacillus* sp. N137 enzyme has a lysine-rich region at the C-terminus not found in the rest of lichenases. Genes encoding 1,3-1,4-β-glucanases have also been isolated from non-*Bacillus* species. Their catalytic domains share high degree of sequence similarity to *Bacillus* enzymes but they often have additional regions with a different function. The *xynD* gene of *Ruminococcus flavofaciens* (Flint et al., 1989 and Flint et al., 1993) encodes for a bifunctional enzyme having separate xylanase (N-terminus) and 1,3-1,4-β-glucanase (C-terminus) domains linked by a 309 amino acid region of unknown function that includes a 30-amino-acid threonine-rich sequence. The isolated β-glucanase domain exhibits some activity against the homopolymeric 1,3-β-glucan laminarin as opposed to the *Bacillus* 1,3-1,4-β-glucanases but this activity is much lower than that on lichenan or cereal β-glucans. The *Clostridium thermocellum licB* gene expresses a lichenase (Schimming et al., 1991; 1992; Zverlov and Velikodvorskaya, 1990; Zverlov et al., 1991; 1994) in which its catalytic region is homologous to the *Bacillus* enzymes. In addition, it has a C-terminal reiterated domain homologous to the duplicated non-catalytic domain of clostridial cellulases and xylanase Z, suggesting that this enzyme is also part of the cellulosome or cellulosome-like enzyme complex (Schimming et al., 1991).

The lichenases gene product sequence alignment of the catalytic domain from *Fibrobacter* (formerly *Bacterioides*) *succinogenes* (Erfle et al., 1988; Teather and Erfle, 1990) with that of *Bacillus* and *Clostridium thermocellum* indicates that the catalytic

sequence can be divided into two segments, the order of which is reversed in the *F. succinogenes* enzyme (Schimming et al., 1992).

The non-homologous part includes a serine-rich region common to other endoglucanases from the cellulolytic bacteria *Clostridium thermocellum* (Grepinet and Beguin, 1986) or fungi such as *Trichoderma* (Penttila et al., 1986; Teeri et al., 1987), or *Cellulomonas* (Wong et al., 1986). The gene cloned from *Streptococcus bovis* (Ekici et al., 1997) expresses a 1,3-1,4-β-glucanase with high sequence similarity and is about the same size as the *Bacillus* enzymes. Just recently 1,3-1,4-β-glucanases have been identified in fungi. Lichenase gene from *Orpinomyces* has also been cloned (Chen et al., 1997). The amino acid sequence has high homology with bacterial 1, 3-1,4-β-glucanases and it does not contain the non-catalytic peptide domain common in most of the hydrolytic enzymes of anaerobic fungi. Because the nucleotide sequence is devoid of introns and its flanking regions are of fungal origin (extremely AT rich), it was suggested that the gene had a bacterial origin. Three enzymes active on cereal β-glucans have been identified from the fungal plant pathogen *Cochliobolus carbonum* (Gorlach et al., 1998) only one of them is probably a true lichenase.

An aerobic fungus *Talaromyces emersonii* secretes a 41 kDa β-glucanase based of its substrate specificity. A related group of enzymes able to depolymerize both 1,3-1,4-β-glucans (lichenan and cereal β-glucans) and 1,3-β-glucans (laminarin) have also been cloned. They show similar substrate specificity than the *Rhizopus arrhizus* β-glucan endo-hydrolase (Parrish et al., 1960, Anderson and Stone, 1975 and Clark et al., 1978) and have been classified as 1,3(4)-β-glucanases (EC 3.2.1.6) which also belong to family 16 glycosyl hydrolases. These enzymes have lower sequence similarity to *Bacillus* lichenases, the catalytic region having a higher degree of identity but with some additional amino acids in the neighbourhood of the catalytic residues. In the *Rhodothermus marinus* enzyme (Spilliart et al., 1994), the primary protein sequence shows limited identity to *Bacillus* 1,3-1,4-β-glucanases and a higher identity to the C-terminal part of the laminarinase of *Bacillus circulans* (Yahata et al., 1990). The enzyme can hydrolyse β-1,4 as well as β-1,3 linkages in barley β-glucan and the end products of hydrolysis on lichenan and laminarin are mostly mono- and di-saccharides in addition to oligosaccharides, an unusual behaviour since lichenases and laminarinases yield mainly

tri- and tetra-saccharides as final products. In addition to a lichenase (*licB gene product*), *Clostridium thermocellum* produces an enzyme (*licA* gene product) active on lichenan and laminarin (Schwarz et al., 1988). Likewise, *Cellvibrio mixus* (Sakellaris et al., 1993) also expresses a 1,3(4)-β-glucanase that differs from the clostridial enzyme by its low activity on lichenan as compared to barley β-glucan.

Hybrid enzymes and thermostability

Engineering increased thermostability in enzymes is a biotechnological target both to broaden their usefulness in industrial applications and to understand the structure-stability relationships that govern protein stability. Bacterial 1,3-1,4-β-glucanases are intrinsically more thermostable than that of the plant (family 17) enzymes and have found applications in the brewing and animal feedstuff industries to depolymerize cereal β-glucans under moderate to high operation temperatures. Mixed-linked β-glucans of the endosperm cell walls of barley have to be degraded during germination as well as in the industrial process of malting in the brewing industry. A large fraction of the endogenous malt β-glucanases are irreversibly heat inactivated during kilning and the remaining activity is rapidly destroyed during mashing. Because remaining high molecular mass β-glucans cause severe problems such as reduced yields of extracts and lower rates of wort separation and beer filtration, thermostable 1,3-1,4-β-glucanases of bacterial origin are often added during mashing. To obtain enzymes with improved thermostability at the pH of industrial operation, hybrid enzymes derived from naturally occurring *Bacillus* 1,3-1,4-β-glucanases that combine different properties suited for industrial processes have been developed. The *B. macerans* enzyme is one of the most thermostable *Bacillus* lichenase at neutral pH with a temperature for optimal enzyme activity of 65°C and retaining 80% of its activity at 75°C. But it is rather labile in an acidic environment while the corresponding enzyme from *B. amyloliquefaciens* is less affected by acidic condition with a pH optimum around 6.0 (Borriss and Zemek, 1981). A number of hybrid enzymes have been produced by domain hybridisation in which different parts of the genes from *B. amyloliquefaciens* and *B. macerans* have been exchanged. The first reported hybrids were constructed by reciprocal exchange of the two halves of the parental genes (Borriss et al., 1989). The hybrid protein H(A107-M) (containing the 107 amino terminal residues of the mature *B. amylolyquefaciens* enzyme and the last 107 C-terminal amino acid

residues of *B. macerans*) showed slightly improved thermostability especially in an acidic environment. A more systematic study have produced a repertoire of hybrid genes that were expressed in *E. coli* to yield enzymes with a range of different properties (Olsen et al., 1991 and Politz et al., 1993). The hybrid H(A16-M) has a remarkably improved thermostability in both neutral and acidic environments with a specific activity 40% higher than that of the *B. macerans* wild type enzyme and retains at least 80% optimal activity at 80°C. Since substitution of only 16 N-terminal amino acids in the *B. macerans* enzyme with the homologous part of the *B. amyloliquefaciens* protein produced such large effects, a series of hybrids at the N-terminal end were constructed to delineate the amino acid residues that affect protein stability (Politz et al., 1993). It was concluded that the N-terminal eight amino acids from *B. amyloliquefaciens* was sufficient to significantly enhance thermostability whereas site-directed mutagenesis revealed that the combined effects of Gln1, Thr2, Ser5 and Phe7 confer improved stability probably by optimizing the hydrogen bonding between the N-terminal and the centre of the folded protein and between the two termini of the polypeptide chain. Moreover, deletion of Tyr13 in the hybrid H(A12-M) resulted in an even more thermostable enzyme with a two-fold higher half-life at 70°C and at pH 5 than the H(A16-M) hybrid. It is precisely this hybrid that was chosen for production of transgenic barley (Jensen et al., 1996) because it had a better suited thermostability profile for potential industrial applications. Some of the hybrids were more thermotolerant when expressed in yeast due to *N*-glycosylation as compared to the unglycosylated forms expressed in *E. coli* (Meldgaard and Svendsen, 1994). The thermostability of a series of alanine mutants in the major loop involved in substrate binding of the *B. licheniformis* 1,3-1,4-β-glucanase (from Asp51 to Arg64) were also analysed (Pons et al., 1997).

Biotechnological importance of lichenase

The bacterial 1,3-1,4-β-glucanases are important biotechnological aids in the brewing and animal feedstuff industries. During malting, endogenous 1,3-1,4-β-glucanases are heat inactivated and large contents of remaining high molecular weight β-glucans may cause severe problems such as reduced yields of extracts and lower filtration rates, as well as the appearance of gelatinous precipitates in the finished beer. Thus the

level of activity of glucan hydrolases achieved during germination and the amount of their substrates are important factors for good quality brewers malt and thermostable bacterial 1,3-1,4-β-glucanases are often added to reduce viscosity during mashing (Stone and Clarke, 1992 and Godfrey et al., 1983). In animal feedstuff, specially for broiler chickens and piglets, addition of enzymatic preparations containing bacterial β-glucanases improves digestibility of barley-based diets and reduces sanitary problems such as sticky droppings (White et al., 1983 and Stone and Clarke, 1992).

CHAPTER II

PRODUCTION OF LICHENASE AND XYALANSE FROM *THERMOMONOSPORA* SP.

Abstract

The isolate secreting lichenase was identified to be a new species of *Thermomonospora* by 16S rDNA sequencing. It showed 98% homology toward *Thermomonospora mesouviformis*. The medium was optimized for the production of lichenase from *Thermomonospora* sp. by surface response method. The optimized medium produced 2.5 to 3.0 times lichenase activity as compared to the basal medium. Cellulase and xylanase enzyme were co produced during the fermentation. A proportional increase in activities of cellulase and xylanse were observed during media optimization for lichenase. The optimum medium composition per 100ml obtained from factorial was Tween 80, 0.025µl; yeast extract, 2g; Ammonium sulphate, 0.5g; Urea, 0.05g and CPP, 2g. The methodology as a whole proved to be quite adequate for the design and optimization of a bioprocess. Using this method of experimental factorial design and response surface analysis it was possible to determine optimal cultivation medium to obtain high lichenase production. This method not only helps in establishing optimal fermentation media for operation but also predicts the possible yield and productivity which the system is disturbed in some way making it useful too to understand the potential for process control.

Introduction

1-3, 1-4 β-Glucans (mixed-linkage glucans) are polysacchrides found in the cell walls of cereals. They play the role of storage polysaccharides and are found to be abundant in the endosperms of barley, oat, rye, rice, sorghum and wheat grains (Stone and Clarke, 1992). Mixed-linkage (1-3),(1-4)-β-D-glucans are also produced by some bacteria (Anderson and Stone, 1975, Hou et al., 1996), lichen (da Silva et al., 1993; Kramer et al., 1995), and fungi (Allard and Tazi, 1993). β 1-3,1-4 glucan 4 glucanohydrolase (E.C. No. 3.2.1.73) is an inducible, endo glucanase that specifically hydrolyzes β-1,4- linkages adjacent to a β-1,3 glycosidic moiety on its non-reducing end to yield 3-O-β-D-cellobiosyl-D-glucose and 3-O-β-D-cellotriosyl-D-glucose (Malet et al.,1993; Tang et al., 2004). A number of microorganisms (bacteria and fungi) secrete 1, 3-1,4-β- glucanases, involved in the degradation of polysaccharides that can be present in their natural environment and be used as an energy source. The plant and microbial enzymes have neither amino acid sequence similarity nor related three-dimensional structure, being an example of convergent evolution towards the same substrate specificity. Whereas the plant enzymes belong to the glycosyl hydrolase family 17 (Henrissat, 1991; Herissat and Bairoch, 1993; Henrissat and Bairoch, 1996) with an $(\alpha/\beta)_8$ barrel three-dimensional structure, the microbial enzymes are classified as members of Family 16 with a jellyroll β-sandwich structure. Lichenases have been found in several *Bacillus* species, including *B. subtilis* (Murphy et al., 1984; Cantwell and McConnell, 1983), *B. amyloliquefaciens* (Hofemeister et al., 1986), *B. macerans* (Borris et al., 1990; Heng et al., 1997), *B. licheniformis* (Lloberas et al., 1991), *B. brevis* (Louw et al., 1993), and *B. polymyxa* (Gosalbes et al., 1994), and in *Clostridium thermocellum* (Schimming et al., 1992), *Fibrobacter succinogenes* (Teather and Erfle, 1990), *Ruminococcus flavefaciens* (Flint et al., 1993), *Rhizobium meliloti* (Becker et al., 1993), and *Cellvibrio mixtus* (Sakellaris et al., 1993), *Streptococcus bovis* (Ekinci et al., 1997), and anaerobic fungus *Orpinomyces* sp. (Chen et al., 1997). Lichenase production by some microorganism studied recently have been enlisted in Table 1.

Table 1: Representative microorganisms producing lichenase

Organism	**Lichenase (IU/ml)**	**Ref**
Bacillus subtilis	123 IU/ml	El-Helow and El-Ahawany, 1999
Rhizopus microsporus	0.197 units/mg	Celestino et al., 2006
Clostridium acetobutylicum ATCC 824	119.2 IU/ml	Lo´pez-Contreras et al., 2003
C. stellata	6±190 nkat units	Strauss et al., 2001.
C. pulcherrima	1±64 nkat units	Strauss et al., 2001.
K. apiculata	09±306 nkat units	Strauss et al., 2001.

The conventional method of medium optimization (variation of one variable at a time) is time-consuming, expensive, and inaccurate, especially when interactions between different components are present. Statistical experimental designs allow simultaneous, systematic, and efficient variation of all components (Box et al., 1978; Davies, 1993). The use of user-friendly software packages has made this technique increasingly popular for media optimization (Achary et al., 1997; Hounsa et al., 1996; Lee and Chen 1997; Zhu et al., 1996). In the present study, we used a surface response strategy to increase lichenase production by shake flask submerged fermenation. In the first step, optimum medium composition was determined in shake flasks by using fractional factorial designs with five variables. Xylanases and CMCases were co-produced by *Thermomonospora* sp. along with lichenase. The present study concentrates on the optimization of media component for the production of lichenases from *Thermomonospora* sp.

Materials and Methods

Microorganism

The microorganism used in these studies is an alkalothermophilic actinomycete, *Thermomonospora* sp. isolated from self heating compost from Barabanki district, Uttarpradesh, India (George et al., 2001a).

Isolation of genomic DNA

The bacterial culture was grown in 100 ml of *Luria Broth* (pH 9.0) at 50°C for 48h with continuous shaking at 200rpm. DNA was isolated according to the method described by Hopwood et al. (1985) with slight modifications. Mycelium (1g) washed in 5ml TE buffer (pH 8.0) was centrifuged at 10,000rpm for 10min. at 4°C. The pellet was freezed in liquid nitrogen and powdered for better access by lysozyme. The powdered pellet was resuspended in 5ml TE containing 2mg/ml lysozyme. It was incubated at 30°C, triturating every 15min. until a drop of suspension on a microscopic slide was completely cleared with a drop of 10% SDS (1 hour). Further, 1.2ml of 0.5 M EDTA was added to the mixture followed by 0.7 ml 10% SDS. The solution was swirled and incubated at 37°C for 2h. Proteins were extracted from the aqueous phase by adding 6ml of a mixture of phenol:chloroform: IAA (isoamyl alcohol) (25:24:1). The mixture was shaken for 5min. and then centrifuged at 10,000 rpm, 4°C for 10min. This step was repeated again for the aqueous phase in a new tube. Finally, 40µg/g of RNAase from a stock of 10mg/ml was added to pre-weighed aqueous phase and incubated at 37°C for 1h. The genomic DNA was precipitated by adding equal volume of isopropanol and was stored at room temperature for 1h. DNA was spooled with a glass rod and transferred to a fresh tube and dissolved in 5ml of TE buffer, pH 8.0.

PCR Amplification of 16S rDNA

The primers used for the identification of *Thermomonospora* sp. were universal eubacterial 16S rDNA primers (16F27N- 5'CCA GAG TTT GAT CMT GGC TCA G, 16F536 (5'GTG CCA GCA GCC GCG GTR ATA), 16R1525XP (5'TTC TGC AGT CTA GAA GGA GGT GWT CCA GCC) commercially obtained from Life technologies, India. The 25µl Polymerase chain reaction (PCR) was set by using the genomic DNA

and the reaction mixture typically contained Genomic DNA 0.50µl, 10X Buffer 2.50µl, 0.2mM deoxyribonucleoside triphosphate (dNTP) 2.5µl, Forward and Reverse Primers 10-20pmoles, (1.25µl each), distilled water 16.87µl, and 1unit of *Taq DNA polymerse* 0.25µl (Bangalore Genei). All the additions were done on ice and the PCR reaction was performed on Gene Amplifier PCR System 9700 (Perkin Elmer, USA). The PCR conditions for 16SrDNA gene amplification were: Initial denaturation 95°C for 3min.; followed by 35cycles of 94°C for 1min., 55°C for 1min., 72°C for 1min. and final extension at 72°C for 10min. 5µl of the above PCR amplified product was used for electrophoresis using 1.0% agarose gel in 1X TBE buffer (Working solution: 0.5X; Stock: 5X, 54g Tris base, 27.5g boric acid, 20ml 0.5M EDTA [pH 8]). The gel was run at 80V for 90min. using 1X TBE as running buffer. The gel was stained in 1% ethidium bromide for 45min. and was observed under UV illumination.

Purification of PCR amplified product

To 20µl PCR amplified product, 0.6volume of 20% PEG-NaCl (Polyethylene glycol - NaCl) solution was added and incubated at 37°C for 30min. It was then centrifuged at 12000rpm for 20min. The supernatant was discarded and the pellet was washed twice with 70% ethanol and separated by centrifuging at 12000rpm for 20min. The pellet was dried and dissolved in 10 µl of double distilled water and stored at -20°C.

Sequencing of the purified PCR product

The sequencing reactions were carried out using *Taq* DNA polymerase dye terminator cycle sequencing using the 'ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit' (Perkin Elmer Applied Biosystems Division, Foster City, CA) according to the manufacturer's protocol. This Kit contains the four ddNTPs with different fluorescence labels termed as BigDye Terminators. 2µl PCR product and 3pmol of the sequencing primer were used in a 20µl sequencing reaction. The sequencing reaction mixes were subjected to 25cycles in a Perkin Elmer thermal cycler 9700. Each cycle consisted of 95°C for 10min., 50°C for 5min. and 60°C for 4min. DNA sequencing was carried out on ABI 1500 Automated Sequencer at the DNA sequencing facility in

National Centre for Cell Science (NCCS), Pune. The analysis of nucleotide sequence was done at NCBI server using BLAST-n (www.ncbi.ncm.gov/blast).

Production of lichenase

The lichenase production was studied in 500ml Erlenmeyer flasks containing 100ml of medium with a composition similar to that used by George et al. (2001). Cellulose paper powder (CPP) (4%) was used as a carbon source. Sterile 10% sodium carbonate was used to adjust the pH of the medium to 9. A 10% inoculum was added from an inoculum flask grown for 48h at 50°C. The microorganism was grown at 50°C for 120h on a rotary shaker maintained at 200rev min^{-1}. At the end of 120h the fermentation broth was centrifuged at 10,000rpm for 10min. The supernatant was used as the source of enzyme. Lichenase production was studied in the presence of different carbon, nitrogen sources and surfactants. All experiments were performed in triplicate.

Table 1: Composition of modified Reese medium used as basal medium

Ingredients	g/100ml
Cellulose paper powder	4.000
Potassium monohydrogen phosphate	0.200
Ammonium sulphate	0.700
Urea	0.150
Magnesium sulphate	0.030
Peptone	0.125
Yeast extract	1.000
	µl/100ml
Tween 80	100
*Ferrous sulphate	50
*Manganese sulphate	15.6
*Zinc suphate	14
*Cobalt sulphate	20

* 1% stock solution were used.

Enzyme Activity Assay

The activity of lichenase / endoglucanase / xylanase was measured by incubating 1ml of assay mixture containing 0.5ml of Lichenan (1%) / carboxymethylcellulose (CMC) (1%)/ xylan (1%) and 0.5ml of suitably diluted enzyme in 50mM, pH 7, phosphate buffer for 30min at 50°C. Enzyme and reagent blanks were also simultaneously incubated with the test samples. The reducing sugar formed was estimated by dinitrosalicylic acid (Miller, 1959).

One international unit of lichenase/ cellulose is defined as the amount of enzyme required to liberate 1µmole of glucose from lichenan / carboxymethyl cellulose respectively in 1 min under the said assay conditions. One international unit of xylanase is defined as the amount of enzyme required to liberate 1µmole of xylose from xylan in 1min under the said assay conditions.

Protein concentration was determined according to the method of Bradford (1976), using bovine serum albumin as standard.

Factorial Designs and Analysis of Results

Factorial experiments were designed using the approach given in standard texts on design of experiments (Box et al., 1978; Davies, 1993). To estimate standard error in enzyme activity measurement, an experiment was performed using six flasks containing identical medium (at the center of the first factorial). Effect of five variables, namely, Tween 80, ammonium sulphate, urea, CP powder, and yeast extract concentrations on lichenase production were studied using a two level fractional design (Table 2). The experimental data obtained from the factorial design were fitted to the following polynomial:

$$\text{Activity} = \alpha_0 + \alpha_1 x_1 + \alpha_2 x_2 + \alpha_3 x_3 + \alpha_4 x_4 + \alpha_5 x_5 + \alpha_6 x_1 x_2 + \alpha_7 x_1 x_3 + \alpha_8 x_1 x_4 + \alpha_9 x_1 x_5 + \alpha_{10} x_2 x_3 + \alpha_{11} x_2 x_4 + \alpha_{12} x_2 x_5 + \alpha_{13} x_3 x_4 + \alpha_{14} x_3 x_5 + \alpha 15 x_4 x_5$$

Here the α's are fitted constants and x_1, x_2, x_3, x_4, and x_5 are the variables for Tween 80, ammonium sulphate, urea, CP powder, and yeast extract, respectively.

Table 2: Variable influencing the production of lichenase selected for factorial design

Variables (g%)	Code	Factorial 1		Factorial 2	
		-	+	-	+
Tween 80*	x_1	50	150	25	75
$(NH_4)_2SO_4$	x_2	0.1	1.0	0.5	1.5
Urea	x_3	0.1	0.2	0.05	0.15
CP powder	x_4	3	5	2.0	4.0
Yeast extract	x_5	0.5	1.5	1.0	2.0

*= Used in µl and not grams

Time Course of lichenase production

The time course of lichenase production was studied with the optimized medium in shake flask for 120h. A 10% inoculum was added to 100ml of optimum medium in a 500ml Erlenmeyer flask and incubated at 50°C at 200rpm on a rotary shaker for 120h. Samples were removed periodically and cell growth as well as lichenase activity was determined in each sample as stated earlier.

Results and Discussion

Identification of the isolate by16SrDNA sequencing

The genomic DNA was isolated, loaded on agarose gel and was found to be high molecular weight, intact DNA (Figure 1). The spectrophotometric analysis of the DNA showed that the DNA had a A_{260}:A_{280} ratio of 2.0, which indicates the purity of the DNA preparation.

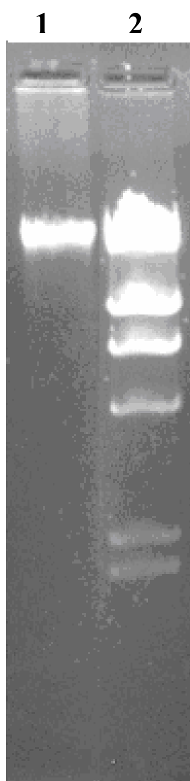


Figure 1: Agarose gel (1%) electrophoresis of genomic DNA isolated from alkalothermophilic *Thermomonospora* sp. Lane 1: *Thermomonospora* sp. genomic DNA ; Lane 2: λ *Hind* III marker.

The 16SrDNA sequence (1493bp) of *Thermomonospora* sp. was obtained by sequencing with the primers 16F27N, 16R1525XP and 16F536. The sequences so obtained by each set of primer were then overlapped to get a 1493bp 16SrDNA sequence.

5'AGCTGGCGGCGTgcTTAACACATGCAAGTCGAGCGGTAAgGCCCCTTCGGGG
GTACACGAGCGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCCTGACTC
TGGGATAAGCCCGGGAAACCGGGTCTAATACCGGATACGACTTCTCACCGCA
TGGTGGGGGGTGGAAAGGTTTGTTCCGGTTGGGGATGGGCTCGCGGCCTATC
AGCTTGTTGGTGGGGTGATGGCCTACCAAGGCGATGACGGGTAGCCGGCCTG
AGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTGCGGG
AGGCAGCAGTGGGGAATATTGCGCAATGGGCGGAAGCCTGACGCAGCGACG
CCGCGTGGGGGATGACGGCCTTCGGGTTGTAAACCTCTTTACCACTCACGAA
GGCTCCCAGTGTTCTGGGGGTTGACGGTAGGTGGTGAATAAGGACCGGCTAA
CTACGTGCCAGCAGCCGCGGTAATACGTAgGGTCCGAGCGTTGTCCGGAATT
ATTGGGCGTAAAGAGCTCGTAGGCGGCCTGTCGCGTCTGCTGTGAAAGTCCG
GGGCTTAACTTCGGGTGTGCAGTGGATACGGGCAGGCTTGAGGTAGGTAGGG
GAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACA
CCGGTGGCGAAGGCGGGTCTCTGGGCCTTACCTGACGCTGAGGAGCGAAAGC
GTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGG
GCGCTAGGTGTGGGGACTTTCCACGGTTTCCGTGCCGTAGCTAACGCATTAAG
CGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACG
GGGGCCCGCACAAGCGGCGGAGCATGTTGCTTAATTCGACGCAACGCGAAG
AACCTTACCAAGGTTTGACATCACCGGTAATCCTCCAGAGATGGGGGGTCCT
TCGGGGATCGGTGACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGA
TGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGTTCCATGTTGCCAGCAC
GTGATGGTGGGGACTCATGGGAGACTGCCGGGGTCAACTCGGAGAAAGATG
GGGACGACGTCAAGTCATCATGCCCCCTATGTCTTGGGCTGCAAACATGTTAC
AATGGCCGGTACACAGAGCGTGCGATGTCTTGAGACGGAGAGAATCTCTTAA
AGCCGGTGTCAGTTCGGATTGGGGTGTGCAACTCTACCCCATAAAGGTGGAG
TCGCTAGTAATCTCGGATCAGCAACGCCCCGGTGAATACGTTCCCGGGCCCT
GTACACACCGCCCGTCACGTCTCGAAAGTCGGCAACACCCGAAACGCGTGGC
GCTAACCCCTTTTGGGGAGGGAGCGTGTGAAGGTGGGGCTGGTGATTGGGAC
GAAGTCGTAACAAGGTAGCCGTACCGGAAGGCCGGTTCC3'

Figure 2: 16SrDNA sequence of *Themomomospora* sp. NCBI accession No. EF205152.

Sequences producing significant alignments:		Score (bits)	E Value
gi|4103855|gb|AF028247.1|AF028247	*Thermomonospora mesouvifo*...	2716	0.0
gi|2465537|gb|AF002265.1|AF002265	*Thermomonospora mesouvifo*...	2702	0.0
gi|2465532|gb|AF002260.1|AF002260	*Thermomonospora alba* 16S ...	2670	0.0
gi|6539498|dbj|AB006170.1|	*Thermomonospora mesophila* 16S rR...	2646	0.0
gi|23017464|ref|NZ_AAAQ01000024.1|	*Thermobifida fusca* Tfus_...	2539	0.0
gi|23017322|ref|NZ_AAAQ01000021.1|	*Thermobifida fusca* Tfus_...	2531	0.0
gi|2465536|gb|AF002264.1|AF002264	*Thermomonospora fusca* 16S...	2488	0.0
gi|11602788|emb|AJ298058.1|TCE298058	*Thermobifida celluloly*...	2458	0.0
gi|4103853|gb|AF028245.1|AF028245	*Thermomonospora fusca* 16S...	2405	0.0
gi|6539499|dbj|AB006171.1|	*Thermomonospora fusca* 16S rRNA g...	2355	0.0

Figure 3: First ten BLAST result hits obtained from NCBI for *Thermomonospora* sp. 16SrDNA sequence.

On NCBI BLAST, this sequence showed closest homology (98%) to *Thermomonospora mesouviformis*. The first ten blast hits have been shown in Figure 3.

Using homology of the 16SrDNA sequence obtained with the known organisms, a distance based phylogenetic tree was constructed. It revealed that the isolate of *Thermomonospora* had maximum similarity to *Thermomomospora mesouviformis.* Since *Thermomomonspora mesouviformis* grows optimally at neutral pH and the present isolate is an obligate alkalophile (optimum growth at pH 9.0), the isolate was classified to be a new species of *Thermomonospora*.

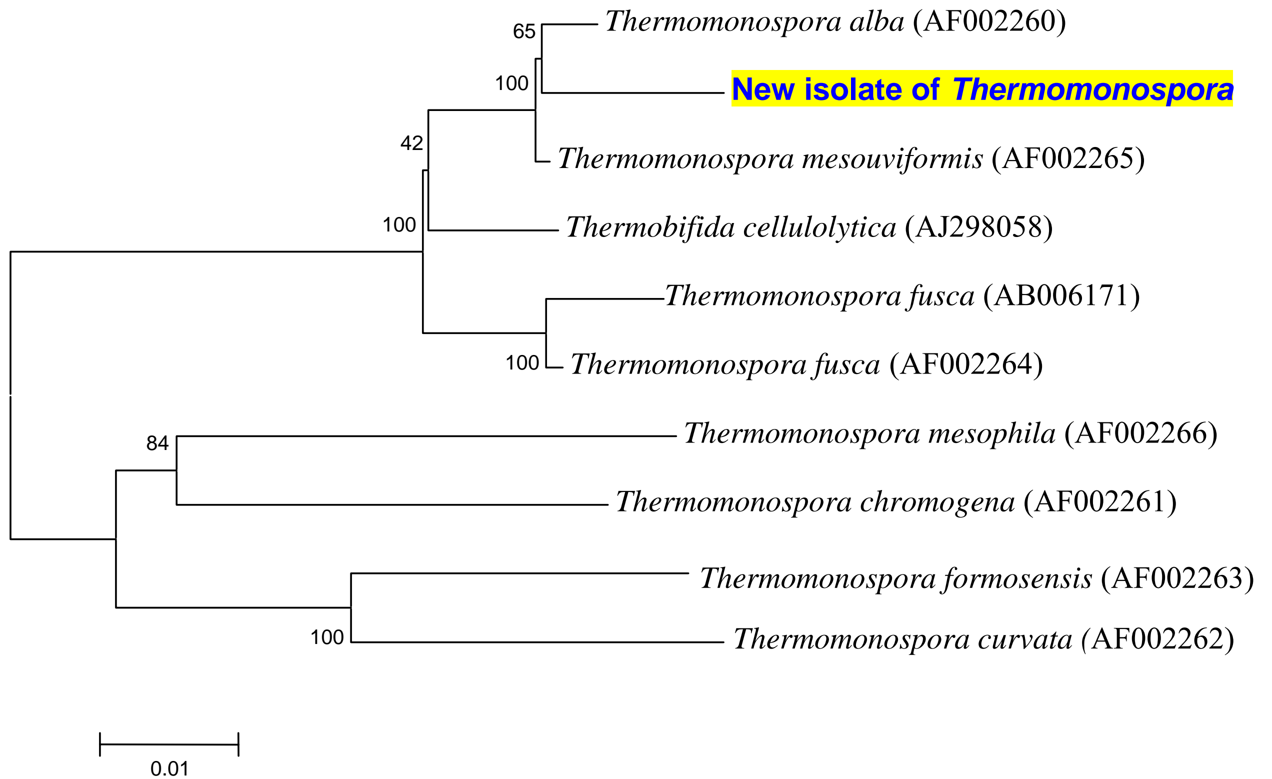


Figure 4: Dendogram showing the similarity between different known *Thermomonospora* species with the new isolate.

Effect of Carbon Source

Effect of different carbon sources on lichenase, xylanase and cellulase production was studied by substituting soluble CPP in the basal medium with various carbon sources. It was observed that enzyme could be produced only when CPP, Corn cob, Tamarind seed and wheat bran were used as carbon source; maximum activity was obtained with CPP (Table 3). When simple sugars like glucose, xylose, sucrose were tested, there was no enzyme production suggesting that the production of lichenase from *Thermomonospora* sp. is not a constitutive.

Table 3: Effect of carbon source on lichenase production by *Thermomonospora* sp.

Carbon source (4%)	Lichenase activity (IU/ml)
Wheat Bran	50
Rice Bran	24
Corn cob	145
Cellulose paper powder (CPP)	170
Coconut stalk	Negligible
Saw dust	Negligible
Tamarind seed	140
Xylan	43

Effect of Nitrogen Source

Influence of organic and inorganic nitrogen sources on enzyme production was tested. The results indicated that CGTase production was good when an organic nitrogen source was present in the medium (Table 4). Among the organic sources tested, highest enzyme production was observed with yeast extract, whereas among the inorganic sources tested urea proved to be the best. Enzyme production was high when an organic nitrogen source in the form of yeast extract was introduced in the medium rather than inorganic nitrogen source as urea. As compared to peptone yeast extract gave promising yield of enzyme. This suggests that yeast extract contains some micronutrients essential for the production of the lichenase, xylanase and cellulase.

Table 4: Effect of nitrogen source on Lichenase production by *Thermomonospora* sp.

Nitrogen source (1%)	Lichenase activity (IU/ml)
Organic source	
Tryptone	144
Peptone	155
Casamino acid	138
Yeast extract	170
Soya meal	152
Inorganic source	
Ammonium chloride	115
Sodium nitrate	100
Ammonium sulphate	127
Di ammonium hydrogen sulphate	118

Factorial Design

A fractional factorial experiment was designed with five significant variables namely Tween 80, yeast extract, ammonium sulphate, urea and cellulose paper powder (CPP) (Table 5). Using six flasks containing identical medium at the center of the factorial, the standard error in enzyme production was calculated to be 0.09. The experimental results obtained from the first factorial design showed that all the variables had significant effect on enzyme production with Yeast extract having the maximum effect.

Table 5: Experimental results of 1st fractional factorial design.

x_1	x_2	x_3	x_4	x_5	Experimental activity (IU/ml)
-	-	-	-	-	190
-	-	-	+	+	147
-	-	+	-	+	151
-	-	+	+	-	208
-	+	-	-	+	217
-	+	-	+	-	118.4
-	+	+	-	-	131.42
-	+	+	+	+	128.71
+	-	-	-	-	159.56
+	-	-	+	+	74.22
+	-	+	-	+	88.71
+	-	+	+	-	26.41
+	+	-	-	+	153.46
+	+	-	+	-	107.38
+	+	+	-	-	119.54
+	+	+	+	+	163.87

Media composition showing the highest activity was chosen as the basal media for designing the 2nd factorial. A central media of composition Tween80, 50 µl; yeast extract, 1.5g; Ammonium sulphate, 1g; Urea, 0.1g and CPP, 3g was obtained for the 1st factorial. Highest production of lichenase with 217IU/ml was obtained using this media. A 2nd factorial was constructed based on this media as a central medium (Table 6).

Table 6: Experimental results of 2nd fractional factorial design

x_1	x_2	x_3	x_4	x_5	Activity(IU/ml)	
					Experimental	**Calculated**
-	-	-	-	+	263	264
-	-	-	+	-	207	205
-	-	+	-	-	132	134
-	-	+	+	+	186.4	187
-	+	-	-	-	40.4	40
-	+	-	+	+	180.6	180
-	+	+	-	+	150.9	152
-	+	+	+	-	179.45	179.4
+	-	-	-	-	170.68	171.6
+	-	-	+	+	112.7	113.8
+	-	+	-	+	105.75	104.9
+	-	+	+	-	78.67	79.9
+	+	-	-	+	170.24	170.45
+	+	-	+	-	146.12	144.67
+	+	+	-	-	152.16	152.43
+	+	+	+	+	143.2	144

Evaluation of the effect of CP powder and urea on lichenase production revealed that the production of lichenase was directly proportional to the concentration of CP powder and urea in the mixture (Figure 5). But it was observed that lower concentration of CP powder with higher concentration of yeast extract yield better production of lichenase.

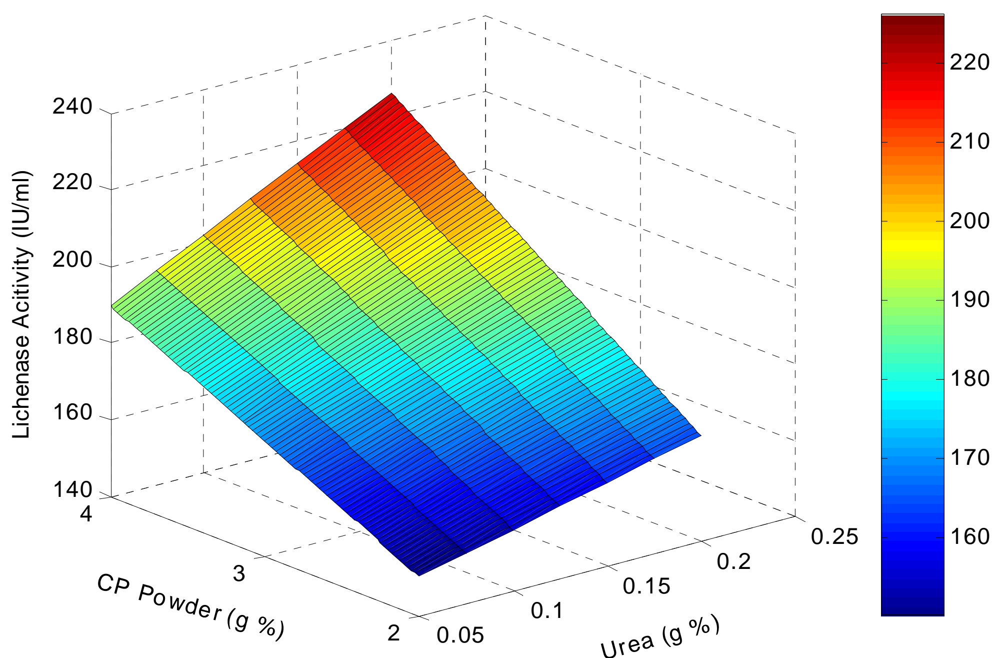


Figure 5: Surface plot of lichenase activity of *Thermomonospora* sp. as a function of urea and cellulose paper powder.

Lichenase production was studied as a function of yeast extract and ammonium sulphate. It was found that both ammonium sulphate and yeast extract were able to support the production of lichenase as nitrogen source. Increasing concentrations of these ingredients increased the production of the enzyme (Figure 6). 4g of yeast extract, 1.5g of ammonium sulphate along with 2g of CP powder supported the production of lichenase. A decrease in Tween80 concentration increased the production of lichenase but eliminating this ingredient from the medium composition did not yield proper growth of the organism and thus production of lichenase. A trace amount of Tween80 was thus required for lichenase production which was fixed to 50µl per 100ml of medium.

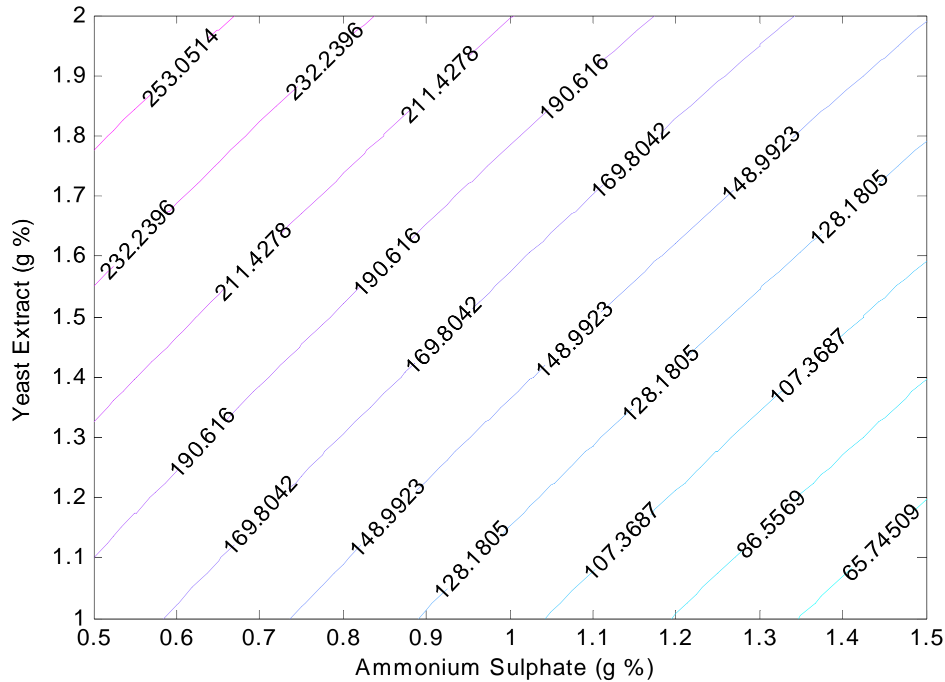


Figure 6: Contour plot for lichenase as a function of yeast extract and ammonium sulphate.

A similar pattern of production profile was observed in case of xylanase in which a concentration of 2g of CP powder in combination with a concentration of 4g of yeast extract exhibited better production of xylanase than the basal medium (Figure 7) . This indirectly also could conclude that the amount of nitrogen source provided in the basal medium was not proportional to the amount of carbon source present in the medium.

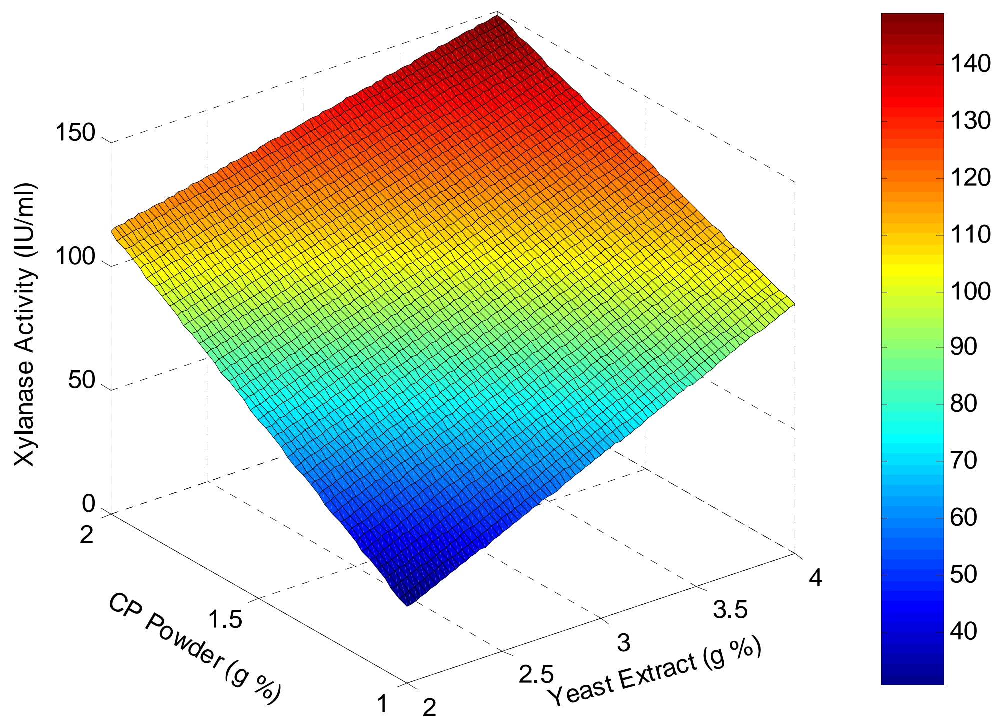


Figure 7: Surface plot of xylanase activity of *Thermomonospora* sp. as a function of yeast extract and cellulose paper powder.

Xylanase production increased with increasing concentration of CP powder and urea (Figure 8). But as compared to the previous results with CP powder and yeast extract a lower concentration of CP powder with higher concentration of yeast extract was responsible for enhanced production of xylanase. This indicated that urea was not a proper nitrogen source for production of the enzyme. Instead yeast extract was used as a better nitrogen source. It may also be providing trace nutrients in the form of amino acids for the growth of *Thermomonospora* sp.

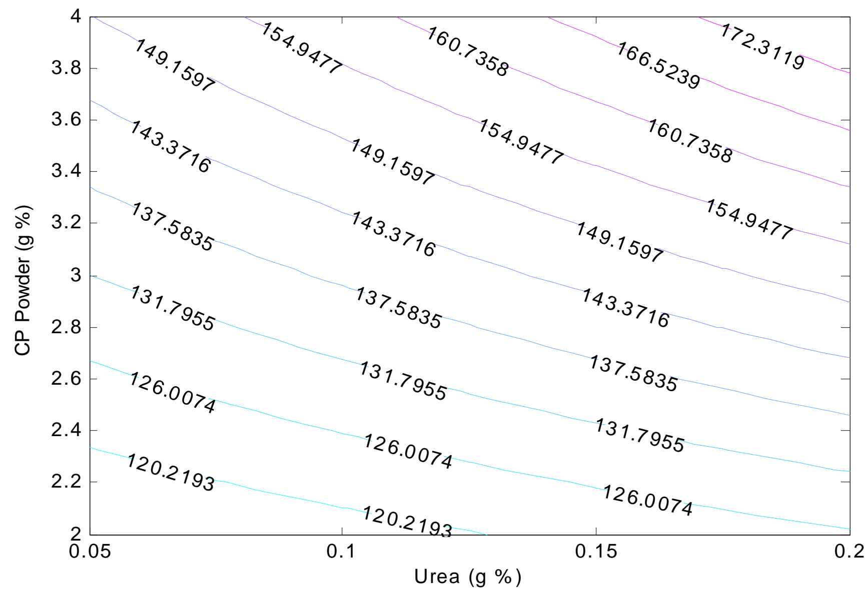


Figure 8: Contour plot for xylanase as a function of urea and CP powder

Cellulase was found to be co produced along with Lichenase and xylanase in the medium. A similar pattern of production was exhibited for lichenase (Figure 9, 10).

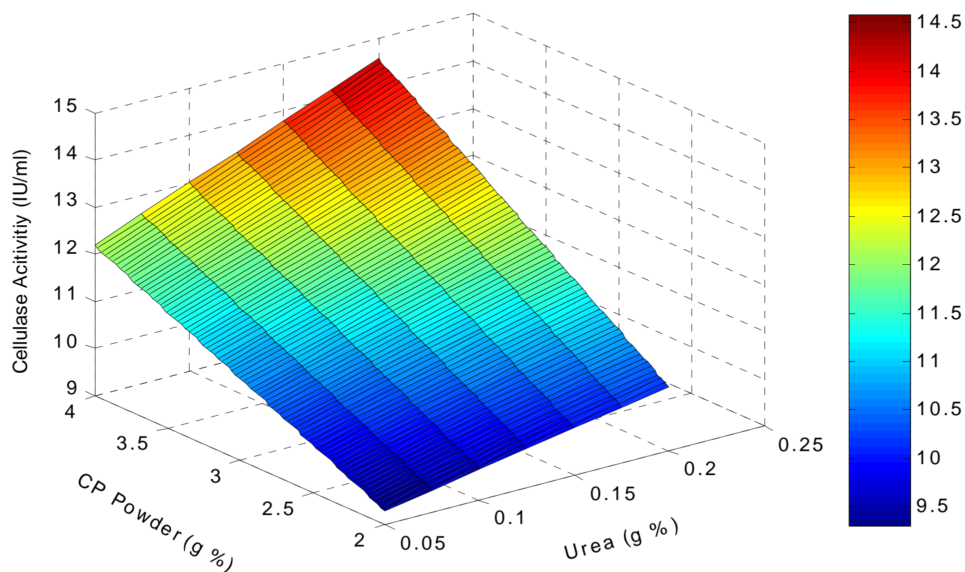


Figure 9: Surface plot of cellulase activity of *Thermomonospora* sp. as a function of yeast extract and cellulose paper powder concentrations when the variables for Urea, Ammonium sulphate, and Tween80 were kept constant.

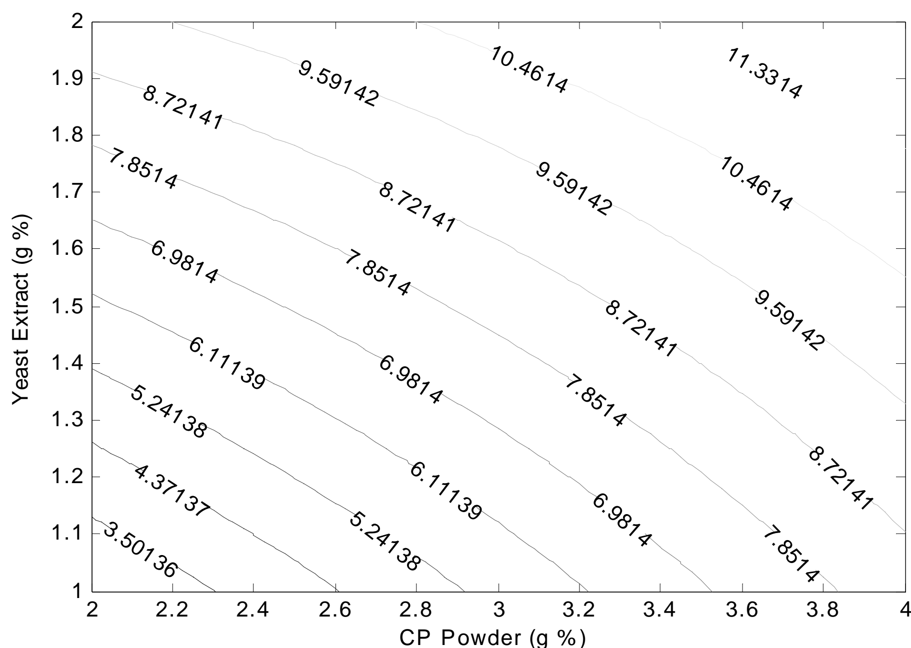


Figure 10: Contour plot for cellulase as a function of yeast extract and CP powder

Thus, the optimum medium composition per 100ml obtained from factorial was Tween 80, 0.025 µl; yeast extract, 2; Ammonium sulphate, 0.5g; Urea, 0.05g and CPP, 2 g.

Cell Growth and lichenase Production Profile

Time course of lichenase production from *Thermomonospora* sp. was studied in shake flask (Fig.11). It was found that lichenase production was growth associated. The enzyme activities obtained with optimized medium in shake flask was 264, 213, 18IU/mL for lichenase, xylanase and cellulase activities respectively as compared to170, 120 and 7.0IU/mL of lichenase, xylanase and cellulase activities respectively in the basal medium. The pH of the medium was found to decrease from 9.0 to 7.5. A maximum protein concentration of 600mg/ml was obtained in 120hours during lichenase production.

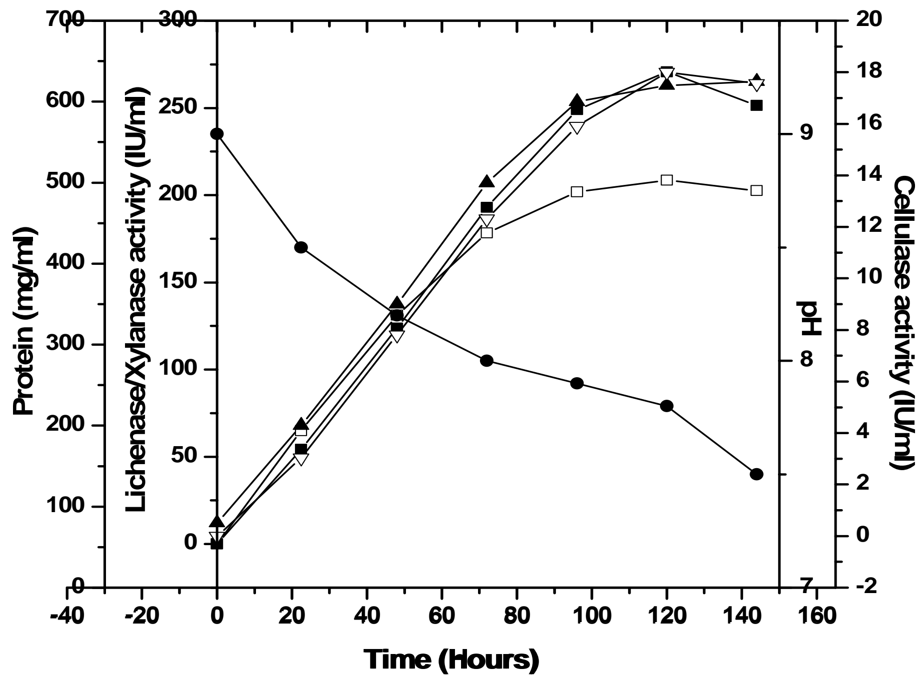


Figure 11: Time course of enzyme production: (▲) Protein; (•) pH; (■) Lichenase activity; (□) Xylanase activity; (▽) Cellulase activity.

The optimization method can be applied for different enzyme production by which the interaction between individual media ingredients can be studied. Tang et al. (2004) has optimized lichenase production from Bacillus with 1.4time increase in production of the enzyme. El-Helow and El-Ahawany (1999) have employed steepest ascent method to optimize the production of lichenase from *B. subtilis.* to achieve about 5times increase in production.

Conclusions

The isolate secreting lichenase was identified to be a new species of *Thermomonospora* by 16SrDNA sequencing. It showed 98% homology toward *Thermomonospora mesouviformis*. The medium was optimized for the production of lichenase from *Thermomonospora* sp. by surface response method. The optimized medium produced 2.5 to 3.0times lichenase activity as compared to the basal medium. Cellulase and xylanase enzymes were co produced during the fermentation. A proportional increase in activities of cellulase and xylanse were observed during media optimization for lichenase. The optimum medium composition per 100ml obtained from factorial was Tween80, 0.025µl; yeast extract, 2g; Ammonium sulphate, 0.5g; Urea, 0.05g and CPP, 2g. The methodology as a whole proved to be quite adequate for the design and optimization of a bioprocess. Using this method of experimental factorial design and response surface analysis it was possible to determine optimal cultivation medium to obtain high lichenase production. This method not only helps in establishing optimal fermentation media for operation but also predicts the possible yield and productivity which the system is disturbed in some way making it useful too to understand the potential for process control.

CHAPTER III

PURIFICATION AND MOLECULAR CHARACTERIZATION OF β-1, 3-1, 4-GLUCAN 4-GLUCANOHYDROLASE

SECTION A

PURIFICATION AND MOLECULAR CHARACTERIZATION OF β- 1-3, 1- 4 GLUCAN 4-GLUCANOHYDROLASE

Abstract

A bifunctional high molecular weight (Mr, 64,500 Da) β-1-3, 1-4 glucan 4-glucanohydrolase was purified to homogeneity from *Thermomonospora* sp., exhibiting activity towards lichenan and xylan. A kinetic method was used to analyze the active site that hydrolyzes lichenan and xylan. The experimental data was in agreement with the theoretical values calculated for a single active site. Probing the conformation and microenvironment at active site of the enzyme by fluorescent chemo-affinity label, OPTA resulted in the formation of an isoindole derivative with complete inactivation of the enzyme to hydrolyse both lichenan and xylan confirmed the results of kinetic method. OPTA forms an isoindole derivative by cross-linking the proximal thiol and amino groups. The modification of cysteine and lysine residues by DTNB and TNBS respectively abolished the ability of the enzyme to form an isoindole derivative with OPTA, indicating the participation of cysteine and lysine in the formation of isoindole complex.

Introduction

1-3, 1-4 β-Glucans (mixed-linkage glucans) are polysaccharides found in the cell walls of cereals. They play the role of storage polysaccharides and are found to be abundant in the endosperms of barley, oat, rye, rice, sorghum and wheat grains (Stone and Clarke, 1992). Mixed-linkage (1-3),(1-4)-β-D-glucans are also produced by some bacteria (Anderson and Stone, 1975, Hou, 1996), lichen (da Silva et al., 1993; Kramer et al., 1995), and fungi (Allard and Tazi, 1993). β 1-3,1-4 glucan 4 glucanohydrolase (E.C. No. 3.2.1.73) is an inducible, endo glucanase that specifically hydrolyzes β-1,4-linkages adjacent to a β-1,3 glycosidic moiety on its non-reducing end to yield 3-O-β-D-cellobiosyl-D-glucose and 3-O-β-D-cellotriosyl-D-glucose (Malet et al.,1993; Tang et al., 2004). This enzyme has important application in the brewing industry to reduce the large fraction of high molecular mass b-glucans during mashing, which causes a severe filtration problem and may lead to gelatinous precipitates in the finished beer (Bamforth, 1982). Also important is the use of enzyme preparations containing β-glucanases in the formulation of broiler chicks feedstuff in order to improve its digestibility and reduce ‘‘sticky dropping’’ problems (White et al., 1983). These biotechnological applications make the enzyme a focus of interest.

Bacillus lichenases are monodomain proteins with a temperature optima of 45°C is exhibited by *B. polymyxa* (Borris and Zamek, 1991), 55°C by *B. subtilis* (Olsen et al., 1991), *B. amyloliquefaciens* (Olsen et al., 1991), *B. licheniformis* (Lloberas et al., 1988) and 65°C by *B. macerans* (Olsen et al., 1991), *B. brevis* (Louw et al., 1993), *Bacillus*. sp. N137 (Tabernero et al., 1994). A basic pI ranging from 7.5–9.1 and pH optimum around neutrality (6–7.5) is a characteristic property of *Bacillus* lichenases, *B. brevis* being an exception with a pH optimum of 9. The lichenase also exhibit the K_m values in the range of 1.2–1.5 mg ml^{-1} for barley β-glucan and 0.8–2 mg ml^{-1} for lichenan and specific activities of 1200 to 4500 μmol min^{-1} mg^{-1}.

The non-Bacillus 1,3-1,4-β-glucanases are usually larger than the *Bacillus* enzymes because of additional domains with different function, but have similar biochemical properties: *C. thermocellum* [(38 kDa, pH_{opt} 6.6–10, T_{opt} 80°C (Schimming et al., 1991)], *Fibrobacter. succinogenes* [(37 kDa, pH_{opt} 6.0, Topt 50°C (Erfle et al., 1988)], *R. flavofaciens* [90 kDa (Flint et al., 1993)], *S. bovis* [25 kDa, pHopt 6.5, Topt 50°C (Ekinci et al., 1997)]. The *Orpinomyces* lichenase with a M_r of

26 kDa, pH_{opt} 6 and T_{opt} 45°C (Chen et al., 1997)] and *Talaromyces emersonii* (40.7 kDa, pH_{opt} 4.8, T_{opt} 80°C, are some of the fungal lichenases characterized. Some of the lichenase have also been enlisted in the Table1.

Table 1: Lichenase from some organisms and their characters.

Organism	M_r, kDa	pH	Temperature (°C)	Reference
Enterococcus faecium	130, 83, 63, 48, 30.5	6.5	40	Beckmann et al., 2000
Streptococcus bovis	60	6.5	50	
Clostridium perfringens	15, 85, 55	4.0, 6.3	45	
Bacteroides ovatus	31, 32	4.0, 6.5	50	
Bacillus uniformis	31	-	50	
Bacillus capillosus	48	4.0	50	
Clostridium acetobutylicum ATCC 824	-	-	45	Lo´pez-Contreras et al., 2003
Bacillus N137	29	7.5	60-70	Tabernero et al., 1994
Bacillus halodurans	31	6-8	60	Akita et al., 2005
C. stellata	-	-	50	Strauss et al., 2001.
C. pulcherrima	-	-	50	Strauss et al., 2001.
K. apiculata	-	-	50	Strauss et al., 2001.

Rhizopus microsporus var. microsporus	33.7	4-5	50-60	Celestino et al., 2006

The present chapter deals with the biochemical characterization lichenase produced by *Thermomonospora* sp., studies of conformation at and near the active site of the enzyme by chemo affinity labeling studies and chemical modification.

Materials and Methods

Microorganism and culture conditions

An alkalothermophilic actinomycete, *Thermomonospora* sp. with characteristics as mention in Chapter II has been used.

Enzyme assays

Lichenase and xylanase activities were measured as described in the previous chapter by measuring the reducing sugar formed (Miller, 1959).

One unit of lichenase / xylanase activity was defined as the amount of enzyme that produced 1μmole of glucose / xylose equivalent per min. from lichenan / xylan respectively, under assay conditions.

Protein concentration was determined according to the method of Bradford, using Bovine Serum Albumin as standard (Bradford, 1976).

Production and purification of lichenase

Thermomonospora sp. was grown for 120h in a modified Reese media (George and Rao, 2001; George et al., 2001a). Sterile 10% Na_2CO_3 was used to adjust the pH of the medium to 9 after autoclaving. The culture filtrate was obtained by centrifuging at 10,000 rpm for 15min. The detailed procedure of production of lichenase has been described in Chapter 1. All purification steps were carried out at 4°C unless otherwise stated. The culture filtrate was subjected to fractional ammonium sulphate precipitation (30-55%). The precipitate dissolved in 0.05M sodium phosphate buffer pH7 was dialyzed and was applied to DEAE Sephadex A50 column (3 x 25cm) previously equilibrated with 0.05M sodium phosphate buffer pH7. The elution was carried out by a linear gradient of NaCl (0 - 1.0M) in same buffer. The fractions having maximum specific activity were pooled and concentrated by ultrafiltration through Amicon UM-10 membrane. The concentrated sample was applied to Sephacryl S-200 column (2.0 x 100cm) previously equilibrated with 0.05M sodium phosphate buffer pH7. Elution was carried out using 0.05M sodium phosphate buffer pH7 at a flow rate of 10ml/h. The fractions (2ml each) having maximum specific activity were pooled and concentrated. The purity of the enzyme was checked by SDS PAGE (Laemmli, 1970) followed by silver staining.

Determination of molecular weight of lichenase

The molecular weight of the lichenase was determined by gel filtration chromatography using Sephadex G-100 previously equilibrated with 50mM Phosphate buffer pH 7. The column was calibrated using the following marker proteins: bovine serum albumin (67,000Da), ovalbumin (43,000Da), carbonic anhydrase (29,000Da), lysozyme (14,200Da) and cytochrome C (12,384Da) obtained from Amersham. The Void volume of the column was determined by the elution volume of Blue dextran. The presence of subunits in the protein was determined by 12% SDS-PAGE.

Determination of optimum pH, temperature, and stability of purified lichenase

Estimation of lichenase and xylanase activities at different pH (4-10) and temperatures (40-100°C) were carried out under standard assay conditions to determine optimum pH and temperature for enzyme activity. The pH stability of the enzyme was measured by incubating 5IU of enzyme at 50°C in buffer of desired pH for 1h. The temperature stability was determined by incubating 5IU of enzyme at different temperatures. The samples were removed at regular intervals and the residual activity was estimated under standard assay conditions.

Substrate specificity of the lichenase

The lichenase was used to hydrolyse different substrates (500µg) with varying glycosidic bonds of glucose and xylan. The substrates used were gum arabic, laminarin, lichenan, oat spelt xylan, avicel, PNPG and PNPX.

Amino acid composition of Lichenase

The amino acid analysis was carried out using amino acid analysis kit AccQ-Fluor supplied by Waters Corporation. Salt free lyophilized lichenase (50µg) was hydrolyzed using 6N constant boiling HCl, in a vacuum-sealed hydrolyzing tube for 24h at 110°C. The sample was then derivatized by 6-aminoquinolyl-N- hydroxyl succinimidyl carbamate (AQC) in borate buffer at pH9; and 10picomoles of the hydrolysate was loaded on AxxQ-Tag column equipped with a fluorescent detector. Total cysteine and tryptophan were determined according to Cavallini et al. (1966) and Spande and Witkop (1967) respectively.

Determination of Michaelis -Menten constant

The effect of substrate concentration on xylanase activity was determined by incubating the enzyme with different amounts of xylan (1-15mg) under the assay conditions. The value of K_m and V_{max} was determined from Lineweaver Burk plot.

Kinetic analysis

Kinetic method elaborated by Keleti et al., (1987) was employed to evaluate whether lichenase hydrolyzed both lichenan and xylan by one or two active sites. The initial velocities are calculated as $\mu mole.min^{-1}.ml^{-1}$ in the simultaneous presence of both substrates at concentrations obeying the relationship.

$$\textbf{(Lichenan) } K_m^{xylan} + \textbf{(xylan) } K_m^{lichenan} = K = 10 \text{ (mg/ml)}^2$$

were the value of K was chosen arbitrarily to achieve concentration ranges around K_m values. For computations K_m^{lichen} = 3mg/ml and K_m^{xylan} = 2mg/ml determined separately were used. The Michaelis constants and maximum velocities (V_{max}) of the lichenase and xylanase activities of the enzyme were estimated from Lineweaver-Burk plots.

Assuming single active center acting concomitantly with substrate S_1 and the alternative substrate S_2, the two substrates will compete with each other for the same active site the equation for initial velocity (V_0) is represented by

$$V_0 = (V_1K_2 (S_1) + V_2K_1 (S_2) / K_1K_2 + K_1 (S_2) + K_2 (S_1)) \qquad \text{-------(I)}$$

were V_1 and V_2 are maximum velocities, and K_1 and K_2, the dissociation constants for lichenan and xylan respectively.

Considering two different active sites for the substrates S_1 and S_2 independent of each other, the initial velocity can be represented by

$$V_o = \frac{V_1K_2 (S_1) + V_2K_1 (S_2) + (V_1 + V_2) (S_1) (S_2)}{K_1K_2 + K_1 (S_2) + K_2 (S_1) + (S_1)(S_2)} \qquad \text{---------(II)}$$

If there are two active sites and the two substrates mutually influence each other's binding

$$\mathbf{V_o = V_1 K_2 (S_1) + V_2K_1 (S_2) / K_1K_2 \quad + K_1 (S_2) + K_2 (S_1) + (S_1) (S_2)}$$
$$+$$
$$\mathbf{(V_1 + V_2) (S_1) (S_2) / K_1K_3 + K_4 (S_2) + K_3 (S_1) + (S_1) (S_2) \qquad ------(III)}$$

were K_3 and K_4 are the dissociation (or Michaelis) constants of S_2 and S_1 in the presence of S_1 and S_2, respectively.

Chemo- affinity labeling studies using OPTA

Lichenase (10μg), in 0.05M phosphate buffer pH7 was incubated with OPTA in methanol at 25°C. The formation of isoindole derivative was followed spectrophotometrically by monitoring the increase in fluorescence at 420nm with excitation wavelength fixed at 338nm (Simons and Johnson, 1978). The aliquots were removed at intervals to check the residual activities of lichenase and xylanase.

Modification of lichenase with TNBS

Lichenase (100μg) was incubated with varying concentrations of TNBS (10 to 40 mM) in the presence of 0.25ml 4% sodium bicarbonate at 37°C in a reaction volume of 0.5ml. Aliquots were withdrawn at suitable time intervals and the reaction was terminated by adjusting the pH to 4.5. The extent of inactivation in presence of 1% lichenan was also determined. Control tubes with only enzyme, inhibitor and inhibitor/ substrate were incubated under identical conditions (Habeeb, 1996).

Modification of lichenase with DTNB

The enzyme (300μg) in 50mM phosphate buffer, pH7.2, was incubated with 0.1mM of DTNB at 25°C for one hour. Aliquots were removed at different time intervals and the residual activity determined. The modification reaction was followed by monitoring the increase in absorbance at 412nm and the number of sulphydryl group modified were calculated using a molar absorption coefficient of $13{,}600 M^{-1}cm^{-1}$ (Cavallini et al., 1966; Ellman, 1959).

Kinetics of inactivation

The apparent first-order constant of inactivation depends on the concentration of the modifier and can be expressed as

$$\mathbf{K_{app} = K\,(M)_n}$$

were, K_{app} is the apparent first order rate constant, M is the concentration of the modifier and n is the number equal to the average order of reaction with respect to the concentration of modifier

$$\mathbf{\log K_{app} = \log K + n \log (M)}$$

K_{app} can be calculated as a slope from a semi-logarithmic plot of residual enzyme activity as a function of time. The second order rate constant for inactivation was determined from the slope of the plot of pseudo-first order rate constant against inhibitor concentration. The order of the reaction (n) can be experimentally estimated by determining the K_{app} at different concentrations of the modifier. A plot of log K_{app} against log (M) gives a straight line with a slope equal to n, where n is the number of the molecules of the modifier reacting with each active unit of the enzyme to produce an enzyme inhibitor complex (Levy et al., 1963).

CD measurements

CD spectra were recorded in a Jasco-J715 spectrophotometer at ambient temperature using a cell of 1mm path length. Replicate scans were obtained at 0.1nm resolution, 0.1nm bandwidth, and a scan speed of 50nm/min. Spectra were average of six scans with the baseline subtracted spanning from 260 to 190nm in 0.1nm increments. The CD spectra of the native, TNBS (10mM), DTNB (100mM) and OPTA (1mM) modified lichenase (400µg/ml) were recorded in 50mM sodium phosphate buffer, pH7 and analyzed by CDPRO (Sreerama and Woody, 2000).

Results

Purification of lichenase

Lichenase was purified from the extracellular culture filtrate by fractional ammonium sulphate precipitation (35 to 55%) followed by chromatography on DEAE Sephadex ion exchange column and Sephacryl S-200 gel filtration column (Figure 1, 2).

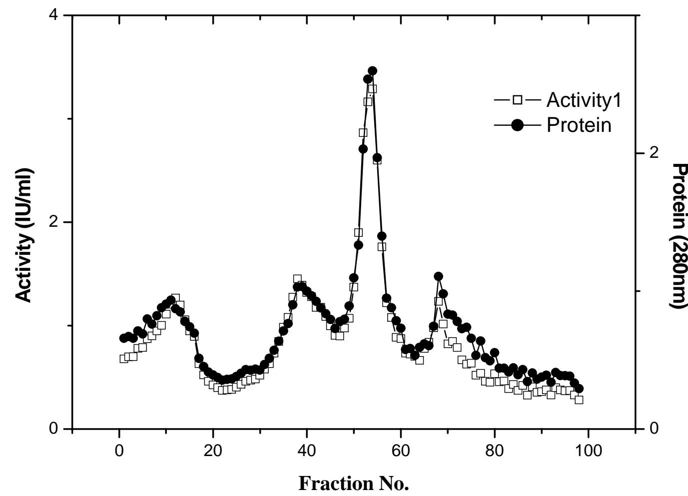


Figure 1: Ion exchange chromatography of crude culture filtrate: (□) lichenase and (•) Protein;

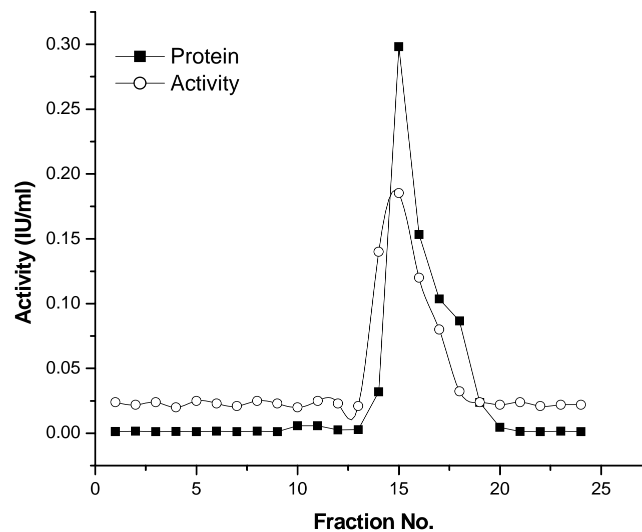


Figure 2: Sephadex G 100 gel filtration chromatograph of 2nd fractions (o)β- 1-3, 1-4 glucan 4-glucanohydrolase activity and (■) Protein.

Table 2: Purification Chart

	Volume (ml)	Lichenase activity (IU/ml)	Total Lichenase Activity (IU/ml)	Xylanase activity (IU/ml)	Total Xylanase Activity (IU/ml)	Total Protein (mg/ml)	Specific activity Lichenase (IU/mg)	Specific activity Xylanase (IU/mg)	Fold Purification Lichenase	Fold Purification Xylanase	Recovery Lichenase	Recovery Xylanase
Crude	**100**	**158**	**15875**	**120**	**12000**	**635**	**25**	**18.89**	**1**	**1**	**100**	**100**
$(NH_4)_2SO_4$ ppt.	**25**	**493**	**12323**	**372.4**	**9310**	**236**	**52.22**	**39.451**	**2**	**2.088**	**77.62**	**77.58**
Ion-exchange DEAE Sephadex–(II)	**40**	**125.39**	**5015.69**	**94.75**	**3790**	**18.47**	**271.559**	**205.19**	**10.84**	**10.86**	**31.59**	**31.58**
Gel Filtration Sephacryl S-200	**15**	**149.99**	**2249.848**	**113.3**	**1700**	**2.84**	**792.20**	**598.59**	**31.6**	**31.65**	**14.17**	**14.17**

The lichenase from *Thermomonospora* sp. was purified to homogeneity with thirty one fold purification and specific activity of 792 IU/mg (Table 2).

The lichenase and xylanase activities were almost coincident with each other and with the protein peak. SDS-PAGE revealed a single homogeneous 64,565 Da band (Figure 3, 4).

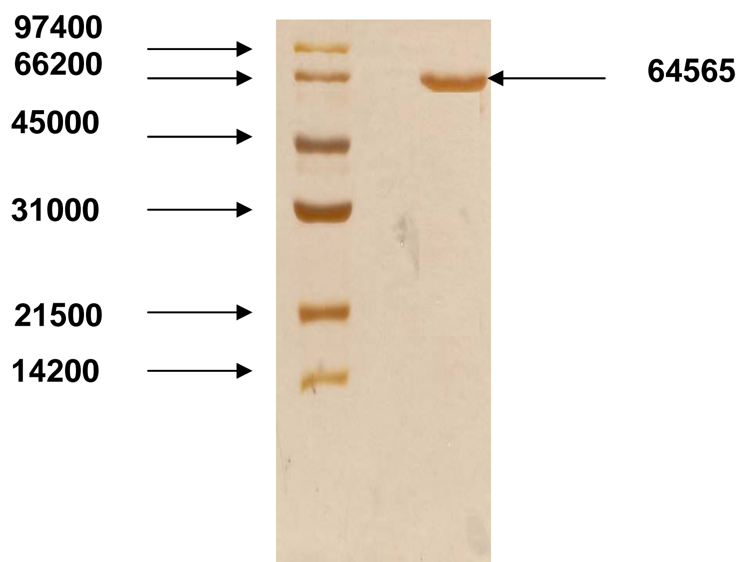


Figure 3: 12% SDS–PAGE visualized by silver staining: lanes 1, standard molecular marker proteins (Da) and lane 2, purified lichenase.

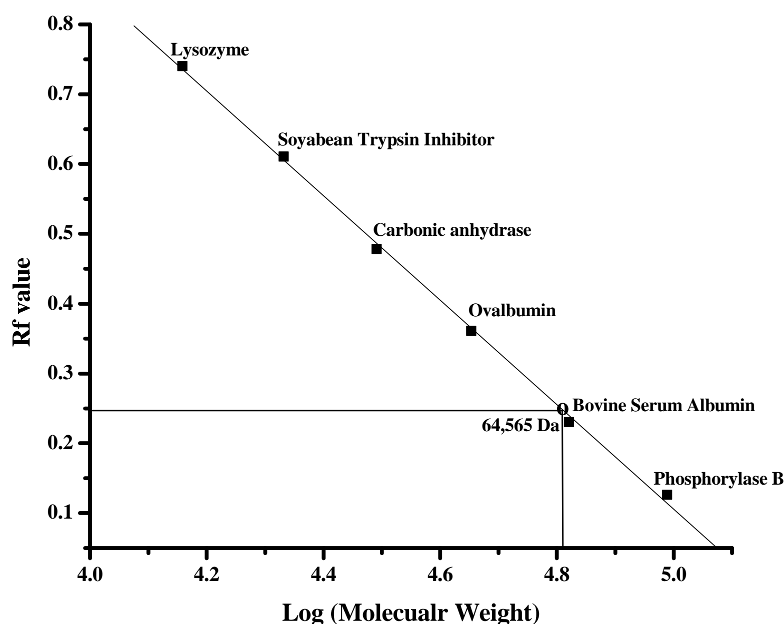


Figure 4: Mr determination of lichenase by 12% SDS PAGE: (1) Phosphorylase B (Mr, 97400), (2)Bovine serum albumin (Mr, 66200), (3) Ovalbumin (Mr, 45000), (4) Carbonic anhydrase, (Mr,31000) (5) Soyabean trypsin inhibitor (Mr,21500) and (6) Lysozyme (Mr,14200) were used as standard molecular weight markers (Da).

A sharp peak of an average molecular mass of 64,120 Da by gel filtration confirmed the homogeneity of the enzyme and its monomeric nature (Figure 5).

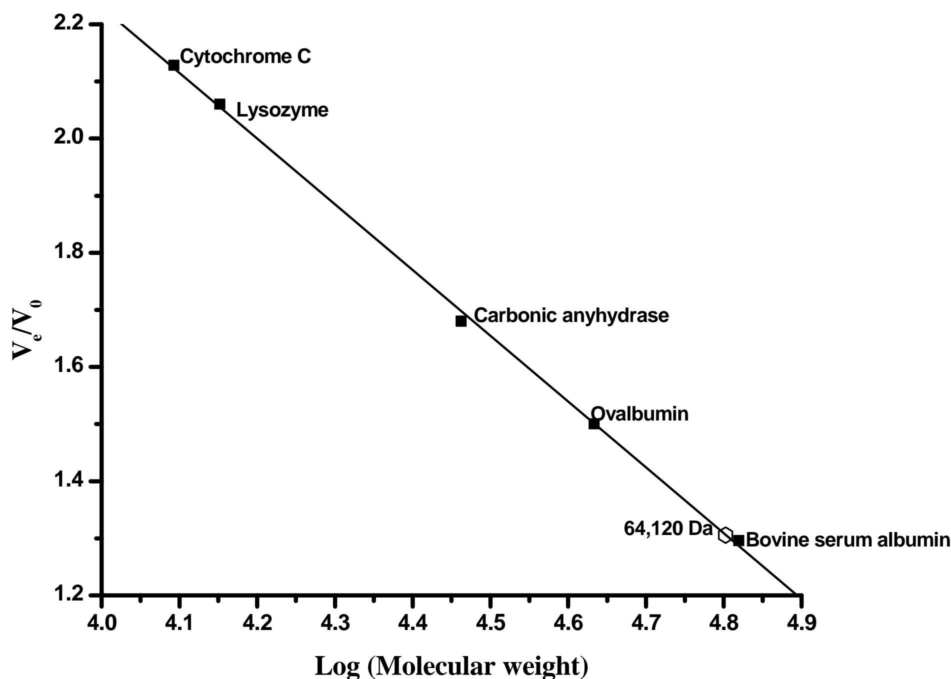


Figure 5: Mr determination of lichenase by gel filtration sephadex G 100: (1) bovine serum albumin (Mr 66,000), (2) ovalbumin (Mr 43,000), (3) carbonic anhydrase (Mr 29,000) and (4) cytochrome c (Mr 12, 384) were used as standard molecular weight markers (Da).

This is the first report of a high molecular weight lichenase from a *Thermomonospora* sp. with activity towards lichenan and xylan.

Biochemical characterization

The enzyme exhibited optimum activity at pH7 for both lichenase and xylanase activities even though it remained active in an expansive pH range of 5 to 8 (Figure 6) . It was stable in a broad range of pH 4 to 8 with more than 75% activity (Figure 6). The enzyme was highly thermostable with an optimum of 50°C (Figure 7). The enzyme retained complete activity of hydrolyzing xylan and lichenan at 50°C for more than 24hrs and half-life of 5min at 80°C (Figure 8). Amino acid analysis revealed the presence of 584 amino acids corresponding to a molecular weight of (64,240Da) (Table 3, Figure 9) which supported the molecular weight estimated by

SDS-PAGE and gel filtration chromatography. The isoelectric point of the enzyme was determined to be 4.27 by isoelectric focusing, indicating its acidic nature.

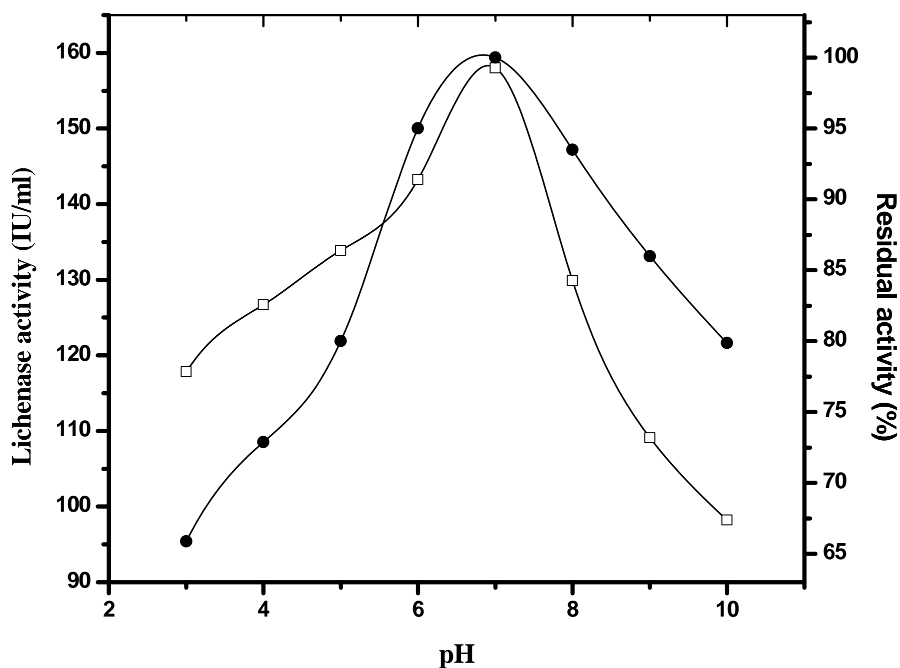


Figure 6: Effect of pH on lichenase activity: (□) optimum pH of lichenase; (•) pH stability of lichenase.

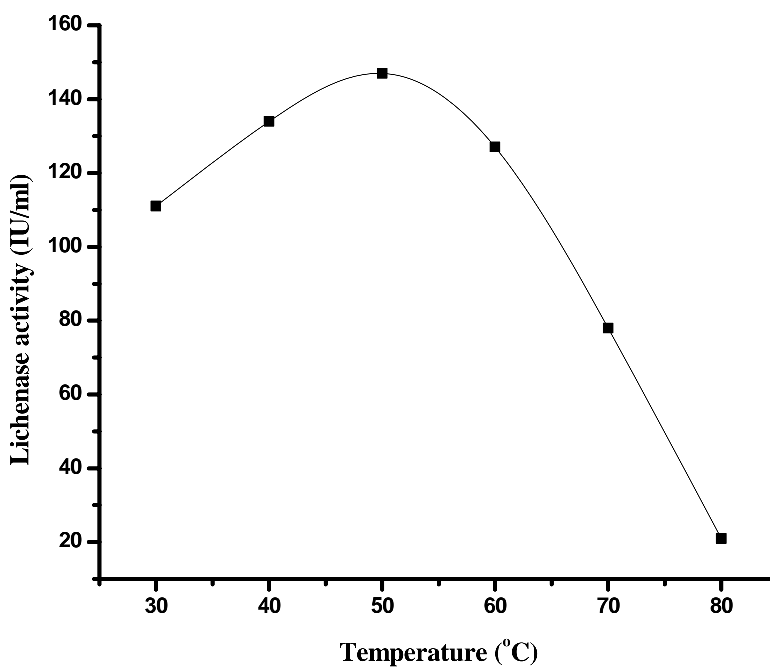


Figure 7: Temperature optimum of lichenase.

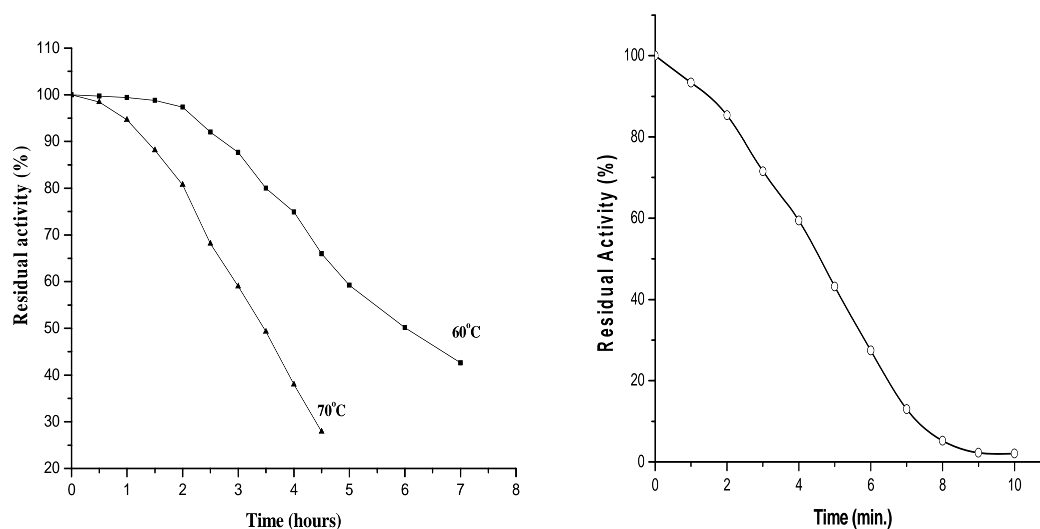


Figure 8: Temperature stability of lichenase: (■) 60°C; (▲) 70°C and (○) 80°C.

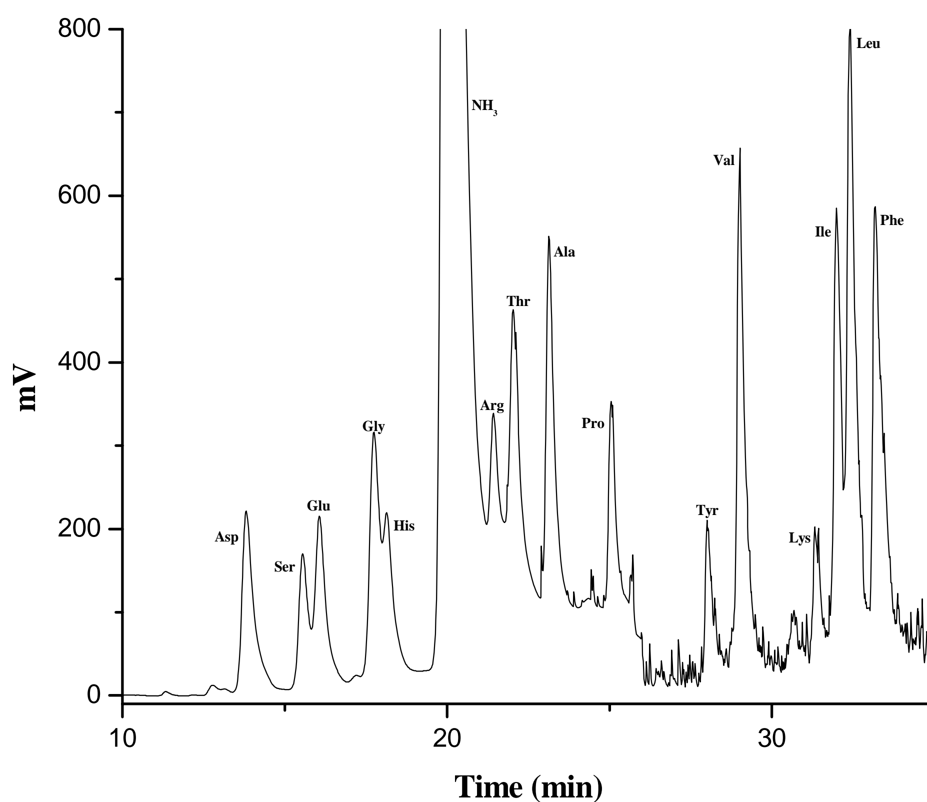


Figure 9: HPLC profile of amino acid analysis of purified lichenase.

Table 3: Amino acid composition

Amino acids	No. of residues
Alanine	47
Arginine	26
Aspartic acid	45
Cysteine*	1
Glutamic acid	49
Glycine	47
Histidine	9
Isoluecine	53
Leucine	37
Lysine	27
Methionine	0
Phenylalanine	31
Proline	43
Serine	49
Threonine	41
Tryptophan**	34
Tyrosine	7
Valine	38

*: Determined by DTNB; **: Determined by NBS

Substrate specificity

The enzyme was active towards lichenan and xylan. The enzyme had no activity towards avicel, laminarin, gum arabic, PNPG and PNPX (Table 3).

Kinetic analysis

The ratio of lichenase to xylanase activity remained constant during the entire purification process indicating that both the activities reside on the same protein. Since the following data indicated that the enzyme hydrolyzes lichenan and xylan; at the same active site like (i) the enzyme exhibited the same pH and temperature optimum for lichenan and xylan hydrolysis. (ii) both the activities were stable in and

expandable pH range 4 to 8 (iii) both the activities were stable at 50°C for 24h for both substrates (iv) both activities were inhibited by O- pthaldehyde, the question arises whether the two activities of the enzyme reside in the same or two different active sites. However the Km for the two activities is varied widely, viz. 2 mg/ml for Lichenan (Figure 10) and 3 mg/ml for Xylan.

Table 3: Activity of lichenase against different substrates

Substrate	Glycosidic Linkage	Monomers	Specific Activity (IU/mg)
Avicel	β- 1,4	Glucose	Nil
Xylan	β- 1,4	xylose	18
Laminarin	β- 1,3	Glucose	Nil
Lichenan	β- 1,3 – 1,4	Glucose	25
Gum arabic	β- 1,4	Galactose	Nil
p-nitrophenyl glycoside	β- 1,4	p-nitrophenol, glucose	Nil
p-nitrophenyl xylobioside	β- 1,4	p-nitrophenol, xylose	Nil

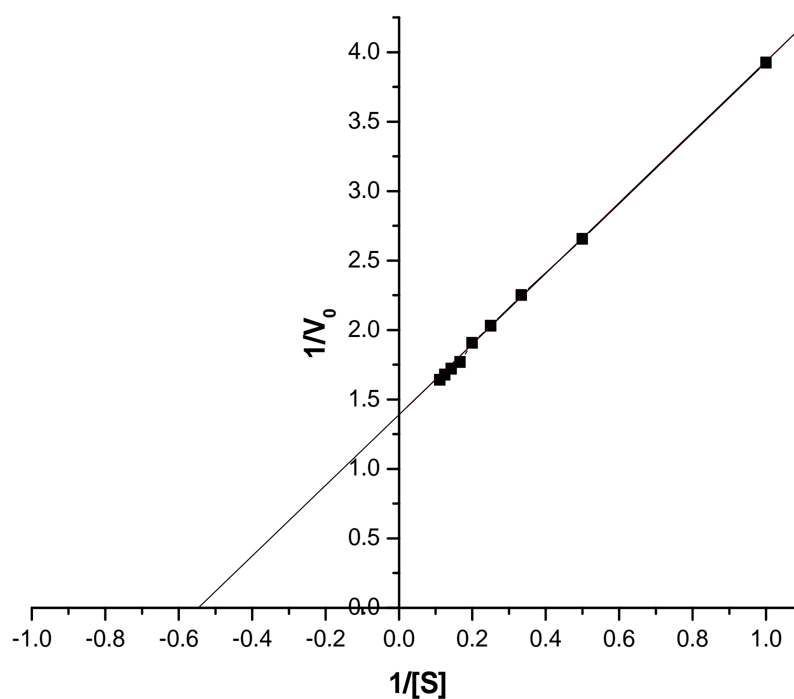


Figure 10: Lineweaver Burk Plot for lichenase.

The kinetic analysis by the method of Keleiti was employed to analyze if one or two sites were responsible for hydrolysis of lichenan and xylan.

If lichenan and xylan were hydrolyzed at two different active sites, the rate of hydrolysis of two substrates should be equal to the sum of their individual rates. Conversely if there is single site, these two substrates will show competitive inhibition with respect to each other and hence the rate of hydrolysis by the enzyme with the mixture of substrates would be lower than the sum of the rates with individual substrates. The Table 5 summarizes the calculated and the actual experimental values.

The observed values of overall rate of hydrolysis fitted well with the theoretical values calculated from Eqn.1 than with those calculated from Eqn.2 and Eqn.3 (Figure 11). The results of the kinetic analysis are consistent with assumption of a single active site.

Table 5: Overall reaction velocities of lichenase with mixtures of various concentration of xylan and Lichenan.

Individual rates				**Rate of mixtures**	
Lichenan		**Xylan**		**Observed**	**Calculated**
Concentration (mg/ml)	**A_{540}**	**Concentration (mg/ml)**	**A_{540}**	**A_{540}**	**A_{540}**
2	0.0868	8	0.1243	0.0921	0.2111
4	0.1247	6	0.1090	0.1055	0.2342
6	0.1510	4	0.0995	0.1128	0.2505
8	0.1758	2	0.0943	0.1188	0.2813
9	0.1945	1	0.0788	0.1233	0.3045

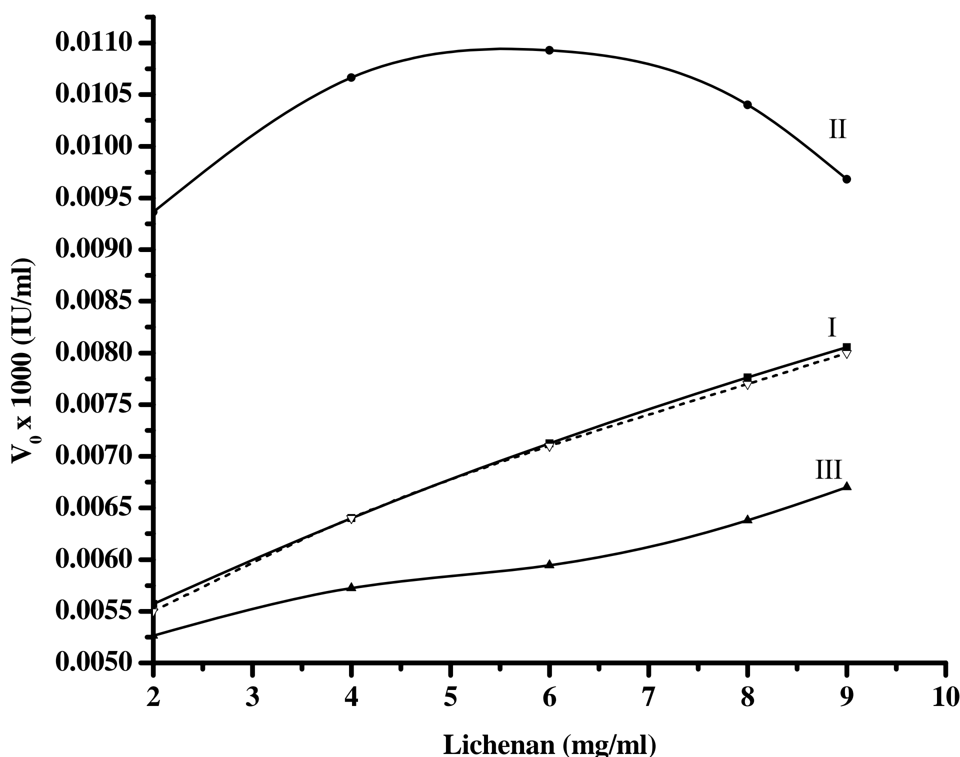


Figure 11: Plot of V_0 vs. (S) at different concentrations of lichenan and xylan. In each case, 0.5 U/µg of the enzyme was added to reaction mixture. The concentration of only one substrate is indicated on the abscissa. (▽) Experimental points, (■) single active site Eq. (I), (•) two independent active sites Eq. (II) and (▲) two substrates mutually influencing each others binding Eq. (III).

Chemo- affinity labeling studies

Conformation and microenvironment at the active site of lichenase was probed with fluorescent chemo affinity labeling using o-phthalaldehyde as the chemical initiator. O-phthalaldehyde, a bifunctional reagent forms isoindole derivative by cross-linking the proximal thiol and amino groups. OPTA reacted with lichenase yielding a stable fluorescent derivative, which exhibited an absorbance characteristic of isoindole derivative (420nm) (Figure 12) with the complete loss of activity towards both lichenan and xylan. Complete inactivation by OPTA might be due to the formation of isoindole derivative by cross-linking the proximal thiol and amino groups located at or near the active site of the enzyme. Modification of lysine by TNBS and of cysteine residue by DTNB of the enzyme abolished the ability of the

enzyme to form an isoindole derivative with OPTA (Figure 12) supporting the above assumption.

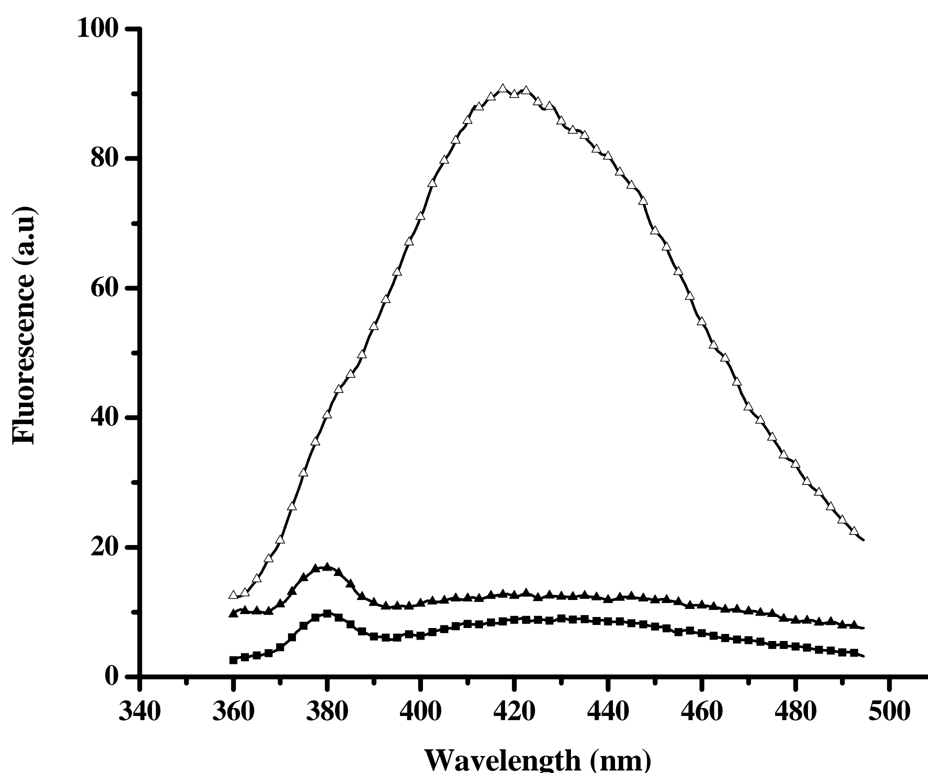


Figure 12: (A) Isoindole fluorescence of (400µg) lichenase monitored at k_{excit} 338 nm and k_{emiss} 415 nm: (△) Native lichenase, (▲) DTNB modified lichenase and (▼) TNBS modified lichenase, incubated with OPTA (1mM).

Chemical modification

A time and concentration dependent inactivation of enzyme towards substrates, lichenan and xylan were observed with different concentrations of TNBS (Figure 13A). The reaction followed pseudo first order kinetics. The pseudo first order rate constants (K) were linearly correlated to the concentrations of the reagent, suggesting that no reversible complex was formed during the inactivation process. A reaction order of 1.2 with respect to the modifier was determined from the slope of the double logarithmic plots (Figure 13B) indicating that 1mole of TNBS inactivated 1 mol of enzyme.

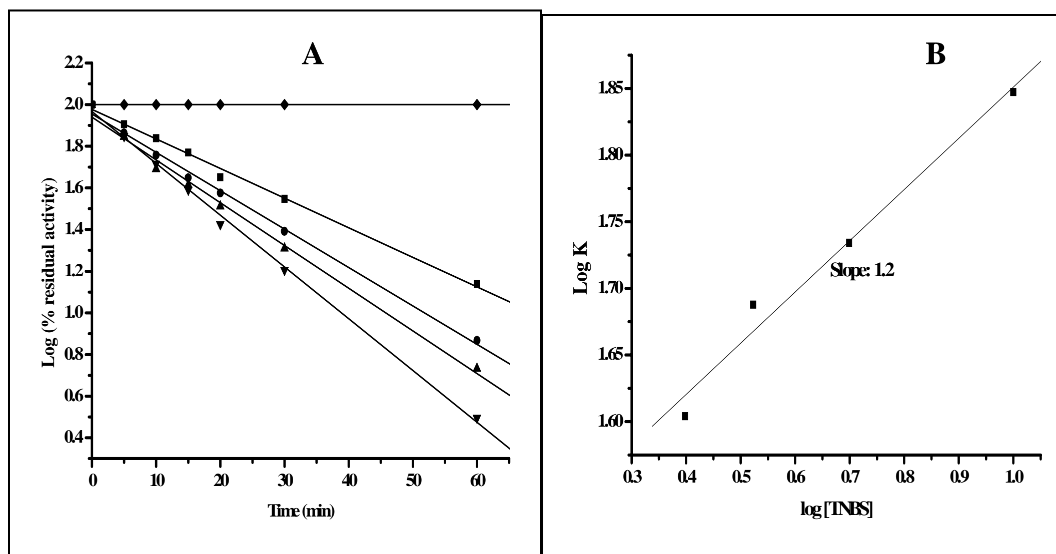


Figure 13: A) Kinetics of inactivation of lichenase by TNBS. Pseudo first order plots for the inactivation of lichense by TNBS. Enzyme was incubated with 0.01M (■), 0.02M (●), 0.03M (▲), 0.04M (▼), and control- 0 M (◆) at 37 °C. Aliquots were removed at indicated time intervals. (B) Double logarithmic plots of pseudo first order rate constants as a function of TNBS concentration.

Inhibition of lichenase DTNB on incubation, was time and inhibitor concentration dependant and followed first order kinetics (Figure 14A). Double logarithmic plots calculated a reaction order of 0.91 (Figure 14B) indicating that 1mole of TNBS was inactivated per mole of enzyme.

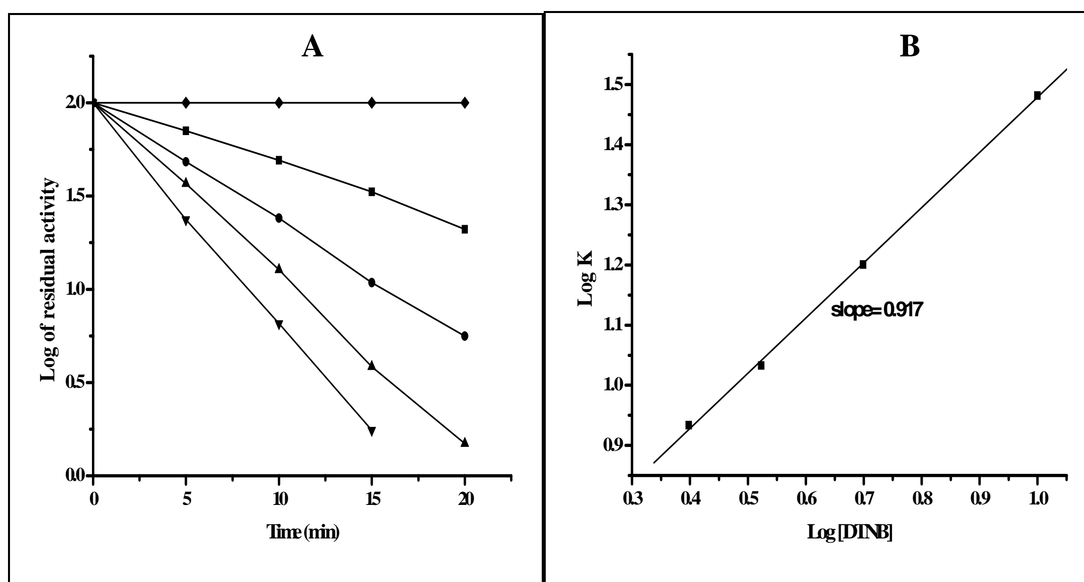


Figure 14: (A)Kinetics of inactivation of lichenase by DTNB. Pseudo first order plots for the inactivation of lichenase by DTNB. Enzyme was incubated with 0.1M (■), 0.2M (●), 0.3M (▲), 0.4M (▼), and control- 0 M (◆) at 37 °C. Aliquots were removed at indicated time intervals. (B) Double logarithmic plots of pseudo first order rate constants as a function of DTNB concentration.

These results were in accord with the formation of isoindole derivative by OPTA that crosslinked the proximal thiol group of cysteine and amino group of lysine present at the active site of the enzyme

Substrate protection

The complete protection of enzyme activity against inactivation by OPTA, TNBS and DTNB was observed in presence of higher concentration of lichenan (Table 6). This confirmed the participation of lysis and cysteine in the active site of the enzyme.

Table 6: Substrate protection of lichenase against inhibition by OPTA and TNBS

Reaction	Residual Activity (%)	Reaction	Residual Activity (%)	Reaction	Residual Activity (%)
None	100	**None**	100	**None**	100
OPTA (1mM)	0	**TNBS (10 mM)**	0	**DTNB (100 mM)**	0
Lichenan (1mg) + OPTA(1mM)	69.8	**Lichenan (1mg) + TNBS (10 mM)**	70.3	**Lichenan(1mg) + DTNB (100 mM)**	79.4
Lichenan (3mg) + OPTA(1mM)	89.5	**Lichenan (3mg) + TNBS (10 mM)**	85.2	**Lichenan (3mg) + DTNB (100 mM)**	92.1
Lichenan (5mg) + OPTA(1mM)	100	**Lichenan (5mg) + TNBS (10 mM)**	100	**Lichenan (5mg) + DTNB (100 mM)**	99.8

CD analysis

Analysis of CD spectra indicated that lichenase was maximally composed of β-sheets (71.9%). Remaining 25.2% was unordered structure and 2.9% consisted of α-

helix. CD measurements revealed no effect of the modifier on the α-helix and β-sheet content of lichenase (Figure 15).

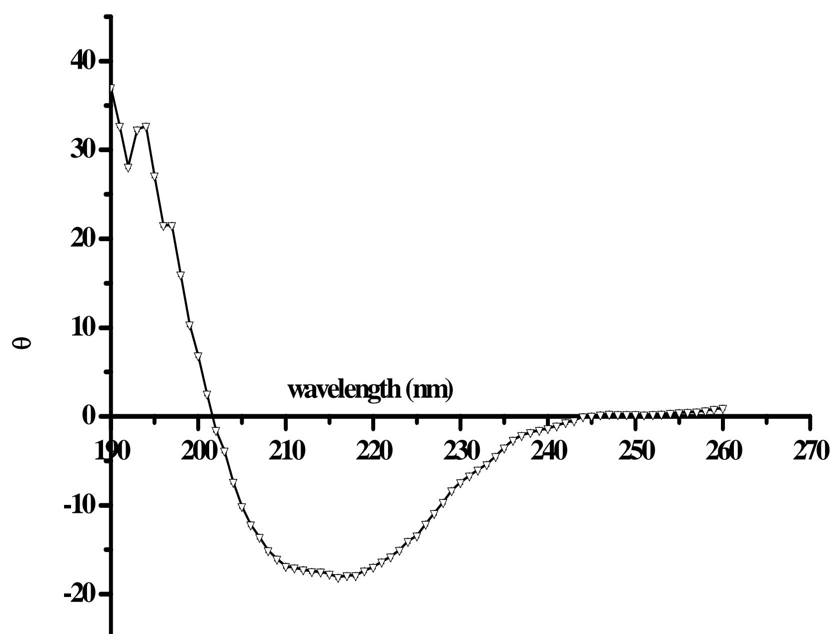


Figure 15: CD spectra of native, TNBS, OPTA and DTNB-modified lichenase. Far-UV CD spectra were recorded for native (▼), TNBS-modified (▲), DTNB-modified, (•), OPTA-modified and (■) native lichenase from 260 to 195 nm at 30°C. Each spectrum represents the average of six scans.

Discussion

Every potential action taken by a cell is guided by desire to economize. Thus the concept of one enzyme- one activity is rapidly vanishing as more and more multifunctional proteins are being identified (Kirschner and Bisswanger, 1976). The bifunctional lichenase in the present investigation was purified to homogeneity exhibited a molecular weight of 64,500Da. Kinetic analysis and chemoaffinity labeling of the protein confirmed the participation of a single active site for the hydrolysis of xylan and lichenan. OPTA, a bifunctional chemical initiator employed, formed a fluorescent isoindole derivative by crosslinking the thiol and amino groups near or at the vicinity of active site inhibiting enzyme activity towards lichenan and xylan. DTNB and TNBS modification inhibited the lichenan and xylan hydrolyzing activity indicating the involvement of cysteine and lysine in the active site of the enzyme. This was confirmed by substrate protection studies. These results were in corroboration with the chemoaffinity labeling studies indicating the participation of –SH group of cysteine and $-NH_2$ group lysine in isoindole formation. These result have both evolutionary and ecological significance. The identical pH and temperature optima, stability, kinetic analysis and inhibition by OPTA, DTNB and TNBS support the hypothsis of single active site for the hydrolysis of lichenan and xylan.

SECTION B

THERMAL INACTIVATION OF β-1, 3- 1, 4 D-GLUCAN 4 GLUCANOHYDROLASE FROM AN ALKALOTHERMOPHILIC *THERMOMONOSPORA* SP.

Abstract

An insight in to the mechanism of thermal inactivation of lichenase (β-1, 3- 1, 4 D-glucan 4 glucanohydrolase) from *Thermomonospora* sp. was elucidated using chemoaffinity labeling studies, intrinsic fluorescence and CD analysis. Conformational integrity of the active site of an enzyme is essential for its catalysis and activity. OPTA a bifunctional, fluorescent chemoaffinity label, is known to have absolute specificity for amino and thiol groups for the formation of an isoindole derivative and give a characteristic fluorescence at 420nm on excitation at 338nm. OPTA which binds at / near the active site of lichenase exhibited the characteristic fluorescence of the isoindole derivative with lichenase on exposure to temperatures varying from 30 to 70°C. But OPTA was unable to form isoindole derivative with lichenase at 80°C. In presence of lichenan OPTA was able to form isoindole derivative at 80°C. Acrylamide, KI and $CsCl_2$ quenching studies of the enzyme revealed 75% of the total tryptophan of lichenase to be responsible for the intrinsic fluorescence of the the enzyme. Intrinsic fluorescence and CD analysis of lichenase at varying temperatures (30 – 80ºC) indicated the conservation of the tertiary and secondary structure of the enzyme. Thus it was concluded that the thermal inactivation of the lichenase at 80°C occurred due to distortion in active site and not due to any major structural changes as confirmed by CD and fluorescence analysis.

Introduction

Lichenases (β-1, 3-1, 4-D-glucan 4-glucanohydrolase) have raised enormous interest in the past decades in view of their specific action and biotechnological importance in brewing and animal feedstuff industries. Lichenases specifically catalyses the hydrolysis of β-1,4- linkage adjacent to a β-1,3 glycosidic moiety on its non-reducing end to yield 3-O-β-D-cellobiosyl-D-glucose and 3-O-β-D-cellotriosyl-D-glucose (Malet et al.,1993; Tang et al., 2004). Though studies have carried out on purification and general characterization of lichenases, there are comparatively fewer reports on the molecular enzymology of these enzymes.

Structure function relationships are on to the central issues in the investigation of biological macromolecules. Conformational integrity of the active site of an enzyme is essential for its catalysis, and investigations on the molecular orientation of the functional groups of active site as well as their microenvironment are the areas of growing scientific interest. Few attempts have been made to correlate the conformational changes for an enzyme to changes in its catalytic activity. (George and Rao, 2001). Interactions of proteins with perturbants like salts, temperature, pH, and solvent delineate the relative role of proteins. A number of noncovalent interactions such as hydrogen bonds, Van der Waals, ionic, and hydrophobic interactions contribute to the structure and stability of proteins (Gouda et al., 2003).

The present study investigates the structural relationship of inactivation of lichenase at 80°C with its functional activity using chemoaffinity labeling technique which is a powerful tool to assign ligand-macromolecule complex. Lichenase in the present investigation is found to have cysteine and lysine at or near the active site of the enzyme. OPTA a bifunctional fluorescent chemoaffinity label has been demonstrated to have affinity towards the active site of lichenase (previous section) from *Thermomonospora* sp. as it binds to the cysteine and lysine residues at or near the active site of the enzyme to emit a characteristic fluorescence at 420nm on exitation at 318nm. OPTA was thus applied to probe the active site of lichenase during its thermal inactivation.

Material and Methods

Microorganism

An alkalothermophilic actinomycete, *Thermomonospora* sp. optimized for lichenase production in Chapter II has been used.

Enzyme assays

Lichenase assays were carried out as mentioned in the previous section.

One unit of lichenase activity was defined as the amount of enzyme that produced 1µmole of glucose equivalent per min from lichenan under assay conditions respectively. Protein concentration was determined according to the method of Bradford, using Bovine Serum Albumin as standard (Bradford et al., 1976).

Production and purification of lichenase

The lichenase was purified to homogeneity as mentioned in the previous section.

Fluorescence measurements

All fluorescence studies were performed on a Aminco SPF-500 spectrofluorometer at varying temperatures (30–80ºC). The emission spectra were obtained with the spectrofluorometer in the ratio mode and an emission and excitation band pass of 4nm each. The fluorescence signal was corrected for dilution background and inner filter effect (Hill et al., 1986). The residual activity of lichenase was determined after exposure for 10min at various temperatures.

Acrylamide, KI and $CsCl_2$ quenching studies

Titration of the enzyme in the presence was accomplished by sequential additions of small aliquots of the quencher acrylamide from 5M stock solution. Fluorescence intensities were determined by excitation of the sample at 295nm and continuous monitoring of the emission at 326nm. The spectra were corrected for inner filter effects due to the quencher. Quenching data was analyzed according to Stern-Volmer (1919) and modified Stern-Volmer equation (Lehrer, 1971).

Chemo- affinity labeling studies using OPTA

Lichenase (50µg), in 0.05M phosphate buffer pH7 was incubated with o-phthalaldehyde in methanol at varying temperatures (30 – 80ºC). The formation of enzyme – isoindole derivative was followed spectrophotometrically by monitoring the increase in fluorescence at 420nm with excitation wavelength fixed at 338nm.

CD measurements

CD spectra were recorded in a Jasco-J715 spectropolarimeter at varying temperature (30 – 80ºC) using a cell of 1mm path length. Replicate scans were obtained at 0.1nm resolution, 0.1nm bandwidth and a scan speed of 50nm/min. Spectra were average of 6 scans with the baseline subtracted spanning from 260 to 190nm in 0.1nm increments. The CD spectrum of the lichenase (25mg/ml) was recorded in 50mM sodium phosphate buffer (pH 7).

Results

Acrylamide, KI and $CsCl_2$ fluorescence quenching studies

Quenching titrations with either acrylamide or $CsCl_2$ or KI were performed at 30ºC by sequentially adding aliquots of the concentrated quencher stock solutions (5M) to the enzyme solution to study the conformation of the enzyme. Sodium thiosulfate (0.1mM) was added to the KI stock solution to prevent I^{3-} formation. The excitation wavelength was set at 295nm and the fluorescence emission spectra were scanned from 300-400nm.

The fluorescence quenching data in the presence of acrylamide, $CsCl_2$ or KI were analyzed by the Stern-Volmer equation (Eftink and Ghiron., 1976).

$$\mathbf{F_0/F = 1 + K_{sv} X (Q)}$$

In which F_0 and F are the fluorescence intensities in the absence or the presence of the quencher, respectively. K_{sv} is the Stern-Volmer quenching constant and (Q) is the quencher concentration (Stern Volmer, 1919).

For proteins containing more than one fluorescing tryptophan residues differing in their accessibility to the quencher, the Stern-Volmer plot will be non linear and hence a modified Stern-Volmer equation was applied.

$$\mathbf{F_0/\triangle F = 1/fa.\ K_{sv} [Q]}$$

$$\mathbf{\triangle F = F_0 - F}$$

fa is fraction of the quenchable fluorescence , obtained from the ordinate intercept of the linear portion of the $F_0/\triangle F$ vs 1/Q plot (Lehrer, 1971).

The intrinsic fluorescence measurement in presence of acrylamide, KI and $CsCl_2$ were measured and the quenching was calculated by Stern Volmer and modified Stern Volmer's plots. The percent Q value for KI was calculated to be 52 which were higher as compared to that of $CsCl_2$ (19.92). Quenching by acrylamide was estimated to be 103% (Table 1).

Fluorescence studies indicated 103% of the total tryptophan of the protein responsible for the intrinsic fluorescence. The KI and $CsCl_2$ quenching studies

indicate the Tryptophan to be present in a more electropositive environment on analysis by Stern Volmer plot and modified Stern Volmer's plot.

Table 1: Tryptophan environment as detected by quenchers.

	K_{SV}	%Q	Fa	KQ
KI (5M)	2.7	52	0.522	21.88
$CsCl_2$ (5M)	1.41	19.92	0.1992	15.9
Acrylamide (5M)	24.87	103	1.03	14.9

Fluorescence measurements

Intrinsic fluorescence measurement at different temperatures showed quenching of fluorescence. A linearity in quenching of fluorescence was observed with increasing temperature (30 – 80°C) (Figure 1 inset) without a red or blue shift (Figure. 1). The residual activity of the enzyme decreased with an increase in temperature (Table 2). The oxidation of indole group of tryptophan has been implicated during thermal inactivation of proteins (Gupta, 1991).

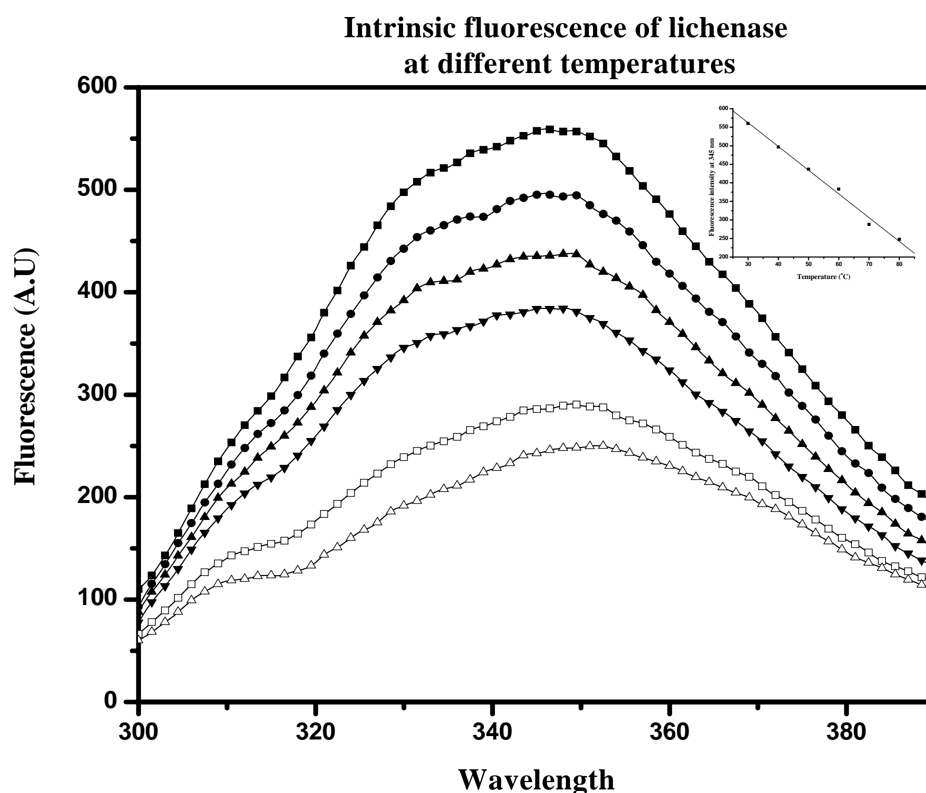


Figure 1: Instrinsic fluorescence of lichenase at different temperatures: (■) 30°C (●) 40°C, (▲) 50°C, (▼) 60°C, (□) 70°C and (▽) 80°C. Inset: Quenching in fluorescence with respect to temperature.

Chemo- affinity labeling studies using OPTA

Chemoaffinity labeling is a powerful technique to assign the binding sites of ligand-macromolecule complexes, which exhibits some of the advantages of both the photoactivated and electrophilic affinity labeling. OPTA is a bifunctional, fluorescent chemoaffinity label, which is known to have absolute specificity for amino and thiol groups for the formation of an isoindole derivative. However, application of OPTA as a probe to ascertain the conformational flexibility and polarity of the active site of lichenase by the formation of a fluorescent isoindole derivative with the lysine and

Table 2: Effect of temperature on lichenase activity

Temperature (°C)	Residual activity (%)
30	75.5
40	91.15
50	100
60	86.39
70	53.06
80	14.28
85	0

cysteine residue have been reported in our laboratory. OPTA contains two aldehyde groups; one of which reacts with the primary amine of lysine, whereas the second reacts with the sulphydryl group of cysteine, resulting in the formation of the isoindole derivative located at or near the active site of the enzyme. From the consideration of bond angles and bond distances of the two aldehydic functions in o-opthaldehyde the SH and NH_3 groups participating in isoindole formation in the reaction should be about 3Å apart (Puri et al., 1985 a; b). OPTA was able to form the fluorescent isoindole complex with lichenase at various temperatures (30 – 70°C). OPTA failed to form an isoindole derivative at 80°C indicating a distortion in the position of cysteine and lysine residues present at the active site of the enzyme

(Figure 2) but in presence of lichenan OPTA was able to form isoindole derivative exhibited by fluorescence at 420nm.

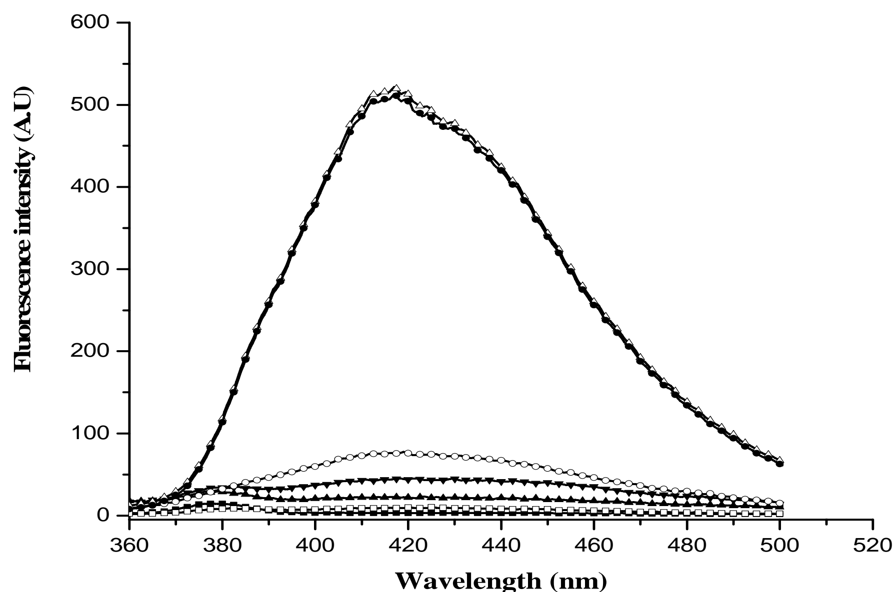


Figure 2: A) OPTA fluorescence of lichenase at 420nm when exited at 318nm: (■) Buffer, (□) OPTA in buffer, (▲) native enzyme, (△) native enzyme incubated with OPTA for 5 min, (▼) enzyme exposed to 80°C incubated with OPTA (○)enzyme exposed to 80°C incubated with OPTA for 15min and (•) enzyme exposed to 80°C incubated with OPTA for 15min in presence of lichenan. Each spectrum represents the average of six scans.

CD measurements

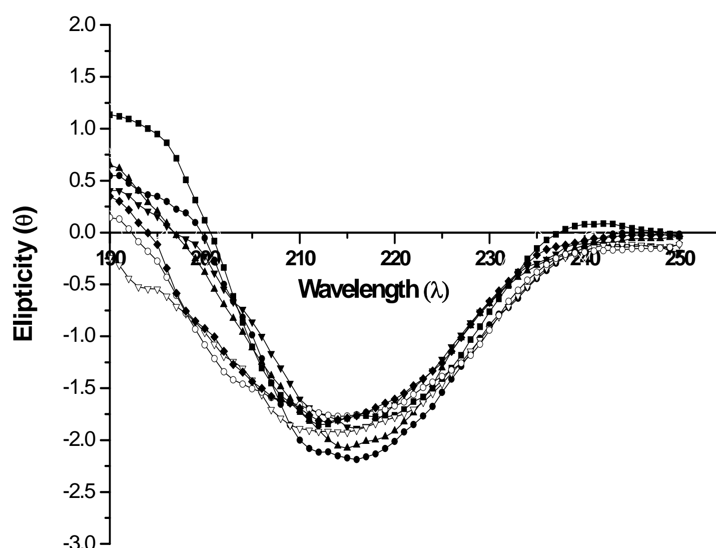


Figure 3: CD spectra of lichenase at various temperatures. Far-UV CD spectra were recorded for (■) 30°C (▲) 40°C, (•) 50°C, (▼) 60°C, (◆) 70°C, (o) 80°C and (▽) 85°C from 260 to 195 nm at 30°C. Each spectrum represents the average of six scans.

The CD spectra obtained at different temperatures for lichenase exhibited no major shift or deviation in the spectra at different temperatures (Figure 3). This indicated the conservation of the secondary structure of lichenase.

Discussion

The conformational integrity of an enzyme is essential for its activity. However, few attempts have been made to correlate the changes in the conformation of an enzyme to its catalytic properties. The unfolding of protein molecules during denaturation is usually accompanied by loss in biological activity before significant conformational changes can be detected. Studies have shown that in case of creatine kinase and glyceraldehydes 3 phosphate dehydrogenase the change in conformation at the active site parallels the inactivation of the enzyme by denaturant suggesting that the active site is located in relatively fragile part of the enzyme (Xie and Tsou, 1987, Zhou et al., 1993). Hence a slight disturbance in the spatial arrangement of the active site destroys the enzyme activity before any gross conformational change can be detected. However in this case thermal inactivation is proportional to the conformational changes in the active site of the protein and precedes conformational changes of the protein. A relatively high temperature was required to perturb the active site of the enzyme. Our present studies reveal that the active site of lichenase is located relatively in a less fragile environment. The inactivation may be due to a slight distortion in the active site of the enzyme rather than a major tertiary and secondary structural change by temperature.

CHAPTER IV

INTRODUCTION CELLULASE

The rapid depletion of fossil fuels coupled with the increasing demands for transportation fuels has necessitated the research focus on alternative renewable energy source. Despite its recalcitrant nature, in the guise of dead or dying plant matter, the polysaccharides of the plant cell wall provide an exceptional source of carbon and energy and a multitude of different microorganisms have evolved which are capable of degrading plant cell wall polysaccharides. Lignocellulose is the most abundant renewable resource abundantly available for conversion to fuels. On a worldwide basis, terrestrial plants produce 1.3 x 10^{10} metric tons of wood/year which is equivalent to 7 x 10^{9} metric tons of coal or about two third of the world's energy requirement. (Demain et al., 2005) Annually agriculture and other sources provide about 180 million tons of cellulosic feedstock.

Furthermore, tremendous amounts of cellulose are available as municipal and industrial wastes causing pollution problems. Various types of plant cell tissues exhibit different ratios of the three major types of cell wall component on the average, the cell wall contains roughly 40% cellulose, 30% hemicellulose and 20% lignin but the exact composition of an individual type of plant varies greatly.

Structure of cellulose

Cellulose is the main constituent of plant cell wall and is closely associated with hemicelluloses and lignin. Cellulose consists of D-glucopyranose monomer units bound by β 1,3 and 1-4-glycosidic linkages. The successive glucose residues are rotated by 180º relative to each other, and thus the repeating unit of the cellulose chain is the cellobiose unit (Figure 1). The average degree of polymerization (DP) of plant cellulose varies between 7000 and 15000 glucose units, depending on the source (Fengel and Wegener, 1983). The functional groups in the cellulose chain are the hydroxyl groups. These OH groups are able to interact with each other or with 0-, N-, and S-groups, forming hydrogen bonds. H-bonds also exist between OH-groups of cellulose and water molecules. These hydroxyl groups make the surface of cellulose largely hydrophilic. The cellulose chain has OH- groups at both ends. The Cl^- end has reducing properties. The cellulose chain is stabilized by strong hydrogen bonds along the direction of the chain. In

native cellulose found in plant sources, cellulose chains are packed together to form highly crystalline microfibrils in which the individual cellulose chains are held together by hydrogen bonds. An individual cellulose crystal contains tens of glucan chains in a parallel orientation. (O'Sullivan, 1997). In addition to highly crystalline regions, native cellulose contains less ordered amorphous regions. In wood fibers, the winding direction of cellulose microfibrils varies in different cell wall layers.

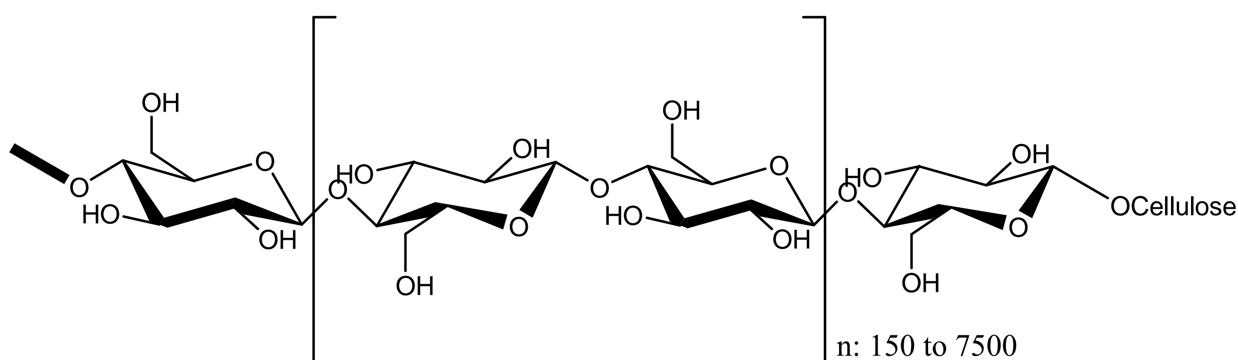


Figure1: Unit of cellulose showing the linkage existing between two glucose moiety.

Cellulase

Cellulases are enzymes responsible for the hydrolysis of cellulose and are composed of a complex mixture of enzyme proteins with different specificities to hydrolyse glycosidic bonds. The complete cellulase complex consists of (1) endoglucanase (EG, endo-1,4-β-glucanohydrolase, EC 3.2.1.4.) which attacks regions of low crystallinity in the cellulose fiber, creating free chain-ends; (2) exoglucanase or cellobiohydrolase (CBH, 1,4-β-D-glucan cellobiohydrolase, EC 3.2.1.91.) which degrades the molecule further by removing cellobiose units from the free chain-ends; (3) β-glucosidase (EC 3.2.1.21) which hydrolyzes cellobiose to produce glucose (Coughlan and Ljungdahl, 1988). For the complete degradation of cellulose to reducing sugars a synergistic action of the three enzymes is required.

Enzymes were usually named and grouped according to the reactions they catalyzed. Despite the sequence similarity of both enzymes and their classification as family-6 glycosyl hydrolases, their respective active-site architecture is different.

The endoglucanase E2 from the bacterium, *Thermomonospora fusca* (PDB code 1TML) is characterized by a deep cleft to accommodate the cellulose chain at any point along its length whereas the active site of the exoglucanase (cellobiohydrolase CBHI) from the cellulolytic fungus, *Trichoderma reesei* (PDB code 1CEL) bears an extended loop that forms a tunnel through which one of the termini of a cellulose chain can be threaded.

The ribbon diagrams, showing the secondary structures (α-helices and β-strands) of the two enzymes drawn using RasMol 2.6. (Figure 2).

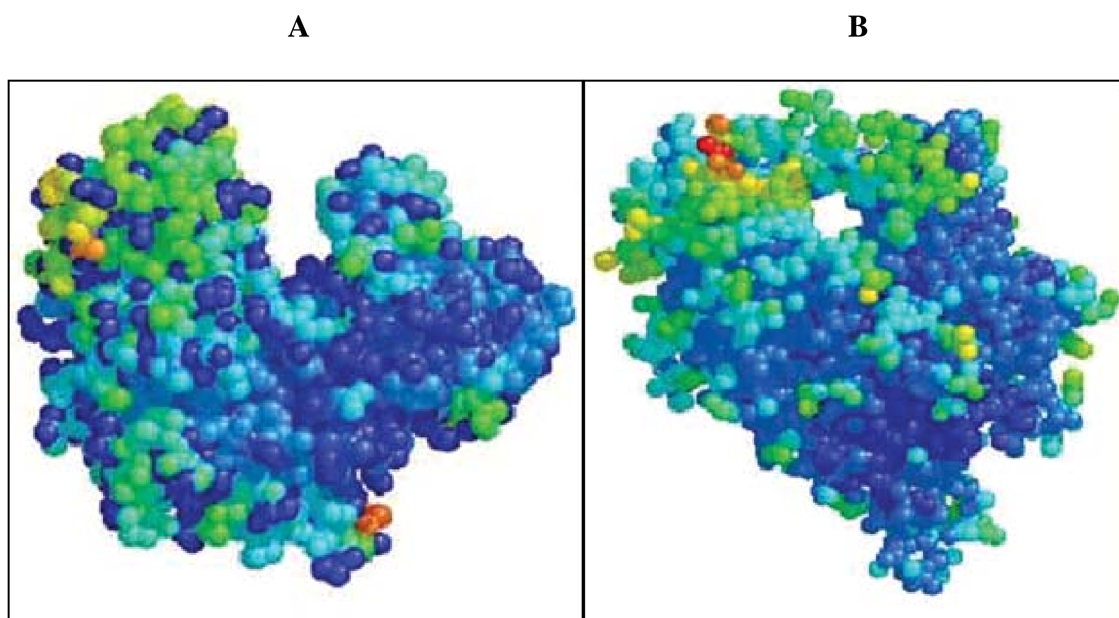


Figure 2: 3-D structure of exoglucanase from *Trichoderma reesei* (A) and endoglucanase from *Thermomonospora fusca* (B).

The cellulolytic microorganisms are ubiquitous in nature which include protozoa, fungi and bacteria. Bacteria and fungi both can produce cellulases which can hydrolyse the lignocellulosic materials. These microorganisms can be aerobic or anaerobic,

mesophilic or thermophilic. Bacteria belonging to *Clostridium*, *Cellulomonas*, *Bacillus*, *Thermomonospora*, *Ruminococcus*, *Bacteriodes*, *Erwinia*, *Acetovibrio*, *Microbispora*, and *Streptomyces* can produce cellulases (Bisaria, 1991). *Cellulomonas fimi* and *Thermomonospora fusca* have been extensively studied for cellulase production. Although many cellulolytic bacteria, particularly the cellulolytic anaerobes such as *Clostridium thermocellum* and *Bacteroides cellulosolvens* produce cellulases with high specific activity, they do not produce high enzyme titres (Duff and Murray, 1996). Because the anaerobic bacteria have a very low growth rate and require anaerobic growth conditions, most research for commercial cellulase production has been focused on fungi (Duff and Murray, 1996). Fungi secreting cellulases include *Sclerotium rolfsii*, *P. chrysosporium* and species of *Aspergillus*, *Schizophyllum* and *Penicillium*. However, *Trichoderma* spp. has been most extensively studied for cellulase production (Sternberg, 1976). Cellulolytic bacteria also have been found in the gut of wood-eating worms, termites and vertebrate herbivores, all of which exploit anaerobic symbionts for the digestion of wood and fodder.

Mechanism of cellulase action

There are two major catalytic mechanisms of glycosidic bond hydrolysis. The retaining mechanism involves initial protonation of the glycosidic oxygen via the acid/base catalyst with concomitant formation of a glycosyl-enzyme intermediate through the nucleophile. Hydrolysis of the intermediate is then accomplished via attack by a water molecule resulting in a product that exhibits the same stereochemistry as that of the substrate. The inverting mechanism involves the single step protonation of the glycosidic oxygen via the acid/base catalyst and concomitant attack of a water molecule activated by the nucleophile. The resultant product exhibits a stereochemistry opposite to that of the substrate. The type of mechanism is conserved within a given glycosyl hydrolase family and dictated by the active site architecture and atomic distance between the acid/base and nucleophilic residues (aspartic acid and/or glutamic acid) (Figure 3). During the degradation of cellulose, a retaining enzyme would produce a product in the β-configuration whereas an inverting enzyme would yield the α-sugar.

One of the major recent conceptual advances in assessing the mode of enzymatic action of a cellulase is the concept of "processivity." Processive enzyme action can be defined as the sequential cleavage of a cellulose chain by an enzyme. In effect, exo-glucanases are by nature and structure processive enzymes. Their tunnel-like active site thus allows processive action on the cellulose chain. Endo-glucanases, however, were thought to be intrinsically nonprocessive. However, the traditional distinction between exo- and endo-cellulases was modified recently.

Experiments combining two or more purified cellulases have shown that synergism can even be detected upon mixing two different types of exo-acting enzymes. Such experiments led to the recognition that the exo-enzymes can operate on both ends (i.e., the reducing and non-reducing ends) of the cellulose chain. Some enzymes, however, exhibit both endo and exo activities, although in such cases, the endo-cellulase activity is usually very low. In attempts to explain these phenomena, the concept of processivity was proposed by which the activity of the enzyme is characterized by the sequential hydrolysis of the cellulose chain. Implicit in this concept is the notion that the catalytic site of the enzyme remains in continual and intimate contact with a given chain of the cellulose substrate.

A more complete mechanistic picture of the processive nature of such cellulases was revealed with the advent of high resolution 3-D structures. It was thus demonstrated that the cellulose chain makes contact with the protein at multiple sites, either via a tunnel-shaped structural element (such as that observed in the family-48 enzymes) or by a special type of CBM (such as the family-9 theme B cellulases). These arrangements allow the threading of the cellulose chain into the active site and following initial cleavage at the end of the chain, the enzyme can move along the chain and position itself for the next cleavage. In addition to this processive nature of the active site, these enzymes also can make classic endo cleavages thus generating new ends.

Biochemically processive enzymes exhibit characteristics between endo- and exo-enzymes. They have low but detectable endo activity towards soluble derivatives of CMC and may or may not possess exo activity on such substrates. With insoluble substrates,

they will generate reducing power with a ratio between the soluble to the insoluble fractions of about 7. Endocellulases usually give a ratio of less than 2, whereas exocellulases produce a ratio of 12 to 23.

Once the processive nature of an enzyme has been indicated experimentally, molecular insight into the mechanisms responsible for this feature can be gained by determining the 3-D crystal structure of the active site together with model cello-dextrins. In the case of the cellulases, the crystal structure of the catalytic domain together with the fused module, combined with accumulating enzymatic activity data, allowed further postulation as to the accessory role of the fused module. The fused CBM presumably interacts with a single cellulose chain and feeds it into the active site. Interestingly, this domain does not bind crystalline cellulose but is inferred to act in dynamic binding of the single cellulose chain prior to its hydrolysis, thereby imparting the quality of processivity to the enzyme. Once such a property is associated with a given type of enzyme, the primary structure of the protein can now be used as an indication for all such enzymes. In the case of the family-9 theme B enzymes, it is now possible to identify the catalytic domain (e.g. glycosyl hydrolase family 9) and the additional accessory domains (in this case, family-3c CBM). Thus, the primary structure may by itself give a strong indication of the nature of the enzyme itself. Of course, the ultimate identification as to the mechanism of enzyme activity will come from the detailed 3-D structure of the enzyme-substrate complex.

Genetic engineering of cellulases

Cellulases are biotechnologically important and are produced by various microorganisms at low levels and in multiple forms with various properties. This drove the isolation, cloning and the expression of cellulase genes. The cellulase genes from fungi, bacteria and actinomyces have been cloned, expressed and the recombinant enzymes purified and characterized [Begnin et al., 1988; Beguin and Anbert 1993]. Expression of cellulases from different microorganisms has been published [Azevedo et al., 1990; Beguin and Anbert 1993; Beguin et al., 1983; 1985; Cheng et al., 1990; Durand et al., 1988; Entian et al., 1985; Flint et al., 1989; Grepinet and Beguin 1986; Lewin, 1987, Stahlbrand et al., 1995, Wang et al., 1993; Wong et al., 1986; Xue et al., 1992;

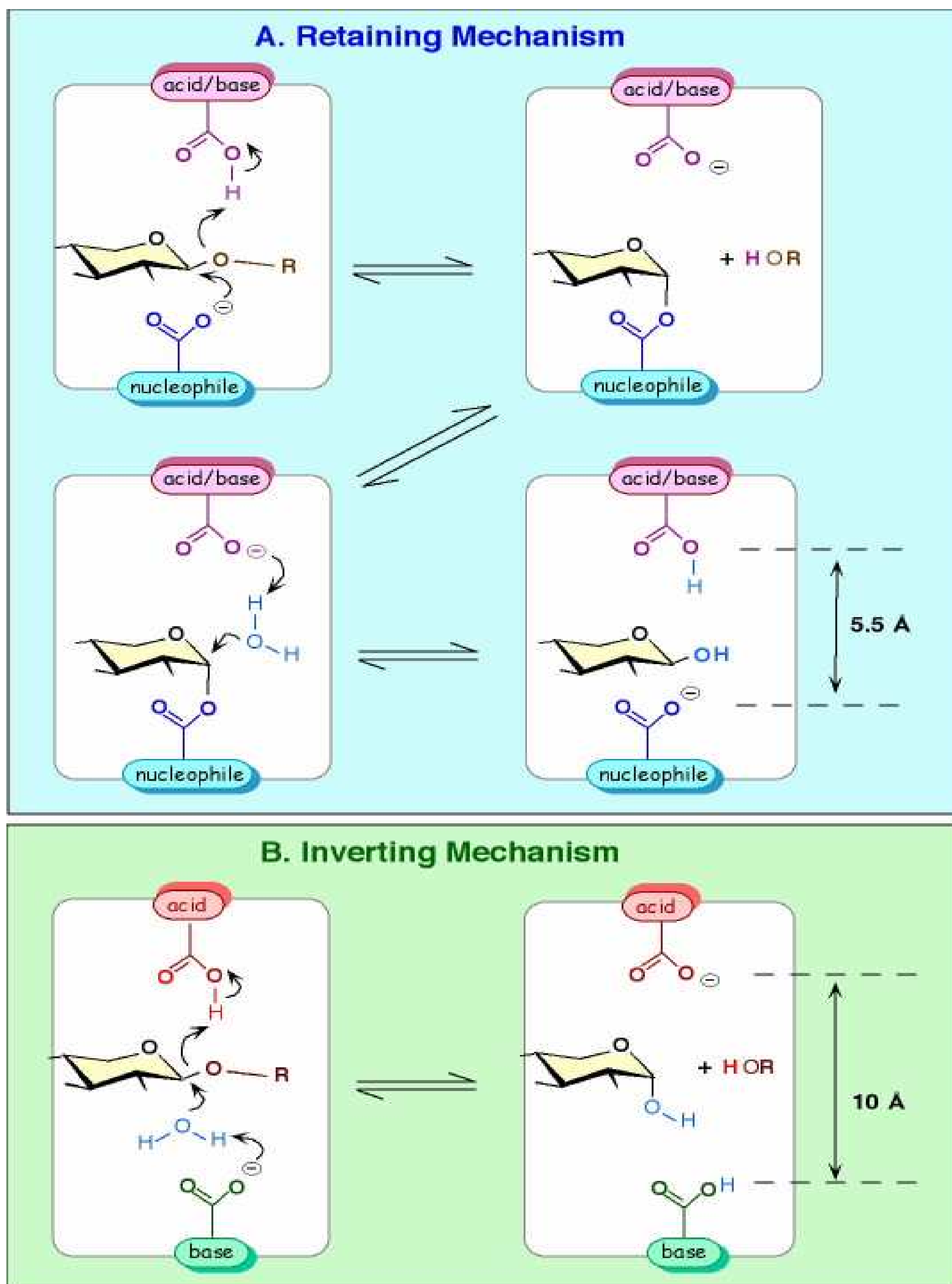


Figure 3: Mechanism of cellulase action: A) Retaining mechanism B) Inverting mechanism.

Yague et al., 1992; Yagiie et al., 1994; Zhu et al., 1982]. An expression vector, pAMH110, containing the promotor sequences of the strongly expressed cbh 1 gene has been used to over-express a cDNA coding for EGI of *T. reesei* [Harkki et al., 1991]. Novel strains with either mixtures of CBHs, endoglucanases and xylanases or mixtures completely free of CBHs and strains over-expressing the endoglucanase have also been constructed [Harkki et al., 1991]. Hybrid strains are likely to be produce using genetic engineering capable of producing either one or all cellulase components at high levels and could solve some of the current industrial problems. Attempts have been made to change the proportion of different cellulases produced by *T. reesei* [Harkki et al., 1991].

Application of cellulases

Cellulases have a wide range of applications. The main potential applications are in food, animal feed, textile, fuel and chemical industries [Beguin and Anbert, 1993; Coughlan et al., 1985a; b; Mandels 1985]. Other areas of application include the paper and pulp industry, waste management, medical/pharmaceutical industry, protoplast production, genetic engineering and pollution treatment [Beguin and Anbert, 1993; Coughlan et al., 1985a; b; Mandels 1985]. The application of cellulases has been discussed in detail in Chapter V.

MOLECULAR CLONING AND EXPRESSION OF A LOW MOLECULAR WEIGHT β-1, 4-D GLUCAN GLUCANOHYDROLASE

Abstract

In the present study cloning of the gene encoding a low molecular weight 1, 4-β-D-glucan glucanohydrolase (TSC) from *Thermomonospora* sp. was attempted. The peptide sequences of the tryptic digest of TSC was used to design primers for amplification of the gene from the genome of *Thermomonospora* sp. The gene was partially amplified by PCR using the forward and reverse primers to yield an amplicon of 400bp. The predicted amino acid sequence of the 400bp amplicon revealed the presence of all the peptides from the tryptic digest indicating that the amplified fragment is of TSC. A characteristic 14bp inverted repeat sequence that lies in the 5' upstream region of the cellulase genes from *Thermomonospora fusca* has been reported by Spiridonov and Wilson (1999). The genomic DNA was amplified using the forward primer to obtain an amplicon of 700bp containing the complete ORF of TSC gene. This was subsequently cloned in to pGEMT^{+} vector and transformed in *E. coli*DH5α. The ORF was amplified from pGEMT^{+} vector and was subcloned into an expression vector pET28(a)$^{+}$ and transformed in to *E. coli*BL21. The gene was expressed as a recombinant protein with a N-terminal His-tag for the ease of purification and a thrombin cleavage site for the separation of pure protein. The soluble recombinant protein was purified by Ni-NTA affinity chromatography. The purified protein has a molecular weight of 21kDa in contrast to 14.2kDa of TSC. The discrepancy in molecular weight may be due to post translational modification. The expressed protein did not exhibit any detectable endoglucanase activity probably due to misfolding of the protein due to its expression in a heterologous host.

Introduction

The cloning and sequencing of the structural gene of an enzyme is useful for the overproduction of the protein for its potential biotechnological application and for mutagenesis, x-ray crystallography and structure function studies. The cloning of cellulases are important since multiple cellulase genes are present in most of the cellulolytic organisms which makes it difficult to isolate cellulases without the other associated cellulase activities.

The cellulases from two species of *Thermomonospora* namely *T.fusca* and *T.curvata* have been cloned (Table 1) (Lao et al., 1991; Collmer and Wilson, 1983; Hu and Wilson, 1988). The cloning of cellulases from *Thermomonospora* sp. using phage vector appeared to be more effective than plasmid vectors since five cellulase genes from *Thermomonospora* were cloned using phage vectors (Hu and Wilson, 1988) as compared to two by plasmid vectors (Rouviner et al. 1990). Expression of cellulase genes in *E.coli* was poor. The expression of cellulase gene of E4 from *Thermomonospora fusca* in *Streptomyces lividans* resulted in production of active enzyme (Lao et al., 1991). The cellulase E_5 from *Thermomonospora fusca* has been expressed in *E. coli, Enterobacteria agglomerans, Erwinia hervicula, Erwinia amolovera* and *Streptomyces lividans* (Ghangas and Wilson, 1987). It could not be expressed in *Bacillus subtilis, Streptococcus faeclis* or *Saccharomyces cerevisia.* All the cellulase genes from *T. fusca* exhibited 67-68% G+C content which was very close to the value of 67% reported for *T.curvata* chromosomal DNA (Petricek et al., 1989). The region coding for the N-terminal sequence determined from the pure protein is preceded in each cellulase gene by a region coding for a potential signal sequence. This was expected as all of these cellulases were secreted by *T. fusca.* The length of the signal sequences varies form 31 to 46 amino acids with atleast two basic residues near the N-terminal end. The presence of a characteristic 14bp inverted repeat sequence TGGGAGCGCTCCCA in the 5' upstream (20 to 60 bases preceding the initiation codon) of all cellulases genes from *Thermomonospora fusca* has been reported and is absent in the xylanase gene from the same organism (Spiridonov and Wilson, 1999). This inverted sequence has also been found in *Streptomyces* and *M. bispora* cellulase genes. Cellulase sysnthesis in *Thermomonospora* is regulated by induction and repression. Both induction and repression of E5 are involved in controlling the translation

and transcription of mRNA. Three endoglucanases form *Thermomonospora curvata* have been cloned in to *E. coli* using cosmid vector pHC79 which were able to hydrolyse CMC (Presutti et al., 1991). Cellobiose is a good inducer of cellulase activity.

Table 1: Different cellulases cloned from *Thermomonospora* sp.

Wild organism	Cellulase	Host	Reference
T. fusca	E1, endoglucanase	*E. coli* HB101	Hu and Wilson, 1988
	E2, endoglucanase	*E. coli* HB101, *S. lividans*	
	E5, endoglucanase	*E. coli* HB101	
	E3, exocellulase	*E. coli. S. lividans*	Zhang S et al., 1995
	E4, endo/exocellulase	*E. coli*	Sakon et al., 1997
T. curvata	Endoglucanase 1	*E. coli*	Presutti et al., 1991
	Endoglucanse 2	*E. coli*	
	Endoglucanse 3	*E. coli*	

The present study describes the cloning of a low molecular weight endoglucanase (TSC) from *Thermomonospor*a sp. in *E. coli* and its expression. The native enzyme has been purified, characterized (Jagtap and Rao, 2005, 2006) and crystallized (Manikandan et al, 2006).

Materials and Methods

Microorganisms and vectors

The competent cells, *E. coli*DH5α and *E. coli*BL21DE3 were obtained from Invitrogen. pGEMT vector and ligation kit were from Promega wereas pET28a^{+} was from Novagen. DNA marker was obtained from Banglore Genei.

Isolation of *Thermomonospora* sp. genomic DNA.

Thermomonospora sp. genomic DNA was isolated as described in Chapter 2.

Quantitation of DNA

Absorption spectra of suitably diluted DNA solution was recorded on a Varian Cary Win UV spectrophotometer in the range 220-320nm. The preparations with A_{260}:A_{280} ratio > 1.8 - 2 was considered suitable for further work. The DNA was quantified according to the following equation.

Absorbance of 1.0 at 260 nm= 50µg/ml DNA (Maniatis *et al.*, 1982)

Isolation of plasmid DNA

A 30ml culture of *E. coli* containing the plasmid was grown to late log phase (OD_{600} = 0.6) in Luria Bertani (LB) media (500ml) containing either ampicillin or kanamycin (50µg/ml) in a 2L flask was inoculated with 25ml of late log phase culture. The culture was incubated at 37°C for 2.5h with vigorous shaking. In order to get high yields of plasmid (2-5mg/500ml culture) the culture was grown in the presence of chloramphenicol (170µg/ml). The culture was incubated for a further 12-16h at 37°C with vigorous shaking. The bacterial cells were harvested from a 500ml culture by centrifugation at 4,000rpm for 15min at 4°C. The bacterial pellet was resuspended in 10ml of solution I [50mM glucose, 25mM TrisCl (pH8), 10mM EDTA (pH8)]. 1ml of a freshly prepared solution of lysozyme (10mg/ml in 10mM TrisCl, pH8) was added to the solution followed by the addition of 20ml of freshly prepared solution II [0.2N NaOH, 1%SDS]. The centrifuge bottle was closed and the contents were mixed thoroughly by gently inverting the bottle several times. The bottle was stored at room temperature for 5-10min. 15ml of ice-cold solution III [5M potassium acetate (60ml), glacial acetic acid

(11.5ml), H_2O (28.5ml)] was added and the contents were mixed by shaking the bottle several times till there was no longer two distinguishable liquid phases. The bottles were stored on ice for 10min followed by centrifugation of the bacterial lysate at 4,000rpm for 15min at 4°C. The supernatant was filtered through four layers of cheesecloth. To the filtrate 0.6volumes of isopropanol was added and mixed well. The mixture was stored at room temperature for 10min. The plasmid was recovered by centrifuging at 5,000rpm for 15min at room temperature. The pellet of nucleic acid was dissolved in 3ml of TE, pH8.0.

Purification of plasmid by polyethylene glycol

The plasmid solution was transferred to a 15ml centrifuge tube and 3ml of ice-cold solution of 5M LiCl was added. The solution was mixed well and centrifuged at 10,000rpm for 10min at 4°C. The supernatant was transferred to a fresh tube and an equal volume of isopropanol was added to it. The solution was mixed well and the precipitated nucleic acid was recovered by centrifugation at 10,000rpm for 10min at room temperature. The pellet was dissolved in 500µl of TE (pH8) containing RNAase (20µg/ml). The solution was transferred to a microfuge tube and stored at room temperature for 30min. 500µl of 1.6M NaCl containing 13% (w/v) polyethylene glycol (PEG8000) was added and mixed well. The plasmid DNA was recovered by centrifugation at 10,000rpm for 5min at 4°C. The pellet was dissolved in 400µl of TE (pH8) and the solution was extracted with phenol: chloroform (1:1). To the aqueous layer, 100µl of 10M ammonium acetate was added followed by addition of 2volumes of ethanol, mixed well and the tube was stored for 10min at room temperature. The plasmid DNA was recovered by centrifugation at 10,000rpm for 5min at 4°C. The pellet was washed with 70% ethanol and dried to remove the last traces of alcohol. The pellet was dissolved in 500µl of TE (pH8).

Primer design for the cellulase gene and its amplification

The genomic DNA was used as a template for the amplification of the low molecular weight endoglucanase gene of interest. The primers TS forward (5'AAC TAC GGT TGC TGG CTG CGT 3'), NTS forward (5' CAT GGG AGC GTT CCC ATG CC

3') and TS reverse (5' GAC GAA GTT CAC GTC GCT GCA 3') were designed based on the tryptic digest sequences obtained for the low molecular weight endoglucanase (Jagtap BBRC). Primers with restriction site to be incorporated in the amplified product were flanked with restriction sites on the 5' end of the respective primers. The forward primer with Sac I site TSRT forward (5'GAG CTC ATG CAT CGT CAT GTT CAC 3') and reverse primer with Hind III site TSRT reverse (5'AAG CTT CTA GTG ATT GAC GAA GTT C 3') were used for the study.

Ligation reaction

The pGEM-T vector and PCR amplified product were mixed in a molar ratio of 1:2 and suspended in 5µl of DNA ligase buffer. To the mixture, 1unit of ligase was added and incubated for 4h at 16°C.

Transformation of *E. coli* cells

To 200µl of competent *E. coli* 5µl of the ligation mixture was added, mixed by gentle swirling and stored on ice for 30min. The cells were given heat shock in a water bath at 42°C exactly for 45 seconds and immediately transferred to an ice bath to chill for 1-2 min. To these cells, 800µl LB was added and was incubated at 37°C for 45 min. The culture was centrifuged at 3000rpm for 5 min, the supernatant was discarded and the pellet was resuspended in 200µl LB broth. The transformed competent cells were plated onto LB agar medium containing 60µg/ml of antibiotic (ampicillin or kanamycin). The plates were incubated at 37°C for 12-16 h.

Screening of positive transformants

In order to differentiate between the colonies containing recombinant plasmids from the non-recombinant colonies, blue white selection of the colonies was performed. To the LB plates containing ampicillin (60µg/ml), 40ml of a stock solution of X-gal (20µg/ml in dimethylformamide) and 4ml solution of isopropylthio-β-D-galactoside (IPTG) (200µg/ml). The mixture of X-gal and IPTG were spread uniformly onto the surface of agar plates and incubated at 37°C. The transformants were plated onto the

plates and incubated at 37°C for 12-16h. After the appearance of the colonies, the plates were placed at 4°C for several hours to allow the blue colour to develop fully.

Cloning Strategy

The primers TS forward (5'AAC TAC GGT TGC TGG CTG CGT 3'), NTS forward (5' CAT GGG AGC GTT CCC ATG CC 3') and TS reverse (5' GAC GAA GTT CAC GTC GCT GCA 3') were designed based on the tryptic digest sequences obtained for the low molecular weight endoglucanase (Jagtap, 2006). PCR was performed with genomic DNA of *Thermomonospora* sp. as template using these primers at 94°C denaturation for 1 min, 55°C annealing for 1 min followed by 72°C extension for 1min. for 35 cycles in a eppendorf Thermal cycler to dissect the full ORF of the endoglucanase gene from the genome. The details of the cloning strategy is as depicted in Figure 4. The full length gene amplified was subsequently cloned in to pGEMT vector through TA cloning and designated as pTSC1. The forward primer with Sac I site TSRT forward (5'GAG CTC **ATG** CAT CGT CAT GTT CAC 3') and reverse primer with Hind III site TSRT reverse (5'AAG CTT **CTA** GTG ATT GAC GAA GTT C 3') were used for the cloning of endoglucanase gene in to pET28a^{+} expression vector with an N-terminal hexahistidine tag and a thrombin cleavage site. The recombinant clone was confirmed for the complete coding sequence with the in frame histidine tag by sequencing. The resultant clone was named as pET28-TSC which was further utilized for expressing this gene as a recombinant protein in *E. coli*. All the constructs were propogated in *E. coli*DH5α.

Expression of pET28-TSC

The recombinant pET28-TSC plasmid was prepared and subsequently transformed in to *E. coli*BL21DE3 competant cells. The culture was grown in LB with 50µg/ml kanamycin at 37°C under constant shaking till the A_{600} reaches 0.6. The culture was induced by 1mM IPTG and was further allowed to grow for another 4h at the same conditions. The aliquots were collected in each hour for checking the expression profile as compared to the uninduced fraction.

SDS –PAGE analysis.

For checking the expression profile the 1ml of culture aliquot was taken in each hour, centrifuged and the pellet was resuspended in 50ul 1X SDS sample buffer. Samples were heated at 95°C for 15min. and subjected to 10% SDS PAGE (Laemili, 1976). 5ul samples were loaded and electrophoresed. Gels were stained by Commassie Blue-R-250 for visualization of the protein bands.

Protein purification

The bacterial cells were harvested after 3h of induction with IPTG, spun at 5000rpm for 15min. The pellet was dissolved in 200ml lysis buffer (PMSF, 1µl from 200mM stock; Lysozyme, 10µl from 10mg/ml stock; Na-deoxycholate- 1mg) and disrupted by sonication (60Hz, 1second pulse for 5min) at 4°C. The lysate was centrifuged at 10000rpm for 15min at 4°C. Both the supernatant and pellet fractions were analysed for the presence of recombinant protein. The supernatant was applied to Ni-NTA matrix preequilibriated with equilibration buffer (50mM Na_2HPO_4; 300mM NaCl; 20mM Imidazole). The recombinant protein containing the N-terminal his-tag was allowed to bind to the column. The unbound proteins were washed out using the wash buffer. Finally the bound protein was eluted using a elution buffer (50mM Na_2HPO_4; 300mM NaCl; 20mM imidazole). Imidazole competes with histidine to bind to Ni thereby releasing the tagged protein. All the fractions collected were analysed on SDS-PAGE to check the homogeneity of the protein. The eluted fractions were pooled and subjected to Thrombin cleavage to obtain the pure protein devoid of his tag. Finally the fraction was dialysed and checked for endoglucanase activity.

Results and discussion

Disection of the endoglucanase gene from *Thermomonospora* sp.

On digesting the native endoglucanase (TSC) from *Thermomonospora* sp. using trypsin six sequences NYGCWLR, LPDGQLCSGGLAEGGR, PLTWGSLDLVHR, HVVFTIWK, ASHMDQTYYL including C-terminal sequence CSDVNFV was obtained (Jagtap and Rao, 2006; Jagtap, 2006). Primers were designed based on the C-terminal (TS reverse) and NYGCWLR (TS forward) sequences using the codon usage chart for *Thermomonospora* sp. PCR amplification of the cellulase gene using the genomic DNA of *Thermomonospora* sp. as a template yielded a 400bp amplicon with these primers (Figure 1).

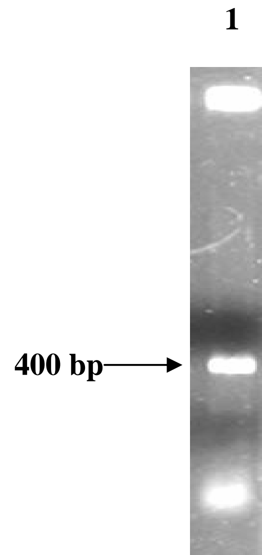


Figure 1: PCR amplified product obtained by primers TS forward and TS reverse using *Thermomonospora* sp DNA as a template. Lane 1: 400bp PCR amplified product.

5’TGCGTTTACATGGTCTCTGCATTAGTGACTTCATACATCGTCATGTTCACGC
CCGGACCTGCCGGTGGACCGTGCGAGCAGACCTCTGTTCGCGGCCCTGCTGG
GCTGACCCAGGGACCGACCCCGCGGCCGCCCACGGGTCGGTCATCAACCCGG
CGACGCGCAACTACGGCTGCTGGCTGCGGTGGGGCAACGACCACCTCAACCC
CAACATGCAGCACGAAGACCCCATGTGCTGGCAGGCCTGGCAGGACAACCCC
AACGCCATGTGGAACTGGAACGGCCTGTACCGCGACAACGTGGGCGGCAAC
CACCGGGCCGCGCTGCCCGACGGGCAGCTGTGCAGCGGCGGTCTGGCCGAGG

GCGGCCGCTACCGCTCCATGGACGCCGTGGGCCCGTGGAAGACCACCGACAT
CAACAACACCTTCACGATCCACCTGTACGACCAGGCCAGCCACGGGGGGGAC
TACTTCGAGGTCTACGTCACCAAGCAGGGCTTCGACCCGACCACCCAGCCGC
TGACCTGGGGCAGCCTGGACCTGGTCCACCGGACCGGCAGCTACGCGCCCAG
CCAGAACATCCAGTTCACGGTCAACGCGCCCAACCGCAGCGGCCGCCACGTG
GTCTTCACCATCTGGAAGGCCTCGCACATGGACCAGACCTACTACCTGTGCA
GCGACGTGAACTTTCGTCA 3'

Figure 2: Sequence of the partial TSC gene (400bp) amplicon.

On theoretical translation of the 400bp sequence (Figure 2) to its corresponding aminoacid sequence, all the six peptide sequences could be located in the protein sequence indicating the amplicon to be the gene encoding the endoglucanase (TSC). But this was a partial sequence and lacked the N-terminal coding region. The 400bp sequence on BLAST analysis was found to have close homology to a cellulose binding protein E7 from *Thermomonospora* fusca. To excise out the complete ORF of endoglucanase from *Thermomonospora* sp., another primer (NTS forward) was designed based on the 14 bp inverted 5' upstream conserved *cis* regulatory element from *Thermomonospora fusca* cellulases as reported by Spiridonov and Wilson (1999). PCR using primers NTS forward and TS reverse yielded a 700bp amplicon which on sequencing indicated the presence of the complete gene (Figure 3, 4). The sequence has been deposited at NCBI with the accession No. EF205153.

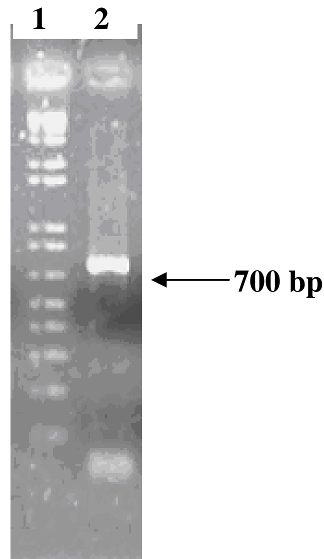


Figure 3: PCR amplified product obtained by primers NTS forward and TS reverse using *Thermomonospora* sp DNA as a template. Lane 1, 1kb plus DNA marker and Lane 2: 700bp PCR amplified product.

5'CTACGGGGAGCGTCCATCCTGTACTCCCTATAGTTCGGTGCAGTCTAGCTAT
CGAACCTAAGCGATCTATAGATCTAGCGGAATGCGGAAACTGGAGAAAAGA
GGAAAAAAGCGAATGGAGCGGTCCTAGTCCTTGCCATGTTAGCGTCACGTTG
CGGTAACCACCACACCGCGCGCTAATGCGCCGCTACAGGGCGCGTCATCGCA
TCAGGCTGCGCAACTGTGGGAAGGGCGATCGGTGCGGGCTCTCGCTATTACG
CCAGCTGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTGGGTAACGCCAG
GGTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTGTAATACGAC
TCACTATAGGGCGAATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCC
GCGGGATTCATGGGAGCGTTCCCATGCCCGGAACCCCCACATCCGGAATTTG
GAGATCAGACAATGCATCGTCATGTTCACGCCGGGACCTCCCGGTGGACCGT
GCGCGCTCTGGCCGTGCTGTTCGCGGCCCTGCTGGGCCTGACCCAGTGGACC
GCCCCCGCGGCCGCCCACGGGTCGGTCATCAACCCGGCGACGCGCAACTACG
GCTGCTGGCTGCGGTGGGGCAACGACCACCTCAACCCCAACATGCAGCACGA
AGACCCCATGTGCTGGCAGGCCTGGCAGGACAACCCCAACGCCATGTGGAAC
TGGAACGGCCTGTACCGCGACAACGTGGGCGGCAACCACCGGGCCGCGCTGC

CCGACGGGCAGCTGTGCAGCGGCGGTCTGGCCGAGGGCGGCCGCTACCGCTC

CATGGACGCCGTGGGCCCGTGGAAGACCACCGACATCAACAACACCTTCACG

ATCCACCTGTACGACCAGGCCAGCCACGGTGCGGACTACTTCGAGGTCTACG

TCACCAAGCAGGGCTTCGACCCGACCACCCAGCCGCTGACCTGGGGCAGCCT

GGACCTGGTCCACCGGACCGGCAGCTACGCGCCCAGCCAGAACATCCAGTTC

ACGGTCAACGCGCCCAACCGCAGCGGCCGCCACGTGGTCTTCACCATCTGGA

AGGCCTCGCACATGGACCAGACCTACTACCTGTGCAGCGACGTGAACTTCGT

CAATCACTAGTGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCAACGC

GTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTGCGATA3’

Figure 4: Sequence of the 700bp amplicon containing the complete ORF of TSC gene. The red alphabets indicated the ORF of TSC, yellow highlighted area indicates the complete gene of TSC and the pink highlighted area indicates the sequence of the conserved inverted conserved bp sequence. NCBI accession No. EF205153.

The sequence of the 700bp amplicon containing the complete ORF of TSC gene has been deposited in NCBI data bank with the accession No. EF205153. The predicted amino acid sequence of the 700bp amplicon showed the presence of peptide sequences from the tryptic digest of TSC (Figure 3).

LRGASILYSL@FGAV@LSNLSDL@I@RNAETGEKRKKANGAVLVLAMLASRCG

NHHTAR#CAATGRVIASGCATVGRAIGAGSRYYASCERGMCCKAIKWVTPGFPS

HDVVKRRPVNCNTTHYRANWARRRMLPAAMAAGFMGAFPCPEPPHPEFGDQT

MHRHVHAGTSRWTVRALAVLFAALLGLTQWTAPAAAHGSVINPATRNY**GCWL**

RWGNDHLNPNMQHEDPMCWQAWQDNPNAMWNWNGLYRDNVGGNHRAA**LP**

DGQLCSGGLAEGGRYRSMDAVGPWKTTDINNTFTIHLYDQASHGADYFEVYV

TKQGFDPTTQ**PLTWGSLDLVHR**TGSYAPSQNIQFTVNAPNRSGR**HVVFTIWKA**

SHMDQTYYLCSDVNFVNH@CGRLQVDHMGELQRVGCIA&VFYSVT#IAAI

Figure 5: The predicted aminoacid sequence of the 700bp gene amplicon of TSC from *Thermomonospora* sp. The highlighted (yellow) area indicated the complete TSC amino acid sequence. The highlighted area (pink) indicates the N-terminal sequence obtained for TSC.

Cloning and Subcloning of endoglucanase gene

The amplicon obtained by primers NTS forward and TS reverse was cloned in to pGEMT vector by TA cloning and transformed in to *E. coli* DH5α. The fact that *Taq* polymerase adds up poly A tail to the amplicon has been exploited in TA cloning. The pGEMT has poly T at the cloning sites which make it easy to bind to the poly A of the PCR amplified fragment with poly A tail. The positive clones were selected based on the blue-white selection using IPTG and X-Gal. The positive clones were then cross checked for the presence of the gene by isolating the plasmid and sequencing it. For expression of the gene, it was necessary to subclone the gene in to an expression vector (pET28a$^+$). For this, primers were designed based on the N-terminal sequence and C-terminal sequence obtained from the earlier clones. The primers were flanked with *Sac*I restriction site to forward primer (TSRT forward) and *Hind*III restriction site to the reverse primer (TSRT reverse) so as to introduce these sites in the amplicon facilitating the excision of the complete gene for subcloning in to pET28a$^+$. The pGEMT clone with complete gene was subjected to PCR with TSRT forward and TSRT reverse primers to obtain an amplicon of 500bp (Figure 6) corresponding to the full ORF of TSC which was further sequenced and cloned in to pGEMT vector system (Figure 7).

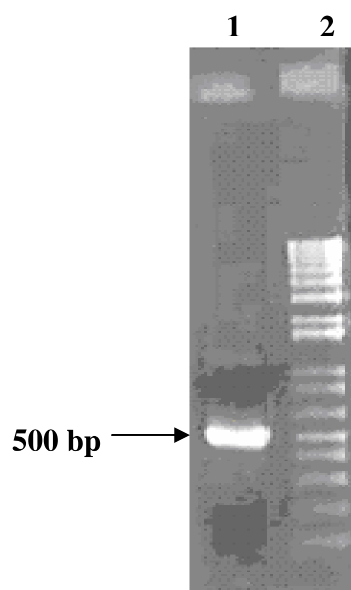


Figure 6: PCR amplified product obtained by primers TSRT forward and TSRT reverse using *Thermomonospora* sp DNA as a template. Lane 1, 2: 500bp PCR amplified product and Lane 2: 1kb plus DNA marker.

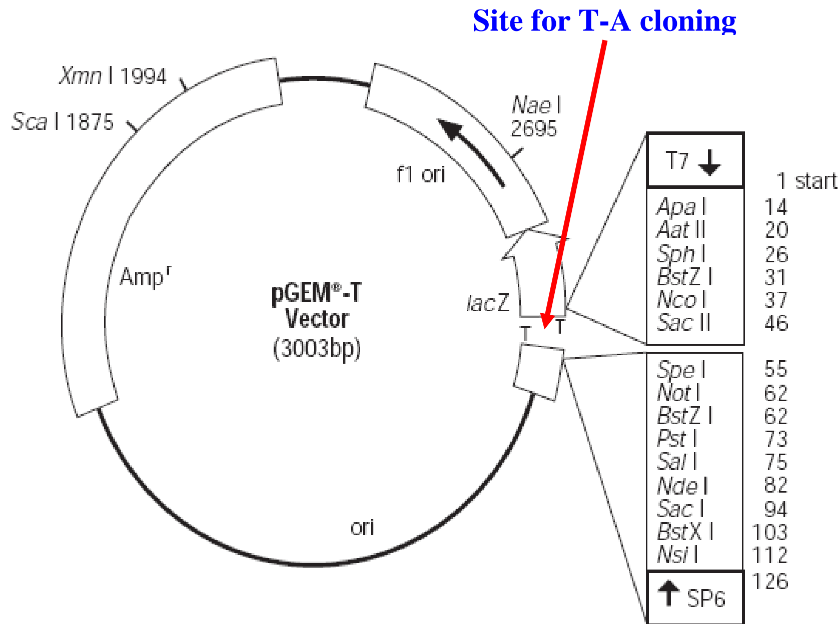


Figure 7: Map of pGEMT vector showing the ampicillin resistance, cloning and restriction sites. The PCR amplicons containing A-tails were cloned in to T- ends of the pGEMT vector there by facilitating T-A cloning.

The positive clone so obtained was double digested with restriction enzymes *Sac*I and *Hind*III to excise the complete ORF of the endoglucanase gene. Similar digestion was carried out with pET28a$^+$ to create sticky ends (Figure 8).

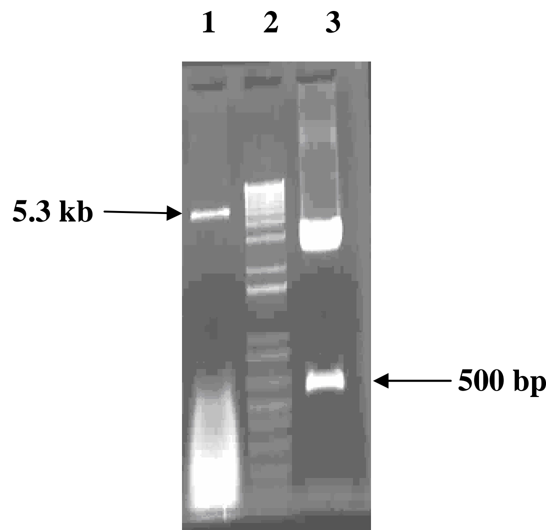


Figure 8: PCR amplified product obtained by primers NTS forward and TS reverse using *Thermomonospora* sp DNA as a template. Lane 1: pET28a$^+$ digested with *Sac*I and *Hind*III, Lane 2: 1kb plus DNA marker and Lane 3: pGEMT clone digested with *Sac*I and *Hind*III showing the 500bp insert.

The excised complete ORF was then subcloned into the digested pET28a^{+} and transformed in to *E. coli*BL21DE3. The site where the ORF was cloned has been indicated in Figure 9.

Figure 9: Map of pET28a^{+} vector showing the restriction sites, kanamycin resistance site, His-tag and Thrombin digestion site. The highlighted areas show the position where in the complete ORF of the TSC was cloned.

A schematic representation of the complete cloning strategy has been described in the Figure 10.

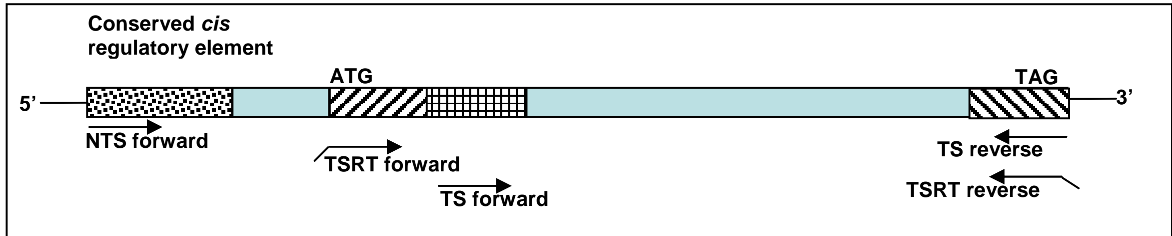


Figure 10: Schematic representation of cloning strategy for cloning of endoglucanase from *Thermomonospora* sp. (TSC).

Expression of TSC in *E. coli* BL21 DE3

For the expression of TSC gene in *E. coli* BL21 DE3, the log phase culture (A_{600} = 0.6) was induced with 1mM IPTG and samples were removed every hour for checking the induction of the protein. The host strain *E. coli*BL21DE3 has a T7 RNA polymerase which can be induced by IPTG. Thus RNA polymerase will be produced by the host which will bind at the T7 promotor. It inturn drive the expression of all the genes in the downstream region including the recombinant gene. A progressive increase in the induced protein of around 21kDa was observed on the SDS PAGE for 5 hours after induction (Figure 11). This protein band was absent in the uninduced sample.

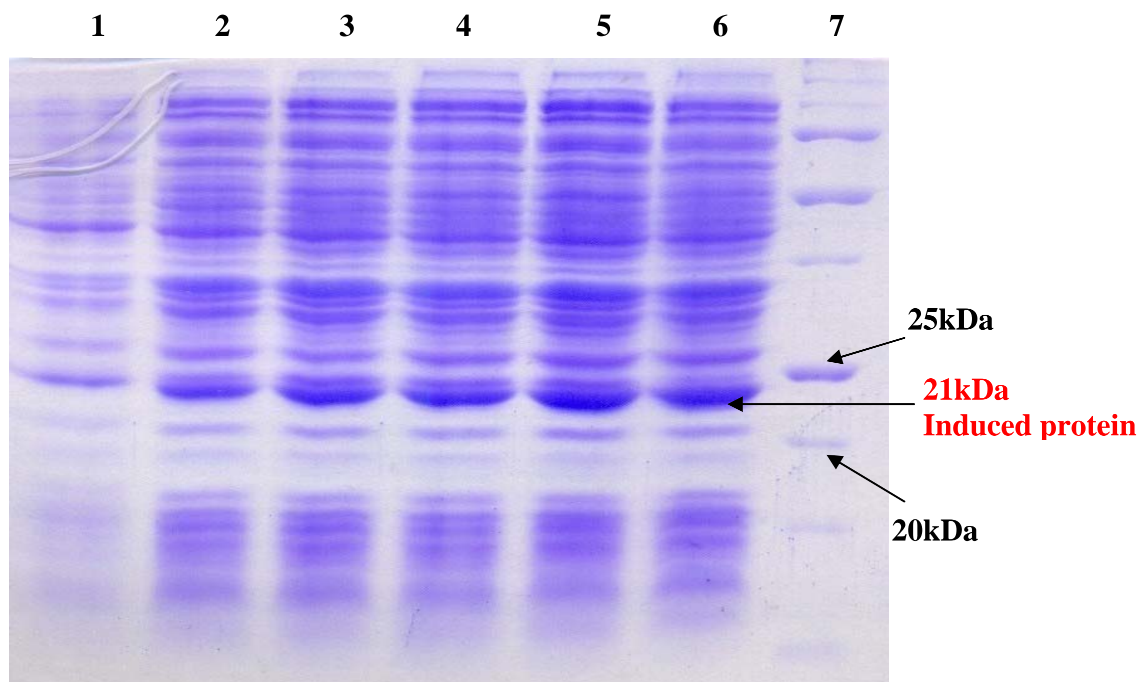


Figure 11: 10% SDS-PAGE showing induction profile of recombinant protein in *E. coli*BL21DE3. Lane1: Uninduced sample; Lane 2 to 6: 1, 2, 3, 4, 5 hours respectively; Lane 7: Protein Markers.

It was also found that the induced protein was secreted as a soluble fraction and did not form inclusion bodies.

Purification of recombinant TSC

E. coli BL21 DE3 cells were harvested by centrifugation after 4 hours of induction by IPTG and subjected to sonication. The lysate was centrifuged and the supernatant was applied to Ni-NTA column. 1ml fractions of the bound protein were collected on elution by imidazole buffer. The fractions were separately checked on 10% SDS PAGE. A single homogenous band of 21kDa was observed in the first 3 fractions (Figure 12). These fractions were pooled, dialyzed and subjected to Thrombin digestion at 12°C for 5h to remove the His-tag from the purified recombinant protein. The protein thus obtained was checked for CMCase and xylanase activities. The purified protein was not exhibiting any detectable activity towards xylan and CMC.

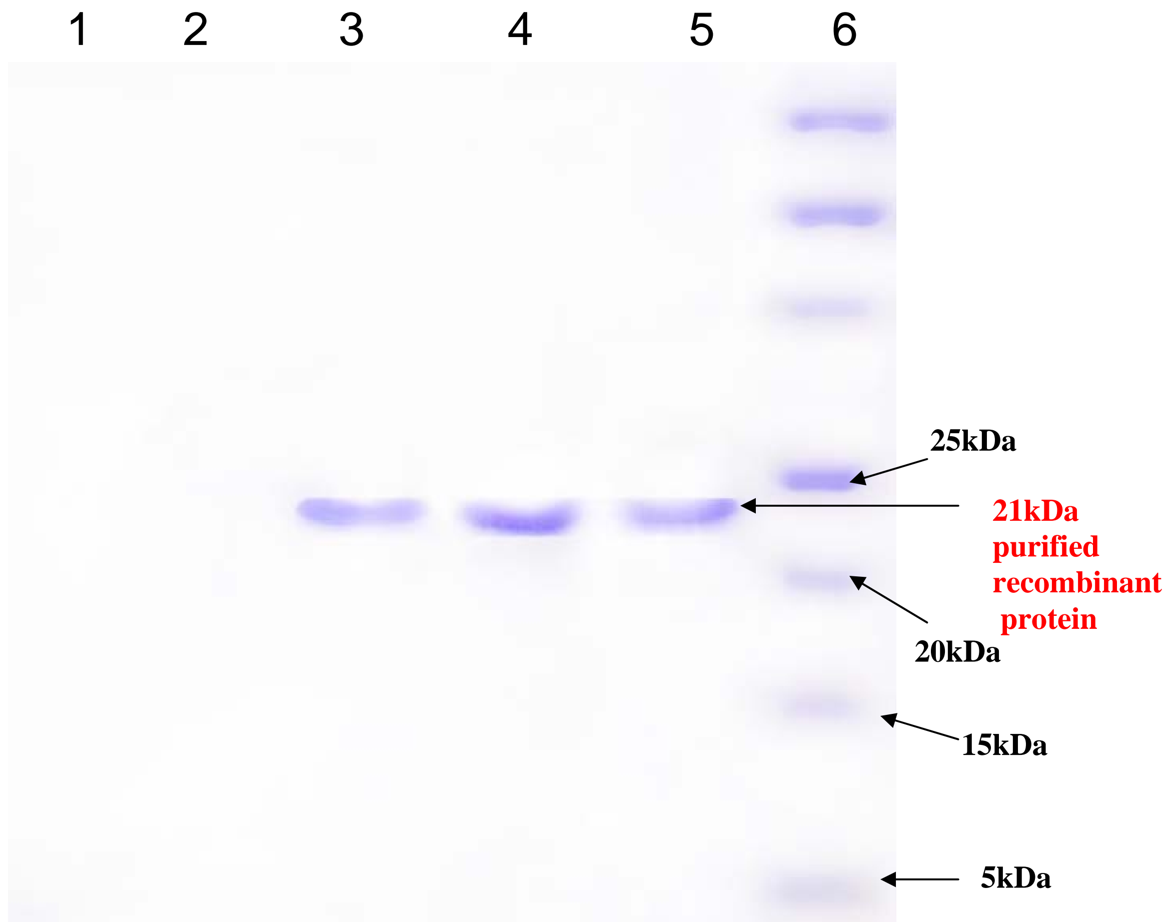


Figure 12: 10% SDS-PAGE showing purification profile of recombinant protein on Ni-NTA column. Lane1 to 5 : Fraction 5 to 1 eluted using 250 mM imidazole elution buffer; Lane 6: Protein Markers.

There could be many possible explanations for the lack of biological activity by the recombinant TSC. One of the factors could be that the TSC gene was expressed in a heterlogous host. Certain post translational modification might be required for the protein to attain its active form. The protein might not have accomplished its original conformation in which it remains active after being translated since it was devoid of the native environment in which different molecules assist the trafficking as well as proper folding of the protein to its active form. The possibility of the presence of a signal peptide which aids in the targeting of TSC outside the cell cannot be ruled out. Thus TSC endoglucanase from *Thermomonospora* sp. has been cloned and expressed as a recombinant protein in *E. coli*.

CHAPTER V

INDUSTRIAL APPLICATION OF CELLULASE

Introduction

Active research on cellulases and related polysaccharidases began in the early 1950s owing to their enormous potential to convert lignocellulose, the most abundant and renewable source of energy on the earth, to glucose and soluble sugars (Coughlan 1985a,b; Mandels 1985; Reese 1976 and Reese and Mandel, 1984). Extensive basic and applied research during the 1970s and 1980s demonstrated that the enzyme induced bioconversion of lignocellulose to soluble sugars was rather difficult and uneconomical (Coughlan, 1985a; Ladisch et al., 1983; Mandels, 1985 and Ryu and Mandels, 1980). Nevertheless, continued research on cellulases revealed their biotechnological potential in various industries, including food, brewery and wine, animal feed, textile and laundry, pulp and paper, agriculture as well as in research and development (Bayer et al., 1994, 1995; Beguin and Aubert, 1993; 7; Gilbert and Hazlewood, 1993; Godfrey, 1996; Harman and Kubicek, 1998; Lamed, 1998; Mandels, 1985; Poutanen, 1997; Saddler 1993; Uhlig 1998; Viikari et al., 1986; Visser et al. 1992; Visser and Voragen, 1996; Wong and Saddler, 1992). The enzymes are commonly used in many industrial applications.The demand for more stable, highly active and specific enzymes is growing rapidly.

During the last two decades, the use of cellulases has increased considerably, especially in textile, food, brewery and wine as well as in pulp and paper industries (Godfrey, 1996;; Harman and Kubicek, 1998; Saddler 1993; Uhlig 1998).

Food biotechnology

Cellulases have a wide range of biotechnological applications in food industry. Initially when food industries began to produce juice, the yields were low and many difficulties were encountered in filtering the juice to an acceptable clarity (Uhlig, 1998). Subsequently, research on industrially suitable enzymes from food-grade micro-organisms (*Aspergillus niger* and *Trichoderma* sp.) along with increased knowledge on fruit components, helped to overcome these difficulties (Grassin and Fauquembergue, 1996a). Currently, a combination of enzymes, collectively called macerating enzymes cellulases (endoglucanases, exoglucanases and cellobiases), (pectin lyase, pectin methylesterase, endo and exo-polygalacturonases, pectin acetylesterase, rhamnogalacturonase, endo- and exo-arabinases) and hemicellulases (endo- and exo-xylanases, galactanases, xyloglucanases and mannanases) are used in the extraction and clarification of fruit and vegetable juices (Galante et al., 1998 and

Grassin and Fauquembergue,1996b). Macerating enzymes containing cellulases are generally used to improves the extraction of valuable fruit components and for the clarification of fruit juice prior to concentration and increasing the filtration rate and stability of the final product. In addition, the infusion of pectinases and β-glucosidases increases the aroma and volatile characteristics of specific fruits and vegetables (Krammer et al., 1991; Marlatt et al., 1992; Pabst et al., 1991). Olivex and Cytolase O are macerating enzyme used to increase the anti-oxidants in extra-virgin olive oil and reduced the induction of rancidity (Galante et al., 1998b). The main advantages of using macerating enzymes during olive oil extraction are (1) increased extraction (up to 2 kg oil per 100 kg olives) under cold processing conditions (2) better centrifugal fractionation of the oily must (3) oil with high levels of anti-oxidants and vitamin E (4) slow induction of rancidity (5) overall improvement in plant efficiency and (6) low oil content in the waste water (Galante et al., 1998b).

Animal feed biotechnology

Cellulases and hemicellulases have a wide range of potential applications in the animal feed industry. These are used to (1) eliminate anti-nutritional factors present in grains or vegetables (2) degrade certain cereal components in order to improve the nutritional value of feed and (3) supplement animal's own digestive enzymes (e.g. proteases, amylases and glucanases), whenever these enzymes are inadequate during post-weaning period, as it is often the case with broilers and piglets (Galante et al., 1998b). Addition of β-glucanases markedly improve the digestion and absorption of feed components as well as weight gain by broiler chickens and egg laying hens (Cowan 1996; Hesselman et al., 1982; Rexen, 1981). Besides, the supplementation of xylanase along with cellulase reduced the overall cost of pig feed and facilitated the use of inexpensive feed.

Beer and wine biotechnology

The addition of exogenous glucanases and related polysaccharidases are known to improve not only the beer and wine qualities but also their overall production efficiency (Galante et al., 1998b). Malting of barley depends on seed germination which initiates the biosynthesis and activation of α- and β-amylases, carboxypeptidase and β-glucanase that hydrolyse the seed reserve which should act in synergy under optimal conditions to produce high quality malt. If unmalted or poor

quality barley in combination other cereals have the presence of non-starch polysaccharide, mainly a soluble β-glucan. This forms gels during the brewing process and leads to poor filtration, low extract yields and the development of haze in the final product. To overcome these problems, microbial β-glucanases, which hydrolyse β-glucan and reduce the viscosity of the wort are added either during mashing or primary fermentation. The main benefits of using these enzymes during wine making include (1) better skin maceration and improved colour extraction (2) easy must clarification and filtration and (3) improved wine quality and stability (Galante et al., 1998b). β-glucosidase is used in the wine industry for its ability to improve the aroma of wines by modifying naturally present, glycosylated precursors (Caldini et al., 1994; Gunata et al., 1990).

Pulp and paper biotechnology

The mechanical pulping processes such as refining and grinding of the woody raw material lead to pulps with high content of fines, bulk and stiffness which leads to high energy consumption. Biomechanical pulping using white rot fungi resulted in substantial energy savings during refining and improvements in hand-sheet strength properties (Akhtar, 1994; Leatham et al., 1990). Unrefined wood chips are generally less accessible to enzymatic modification, hence the addition of an enzyme in mechanical pulping can be effective only after the initial refining (Pere et al., 1996). The enzymatic treatment was performed either before or after beating of the pulps.

Endoglucanases have been identified to improve the drainage rates of recycled fibres (Kamaya, 1996) using purified endoglucanases from *Trichoderma*. Pergalase-A40, a commercial cellulase/hemicellulase preparation from *Trichoderma* is being used world wide in many paper mills.

The application of enzymes in de-inking has been intensively studied in both laboratory and pilot scales but the technique has not yet been commercialised (Buchert et al., 1998). The two principal approaches in using enzymes for deinking include the (1) hydrolysis of soy-based ink carriers by lipase and (2) the release of ink from fiber surfaces by cellulases, xylanases and pectinases. Most applications proposed so far use cellulases and hemicellulases for the release of ink from the fiber surface by partial hydrolysis of carbohydrate molecules. The main advantage of enzymatic deinking is the avoidance of the use of alkali. Deinking, using enzymes at acidic pH also prevents the alkaline yellowing, simplifies the deinking process,

changes the ink particle size distribution and reduces the environmental pollution. In addition, the enzymatic de-inking improves the fibre brightness, strength properties, pulp freeness and cleanliness as well as reduces fine particles in the pulp. Xylanase treatment has been reported to increase the strength properties while cellulase treatment improved the brightness and freeness of the pulp (Prasad et al., 1993). Vyas and Lachke (2003) have demonstrated the application of Fusarium cellulase in biodeinking of paper. The enzymatic deinking has a great potential both from commercial and environmental standpoints and expected to be commercialised in the near future.

Textile and laundry biotechnology

The cellulase preparations capable of modifying the structure of cellulose fibrils are added to laundry detergents to improve the colour brightness, hand feel and dirt removal from cotton and cotton blend garments. Chapter V section I, puts some light on the application of cellulases in detergent industry.

Cellulases have achieved their worldwide success in textile and laundry because of their ability to modify cellulosic fibers in a controlled and desired manner so as to improve the quality of fabrics. Although cellulases were introduced in textile and laundry only a decade ago, they have now become the third largest group of enzymes used in these applications. Bio-stoning and bio-polishing are the best-known current textile applications of cellulases which have been discussed in detail in Chapter 5, section II. Cellulases are also increasingly used in household washing powders since they enhance the detergent performance and allow the removal of small, fuzzy fibrils from fabric surfaces and improve the appearance and colour brightness. The main advantages of using cellulases are (1) removal of short fibres and surface fuzziness; (2) smooth and glossy appearance (3) improved colour brightness and uniformity (4) high hydrophilicity and moisture absorbance (5) new and improved finishing and fashionable effects and (6) environmentally friendly process. In fact, bio-polishing is currently a key step in the textile industry for producing high quality garments.

Research and development in agriculture

Mixture of cellulases and other polysaccharidases produced by fungal strains of *Trichoderma* and *Penicillium* are used for the production of plant and fungal

protoplasts. These protoplasts can be fused to produce either hybrid or mutant strains with desired characteristics. Brown and co-workers (1986) evaluated a number of commercial and in-house cellulase preparations for the production of protoplasts from wild and mutant strains of *Penicillium*. They reported that the enzymes from *Trichoderma viride* Persoon (strain BIA), grown on solid-state culture using wheat bran and *Penicillium pinophilum* 87160iii, grown as submerged culture on a mixed substrate (laminarin and *P. pinphilum* cell walls), were the best for the production of fungal protoplasts. Thus, a combination of enzyme preparations containing cellulase and hemicellulase activities can be successfully used for the production of plant and fungal protoplasts. Cellulose-binding domains (CBD) of fungal cellulases, which functions normally when fused to heterologous proteins, have been successfully used either as an affinity tag for the purification of proteins or immobilisation of fusion proteins (Greenwood et al, 1989; 1992; Ong et al., 1989 and Tomme et al, 1994). Similarly, using the scaffoldin CBD of the *Clostridium thermocellum* cellulosome, a novel affinity column was prepared for the purification of antibodies. The biotinylated CBD bound to cellulose was attached to biotinylated protein A via avidin and used successfully for the purification of antibodies (Bayer et al., 1995). Thus, the CBD has a great potential in biotechnology.

Section A

ENTRAPMENT OF CELLULASE IN A MATRIX FOR ITS CONTROLLED RELEASE

Abstract

Gum arabic, a biodegradable natural polymer was used as a matrix to encapsulate endoglucanase from *Thermomonospora* sp. The modified enzyme was found to retain complete biocatalytic activity. The modified enzyme exhibited a shift in the optimum temperature [50 – 55°C] and considerable increase in the pH and temperature stabilities as compared to the free enzyme. Encapsulation of the enzyme also protected the activity in presence of detergents and enhanced the shelf life. A 3-fold decrease in the initial rate of reaction indicated a controlled release of the enzyme conferring properties preferred for its potential application in the manufacture of detergents.

Introduction

Microencapsulation is a rapidly emerging area with multitude of applications in biotechnology, one of them being the controlled release of active biomolecule. Microencapsulation is a technological approach used to protect sensitive oils, mask or preserve flavors and aromas and transfer liquids into easily handled solid ingredients (Balassa and Fanger, 1971; Dziezak, 1988). For instance, proteolytic enzymes entrapped in liposomes are used to increase the ripening of cheese (Kirby et al., 1987; Alkhalaf et al., 1988). Microencapsulation has been applied to fish oils to improve oxidative stability and overcome palatability problems due to unpleasant taste or odour (Dziezak, 1988; Newton, 1996). Microencapsulation is considered to be promising systems for oral protein drug delivery because they ensure physical protection to the encapsulated proteins against inactivation during the gastrointestinal transit. The microencapsulation of α-chymotrypsin in multiplayer alginate/protamine as a model for drug delivery system for controlled release properties has also been reported (Tiourina & Sukhorukov, 2002). An increase in transesterification rate by encapsulation of lipase has been reported (Rassy et al., 2004). Another significant example is the encapsulation of proteases in liquid detergent in order to protect the other enzymes [i.e. lipases and cellulases] from proteolysis during storage. Disruption of the microcapsules takes place on washing releasing the enzyme. During encapsulation it is necessary that the enzyme maintains its activity while it is inoperative during processing and storage. Granulation converts liquid enzyme concentrate in to a dry particular form, stabilizing and protecting the active hydrolase, increasing shelf-life, and improving product safety during handling by workers, formulators and end users (Ariel et al, 2005). One of the factors that govern enzyme activity is its water activity and reactivation of an enzyme can be achieved by increasing the water activity upon water uptake (Mathewson, 1998). In a controlled release system of encapsulation degradation of matrix material occurs as a determining factor for release of the encapsulant (Pothakamury and Barbosa-Canovas, 1995, Imam et al, 1998). Thus research activities are focused on identifying matrices which impart properties for the controlled release of the encapsulated biomaterials. The advantages of controlled release are i) the active ingredients are released at controlled rates over prolonged periods of times ii) loss of ingredients, such as

vitamins and minerals, during processing and cooking cand be avoided or reduced iii) reactive or incompatible components can be separated. The active ingredient is released from the controlled release delivery system by diffusion, biodegradation, swelling or osmotic pressure (Dziezak, 1988). Extensive work has been carried out on encapsulation using synthetic polymers such as eudragit polymers, polyvinyl alcohol, polyethylene glycol, etc. However, due to ever growing environmental concerns, uses of biopolymers such as starch, gelatin, agar, cellobiose, alginate, etc. are being favoured. Gum arabic is a naturally produced biopolymer by *Acacia* trees growing in arid regions and one of the largest known emulsifiers (Whistler, 1993). Structurally gum arabic is a branched molecule with a main chain of (1→3) β-D galactopyranosyl units having side chains consisting of (1→3) β-D galactopyranosyl units, joined to it by (1→6) linkages (Anderson et al., 1972). The side chains are two to five units in length. Both the main chain and the numerous side chains have attached units of α-L-arabinofuranosyl, α-L-rhamnopyranosyl, β-D-glucuronopyranosyl and 40 O-methyl-β-D-glucuronopyranosyl unts. The latter two uronic acid uits occur most often as terminal ends of the chain branches. The carboxyl groups of uronic acid are deprotonated in its normal ionized form near neutral pH values. Being a salt of polycarboxylic acid, it undergoes a degree of crosslinking if allowed to stand for a length of time (Whistler, 1993). The Gum Arabic also contains proteinaceous material covalently attached to the polysaccharide moieties (Akiyama et al., 1984). This protein component makes gum arabic surface active nd it is mainly associated with a high molecular weight fraction representing less than 30% of the total gum (Vandevelde and Fenyo, 1985).

Most cotton or cotton blend garments, during repeated washings, tend to become fluffy and dull. This is mainly due to the presence of partially detached microfibrils on the surface of garments that can be removed by cellulases in order to restore a smooth surface and original colour to the garment. The cellulase preparations capable of modifying the structure of cellulose fibrils are added to laundry detergents to improve the colour brightness, hand feel and dirt removal from cotton and cotton blend garments. The degradation of microfibrils by cellulase, softens the garment and removes dirt particles trapped in the microfibril network. This is currently accomplished by adding a commercial cellulase preparation from *H.*

insolens, active under mild alkaline conditions (pH 8.5–9.0), and at temperatures over 50°C in washing powders (Uhlig, 1998).

In the present chapter deals with the use of gum arabic a naturally charged biodegradable polymer for the first time as a matrix to entrap thermostable endoglucanases from *Thermomonospora* sp. for their potential application in detergent and textile industries. A thermostable enzyme was used in order to tolerate temperatures required for processing. A controlled release of the enzyme is a prerequisite for use in detergents which could be achieved by the use of gum arabic imparting enhanced shelf life and stability to endoglucanase.

Materials and Methods

Production of endoglucanase

The production of endoglucanase by *Thermomonospora* sp. was carried out in 500ml Erlenmeyer flasks containing 100ml of medium of the composition as described in Chapter 2. The inoculum (10%) was added from an inoculum flask grown for 48h at 50°C. The culture was grown with continuous shaking on rotary shaker at 50°C for 120h. The biomass was separated from the fermented broth by centrifugation and the filtrate was used as a source of endoglucanase.

Encapsulation of endoglucanase in Gum Arabic

Gum arabic (Commercial grade Gum acacia from Burgoyne and Burbidges company) was suspended in distilled water at 50°C and cooled to room temperature. Endoglucanase was added to the above solution and mixed properly. This mixture was freezed at 4°C for 48h for the retrogadation of the polymer. After 48h, the polymer was kept in air-draft oven with continuous flow of air for drying at room temperature to obtain a thin film. The film of the mixture was crushed to make a fine powder, which was used for further studies.

Scanning electron microscopy (SEM) of the encapsulated endoglucanase

Morphological examination of the fine powder of gum arabic and that of the encapsulated endoglucanase in Gum Arabic was carried out using SEM which was performed on a Leica Stereoscan-440 scanning electron microscope. Samples for SEM analysis were prepared by placing the powder on copper grids and then coating with gold.

Endoglucanase assay

The activity of endoglucanase was measured by the amount of reducing sugar formed (Miller, 1959) as mentioned in Chapter II.

One international unit (IU) of enzyme activity for endoglucanase was defined as the amount of enzyme releasing 1μmol of reducing sugar from Carboxy methyl cellulose (CMC) per min using glucose as standard.

Stability of endoglucanase in commercial detergents

The stability of endoglucanase in the presence of the commercial detergents Ariel, Surf Excel and Henko was investigated by incubating the enzyme in the presence of the detergent (7mg/ml) at 40°C. Aliquots of enzymes were removed at intervals of 10min for 1h and the residual activity of the enzyme was determined under standard assay conditions.

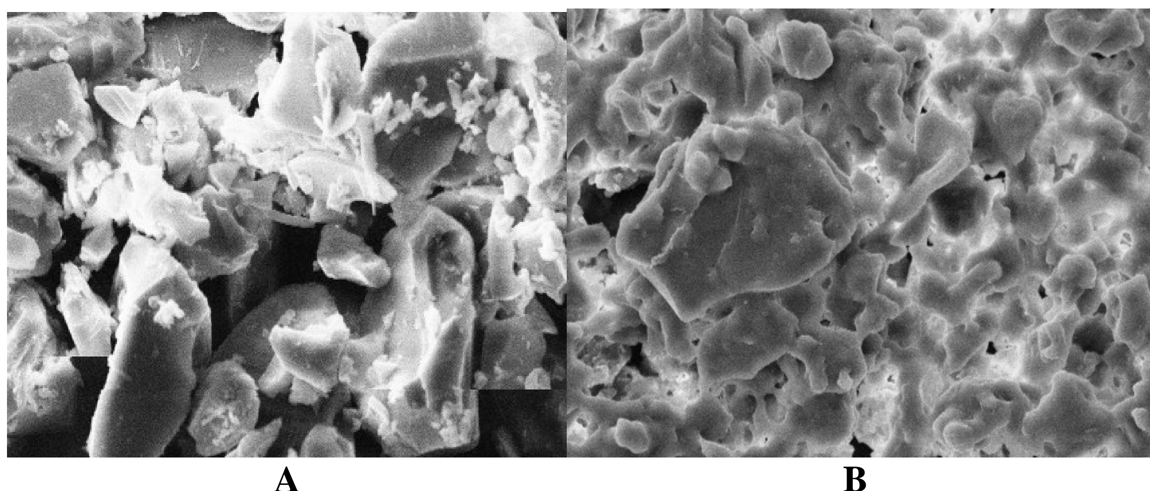
Spray drying of cellulase

CMCase was spray-dried in a laboratory spray-dryer. The dimensions of the drying chamber are 0.5 -0.15m. The spray dryer operates concurrently and has a single-fluid spray-nozzle with an orifice 1 mm in diameter. In the present preparations the inlet gas temperature was 70°C while the outlet gas temperature was kept between 50°C. Liquid feed to the dryer was 4 ml/min. The dried powder was collected in a cyclone at the outlet, yielding approximately 2 g of powder per 100 ml of solution with 3% solids. The powders were stored dry at room temperature in closed containers. The spray drying was also conducted using different additives (1%) (Maltodextrin, sorbitol) to increase the stability and shelf-life of CMCase.

Results and Discussion

Morphology of encapsulated endoglucanase

The endoglucanase was encapsulated in various concentrations of gum arabic (1-15%) and 10% was found to be most suitable. The gum arabic solution (pH 4.8) was mixed with the endoglucanase solution and frozen at 4°C for 48 hrs for the retrogadation of the polymer. SEM of encapsulated endoglucanase in gum arabic showed a coating on the surface of the polymer indicating that the enzyme gets encapsulated in the scaffolds of gum arabic during retrogadation (Figure 1). Previous results have shown that endoglucanase from *Thermomonospora* sp. is cationic in nature (George et al, 2001), and gum arabic being an anionic polysaccharide probably interacts electrostatically with the enzyme macromolecules. Encapsulation of endoglucanase in gum arabic provides an example of swelling- controlled system for the controlled release (Pothakamury and Barbosa-Canovas, 1995). Endoglucanase is dispersed evenly throughout the matrix and is unable to diffuse to any significant extent with in the matrix. But when the polymer matrix is placed in a thermodynamically compatible medium (buffer), the gum arabic swells owing to absorption of buffer and endoglucanase in the swollen part diffuses out of Gum Arabic. The release of enzyme from the polymer matrix has been schematically described (Figure 2).



A B

Figure 1: Scanning electron micrograph under 1000X magnification of gum arabic A) without enzyme; B) entrapped with endoglucanase.

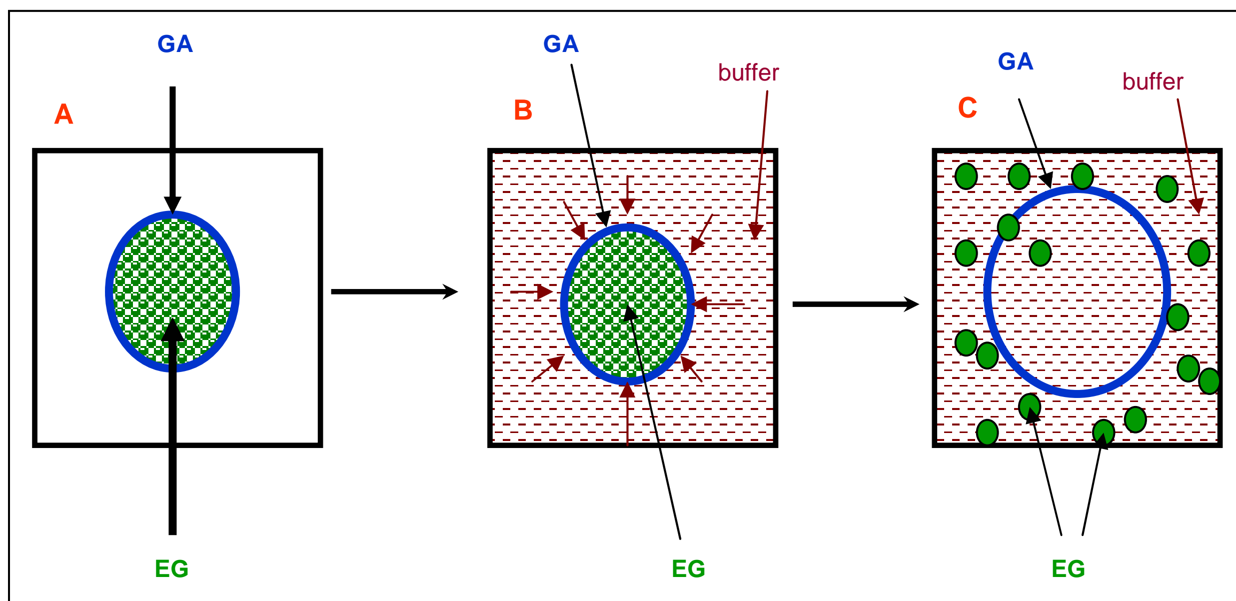


Figure 2: Schematic representation of swelling type controlled release system of endoglucanase. A) Endoglucanase (EG) entrapped in Gum Arabic (GA); B) Endoglucananse entrapped in Gum Arabic placed in thermodynamically stable system: buffer diffuses in to the polymer; C) Endoglucanase released in to the buffer system on swelling of the polymer matrix.

Properties of encapsulated endoglucanase

The activity of the encapsulated endoglucanase on dispersal in to a buffer solution was compared to that of free enzyme. A significant 3-fold decrease in initial rate of reaction was observed on entrapment (Figure 3 inset). A similar final plateau was reached indicating that the same amount of reducing sugar was liberated as a function of time (Figure 3) indicating a controlled release of the enzyme.

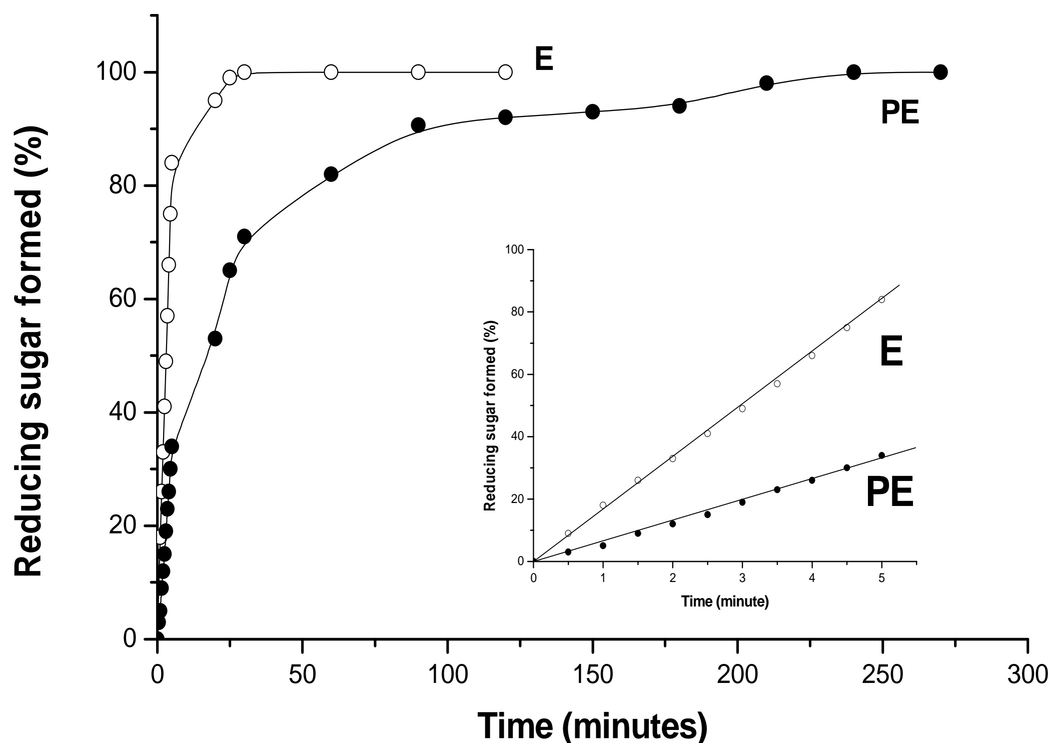


Figure 3: Comparison of the enzymatic activity of free (**E**) (○) and entrapped enzyme (**PE**) (●). The enzyme release has been evaluated based on the formation of reducing sugar. Formation of reducing sugar monitored for a broad time course. Focus on the short times (Inset). The slopes are representative of the initial rates.

The encapsulated endoglucanase retained 97, 91 and 85% activities at pH 9, 10 and 11 respectively whereas the free enzyme retained 71, 52 and 35% of the residual activities respectively. Thus pH stability of endoglucanase was considerably increased after encapsulation (Figure 4). A shift in optimum temperature to 55°C was observed in case of entrapped enzyme whereas the free enzyme had an optimum temperature of 50°C (Figure 4).

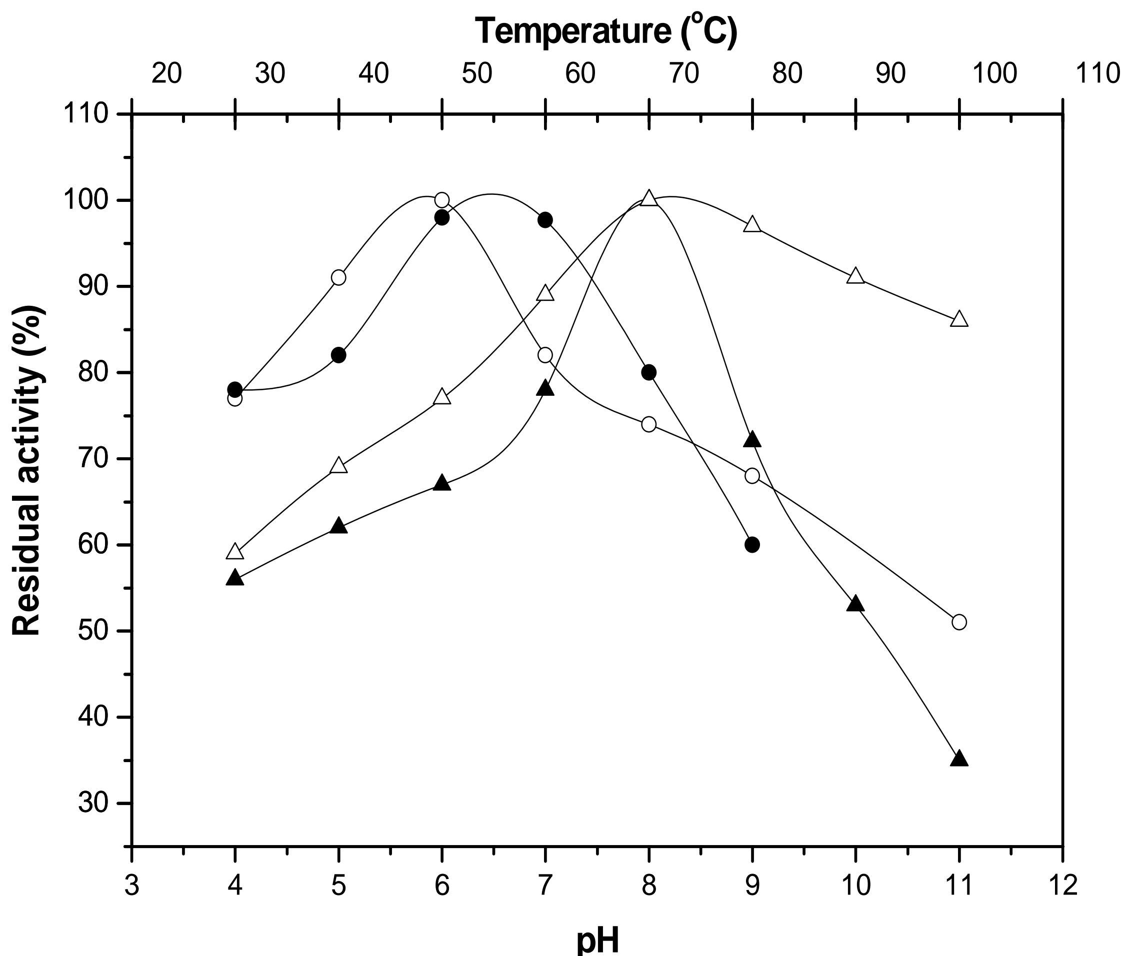


Figure 4: The pH stability of endoglucanase action for free (○) and entrapped enzyme (●) at 50°C. 0.05M acetate buffer (pH 4-5), 0.05 M phosphate buffer (pH 6-7), 0.05 M Tris-HCL buffer (pH 8), 0.05 M carbonate-bicarbonate buffer (pH 9-11) were used. Temperature optima of endoglucanase action of free (△) and entrapped enzyme (▲) at various temperatures: 30°C, 40°C, 50°C, 60°C, 70°C, 80°C and 100°C.

The entrapped enzyme retained 100% activity after 2h whereas the free enzyme exhibited 74% residual activity after 1 hour and negligible activity after 2h at 70°C (Figure 5A). The entrapped endoglucanase retained 70% of the residual activity after 4h at 60°C (Figure 5A). The polymer-entrapped enzyme exhibited a half life of about 3 $^{1}/_{2}$h at 80°C where as the free endoglucanase had a half life of only 8 min (Figure 5B). As compared to the free enzyme the encapsulated enzymes demonstrated higher temperature stability at 50, 60, 70 and 80°C.

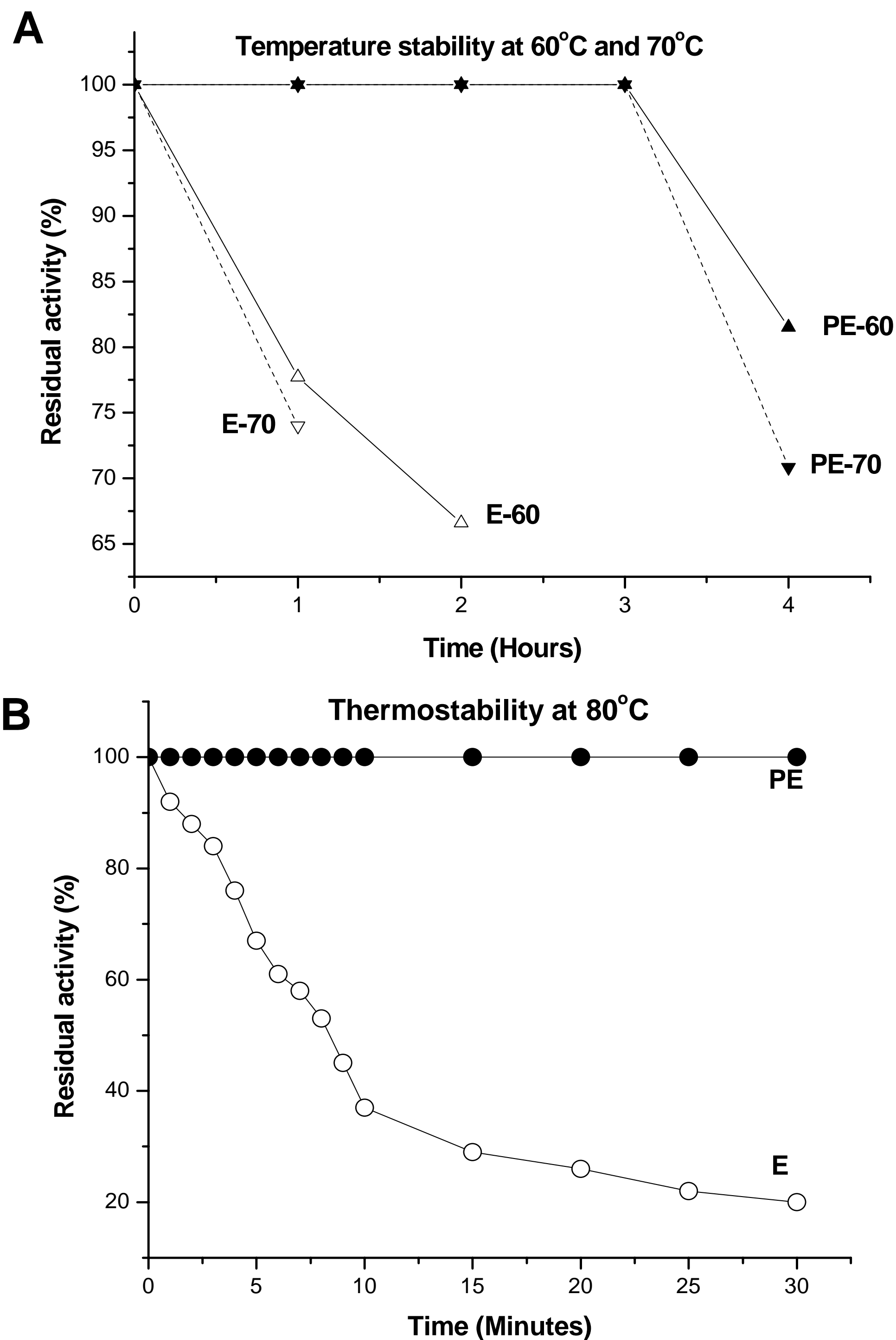


Figure 5: Temperature stability of free (hollow symbols) and entrapped endoglucanase (solid symbols) at (A) 60°C, (△,▲) and 70°C (▽,▼) (B) 80°C (○,●). 2.5 IU of endoglucanase was incubated in 0.05M acetate buffer at various temperatures.

Stability of entrapped endoglucanase in the presence of commercial detergents

The free endoglucanase was stable for 1 hour in presence of different detergents like Ariel, Surf Excel and Henko and retained about 85%, 72% and 66% residual activities respectively (George et al., 2001). The entrapped enzyme showed higher stability in the presence of Henko (95%), Ariel (93%), Tide (86%) and Surf Exel (84%). The encapsulated endoglucanase retained 100% activity up to 60 days whereas the free enzyme exhibited 50% residual activity at the end of 15 days (Table 1). The stability of the encapsulated enzyme in the presence of the commercially used detergents is an added advantage for its application.

Table 1: Shelf life of the endoglucanase

No. of Days	Residual activity (%)	
	Endoglucanase	Endoglucanase (Entrapped)
0	100	100
5	96	100
10	80	100
15	50	100
20	36	100
25	-	100
30	-	100
60	-	100

Spray drying of cellulase

The spray dried CMCase retained 100% activity after spray drying at 70-50ºC. But the powder so obtained was hygroscopic and had a half life of 10 min at 80ºC. Further studies using additives, enhanced the stability of the enzyme at 80ºC. Using Maltodextrin as an additive, the spray drying process could be carried out at a relatively higher temperature 90-50ºC which gave better CMCase powder. Maltodextrin also imparted better stability properties to the enzyme. The shelf life of the enzyme increased in presence of Maltodextrin and the inlet temperature of spray drying could be increased to 90°C (Table 2).

Table 2 : Stability of cellulase during spray drying processing

Sample	Temperature (°C)		Activity (%)	Stability
	Inlet	Outlet		
Enzyme	70	50	100	1 month
Enzyme	90	50	0	-
Enzyme + Sorbitol	70	50	100% but hygroscopic	-
Enzyme + sorbitol	90	70	0	-
Enzyme + Maltodextrin	70	50	100	3 Months
Enzyme + PEG	90	50	75	15 days

Few biopolymers such as starch, alginates have been used for entrapment of enzymes and proteins. Starch being neutral in charge has to be either artificially charged or can be used to entrap proteins such as amylase that binds to starch (Ongen et al, 2002). Alginates are hydrophilic polymers having ion binding properties and are used for encapsulation in combination with Ca^{2+} ions (Bregni et al, 2000). Xanthan-alginate spheres and Ca^{2+}-alginate hydrogels are used to encapsulate proteins such as urease, subtilisin, bovine serum albumin and hemoglobin. The controlled release of protein from alginate matrix was achieved by dissociation of physical hydrophobic network (Elcin, 1995; Leonard et al., 2004; Ariel et al, 2005). Subtilisin encapsulated in alginate showed enhanced temperature stability with potential for use in detergents (Aerial et al, 2005; 2006). The temperature and pH stability of urease has been shown to increase on encapsulation in xanthan-alginate spheres. The optimum temperature for the activity of urease also shifted from 50 to 60°C (Elcin, 1995). Spherulites are also demonstrated for encapsulation of alkaline phosphatase and lipase. (Bernheim-Grosswasser et al, 2000). Though alginates and vesicular systems can be used for encapsulation, only a limited increase in shelf life of the enzyme was achieved since the water activity of these systems could not be controlled.

Conclusions

The encapsulation of endoglucanase is successfully carried out for the first time in a biodegradable and naturally charged polymer, gum arabic, with slow release of active enzyme suitable for application in detergents and textile industry. The results of the entrapment of the endoglucanase in gum arabic support the hypothesis that it is possible to obtain a system, which can be triggered to degrade, when the water activity is increased upon water uptake thus resulting in reactivation of the entrapped enzyme. In addition, water activity can indeed be used as a tool to inhibit the enzyme activity during storage and increase shelf life of the enzyme. In the present study use of gum Arabic for controlled release of endoglucanase increased thermal and pH stability with extended shelf life of the enzyme which is a preferable trait for biotechnological applications. Spray drying of the enzyme also enhanced its shelf life.

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SECTION B

EVALUATION OF THE CELLULASE ACTIVITY IN TEXTILE INDUSTRY ON BIO-FINISHING OF DENIMS

Abstract

Use of cellulase for denim washing is a standard eco-friendly technique to achieve desirable appearance and softness for cotton fabrics and denims. But enzymatic washing of denim till date involved acid cellulase (*Trichoderma reesie*) and neutral cellulase (*Humicola isolens*) the use of which has a drawback of back staining of the indigo dye on to the fabric. Though it has been suggested that pH is a major factor in controlling back staining there are no reports on use of cellulase under alkaline conditions for denim washing. In this paper for the first time an alkali stable endoglucanase from alkalothermophilic *Thermomonospora* sp. (T-EG) has been used for denim biofinishing under alkaline conditions. T-EG is effective in removing hairiness with negligible weight loss and imparting softness to the fabric. Higher abrasive activity with lower backstaining was a preferred property for denim biofinishing exhibited by T-EG. The activities were comparable to acid and neutral cellulases that are being regularly used. The enzyme was also effective under non-buffering conditions which is and added advantage for use in textile industry. A probable mechanism of enzymatic finishing of cotton fabric has been represented based on the unique properties of T-EG.

Introduction

Cotton fabrics are usually preferred due to the good physico-mechanical properties and comfort they provide. Cotton chemically is pure celluose. Cellulose is a polymer of glucose linked by β-1, 4 glycosidic linkages. Endoglucanases (EG), cellobiohydrolases (CBH) and beta-glucosidases are cellulolytic enzymes, involved in the degradation of cellulose which act synergistically to hydrolyze cellulose (Teeri and Koivula, 1995). Cellulases have applications in brewing, paper pulp and detergent industries (Bhat and Bhat, 1997). In textile industry the traditional use of pumice stone in a water- loaded tumbling machine produces severe wear & tear and loss of tensile strength when used to achieve high degree of indigo fading (Kochavi, et al., 1990; Kumar and Harnden, 1999; Tyndall, 1990a). Due to increasing environmental concerns and constraints being imposed on textile industry, cellulase treatment of cotton fabrics is an environmentally- friendly way of improving the property of the fabrics (Belghiht et al., 2001). A more recent trend is the use of cellulases for improving the surface properties of cellulose-based products such as textile and paper (Prasad et al., 1992; Zeyer et al., 1993). In the textile industry, cellulases are widely replacing the pumice stones for the 'stone washing' of denim (Olson, 1988; Tyndall, 1990b). 'Enzymatic biopolishing' is another biological process that is currently being developed to reduce the pill of cotton fabric (Kochavi et al., 1990; Pedersen et al., 1992; Chikkodi et al., 1995). In this case, cellulases achieve a controlled surface hydrolysis that essentially reduces the fuzziness of the fabric with the consequence of clearer surface structures and more vivid colours. The advantages in the replacement of pumice stones by a cellulase-based treatment include: (1) reduced wear and tear of washing machines and short treatment times; (2) increased productivity of the machines because of high loading; (3) substantial decrease of second quality garments; (4) less work-intensive and safer working conditions; (5) safe environment, since pumice powder is not produced; (6) flexibility to create and consistently reproduce new finished products; and (7) the possibility to automate the process with computer-controlled dosing devices when using liquid cellulase preparations (Galante et al., 1998).Evaluation of abrasion and back-staining of denim garments by reflectance measurement using neutral (from *Humicola insolens*) and acidic (from *Trichoderma reesei*) cellulases revealed that the former caused higher abrasion and less back-staining than the latter (Galante et al., 1998a). The exact reason for the

differential levels of back-staining by the acid and neutral cellulases is not known. Initially, the acidic pH during treatment was believed to be responsible, but this was found not to be the case. In fact, there are indications that some acid cellulases facilitate low levels of back-staining, while some neutral cellulases show high re-deposition of indigo (Galante et al., 1998a). Hence, these results cautioned that the pH profile alone should not be considered as the sole reason for its potential performance during bio-stoning.

Denim washing with cellulases is a technique, providing an environmentally friendly process to achieve a desirable appearance and soft handle for fabrics (Sinistyn et al., 2001). The application of *Trichoderma reesei* (acid) and *Humicola insolens* (neutral) cellulases have been demonstrated in denim biofinishing but during the enzymatic treatment, backstaining of the white yarn of denim fabric is a disadvantage. The basic mechanism of indigo redeposition or backstaining involves binding the dye to cellulase molecules adsorbed on the surface of cellulose fibers. Experimental evidence indicates that *Humicola insolens* enzyme (neutral) gives less dye redeposition than *Trichoderma reesei* enzymes (acidic) suggesting that pH is the major factor in controlling backstaining (Cavaco-Paulo et al. 1998; Gusakov et al., 2000a; b). Thus, in the textile industry, there is a need for novel cellulases that are active at neutral and alkaline pH values, have short reacting time, do not compromise the strength of fabric and have good denim finishing properties, including low backstaining. An alkalothermophilic *Thermomonospora* sp. has been isolated in our laboratory, secreting maximally endoglucanases (T-EG) and xylanases, active and stable in alkaline conditions with negligible exoglucanase activity. The present chapter deals with the production, and the practical evaluation of T-EG for its effectiveness with respect to the removing hairiness with negligible weight loss, softness, backstaining and abrasive acitivity under alkaline condition for the first time. A schematic mechanism of enzyme action in biofinishing based on the unique properties of the enzyme has also been proposed.

Materials and methods

Production of endoglucanase

An inoculum of *Thermomonospora* sp. in a medium as described by George et al. containing 2% wheat bran (2001 a) grown for 48h at 50°C was added (10%) to the fermentation medium (10L in 14L fermentor). The pH of the fermentation medium was adjusted to pH9 using sterile 10% sodium carbonate as described in chatper II. The culture was at 50°C for 120h. An aeration of 2.5L of air/hour was continuously supplied with an agitation of 200rev/ min. After 120h of aerobic cultivation under the specified growth conditions, the cells were harvested by centrifugations at 10,000rpm for 10min. The culture filtrate (10L) so obtained was ammonium sulphate precipitated and used as a source of enzyme. Enzyme production was also carried out in shake flasks as mentioned in Chapter II.

Trichoderma cellulase and *Humicola* cellulase were obtained from Novozyme company, Denmark.

Enzyme activity assay

The activities for endoglucanase / xylanase was measured as mentioned in Chapter II. One international unit is defined as the amount of enzyme required to liberate 1μmole of glucose or xylose from carboxymethyl celllose or xylan respectively in one min under the said assay conditions. Protein concentration was determined according to the method of Bradford (1976), using bovine serum albumin as standard.

Identification of optimum pH, temperature, and stability of endoglucanase

Estimation of carboxymethyl cellulase and xylanase activities at different pH(4–10) and temperature (40–100°C) values was carried out under standard assay conditions to determine optimum pH and temperature for enzyme activity. The pH stability of the enzyme was measured by incubating 5 IU enzyme at 50°C in buffer of desired pH for 1h. The following buffer systems were used: 0.05M acetate buffer (pH 4-5), 0.05M phosphate buffer (pH6-7), 0.05 M tris-HCl buffer (pH8), and 0.05M carbonate-bicarbonate buffer (pH9-10). The temperature stability was determined by incubating 5IU enzyme in 0.05mM phosphate buffer, pH7 at different temperatures.

The samples were removed at regular intervals and the residual activity was estimated under standard assay conditions.

Fabric Treatment

The evaluation of application of T-EG on Denim fabric was carried in a Siemens front-loading washing machine. 200g Denim fabrics were treated with different concentrations of T-EG and commercially available Novozyme (*Trichoderma* cellulase and *Humicola* cellulase) separately in water of hardness 50ppm (parts per million). The fabric was incubated at 55°C for 1 hour wherein a material to liquor ratio of 1: 50 was maintained. The experiments under buffering conditions were carried out at pH 5 for *Trichoderma reesie* cellulase, pH7 for *Humicola* cellulase and pH 8 for T-EG. Experiments under non- buffering conditions were carried out in normal Tap water (pH 7.8) for T-EG enzyme. After one hour, the enzyme was inactivated by raising the temperature of the treating medium to 95°C for 10min. The treated fabric was rinsed thrice with normal tap water and dried in normal air continued by drying in air oven at 100°C. Evaluation of the effectiveness of the enzyme was judged through comparative study of both the treated and un-treated fabric.

Hairiness measurement

Hairiness measurement by microscopic methods

Determination of the number of hairs in the specified hair length group in a pre-selected area of the control and enzyme treated fabric is counted by microscopic examination.

The total hair (fibre) length is another way of calculation which shows an average total length based on the length and number of fibres in each group in the pre-selected area of the treated and untreated fabric.

Hairiness measurement by American society of testing materials (ASTM) D 5647 – 01 method

In this method the indigo dyed denim yarn is passed through a photo-electric devise containing a light beam that is interrupted by the occurrence of protruding

fibers or filaments. By the use of digital volt meter, the number of protruding fibers or filaments of a specific length along a specified surface length of yarn are counted and reported as hairiness.

Measurement of cellulase abrasive activity

The abrasive activity (denim-washing performance) of cellulases was determined using model microassays developed by Gusakov et al. (Gusakov et al., 2000c). The analysis of color intensity on denim fabric samples was carried out using a scanner (hp Scanjet 5470c). An area selected on the treated denim fabric was scanned at 300 dpi resolution. The JPEG images obtained as a result of scanning were analyzed using Adobe Photoshop software. A histogram of color intensities was calculated for each sample using a blue channel of the software, and a percentile at level 32(P32) was calculated. The difference between the average value of P32 obtained in the control and on the enzyme treated fabric was taken as an indicator of the abrasive activity of the enzyme, and was expressed in relative units.

Measurement of indigo backstaining activity

The back staining activity of cellulases was determined using model microassays developed by Gusakov et al. (Gusakov et al., 2000c). The analysis of color intensity on denim fabric samples was carried out using a scanner (hp Scanjet 5470c). An area selected on the treated denim fabric was scanned at 300 dpi resolution. The JPEG images obtained as a result of scanning were analyzed using Adobe Photoshop software. A histogram of color intensities was calculated for each sample using a blue channel, and a percentile at level 120 (P120) was used as back staining index. An average value and standard deviation were calculated for each enzyme preparation under testing, and they were expressed in relative units.

Measurement of Flexural rigidity

The overall flexural rigidity of the fibers was determined by American society of textile materials (ASTM) D-1388-64 method. The stiffness of the fiber is determined by the Cantilever method. A strip of the fabric is slid at a specified rate in a direction parallel to its long dimension, until its leading edge projects from the edge of a horizontal surface. The length of the overhang is measured when the tip of the

specimen is depressed under its own mass to the point where the line joining the top to the edge of the platform makes a 41.5º angle with the horizontal. From this measure length the bending length and flexural rigidity are calculated.

Subjective assessment of performance of enzyme

A panel of three experts rated the fabric based on the following parameters:- i) Reducing hairiness, ii) Total weight loss, iii) Impartation of softness, iv) Wash down effect, v) Back staining, vi) Colour contrast and vii) Seam puckering.

Results and Discussion

The maximum endoglucanase production of 12 IU/ml and xylanase of 200IU/ml was obtained at 120h (Figure 1.)

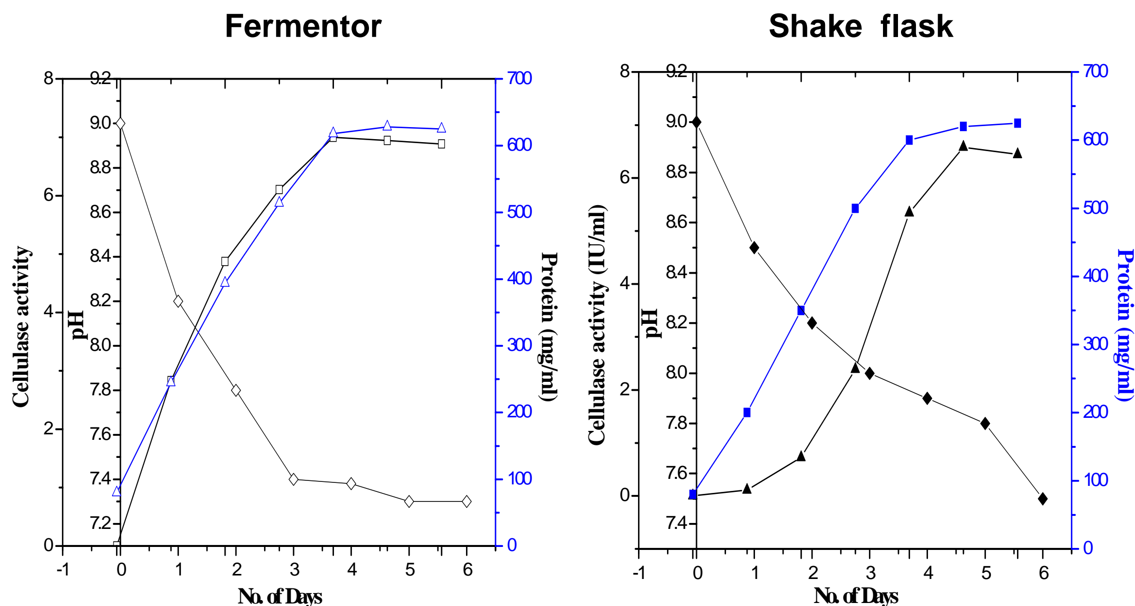

Figure 1: The time course of T-EG production by the alkalothermophilic *Thermomonospora* sp. by shake flask (100ml) (solid symbols) and fermentor (10L) (hollow symbols). The extracellular culture filltrate was estimated for: protein (△,▲), cellulase activity (□, ■), pH of medium (◇,◆).

The extracellular culture filtrate was precipitated by ammonium sulphate and was used as a source of enzyme (T-EG) for the further work. It showed upto % activity in the pH range 4-10. The endoglucanase had pH stability in a broad range (4-10). It was most stable at pH8. The enzyme was thermostable showing a half life of 20min at 80°C at a protein concentration of 50mg/ml (George et al., 2001a). The cellulases from *Trichoderma reesei* (acid), *Humicola isolens* (neutral) and T-EG were used at a concentration of 40IU/ml for the experiments.

Denim biofinishing under buffering conditions

Hairiness of the fabric

The fabric (200g) was treated with T-EG at pH8 and with commercial enzymes, *Trichoderma* cellulase and *Humicola* cellulase at pH5 and 7 respectively (as recommended by the manufacturers). The other treatment conditions are as mentioned

in Table 1. T-EG was effective in removing the protruding hairs of the length of 1-2mm and 2-3mm in the preselected area of the fabric. As seen from the table. 2 the activity of T-EG was comparable to that of *Humicola* cellulase and better than *Trichoderma* cellulase.

Table 1: Condition maintained during treatment of cotton fabric with the enzymes.

1.	Fabric type	Denim
2.	Weight of fabric	200 gms, 420g, 1.2Kg
3.	Enzyme used	a) *Thermomonospora* cellulase (T-EG) Commercial enzymes b) *Humicola* cellulase c) *Trichoderma* cellulase
4.	Material to liquor ratio	1: 50
5.	Hardness of water used	50 ppm
6.	Temperature	55°C
7.	Time of treatment	1h
8.	Rinsing (with normal tap water)	Three times
9.	Drying conditions	Normal air-drying.

Table 2: Measurement of hairiness of the fabric by microscopic method.

Enzyme used	**Average no. of fiber (hair) per 10cm length of fabric.**							
Ratio of enzyme to fabric (v /w) – ml/g	**0.5%**		**1.0%**		**1.5%**		**3.0%**	
Length of fibre in mm	**1-2**	**2-3**	**1-2**	**2-3**	**1-2**	**2-3**	**1-2**	**2-3**
***Trichoderma* cellulase**	151	28	101	22	92	18	36	11
***Humicola* cellulase**	110	35	82	12	71	13	63	11
T-EG enzyme	87	17	86	16	68	14	62	10
No enzyme	1-2mm =177				2-3mm = 58			

IU/ml of enzymes used were *Trichoderma* cellulase, 40IU/ml; *Humicola* cellulase, 40IU/ml and T-EG, 40IU/ml.
The enzyme treatments were carried out at pH5 for *Trichoderma* cellulase, pH7 for *Humicola* cellulase and pH8 for T-EG

The efficiency of the T-EG in reducing hairiness of the fabric during biofinishing was also confirmed by calculating the average length of the hairs in a pre-determined area before and after treatment with the enzyme. It is observed from Figure 2 that there is a decrease in hairiness on treatment with T-EG. A steady decrease in hairiness of the fabric is seen on increasing the dosage of the enzyme up to 1.5%. It is also observed to be as effective as *Humicola* cellulase and better than *Trichoderma* cellulase.

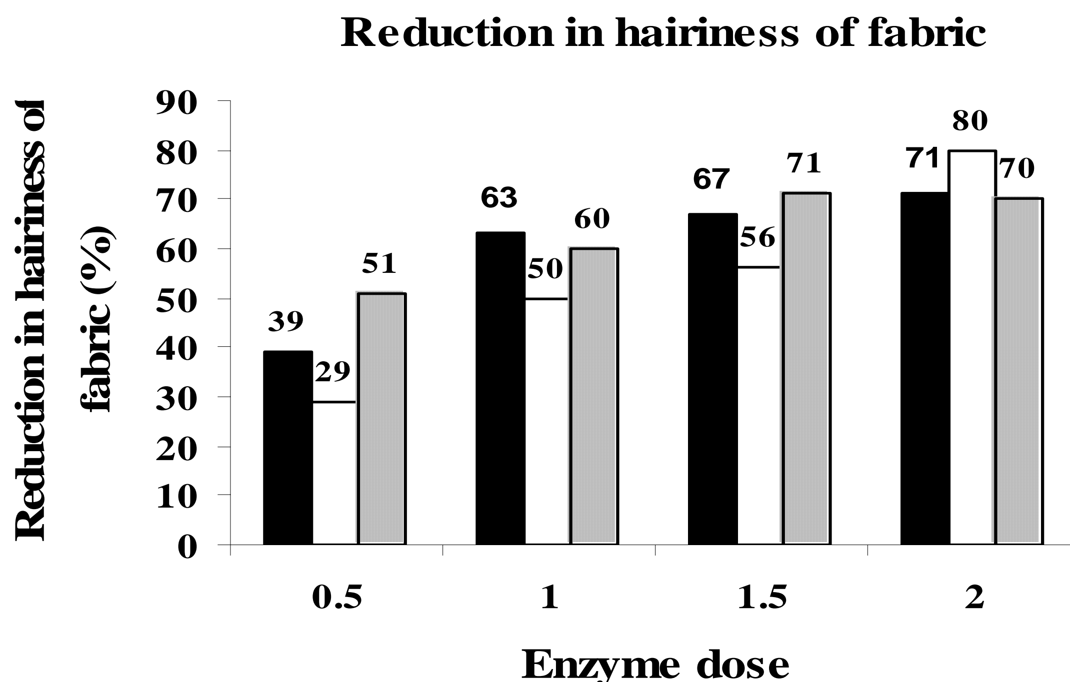

Figure 2. Measurement of hairiness of the denim fabric by calculating the total length of hairs in the preselected area of the fabric after treatment with the following enzymes : (■) *Humicola* cellulase; (□) *Trichoderma* cellulase and ( ) T-EG.

The effectiveness of T-EG in reducing hairiness was confirmed by determining the ability of the enzyme to reduce the number of protruding hairs of the indigo dyed yarn. This was done by ASTM D 5647 – 01 method. It was found that T-EG had better action on the protruding fibres of the length of 3-10 mm than the 1-2mm fibers. It was also observed that T-EG exhibit

Table 3: Determination of hairiness of yarn by ASTM D 5647 – 01 method.

Yarn sample treated with	Ratio of enzyme to fabric (IU/w) – ml/g	S_3 ***	No. of hairs of the preselected length zones (Per 100 m)						
			1 mm	2 mm	3 mm	4 mm	6 mm	8 mm	10 mm
No enzyme	-	2997	15284	4165	1724	979	228	58	8
Humicola cellulase	40	1222	8682	1956	789	350	67	13	2
T-EG	40	1259	12917	2744	850	364	39	6	-

S_3 ***: Sum of all hairs from 3 mm to 25 mm length zones.
The enzyme treatments were carried out at pH 7 for *Humicola* cellulase and pH 8 for T-EG

comparable activity in reducing hairiness of the fabric to that of *Humicola* cellulase (Table 3).

Total weight loss of the fabric

Loss of tensile strength of the fabrics is directly related to the degradation of crystalline cellulose which is highly ordered. Amorphous cellulose is less structured and easily accessible; hence a single type of cellulase preferentially endoglucanase will be sufficient for degradation of amorphous cellulose causing fuzz and piling of the fabric. The weight loss was less than 2% suggesting that the weight loss may be only due to the removal of surface fibrils, seed coat fragments, water extractable material and other natural impurities from the denim fabric. Considerable decreases in the hairiness of the fabric on treatment with T-EG only lead to negligible weight loss of the fabric after biofinishing (Table 4). These results were comparable to treatment of denim fabric with *Humicola* cellulase (Table 4).

Table 4: Total weight loss of the fabric.

Enzyme	**Ratio of enzyme to fabric (v /w) – ml/g**	**% weight loss of fabric.**
T-EG	0.5 %	0.65
	1.0 %	0.99
	1.5 %	1.19
***Humicola* cellulase**	0.5 %	0.59
	1.0 %	1.02

The percentage decrease in weight of the fabric was calculated to estimate the total weight loss during the treatment with the respective enzymes.
The enzyme treatments were carried out at pH 7 for *Humicola* cellulase and pH 8 for T-EG

Softness of the fabric

Impartation of softness to the fabric was estimated indirectly by measuring the flexural rigidity of the fabric. The flexural rigidity of the fabric is inversely proportional to the softness of the fabric. It is observed from table 5 that T-EG imparts softness to the fabric and is comparable to commercial enzyme, *Humicola* cellulase.

Table 5: Determination of Overall Flexural Rigidity (Method:ASTM D-1388-64).

Enzyme	Ratio of enzyme to fabric (IU /w) – ml/g	Stiffness (mg.cm)
No.enzyme (control)	-	1165
Humicola cellulase	40	1100
T-EG	40	1145
T-EG	50	1040

The yarn is treated with the enzymes under the same conditions as mentioned in table 1.
The stiffness of the fibre is inversely proportional to the softness of the fabric.
The enzyme treatments were carried out at pH 7 for *Humicola* cellulase and pH 8 for T-EG

Abrasive activity and back staining index

An ideal biostoning enzyme would pocesses high abrasive activity as well as low backstaining (Gusakov et al., 2000). Higher abrasive activity with a lower backstaining index is a pre-requisite for application of cellulase in biofinishing of denim. Figure 3A shows that T-EG exhibits higher abrasive activity as compared to *Humicola* cellulase with a marginally lower backstaining index (3B).

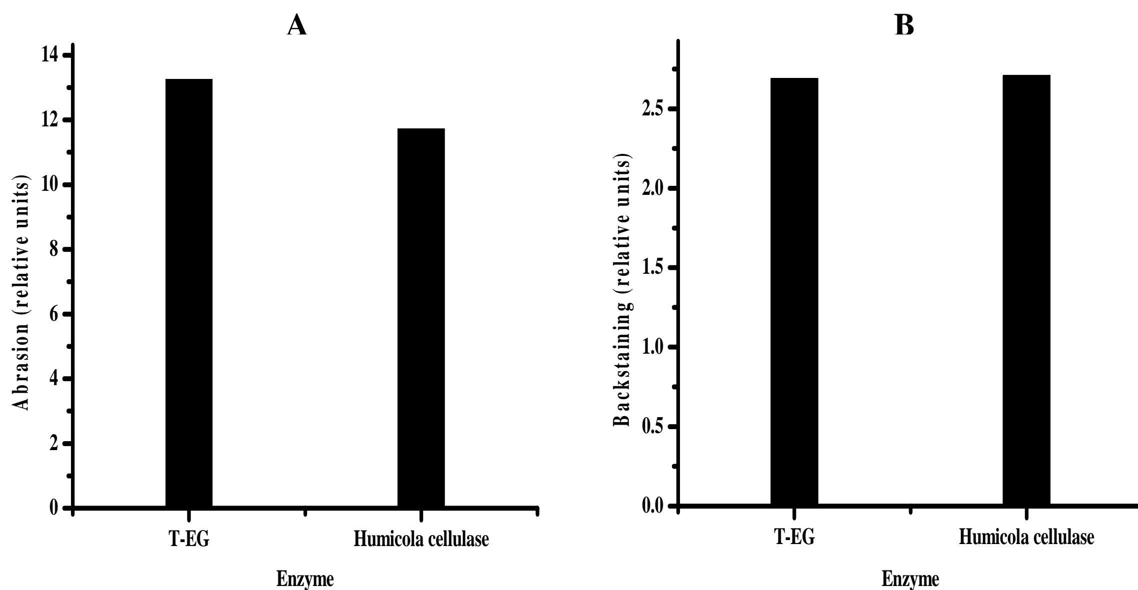

Figure 3: Abrasive activity (A) and backstaining (B) for T-EG and *Humicola* cellulase in the process of Denim treatment at 1.5% concentration each under buffering conditions.

Subjective evaluation of the fabric

Subjective evaluation of the denim fabric (200g) after treatment with T-EG rates it to be effective in reduction of hairiness, impartation of softness, wash-down effect and back Staining. T-EG works better than *Trichoderma* cellulase and comparable to *Humicola* cellulase (Table 6).

The performance of effectiveness of T-EG on bio-finishing (420 g of fabric) also yielded similar results. The enzyme was active in reducing hairiness, back staining, wash down effect and imparting softness to the fabric (Table 7 A). These results were reproducible on treating larger amount of fabric (1.2 Kg constituted by a trouser and a jacket) under the same condition (Table 7 B) thus establishing the reproducibility of results from fabric to garment.

Table 6: Subjective assessment of performance of enzyme in biopolishing of Denim (200g) treated under buffering conditions.

Enzyme	**Ratio of enzyme to fabric (IU /w) – ml/g**	**Expert**	**Expert Rating of Properties**					
			a	**b**	**c**	**d**	**e**	**Total**
T-EG	40	**A**	3	1	2	1	2	**9**
		B	3	2	1	2	3	**11**
		C	2	2	1	1	3	**9**
***Humicola* cellulase**	40	**A**	2	2	1	2	3	**10**
		B	2	1	2	1	2	**8**
		C	3	1	2	2	2	**10**
***Trichoderma* cellulase**	40	**A**	1	3	3	3	1	**11**
		B	1	3	3	3	1	**11**
		C	1	3	3	3	1	**11**

a- Reduction of Hairiness, b-Impartation of Softness, c-Wash-Down Effect, d-Back staining, e-Colour Contrast.
Rating: 1- best, 2- better, 3- good……
The enzyme treatments were carried out at pH 5 for *Trichoderma* cellulase, pH 7 for *Humicol*a cellulase and pH 8 for T-EG

Table 7: Subjective evaluation of the denim fabric.

	Rating for fabric treated with different enzyme and conditions					
Experiment **Quantity of fabric**	**A** **420 g of fabric**			**B** **1.2 kg fabric**		**C** **1.2 Kg fabric**
Enzyme	***Humicola* cellulase**	**T-EG**	**T-EG**	***Humicola* cellulase**	**T-EG**	**T-EG**
pH	7.0	8.0	8.0	7.0	8.0	7.8 (pH of tap water)
Ratio of enzyme to fabric (IU /w) – ml/g	40	40	50	40	40	40
Properties						
Impartation of softness	ND	ND	ND	4	4	ND
Back staining	3	4	4	4	4	4
Depth of colour	3	4	4	3-4	4	4-5
Colour contrast	4	3-4	4	4	3-4	2-3
Wash down effect	4	3	3	4	3-4	3
Reduction of surface hairiness	4-5	4	4-5	4	4	3-4

Rating: 1- worst; 2- better; 3- …. 5 best; ND- not determined
A jacket and a trouser constitute 1.2 Kg of the fabric which was used.

Denim biofinishing under non- buffering conditions

Measurement of hairiness of the fabric

T-EG is also active under non-buffering conditions i.e in normal tap water (pH 7.8). A stable decrease in the number of protruding hairs (fibres) of the fabric is clearly seen in table VIII. It exhibits better action on the protruding fibres of length 2-3mm than 1-2mm. These results were comparable to *Humicola* cellulase treated at pH 7.

Table 8: Hairiness measurement of denim fabric treated under non-buffering condition.

Treatment	Ratio of enzyme to fabric (v /w) – ml/g	Average Number of Hair in pre-selected length group/ 100 cm of fabric.	
		1-2 mm	**2-3 mm**
No enzyme	-	562	133
T-EG	0.5 %	500	71
T-EG	1.0 %	486	48
T-EG	1.5 %	428	38
***Humicola* cellulase**	1.0 %	390	57

Biofinishing was carried out in tap water pH (pH 7.8) for T-EG and at pH 7.0 for *Humicola* cellulase

Measurement of abrasive activity and back staining

T-EG shows better abrasive activity than the commercially used enzyme, *Humicola* cellulase (Figure 4A) where as a marginally lower backstaining activity is exhibited by T-EG as compared to *Humicola* cellulase (Figure 4B). This is a preferred character for industrial application.

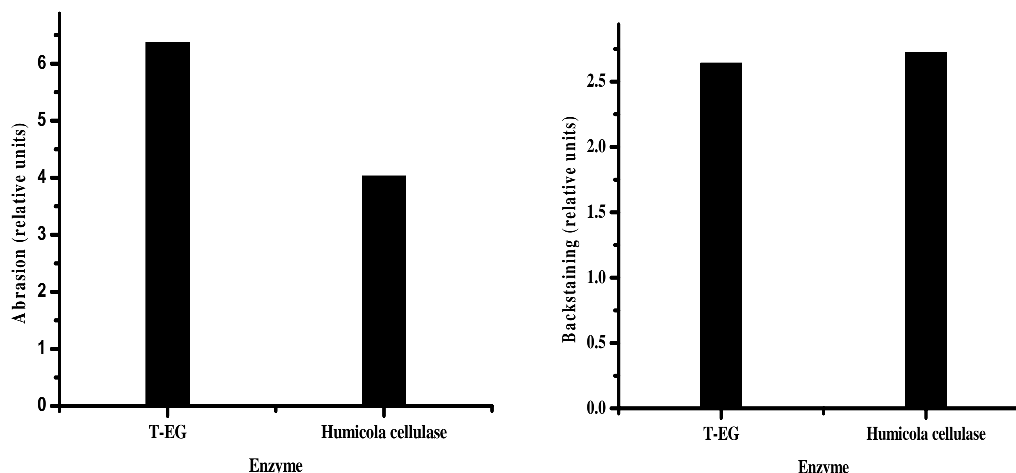

Figure 4: Abrasive activity (A) and backstaining (B) for T-EG and *Humicola* cellulase in the process of Denim treatment at 1.5% concentration each under non-buffering conditions.

Subjective evaluation

T-EG is found to show low backstaining, good colour contrast and wash down effect with better depth of colour of the denim fabric on subjective evaluation (Table 7C).

Mechanism of biofinishing

Understanding the behavior of the cellulolytic enzymes towards the cotton fibre is essential to minimize the related negative effect on the strength of the cotton fabric. Cellulose is made up of highly ordered crystalline region and less structured amorphous region. The degree of departure from crystallinity is variable to form the less structured purely amorphous region with all degrees of order in between. The amorphous region is generally responsible to form the fuzz and pilling in cotton fabric. Hence a single type of cellulase preferentially endoglucanase may probably be sufficient for degradation of amorphous cellulose with minimum weight loss. It has also been shown that *Trichoderma* cellulase and cellulase which exhibited the highest filter paper activity i.e. action on crystalline cellulose, showed the most aggressive action on cotton in agitated system (Csiszar et al., 2001). This may also lead to the weight loss of the fabric. There is also evidence that during biosynthesis of cellulose the associated hemicelluloses regulate the aggregation process. Thus application of cellulase for biofinishing along with xylanases may probably have an added

advantage mainly in removal of the surface fibrils, small protruding fibres, seed coat fragments and other natural impurities of cotton fibre. Use of xylanase along with cellulase in optimum concentration at suitable conditions for enzymatic action will prove to be beneficial with minimum harm to the fabric during biofinishing**.**

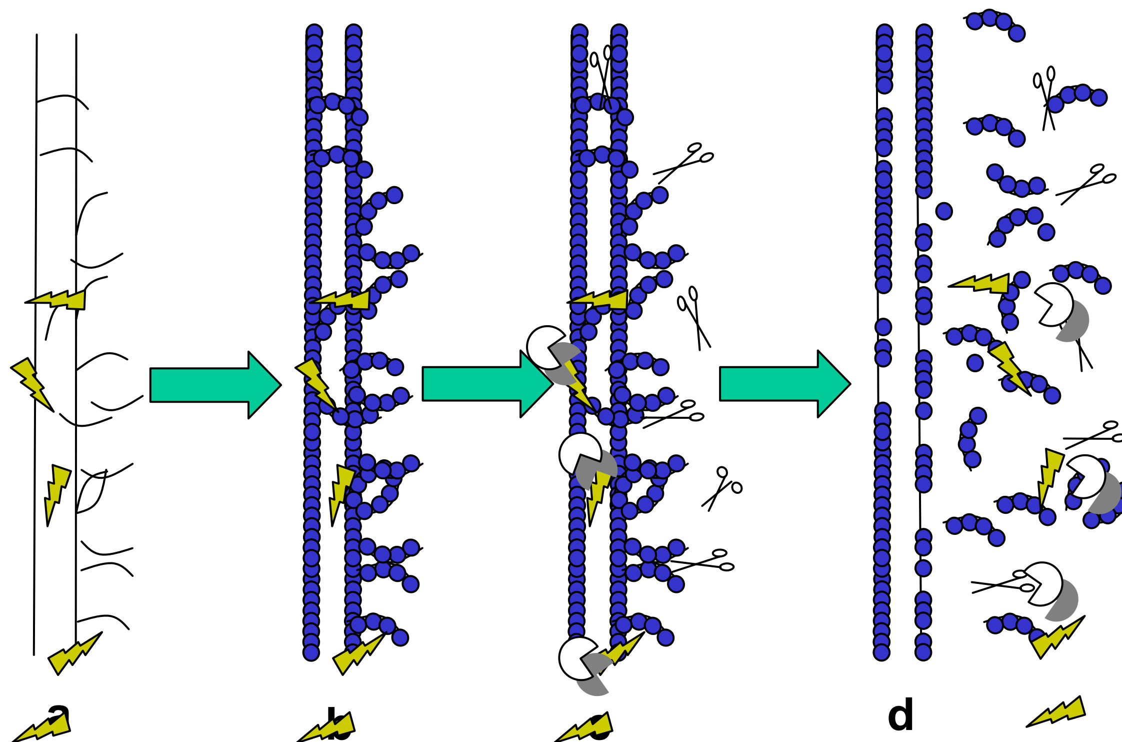

Figure 5: Mechanism of action of cellulase on Denim fabric: A) Desized Denim fabric without indigo stain; B) Indigo dyed denim fabric; C) Action of endoglucanase (represented by scissors) on the protruding fibres of the denim fabric during biofinishing process and xylanase ( ) on seed coat and other impurities D) Biofinished denim Fabric.

The present work describes the use of alkali stable cellulase with negligible activity on crystalline cellulose from alkalothermophilic *Thermomonospora* sp. for biofinishing of denim fabric. We represent here a schematic diagram that indicates the probable mechanism of enzymatic finishing and stone washing of denim garment by endoglucanase (Figure 5). During indigo staining of denim garment the indigo dye particles get adhered to the microfibrils that are present on the surface of the garment (Figure 5. b). Endoglucanase acts on the amorphous region of the cellulose forming the protruding hairs on the fabric generally responsible for causing fuzz and pilling on the fabric as seen in the scanning electron micrograph and loosens it. The mechanical action in the washing machine in turn removes the loosened fibers to give a final finished product (Figure 5 d; 6 b). Also as the protruding fibers are removed the

indigo particles adhereing to the surface of the protruding fibers are also removed thus giving the fabric a patchy appearance which is preferred (Figure 5 d). Xylanases will act on the seed coat fragments and other natural impurities thus giving a final finished touch to the cotton fabric.

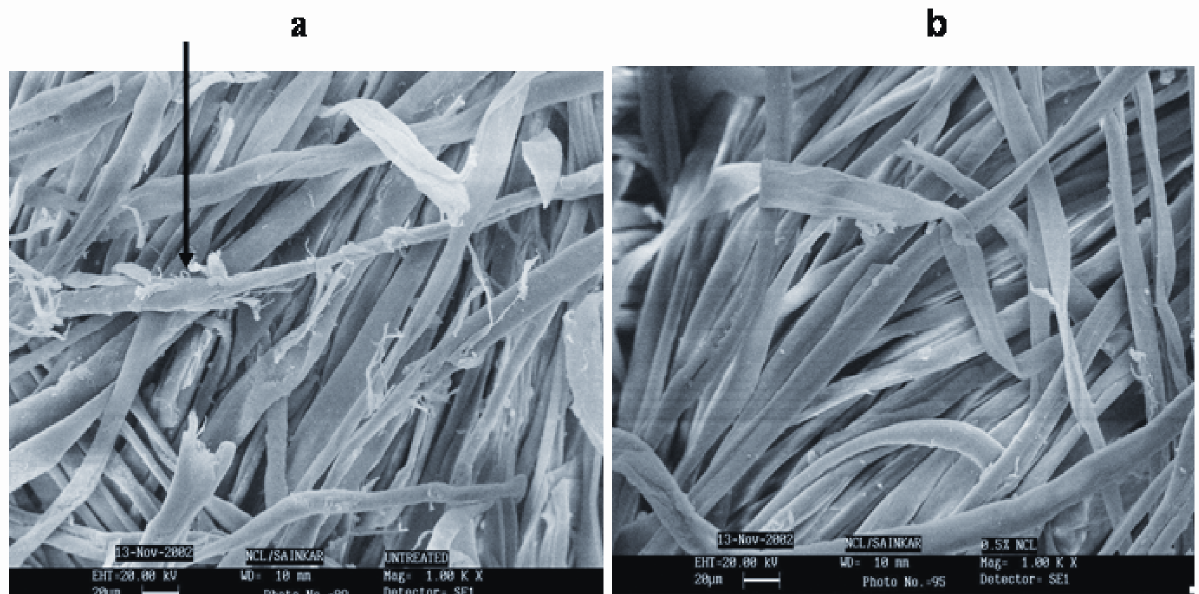

Figure 6: Scanning electron micrograph of Denim fabric under 1000 X magnification: a) Untreated denim fabric – arrow indicates the protruding fibers; b) Denim fabric treated with T-EG.

Conclusion

Cellulase treatment of cotton fabrics is an environmentally- friendly way of improving the property of the fabrics [Belghiht et al, 2001]. Although, traditionally, denim jeans manufacturers have washed their garments with pumice (P) stones to achieve a soft handle as well as a desirable worn look, nowadays, the aged look is obtained by non-homogeneous removal of the indigo dye trapped inside the fibres by the cooperative action of the enzymes and mechanical factors such as beating and friction (Cavaco-Paulo, 1998 and Csiszár, et al., 1998). In addition, enzymatic stonewashing allows up to 50% higher jeans load (in replacement to the 70kg of stones) and yields the desired look and a softer finish (Belghith., 2001). Cellulases is also be used to improve the appearance of cellulosic fabrics by removing fuzz fibre and pills from the surface, reducing pilling propensity or delivering softening benefits (Cavaco-Paulo et al., 1996; Tyndall., 1990b). Since cellulose is made up of highly ordered crystalline region and less structured amorphous region, a single type of cellulase preferentially endoglucanase will be sufficient for degradation of amorphous cellulose causing fuzz and piling of the fabric. Treatment of cotton fabric with cellulases along with an appropriate dose of xylanase will help in removal of seed coat fragments and other natural impurities of cotton fibre (Csisz´ar et al, 2001). Similarly xylanase activity associated with T-EG can help in the removal of natural impurities like seed coat from the denim fabric. But use of enzyme for finishing leads to backstaining due to the affinitity of indigo dye towards the cellulose fibre bound enzyme. Alkaline or neutral conditions are preferable for enzymatic processing of denim since lower backstaining takes place under these conditions. Hence use of *Thermomonospora* sp. cellulase rich in endoglucanase and xylanase and having negligible activity towards crystalline cellulose, active and stable under alkaline condition appears to be an excellent alternative for biostoning and biofinishing of denim garments and compare well with commercial cellulases under alkaline conditions. In addition T-EG caused less back-staining and is effective under non-buffering condition which is more preferred for industrial application.

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