XYLANASE FROM AN ALKALOTOLERANT *CEPHALOSPORIUM* SP.,: PRODUCTION, CHARACTERIZATION AND POTENTIAL APPLICATION.

A THESIS
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DECLARATION

Certified that the work incorporated in the thesis entitled "**XYLANASE FROM AN ALKALOTOLERANT *CEPHALOSPORIUM*SP.,:PRODUCTION, CHARACTERIZATION AND POTENTIAL APPLICATION**" submitted by **Miss. Snehal Bansod** was carried out under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

MRele

(Dr. M.V.Rele)

Research Guide

Abstract

Xylanases (1,4-ß-xylan xylanohydrolase; EC 3.2.1.8) catalyze the random hydrolysis of ß-1,4 linkages in xylan, one of the main components of hemicellulose. Cellulase-free xylanases have attracted considerable attention due to their application in pulp and paper industries, where they have been shown to effectively reduce the requirement for toxic chemicals, such as chlorine and chlorine dioxide, in the bleach sequence. Although xylanases have been reported from a wide variety of microbial systems, the enzymes from fungal sources have received more attention. Moreover, these enzymes are often co-secreted along with high levels of extracellular cellulase and exhibit pH optima on the acidic side, thus making them unsuitable for the aforementioned application. In this respect xylanases exhibiting high activity and stability at alkaline pH have enormous commercial significance.

A number of alkalophilic bacteria, especially belonging to the genus *Bacillus*, have been intensively investigated for their ability to produce extracellular enzymes including xylanases. However fungi capable of growing at alkaline pH and secreting extracellular xylanases have not been investigated. Hence, the present investigation was carried out (a) to isolate fungal cultures capable of growing and secreting xylanase under alkaline conditions (b) optimize the fermentation conditions for maximum enzyme production (c) purify and characterize the enzyme and (d) evaluate the potential application of the enzyme in paper and pulp biotechnology.

Chapter 1: General Introduction

This part comprises of literature survey with reference to xylanases from alkalophiles and a comparative study of fungal xylanases.

Chapter 2 : Isolation, Strain Conservation and Media Optimization for Production of *Cephalosporium* Xylanase

An alkalotolerant fungus, identified as *Cephalosporium* sp., (NCL 87.11.9) was isolated from local soil samples. The fungus produced extra-cellular cellulase-free xylanase (18-20 IU/ml in 96

h) when grown at pH 10, in a medium containing 3% (w/v) wheat bran and 1% (w/v) yeast extract as the carbon and nitrogen source respectively. Influence of carbon sources showed that xylan and xylan-rich sources such as wheat bran or corn cobs supported optimal enzyme production. Organic nitrogen supplements such as peptone, tryptone, malt extract, ground nut cake, ProFlo or Baker's yeast were found to support growth and enzyme production. However, maximum enzyme levels were obtained with yeast extract. Xylanase secretion was observed even when the fungus was cultivated in semi-solid fermentation (SSF), with moist wheat bran. Enzyme activity was 25% higher in SSF when activity was calculated on the basis of IU/g weight of substrate.

Chapter 3: Purification and Characterization of Xylanase

The extracellular broth (cell free culture filtrate) when subjected to gel filtration separated into two active fractions khaving a Mr of 28,000 and 70,000 respectively. The major xylanase fraction (90%) having a Mr of 28,000 was taken up for detailed studies. This enzyme was purified to homogeneity by ammonium sulfate precipitation, polyethyleneimine treatment followed DEAE-Sephacel chromotography. The purified enzyme has a molecular weight of 28,000. It is an acidic protein with a pI of 4.05. The optimum pH was found to be 8.0, while the optimum temperature was 40°C. The Km and Vmax values, using oat spelts xylan, were 3.52 mg/ml and 3.3 X 10^3 µm/mg/min respectively. The enzyme was inhibited by heavy metals like Ag^{++}, Hg^{++} and Pb^{++}. However, Hg^{++} inhibition could be reversed by addition of mercaptoethanol. Product analysis of xylan hydrolysis indicated the *Cephalosporium* xylanase to be an endo-enzyme. The hydrolytic products ranged from X1 to X6 with X2 and X3 being the predominant species. A substantial amount of arabinose was also released, suggesting that the enzyme belongs to the class of debranching xylanases. Chemical modification studies revealed the involvement of a single tryptophan and histidine and two carboxylic acid residues in the catalytic activity of the enzyme.

Chapter 4: Application of the enzyme in Pulp Bleaching

The application of *Cephalosporium* xylanase in biobleaching was evaluated using commercially available bagasse pulp obtained from a local paper mill. Enzyme pretreatment reduced the kappa number by 5-6 units, when a condition similar to those reported in literature were employed. The

enzyme treatment was effective over a pH range of 7 - 10 at 40 - 50 °C using 10 - 12 IU of enzyme per 10 g of pulp.

PUBLICATIONS/PATENTS

1. Bansod, S. M., Dutta Choudhary, M. Srinivasan, M.C. and Rele, M.V. (1993) Xylanase active at high pH from an alkalotolerant *Cephalosporium* species. Biotechnol. Letts. **15**:9, 965-971.

2. Rele, M.V., Bansod, S. M. and Srinivasan, M.C. (1994). A process for the production of extra-cellular endoxylanase active at high alkaline pH from an alkalotolerant fungal strain *Cephalosporium* NCL 87.11.9. Ind. Pat. Appl. No. 705/DEL/94.

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ABBREVIATIONS

AOX	Adsorbable organic halogen
BSA	Bovine serum albumin
CMC	Carboxy methyl cellulose
DEP	Diethyl pyrocarbonate
DNS	3,5-Dinitro salicylic acid
DEAE-cellulose	Diethyl amino ethyl cellulose
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
EDC	1,-Ethyl-3-(3-dimethyl amino propyl)-carbodiimide
GEE	Glycine ethyl ester
HNBB	2,-Hydroxy-5-nitrobenzyl bromide
Km	Michaelis-Menten constant
NBS	n-Bromosuccinimide
NAI	n-Acetyl imidazole
PAGE	Polyacrylamide gel electrophoresis
PEI	Polyethyleneimine
PMSF	Phenyl methyl sulphonyl fluoride
PNPX	p-Nitrophenyl β-D xylopyranoside
PNPA	p-Nitrophenyl xyloarabinoside
pI	Isoelectric point
SDS	Sodium dodecyl sulphate
SSF	Solid state fermentation
TCF	Totally chlorine-free
TEMED	N,N,N,'N'-Tetra methyl ethylene diamine
IU/ml	International units/ml

CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Plant cell walls are composed of three major structural components namely cellulose, hemicellulose and lignin in a ratio of 4:3:3 (Brauns and Brauns, 1960). Cellulose and hemicellulose represent the major products of carbon fixation which are useful as substrates for bioconversion. Crop residues such as cereal stalks, sugarcane bagasse and forest residues form the major renewable resoures produced by photosynthesis amounting to several million tonnes per annum globally.

Enzymatic hydrolysis of cellulose to glucose as well as fermentation to produce ethanol gained considerable momentum during the seventies and the eighties and several schools of research established the technical feasibilty of these processes. Along with these studies, considerable attention was also focussed on the bioconversion of hemicellulose especially xylan to alcohol using yeast strains such as *Pachysolen tannophilus* (Schneider *et al.*, 1981) and *Candida shehatae* (du Preez and van der Walt., 1983). More recently, hemicellulases such as xylanases and mannanases which are essentially free of associated cellulase activity have attained commercial significance in the pulp and paper industries. Microbial xylanases having properties compatible with the operating conditions in the pulp and paper industry have been successfully used to achieve pulp bleaching and Kappa number reduction with partial or almost total elimination of toxic chlorine compounds.

Use of xylanases in various other applications such as retting of fiber crops, food industry for improving the quality of bread etc. and poultry feed are also gaining significance in recent years.

XYLAN STRUCTURE

Schulze in 1891, first introduced the term "hemicellulose" for polysaccharides that are extractable with aqueous alkali. The isolated hemicellulose fractions were heterogeneous in nature and composed of sugar residues such as D-xylose, D-glucose, D-galactose, L-arabinose, D-mannose, 4-O-methyl glucuronic acid, D-galacturonic acid and D-glucuronic acid. Hemicelluloses are classified according to the predominant sugar residues present. The commonly occuring heteroglycans are L-arabino-D-xylan, L-arabino-D-glucurono-D-xylan, 4-O-methyl-D-glucurono-D-xylan, L-arabino-(4-O-methyl-D-glucurono)-D-xylan, D-gluco-D-mannan, D-galacto-D-glucurono-D-mannan and L-arabino-D-galactan (Whistler and Richards, 1970). In tropical hardwoods as well as grasses, heteroxylan constitutes the major hemicellulose components whereas in gymnosperm, ß-D-mannan is the principal hemicellulose component.

Xylan essentially consists of a backbone made up of ß-1,4 linked D-xylose residues to which are attached other sugar residues *viz.* α-L-arabinofuranose, α-D-glucuronic acid or α 4-O-methyl-D-glucuronic acid as side chains, the frequency and composition of the side chains showing considerable variation in the different plant sources (Puls and Schuseil, 1992).

Aspinall *et al* (1954) studied the structure and composition of beechwood xylan and showed that the xylan was composed of a linear frame work of a minimum of seventy xylose residues and on an average every tenth residue had a terminal 4-O methyl-α-D-glucuronic acid attached at the second position. Subsequently Timell (1964) showed that all wood xylans had a common structure with slight differences in the average molecular size and proportion of glucuronic acid side chains. The ß-linkage of xylan is characterized by a high laevorotation, ranging from -78.2° to -109.5° in alkaline solution. Periodate oxidation confirmed the presence of ß-1-4 linkage in xylan (Whistler and Richards, 1970). While in the majority of plants, xylan is a heteropolymer, in a few instances such as esparto grass (Chanda *et al.*, 1950), tobacco (Wilke, 1979) and *Tamarindus indicus* seed (Savur, 1956), the xylan was a homopolymer devoid of side chains.

Xylan from hardwoods

The xylan content in hardwood lies in the range of 20-25%. The polysaccharide consists of, on an average approximately two hundred ß-1,4 linked D-xylose residues and approximately every tenth xylose unit carries a 4-O methylglucuronic acid (at position 2) as side chain. Generally xylans in many hardwood species are acetylated, and 7 out of 10 xylose residues contain O-acetyl groups with the acetylation occurring more frequently at the O-3 position than at O-4 (Puls and Schuseil, 1992). The presence of acetyl groups in xylan makes it more soluble in water. Besides, hardwood xylan also consists of minor amounts of rhamnose and galacturonic acid (Johannson and Samuelson, 1977). Xylans from hardwood are devoid of arabinose residues (Timell, 1964), while xylans from softwood as well as grasses contain substantial quantities of arabinose. Hardwood also contain 2-5% glucomannan and the mannans are devoid of galactose.

Xylan from Softwoods

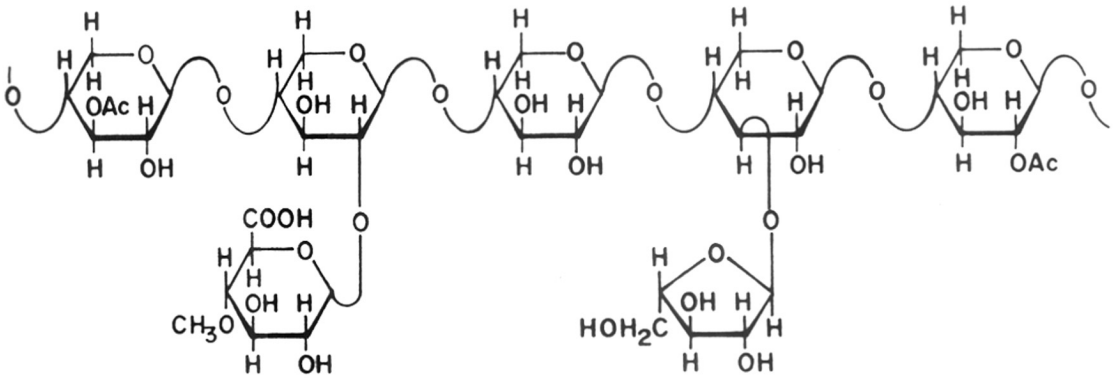
The xylan content of softwood varies from 7-12 % and is characterized by a higher proportion of 4-O-methyl-α-D-glucuronic acid. Approximately 7 out of 10 xylose residues are substituted by terminal 4-O methyl-α D glucuronic acid residues as compared to every tenth residue in hardwood. Softwood is non acetylated at the C-3 position of the xylosyl residue and carries an arabinofuranose residue instead of O-acetyl substituents. The arabinose substituents occur after 7-12 D-xylose residues. In contrast, the glucomannans have acetyl substituents The acetyl content of glucomannans in softwood varies between 6 to 9%. Softwood contains atleast two galactoglucomannans with different sugar ratios. The major water soluble polysaccharide consists of a chain with mannose, glucose and galactose residues present in a ratio of 3:1:1 respectively and the alkali soluble polysaccharide, glucomannan consists of mannose, glucose and galactose in the ratio 3:1:0.1 (Puls and Schuseil, 1992). The arabinogalactan from softwood has been shown to have a high degree of branching.

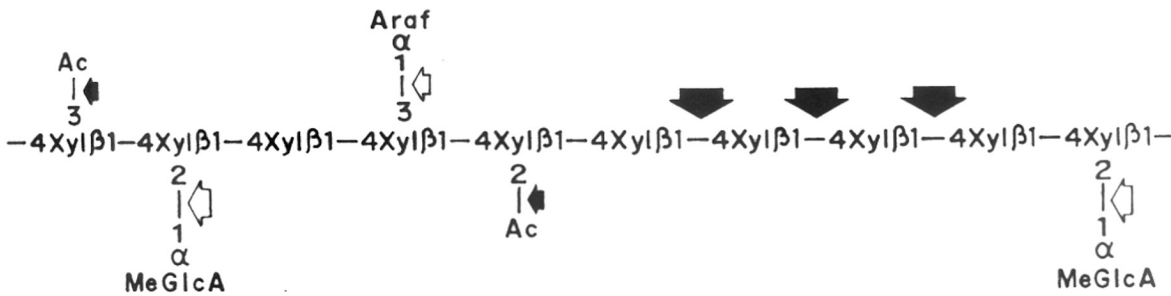
Xylan from Grasses

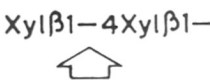
The xylan content of cereals and grasses range from 20-40 percent, the xylan structure varying with the species and the tissue (Puls and Schuseil, 1992). These xylans consist of L-arabinofuranose residues linked to xylan backbone and occur as single unit side chains attached to C-3 position of xylose. Other than arabinose, D-glucuronic acid or 4-O methyl-α D glucuronic acid or both can be present in a smaller proportion. Although the backbone structure of wheat straw xylan is the same, variations in the attachment of uronic acid residues to xylose by α 1-2 or α 1-3 linkages have been reported (Aspinall, 1959).

The hemicelluloses from corn cobs, maize fibre, wheat bran and barley husk are more complex and exhibit variations from the general structure. The xylan from corn cobs, apart from containing arabinose as side chain also has small amounts of glucose residues as substituents linked at positions 1, 3 and 4. Maize fiber hemicellulose contains L-galactose in addition to D-galactose (Whistler and Corbett, 1955), while wheat bran hemicellulose contains a higher proportion of L-arabinose than D xylose and these residues not only occur as nonreducing end groups but also in non-terminating positions linked through positions 1 and 3.

It is obvious from the foregoing discussion that unlike cellulose, xylan isolated from diverse plant sources exhibits considerable variation in its structure, especially in the composition of the sugar residues as well as in the degree of branching of the side chain. Hence enzymatic hydrolysis of xylan is a topic beset with complexity, particularly when one attempts to study and characterize xylan degradation by a specific enzyme fraction on xylan derived from different plant sources.







ENDO-1,4-β-XYLANASE (EC 3.2.1.8)

β-XYLOSIDASE (EC 3.2.1.37)

α-GLUCURONIDASE (EC 3.2.1)

α-L-ARABINOFURANOSIDASE (EC 3.2.1.55)

ACETYLESTERASE (EC 3.1.1.6) OR ACETYL XYLAN ESTERASE ?

A HYPOTHETICAL PLANT XYLAN AND THE SITES OF ITS ATTACK BY MICROBIAL XYLANOLYTIC ENZYMES.

Biely P., (1985)
Trends in Biotechnology, 3, 11.

FIG. 1·1

ENZYMATIC HYDROLYSIS OF XYLAN

Hemicellulose degrading enzymes are generally referred as Glycan hydrolases (EC 3.2.1). These include xylanases, mannanases, galactosidases etc. Among the glycan hydrolases, enzymes which hydrolyze ß-1,4 linkages of D-xylan *viz.* arabinoxylan, glucuronoxylan, 4-O methyl α-D glucuronoxylan have been assigned the EC number (3.2.1.8). Endoxylanase is also termed as 1,4-ß-D xylan xylanohydrolase by the Enzyme Commission.

Hydrolysis of Xylan

Xylan can be hydrolyzed either by acid or enzyme yielding xylo-oligomers and the monomeric sugar D-xylose. Enzymatic hydrolysis yields the products under ambient conditions with a high degree of specificity while acid hydrolysis is non-specific and yields an uncontrolled mixture of oligomeric products.

Xylanases

Enzymes which hydrolyse the backbone have been broadly classified into the following three main groups and are described briefly

1) **Endo-ß-(1-→4)-D-xylanase**: It is also termed as ß-(1-→4)-D-xylan xylanohydrolase, and corresponds to the EC No.(3.2.1.8). These enzymes hydrolyse the xylan randomly to produce large amounts of xylo-oligosaccharides of various chain lengths. The endo-xylanases are further classified into four types: (Reilly, 1981)

a) Non-arabinose liberating endo-xylanases: These enzymes cannot cleave L-arabinosyl initiated branch points. The major end products formed are xylobiose and xylose. They can act on xylo-oligosaccharides as short as xylotriose, the rates being slower with decreasing chain length.b) Non-arabinose liberating endo-xylanases: These enzymes cannot cleave the branch points, and the major end products formed are the xylo-oligosaccharides larger than xylobiose. These cannot act on xylotetrose and smaller substrates.

c) Arabinose liberating endo-xylanases: These enzymes can cleave the xylan side branch and the end products are mainly xylobiose, xylose and arabinose.

d) Arabinose liberating endo-xylanases: These enzymes can hydrolyze the branch points and produce predominantly xylo-oligosaccharides of intermediate size and arabinose.

2) **Exo-ß-(1-→4)-D-xylanase**: ß-(1-→4)-D-xylan xylohydrolase (E.C.No. 3.2.1.37). This enzyme cleaves single D-xylose units from the non-reducing end of the xylan chain and brings about inversion of product configuration.

3) **ß-xylosidase or xylobiase**: (E.C. 3.2.1.37), Hydrolyses xylobiose and the higher xylo-oligosaccharides. The reaction rate falls with increasing chain length. These enzymes show high transferase activity and retain product configuration.

ASSAY OF XYLANASE

Several methods are available for the detection and assay of xylanase activity, which has resulted in several research groups following different protocols using xylans from various sources. This is further complicated by the non-availabilty of pure xylan as many of the commercially available samples have varying degrees of contaminants such as starch ß-glucan and lignin. The xylanase activities of the same enzyme sample was also found to vary significantly with different lots of xylan (Khan *et al.*, 1986). The authors have attributed this variation to the degradable xylo-oligosaccharides present in the substrate. As a result it is difficult to compare activities of different enzyme samples (Khan *et al.*, 1986). This section describes the various assay procedures followed at several laboratories. A brief description of the round robin method, proposed during the collaborative testing of xylanase activity (IEA network programme entitled "Biotechnology for Conversion of Lignocellulose," 1989) is given which is intended to bring about the uniformity of the assay system (Bailey and Poutanen, 1992).

Assay by reducing sugar estimation

Reducing sugar methods can be used to assay enzyme activities in preparations which have negligible levels of background sugar or reducing compounds. A number of methods are described in the literature for measurement of reducing sugars. Some of the commonly used procedures are described below.

3,5-Dinitrosalicylic acid method [DNS]: This is the most preferred and commonly used assay procedure due to its simplicity (Miller, 1959). There are variations in the reaction volumes but the routinely used system consists of using an equal volume (1 ml) of the substrate and the enzyme followed by addition of 2 ml of DNS reagent after the desired incubation period. The reaction mixture is then heated at 80-100°C in a boiling water bath to develop the colour and diluted with 20 ml of water before the absorbance is read at 540 nm.

A major drawback of the DNS procedure is the non-stoichiometry of the colour developed with equimolar concentrations of oligosaccharides of increasing chain length. Xylobiose gives a greater colour than xylose when taken in equivalent amounts. The problem can be minimized to a certain extent by the use of xylobiose as standard instead of xylose or glucose (McCleary, 1992). The non-linearity of the DNS reaction has been attributed to two factors *viz.*, the practice of diluting the reaction products before quantification and the insufficiency of the substrate (Bailey 1988). The method is sensitive in the range of 1 to 5 mg of xylobiose equivalent per assay. Therefore, a high concentration of the substrate used can result in product inhibition and transglycosylation which in turn leads to inflated activities. The problem of insufficiency of the substrate is much more, when a highly branched arabinoxylan, such as 4-O-methyl-glucurono-xylan is used instead of beechwood xylan, which has a lower degree of branching.

Somogyi Nelson's Method: The procedure (Somogyi, 1952) is followed by a number of workers mainly for two reasons (a) the colour developed is stoichometric with increasing chain length of oligosaccharides (b) and the assay is very sensitive (10-50 μg). Therefore, lower amounts of the substrate can be used and this minimizes the likelyhood of product inhibition and transglycosylation. The method was found to be suitable for comparison of the initial rate of hydrolysis when different substrates and xylanases were evaluated (McCleary, 1992).

A major drawback of the Somogyi Nelson's procedure is the precipitation of xylan on addition of the copper reagent.

Viscometric Method

Although the viscometric method is used routinely for measurement of cellulase activity, it is not routinely used for measurement of xylanase activities due to the non-availability of xylan substrates having a high degree of polymerization. The viscometric method is nevertheless specific for endo-xylanases as exo-xylanases and side chain cleaving enzymes do not contribute to decrease in viscosity. The method is highly sensitive and the hydrolysis of as little as 0.1 % of the glycosidic bonds in the D-xylan backbone can be detected. The method is suitable for substrates such as arabinoxylan from rye where the initial solution viscosity is high. The assays are unaffected by presence of high background sugar.

The disadvantage of this procedure is that it is time consuming and hence fewer assays can be carried out at a given time. Furthermore the increase in activity with increasing enzyme concentration is exponential and not linear.

Xylanase assay with Chromogenic substrates

This method has several advantages over the conventional reducing sugar methods. The values are unaffected by the presence of background sugar. The method is specific, even when high concentrations of side chain cleaving enzymes such as α-L-arabinofuranosidase are present in the sample. These assays can be used to directly compare xylanases from different sources, and

are relatively unaffected by change in pH and temperature. As compared to other methods the test is simple and reproducible (McCleary, 1992). There are two types of chromogenic substrates commercially available a) soluble chromogenic xylan b) insoluble chromogenic xylan. Although soluble dye bound substrates using xylan from oat spelts, beechwood, birchwood and wheat arabinoxylan are available. The most commonly used chromogenic substrate is wheat arabinoxylan coupled to Remazol Brilliant Blue R. The method involves incubation of an aliquot of the dye bound substrate with enzyme solution at desired temperature and pH. The reaction is terminated by the addition of an organic solvent, preferably ethanol to precipitate the unreacted substrate. The colour is read after contrifugation at 590 nm. The concentration of the enzyme is calculated by reference to a standard curve.

Insoluble substrates are prepared by covalent cross-linking of soluble dye bound xylan with epichlorohydrine. In order to add uniform amounts of the solid substrate, the dye bound substrate is available in the form of tablets marketed by MegaZyme Pvt. Ltd. (Austria) under the trade name XylaZyme. A typical assay is described below.

The assay mixture contains suitably diluted enzyme solution (1 ml) in desired buffer to which a tablet (XylaZyme) is added. The tablet is easily soluble within a short span of 20 sec. The reaction is terminated by the addition of 10 ml of alkaline solution, normally Trizma-base (Sigma Chemical Co.). The suspension is filtered and the absorbance of the filtrate is measured at 590 nm. Enzyme activity is determined with reference to a standard curve (McCleary, 1992).

The major advantage in using insoluble substrates over soluble chromogenic substrates are greater stability, sensitivity and ease of use. The susceptibility of the substrate to enzyme attack is dependent on the degree of cross-linking and length of the cross-linker. Hence it is necessary to use a standardized product to compare enzyme activities.

The major disadvantage of chromogenic substrates is that batches of substrates vary in their dye content and hence each batch needs to be standardized before arriving at the correct enzyme activity.

Round robin method

As the xylanase assay procedures vary with the type of substrates and protocols used, no standard method was available for comparison of results obtained from different laboratories. Several laboratories in 1989, agreed to undertake a collaborative programme to arrive at a standard procedure for xylanase assay. This involved the use of a common protocol employing the same substrate. The procedure arrived at centers mainly around the estimation of fungal xylanases (Bailey and Poutanen, 1992). However the method can be modified to suit the conditions applicable for other xylanases, provided the same substrate is available readily. The substrate chosen was 1% birchwood 4-O-methyl glucoronoxylan marketed by (Roth product No.750). The enzyme to substrate ratio was changed from 1:1 to 1:9, to ensure that maximum substrate was available for reaction. The incubation period was chosen as 300 sec (5 min) in order to obtain accurate values for those enzymes which are not stable for prolonged incubation periods. The temperature was fixed at 50°C as most fungal xylanases had an optimum temperature of 50°C. The reaction was terminated by the addition of 3 ml of DNS followed by boiling for 5 min. The colour developed was measured at 540 nm without dilution with water. The xylanase activities were expressed in SI units in terms of katals (1katal=1mol sec^{-1} and 16.6 nkat=1IU).

The deviation among the various procedures was found to be as high as 108 % while it was reduced to 10 % when the aforementioned standard method was compared. Thus the round robin method, can be used for comparing activities from different laboratories and samples.

OCCURRENCE AND PRODUCTION OF D-XYLANASE

Xylanases are ubiquitous in distribution and they have been reported from prokaryotes as well as eukaryotes. They occur in bacteria of marine and terrestrial environment, fungi (saprophytes as well as phytopathogens and mycorhizea), rumen bacteria and protozoa, insects, snails, crustaceans, marine algae and germinating seeds (Dekker and Richards, 1976; Reilly, 1981). Majority of the xylanases are secreted extracellularly but reports have also been published on intracellular xylanases from rumen bacteria (Walker, 1961; Bailey and Clarke, 1963; Koch and Christina 1964) *Sporocytophaga myxococcoides* (Clermont *et al*, 1970) and *Aspergillus niger* (Ivamato *et al*, 1973a).

There are fewer reports on bacterial and actinomycetes xylanases in comparison to xylanases of fungal origin. In recent years, focus on xylanases from extremophile microorganisms, particularly alkalophiles and thermophiles has been on the increase (Horikoshi and Akiba, 1982; Honda *et al*, 1985; Okazaki, 1985; Balakrishnan *et al*, 1992; Dey *et al*, 1992, 1993; Chandra and Chandra, 1995). A more detailed description on occurrence and production of xylanases from extremophiles is discussed later.

Microbial xylanase production is largely induced by xylan or xylan-rich substrates. A few reports are published claiming constitutive production (Lyr, 1960; Strobel, 1963; Esteban *et al.*, 1983; Berenger *et al.*, 1985; Smith and Wood, 1991) while others have shown them to be inducible (Kawaminami and Iizuka, 1969; Kitpreechavanich *et al.*, 1984; Leathers, 1986; Keskar, 1989; Royer and Nakas, 1991; Balakrishnan *et al.,* 1992; Chandra and Chandra, 1995). Xylanases in general are produced when either pure xylan or xylan containing natural substrates are present in the medium. It has been shown that the hydrolyzed products of xylan, namely xylo-oligosaccharides are the actual inducers of the enzyme. Very low levels of the enzyme present constitutively bring about the hydrolysis of the substrate in extremely small quantities and the products thus formed activate the gene to secrete larger amounts of the enzyme (Biely, 1985).

Xylan has been the most prefered carbon source for the production of xylanases (Ishaque and Kluepfel, 1981; Esteban *et al.*,1982; Morosoli *et al.*, 1986; Thaker *et al.*,1986; Kelly *et al.*,

1989; Yoshida *et al.*,1989; Yu *et al.*, 1987). However the use of pure xylan is not economically feasible for commercial production; therefore agricultural residues rich in hemicellulose such as wheat bran, rice bran, rice stalk, corn cobs, bagasse, corn stalk, canola meal *etc.* have been used as substrates for microbial xylanase production (Panbangred *et al.*, 1983; Paul and Verma, 1990; Keskar, 1992; Brown *et al.*,1987; Grajek, 1987; Gattinger *et al.*, 1990; Balakrishnan *et al.,* 1992; Chandra and Chandra, 1995). In most cases glucose represses the production of xylanases. Xylanase activities were undetected in glucose grown cultures of *Bacillus circulans* WL-12 (Esteban *et al.*, 1982) but xylose, mannose and cellobiose supported growth and xylanase production. In *Bacillus pumilus* (Panbangred *et al.*, 1985), the xylanases were induced more effectively by xylose and xylobiose.

In actinomycetes the results have varied widely. In *Streptomyces lividans*-66, Kluepfel *et al.*,(1990) obtained higher enzyme yields in presence of xylose than with xylan. Lower yields with xylan was attributed to the enzyme forming a complex with the substrate. In *Streptomyces lividans*-1326 (Bertrand, 1989) xylose was shown to repress enzyme synthesis. However in some thermophilic actinomycetes xylose did not influence the regulation of xylanase expression (McCarthy *et al.*, 1985). Some interesting observations have also been made on induction of xylanase by microcrystalline cellulose. In *Streptomyces* sp. (Okeke and Paterson, 1992) xylanase activities were the highest when the organism was grown on microcrystalline cellulose and the enzyme activity increased with the increase in degree of crystallanity.

Among fungi *Aspergillus* (Frederick *et al.*, 1985; Bailey and Poutanen, 1989), *Schizophyllum commune* (Steiner *et al.*, 1987) as well as *Sclerotium rolsii* (Sadana *et al.*, 1980) and *Trichoderma*, (Wong and Saddler, 1992) are reported to be excellent producers of xylanases. Fungi are generally found to co-secrete xylanases and cellulases and the relative amounts of the enzymes produced varies with the ratio of cellulose and xylan present in the growth medium. For example, when *Trichoderma longibrachiatum* was grown on xylan, very low levels of cellulases were detected (Royer and Nakas, 1989). Similar results were obtained in the case of *Trichoderma harzianum* (Saddler *et al.*, 1985) unlike in *Trichoderma reesei* C-30 (Wong *et al.*, 1992)

which produced high levels of xylanase activity on cellulosic substrates rather than on xylan. Gamerith *et al.* (1992) have made similar observations in *T.reesei* Rut C-30 when grown on hemicellulosic waste substrates. The xylanase production in *Schizophyllum commune* was also linked to cellulase production and was induced in the presence of cellulosic substrates. Upto 1244 IU/ml activity was reported on an Avicel containing medium (Steiner *et al.*, 1987). These observations indicate that the ratio of xylanase to cellulase production is species dependent.

There are a few reports on constitutive production of xylanases in fungi. In the case of *Aureobasidium pullulans* xylanase production has been reported to occur in the presence of xylose (Leathers, 1986). A productivity of 300 IU/h was obtained when *Aureobasidium pullulans* (Priem *et al*, 1991) was grown in a medium containing xylose, glucose, yeast extract and corn steep liqour. In contrast, in the yeast *Cryptococcus albidus* (Biely, 1985) xylose repressed xylanase production. Purkarthofer and Steiner (1995) have studied the effect of several sugars on xylanase production in *Thermomyces lanuginosus* and observed that xylanase secretion in the presence of xylose occured approximately two hours earlier than that obtained with xylan. Other pentoses were also found to induce xylanase production, while glucose was found to be repressive.

Several non-metabolizable inducers such as ß-methyl xyloside and 4- thioxylobiose, have been successfully used in some of the *Trichoderma* strains, to obtain enhanced xylanase levels (Wong *et al.*, 1992). In *Aspergillus ochraceus*, ß-methyl xyloside was found to be an inducer for xylanase production and the xylanase induction was approximately 4.2 times more than with xylan (Biswas *et al.*, 1988). In the yeast *Cryptococcus flavus* the xylanase production was 15-20 fold greater in presence of ß-methyl xyloside as compared to that obtained with xylan or xylose (Yasui *et al.*, 1984). ß-methyl xyloside was also shown to effectively induce xylanase production in several other organisms such as *Cryptococcus albidus* species (Biely *et al.*,1980)*Trichosporon* (Biely and Poutanen, 1991), in hyper-producing colour variants of *Aureobasidum pullulans* (Leathers, 1986) and in several strains of *Aspergillus* (Bailey and Poutanen, 1989). However ß-methyl xyloside and thio-xylobiose were shown to be only weak inducers in *Trichoderma lignorum* (Defaye *et al.*,1985).

Lactose, a non-repressive carbon source was shown to induce xylanase in *Trichoderma lignorum* (Defaye *et al.*, 1985), *T. reesei* QM 9414 (Hrmova *et al.*, 1986) and in *T. longibrachiatum* (Royer and Nakas, 1990). In general the inducers of xylanases are xylobiose, xylotriose, higher xylo-oligosaccharides and non-metabolizable inducers such as 4-thioxylobiose and ß-methyl xyloside.

Xylanases from Extremophiles

The word extremophile refers to those organisms which can survive and grow in habitats where the environment is hostile to a majority of the microorganisms. The extreme conditions could be variations in pH, temperature, osmolarity and high pressure. Generally, extremophile is assigned to a few restricted species which have adapted to the rigours of the extreme environment. However newer species are being discovered and biodiversity of extreme environments remains relatively unexplored. These extreme environments can also be found in pockets, or niches in soil and mesophilic habitats. Extremophiles are broadly represented by Alkalophiles, Thermophiles, Psychrophiles, Halophiles and Basophiles. Interest in the potential of extremophiles as source of enzymes with novel properties has intensified in recent years and the major focus has been in the exploration of alkalophiles for such enzymes including xylanases.

Alkalophilic microorganisms: Majority of the microorganisms have an optimum pH for growth between 4.5 to 7.5. Alkalophiles are those microorganisms which have an optimum pH for growth above pH 8.0. Obligate alkalophiles are incapable of growth at neutral pH and can grow best around pH 10. They also generally have an obligate requirement for sodium ions. Alkalotolerant organisms on the other hand are those which can grow over a broad pH range, the optimum being more towards the alkaline pH. Alkalophiles are widely distributed in natural environment and have been isolated through selective enrichment techniques both from high alkaline habitats such as soda lakes and also from mesophilic substrates such as decomposing litter, soil etc. Niches for exploring alkalophiles include environs in which ammonium ion fixation or sulphate

reduction is taking place and also effluents discharged from cement factories, paper pulp industries etc. Species of alkalophilic bacilli are easily isolated in screening programmes and have been widely investigated for their potential in enzyme technology.

Among the earliest investigations on alkalophilic bacilli leading to enzyme technology may be mentioned the commercialization of an alkaline protease developed by NOVO-industry for application in detergents and washing powders (NOVO,1970). Pioneering investigations in Japan by Koki Horikoshi and his associates has contributed to significant advances in the understanding of alkalophilic bacteria and their enzymes. In recent years the subject of alkalophilic bacteria and their enzymes has considerably widened and several publications and review articles have been published giving a comprehensive account of the importance of this group in Biotechnology (Horikosi and Akiba, 1982)

Studies by Horikoshi and his collaborators during the last two decades has resulted in identification of strains of alkalophilic bacteria producing a wide range of enzymes having industrial importance such as alkali-stable protease, amylase, xylanases and cyclodextrinases (Akiba and Horikoshi, 1982). In recent years screening has been intensified for xylanases active at high pH which is a pre-requisite for compatibility with the operating conditions prevailing in paper and pulp industry. Such xylanases being free of associated cellulase activity could be an added advantage since the cellulose pulp will be unaffected by treatment with crude culture filtrate rich in xylanase activity. In the following paragraphs a brief account is presented on some of the salient publications related to xylanases from alkalophilic microorganisms published in the literature.

Horikoshi and Atsukawa (1973) were the first to report on an alkalophilic *Bacillus*, exhibiting xylanase activity over a broad pH range (pH 5.5-9.0). Following this, there have been several studies on xylanases mainly from the genus *Bacillus* (Honda *et al.*, 1985; Srinivasan *et al.*, 1988; Balakrishnan *et al.*, 1992; Dey *et al.*, 1992; Rajaram and Varma, 1990). An obligate alkalophilic *Bacillus* (NCL 87.6.10) secreting substantial amounts of thermoactive alkaline cellulase-free xylanases was identified by Srinivasan *et al*, (1988), from decomposing coconut fibre. The xylanase was cellulase-free and, in a media containing wheat bran and organic nitro-

gen source such as yeast extract, the culture secreted 150 IU/ml of xylanase (Balakrishnan *et al.*, 1992). Xylanase activity of 400 IU/ml was reported by Ratto *et al.* (1992) from an alkalotolerant strain of *Bacillus circulans* using xylan as the carbon source. Chauthaiwale (1994) found that in the case of the alkalophilic thermophilic *Bacillus*-NCIM 59, washed wheat bran induced substantial activities as compared to unwashed wheat bran.

Okazaki *et al.*, (1985) observed that in alkalophilic-thermophilic *Bacillus* strains W1, W2, W3 and W4 the xylanase production was more in presence of xylan and wheat bran as the carbon source. Xylose supported enzyme production in strain W2 and W4 while glucose repressed enzyme synthesis in other strains. In the alkalophilic *Bacillus* strain 41 M-1, the xylanase production was carried out in a medium containing oat spelts as the carbon source, while it was suppressed by the addition of xylose, arabinose or glucose to the medium. The xylanase was also found to be free of cellulases (Nakamura *et al.*, 1993). A novel feature of the alkalophilic *Bacillus* NCL 87.6.10 is that significant quantities of the xylanase was secreted even in the presence of soluble sugars without xylan inducers under appropriate fermentation conditions (Balakrishnan, 1993).

Most of the alkalophilic microorganisms required sodium for growth which is normally supplied in the form of sodium carbonate (Srinivasan and Rele, 1992). Balakrishnan *et al* (1992) have noted that when sodium carbonate was replaced by potassium carbonate, no growth and activity was observed. A mixture of potassium carbonate and sodium ions added in the form of sodium chloride gave comparable activities to those obtained in the presence of sodium carbonate.

Ikura and Horikoshi (1987) in *Bacillus* sp. have shown that addition of glycine, DL-Norvaline and D-alanine enhanced production of xylanases. The increased enzyme levels in the presence of glycine and DL-norvaline was correlated to the concomitant depression of protease production. Similar results were obtained in the case of alkalophilic *Bacillus* NCL 87.6.10. Norvaline enhanced enzyme activity while glycine was found to be inhibitory (Balakrishnan, 1993). There are very few reports on xylanases from alkalophilic actinomycetes. The first such

report on xylanase production at an alkaline pH was from *Streptomyces* VP-5, isolated in our laboratory (Vyas *et al.* 1990). The xylanase had no accompanying cellulase activity. Tsujibo *et al.* (1990) also studied xylanase production from an alkalophilic actinomycete, *Nocardiopsis dassonvillei*. In this case the enzyme production was induced by xylan and three types of xylanases were produced (isoenzymes).

Fungal strains capable of growing under high alkaline pH conditions are rarely reported. Alkaline protease from mesophilic strains of fungi have been reported (Jonsson and Martin, 1964; Srinivasan *et al.*,1983). However there are no published reports available on fungi growing at high alkaline pH and secreting a xylanase active and stable under such conditions. The present work is perhaps the first report describing a xylanase from an alkalotolerant *Cephalosporium* species. Recently an alkalotolerant *Aspergillus fischeri* was reported to produce a cellulase-free xylanase under fermentation conditions over a wide pH range (Chandra and Chandra, 1995). Kang *et al.* (1995) have published cellulase production from an alkalotolerant *Cephalosporium* species.

Xylanase from Thermophilic Microorganisms

Studies on thermophilic as well as thermotolerant microorganisms for their xylanase activity have been widely published in literature and currently the focus has been to look for greater thermostability to suit operative conditions for pulp biotechnology. Among the bacteria, considerable studies have been carried out on an anaerobe *Dictyoglomus* (Mathrani and Ahring, 1992), who observed that the strain is capable of producing xylanase active at 90-100°C. Simpson *et al.* (1991) identified a *Thermotoga* producing xylanase with a half life of 90 min at 90°C. However, the units of xylanase activities obtained have been extremely low and need considerable improvement before the discovery can become industrially relevant.

Several thermophilic/thermotolerant actinomycetes and fungi have been studied for their xylanase activity and the published literature is presented in a tabular statement (Table 1.1). So far little success has been achieved to combine high activity at alkaline pH with a high degree of

TABLE I.1 :

A. XYLANASE PRODUCTION FROM MESOPHILIC-NEUTROPHILES

Organism	Carbon Source	Temp °C	pH	Incubation (h)	Activity IU/ml	References
BACTERIA						
Bacillus circulans WL 12	Xylan	30	6.5	70	121.6	Esteban *et al.*, 1982
Bacillus coagulans 26	Xylan	37	-	20	40.2	Esteban *et al.*, 1983
Bacillus sp.	Rice husk	38	7.2	48	5.0	Paul and Varma, 1990
Cellulomonas flavigena	Kaller grass straw	30	7.3	72	16.0	Mahomad *et al.*, 1984
Chainia (NCL 82-5-1)	Xylan	28	6.8	72	28.0	Srinivasan *et al.*, 1984
Streptomyces lividans	Xylan	40	7.0	72	50.0	Kluepfel *et al.* 1986
Streptomyces olivochromogenes	Xylan	37	7.0	96	30.7	Mackenzie *et al.*, 1987
Streptomyces roseiscleroticus	Xylan	28	7.0			Grabski and Jeffries, 1991
Streptomyces wedmorensis	Xylan	37	7.0	56	41	Tripathi *et al.*, 1992

Organism	Carbon Source	Temp °C	pH	Incubation (h)	Activity IU/ml	References
FUNGI						
Aspergillus foetidus VTT-D-71002	Solka Floc	30	4.8	168	99	Bailey & Poutanen, 1989
Aspergillus fumigatus VTT-D-82195	Solka Floc	30	4.8	168	150	Bailey & Poutanen, 1989
Aspergillus oryzae VTT-D-85248	Wheat bran	30	4.8	168	78	Bailey & Poutanen, 1989
Aspergillus terreus VTT-D-82209	Solka Floc	30	4.8	168	87	Bailey & Poutanen, 1989
Cephalosporium sacchari	Hemicellulose	32	5.6	150	0.27	Richards & Shambe, 1976
Fusarium oxysporium SUF 850	Xylan	27	-	96	42	Yoshida *et al.*, 1989
Penicillium funiculosum	Cellulose powder wheat bran	28	5.0	240	16	Mishra *et al.*, 1985
Penicillium janthinellum	Sugarcane bagasse	30	6.0	96	37	Milagres and Duran 1992
Penicillium pinophilum	Barley straw	28	-	240	27	Brown *et al.* 1987

TABLE I.1

B. XYLANASE PRODUCTION FROM ALKALOPHILES

Organism	Carbon Source	Temp °C	pH	Incubation (h)	Activity IU/ml	References
BACTERIA						
Aeromonas sp. 212	Xylan	37	10	48	1.95	Ohkoshi *et al*. 1985
Bacillus C-59-2	Wheat bran	37	10.2	72	3.50	Horikoshi and Atsukawa 1973
Bacillus sp. C-125	Wheat bran	37	10.0	72		Honda *et al*. 1985
Bacillus sp. NCL 87-6-10	Wheat bran	28	10.0	48	150.	Balakrishnan *et al*., 1992
Bacillus sp. NG-27	Xylan	27	9-10	24	-	Gupta *et al*. 1992
Bacillus sp. 41 M	Xylan	27	10.0	120	20.	Nakamura *et al*. 1992
Bacillus sp. YC 335	Xylan	37	10.0	-	1.2	Park *et al*., 1992
Bacillus SP-91	Xylan	65	7.8	48		Biswas *et al*., 1986
Alkalitolerant *Bacillus circulans*	Xylan	30	8.0	48	400	Ratto *et al*. 1992

Organism	Carbon Source	Temp °C	pH	Incubation (h)	Activity IU/ml	References
Alkalophilic thermophile (AT)						
AT *Bacillus* sp. W	Xylan	45	10.0	48	112	Okazaki *et al.*, 1984
AT Bacillus	Bagasse	60	9.0	24	57	Rajaram and Varma, 1990
AT *Bacillus* sp. NCIM-59	Wheat bran	48	10.0	48	50.	Dey *et al.* 1992
AT *Bacillus* sp. TAR 1	Xylan	27	10.5	48	10	Nakamura *et al.* 1994
Bacillus stearothermophilus	xylose	60	7.0	18	1.45	Khassin et al., 1993
ACTINOMYCETES						
Nocardiopsis dassonvillei	Oatspelts xylan	27	10.0	168	23.3	Tsujibo *et al* 1990
Alkalophilic *Streptomyces* VP-5	Wheat bran	37	10.0	120	12.0	Vyas *et al.* 1990
FUNGI						
Alkalotolerant* *Cephalosporium* sp. NCL 87-11-9)	Wheat bran	28	10.0	96	20	Bansod *et al.*, 1993
Alkalotolerant *Aspergillus fischeri*	Wheat bran	30	10.0	48	40	Chandra and Chandra 1995

* Present work

TABLE I.1

C. XYLANASE PRODUCTION FROM THERMOPHILIC/THERMOTOLERANT ORGANISMS

Organism	Carbon Source	Temp °C	pH	Incubation (h)	Activity IU/ml	References
BACTERIA						
Dictyoglomus	Xylan	68	7.0	20	1.5×10^{-4}	Mathrani and Ahring, 1992
Thermatoga sp.	Xylan	80	-	26		Simpsom *et al.* 1991
Thermatoga maritima	Xylose/xylan	80	7.0	24		Winterhalter and Liebl 1995
Actinomadura	Xylan	55	7.4	192	59	Zimmerman *et al.* 1988
Thermomonospora fusca KW3	Xylan	48	7.2	24	3.5	Rothlisberger *et al.*, 1992
Streptomyces T-7	Wheat bran	50	7.0	72	71	Keskar, 1989
FUNGI						
Gleophyllum trabecum	Spruce saw dust	60	-	216	1.1	Ritschkoff *et al.* 1994
Humicola grisea var. Thermodea	Xylan	70	6.0	24	79	Monti *et al.* 1991
Humicola lanuginosa	Sugar beet pulp	45	6.5	72	18.7	Grazek, 1987
Thermoascus aurantiacus	Xylan	45	-	240	576	Yu *et al.*, 1987
Thermomyces lanuginosus	Xylan	50	6.0	96	494	Gomes *et al.* 1993

thermal stability in any of the microbial strains studied so far and there is a need for screening newer strains from diverse ecosystem for identifying potential strains to meet such requirements.

Xylanase Production by Semi-solid Fermentation (SSF)

Xylanase production has been largely studied in submerged fermentation and there are relatively fewer reports on enzyme production by semi-solid fermentation. Deshamps and Huet (1985) compared xylanase production by *Aspergillus niger* in submerged as well as semi-solid fermentations and indicated differences in enzyme properties. The enzyme produced by SSF had a lower pH optima (3.8) as compared to that by submerged fermentation which had a pH optima of 4.8. A difference in temperature optima was also observed, the enzyme from SSF had an optima at 50°C which was higher by 5°C to the one obtained by submerged fermentation. Similar differences in temperature and pH optima were also reported for B-glucosidase production in *Aspergillus niger*, by the same authors (Deshamps and Huet, 1984).

Purkarthofer and Steiner (1993) in their studies on xylanase production in *Thermomyces lanuginosus* observed higher productivity under SSF conditions while the source of added nitrogen strongly influenced xylanase activities obtained by either method.

Multiplicity of Xylanase

Multiciplity of the xylanases has been observed in most of the xylanolytic microorganisms (Wong *et al.*,1988). This phenomena has been attributed to glycosylation and/or proteolysis. Hence it is possible that the multiple xylanases detected could be due to post translational modification, such as proteolytic action on the major xylanase or precursors of the main enzyme.

Post translational modifications have been reported in several xylanases (Yoshioka *et al.*, 1981; Frederick *et al.*, 1981; Bernier *et al.*, 1983; Berenger *et al.*, 1985; Frederick *et al.*, 1985). Glycosylation or proteolysis or both are the general modifications encountered.

The xylanase from *Aeromonas* sp. strain 212 has been reported to have a molecular weight of 145 kDa. However when the gene was cloned into *E. coli* it produced a xylanase of

135 kDa. As both the xylanases had similar hydrolytic, immunological and physiochemical properties, they appear to be differentially modified protein products of the same gene (Kudo *et al.*, 1985).

In some fungal xylanases such as *Thermoascus aurantiacus*, the N-terminal aminoacid is acetylated (Naren., 1992). The biological significance of acetylation is not yet understood but it has been proposed that this post translational modification protects the enzyme from proteolysis (Joranvall, 1975).

Although microheterogeneity can be attributed to proteolysis, there are evidences suggesting that atleast some of the multiple xylanases of microbial origin are distinct gene products. The aminoacid composition of three xylanases of *Trichoderma harzianum* E58 indicates that they are different gene products (Wong *et al.*, 1986). The amino acid composition data of xylanases from *Aspergillus niger* and *Talaromyces byssochlamydoides* YH-20 indicate that they are evolved from different genes (Yoshika *et al.*, 1985).

Purification:

Xylanases from microbial sources in general are extracellular enzymes. However irrespective of the source, conventional procedures have been utilized to obtain homogeneous preparations. Most of the procedures involve concentration of culture filtrates either with salt or membrane filtration, followed by chromatographic procedures like ion-exchange chromatography and gel filtration, (Wong and Saddler, 1992). An immobilized technique using Con-A Sepharose has been used to purify xylanases which are glycoprotein in nature (Paul & Verma, 1992). Hydrophobic interaction chromatography was used by Holden and Walson (1992) to purify a xylanase from a fungal maize pathogen *viz. Cochliobolus carbonum*. Lappalainen (1986) purified *Trichoderma reesei* xylanase using immunoaffinity chromatography. Rozie *et al.* (1992) have reported a one step purification technique using cross linked xylan as an affinity adsorbant. An endoxylanase from the commercial preparation Pectinoll A-1 (Rohm GmbH, Damstadt, FRG) of fungal origin was purified 80 fold in a single step using epichlorohydrine cross-linked xylan. The capacity of the matrix was 1.5 mg of endoxylanase per gram of the adsorbant. The matrix was also

used to purify an endoxylanase and an endopolygalacturonase from a commercial preparation, KBP-001 marketed by NOVO AG, (Basel, Switzerland). Other enzymes such as B-xylosidase were unadsorbed. Apart from the above procedures more sensitive techniques such as iso-electric focussing (Wong *et al.*, 1986), preparative PAGE (Dey *et al.*, 1992) FPLC, (Simpson *et al.*, 1991) have all proved extremely efficient for the purification of xylanases from various sources, especially when microheterogeneity has posed problems in obtaining homogeneous preparations.

In a few cases the xylanases have been purified using Dextran based gel filtration matrix such as Biogel P-6, Biogel P-60, Sephadex G-25 and Biogel A-0.5m. This method is based on the adsorptive interaction of these apparant low molecular weight xylanases (Less than 20 kD) with gel filtration resin matrices. Apparent low molecular weight xylanases have been reported from *Trichoderma lignorum* (John and Schimdt, 1988), *Trichoderma reesei* (Lappalainen, 1986), *Trichoderma longibrachiatum* (Royer and Nakas, 1991) and *Trichoderma koningii* (Wood and McCrae, 1986). In the adsorptive interaction of low molecular weight xylanases with gel filtration matrix, the enzyme is eluted later, with an apparant molecular weight of 10-12 kD (Wong and Saddler, 1992). Low molecular weight xylanases have also been purified using methods such as ultrafiltration as in *Trichoderma viride* (Dean and Anderson, 1991) and *Trichoderma harzianum* (Tan *et al.*, 1985).

FUNCTIONAL PROPERTIES

The pH optima of xylanases from neutrophilic prokaryotes are generally in the range of 5.0-7.0. Exceptions to this are the acidophiles and alkalophiles (Table 1.2). Uchino and Nakane (1981) have reported an acidic xylanase from *Bacillus* species 11-1S which is optimally active at pH 3.0 to 4.0. Enzymes from alkalophilic microorganisms are active in the pH range of 6.0 to 8.0. In a few cases where the crude enzyme exhibits a broad pH optima, for example in alkalophilic-thermophilic *Bacillus*, (Okazaki *et al.*,1985) and *Bacillus stearothermophilus* (Khasin et al., 1993), it was found that on purification, only one of the enzymes characterized was observed to have a higher pH optima i.e., between pH 8.0 to 9.0.

TABLE I.2:

A. PROPERTIES OF XYLANASES FROM MESOPHILIC-NEUTROPHILES

Organism	Molelcular weight kDa	Optimum pH	Temp.	pI	References
BACTERIA					
Aeromonas caviae W61	22	7.0	55	9.2	Viet *et al.*, 1991
Bacillus circulans A	85	5.5 - 7.0	-	4.5	Esteban *et al.* 1982
B	15	5.5 - 7.0	-	9.1	
Bacillus coagulans	22	6.0	37	10.0	Esteban *et al.*, 1983
Bacillus pumilus	24	6.5	40	-	Panbangred *et al.* 1983
Bacillus subtilis	32	5.0	50	-	Benier *et al.* 1983
ACTINOMYCETE					
Chainia	6	6.0	60	8.9	Bastawde *et al.*, 1990
Streptomyces lividans	43	6.0	60	5.2	Morosoli *et al.*, 1986
Streptomyces lividans 66	31	6.5	55	8.4	Kluepfel, 1990 Srinivasan *et al.*, 19
Streptomyces sp. KT-23	42	5.5	55	6.9	Nakajima *et al.*, 1984
Streptomyces roseiscleroticus	22.6	6.5 - 7.0	60	9.5	Grabaski & Jeffries, 1991

Organism	Molelcular weight kDa	Optimum pH	Optimum Temp.	pI	References
FUNGI					
Aspergillus kawachii IA	35	5.5	60	6.7.5	Ito et al., 1992
IB	26	4.5	55	4.4.6	
IC	29	2.0	50	3.5.7	
Aspergillus niger 14	33	4.0	50	4.2	Gorbocheva & Rodinova, 1977
Aspergillus niger	28	5.0	40 - 45	3.65	Shei *et al.*, 1985
Aspergillus niger I	13	6.0	45	8.6	Frederick *et al.*, 1985
II	13	6.5	45	9.0	
Aspergillus niger	14	4.7	45	4.50	Biswas *et al.*, 1990
Aspergillus ochraceus NG-13	48	6.0	50	-	Biswas *et al.*, 1990
Basidiomycete Irpex lacteus	38	4.6 - 5.2	60	7.6	Hoebler & Brillouet, 1984
Basidiomycetes lentinula	41	4.5 - 5.0	60	3.6	Mishra *et al.*, 1990

Organism		Molelcular weight kDa	Optimum pH	Temp.	pI	References
Cephalosporium sacchari		10.7 9.5	6.0 - 6.5 6.0 - 6.5	55 55	9.4 6.0	Dekker & Richards, 1976
Neocallimastix frontalis	1 2	45 70	5.5 6.5	55 55	- -	Gomes *et al.*, 1993
Neocallimastix frontalis		30	6.0 - 6.6	50	-	McCrae & Wood, 1994
Paecilomyces varioti Bainier		25	5.0 - 7.0	60	3.	Krishnamurthy & Vithayathil 1989
Penicillium herqui		11	3.0	50		Funugama *et al.*, 1991
Phanerochaete chrysosporium ME446			5.0	50		Lopa-Patino *et al.*1993
Robillarda sp. V-20	1 2	17.6 59	4.5 - 6.0 4.5 - 6.0	50 50	9 3	Koyama *et al.*, 1990
Schizophyllum commune		33	5.0	55		Paice *et al.*, 1978
Schizophyllum radiatum		25.7	4.9	55		Cvazzoin *et al.*, 1989
Sclerotium rolfsii UV-8		-	4.5	65	7	Deshpande *et al.*, 1987
Trichoderma harzianum		22	4.5 - 5.0	45 - 50	8	Wong *et al.*, 1986
Trichoderma harzianum	1 2	20 29	5.0 5.0	50 60	9 9.5	Tan *et al.*, 1987

Organism	Molelcular weight kDa	Optimum pH	Temp.	pI	References
Alkalotolerant *Bacillus circulans*	--	7.0 - 8.0	45		Ratoo *et al.*, 1992
Alkalophilic thermophile AT					
AT *Bacillus* sp.W1	21.5	6.0	65	8.5	Okazaki *et al.*, 1985
	49.5	7.0 - 9.0	70	3.6	
W2	22.5	6.0	65	8.3	
	50	7.0 - 9.5	70	3.7	
AT *Bacillus*		6.0 - 7.0	60, 70		Rajaram and Varma, 1990
AT *Bacillus* NCIM-59	35	6.0 - 8.0	50 - 60	4.0	Dey *et al.* 1992
	15.8	6.0 - 8.0	50	8.0	
AT *Bacillus* TAR 1	40	5.0 - 9.5	70 - 75	4.1	Nakamura *et al.*, 1994
B. Stearothermophillus	43	6.0 - 7.0	65 -70	9.0	Khassin *et al.*, 1993

Organism	Molelcular weight kDa	Optimum pH	Optimum Temp.	pI	References
ACTINOMYCETES					
Nocardiopsis dassonville	23	7.0	60	4.	Tsujibo *et al.*, 1990
	23	7.0	60	5.	
	37	7.0	50	4.	
Streptomyces VP-5		4.8 - 10	50		Vyas *et al.*, 1990
FUNGI					
Cephalosporium sp.*	28	7.0 - 9.0	40	4	Bansod *et al.*, 1993
Aspergillus fischeri	-	6.0	60		Chandra & Chandra, 1995

*Present work

TABLE I.2:

C. PROPERTIES OF XYLANASES FROM THERMOPHILES

Organism		Molelcular weight kDa	Optimum pH	Temp.	pI	References
BACTERIA						
Clostridium thermolacticum		39	6.5	80		Debeire *et al.*, 1990
Dictyoglomus		--	5.5 - 9.0	80		Mathrani & Ahring, 1992
Thermatoga sp.		31	5.5	80		Simpson *et al.*, 1991
Thermatoga maritima MS-B8	A	120	6.2	92	-	Winterhalter & Liebl, 1995
	B	40	5.6	105		
ACTINOMYCETES						
Streptomyces T-7		20	4.5 - 5.5	60	7.8	Keskar *et al.*, 1989
Streptomyces thermoviolaceus OPC-520	STX1	54	7.0	70	4.2	Tsujibo *et al.*, 1992
	STX2	33	7.0	60	8.0	

Fungal xylanases in general exhibit pH optima in the acidic pH range (pH 3.5-6.5). The xylanases from *Penicillium herquei* (Funugama et al.,1991), and one of the xylanases from *Aspergillus kawachii* (Ito et al.,1992) exhibit pH otima at 3.0 and 2.0 respectively. The *Cephalosporium sacchari* xylanases have been reported to have a pH optima in the range of 6.0-6.6 (Richards and Shambe, 1976; Richards and Dekker,1975).

Most of the fungal xylanases are stable over a wide pH range of 3.0 to 10.0. The xylanases from prokaryotes are comparable to those from the fungal ones. *Streptomyces* T-7 xylanase was found to be active when tested after incubation at 37°C at pH 7.0 for 11 days (Keskar, 1989).

The tempereture optima of most of the bacterial xylanases is in the range of 50 to 65°C. Exceptions to these are the enzymes from *Bacillus coagulans* which has a low temperature optima of 37°C (Esteban *et al.*, 1983). The endo-xylanase from acidic *Bacillus* species 11-1S was reported to have a high temperature optima of 80°C (Uchino and Nakane, 1981). Many of the thermophilic actinomycetes produce highly stable xylanases which have an optimum temperature in the range of 60-75°C. The fungal xylanases have a comparatively lower optimum temperature in the range 40-50°C. A number of thermophilic fungi are known to exhibit a higher temperature optima in the range of 60-70°C. The xylanases from thermophilic fungus *Taloromyces byssochlamydoides* YM-50 (Yoshoika et al., 1981) and *Thermoascus aurantiacus* (Tan et al.,1987) exhibited a comparativly higher temperature optima at 70-75°C and 80°C respectively. The mesophilic fungus Y-94 has also been reported to have a high temperature optima at 80°C.

Many of the bacterial xylanases are stable upto 55°C, while the fungal xylanases have a lower stability and are stable in the range of 40-50°C. The enzymes from the thermophilic fungi such as *Humicola lanuginosa* are stable upto 60°C (Kitpreechavanich et al.,1984). Alkalophiles exhibit a similar temperature optima profile. An interesting feature of the alkalophiles has been that the optimum temperature has an inverse relation with the pH, for example in *Bacillus* sp., TAR-1, the optimum temperature was 75°C when assayed at pH 7.0 while at pH 9.0 the enzyme was optimally active at 70°C (Nakamura *et al.*, 1994).

Specificity of Xylanases

The majority of xylanases are specific for xylan. There are reports of a few enzymes being non-specific in action. Non-specific glycanases are those which can hydrolyse xylan as well as cellulose, CMC, *p*-nitro-phenyl-ß-glucoside, including laminarin. The non-specific glucanases have been well characterized in the genus *Trichoderma* and it has been possible to classify these enzymes into a separate group as they appear to have certain common physico-chemical properties such as pI and molecular weight. The non-specific xylanases have relatively high molecular weight (32-57 kD) and pI of acidic values, ranging from 3.2 to 5.3 (Wong and Saddler, 1992). Moreover they are induced by sophorose and not by xylobiose suggesting that they are more closely related to cellulolytic families rather than the xylanolytic system (Wong and Saddler, 1992). Non specific xylanases have also been reported from *Penicillum funiculosum* and *Myrothecium verrucaria* (Coughlan, 1992).

Effect of Metal ions

Xylanases in general are inhibited by heavy metals such as Hg^{2+}, Cu^{2+}, Fe^{2+}, Mo^{2+}, As^{2+} etc., An interesting feature of metal ion inhibition of xylanases is that enzymes devoid of cysteine are inhibited by Hg^{2+} and the inhibition is reversed on addition of reducing agents such as mercapto-ethanol, dithiothritol and cysteine. However these enzymes are not inhibited by cysteine specific reagents such as DTNB, NEM, iodoacetamide etc., indicating that Hg^{2+} probably binds to group other than Cysteine (Fournier *et al.*, 1985; Frederick *et al.*, 1985; Shei *et.al.*, 1985).

PHYSICOCHEMICAL PROPERTIES

Molecular weight:

Most microbial xylanases have a molelcular mass in the range of 20-50 kDa. The enzymes from *Melanocarpus albomyces* and *Aeromonas* sp. Y-20 however have very high molecular weights 104,000 and 145,000 respectively (Chaudhari *et al.*, 1988; Ohkoshi *et al.*, 1985). Bacterial xylanases are distinctly classified into two catogories. The low molecular weight enzymes range from 16,000 to 22,000 and the high molecular weight xylanases are in the range of 43,000-50,000. In the genus *Bacillus* there appears to be a relation between the molecular weight and pI of xylanases. The high molecular weight xylanases appear to have an acidic pI, while the low molecular weight ones have basic pI (Wong *et al.*, 1988).

The above dictum on molecular weight data is also apparant in the case of xylanases from the alkalophilic bacilli such as, *Bacillus* NCIM No. 59 (Dey *et al.*, 1992), alkalophilic *Bacillus* NCL 87.6.10 (Balakrishnan *et al.*, 1992). Thus the low molecular weight and the high molecular weight xylanases appear to be conserved in the *Bacillus* sp.

The molecular weight of xylanases of fungal origin generally lie in the range of 16,000-50,000. Among the lowest molecular weight xylanases reported from fungi are the ones from *Chaetomium thermophile* var. *coprophile* (Ganju et al., 1989) and the xylanases from *Cephalosporium* (Richards and Shambe, 1976).

However the above studies are based on gel filtration, where in the protein is retarded either due to interaction with the matrix, or due to the shape of the enzyme.

Km and Vmax

The affinity of D-xylanases towards xylan differs widely depending on the source of xylan. The Km of different xylanases varies between 0.27 and 14 mg/ml (Wong *et al.*,1988).

pI

The pI values of several xylanases have been determined and range from 3.75 to 10.3 (Wong and Saddler, 1992). The relationship between the pI and molecular weight have been discussed in the earlier section.

Glycoprotein nature

Some of the well characterized fungal xylanases are glycoproteins and their carbohydrate content varies from 1-40%. The carbohydrate moiety is either covalently bound or present in the form of a complex. Non-covalently associated carbohydrate moiety has been reported in the xylanases from alkalophilic/thermophilic *Bacillus*, in which they obtained around 40% of the protein to be the carbohydrate moiety (Chauthaiwale, 1993). The same is true of the xylanases from *Trichosporon cutaneum* and *Schizophyllum commune* (Paice *et al.*, 1978; Stuettgen and Sahm, 1982). Carbohydrate moiety is believed to have a role in imparting stability (Woodward, 1984) and multiplicity of the enzyme (Wong *et al.*, 1988). A few reports have been published of bacterial and actinomycetes xylanases which are glycoproteins (Bernier *et al.*,1983; Berenger *et al.*,1985; Marui *et al.*, 1985).

Amino acid composition:

Amino acid composition of several xylanases has been reported. In general they contain high amounts of acidic as well as neutral amino acids while the sulphur containing amino acids are low or absent. The xylanases from *A. niger* (Frederick *et al.*, 1981) and *Irpex lacteus* (Hobler and Brillouet, 1984) lack methionine residues. Absence of Cysteine residues has also been reported in *Aspergillus niger* (Frederick *et al.*, 1985; Shei et al., 1985 and Fournier *et al.*, 1985).

CHEMICAL MODIFICATION STUDIES

Information obtained by studying the effects of chemical modifiers on the catalytic activity of the enzyme can lead to important information on the structure-function relationship of the protein (Coughlan, 1992). There are few reports on active site determination by chemical modification.

Involvement of the tryptophan residues at the active site of xylanases have been based on inhibition of xylanases by N-bromosuccinimide. However, quantitative studies to

determine the number of residues involved in catalysis are few. Keskar *et al.* (1989) have shown the involvement of a single tryptophan residue in catalytic activity of the thermotolerant *Streptomyces* T-7 xylanase. A single residue of tryptophan was shown to be essential for activity in *Chainia* xylanase (Bastawde, 1987), whereas the xylanase from alkalophilic thermophilic *Bacillus* had three tryptophan residues at or near the active site.

The involvement of a carboxyl group at the active site of *Schizophyllum commune* xylanase was shown by Bray and Clarke (1990). EAC was found to inactivate the enzyme rapidly in a pseudo-first order kinetics. These studies have shown the involvement of a single carboxyl group. Inhibition by guanidium hydrochloride has shown the presence of carboxylate groups in *Chainia* (Deshpande *et al.*, 1995) and alkalophilic-thermophilic *Bacillus* (Rao *et al.*, 1995) xylanase. Inhibition of the non-specific xylanase from *Myrothecium verrucaria* xylanase was shown to be inhibited by high concentration of DEP (20 mM) (Coughlan, 1992). Since only 40% inhibition of enzyme activity was obtained, it appears that the histidine residue may not be directly involved in the catalytic activity.

Site directed mutagenesis have confirmed the presence of two glutamate residues at the active site of xylanases from *Bacillus subtilis* (Wakarchuk *et al.*, 1992), *Bacillus circulans* and *Streptomyces lividans* (Coughlan, 1992).

Amino acid sequences: More than eighty cellulases and xylanases have been sequenced. Most of the protein sequences have been deduced from the nucleotide sequence. Cellulases and xylanases have been grouped into eight families based on amino acid sequence homology. A to I (Gilkes *et al.*, 1991; Beguin *et al.*, 1990). The xylanases have been presently classified into two faimilies viz. F and G. Family F contains a few cellulases along with xylanases whereas G has only xylanases. Family F is composed of high molecular weight xylanases and cellulases such as the exoglucanase of *Cellulomonas fumi* or the bifunctional cellulase of *Caldocellum saccharolyticum*. Family G has low molecular weight xylanases similar to *Bacillus pumilis* xylanase whose three dimensional structure is known (Okharda, 1989). The members of the individual families show a common fold, active site architecture and catalytic mechanism. However with the excep-

tion of xylanase A of *Caldocellum saccharolyticum* and one of the two catalytic domains present in the bi-functional cellulase, the catalytic domain of xylanases exhibit little homology with those of cellulases, suggesting that they have evolved from distint ancestral genes (Curry *et al.*,1988; Morag *et al.*,1990; Gilkes *et al.*, 1991).

In the case of alkalophiles there are no reports as yet on the complete aminoacid sequence of xylanases. Tsujibo *et al.*, (1992) determined the partial sequence of the xylanase from alkalophilic *Nocardiopsis*.

X-RAY CRYSTALLOGRAPHY

The three dimensional structures of the xylanases from *Bacillus pumilus* and *Trichoderma reesei* have been determined (Moriyama *et al.*,1987; Katsube *et al.*,1990; Wakarchuk *et al.*,1992). The xylanase from *Bacillus pumilus* has been shown to be ellipsoidal in shape and comprises of three beta sheets, two of which are spread apart to form a 'V' shaped substrate binding cleft. Many of the conserved residues such as Asp-21, Glu-78 are oriented towards the interior of the cleft. Glu-93 and Glu 182 which are 7 A° Angstrom apart are considered to be the catalytic residiues (Katsube *et al.*, 1990). This observation is similar to that of the hen-egg white lysozyme. Structural studies on the two major xylanases of *Trichoderma reesei* showed the presence of two glutamic residues in the cleft which are involved in catalysis (Torronen *et al.*, 1993).

Data from X-ray crystallographic studies corroborate the results obtained from chemical modification studies. The involvement of tryptophan and carboxyl groups at the active site appear to be conserved in microbial xylanases.

MODE OF ACTION:

Xylanases can be either endo or exo in action. Exoxylanases liberate xylose or xylobiose from the non reducing end while endoxylanases randomly liberate oligosaccharides of different chain length (from X2 to X7).

There are very few reports on exoxylanases and their mode of action have not been well worked out (Fukui and Sato, 1957; Sasaki and Inaoka, 1967; King and Fuller, 1968). Endo-xylanases on the other hand have been extensively studied and well characterized from a variety of microbial sources.

Fungal xylanases can be classified into two types namely, debranching and non-debranching enzymes depending on the product of hydrolysis. While the non-debranching enzyme do not liberate arabinose from arabinoxylan and arabinoglucoronoxylan, the debranching enzymes liberate arabinose from the above substrates. Both groups degrade glucuronoxylans and D-xylans.

Trichoderma endoxylanases form xylo-oligomers, xylobiose and xylose. Xylose formation is a result of the hydrolysis of xylo-oligomers and is essentially not a major product of initial hydrolysis. However the enzymes from *Trichoderma pseudokoningii* and two enzymes from *Trichoderma viride* did not produce xylose. One of the non-specific xylanases from *T.viride* was shown to be exo in action as it produced xylobiose as an initial product.

Xylobiose is the smallest xylo-oligosaccharide that is hydrolysed by most *Trichoderma* xylanases. Exceptions to this are the enzyme from *T. lignorum* and one of the enzyme from *Trichoderma harzianum*. The release of arabinose in Trichoderma enzymes is species dependent (Wong and Saddler, 1992).

Takenishi and Tsujisaka (1990), have shown arabinose releasing enzymes in *Aspergillus niger*, which in co-operation with the non-arabinose releasing enzyme brought about increased hydrolysis of arabinoxylan. One of the xylanases from *C.paradoxa* (Dekker and Richards, 1975a) liberates L-arabinose from spear grass, hemicellulose B, sugar-cane bagasse and wheat endosperm arabinoxylan.

Bacterial xylanases, in general have been found to be endoxylanases. The two endoxylanases isolated from *Bacillus circulans* were found to have different modes of action (Esteban *et al.*,1982). Endoxylanase-I could degrade xylan to xylose, xylobiose and xylotriose whereas xylanase II predominantly yielded xylobiose, xylotriose and xylotetrose but not xylose. The endoxylanase from acidophilic *Bacillus* sp. 11-15 (Uchino and Nakane, 1981) showed a similar

mode of action as that of *Bacillus circulans* endoxylanase I. Horikoshi and Atsukawa (1973), showed the presence of arabinose in the hydrolysates of rice straw by the alkalophilic *Bacillus* species. No xylose was detected during the initial stages of hydrolysis. Dey *et al*, (1992) reported the formation of trails of xylobiose, xylotriose and traces of xylose from xylan while no arabinose was detected.

The major endproducts of hydrolysis by *Streptomyces* T-7 endoxylanase were xylobiose and xylo-oligosaccharides. However xylose was detected on prolonged hydrolysis. The xylanase from *Streptomyces* sp. KT-23 initially produced large amounts of xylobiose which was subsequently degraded to xylose (Nakajima *et al.*, 1984). The xylan from *Chainia* produced xylotriose, xylohexose during the initial stages of hydrolysis of xylan and extensive hydrolysis did not show the presence of xylose (Bastawde, 1987).

The extent of hydrolysis depends on the size of the sugar binding site of the enzyme. It is assumed that the enzyme with a larger substrate binding site, requires larger regions of the unsubstituted part of the main chain than enzymes with smaller substrate binding sites. As a result enzymes with larger substrate binding site should hydrolyze heteroxylan to a lesser extent and generally produce larger oligosaccharides and vice a versa. The enzymes with larger binding site also have an affinity for xylotetraose and higher oligosaccharides. The degree of substitution of xylans is known to influence the hydrolytic pattern of xylanases (McNeil *et al.*, 1975). The xylanase from *Ceratocystis paradoxa* has been shown to preferentially attack those regions of the xylan molecule which are highly substituted with arabinose and uronic acid leaving less substituted xylan of low molecular weight (Dekker and Richards, 1975).

APPLICATION

Recent studies have established that xylanases of microbial origin, preferably free of associated cellulase activity hold tremendous promise and potential for use in pulp and paper industries. The outcome of such an application has been projected to offer attractive scope in minimizing environmental pollution and also using reduced concentrations of toxic chlorine compounds to achieve pulp brightness. Xylanases can be employed in paper industry for debarking, enzymatic beating of pulp for enhancing fibrillation, improving filterability, biobleaching, preparation of rayon grade pulps and deinking of recycled fibres. Jurasek and Paice (1980) showed the application of xylanases in production of high quality dissolving pulp. Viikari *et al.* (1986) demonstrated significant reduction in the consumption of bleach chemicals in enzyme aided bleaching.

In European countries such as Finland and Sweden as well as in Canada trials using enzyme-aided bleaching have been carried out on a large scale with highly promising results (Viikari *et al.* 1990). The kraft process accounts for 80 to 85% of all paper production and bleached paper is a highly saleable commodity. During kraft pulping, the major portion of lignin and hemicellulose are solubilized. The kraft pulp at this stage has a brown colour and the residual lignin content varies from 2 to 4%. On cooling some of the solubilized xylan reprecipitates on the cellulose microfibrils, which are recalcitrant to further degradation and as a result forms a barrier for the removal of residual lignin and chromatophores during the subsequent bleaching stage.

Pulp bleaching is a multistage process which involves the use of elemental chlorine (Cl_2). Chlorine dioxide (ClO_2) or a combination of the two, followed by alkaline extraction to remove degraded lignin products. Use of chlorinated compounds especially elemental chlorine results in generation of chlorinated aromatic hydro-

carbons such as dioxane, which are highly toxic and carcinogenic. With greater awareness of the pollution hazards as well as imposition of stringent environmental laws in the developed countries,, the shift towards "environmentally friendly" alternative of xylanase technology is gaining rapid momentum. By the turn of the century totally chlorine-free (TCF) paper technology is envisaged (Dutton, 1992) which means that the enzymatic processes for biobleaching could become commercial realities in the near future. The state of the art as well as the future potential of this interesting area in biotechnology has been critically reviewed recently by Viikari *et al.* (1994). Xylanases pretreatment of pulp caused substantial reduction in residual lignin content and savings in bleach chemicals and concommitant reduction in Ao x levels in the effluents. An average reduction of 25% in elemental chlorine consumption and 15% in total chlorine consumption with softwoods have been reported. (Viikari *et al.*, 1994). Optimally the xylanases are active at pH 5-7 and at 40-50°C and it is obligatory that modifications of the existing pulp processing need to be implemented. Xylanase dosage reported for enzyme aided bleaching is of the order of 30 nkat/gm to 8300 nkat/gm (approximately 2.0 IU/g to 520 IU/g). More recently, it has been shown that enzymatic pretreatment resulted in the pulp having a higher degree of brightness. Enzymatic pre-bleaching has been successfully demonstrated on a nill scale in which a pulp with 88% ISO brightness was obtained when used together with reduced levels of chlorine dioxide and hydrogen peroxide (Viikari *et al.*, 1994). In the case of bleach sequences, where only chlorine dioxide have been used as bleach agent, the use of enzyme was found to increase the productivity of the bleaching plant.

Present Investigations

In recent years, there has been a growing interest in cellulase free xylanases due to their potential applications in paper and pulp industries (Viikari *et al.,* 1994). Akalophilic/akalotolerant micro-organisms producing cellulase free xylanases active and stable at alkaline pH are desirable in the pulp operations. In view of the commercial potential of alkaline cellulase free xylanases from fungal systems, (as they are known to be better secretors of enzymes) the present investigation was carried out to (a) isolate alkalotolerant/alkalophilic fungi capable of producing high levels of cellulase free xylanases (b) optimize the production conditions (c) purify and characterize the enzyme and (d) evaluate its use in the treatment of the bagasse pulp.

CHAPTER 2

ISOLATION, STRAIN CONSERVATION AND MEDIA OPTIMIZATION FOR PRODUCTION OF *CEPHALOSPORIUM* XYLANASE

SUMMARY

During a screening programme undertaken to identify microorganisms capable of growing and secreting xylanases active and stable at high alkaline pH several bacteria, actinomycetes, and fungi were isolated. Two fungal isolates showed significant clearance on xylan plates and these were identified as *Cephalosporium* and *Periconia* species on the basis of the morphological characters of their spore-forming structures. Upon further studies, the *Cephalosporium* strain, designated as NCL 87.11.9, gave consistently higher xylanase activity and was taken up for detailed investigation. The fungus was capable of growing over a wide pH range (pH 6.0 to pH 11.0) indicating its alkalotolerant nature. A maximum activity of 15 to 18 IU/ml was obtained in 96 h on a wheat bran-yeast extract medium when cultivated in shake flasks at pH 10. No xylanase secretion was observed when grown in media containing sugars such as glucose or xylose indicating that the xylanase was an inducible enzyme. There was an obligate requirement of xylan or xylan rich residues for enzyme production. The *Cephalosporium* xylanase showed a pH optima at 8.0, while 80 and 60 percent of maximal activity was observed at pH 9.0 and 10.0, respectively. The activity and stability of the enzyme over a pH range of 7.0 to 9.5, makes it potentially useful for pulp and paper biotechnology applications. The secreted enzyme also lacked cellulase activity which is an additional positive feature associated with this fungal enzyme. Extracellular cellulase secretion was not observed even on media supplemented with Solka Floc.

Significant levels of xylanase were secreted over a broad pH range with substantial amounts being produced at pH 10.0. The properties of the enzyme remained the same, irrespective of the initial pH of the medium used for production. When fermentation studies were carried out in presence of known inhibitors of protease activity such as phenylmethylsulphonyl flouride (PMSF), the extracellular xylanase activity was found to increase two fold. Apart from submerged culture, the fungus when cultivated in semisolid state fermentation on moist wheat bran

supplemented with yeast extract, elaborated significant levels of xylanase activity. The properties of the enzyme were by and large similar to the enzyme obtained in submerged culture.

The present study is perhaps the first report of a cellulase-free extracellular xylanase production from an alkalotolerant fungal culture which is active at alkaline pH. So far xylanases stable to and active at high alkaline pH have been reported only from bacteria and actinomycetes, while fungal xylanases reported in literature are largely active in the acidic pH range (4.5-6.5) and are generally co-secreted with cellulase activity.

INTRODUCTION

In the search for xylanases free of cellulase activity which could find ready application in pulp and paper biotechnology, attempts have been made to inactivate the accompanying cellulase activity through treatment with mercurial compounds (Barnoud *et al.*,1986; Noe *et al.*, 1986) or obtain such enzymes by cloning and expression in non-cellulolytic heterologus hosts (Paice *et al.*, 1988; Bertrand *et al.*, 1989) or bulk scale purification (Tan *et al.*, 1987). The search for naturally occuring microbial systems which are xylanolytic without accompanying cellulase activity has been in progress in several laboratories, and one of the first reports of such a strain has been the extracellular cellulase-free xylanase from a sclerotium-forming actinomycete *Chainia* discovered in our laboratory (Srinivasan *et al*, 1984). This was followed by studies on a cellulase-free xylanase from an alkalophilic *Bacillus* (Srinivasan *et al.*, 1988; Balakrishnan *et al.*, 1992) and also from an alkalophilic *Streptomyces* VP-5 (Vyas *et al*, 1990). Other reports in literature on cellulase-free xylanases include the enzymes from *Saccharomonospora viridis* and *Streptomyces* T-7 (Roberts *et al.*,1990; Keskar *et al.*,1989). As cellulase-free xylanases active at alkaline pH are desirable in pulp and paper applications, and since there were no reports on fungi secreting xylanases active at alkaline pH, the possibility of exploring fungi as sources for cellulase-free xylanases with high activity and stability at alkaline pH appeared to be a challenging prospect. In the present study considerable emphasis was laid on establishing culture methods by which alkalophilic/alkalotolerant fungi with xylanolytic potential could be discovered.

The present chapter details the methodologies adapted to isolate such cultures which led to the identification of alkalotolerant *Cephalosporium* sp., NCL 87.11.9, which exhibited significant levels of xylanase secretion at alkaline pH and also to optimise xylanase production in both submerged and semi solid fermentation.

MATERIALS AND METHODS

MATERIALS

All the chemicals used were of analytical grade. The sources of the following chemicals are shown in parentheses :

Glucose, Yeast extract, Malt extract, Peptone and Tryptone (HiMedia, India). 3,5-dinitrosalicylic acid (DNS), sodium salt of carboxymethylcellulose (CMC), Oat spelts Xylan, Bovine Serum Albumin, Carbonic anhydrase, Aprotinin, Cytochrome C and Ovalbumin (Sigma chemical company, USA). Xylose (Fluka, Germany); Tween 80, Tween 20, (Sisco Research laboratories, India); Solka Floc (Brown Co.,Germany). Wheat bran, wheat straw, rice bran, corn cobs, sugar cane bagasse and soyabean meal (defatted) were obtained locally.

Soil Sample

Several soil samples were collected from in and around Poona whose average pH ranged from 6.5 to 7.5. The soil samples were generally rich in humus and decomposing plant litter. After collections, the samples were air dried and sieved to obtain homogeneous samples. The soil was stored in paper bags at room temperature until use.

MEDIA

All glassware used for fermentation experiments were washed, dried, plugged with cotton and sterilized at 15 lb (121°C) for 1 h. Nutrient media were sterilized at 15 lb (121°C) for 20 to 40 min depending on the media constituents. Media containing agricultural residues such as wheat bran, corn cobs etc., were autoclaved for 40 min. All inoculations and sampling were done under aseptic conditions. Prior to inoculation, the pH of the medium was adjusted approximately to 10 by the addition of 10% (w/v) sodium carbonate, which was separately sterilized and added to obtain a final concentration of 1% (v/v) in the medium unless otherwise stated. The composition of media used in this study is given below:

Medium for Isolation

Wheat bran (washed)*	:	3%
Yeast extract	:	1%
Agar	:	2.5%
Sodium carbonate	:	1% (added separately sterilized)

*Wheat bran was autoclaved at 15 lbs (121°C) for 20 min and then washed with tap water repeatedly until free of starch. Addition of the moist bran was equivalent to 3% dry weight (w/v).

Medium for Plate Assay of Xylanolytic cultures

Yeast extract-Malt extract-Xylan [YMX]:

Yeast extract	:	1%
Malt extract	:	1%
Xylan	:	1%
Agar	:	2.5%
Sodium carbonate	:	1% (added separetly sterilized)

The above medium was used for evaluation of the isolated cultures for xylanolytic activity on agar plates using the congo red method according to MacKenzie *et al.* (1987).

Medium for Culture Conservation

Periodic subculture: Routine subculture of the *Cephalosporium* strain was carried out every four months on both, neutral and alkaline YMX slants.

Lyophiles: *Cephalosporium* showing good growth and sporulation on YMX (neutral) slant was used for the preparation of the lyophiles.

Soil and paraffin oil: The starting culture for conservation in soil and under paraffin oil was grown on YMX (neutral) as well as YMX (alkaline) medium.

Medium for revival: The conserved cultures were revived at periodic intervals in the following medium before evaluating them for xylanase production:

Malt extract-Glucose-Yeast extract-Peptone [MGYP]

Malt extract	:	0.3%
Glucose	:	1.0%
Yeast extract	:	0.5%
Peptone	:	0.5%
Agar	:	2.5%
pH	:	6.7

Medium for enzyme production in submerged fermentation

Inoculum :

Wheat bran	:	3%	
Yeast extract	:	1%	
sodium carbonate	:	1%	(added separetly sterilized)
pH	:	10	

A 10 % (v/v) inoculum was built up for 72 h at 28°C and transferred asceptically thereafter into fermentation flasks, the composition of which is given below.

Fermentation medium

Wheat bran	:	5%	
Yeast extract	:	1%	
sodium carbonate	:	1%	(added separetly sterilized)
pH	:	10	

The inoculum tubes and the shake flasks were incubated at 28°C at 180 rpm on a rotary shaker. The broth was harvested after 96 h, centrifuged and the cell-free filtrate taken up for assaying extracellular xylanase activity.

Medium for semi-solid fermentation

Inoculum : The inoculum was prepared as described under submerged fermentation.

Fermentation medium:

Wheat bran	:	10 g
Yeast extract	:	8 ml of 10% solution (w/v)
Sodium carobonate	:	5ml of 20% solution (w/v)

[added separetly sterilized]

METHODS

Isolation: In order to encourage alkalophilic/alkalotolerant fungal strains with xylanolytic activity the following procedure was adapted, which is a modified method based on a procedure as described by Horikoshi and Atsukawa (1973) to isolate alkalophilic bacteria. Wheat bran was suspended in water, autoclaved and after cooling to room temperature, extracted and repeatedly washed to free it of the associated starch residues. Wheat bran (3 %) on dry weight basis was suspended in 100 ml of 0.5% yeast extract solution to which 2 % of Bacto-agar (Difco) was added, the flask was sterilized at 15 lb for 30 min to which 10 ml of 10% sterile sodium carbonate was aseptically added to obtain a pH above 10.0. Tetracycline (5 mg) was added as a 1 ml solution to the cooled media, just before dispensing it in petri plates (to give an effective concentration of 50 μg/ml).

Soil suspension in sterile water was streaked on solidified agar followed by incubation at 28-30°C for ten days. Bacteria and actinomycetes were found to be effectively suppressed by the added antibiotic, while fungal colonies developed after 4-5 days of incubation. Individual, well isolated colonies were transferred to slants of alkaline wheat bran-yeast extract agar, and allowed to grow for 7-10 days.

Detection of xylanolytic activity: Pure cultures of fungal colonies identified to be different on the basis of their mycelial morphology as well as spore bearing structures were transferred individually on YMX plates at pH 10. After varying periods of incubation (generally 5-7 days) the plates showing clearance zones were flooded with congo red as described by MacKenzie, *et al*, (1987). The method, involved flooding the plates with 0.1% aqueous congo red and the plates were allowed to stand for 30 min. The plates were then thoroughly washed with 1 M of NaCl solution. A clearance zone was evident around the growing colonies of xylanase positive cultures against the dark red background, the unreacted xylan taking up the stain while the hydrolyzed portion remained colourless. The contrast could be enhanced by treating the plate with 5% acetic acid (v/v) for 1 to 2 min and then washing off the excess acid with distilled water. Such of those fungi which showed good clearance beyond the area of mycelial growth were selected for further studies as potential xylanase secretors. Among the several fungal colonies isolated, two colonies differing in morphological characters showed distinct clearance zones. These were identified as *Cephalosporium* and *Periconia* species respectively. The two fungal cultures were further evaluated for extracellular xylanase secretion in shake flasks on a wheatbran-yeast extract medium as described earlier. As the *Cephalosporium* species gave consistently higher xylanase activity, it was selected for carrying out intensive investigation on xylanase production as well as characterization of the enzyme. The fungus was identified as *Cephalosporium* sp., and designated as N.C.L. 87.11.9. The culture was deposited in the following depositories and the accession numbers are given in parentheses:

1. National Collection for Industrial Micro-organism

2. American Type and Culture Collection : (ATCC) (ATCC No. 74297)

Culture Conservation: Long term conservation of the fungus was studied by the following standard methods :

1. Freeze drying

2. Soil dispersal of spores

3. Overlay of the fungus with paraffin oil

4. Preservation under 10% glycerol

In addition to the above methods, the culture was also routinely maintained by quarterly subcultures.

Revival of the fungus and its corresponding xylanase production capability was evaluated after every 3 to 6 months over a period of four years.

The fungus was grown on YMX-agar slants and the spore suspension was used for lyophilization, soil dispersal of spores and for paraffin ovrlay. The revival of the fungus was followed by growing it in neutral MGYP liquid medium for 72 h at 28°C in a shaker incubator (180 rpm). After the culture had grown, it was transferred onto YMX agar slants. A seven day old slant was evaluated for xylanase production.

Culture conservation by lyophilization : The fungus was grown on neutral YMX slants for seven days to obtain abundant sporulation. A spore suspension was made in 5 ml of skimmed milk (10 %, w/v) by gently scraping off the spores with the help of a needle. The spore suspension was approximately adjusted to a spore count of 2.2 x 10^{10} per ml. Three hundred microliters of the spore suspension was aseptically dispensed into sterile glass vials, for lyophilization. The spore suspension was ascertained for its purity by streaking on agar media prior to lyophilization. Lyophilized samples were checked for viability and stored at 4-5°C until use.

Conservation in sterile soil: Soil was sieved to get a uniform particle size and moistened with water before autoclaving. The soil was autoclaved at 15 lbs (60 min.) thrice on three consecutive days followed by drying at 160°C for 2-3h. The sterilized soil tubes were checked on random for sterility by inoculating nutrient broth tubes. Spore suspension prepared from agar slant (2.2 x 10^{10} spores/ml) was aseptically centrifuged, dispersed in the sterile soil and left at room temperature for 3 days prior to refrigeration (5-10°C) for conservation.

Conservation under paraffin oil: Liquid paraffin was autoclaved at 15 lb pressure for 60 min., followed by heating in an oven at 100°C for 1-2 hours. Agar slants with good growth and sporulation were overlaid with sterile paraffin oil and stored at 10°C for conservation.

Deep freeze conservation in glycerol: Spore suspension from agar slant culture was aseptically centrifuged and aliquots of 100 μl were aseptically mixed with sterile 10% (v/v) glycerol solution in small vials. These vials were conserved in the deep freeze at -10°C.

Evaluation for viability and xylanase productivity of stock cultures : At six monthly intervals, viability as well as xylanase productivity of stock cultures conserved under different methods was evaluated. It was observed that cultures preserved under oil took longer to revive (5-7 days) while the cultures conserved by the other methods readily revived within 48 hours.

Enzyme production : Subcultures were initiated on a MGYP slant (neutral pH) and seven day old sporulating cultures were employed for studies on enzyme production. Inoculum was developed in a medium containing wheat bran 3%, yeast extract 1% and sodium carbonate 1% added separately sterilized in submerged cultures on a rotary shaker (180 rpm) for 72 h at 28°C. The mycelial inoculum (10%, v/v) was added to experimental flasks containing wheat bran 5%, yeast extract 1% and sodium carbonate 1% (added separetly sterilized) which were similarly incubated. The fermentation was terminated at 96 hours when maximal extracellular enzyme

activity was observed. Culture filtrates having a standard activity of 18-20 IU/ml were taken up for studies on enzyme purification and biotechnological applications related to paper pulp.

Xylanase production by semi-solid fermentation: The semi solid medium containing wheat bran (10 g) and yeast extract (8 ml of 10% w/v) was inoculated with 10 ml of inoculum aseptically and then incubated at 28°C. The Koji culture was harvested after 144 h of incubation as described below.

Extraction of the enzyme from semi-solid cultures: Six day old cultures grown on moist bran in semi solid fermentation (as indicated earlier) were extracted in distilled water. 50 ml of distilled water was added to the biomass and shaken for 1 h at 28°C to extract the enzyme. The extract was decanted followed by a second extraction with additional quantity of distilled water. The two extracts were pooled together, centrifuged to remove suspended particles and assayed for xylanase activity. The preliminary properties of the crude culture filtrate were compared with the crude enzyme obtained from submerged fermentation.

Substrate Preparation: Two grams of oatspelts xylan was suspended in 100 ml of 50 mM potassium phosphate buffer, pH 8 or in 100 ml of glass distilled water when used for evaluation at other pH values. The suspension was kept on a rotary shaker overnight at room temperature to solubilize the xylan. The insoluble material was removed by centrifugation (approx. 9500g) and the soluble fraction corresponding to 0.7% (w/v) was used as the substrate.

Enzyme Assays

Xylanase Assay : The DNS assay procedure was used routinely to monitor enzyme activity. The reaction mixture of 1 ml contained 0.5 ml of suitably diluted enzyme in potassium phosphate buffer (50mM; pH 8.0) and 0.5 ml of (0.7% w/v) xylan solution. The reaction mixture was incubated at 40°C for 30 min and then terminated by the addition of 1.0 ml of DNS. The tubes

were heated in a boiling water bath for 5 min. to develop the colour and the intensity was read at 540 nm after dilution with 10 ml of distilled water (Bernfeld, 1955). In the case of assays carried out at other pH values, the corresponding amount of soluble xylan prepared in water was added and high molarity buffer was added so as to get final concentration of 50 mM.

Unit of activity: One unit of xylanase activity is defined as the amount of enzyme that produced one umol of xylose equivalent per minute under the assay conditions.

Somogyi-Nelson's Method: The method was used to compare activities obtained with the DNS method. The reaction mixture, 1 ml, contained 0.5 ml of suitably diluted enzyme and 0.5 ml of xylan (0.7%) in 50 mM potassium phosphate buffer pH 8.06. The reaction was allowed to proceed for 30 min at 40°C and was terminated by the addition of 1 ml of Somogyi-Nelson reagent A. The tubes were heated in a boiling water bath for 10 min. The reaction tubes were then cooled immediately after which l ml of Somogyi-Nelson reagent B was added, followed by addition of 10 ml of distilled water. The colour developed was read at 520 nm.

Carboxymethyl cellulase activity: The reaction mixture contained an aliquot of 0.5 ml of the enzyme solution and 0.5 ml of 1% carboxymethylcellulose (Na-salt) in 50 mM phosphate buffer pH 6.0 or pH 8.0. The reaction mixture was incubated for 30 min at 40°C. The reducing sugar liberated was measured as glucose equivalent by the DNS method, as described earlier.

Unit of activity: One unit of enzyme corresponds to one umol of reducing sugar produced per minute under the assay conditions.

Filter paper activity: The reaction mixture contained 50 mg of Whatman No. 1 filter paper (1 x 6 cm strip) in 1 ml of 50 mM phosphate buffer and the enzyme. The mixture was incubated for 60 min (18 h in some cases) at 40°C and the reducing sugar liberated was measured as glucose equivalents by the DNS method.

One unit of the enzyme corresponds to one μmol of reducing sugar produced per minute under the assay conditions.

ß-Xylosidase assay : ß-xylosidase assay was carried out according to Berghem and Peterson (1973), using p-nitro phenyl ß-D-xylopyranoside as the substrate. The total volume of the reaction mixture was 1 ml. The reaction mixture consisted of hundred microliters of the enzyme and four hundred microliters either 50 mM citrate phosphate buffer, pH 6.0 or 50 mM potassium phosphate buffer, pH 7.0. The reaction was initiated by the addition of 500 μl of 0.5 M p-nitro phenyl ß-D-xylopyranoside. The tubes were incubated at 40°C for 30 min after which 1 ml of 1% sodium carbonate was added to terminate the reaction and the yellow colour developed was read at 410 nm. One unit of the enzyme corresponds to one μmol p-nitrophenol produced per minute, under the assay conditions.

α-L-arabinofuranosidase assay: α-L-arabinofuranosidase assay was carried out as described above using para nitrophenyl-L-arabinofuranoside as the substrate at pH 6 and pH 7.0.
One unit of the enzyme corresponds to one μmol p-nitrophenol produced per minute, under the assay conditions.

Alkaline protease assay: The protease activity was determined by Kunitz (1947) modified procedure (Srinivasan *et al.*, 1985). The reaction mixture contained an aliquot of suitably diluted enzyme solution and 1 ml of 1% Hammersten casein in 50 mM potassium phosphate buffer, pH 7.0, in a total volume of 2 ml. After incubation at 40°C for 30 min, the reaction was terminated by the addition of 3 ml of 5% (v/v) tricholroacetic acid. The precipitate formed was filtered through Whatman No. 1 filter paper, after standing for 30 min at room temperature. The absorbance of the trichloroacetic acid soluble fraction was measured at 280 nm. One unit of protease

activity is defined as the amount of enzyme required to bring about an increase of 0.001 absorbance/ml of reaction mixture/min. under the assay conditions.

Molecular weight determination: Molecular weight was determined on a Sephadex G-50 column according to Andrews (1965). The column (1 x 100 cm) was pre-equilibrated with 50 mM potassium phosphate buffer and then callibrated with molecular weight markers. The standard protein markers used were bovine serum albumin, carbonic anhydrase, aprotinin, cytochrome-C and ovalbumin.

Three milligram of each standard protein was used to calibrate the column. The ammonium sulphate precipitated culture filtrate equivalent to 33 IU was loaded. Fractions were collected at a rate of 2 ml/10 min., which were then assayed for xylanase activity. The protein samples obtained in the fractions were read at 280 nm. Aprotinin was detected by estimating the trypsin inhibitor activity of the fractions, while cytochrome C was estimated at 550 nm by reduction with dithionite at pH 8.0 (Tsou, 1951).

Isoelectric focussing: Isoelectric focussing (IEF) was carried out in a glycerol gradient according to Pawar et al (1986) using the ampholine in the range of pH 3 to 10. The ammonium sulphate precipitated and dialysed enzyme equivalent to 30 IU was used. Fractions of 200 ul were collected and diluted to 1 ml with deionized water and the pH of each fraction was determined and assayed for xylanase activity.

Preparation of the standard xylo-oligosaccharides: The standard oligosaccharides were prepared by acid hydrolyzing Oat spelts xylan. Oatspelts xylan (200 mg) was hydrolysed with trifluoroacetic acid (total volume=200 μl containing 50 μl of concentrated trifluoroacteic acid) for 20 min at 100°C. The hydrolysate was then neutralized with the help of 1N NaOH and centrifuged at 9700 g for 10 min. to remove the undissolved portion. The supernatant was lyophilized. The products so concentrated were checked by paper chromatography using Whatman No. 1

paper (13 X 40 cm). Preparative paper chromatography for separation of xylo-oligosaccharides was carried out using Whatman No. 3 paper (13x40 cm) 1 ml of the hydrolysate was loaded onto Whatman No. 3 paper and chromatography was carried out for 30-36 h to get good separation, the solvent system being butanol, acetic acid and water in a ratio of 240:80:80. A strip of the paper was cut out and dried at 100°C for 15 min. and was further sprayed with the following reagents containing phthalic acid [0.332 g] and para-anisidine [0.246g] in 20 ml of absolute ethanol as described by Gunther and Sherma, (1972). The paper strip was heated for 30 min and the sugar spots were visualized as pink spots. The paper was cut into strips after aligning the spots, so as to separate the xylo-oligosaccharides. The separated sugars were eluted by soaking the strips in water, and each fraction was concentrated by lyophilization, after which they were checked for purity by HPLC and analytical paper chromatography.

High Performance Liquid Chromatography [HPLC]: A standard Aminex 42 A column was used for HPLC (3000 X 7.8 mm), to check the purity of xylo-oligosaccharides and to moniter enzymatic hydrolysates. The column was eluted with water at a flow rate of 0.5 ml/min at 70°C. The products were determined by the measurement of refractive index changes. 25ul of the standard xylo-oligosaccharide sample equivalent to approximately 25 µg of sugar was loaded on the column.

Analytical Paper Chromatography: Descending paper chromatography was carried out using Whatman No. 1 paper (13X40 cm). 15 µl of the sample was loaded with the help of a capillary tube. The solvent used as the mobile phase consisted of n-butanol, acetic acid, water in the ratio of 3:1:1. Paper chromatography was carried out for 30 h, after which the paper was dried at 100°C for 30 min. The paper was sprayed with the aforementioned reagent.

End product analysis: The action of xylanase on oatspelts xylan was studied and the end products formed were analyzed. Xylan (50 mg) was hydrolyzed with 3 IU of enzyme in 4 ml of

pH 8, 50 mM potassium phosphate buffer for varying time intervals (30 min to 24 h). The reaction was terminated by heating the tubes in a boiling water bath for 10 min. The supernatant was taken and lyophilized.

The hydrolysate was evaluated for the product by analytical HPLC and paper chromatography as described in Materials and Methods of this section.

RESULTS AND DISCUSSION

ISOLATION AND FERMENTATION STUDIES

Screening and identification of the alkalotolerant fungus

Fermentation experiments in shake flasks indicated that the *Cephalosporium* strain gave consistently higher levels of extracellular xylanase activity (Table 2.1) and hence all further investigations were focussed only on the *Cephalosporium* strain designated NCL 87.11.9 and deposited in NCIM under the accession number 1251 and ATCC no. 74297.

Table 2.1: Evaluation Xylanolytic cultures

Genus	Xylanase activity IU/ml (96h)
Cephalosporium sp.	15.0
Periconia sp.	4.7

Medium containing 3% wheat bran and 1% yeast extract at pH 10.0 was used for evaluating the cultures.

Description of the culture

The *Cephalosporium* strain when transferred to YMX agar slants (alkaline and neutral) and incubated at 28-30°C, for 7-10 days was capable of rapid growth and profused sporulation, the latter being better at neutral pH (Fig. 2a, 2b). Mycelial colonies were floccose with considerable aerial mycelium. After 5-6 days of mycelial growth abundant sporulation was observed resulting in the colony appearimg pale-reddish brown in colour (Fig. 2b). Spores were borne in slimy spore heads terminating in short, individual and generally unbranched sporophores predominately arising from the aerial mycelium (Fig. 2d). When mounted in water on a slide, the slimy spore balls disintegrated, releasing large numbers of ovate unicellular, tiny spores measuring 3-5

Fig. 2a: *Cephalosporium* sp. Growth at neutral pH

Fig. 2b: *Cephalosporium* sp. Growth at alkaline pH

FIG 2a

FIG 2b

Fig.2c: *Cephalosporium* colony showing clearance zone on YMX plate

Fig.2d: Microscopic view of *Cephalosporium*

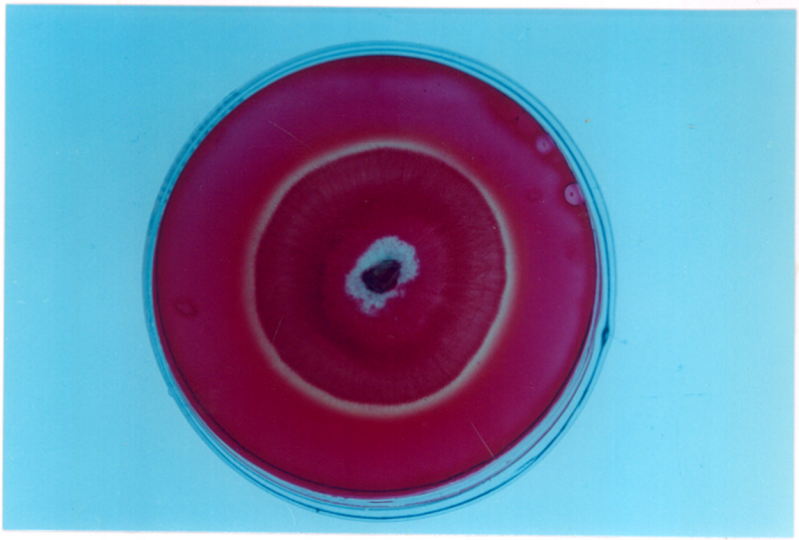


FIG 2c

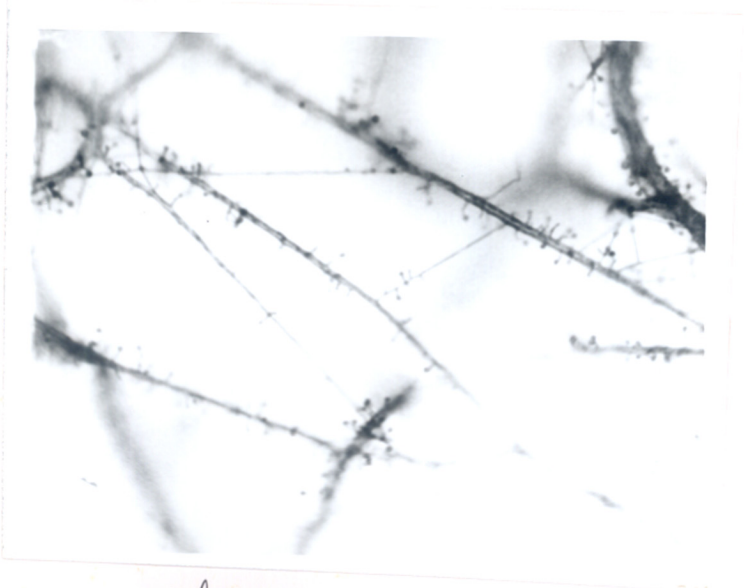


FIG 2d

Fig. 2e: pH growth profile of alkalotolerant *Cephalosporium* sp.

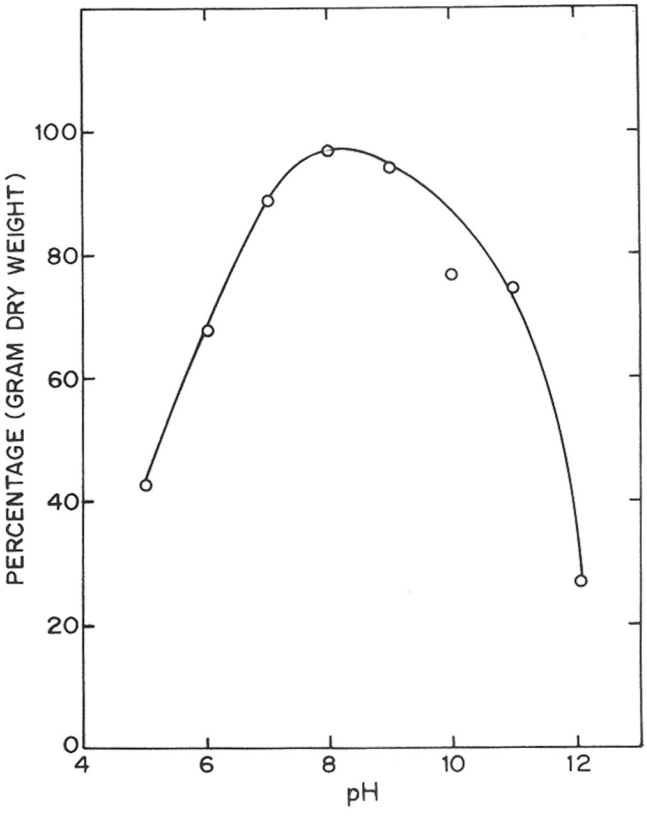


FIG. 2e

microns. The fungus was found to grow equally well on several media such as YMX, wheat bran, MGYP adjusted to neutral pH or high alkaline pH, indicating its alkalotolerant rather than obligately alkalophilic nature. This is in contrast to our earlier studies from this laboratory, where the alkalophilic *Bacillus* NCL-87.6.10 (Srinivasan *et al*, 1988; Balakrishnan *et al.*,1992) was capable of growth and enzyme production only at pH above 9.0 and also showed an obligate requirement for sodium ions.

Effect of pH on growth of the fungus

The growth of the fungus in MGYP liquid medium adjusted to different pH values from pH 4.0-12.0 was evaluated by comparative dry weight determination of the mycelial biomass, in submerged culture (Fig. 2e). It was observed that there was good growth over a broad pH range of 6.0 to 11.0, the growth being maximum around pH 8.0. Growth was very poor at pH 4.0, while at pH 10.0, almost 80% of the maximal growth was observed indicating the preference of the strain for alkaline environment.

Optimum temperature for growth

The *Cephalosporium* species was essentially mesophilic showing maximal growth between 25 to 28°C. Growth and sporulation were poor beyond 30°C.

Culture conservation

The strain could be maintained with ease through periodic subculture once in 3-4 months, followed by conservation at 5-10°C in the refrigerator. However, in order to minimize the onset of genetic variation, strain conservation by techniques such as, lyophilization, paraffin oil overlay, soil conservation and also deep freeze conservation as indicated under Methods (by suspending in 10% (v/v) glycerol) were studied. The results are presented in Table 2.2.

Table 2.2 Conservation of alkalotolerant *Cephalosporium* sp. (NCL 87.11.9.), ATCC No. 74297:

Method of preservation/years	Xylanase Activity IU/ml Years One	Two	Three	Four
Lyophilized culture	19.3	18.7	20.0	21.0
Soil culture	21.6	21.8	21.6	21.0
Paraffin oil culture	21.6	21.0	19.1	18.4
Glycerol (10%)	19.0	18.3	17.6	17.6
Slant culture	19.0	19.3	16.7	18.0

The strain has been observed to be genetically stable, amenable to the different methods of conservation studied. Over a period of four years both viability and xylanase productivity remained stable and consistent as seen from our results.

Standardization of culture conditions for optimum xylanase production

Standarized medium consisted of inoculum preparation containing 3% of wheat bran and 1% yeast extract at pH 10 which was incubated on a shaker with 180 rpm, at 28°C for 72 h the volume being 10 ml. The fermentation medium consisted of wheat bran 5% and yeast extract 1% at pH 10 (100 ml) which was shaker incubated at 180 rpm for 96 h at 28°C and the culture filtrate was evaluated for enzyme production. On the basis of these various studies, details of which are described in the subsequent paragraphs, the formulation of an optimised medium composition for consistent high activity xylanase production was arrived at. All these studies were carried out in shake flasks. In the case of the experiments designed to study the effect of initial pH as well as the effect of protease inhibitors, cultivation of inoculum was carried out at neutral pH.

The following parameters were studied for their individual effects on overall xylanase activity.

a) Period of incubation

b) Concentration of wheat bran

c) Comparative evaluation of alternate xylan-rich residues

d) Comparative evaluation of different organic nitrogen sources.

e) Effect of concentration of yeast extract.

f) Effect of initial pH.

g) Effect of various surfactants on xylanase secretion.

h) Effect of supplementing the control medium with other nitrogen sources.

i) Effect of proteolytic inhibition on xylanase production.

Effect of Incubation period on xylanase production

Shake flask fermentations were carried out for 120 h and the xylanase activity in the culture filtrate was evaluated at intervals of 24 h. Growth in the form of small pellets was observed after 72 h of the fermentation. Maximum xylanase production occured after 96 h at pH 10.0.

Table 2.3: Effect of incubation period on xylanase production:

Time (h)	Xylanase activity[a] IU/ml
24	4
48	13
72	14
96	17
120	16

a: Average value of triplicates

Effect of Wheat bran Concentration

The effect of different concentrations of wheat bran on xylanase production by *Cephalosporium sp.* was evaluated by using untreated wheat bran as the sole carbon source. The concentration of the wheat bran was varied from 3-7 % and the xylanase activity monitored at intervals of 24 h. Maximum activity was obtained with 5% and 7% wheat bran in 96 h. However at higher concentrations the viscosity of the medium increased as growth occured and was not found feasible for enzyme production. 5% wheat bran was used in all further experiments.

Table 2.4: Effect of Wheat bran concentration on xylanase production:

Wheat bran (w/v) %	Xylanase activity[a] IU/m1 [96h]
3	7.5
4	10.2
5	18.5
7	18.7

a: Average value of triplicates
1% yeast extract was used as the nitrogen source.

Effect of other agricultural residues

Xylanase production by the fungus was studied using various xylan rich carbon sources at a concentration of 5% with 1% yeast extract as the nitrogen source. Maximum activity was observed when oat spelts xylan was used as the carbon source, followed by corn cobs (Table 2.5). However, wheat bran was used in all further experiments as it was available around the year, while corn cobs was seasonal. Although, pure xylan gave relatively higher titers, use of pure xylan is not economically feasible.

Table 2.5: Effect of different agricultural residues on xylanase production:

Carbon source (5%) (w/v)	Xylanase activity[a] IU/ml (96h)
Wheat bran (control)	16
Corn cobs	17.4
Bagasse	10.4
Xylan (oat spelts)	18.7
Rice bran	14

a: average values of triplicates
1% yeast extract was used as the nitrogen source.

Effect of different organic nitrogen sources on xylanase production

The effect of different complex nitrogen sources was evaluated for xylanase production (Table [P. T. O] 2.6). Comparable results were obtained with Baker's yeast. Malt extract did not support enzyme production, while other nitrogen sources gave activities in the range of 65-80% of the control. However yeast extract was used for enzyme production in order to obtain a standard preparation for enzyme purification.

Table 2.6: Effect of different nitrogen sources:

Nitrogen source	(%) of nitrogen source	Xylanase activity[a] [IU/ml] in 96 h.
Yeast extract (Control)	1	17
Malt extract	1	4
Tryptone	1	13
Baker's yeast	2.4	19
Peptone	1	12
ProFlo*	1	14
Ground nut	1	13
Soyabean meal (defatted)	1	16

a: Average values of triplicates.
* Cotton seed cake
5% wheat bran was used as the carbon source.

Effect of varying concentrations of yeast extract

The concentration of yeast extract was varied in order to determine optimum xylanase production. The results are presented in Table 2.7.

Table 2.7: Effect of varying concentrations of yeast extract on xylanase production:

Yeast extract concentration (%)	Xylanase activity IU/ml [96 h].
0.5	7
1.0	17
2.0	18
2.5	11

The best results were obtained with 1-2% of the yeast extract.

Effect of pH on xylanase secretion

The effect of initial pH of the fermentation medium on xylanase and cellulase production was studied (Table 2.8). At the end of the fermentation, xylanase, cellulase and filter paper activities were assayed, and the pH of the broth determined.

Table 2.8: Effect of pH on xylanase secretion:

Initial pH	Final pH	Xylanase activity IU/ml [96 h]	Filter paper Activity IU/ml	CMCase activity IU/ml
4	5.3	20.2	0.028	0.51
5	6.7	36.2	0.03	0.047
6	7.6	33	0.03	0.04
7	7.8	27	0.030	0.09
8	8.04	17.2	0.03	0.04
9	8.4	18	0.03	0.03
10	8.9	15	0.03	0.03

All the activities mentioned in the table are average of triplicates.

CMCase refers to carboxylmethylcellulase activity produced.The results showed that while significant levels of xylanase was secreted at pH 10, maximum enzyme secretion occured when the pH of the medium rose to 6.7-7.6 which corresponded to an initial pH of 5.0. From these results and the growth profile it appears that maximum enzyme secretion occurs at a final pH range of 7.0-8.0. Though maximum xylanase production was at pH 5, fermentations were carried out at pH 10, as the initial specific activity was higher for the enzyme produced at pH 10.0. Carboxymethylcellulase and filter paper activities were insignificant under the experimental conditions studied. Supplementing the medium with pure cellulose in the form of Solka Floc also failed to induce extracellular cellulase secretion. The enzyme was found to be inducible requiring xylan or xylan rich substrates for secretion of xylanase and did not give any xylanase activity on

lactose, xylose and glucose, although good growth of the fungus was obtained in the above sugars.

Effect of various surfactants on xylanase production

Since enhancement of enzyme production by non-ionic detergents such as Tween-80 and Tween-20 have been reported for several enzymes such as cellulases (Reese and Maguire, 1969; Takahashi *et al.*,1960; Umesaki *et al.*, 1977; Faith et al., 1971), it was of interest to study the effect of these surfactants, on *Cephalosporium* xylanase production. As can be seen from Table 2.9 only Tween 80 showed enhanced activity at a relatively high concentration of 1 %. Although the activity was enhanced by approximately 70 %, use of such high concentrations of Tween-80 is not practical because of excessive foaming especially at high alkaline pH values. Moreover such high concentrations could hamper membrane filtration during downstream processing of the culture filtrate. Such high concentrations may also have an adverse effect on the organism and hence Tween-80 was not used in the medium for enzyme production. The results with 0.1% of SDS was comparable to the control values, indicating that the enzyme was stable in presence of 0.1% SDS. Tween-20 and Triton X-100 had no significant effect on xylanase production.

Table 2.9: Effect of various surfactants on xylanase production

Surfactant	Concentration of surfactant (%)	Xylanase activity[a] IU/ml [96 h]
Control	--	15
Triton X-100	0.8	15
Tween-20	0.8	15
SDS	0.1	17
Tween-80	0.5	17
	0.8	21
	1.0	26

a: Average of triplicates

Effect of increased nitrogen content

In order to enhance xylanase production, the effect of supplementing the medium with additional nitrogen, was studied (Table 2.10). Addition of 3% Baker's yeast along with 1% yeast extract gave maximal enzyme production of 28 IU/ml as compared to the control value of 17 IU/ml. Similar results were obtained by Balakrishnan *et al* (1993) in the case of the alkalophilic *Bacillus* xylanase. Supplementation with other nitrogen sources gave no significant increase in enzyme production.

Table 2.10 Effect of supplemention of nitrogen :

Nitrogen source	Nitrogen (%)	Xylanase activity [IU/ml]
Yeast extract [control]	1	17
Malt extract	1	11
Tryptone	1	12
Peptone	1	14
ProFlo	1	19
Ground nut cake	1	20
Baker's yeast	1	19
	2.4	25
	3	28
	4	27

All the nitrogen sources employed were alongwith 1% yeast extract.

Effect of protease inhibitors

Enzymes of interest are often co-secreted with proteases. Proteolysis is known to give rise to microheterogeneity, which poses considerable difficulty in obtaining a homogeneous preparation of the enzyme. In order to minimize or nullify the effect of proteases the proteolytic inhibitor, PMSF was added in the medium. Since PMSF is inactive above pH 8.0, its effect on xylanase production could not be evaluated at pH 10.0. Fermentations were therefore carried out at neutral pH. Inhibition of the proteolytic activity led to (at pH 6.0) approximately doubling of xylanase activity in the culture filtrate. Since the addition of PMSF on large scale is not feasible, other nontoxic compounds such as glycine and norvaline, which have been shown to inhibit the proteolytic activity, were incorporated in the medium (Horikoshi *et al.*, 1987, Balakrishnanan 1993). However these compounds did not enhance xylanase production.

Table 2.11: Effect of protease inhibitors on xylanase production:

Expt.	Xylanase activity IU/ml [96 h.]	Protease activity (Kunitz unit)
Control	45	30
PMSF	85	0.0
Norvaline	43	28
Glycine	40	25

All the enzyme activities mentioned above are average of triplicates.

Xylanase production by semi solid fermentation

Xylanase production was evaluated in solid state fermentation (SSF) as it has several advantages over submerged fermentation. SSF is idealy suited to fungi as filamentous growth in Koji fermentation on agricultural biomass mimics growth on natural substrates where extensive ramification of the mycelium occurs, which results in better utilization of the nutrients. Most of the studies on xylanase production has been with respect to submerged fermentation. There are comparatively very few reports on xylanase production in koji fermentation. Deschamps and Huet (1985) have made a comparative study of xylanase production in submerged and solid state fermentation, in *Aspergillus niger*. A concentrated enzyme sample of 2500 IU/g of xylanase activity was obtained using pretreated straw and wheat bran in a ratio of 1:1 in SSF. Untreated straw gave lower enzyme yields. Differences were observed in enzymic properties produced by the two methods. The xylanase production in SSF had a higher pH and temperature optima and was comparatively more heat stable than the enzyme produced by submerged fermentation. The same authors have also shown that the ß-glucosidase produced from *Aspergillus phoenicis* by SSF was significantly more stable and had a higher pH optima than the enzyme produced in shake flasks (Deshamps and Huet, 1984). Purkarthofer *et al.*, (1993) have shown in *Thermomyces lanuginosus* that higher yields were obtained in SSF in comparison to the submerged

fermentation (553 $nkatml^{-1}h^{-1}$ in SSF while 215$nkatml^{-1}h^{-1}$ in submerged fermentation).

SSF studies were undertaken in *Cephalosporium* xylanase, in order to establish whether there were any significant changes in the properties of the enzyme.

Cephalosporium sp.,87.11.9. was grown in a medium containing wheat bran-yeast extract as described in Materials and Methods, in koji fermentation; the results of which are presented below (Table 2.12).

Time profile of xylanase production in semi solid state fermentation

Fermentation was carried out for seven days and the xylanase activity detected by harvesting the flask after an interval of 24 h. The results compiled in Table 2.12 shows that the maximum enzyme production occured in 144 h.

Table 2.12 : Effect of incubation time on xylanase production in Koji fermentation:

Time in hours	Xylanase activity IU/g of wheat-bran
72	92
96	596
120	779
144	903
168	566

Average of triplicates

Effect of extractant on enzyme activity

The enzyme was extracted by three different methods namely, distilled water, 1 M NaCl solution and 0.1% Tween-80. The best yield was obtained with 1 M NaCl solution or distilled water (550 IU/g), while Tween-80 gave marginally lower activities (520 IU/g). Hence water was used for extracting the enzymes.

Effect of agricultural residues on xylanase production

Various agricultural residues were evaluated for the production of xylanase in semi solid culture. Aqueous extracts of the fermented Koji were assayed for xylanase activity.

Table 2.13: Xylanase production in semi-solid fermentation on various Agricultural Residues:

Carbon source [10 g]	Xylanase activity IU/g [144 h]
Corn cobs	540
Corn cobs and wheat bran 1:1	547
Wheat bran	553

Average of triplicates
1% Yeast extract was used as the sole nitrogen source.

No significant differences were observed between the different carbon sources. An evaluation of our results obtained in semi-solid fermentation with the activity obtained in submerged culture brings out some interesting points. Deschamps and Huet (1975) reported higher xylanase productivity in submerged fermentation as compared to solid state fermentation. The results obtained in the present study, however seem to indicate that xylanase productivity is substantially higher in semi-solid state fermentation than in submerged culture. Table 2.14 gives a comparable evaluation of the various parameters compiled from data obtained from both types of fermentations.

Table 2.14 : Xylanase activity by *Cephalosporium*

Productivity\Fermentation	Semi-solid	Submerged
	Xylanase	
Activity IU/ml	78	20
Activity IU/g	750	320
Productivity IU/g/h	5.2	3.3

In view of the advantages of semi-solid fermentation in terms of lower investment costs as well as obtaining enzyme preparations of higher concentrations, the present culture seems to hold some promise for xylanase technology using semi-solid fermentation.

PRELIMINARY CHARACTERIZATION OF THE XYLANASE IN FERMENTATION BROTH:

A preliminary characterization of the crude enzyme was carried out in order to establish that the enzyme was active at alkaline pH and was devoid of cellulase activity. These are important characteristics of the enzyme to be established in screening in order to know its potential application in pulp treatment. Information on important parameters such as pI, molecular weight, inactivation by metal ions, activators etc., are essential to plan the strategy for purification of the enzyme.

pH and temperature optima

The optimum pH and temperature of the broth were determined. It can be seen from Fig. 2e that the enzyme had a broad pH optima from 7.0-9.0, with 80 and 60 percent of maximal activity at pH 9.0 and 10.0, respectively.

The temperature optima for the enzyme was found to be at 40°C, while 50% of the maximal activity was obtained at 50°C. The comparative activities at various pH values and temperatures are given in Fig. 2f.

Fig. 2f: pH optima of the xylanase obtained in the culture filtrate

Fig. 2g: Temperature optima of the xylanase obtained in the culture filtrate

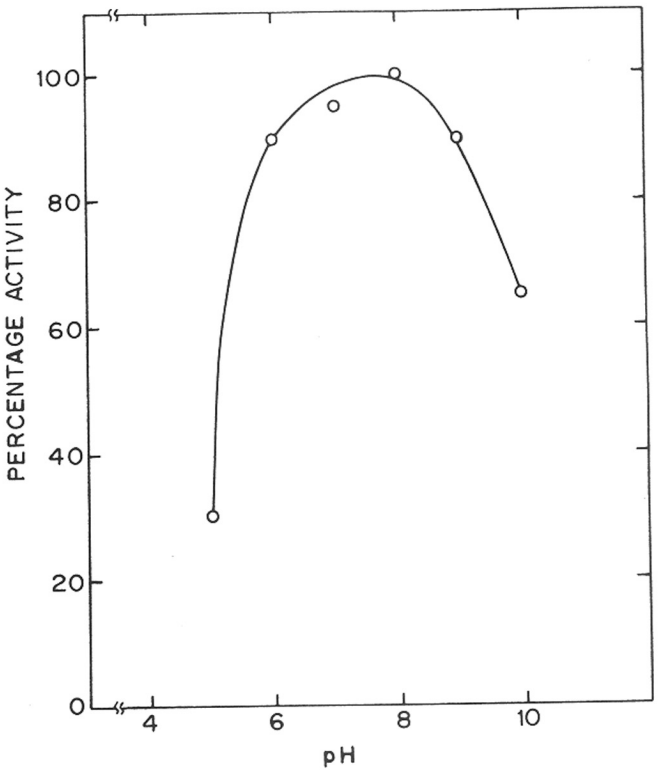

PERCENTAGE ACTIVITY
100
80
60
40
20
0
4
6
8
10
pH

FIG. 2f

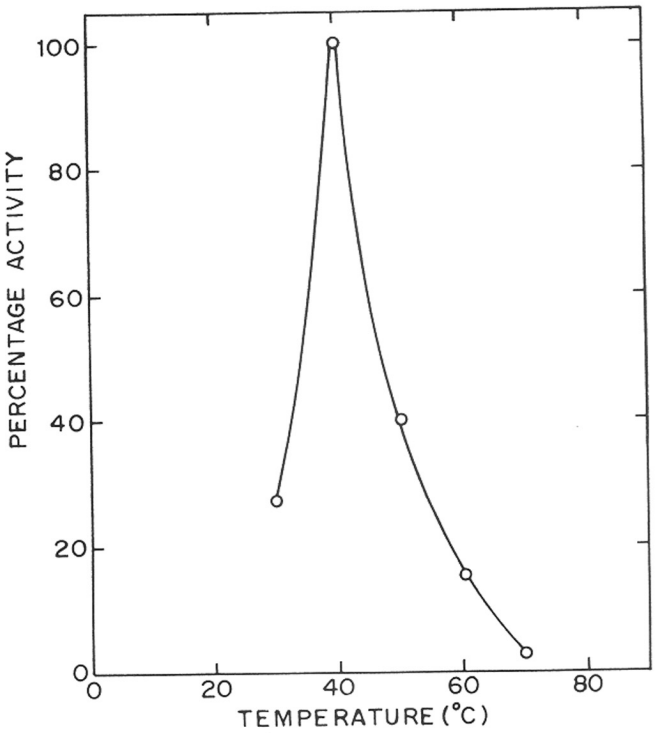

PERCENTAGE ACTIVITY
100
80
60
40
20
0
0
20
40
60
80
TEMPERATURE (°C)

FIG. 2g

pH and temperature stability

The enzyme was more stable in the range above neutrality (pH 7.0-9.5), when incubated at 40°C for 15 min (Fig. 2h, 2i) in absence of the substrate.

The enzyme was found to retain 80% of its initial activity at 40°C while 50% of the activity was lost at 50°C when incubated for the same time.

Determination of the Km

A suitably diluted xylanase sample was incubated with different amounts of oat spelts xylan (0.7 to 7.0 mg) under the assay conditions. K_m was determined by the Lineweaver-Burk plot and was found to be 0.15 mg/ml.

Molecular weight determination

The culture filtrate showed two peaks of xylanase activities, on Sephadex G-50 and Biogel P-100, corresponding to molecular weights of 70,000 and 30,000 in the proportion of 10:90. The pI of the two xylanases in the crude culture filtrate was found to be 9.0 and 4.3 respectively.

Preliminary studies on metal ion inhibition indicated that the enzyme was fairly stable to the metal ions except for Cu^{2+} and Co^{2+}. In the presence of 10 mM Cu^{2+} and Co^{2+} the enzyme activity was drastically reduced to 15 and 30 %, respectively. The enzyme was markedly inhibited by Hg^{2+}.

Specificity of the culture filtrate

The culture filtrate was assayed for various activities, using the following substrates: CM-cellulose, PNPX, PNPA, filter paper, laminarin, casein, starch, sucrose, pectin and chitin (Table 2.15). The broth showed invertase and protease activities. Extracellular ß-xylosidases and arabinosidase activities were comparatively low. The cellulase (carboxymethylcellulase) activity was exceedingly low and is possibly attributable to remenants of xylan present in the cellulose substrate

Fig. 2h: pH stability of the xylanase

Fig. 2i: Temperature stability of the xylanase

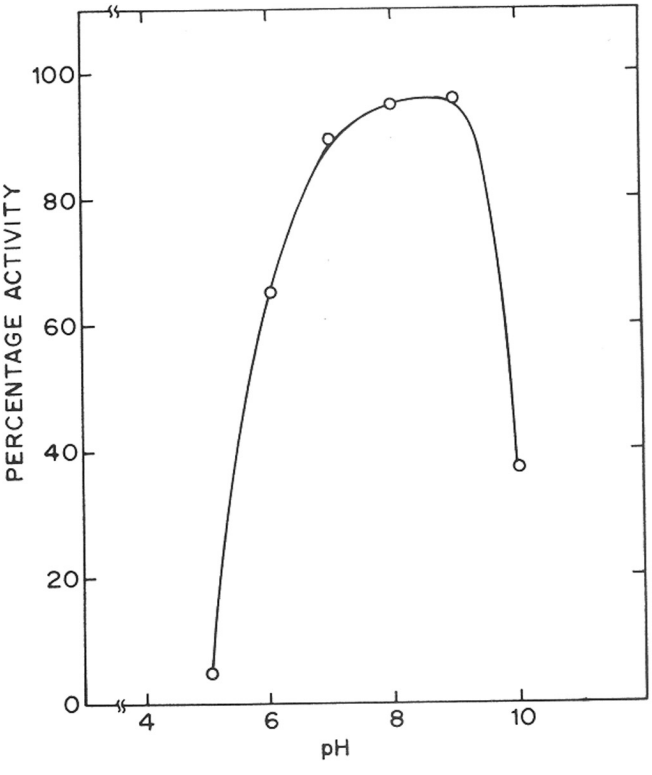

PERCENTAGE ACTIVITY
100
80
60
40
20
0
4
6
8
10
pH

FIG. 2h

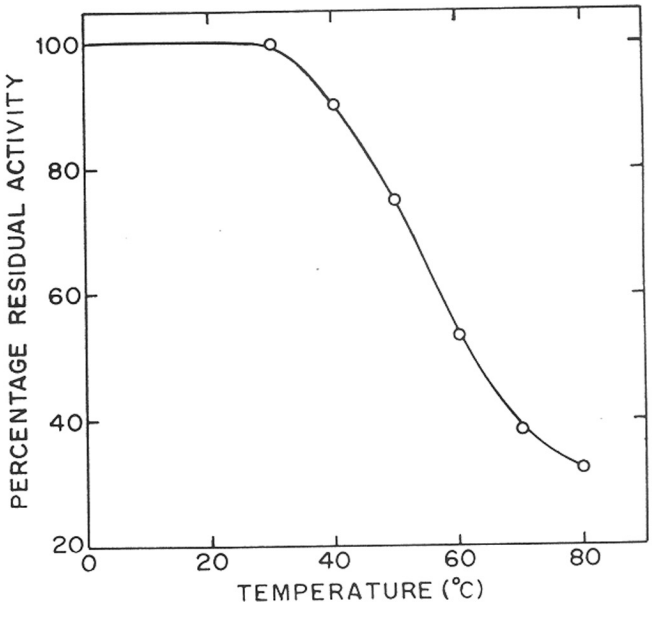

PERCENTAGE RESIDUAL ACTIVITY
100
80
60
40
20
0
20
40
60
80
TEMPERATURE (°C)

FIG. 2i

used. Laminarinase and filter paper activities were not detected. Since the culture filtrate was devoid of cellulase activity, the xylanase was evaluated for its efficacy in reducing Kappa number, which is a measure of the lignin content in pulp.

Table 2.15: Enzymes detected in the culture filtrate

Substrate	Enzyme activity (IU/ml)
CM cellulose[a]	0.03
Filter paper[b]	0.00
Laminarin	0.00
Sucrose	12.0
PNPX	1.65
PNPA	6.0
Chitin	0.00
Pectin	0.00
Protease[c]	3.0

[a, b]: The assay for Filter paper, CM-cellulase was carried out at both neutral and alkaline pH.
[c]: Protease activity is given in Kunitz units as described earlier.

There were no significant differences observed in the enzyme properties produced at neutral or alkaline pH.

A comparative study of xylanase properties produced by submerged and solid state fermentation

Xylanase produced by semi-solid fermentation showed a dual pH optima in contrast to the enzyme produced by submerged fermentation, indicating the possible presence of two enzymes under the semi-solid fermentation conditions. The other properties such as temperature optima, molecular weight etc., were identical.

Fig. 2j: pH optima of the xylanase obtained in culture filtrate in SSF and in submerged fermentation

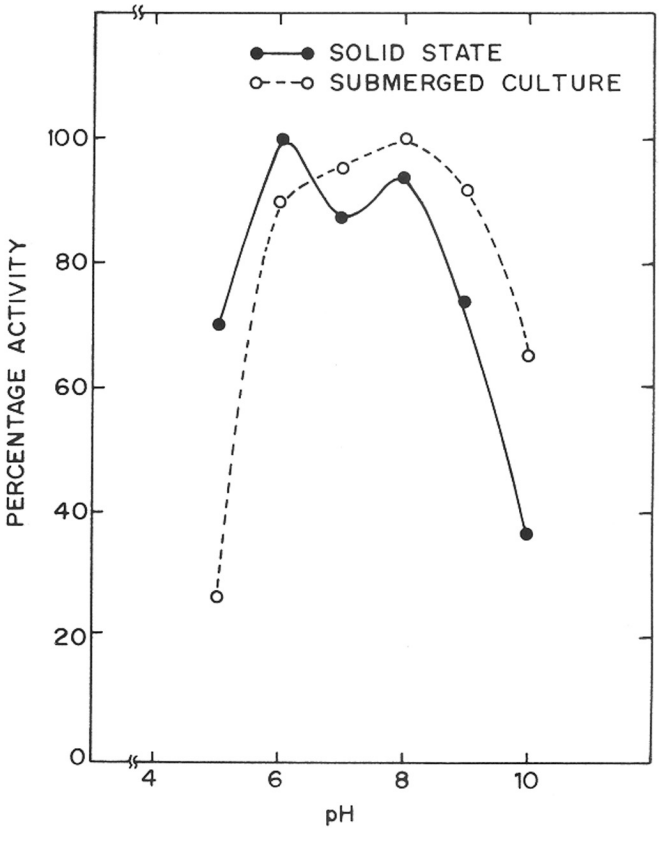

SOLID STATE
SUBMERGED CULTURE
PERCENTAGE ACTIVITY
100
80
60
40
20
0
4
6
8
10
pH

FIG. 2j

In the literature there has been only a single report of xylanase activity from *Cephalosporium sp.,* (Dekker and Richards, 1975; Richards and Shambe, 1975). The xylanase however exhibited an acidic pH optima between pH 5.5-6.5, and was also secreted alongwith cellulase. However the activities reported were extremely low in the range of 0.22-0.27 IU/ml. Recently Chandra *et al.*, (1995) have described a xylanase from *Aspergillus fischeri* active over a broad pH range, with an optimum at pH 6.0 and with 60% activity remaining at pH 8.0. There is a paucity of reports on xylanases of fungal origin which are active under high alkaline pH conditions. To the best of our knowledege, the present study is perhaps the first report of a fungus capable of growing under high alkaline pH conditions and secreting high xylanase activity completely cellulase-free and stable to as well as active at high pH. Such a combination has prompted us to apply for national and international patents covering the unique aspects of the enzyme under study and presently the patent applications are under processing.

CONCLUSION

The alkalotolerant fungal strain isolated from soil was identified as *Cephalosporium* sp. based on morphological characteristics and microscopic examination. *Cephalosporium* was found to produce high levels of xylanase at alkaline pH and the enzyme was active at high pH, in comparison to the other isolates, and hence was taken up for further studies. Standardization of the culture conditions with a view to enhance the xylanase production by the organism, showed substantial increase in activity on a very simple and cost effective medium (wheat bran, Baker's yeast). The organism showed good growth on sugars such as glucose, xylose, sucrose or lactose but did not produce any xylanase. The enzyme was produced in presence of xylan and xylan rich substrate indicating that the enzyme is induced. The culture filtrate exhibiting xylanase activity was devoid of cellulase activity.

Although the pH of the culture medium has been known to play an important role in enzyme production in several organisms such as *Trichoderma viride* (Sternberg, 1976) *Aspergillus fumigatus* (Trivedi and Rao, 1988), and *Paecilomyces Banier varioti* (Krishnamurthy, 1986), the *Cephalosporium* xylanase was found to be produced over a broad pH range. In addition it also exhibited enzyme activity over a wide pH range of 6-10.0, the xylanase being more active in the alkaline range.

CHAPTER 3

PURIFICATION AND CHARACTERIZATION OF XYLANASE

SUMMARY:

A simple procedure involving ammonium sulphate precipitation, polyethyleneimine treatment, followed by chromatography on CM-cellulose and DEAE-Sephacel was developed for the purification of xylanase from *Cephalosporium* sp. to homogeneity, with an overall yield of 35%. The Mr of the enzyme, determined by gel filtration and SDS-PAGE was 28,000. The xylanase is an acidic protein with a pI of 4.05. The optimum pH and temperature were 8.0 and 40°C, respectively. The enzyme was specific only to xylan and did not act on other related hemicellulosic substrates. The Km and Vmax on oatspelts xylan were 3.52 mg/ml and 3.3 X 10^3 μM/mg/min, respectively. The mode of action of the enzyme using oat spelts xylan, showed it to be an endoxylanase liberating arabinose, with xylobiose and xylotriose as the major end products. Chemical modification studies revealed the involvement of a single tryptophan and histidine and two carboxylate residues in the catalytic activity of the enzyme.

INTRODUCTION

Xylanases are important industrial enzymes and have extensive application in pulp and paper industries, as an effective adjuvant for the development of eco-friendly technologies. However for effective application in the above industries, it is desirable to have enzymes which exhibit properties like activity and stability at alkaline pH and also at elevated temperatures. Hence there is a need to look for thermostable enzymes active at alkaline pH. This can be achieved either by screening various organisms to locate such enzymes or by recombinant DNA technology to generate variants of commercially used enzyme with desirable properties. In the preceeding chapter we described the medium optimization for optimal production of xylanase from an alkalotolerant *Cephalosporium* species. As a pre-requisite for gene manipulation studies it is essential to study the structure-function relationship of the enzyme. Hence studies were carried out on the purification and extensive characterization of the *Cephalosporium* xylanase.

MATERIALS AND METHODS

MATERIALS

DEAE-Sephacel, (Pharmacia Fine Chemicals, Sweden), CM-Cellulose (preswollen), (Whatman speciality products Inc., USA), Biogel P-100 (BioRad,USA), polyethyleneimine (BDH, England), Sodium dodecyl sulphate, N,N,N'N'-tetramethylethylene-diamine, Coomassie Brilliant Blue G-250 and R-250, acrylamide, bis-acrylamide, tris, SDS and gel-filtration molecular weight markers, N-bromosuccinimide; diethylpyrocarbonate; 2,hydroxy-5-nitrobenzylbromide; N-acetylimidazole, 5,5'-dithiobis (-2-nitrobenzoic acid); N-ethylmaleimide, p-hydroxymercuribenzoate; ß-mercaptoethanol; dithiothreitol (Sigma Chemical Company, USA); 1-ethyl-3-(3-dimethyl aminopropyl carbodiimide and Ampholine (BioRad, USA) were used. All other chemicals and reagents used were of analytical grade.

METHODS

Enzyme production: This was carried out as described in Chapter 2.

Xylanase Assay: Xylanase was assayed at pH 8.0 and 40°C, by measuring the reducing sugars liberated, at 540 nm, following the hydrolysis of oatspelts xylan, as described in Chapter-2. In some experiments reducing sugars were also determined by Somagyi-Nelson's method (Somagyi 1959).

Protein determination

Protein was determined according to Lowry *et al.* (1951) using Bovine serum albumin (BSA) as standard. The blue colour developed after the addition of Folin phenol reagent was measured at 660 nm. In the column effluents, the protein was determined by measuring the absorbance at 280 nm.

PURIFICATION OF XYLANASE:

Unless otherwise mentioned, all the operations were carried out at 5±1°C.

Ammonium sulphate precipitation: The culture filtrate (500 ml) was brought to 0.9 saturation by the addition of 300 g of solid ammonium sulphate and the mixture was stirred for 3 h. The precipitated protein was collected by centrifugation (9226 g, 30 min) dissolved in minimum amount of 10 mM potassium phosphate buffer, pH 7.0 and dialyzed extensively against the same buffer to remove the ammonium sulphate.

Polyethyleneimine treatment and CM-cellulose chromatography: The dialyzed enzyme was then treated with 0.4% (v/v) polyethyleneimine (effective concentration) for 30 min at room temperature, under mild agitation. The mixture was then centrifuged (3000 g, 30 min) and the supernatant was loaded onto CM-cellulose column (1.5 x 20 cm) pre-equilibrated with 10 mM potassium phosphate buffer, pH 7.0, at a rate of 16 ml/h. The column was then washed with the same buffer and the flow through fractions containing xylanase activity were pooled, concentrated on an Amicon ultrafiltration having a YM-5 membrane and used for the next step.

DEAE-Sephacel chromatography: The concentrated enzyme solution, obtained from the above step, was adsorbed onto a DEAE-Sephacel column (1.5 x 18 cm) pre-equilibrated with 10 mM potassium phosphate buffer, pH 7.0, at a rate of 12 ml/h. The column was then washed with the same buffer, till the flow through fractions showed no xylanase activity. The elution of the bound enzyme was affected with a linear gradient, 120 ml total volume, of potassium chloride (0-1 M) in the above buffer. Fractions of 2.0 ml were collected at a rate of 24 ml/h and those having activity greater than 3 IU/ml were pooled, concentrated by Amicon ultrafiltration and after extensive dialysis against 10 mM potassium phosphate buffer, pH 7.0, was stored at -20°C. No loss of activity was observed over a period of 2 years when the enzyme was stored under these conditions.

Gel electrophoresis: Analytical gel electrophoresis, in slab gels was carried out at pH 8.3 (8 X 10 cm; 1 mm thick) using 7.5% (w/v) acrylamide gel according to Davis (1964). The slab gels were stained by silver staining (Blum *et al.*,1987).

SDS-polyacrylamide gel electrophoresis was performed, at pH 8.3, using 10% (w/v) of the acrylamide, according to Laemmli (1970). After the electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 and destained with methanol, acetic acid, water (20:10:70). Isoelectric focussing was carried out in a glycerol gradient according to Pawar *et al* (1986) using an ampholine over the pH range of 3 to 10.

Analytical IEF gel was prepared as described by LKB [Application note, LKB produkter AB, Stockholm, Sweden] using 7% acrylamide (w/v) solution. Electrofocussing was carried out with a constant power of 4W for 18 h. After termination of the run the gel was stained with Coomassie Brilliant Blue R-250 prepared in methanol, acetic acid, water mixture [20:10:70], for 60 min after which it was destained with the same solution in a proportion of 30:10:60. A second corresponding IEF gel was cut into 24 equal parts and the pH checked by an electrode and matched with the protein band obtained.

Carbohydrate content: Two methods were used to determine the glycoprotein nature of the protein.

i) Carbohydrate content of the protein was determined by the phenol-sulphuric acid method of Dubois *et al.* (1965) using glucose as the standard. 200 g of the protein was taken in 1 ml of water to which was added, 1 ml of 5% (v/v) aqueous phenol. The mixture was allowed to stand at 28+1°C for 10 min. Concentrated sulfuric acid was then rapidly added to the mixture. The tubes were placed in a waterbath at 28$\pm$1°C for 20 min and the colour developed was read at 490 nm.

ii) After polyacrylamide gel electrophoresis the gels were immersed in a fixing solution containing iso-propanol:acetic acid:water (25:10:65) to fix the protein. Thereafter the gels were immersed in 0.2% (w/v) thymol prepared in the above fixing solution. The gels after rewashing

with the fixing solution were transferred in a solution of concentrated sulfuric acid:ethanol (80:20, v/v) and incubated for 3 h at 35°C (Gander, 1984).

Amino acid analysis: Amino acid analysis was carried out on a Pharmacia LKB Alpha-plus amino acid analyzer, by hydrolysis of the protein (100μg) in 6 N HCl at 110°C for 22 h.

Estimation of tryptophan: The tryptophan content of the protein was calculated from an ultraviolet spectrum of the protein in 0.1 N sodium hydroxide according to the method of Goodwin and Morton (1946).

Determination of Molecular Weight

Determination of Mr was carried out by gel filtration on a Biogel P-100 column (1 X 100cm). The column was pre-equiliberated with 10 mM, potassium phosphate buffer pH 7.0. The reference proteins used were bovine serum albumin (66,000); carbonic anhydrase (29,000); soyabean trypsin inhibitor (20,000); myoglobin (17,000); cytochrome C (12,400) and aprotinin (6,500). The Mr was also determined by the SDS-PAGE method as described earlier. (chapter. 2.)

CHEMICAL MODIFICATION STUDIES

Modification of tryptophan residues

Reaction with NBS: Purified xylanase (11 μg) in 50 mM potassium phosphate buffer, pH 7.0, was incubated with varying concentrations of NBS (0.8-2.4mM) at 28±1°C. Aliquots were removed at suitable intervals and the residual activity was determined under standard assay conditions. Enzyme sample incubated in the absence of NBS served as control.

Reaction with HNBB: HNBB was prepared in acetone prior to use and protected from light. Purified enzyme (11 μg), in 50 mM potassium phosphate buffer, pH 7.0, was incubated with

varying concentrations of HNBB (2-10 mM) at 28±1°C for 30 min. Samples were removed at suitable time intervals and the residual activity was determined under standard assay conditions. Enzyme samples incubated in the absence of HNBB were taken as control.

Modification of histidine residues

Photooxidation: This was carried out by exposing the purified protein (100 μg), in 1 ml of 50 mM potassium phosphate buffer pH 7.0, in a glass tube (1x10 cm) containing different concentrations of methylene blue (0.05-0.2%) to 200 W flood light bulb held at a distance of 12 cm for 30 min at 26±1°C followed by estimation of the residual activities. Enzyme samples treated under identical conditions, in dark, served as control.

Reaction with DEP: DEP was prepared in absolute ethanol. The DEP concentration was determined by mixing an aliqout of the sample with 3 ml of 10 mM imidazole buffer, pH 7.0, followed by monitoring the increase in the absorbance at 230 nm. The amount of N-carbethoxyimidazole formed was calculated by using a molar absorption coefficient of 3000 $M^{-1}cm^{-1}$ (Melchior and Fahrney, 1970). The concentration of the diluted DEP solution was 100mM. The ethanol concentration in the reaction mixture did not exceed 1% (v/v) and had no effect on the enzyme activity during the incubation period. The purified xylanase (11 μg) in 50 mM potassium phosphate buffer pH 7.0, was incubated with varying concentrations of DEP (3-7 mM) at 28±1°C for 90 min. Aliqouts were removed at suitable intervals and the reaction was arrested by the addition of 10 mM, imidazole buffer, pH 7.0. Subsequently, the residual activities were determined under standard assay conditions. Enzyme samples incubated in the absence of DEP served as control. The DEP mediated inactivation was also monitored spectrophotometrically, by measuring the change in absorbance at 240 nm as described by Ovadi *et al* (1967).

Reaction with hydroxylamine: Decarbethoxylation was carried out according to Miles (1977). The DEP modified enzyme samples were incubated with 100 mM hydroxylamine, pH 7.0, at 28±1°C for 30 min and the enzyme activity was determined under standard assay conditions.

Modification of tyrosine residues

Purified *Cephalosporium* xylanase (100 μg) in 50 mM potassium phosphate buffer, pH 7.0, was incubated with 10 mM of N-acetylimidazole for 30 min at 26+1°C followed by estimation of the residual activities under standard assay conditions. The enzyme incubated in the absence of N-acetylimidazole was taken as control. The number of tyrosine residues modified were calculated by using a molar absorption coefficient at 1160 $M^{-1}cm^{-1}$ at 278 nm (Means and Feeney, 1971).

Modification of carboxylate groups:

Reaction with EDC: Purified xylanase (10 μg), in 50 mM citrate potassium phosphate buffer, pH 6.0, was incubated with varying concentrations of EDC (5-30 mM) at 28±1°C, for 60 min. Aliqouts were removed at suitable intervals and the residual activity was determined under standard assay conditions. Enzyme samples incubated in the absence of EDC were taken as control.

Substrate protection studies

In all the chemical modification reactions, the effect of substrate protection was studied by incubating the enzyme with excess amounts of xylan followed by treatment with the modifying reagents.

Mode of action

Oat spelt xylan (200 mg) in 4 ml of potassium phosphate buffer, pH 8.0 was incubated with the pure enzyme (6 IU/ml) at 40°C. Samples were withdrawn at various time intervals and assayed for the reducing sugar. The products formed were detected by HPLC and paper chromatography as described in Chapter 2.

RESULTS AND DISCUSSION

The results of a typical procedure for the purification of *Cephalosporium sp.,87.11.9.* xylanase, to homogenity, are summarized in Table 3.1. The enzyme was purified 40 fold with an overall recovery of 30 %. In the present studies, treatment with PEI was essential as it removed substantial amounts (approx. 45 fold) of colour (Table 3.2). The PEI present in the enzyme sample was removed by CM-cellulose chromatography, a step where the enzyme does not bind to the matrix. Removal of PEI was essential at this step as presence of PEI in the sample resulted in poor adsorption of the enzyme onto DEAE Sephacel. Moreover the enzyme also lost its activity on storage in presence of PEI. The enzyme obtained after DEAE-Sephacel chromatography was electrophoretically homogeneous (Fig. 3.a, 3.b, 3.c).

PHYSICAL PROPERTIES

Molecular weight

The Mr of the purified enzyme, determined by gel filtration on BioGel P-100, was 28,000. Similar results were obtained by SDS-PAGE (Fig. 3.b). These values are comparable to those obtained from *Trichoderma harzianum* xylanase C (Wong *et al.*, 1986). However it is considerably higher compared to xylanases from *Cephalosporium acremonium* (Mr. 10,700 and 9,500) as reported by Richards and Shambe (1976). Mr determination based on amino acid composition (Table 3.3) gave a value of 28,123 which is in agreement with the values obtained by gel filtration and SDS-PAGE. Amino acid analysis showed the preponderence of aspartic acid, glutamic acid and glycine. Like the xylanases from *Trichoderma viride* ATCC-52438 and *T. viride*-cellulysin (Dean and Anderson, 1991), *Cephalosporium* enzyme also showed the absence of cysteine and methionine. On the contrary xylanase B and C from *Trichoderma harzianum* showed the presence of both these amino acids (Table 3.3).

pI of Xylanase

The pI of the purified enzyme was 4.05 indicating it to be an acidic protein (Fig. 3.c). Comparable values have been reported for two xylanases from *Myrothecium verrucaria* (Coughlan, 1992).

Table 3.1: Purification of *Cephalosporium* Xylanase

Step	Activity (IU)	Protein (mg)	Specific activity	Fold purifi-cation	Recovery (%)
Crude extract[1]	3,300	-	-	-	100
Ammonium sulphate (0-9 saturation	3,025	350	8.6	-	91
Polyethylene imine[2]	2000	-	-	-	60
CM-Cellulose	1,466	80.6	18.2	2.1	45
DEAE-Sephacel	1,050	2.8	371	43.0	30

1 & 2. Protein detection by Lowry's method was not carried out due to the interference of the pigment and polyethyleneimine respectively.

Table 3.2: Polyethyleneimine treatment

Steps	O.D. 410 nm	Colour removal (fold)
Ammonium sulphate precipitate	6.480	0
PEI treated	0.170	40

Table 3.3a : Amino acid composition of *Cephalosporium* xylanase

Amino acids	No. of Residues	Percent amino acid (%)
Aspartic acid	28	11
Threonine	21	8.2
Serine	19	7.6
Glutamic acid	33	13
Protein	16	6.4
Glycine	33	13
Alanine	24	9.4
Cysteine	0	0
Valine	16	6.3
Methionine	0	0
Isoleucine	9	3.4
Leucine	14	5.5
Tyrosine	12	4.7
Phenylalanine	9	3.5
Histidine	5	2.14
Lysine	5	2.0
Arginine	10	3.94
Tryptophan	6	2.36

Fig. 3a: Polyacrylamide gel electrophoresis (PAGE) of purified xylanase 7.5% (W/V) polyacrylamide gel, protein loaded : 80 μg

Fig. 3b: SDS Polyacrylamide gel electrophoresis (10%), relative mobilities of the reference proteins were plotted against their log molecular weights. Reference proteins were [1] Bovine serum albumin [2] Ovalbumin [3] Glyceraldehyde -3-phosphate dehydrogenase [4] Carbonic anhydrase [5] Trypsinogen [6] Soybean trypsin inhibitor [7] α-Lactalbumin

FIG. 3a

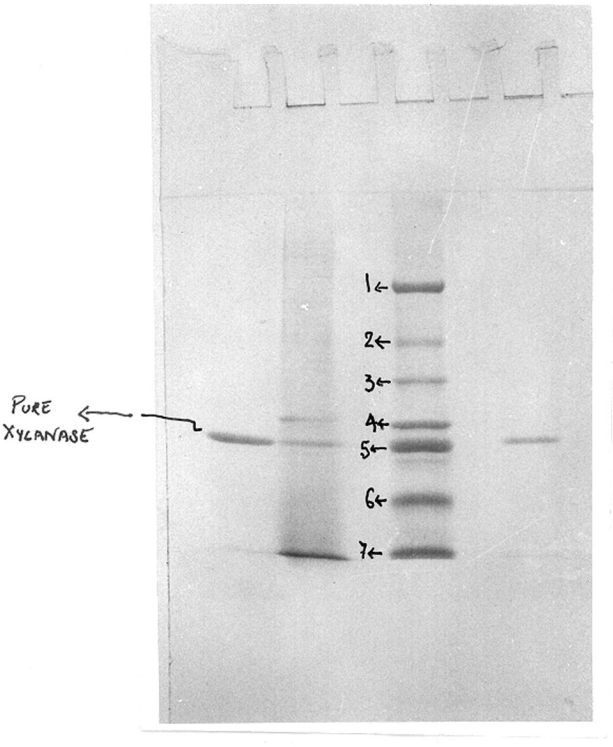


FIG. 3b

Fig. 3b: SDS Polyacrylamide gel electrophoresis (10%), relative mobilities of the reference proteins were plotted against their log molecular weights. Reference proteins were [1] Bovine serum albumin [2] Ovalbumin [3] Glyceraldehyde -3-phosphate dehydrogenase [4] Carbonic anhydrase [5] Trypsinogen [6] Soybean trypsin inhibitor [7] α-Lactalbumin

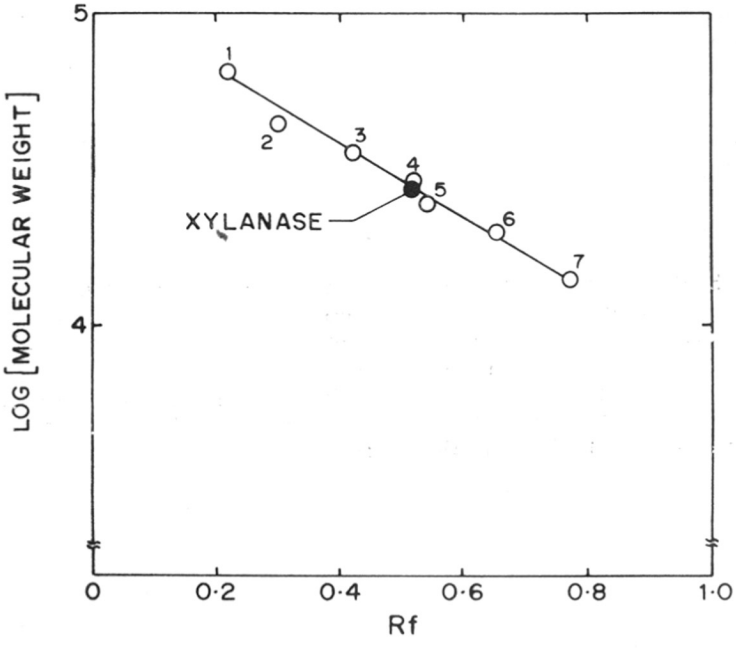


FIG. 3b

Fig.3c: IEF gel showing a single band of *Cephalosporium* xylanase

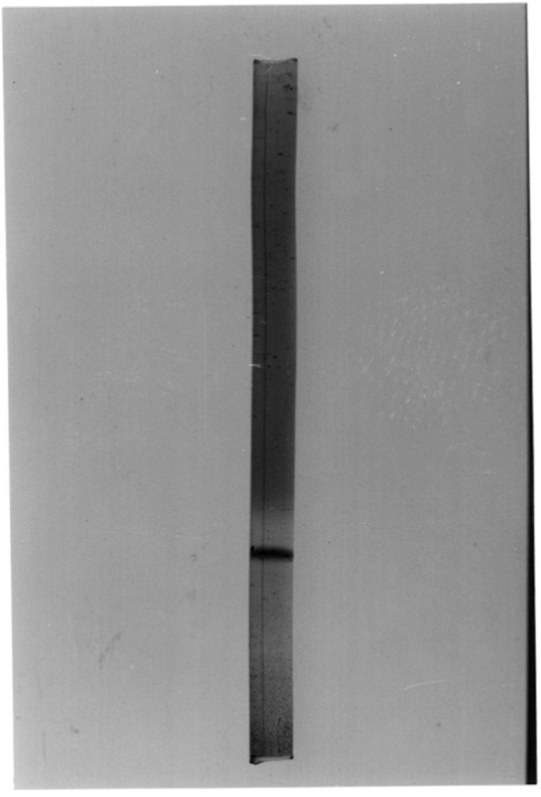


FIG 3c

Glycoprotein nature

Unlike most of the fungal xylanases (Ito *et al.*, 1992; Monti *et al.*, 1991; Lappalainen, 1986; Tenkanen *et al.*, 1992; Paice *et al.*,1978; Stuetgen and Sahm, 1987) the *Cephalosporium* xylanase was not a glycoprotein.

FUNCTIONAL PROPERTIES

Optimum pH and pH stability

Cephalosporium xylanase was found to be active over a broad range of pH i.e., 7.0 to 10.0 with the pH optima being in the range of 7.0 to 9.0 (Fig. 3.d). Moreover, the type of buffer used influenced the enzyme activity and maximum activity was observed in presence of glycine-NaOH buffer. Most fungal xylanases exhibit optimum pH on the acid side including the enzyme from *Cephalosporium sacchari* (Richards and Shambe, 1976). However *Cephalosporium* sp. xylanase showed low activity on the acid side with only 50% of its activity at pH 5.0. This is one of the unique properties of the xylanase described in the present investigation. Additionally this is perhaps the first report of a fungal xylanase active at alkaline pH. The only other report of such an enzyme is of a partially purified xylanase from an alkalotolerant fungi *viz. Aspergillus fischeri* (Chandra and Chandra, 1995). However the enzyme preparation showed an optimum pH of 6.0.

The *Cephalosporium* enzyme was stable over a pH range of 7.0-9.0, The enzyme lost 50% of its activity at pH 6.0 and was totally inactivated at pH 5.0 after 60 min (Fig. 3.e, 3.f). This is in contrast to other fungal xylanases which are stable over a broad pH i.e. 3.0 to 10.0.

Optimum temperature and temperature stabilty

The optimum temperature of the purified enzyme was 40°C and showed 50% of its activity at 50°C (Fig.3.g). Our values are comparable to those obtained in case of xylanases from *Aspergillus niger* (Fournier *et al.*, 1985; Frederick *et al.*, 1985, Shei *et al.*, 1985), *Trichoderma harzianum* and *Trichoderma lignorum* (Wong and Saddler, 1992). Temperature stability studies

Fig.3d: pH optima of Cephalosporium xylanase: The buffer used were
pH 5: Citrate phosphate, 50 mM
pH 6: Citrate phosphate, 50 mM
pH 7: Phosphatephosphate, 50 mM'
pH 8: Phosphatephosphate, 50 mM
pH 9.0 and 10.0 : Glycine NaOH, 50 mM, Carbonate bicarbonate, 50 mM and BorateNaOH, 50 mM

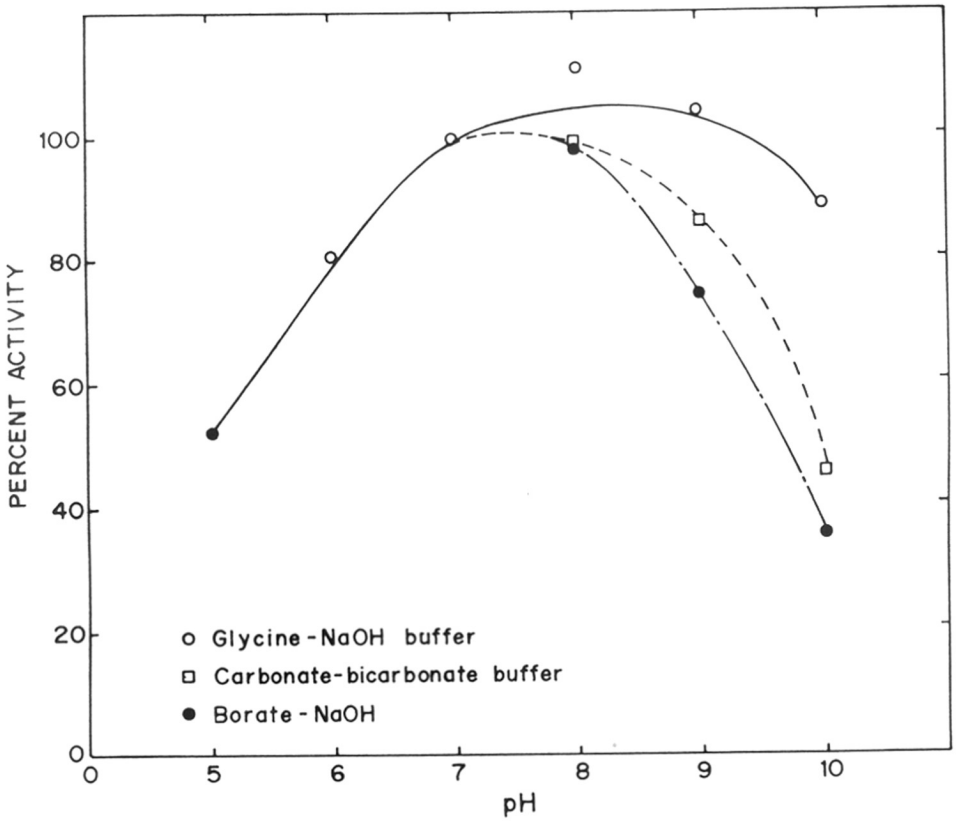

PERCENT ACTIVITY
100
80
60
40
20
0
0
5
6
7
8
9
10
pH
Glycine-NaOH buffer
Carbonate-bicarbonate buffer
Borate-NaOH

FIG. 3d

Fig.3e: pH stability of the *Cephalosporium* xylanase (100 µg) when incubated at pH 8.0 for 60 min

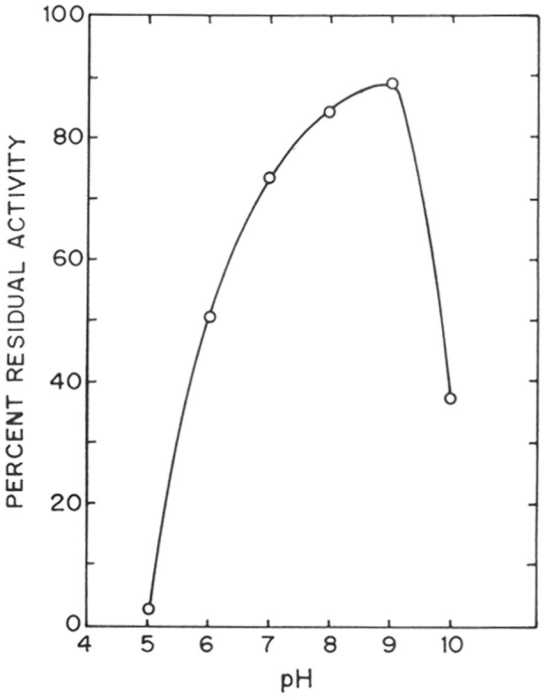


FIG. 3e

Fig.3f: pH stability : time profile

100 μg of the purified protein was incubated at pH values 7, 8 and 9 at 40°C for various time intervals. Buffers were 50 mM potassium phosphate buffer at pH 7 and 8 and 50 mM glycine-NaOH at pH 9, respectively.

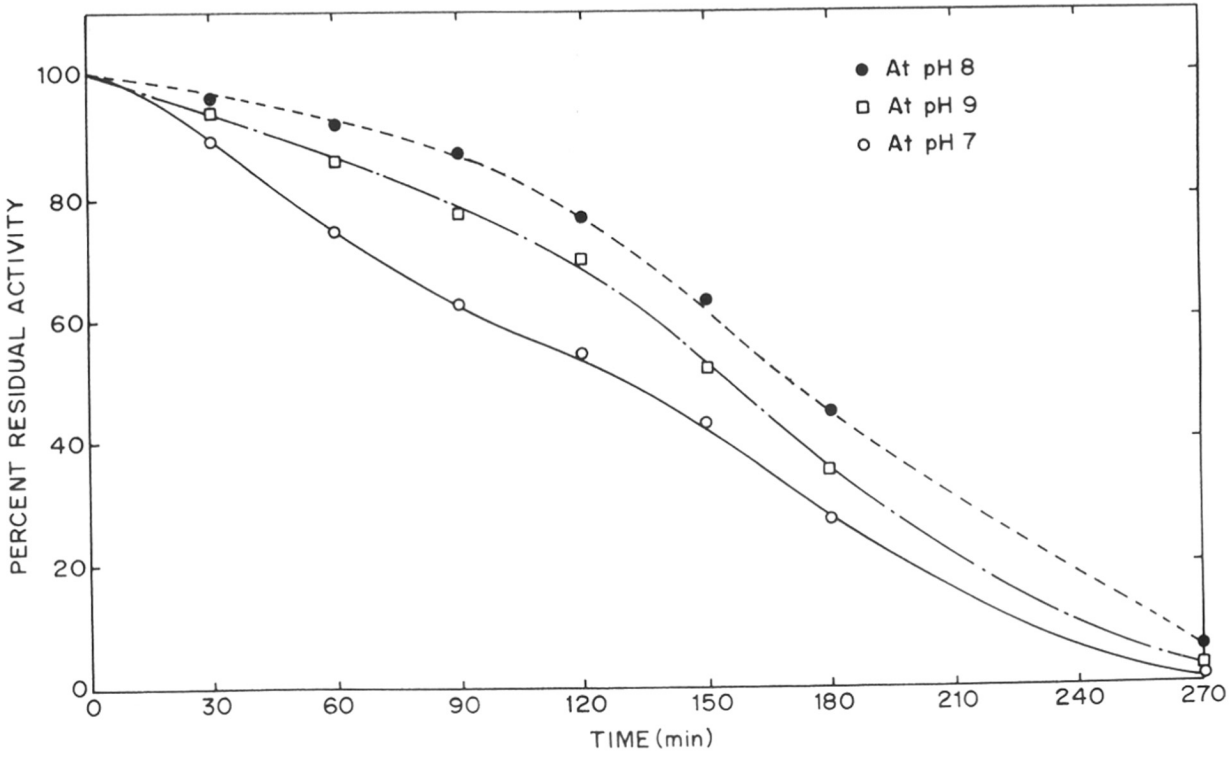

At pH 8
At pH 9
At pH 7
PERCENT RESIDUAL ACTIVITY
100
80
60
40
20
0
0
30
60
90
120
150
180
210
240
270
TIME (min)

FIG. 3f

Fig.3g: Temperature optima of the *Cephalosporium* xylanase

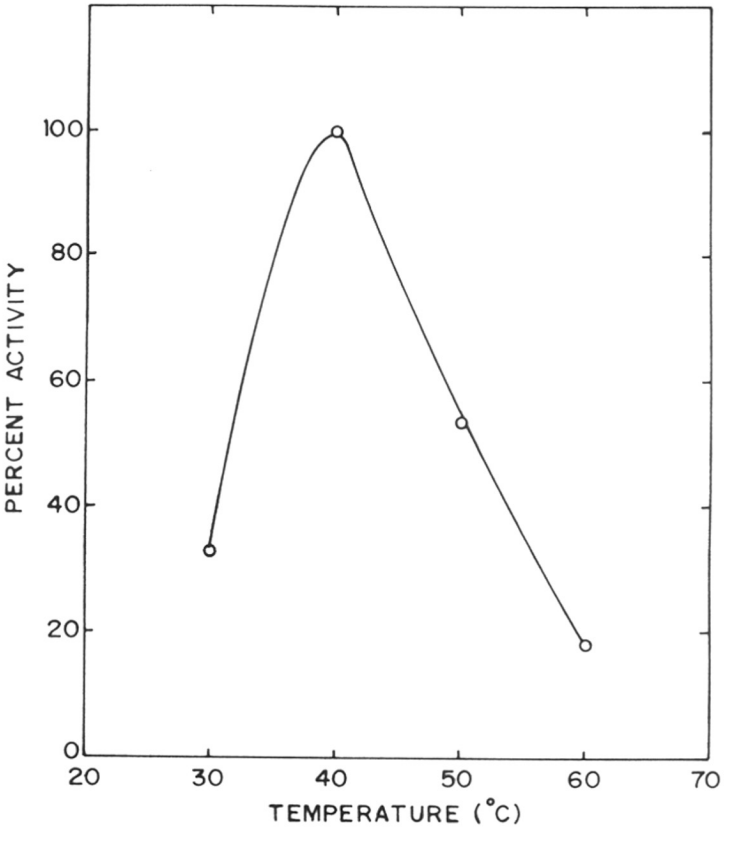

PERCENT ACTIVITY
100
80
60
40
20
0
20
30
40
50
60
70
TEMPERATURE (°C)

FIG. 3g

showed that the pure enzyme comparatively showed low temperature stability and lost 50% of its activity in 2 h at 40°C (Fig. 3.h, 3.i).

Kinetic studies

The Km and Vmax values calculated from Lineweaver-Burk double reciprocal plots were 3.52 mg/ml and 3.3 X 10^3 μM/mg/ml, respectively (Fig. 3.j). These results compare well with the other reports where the Km ranges from 2-7 mg/ml.

Activators and Inhibitors

Enhanced xylanase activity was obtained in the presence of thiol reagents such as cysteine, dithiothritol and B-mercaptoethanol (Fig. 3.k). Similar results were shown in *Myrothecium verrucaria* xylanase where 10 mM cysteine and thioethanol led in increase in enzyme activity ranging from 47 to 116% (Coughlan, 1992). Srinivasan *et al.* (1988) also obtained increased xylanase activity in presence of thiol reagents. Enhanced xylanase activity was obtained in the presence of thiol reagents such as cysteine, dithiothreitol and B-mercaptoethanol (Fig. 3.k).

Divalent cations are known to inhibit xylanases (Coughlan, 1992). In the present study Fe^{2+}, Cu^{2+}, Mn^{2} and Hg^{2+} were found to inhibit *Cephalosporium* xylanase (Table 3.4a). Among them Hg^{2+} was the most potent inhibitor and the enzyme completely lost its activity even in presence of low concentrations (1 mM) of Hg^{2+}.

The Hg^{2+}-mediated inactivation of the enzyme could be reversed by mercaptoethanol (Table 3.4b). These observations are comparable to several fungal and bacterial xylanases (Coughlan, 1992).

Fig. 3h: Temperature stabilty of the purified enzyme when incubated at various temperature for 60 min

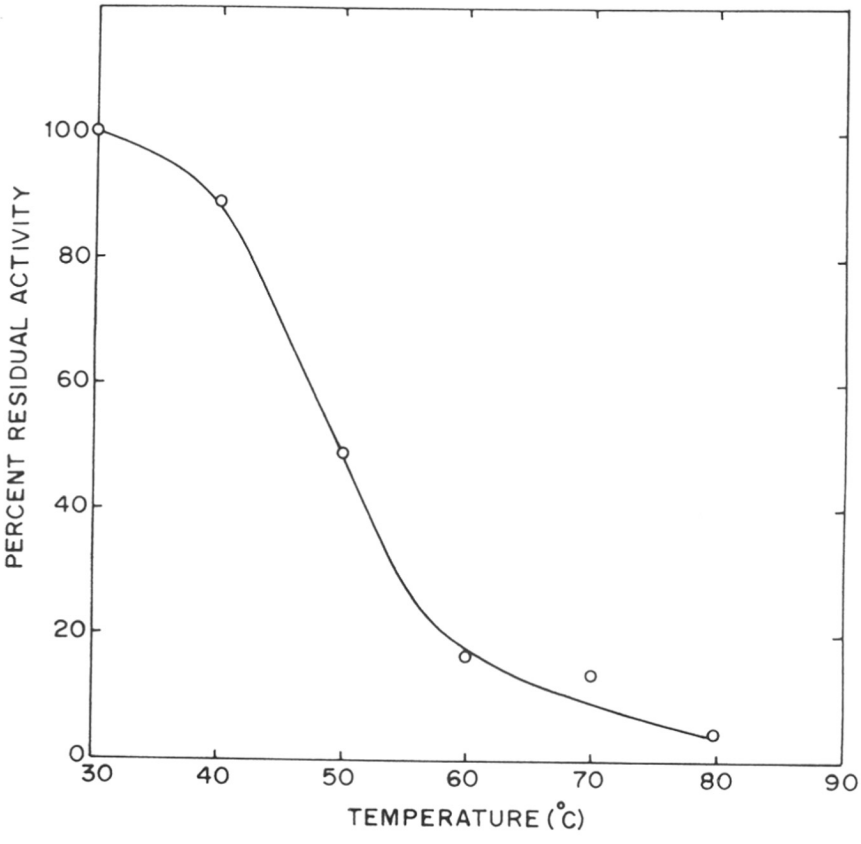

PERCENT RESIDUAL ACTIVITY
100
80
60
40
20
0
30
40
50
60
70
80
90
TEMPERATURE (°C)

FIG. 3h

Fig. 3i: Temperature stability of the pure enzyme: Time profile

100 ug of purified protein was incubated at pH 8 at 40, 45 and 50°C for various time intervals.

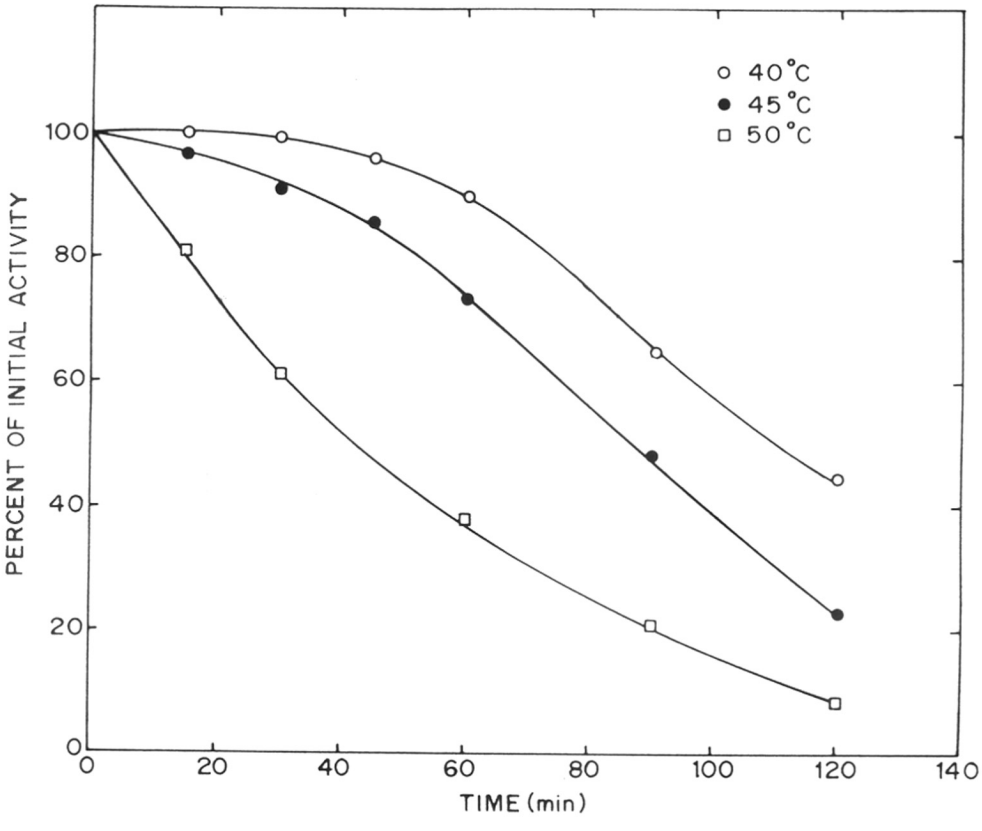

40°C
45°C
50°C
PERCENT OF INITIAL ACTIVITY
100
80
60
40
20
0
0
20
40
60
80
100
120
140
TIME (min)

FIG. 3i

Fig.3j. Lineweaver Burk plot for purified enzyme (6 IU)

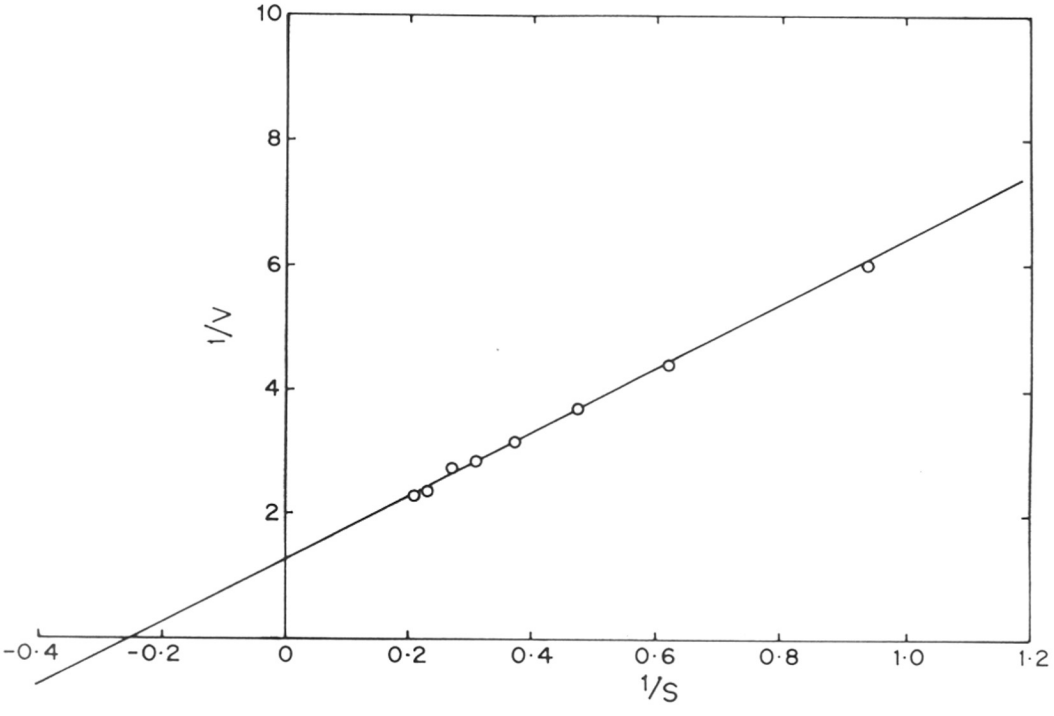


FIG. 3j

Fig.3k: Effect of thiol reagent on the enzyme activity

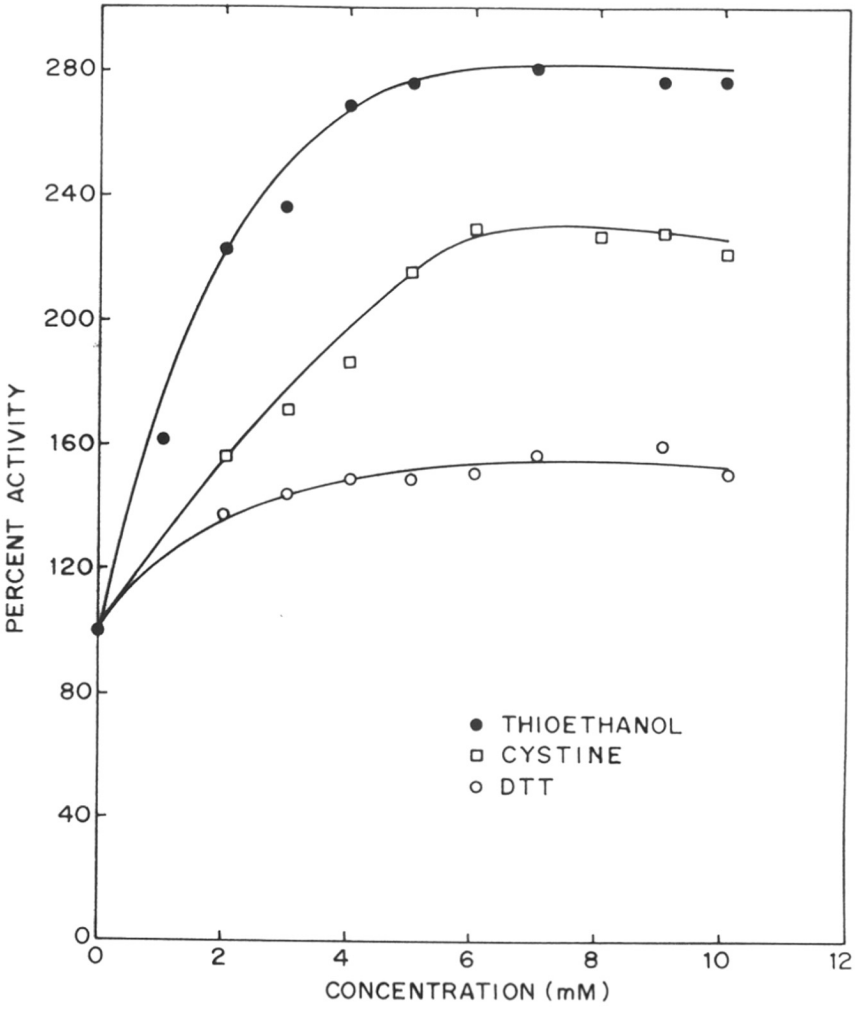

280
240
200
160
120
80
40
0
PERCENT ACTIVITY
0
2
4
6
8
10
12
CONCENTRATION (mM)
THIOETHANOL
CYSTINE
DTT

FIG. **3k**

Table 3.4a : Effect of Metal ion on *Cephalosporium* xylanase

Metal ion	1 mM	5 mM
Control (none)	100%	100%
MO^{3+}	98%	98.3%
Ca^{2+}	83%	83.7%
CO^{2+}	79%	30.8%
Cu^{2+}	72%	12.4%
Mn^{2+}	82%	32%
Fe^{2+}	20.4%	
Mg^{2+}	85.6%	79%
Zn^{2+}	84%	80%
Hg^{2+}	59%	0%

100 µg of the enzymes was incubated with the metal ion at 28°C for 30 min and then residual activity assayed under standard assay conditions.

Table 3.4b: Reactivation of Hg^{2+} treated Xylanase with thioethanol:

Concentration of Hg (mM)	Residual activity (%)	Acitivity in of thioethanol (5 mM)
0.0	100	163
0.1	63	115
0.5	40	97
1.0	0.0	79

Stability to denaturants

The *Cephalosporium* sp. xylanase when incubated with denaturants such as SDS, urea and guanidine hydrochloride was found to be significantly resistant to denaturation (Fig. 3.l, 3.m, 3.n). The enzyme lost approximately 50% activity in presence of 20% SDS, 0.7 M guanidium hydrochloride and 5 M urea.

Substrate specificity

Substrate specificity studies showed that the enzyme has high activity on oat spelts and larch wood xylan in comparison to birchwood xylan (Table 3.5). This can be correlated to low arabinose content of birchwood xylan.

Substrate specificity of the enzyme for various substrates with different types of linkages revealed that the purified enzyme has no activity against microcrystalline cellulose, CM-cellulose, Avicelcellobiose, starch, p-nitrophenyl β-xyloside, p-nitrophenyl B-arabinoside and laminarin (Table 3.5). The above results suggest that *Cephalosporium* xylanase is a true xylanase.

Mode of action

The products of time course hydrolysis of oat spelts xylan were analysed by paper chromatography as well as HPLC (Fig. 3.o). From the initial stages of hydrolysis, reaction products contained low molecular weight xylo-oligosaccharides, xylose and substantial amounts of arabinose (Table 3.6). As shown in Fig. 3.p the maximum degree of hydrolysis was 30%. With increase in the duration of hydrolysis the concentration of low molecular weight xylo-oligosaccharides as well as xylose increased clearly suggesting an endo-type of action. This observation shows *Cephalosporium* xylanase to be an endo-enzyme.

Active site Characterization:

It has been reported that tryptophan and carboxylate are involved in the catalytic activity of xylanases from *Streptomyces* T-7 (Keskar *et al.*, 1989), *Schizophyllum commune* (Bray and

Fig.31: Effect of SDS on the enzyme (100 μg)

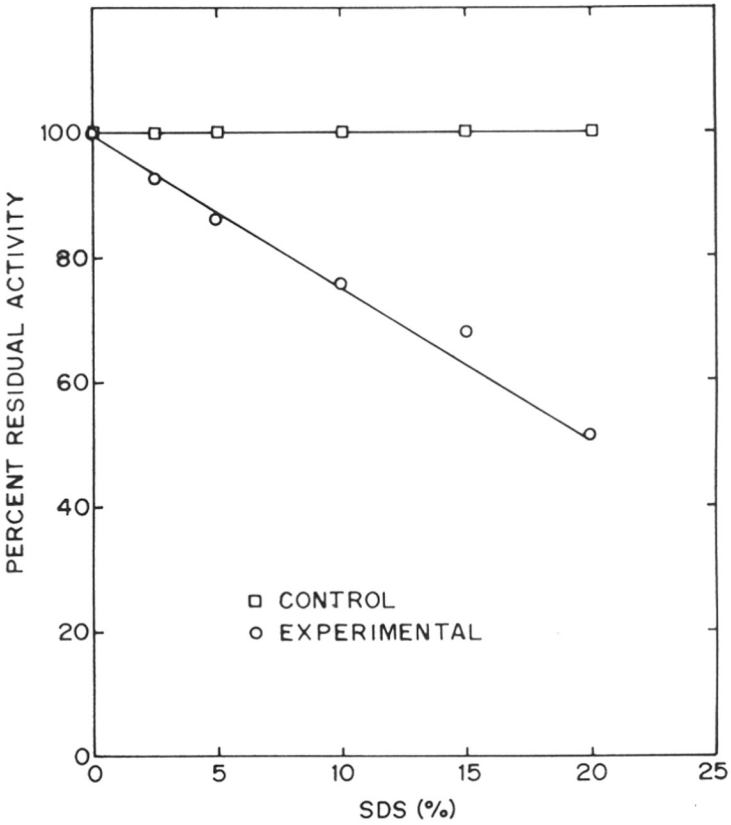

PERCENT RESIDUAL ACTIVITY
100
80
60
40
20
0
0
5
10
15
20
25
SDS (%)
CONTROL
EXPERIMENTAL

FIG. 31

Fig. 3m: Effect of guanidine HCl on the enzyme (100 μg)

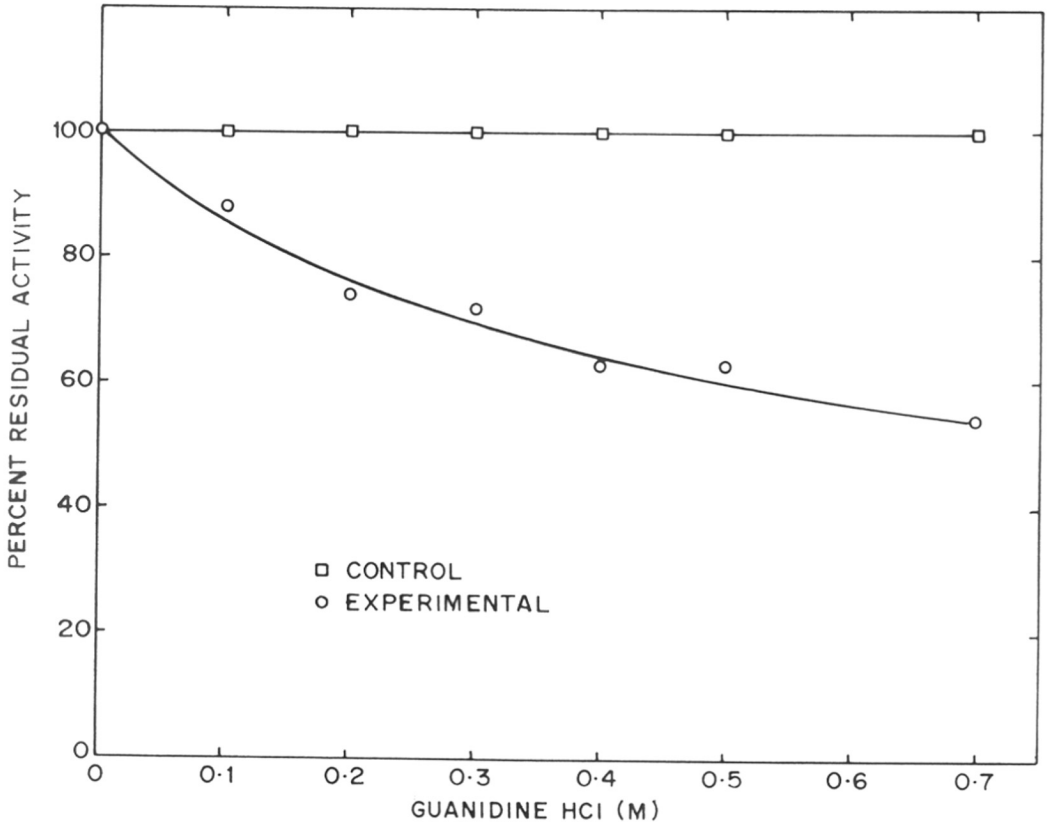

PERCENT RESIDUAL ACTIVITY
100
80
60
40
20
0
0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
GUANIDINE HCl (M)
CONTROL
EXPERIMENTAL

FIG. 3m

Fig.3n: Effect of urea on the enzyme (100 μg)

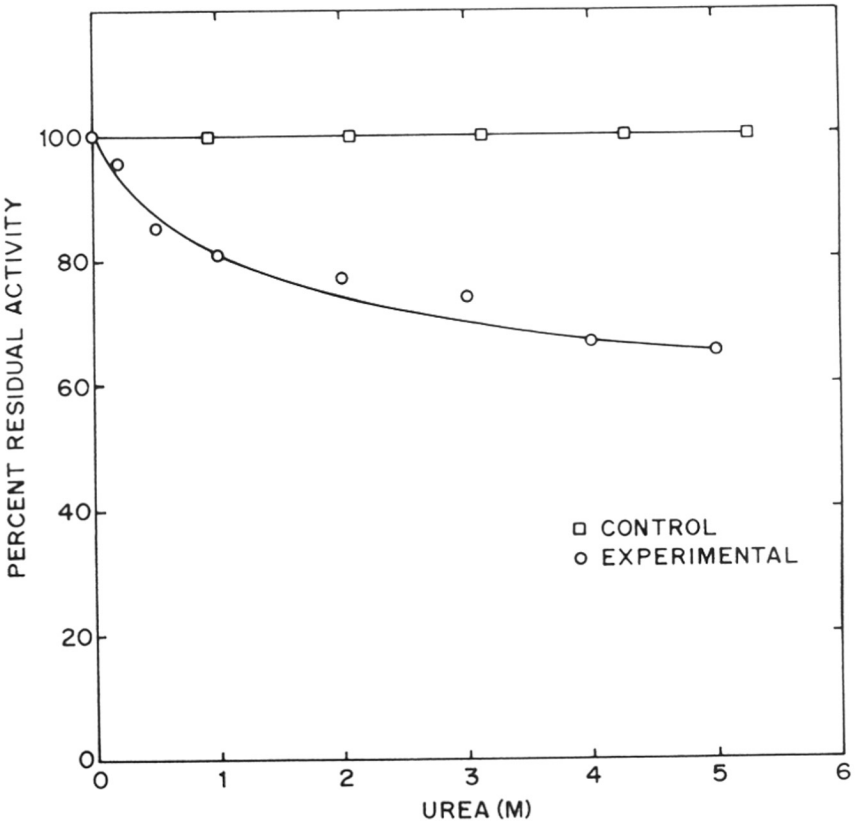

PERCENT RESIDUAL ACTIVITY
100
80
60
40
20
0
0
1
2
3
4
5
6
UREA (M)
CONTROL
EXPERIMENTAL

FIG. 3n

Table 3.5 Substrate specificity

Substrate	Linkage	Activity (%)
Xylan		
Oatspelts	β-1,4	100
Lardwoods	β-1,4	96
Birchwood	β-1,4	53
Cellulose		
Carboxymethyl	β-1,4	Nil
Avicel	β-1,4	Nil
Cellobiose	β-1,4	Nil
p-Nitrophenyl β-D-xyloside	β-1,4	Nil
Soluble	α-1,4	Nil
Starch	α-1,6	Nil
Sucrose		Nil
Laminarin	β-1,3	Nil

Fig. 3o: Figure showing the products formed on the oat spelt xylan hydrolysis by the pure enzyme

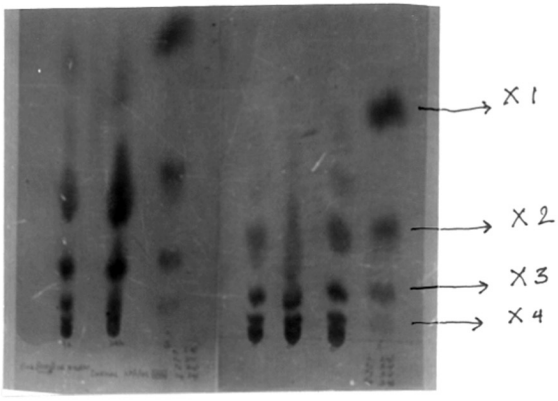


Fig. 30(i): Analysis by paper chromatography

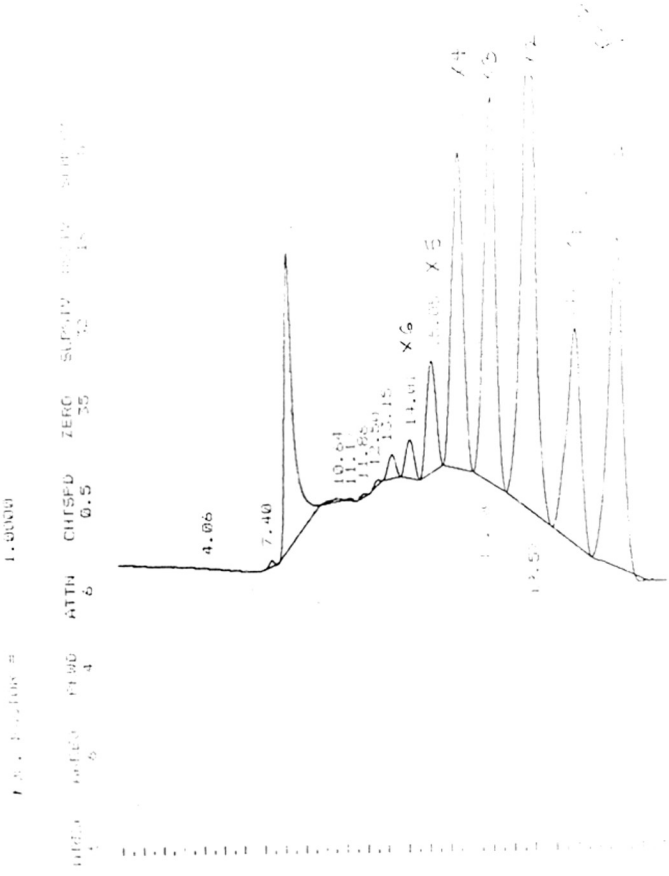


Fig. 30 (ii): Analysis by HPLC

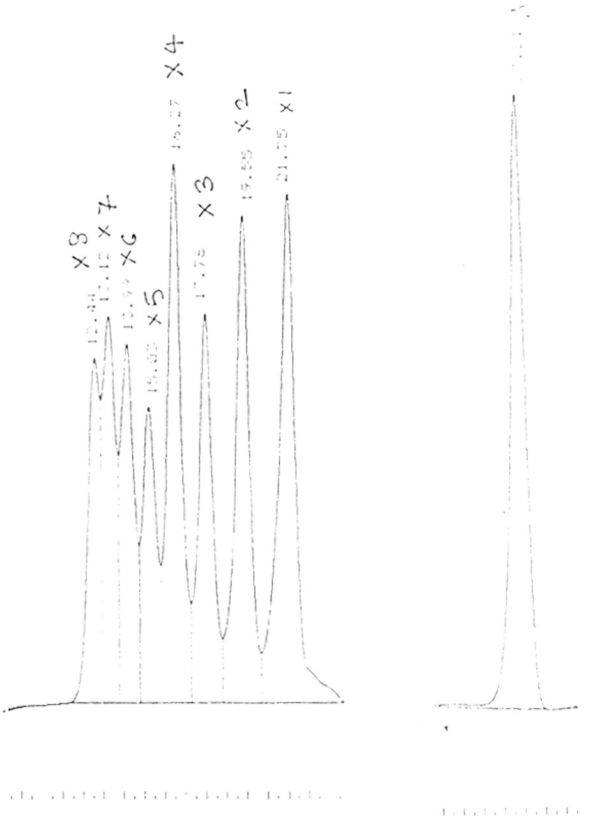


Fig. 3o: Standard xylo-oligosaccharides.

Fig.3p: Degree of hydrolysis of the oatspelt xylan on enzymatic hydrolysis

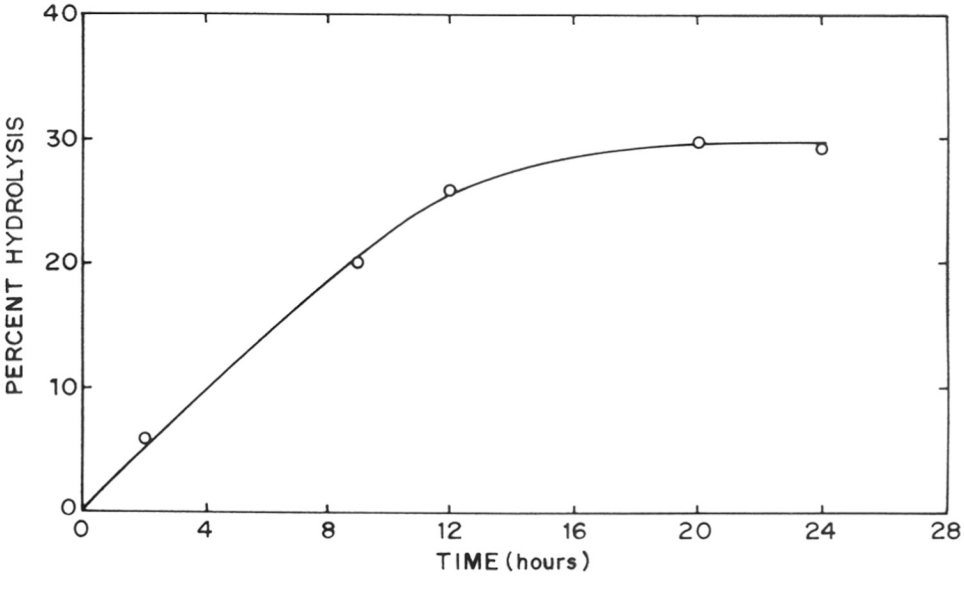


FIG. 3p

Table 3.6 : **Xylo-oligosaccharides formed by *Cephalosporium* xylanase**

Xylo-oligosaccharides (% area)	Time (h) 2	12	24
Arabinose	10	15	19
X1	8.7	12	12.9
X2	18.2	29.6	32.4
X3	27	19	20
X4	18	15	14
X5	4	6	6
X6	0.8	1.3	1.8

The xylo-oligomers (% area from the HPLC graphs) formed with increasing time of hydrolysis

Clarke, 1990), *Chainia* (Deshpande *et al.*, 1990; Rao *et al.*, 1995), alkalophilic-thermophilic *Bacillus* (Chauthaiwale, 1994). Hence modification of these residues were carried out to evaluate their role in the catalytic activity of *Cephalosporium* xylanase.

Modification of tryptophan:

Purified xylanase when incubated with 2.4 μM NBS for 30 min, at pH 7.0 and 28±1°C, lost 65 % of its initial activity. The inactivation was dependent on the concentration of the reagent. However no loss of activity was observed in the control sample. NBS mediated inactivation followed pseudo first-order kinetics at any fixed concentration of the reagent. The first-order rate constants were calculated from the slope of plots of log (percent residual activity) against the reaction time and the order was determined from the slope of the plots of log (K_{app}) against log [NBS]. These plots (inset Fig. 3.q) indicated that the loss of enzyme activity was caused by the modification of a single tryptophan residue per molecule of the enzyme. The NBS-mediated inactivation was also accompanied by a sharp decrease in the absorption of the modified protein at 280 nm, characteristic of tryptophan modification. Based on the molar absorption coefficient of the modified tryptophan, at 280 nm, to be $5500^{M-1}cm^{-1}$ (Spande and Witkop, 1965) and the molecular weight of *Cephalosporium* xylanase to be 28,000, the total number of tryptophan residues modified was found to be 1.6. However, the plot of percent residual activity against the number of tryptophan residues modified, revealed that the loss of enzyme activity resulted from the modification of a single tryptophan residue (Fig. 3.r). The second order rate constant for NBS was 4.0×10^3 M^{-1} min^{-1}.

Though the NBS is specific for tryptophan it also reacts with tyrosine at or around neutral pH. However modification of tyrosine residues of the purified enzyme with N-acetylimidazole though resulting in the modification of 7 residues out of 12 (Table 3.7) did not have any significant effect on the enzyme activity suggesting that tyrosine may not be involved in the catalytic activity of the *Cephalosporium* xylanase.

Fig.3q: (i) Kinetic of inactivation of xylanase by NBS
Inset: First order rate constant

(ii) Second order rate constant for inactivation by NBS

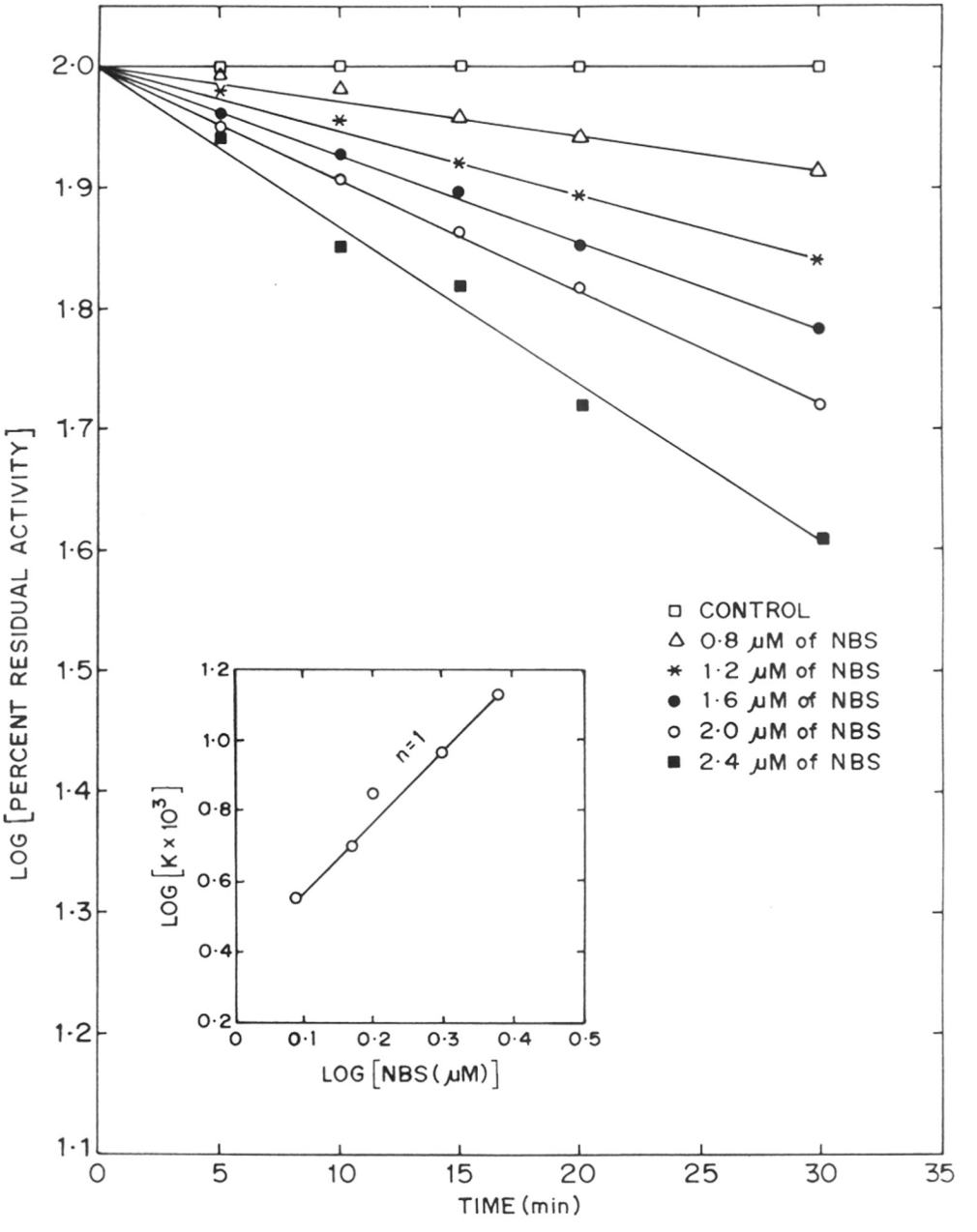

LOG [PERCENT RESIDUAL ACTIVITY]
2·0
1·9
1·8
1·7
1·6
1·5
1·4
1·3
1·2
1·1
0
5
10
15
20
25
30
35
TIME (min)
CONTROL
0·8 μM of NBS
1·2 μM of NBS
1·6 μM of NBS
2·0 μM of NBS
2·4 μM of NBS
LOG [K × 10³]
1·2
1·0
0·8
0·6
0·4
0·2
0
0·1
0·2
0·3
0·4
0·5
n=1
LOG [NBS (μM)]

FIG. 3q (i)

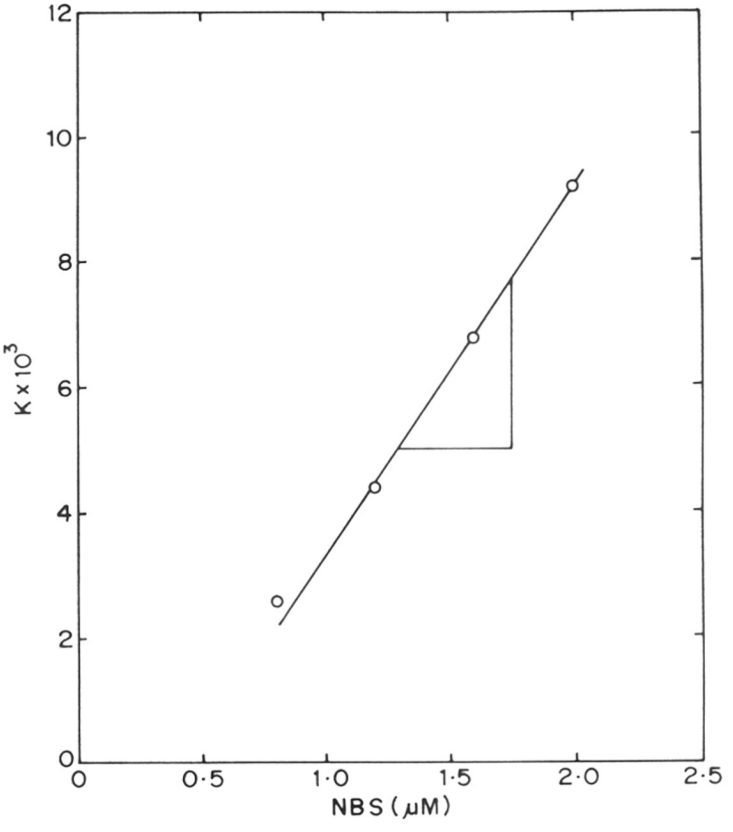


FIG. 3q (ii)

Fig.3r: Titration of NBS with xylanase

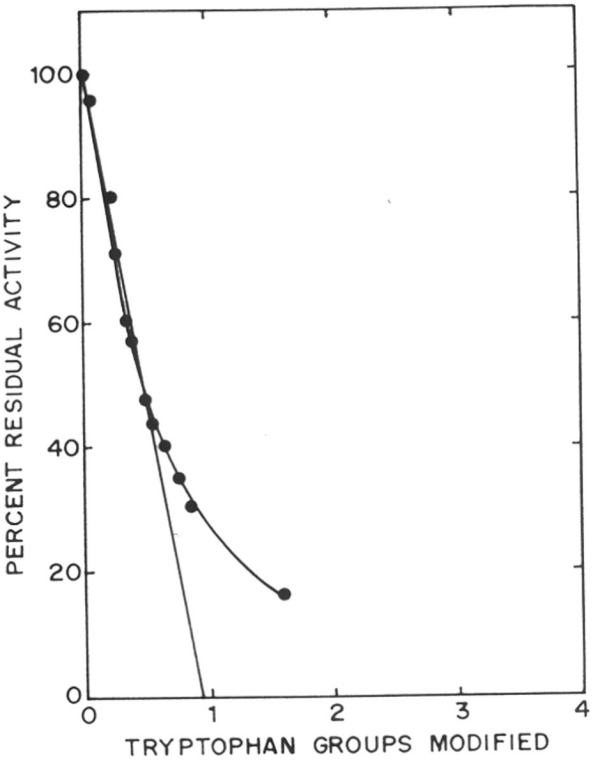

PERCENT RESIDUAL ACTIVITY
100
80
60
40
20
0
0
1
2
3
4
TRYPTOPHAN GROUPS MODIFIED

FIG. 3r

Table 3.7: Effect of NAI on xylanase:

Modification reaction	Number of residues modified	Xylanase activity
Control	0	100
Enzyme + NAI (10 mM)	7	90

The NBS mediated inactivation could be prevented to a significant extent (95%) by pre-incubating the enzyme with excess amount of xylan (5.36 mg). The above results point towards the presence of tryptophan at or near the active site of *Cephalosporium* xylanase.

The involvement of tryptophan in the catalytic activity was also confirmed by modifying the enzyme with HNBB. Purified xylanase when incubated with 8 mM HNBB for 30 min, at pH 7.0 and 28±1°C, lost 75 % of its initial activity and the inactivation was dependent on the concentration of the reagent. However no loss of activity was observed in the control samples. Kinetic analysis and substrate protection studies on HNBB-mediated inactivation of the enzyme also suggested that the modification of a single tryptophan residue is responsible for the loss of activity of the enzyme (Fig. 3.s, Table 3.8). The second order rate constant for HNBB was calculated to be $6.87 \times 10^2\ M^{-1}\ min^{-1}$.

The above observations substantiate the role of tryptophan in the catalytic activity of *Cephalosporium* xylanase.

Modification of carboxyl group

Purified xylanase when incubated with 25 mM EDC for 30 min, at pH 6.0 and 28±1°C, lost 55% of its initial activity and the inactivation was dependent on the concentration of the reagent. The EDC mediated inactivation followed pseudo first-order kinetics at any fixed concentration of the reagent. Pseudo first-order rate constants were calculated from the slope of plots of log (percent residual activity) against the reaction time and the order was determined from the slope of the plots of $\log(K_{app})$ against log[EDC]. These plots (inset Fig. 3.t) indicated that the loss of enzyme activity occured as the modification of two carboxylate groups per molecule of the enzyme while the second order rate constant was $1.8 \times 10^2\ M^{-1}\ min^{-1}$.

The loss of activity as a result of carboxylate modification could be prevented to a significant extent by incubating the enzyme with excess amount of xylan prior to modification reaction (Table 3.9). These results point towards the involvement of carboxylate groups in the catalytic activity of *Cephalosporium* xylanase.

Fig.3s. (i) Kinetics of inactivation of xylanase by HNBB
Inset: First order rate constant

(ii) Second order rate constant for inactivation by HNBB

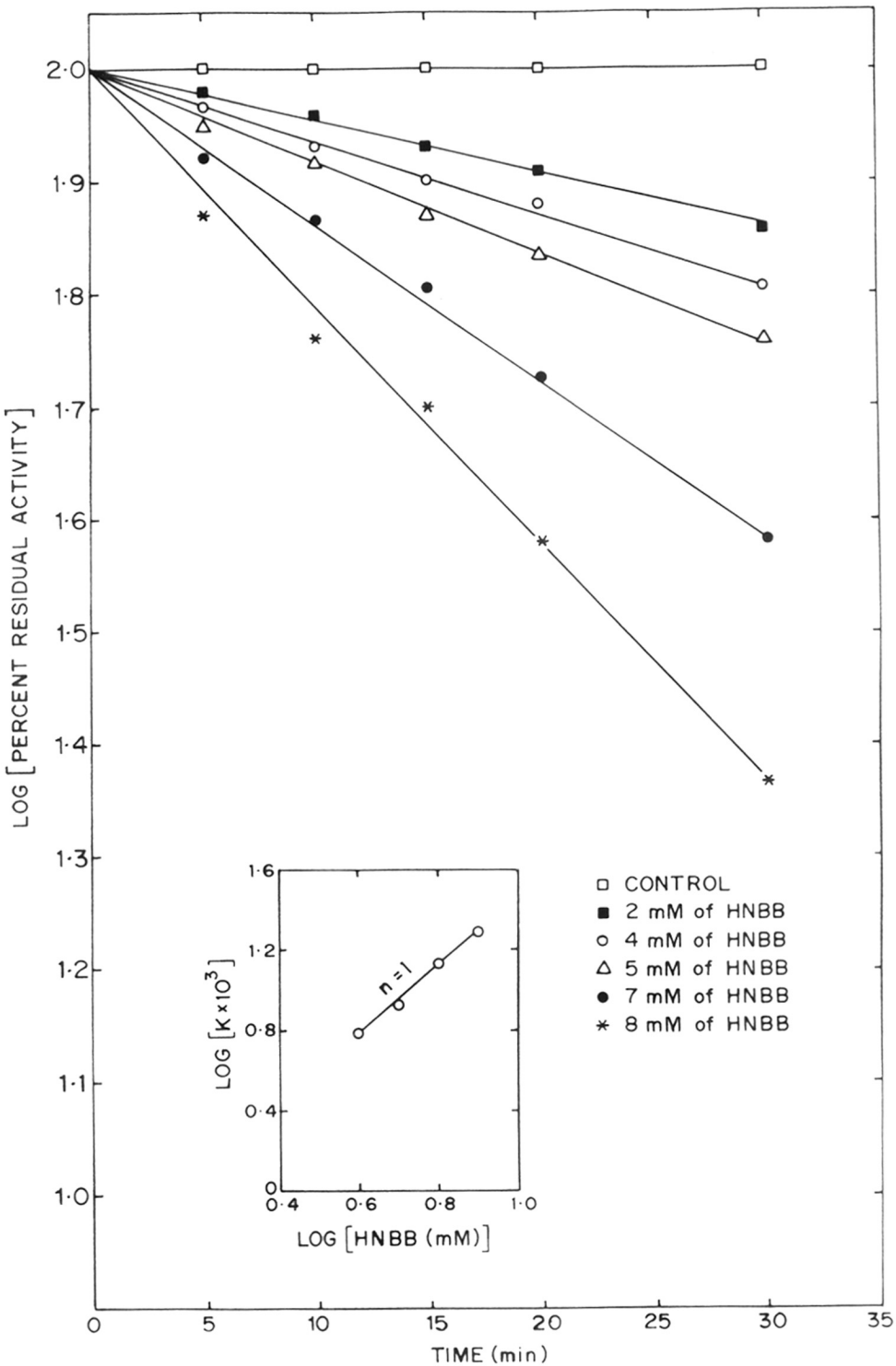

LOG [PERCENT RESIDUAL ACTIVITY]
2·0
1·9
1·8
1·7
1·6
1·5
1·4
1·3
1·2
1·1
1·0
0
5
10
15
20
25
30
35
TIME (min)
CONTROL
2 mM of HNBB
4 mM of HNBB
5 mM of HNBB
7 mM of HNBB
8 mM of HNBB
LOG [K×10³]
n=1
1·6
1·2
0·8
0·4
0
0·4
0·6
0·8
1·0
LOG [HNBB (mM)]

FIG. 3s(i)

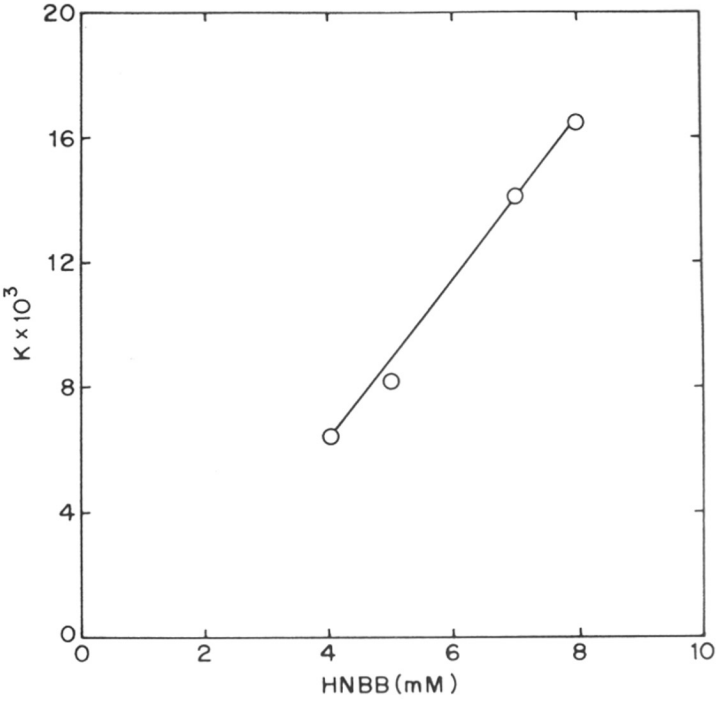


FIG. **3s** (ii)

Fig.3t (i) Kinetics of inacticvation of xylanase by EDC
Inset: First order rate constant

(ii) Determination of second order rate constant for inactivation by EDC

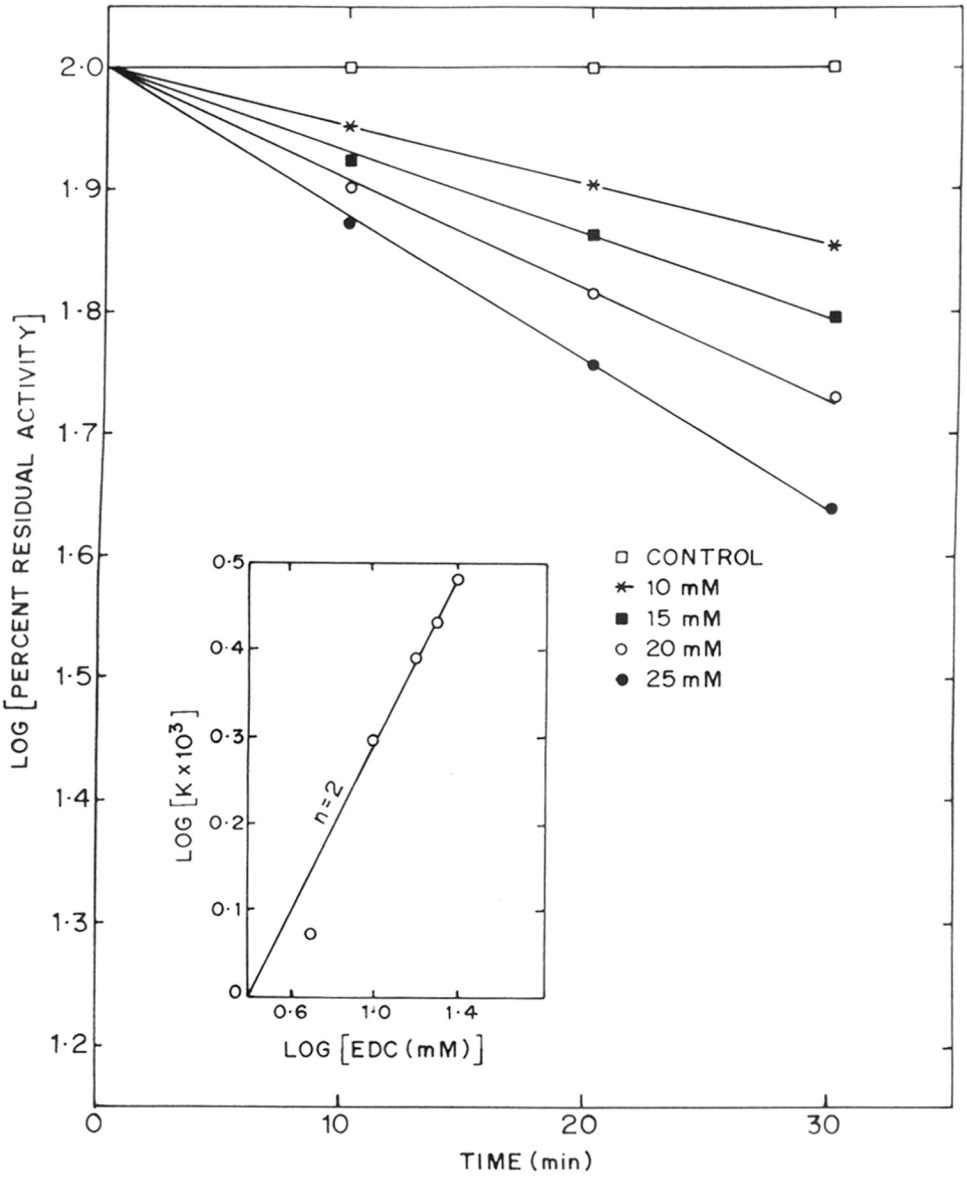

2·0
1·9
1·8
1·7
1·6
1·5
1·4
1·3
1·2
LOG [PERCENT RESIDUAL ACTIVITY]
0
10
20
30
TIME (min)
CONTROL
10 mM
15 mM
20 mM
25 mM
0·5
0·4
0·3
0·2
0·1
0
LOG [K x 10³]
n=2
0·6
1·0
1·4
LOG [EDC (mM)]

FIG. 3t (i)

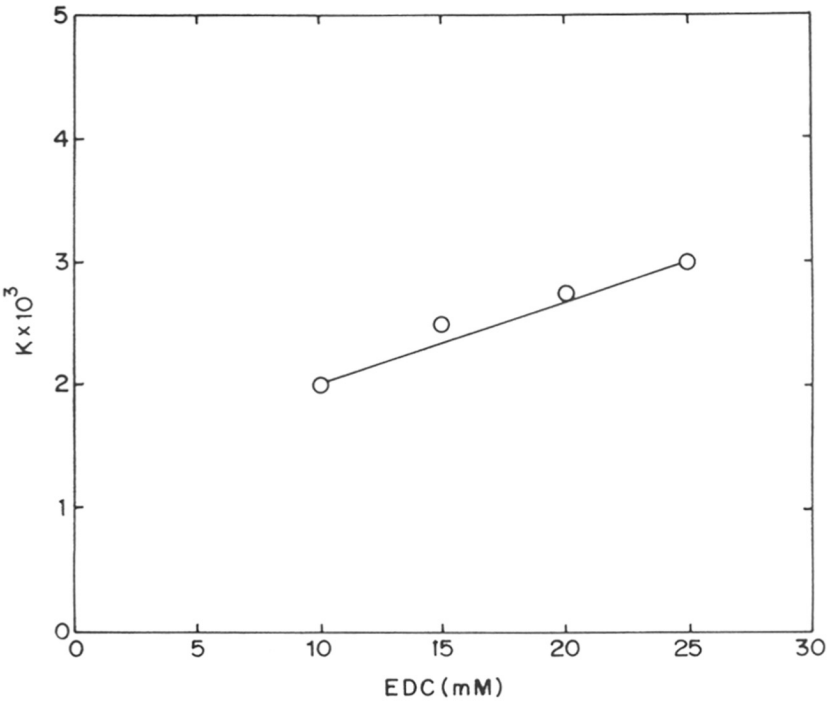


FIG. 3t (ii)

Table 3.8: Substrate protection against inactivation of Xylanase by NBS and HNBB

Experiments	Xylanase (%) Residual Activity
Enzyme	100
Enzyme + NBS (6.4 μM)	0
Enzyme + xylan (5.6 mg) + NBS	95
Enzyme + HNBB (10 mM)	0
Enzyme + xylan (4.9 mg) + HNBB	98

Table 3.9: Influence of Carboxylate group modification on xylanase activity: Substrate protection

Experiments	Xylanase activity (%)
Enzyme	100
Enzyme + EDC (30 mM)	30
Enzyme + oatspelt xylan (3.5 mg) + EDC (30 mM)	94

The EDC mediated inhibition studies were also carried out in the presence of glycine ethyl ester (nucleophile), but this did not cause any increase in the inactivation. This indicated that EDC alone was sufficient for inactivation. Hence, in all the experiments EDC alone was used. Similar observations were also reported by Chan *et al.* (1988) and Bray and Clarke (1990).

Modification of Histidine

Photo-oxidation: When the purified enzyme was irradiated with 0.2 % (w/v) methylene blue at pH 7.0 and 26±1°C for 30 min, lost 55% of its initial activity and the inactivation was dependent on the concentration of the reagent (Fig.3.u). The inhibition of the activity could be prevented by sheilding the enzyme-methylene blue mixture from irradiation, indicating the presence of histidine at or near the active site.

The involvement of histidine was also ascertained by modifying the enzyme with a histidine specific reagent namely DEP.

Carbethoxylation of purified xylanase at pH 7.0 for 90 min resulted in 60% loss of its initial activity and the inactivation was concentration dependent. No loss of activity was observed in the control samples. The logarithm of the percent residual activity plotted as a function of time at various DEP concentration was linear upto 40% of the initial activity (Fig. 3.v). DEP mediated inactivation followed pseudo first-order kinetics at any fixed concentration of the reagent. The pseudo first-order rate constant were calculated from the slope of plots of log (percent residual activity) against the reaction time and the order was determined from the slope of the plots of log (K_{app}) against log [DEP]. These plots (inset Fig. 3.v) indicated that the loss of enzyme activity was due to the modification of a single histidine residue per molecule of the enzyme. Moreover the DEP mediated inactivation of the enzyme was accompanied by an increase in the absorbance of the modified protein at 240 nm, which is characteristic of ethoxycarboxylation of histidine residues. Based on a molar absorption coefficient of carbethoxyhistidine, at 240 nm, to be 3200 $M^{-1}cm^{-1}$ (Ovadi *et al.*, 1967) and the Mr of xylanase to be 28,000, the total number of histidine modified was found to be 3.3 residues per molecule of the enzyme. However

Fig. 3u: Photooxidation of the pure enzyme with methylene blue

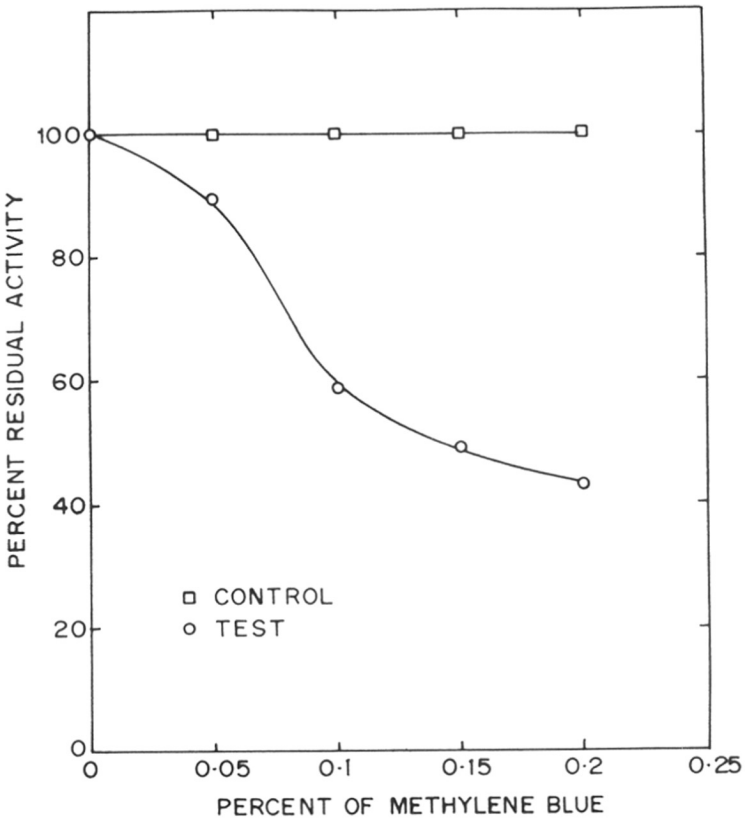

PERCENT RESIDUAL ACTIVITY
100
80
60
40
20
0
CONTROL
TEST
0
0·05
0·1
0·15
0·2
0·25
PERCENT OF METHYLENE BLUE

FIG. 3u

Fig. 3v (i) Kinetics of inactivation of xylanase by DEP
Inset: First order rate constant for inactivation by DEP

(ii) Second order rate constant for inactivation by DEP

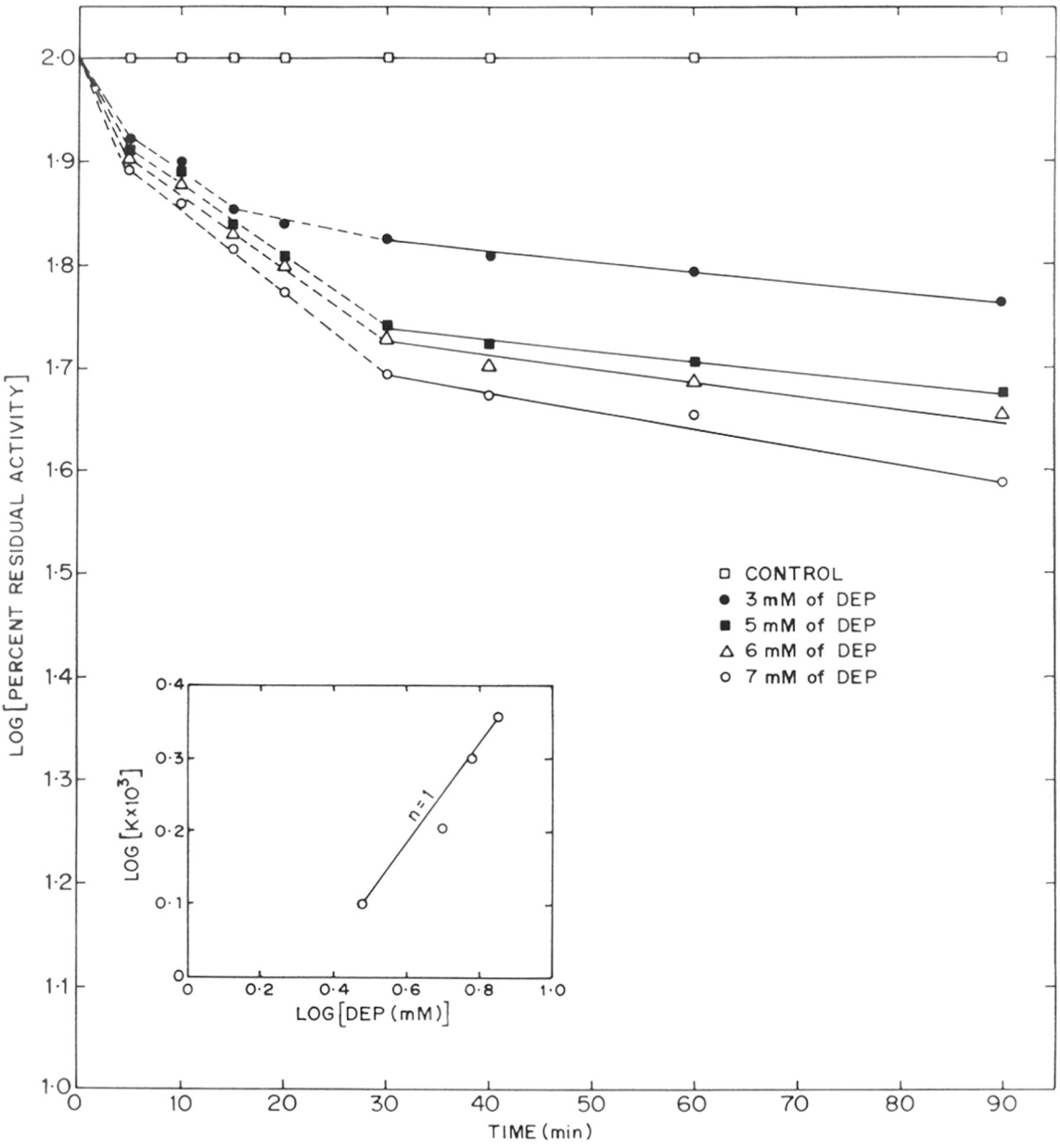

LOG [PERCENT RESIDUAL ACTIVITY]
2·0
1·9
1·8
1·7
1·6
1·5
1·4
1·3
1·2
1·1
1·0
0
10
20
30
40
50
60
70
80
90
TIME (min)
CONTROL
3 mM of DEP
5 mM of DEP
6 mM of DEP
7 mM of DEP
LOG [K×10³]
0·4
0·3
0·2
0·1
0
0·2
0·4
0·6
0·8
1·0
LOG [DEP (mM)]
n=1

FIG. 3v(i)

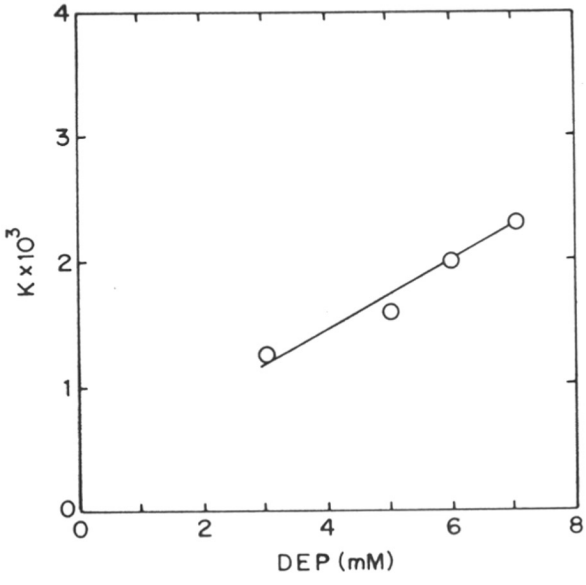


FIG. 3v(ii)

modification in presence of xylan gave a value of 2.2 residues, with significant retention of activity (85-90%) suggesting the involvement of a single histidine residue in the catalytic activity of the enzyme. Incubation of the DEP modified enzyme with 100mM hydroxylamine, at pH 7.0 at 28+1°C for 30 min, restored 95% of its original activity substantiating the role of histidine in the catalytic activity of *Cephalosporium* xylanase (Table 3.10).

Though DEP is specific for histidine at or around neutral pH, it also reacts to a lesser extent with tyrosine, cysteine and lysine residues (Miles, 1977). However modification of tyrosine residues, of the purified enzyme with N-acetyl imidazole though resulting in the modification of 7 residues out of 12, did not have any significant effect on the enzyme activity suggesting that tyrosine may not have a role in the catalytic activity of the enzyme (Table 3.11). The modification of tyrosine, as a result of DEP treatment, was also ruled out by the observation that there was no significant decrease in the absorbance of the modified protein at 278 nm. Though the above observation supports the presence of histidine at or near the active site, they still do not rule out the possible involvement of cysteine. However, in the present case, the DEP mediated inactivation of the enzyme cannot be attributed to the modification of cysteine residues as amino acid analysis of the purified enzyme revealed the absence of cysteine. Furthermore the loss of activity of *Cephalosporium* xylanase as a result of DEP treatment cannot be correlated to lysine modification since DEP modified enzyme could recover significant amount of its activity in presence of hydroxylamine. If the inactivation of the enzyme had been due to lysine modification, then hydroxylamine treatment would not have restored its activity.

Studies on substrate protection revealed that methylene blue and DEP mediated inactivation could be prevented to a significant extent by preincubating the enzyme with excess amounts of xylan (Table 3.12). These results substantiate the involvement of histidine in the catalytic activity of the *Cephalosporium* xylanase.

Table 3.10: Reactivation of DEP inhibited Xylanase

Modification Reaction	Xylanase Residual activity (%)
Enzyme	100
Enzyme + DEP (10 mM)	0
Decarbethoxylation (hydroxylamine hydrochloride)	91

Table 3.11: Substrate protection against inactivation of Xylanase by DEP and methylene blue:

Experiments	Xylanase residual activity (%)
Enzyme	100
Enzyme + DEP (10 mM)	0
Photo-oxidation Enzyme + 0.2% methylene blue	45
Enzyme + xylan (5.6 mg) + DEP	100
Enzyme + xylan (5.6 mg) 0.2% methylene blue	80

CONCLUSIONS

In conclusion the xylanase from alkalotolerant *Cephalosporium* was purified to homogeneity. The pure enzyme was confirmed to be active at alkaline pH (7.0 - 9.0) indicating novelty of the enzyme from a fungal source. The enzyme was shown to release arabinose along with xylobiose and smaller xylo-oligosaccharides on hydrolysis of oatspelts xylan. Structure-function studies indicated the presence of a single tryptophan, histidine and 2 carboxyl groups at the active site.

CHAPTER 4

APPLICATION OF THE ENZYME IN PULP BLEACHING

INTRODUCTION

The conventional bleaching process in paper manufacture uses molecular chlorine and chlorine dioxide resulting in toxic effluents such as dioxane and other chlorinated lignin degradation products. These are resistant to biodegradation or chemical treatment and are also difficult to recycle, thereby causing problems as pollution hazards. Faced with stringent government regulations and legal pressures from environmental protection agencies, the paper companies are presently on the look out for alternative eco-friendly technologies. The concept of using hemicellulases in bleaching technology was first demonstrated by Viikari *et al.* in 1986 and since then use of xylanases as a bleach booster has been successfully achieved in reducing consumption of chlorine based bleach chemicals (Viikari *et al.*, 1986; 1994). Production of Totally Chlorine Free (TCF) paper is envisaged by the turn of the century. Xylanases have been successfully tested as a pre-bleach agent in peroxide bleaching resulting in increased brightness and also reduced consumption of hydrogen peroxide (Lundgren *et al.*, 1993; Viikari *et al.*, 1994).

The xylanases currently being used are active at acidic or near neutral pH at 40-50°C using 2.5-5.0% pulp consistency, with residence times being 12-24 hours (Viikari *et al.*, 1994). In the kraft process the in-coming pulp is at alkaline pH and the residence time between the digester and the bleach plant ranges from 2-4 hours. Enzymes having high activity and stability under alkaline pH conditions would be ideal. There are very few reports on evaluation of xylanase active at alkaline pH in pulp and paper application (Khasin *et al.*,1993) as very few enzymes active at and stable to pH 8-10 are available.

The objective of this study has been to demonstrate that the alkalotolerant *Cephalosporium* xylanase active at alkaline pH is effective in reducing Kappa content at alkaline pH values. Most studies reported are based on soft wood pulp which is the principal raw material used in the paper and pulp industry in the developed countries. No data is available on enzymic treatment of bagasse pulp which is one of the commonly used raw material in India. Hence it was considered worthwhile to undertake the present studies.

SUMMARY

Enzyme-aided bleaching of paper pulp is now becoming a reality and several large scale trials have established the efficacy of xylanase pretreatment in enhancing pulp brightness while reducing chlorine and chlorine dioxide consumption.

Most of the xylanases which have been evaluated are active at acidic or near neutral pH and at 50°C. The kraft pulping process which is carried out at high alkaline pH and temperature necessitates using xylanases active at relatively high pH and temperature and preferably cellulase-free. Keeping in view the above requirements, the xylanase enzyme from *Cephalosporium* (NCL 87.11.9) was evaluated for its efficacy in pulp treatment. Bagasse pulp was chosen as it is one of the principal raw materials used in the manufacture of paper especially in Western India. The crude xylanase preparation from *Cephalosporium* was shown to reduce the Kappa content effectively and the maximum drop in Kappa content was observed with 0.4 IU/g of pulp. The decrease in Kappa content was accompanied by release of sugar, lignin derived material and chromophore.

The enzyme was found to be effective in reducing sugar content over a broad pH range of 7.0 to 10.0 with concomitant reduction in Kappa content. The reduction in Kappa number at 40° and 50°C were comparable.

A noteworthy feature of the *Cephalosporium* xylanase was that the reduction in Kappa content at Stage I (prior to alkali-treatment) was significantly higher than at Stage II which indicates the possibility of eliminating alkali extraction after enzyme treatment.

MATERIALS

All the chemicals used were of analytical grade and the sources are indicated in the parenthesis.

Potassium permangnate, sodium thiosulphate ampoules, (0.1N) (Ranbaxy chemical Co.,Ltd. Bombay, India), potassium iodide and sodium hydroxide (Qualigens, Bombay, India), Bagasse Pulp (unbleached) was kindly provided by Pudumjee Pulp and Paper Mills Ltd., Chinchwad, Pune. The initial Kappa number of the unbleached pulp was 46.

METHODS

Enzyme: A crude enzyme prepared as described in Chapter 2 was used for pulp studies. Enzyme dosages used in this Chapter are as estimated by Somagyi Nelson's method (5 Units as estimated by DNSA corresponds to approximately 1 IU by Somagyi-Nelson's).

Determination of the consistency of the pulp: 1 g wet pulp was dried to constant weight at 110° C and the percentage consistency expressed based on the wet weight-dry weight difference.

Detection of chromophore and lignin derived material: Lignin derived compounds and chromophore release in the pulp filtrate was monitored by recording the increase in the absorbancies at 280 nm and 465 nm, respectively (Patel *et al.*,1992).

Sugar detection : The sugar release on enzymatic treatment of pulp was determined by the Somogyi-Nelson's method (Somogyi, 1952).

Kappa number determination: The Kappa number was determined by the TAPPI std. (Tappi 1984). Air dried pulp equivalent to 1 g dry weight was disintegrated in 500 ml of distilled water in a blender. The suspension was taken in a 2 liter conical flask to which freshly prepared solution of 100 ml each of 0.1 M $KMnO_4$ and 4 N H_2SO_4 was added, and the final volume made to 1000 ml by the addition of distilled water. The temperature of the suspension was recorded to be 26°C and a correction for this was made by the formula stated below. Variations in temperature is corrected for by a factor given in the formula below for calculation of Kappa number as per Tappi standard. The reaction was terminated after 10 min by the addition of 20 ml of 1N potassium iodide solution. The iodine liberated was titrated against 0.2 N sodium thiosulphate. A few

drops of 1 % starch was added and the disappearance of the blue colour indicated the end point. The Kappa content was calculated according to the following formula:

$$K = p \times f/w \; 1 + 0.013 \, (25 - t),$$

$$\text{Where } p = (b-a)N / 0.1$$

Where:

K = Kappa number

f = Factor for correction to a 50% permanganate consumption, dependent on the value of p (Factor to correct for different percentages of permanganate used).

w = grams of moisture free pulp in the specimen.

p = cubic centimeters of 0.1N permanganate actually consumed by the test specimen.

b = cubic centimeters of the thiosulphate consumed in the blank determination.

a = cubic centimeters of the thiosulphate consumed by the test specimen.

N = normality of the thiosulphate.

and 1 + 0.013 (25 - t) is the factor for the correction of the temperature.

The Kappa number of the untreated pulp subjected to the same conditions was taken as the control and the difference in the Kappa values between the enzyme treated pulp and the control pulp gave the actual decrease in Kappa content.

Pretreatment of Bagasse Pulp with *Cephalosporium* Xylanase: Pretreatment of the pulp with xylanase is a two stage process.

1) Stage I: Enzymatic treatment of the pulp.

2) Stage II: Alkali treatment of the pulp.

In Stage I the pulp was treated with the enzyme for a definite period and thereafter washed with water. At Stage II the treated pulp was extracted with sodium hydroxide to remove solubilized lignin degradation products and washed with water extensively. The details of the two stage process are given below:

Stage I

Enzymatic treatment of pulp: Pulp 10 g [dry weight] was taken in self sealing poly bags to which potassium phosphate buffer (50 mM, pH 8.0) was added. The pulp was pre-incubated at 40°C for 30 min to attain the temperature. Appropriate amounts of the enzyme was added and the net weight of the pulp was made to 100 g by addition of buffer. The pulp was incubated at 40°C for 150 min. Thereafter the pulp was filtered, washed and divided into two equal parts one of which was used for Kappa number determination and the other half was treated with alkali (Stage II). The filtrate was checked for lignin, sugar and chromophore release as described earlier. The results were compared with a control, incubated under identical conditions but without the addition of enzyme. The effect of variations in pH, temperature and enzyme concentration on the biobleaching of the pulp was studied in a similar manner.

Stage II

Alkali treatment : The washed pulp equivalent to 5 g from Stage 1 (after the enzymatic treatment) was taken in the self sealing polybags, pre-incubated at 60°C for 30 min after which 0.05% NaOH was added (final concentration 0.0125M) and the weight of the reaction mixture made to 100 g by the addition of distilled water. This was further incubated for 60 min at 60°C and then the Kappa number of the pulp determined after thoroughly washing it alkali-free. The filtrate was assayed for lignin and chromophore release.

RESULTS AND DISCUSSION

The alkaline xylanase from the alkalotolerant *Cephalosporium* species was evaluated for its ability to decrease the Kappa content of bagasse pulp. The Kappa content of the pulp is the measure of the lignin present. The chlorine load used in the bleaching of pulp is determined from the Kappa number of the pulp. A fall in the Kappa number as compared to the control with concomitant release of sugar, 280 and 465 nm absorbing material is a reliable indicator of enzyme action on pulp. Several parameters such as enzyme dosage, residence time, pH and temperature were studied before arriving at an optimized procedure for treating of pulp.

In the first experiment pulp (10 g) was treated with 12 IU of xylanase at pH 8.0 at 40°C for 150 min. The Kappa number was evaluated before and after alkali-extraction. The filtrates were estimated for sugar, chromophore and lignin derived compound release. Figure 1 gives a visual picture of the chromophore release from bagasse pulp on enzymatic treatment (Stage I). At Stage I there was substantial reduction in Kappa content as compared to the untreated pulp. This was further substantiated by the release of sugar, lignin and 280 nm absorbing material. On treatment with alkali there was approximately 30% decrease in the Kappa number as compared to Stage II (Table 4.1). Hence, for the initial standardization of the parameter only Stage I was carried out whereas studies on effect of pH and temperature were carried out both at Stage I and Stage II.

Table 4.1: Xylanase treatment of Bagasse pulp:

Stage	280 nm	465 nm	Reduction in Kappa number
Stage I	122	22	6.5
Stage II	48	2.2	1.8

Stage I: Xylanase treatment of pulp
Stage II: Alkali-treatment of pulp

Fig. 4.1 : Figure showing the colour release on enzymatic treatment

(a) control
(b) On enzymatic treatment

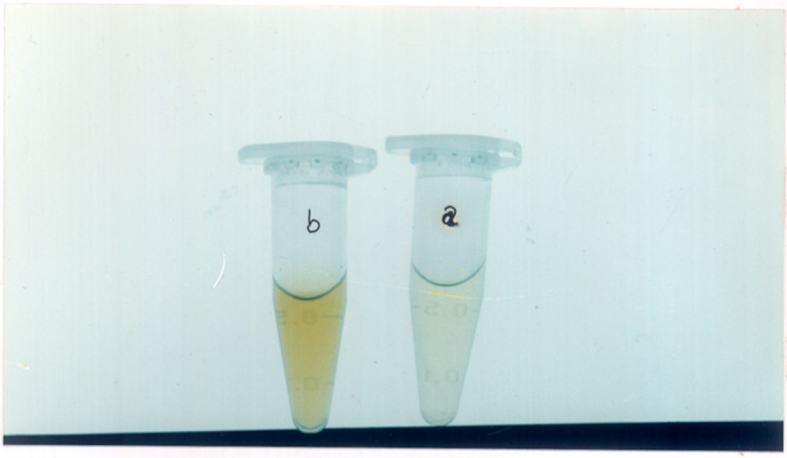


FIG. 4·1

Optimization of Residence time of Enzymatic treatment

The pulp (10 g) was treated with 12 IU of the enzyme (1.2 IU/g) at pH 8.0 (0.05 M potassium phosphate buffer) and 40°C. The pulp was incubated for varying time intervals (30 to 150 min). The chromophore and lignin release were approximately linear upto 30 min and the increase was gradual thereafter and reached a plateau around 120 min (Fig. 4.2a). Maximum release was observed at 150 min. The sugar release was approximately linear upto 60 min and subsequently remained constant (Fig. 4.2b). In all further experiments the enzymatic treatment was carried out for 150 min as this allowed the maximum release of chromophore and lignin, although the sugar release had reached a maximum in 60 min.

Effect of increasing xylanase dosage

Dosage response trials were carried out at 40°C for 150 min at pH 8.0 using 10 g of the pulp. Significant chromophore and 280 nm absorbing material release were obtained with enzyme dosages upto 2.0 IU per gram of the pulp (Fig. 4.3a). This correlated well with the increase in sugar release which was maximum at 2.0 IU/g (Fig. 4.3b). Maximum Kappa number reduction was observed at a concentration of 0.4 IU/ g of the pulp (Fig. 4.3c) and thereafter the drop in Kappa number was negligible. Although the drop in Kappa content was maximum with 0.4 IU/g of pulp, the enzyme dosage was fixed at 1.2 IU/g of pulp in all further experiments taking into consideration the sugar and chromophore release.

Enzyme dosages which have been used in prebleaching studies have varied between 30 nkat to 8300 nkat (1.8 IU to 500 IU per gram) of the pulp (Viikari *et al.*,1994). In the present studies, optimum enzyme dosage for effective pretreatment was 0.4 to 1.2 IU/g of pulp as estimated by Somagyi Nelson's method. The effective enzyme dosage for the *Cephalosporium* xylanase is in a range similar to that described by Viikari *et al.*, (1990) in their review on commercial trials with xylanases.

Effect of pH on the enzymatic treatment of bagasse pulp

The pulp was treated over a wide pH range of 7.0-10.0, for 150 min with 1.2 IU of the *Cephalosporium* xylanase per gram of the pulp. The efficacy of the enzyme treatment was evaluated at

Fig 4.2a : Chromophore and lignin release on enzymatic treatment of the pulp with increasing time intervals

Fig. 4.2b: Sugars release on enzymatic treatment of pulp: time profile

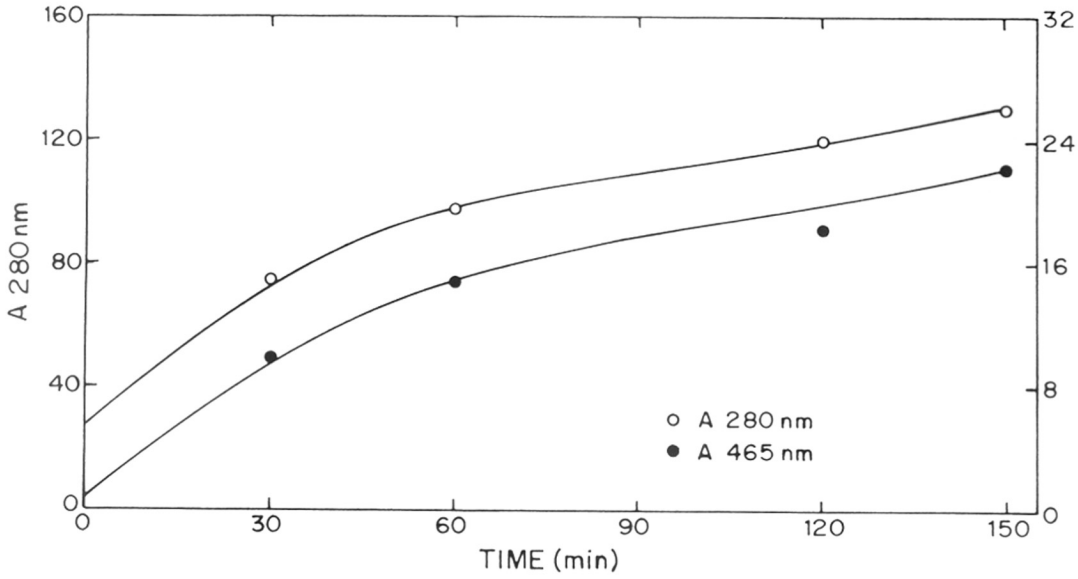


FIG. 4·2a

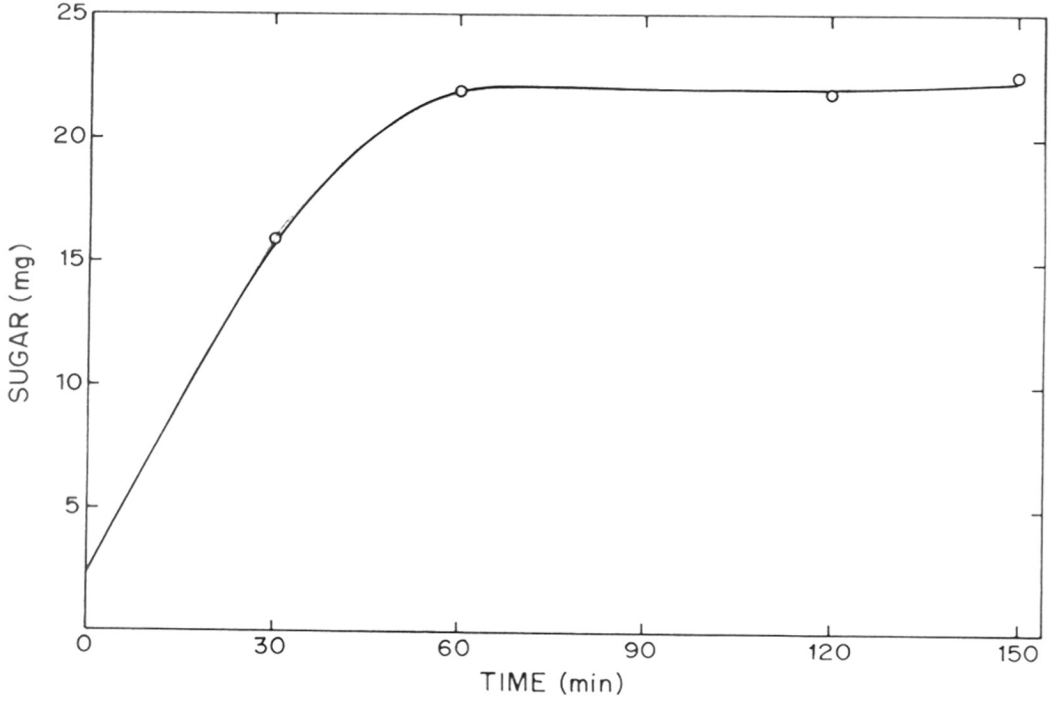


FIG. 4·2b

Fig.4.3a: Effect of enzyme dosages on chromophore and lignin release

Fig.4.3b: Effect of enzyme dosages on the sugar release

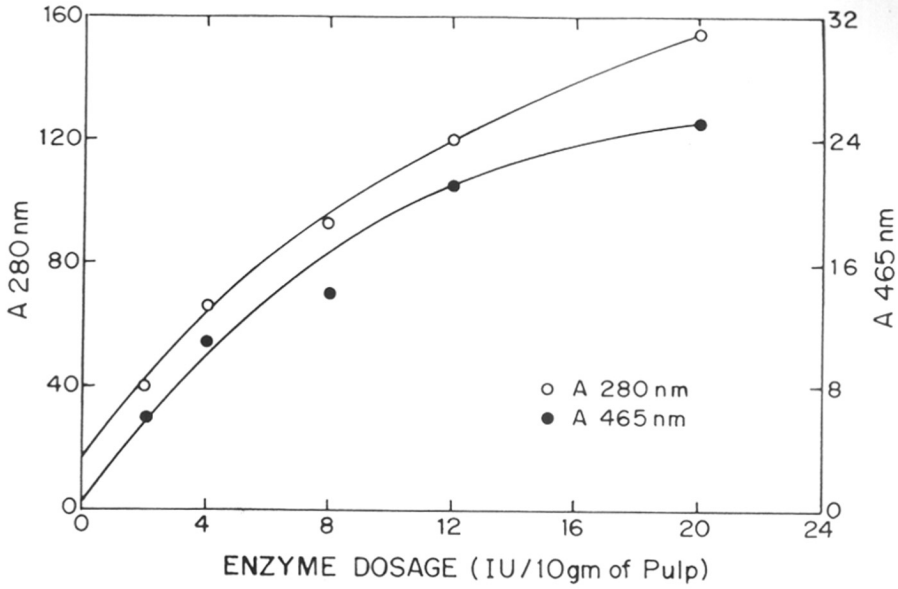


FIG. **4·3a**

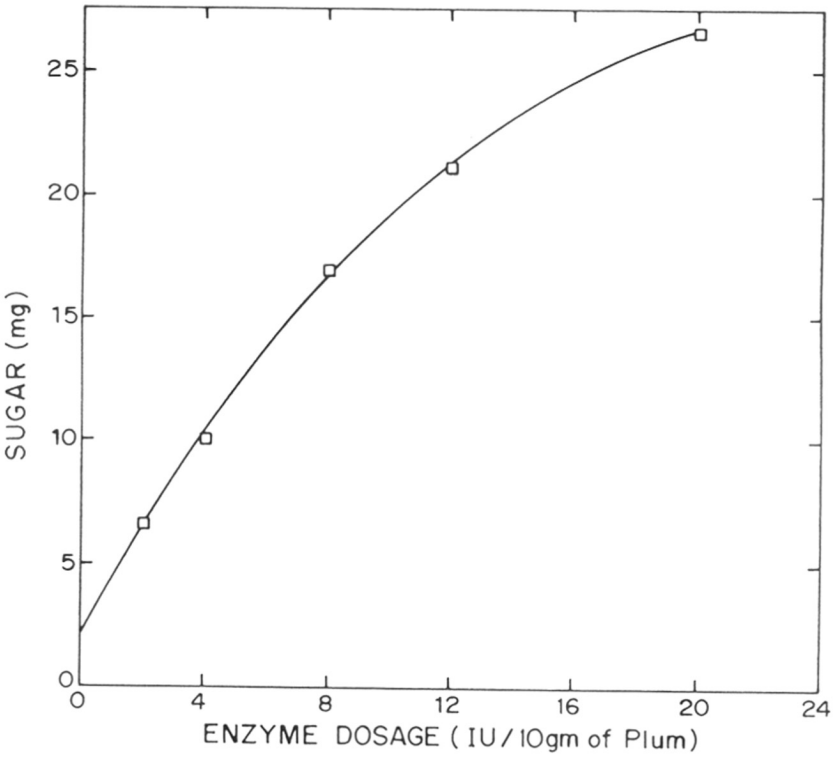


FIG. **4·3b**

Fig. 4.3c: Kappa number reduction with increased enzyme dosages

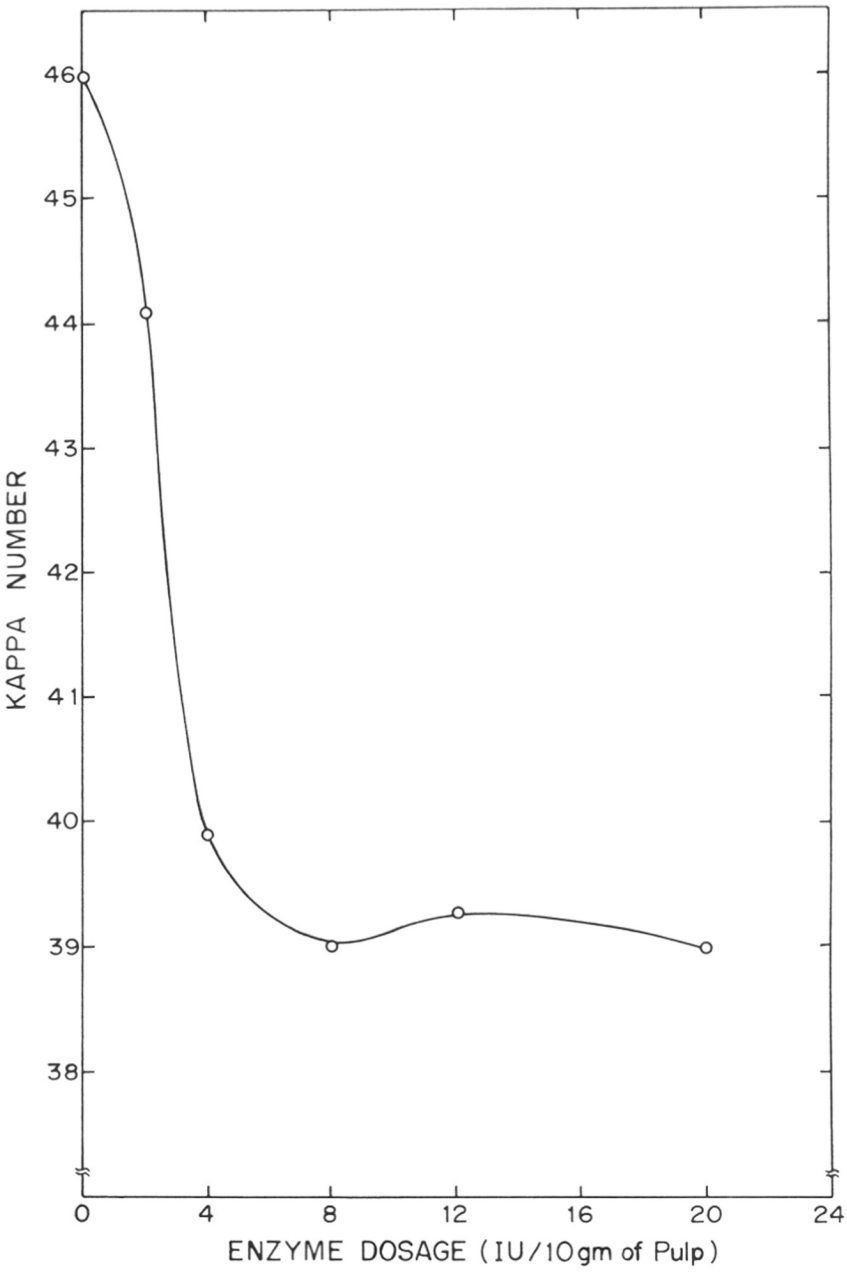

KAPPA NUMBER
46
45
44
43
42
41
40
39
38
0
4
8
12
16
20
24
ENZYME DOSAGE (IU/10gm of Pulp)

FIG. **4·3c**

both Stage I and Stage II as described in the Materials and Methods section. Although the sugar release was comparable at pH values evaluated (7.0 to 10.0) the chromophore release was maximal at pH 8.0 and 9.0. (Fig. 4.4a). The lignin release in comparison was maximum at pH 9.0 (Fig. 4.4a). This was further corroborated by the decrease in Kappa content which followed a similar pattern. There was a marked decrease in Kappa content at pH 8.0 and 9.0 while it was 50% less at pH 10.0 and 40% at pH 7.0 of the maximal decrease repectively (Fig.4.4c). These results indicate that the enzyme is effective in hydrolyzing the pulp xylan at alkaline pH values.

There has been only a single report on enzymatic treatment of pulp with alkaline xylanases (Lundgren *et al.*, 1994). An alkaline xylanase from *Bacillus stearothermophilus* was used at 150 IU/g of pulp at pH 8.7 and 63°C resulting in a pulp brightness of 78% ISO without impairing pulp properties. An effective saving of 20% in the use of hydrogen peroxide was also achieved. More extensive studies on pulp treatment with alkaline xylanases active at high temperature are warranted and the present study appears to be promising for further development.

Effect of temperature on the pulp treatment

The pulp (10 g) was incubated with 12 IU of the enzyme for 150 min at pH 8.0 at 40 and 50°C respectively. The reduction in Kappa content and sugar release were comparable at 40 and 50°C respectively, while the 280 nm absorbing material release was approximately 50% at 50°C of that obtained at 40°C (Fig. 4.5a,b,c).

The kappa number decrease both in the case of pH and temperature experiments, at Stage II was markedly less as compared to the decrease obtained at Stage I, indicating that the enzymatic treatment has effectively contributed to the lignin reduction and further alkali treatment accounts for less removal of the lignin (Table 4.1).

Fig.4.4a: Effect of pH on chromophore and lignin release from the bagasse pulp

Fig.4.4b: Effect of pH on the sugar release

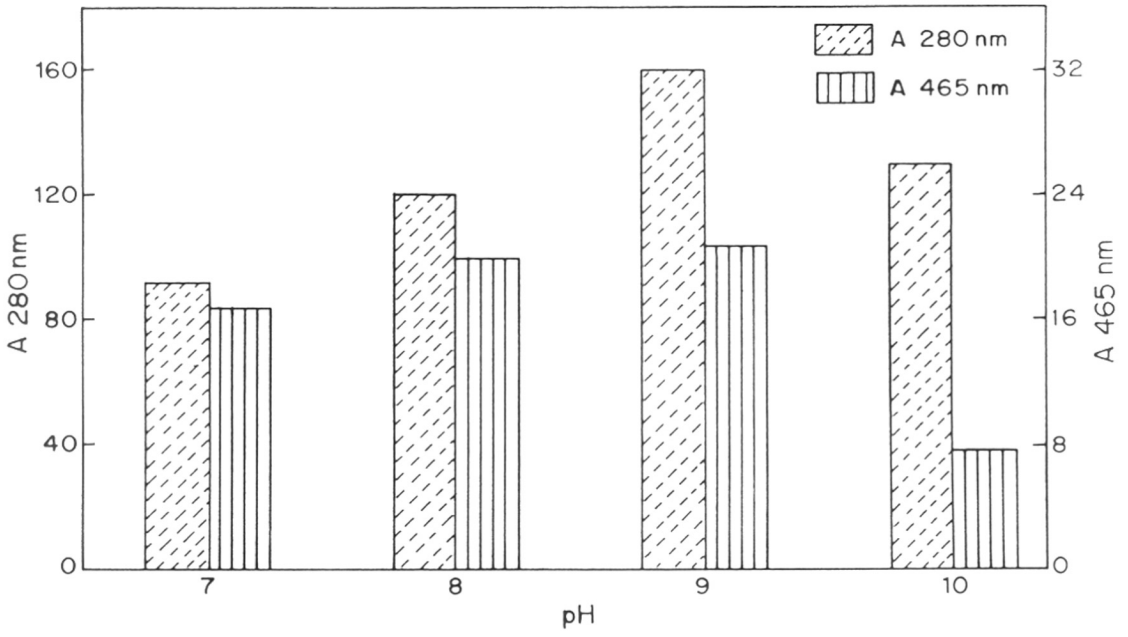


FIG. **4·4a**

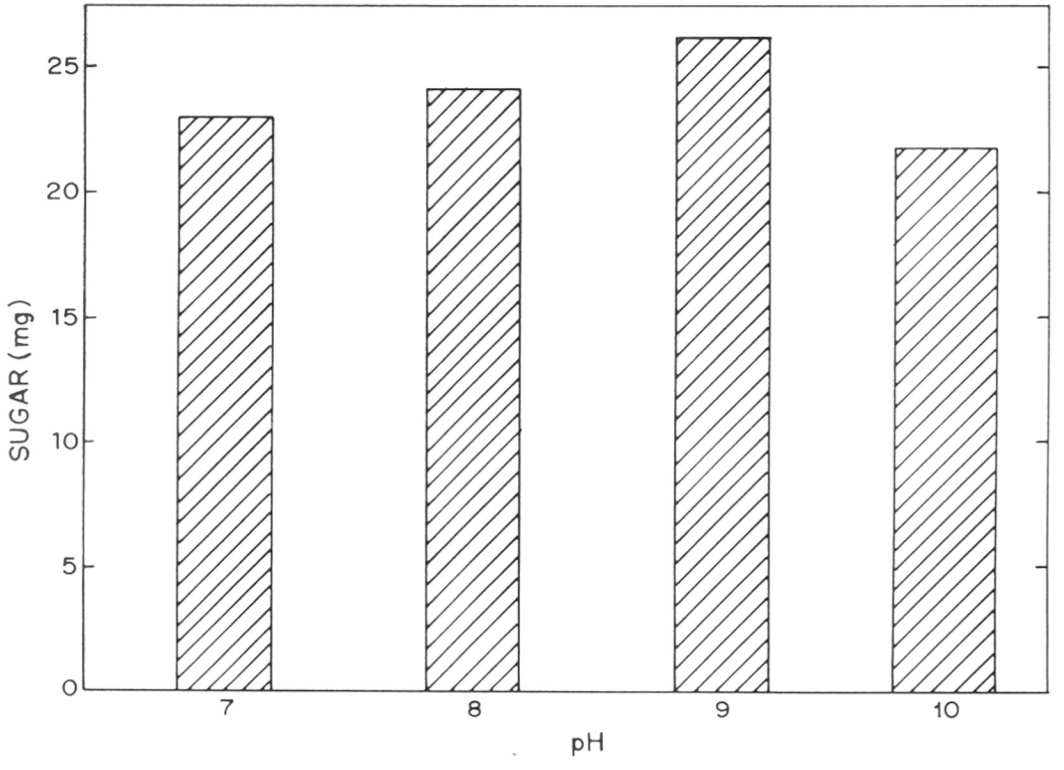


FIG. **4·4b**

Fig. 4.4c: Effect of pH on the Kappa number reduction

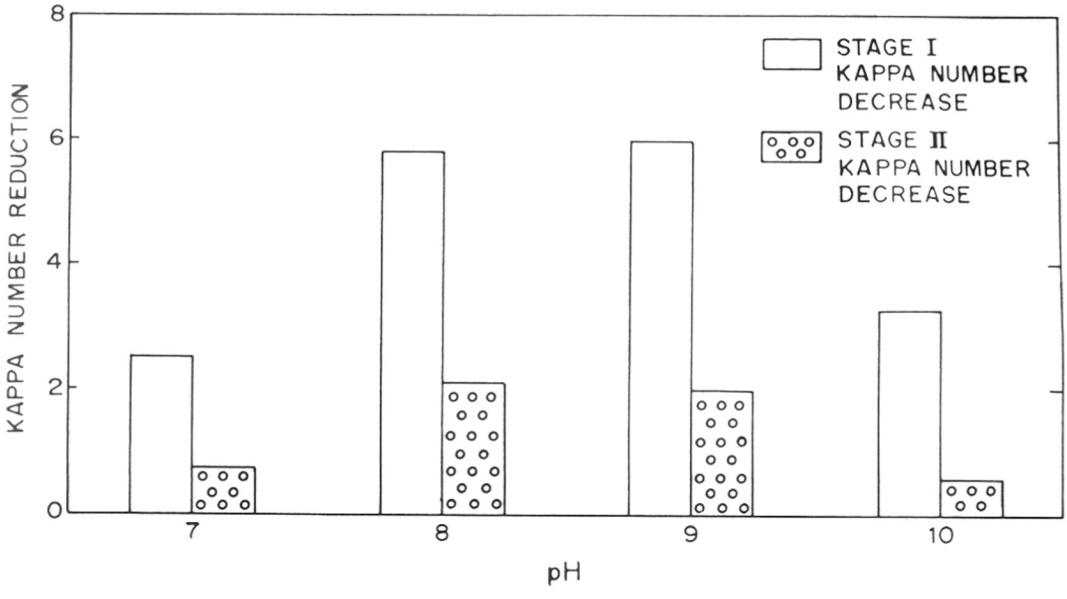

KAPPA NUMBER REDUCTION
8
6
4
2
0
7
8
9
10
pH
STAGE I
KAPPA NUMBER
DECREASE
STAGE II
KAPPA NUMBER
DECREASE

FIG. **4·4c**

Fig.4.5a: Effect of temperature on chromophore and lignin release from the bagasse pulp

Fig.4.5b: Effect of temperature on the sugar release

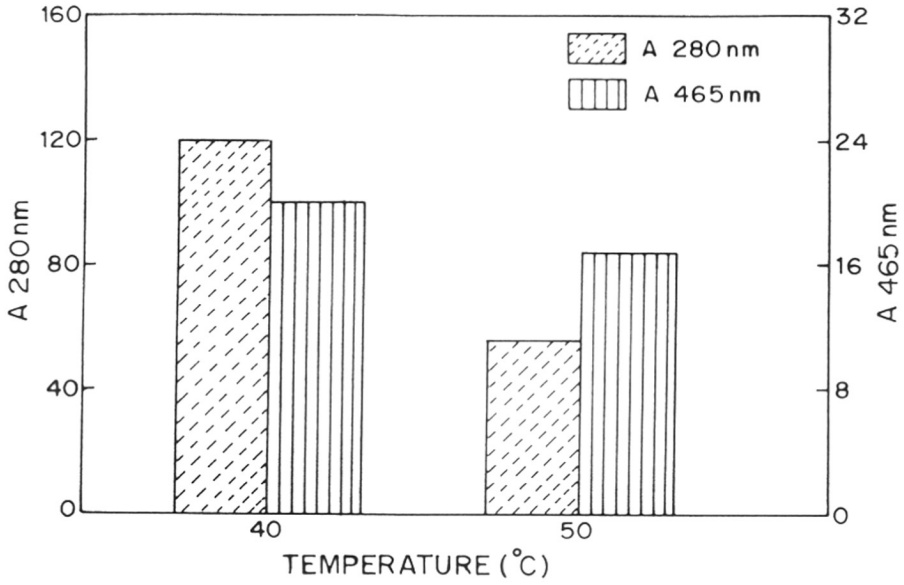


FIG. **4·5a**

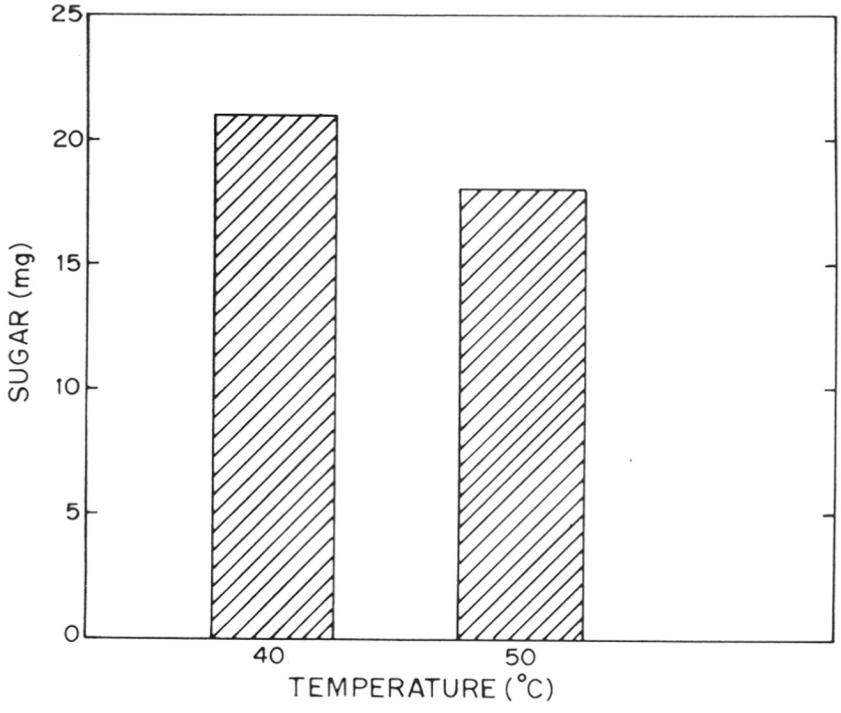


FIG. **4·5b**

Fig. 4.5c: Effect of temperature on the Kappa number reduction

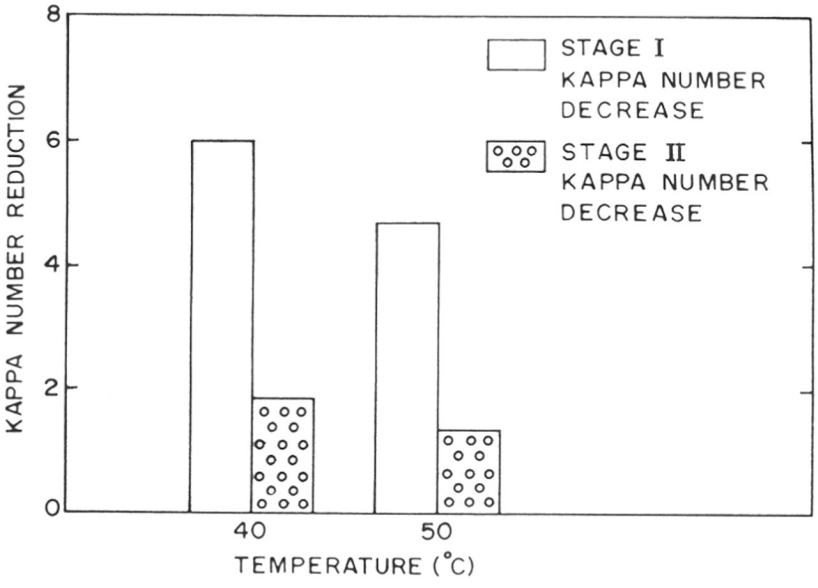

8
6
4
2
0
KAPPA NUMBER REDUCTION
STAGE I
KAPPA NUMBER
DECREASE
STAGE II
KAPPA NUMBER
DECREASE
40
50
TEMPERATURE (°C)

FIG. 4·5c

The optimized conditions thus arrived at for treatment of pulp at bench level are use of 1.2 IU/g of the enzyme per gram of pulp, residence time being 150 min and at temperature of 40°C to 50°C.

Effect of pulp impurities on *Cephalosporium* xylanase:

Senior *et al.* (1991) have shown marked inhibition of *Trichoderma harzianum* xylanase when incubated with commercial grade pulp. Similar results were obtained with xylanases from *S.commune*, *A. pullulans* and *B. circulans*. The authors have attributed the inhibition partly due to the inhibitors present in the pulp and adsorption of the enzyme on the pulp. However *A. niger* xylanase was unaffected when incubated with the pulp (Senior *et al.*,1991).

Hence, studies were carried out to find out as to whether the pulp impurities inhibited the enzyme or adsorbtion of the xylanase occurred on the pulp.

Table 4.2 Effect of pulp and pulp liqour on *Cephalosporium* Xylanase:

Parameters	Incubation time(min)	Activity IU
Control Enzyme	-	12
Enzyme + Pulp	150	12
Enzyme + Pulp liqour	30	11.2

On estimation of the enzyme activity at the end of 150 min, the *Cephalosporium* xylanase did not show any appreciable loss in activity when added to the pulp indicating that it was not inhibited by the impurities present in the pe pulp. This was further substantiated by adding the concentrated pulp liqour to the enzyme and assaying the enzyme in presence of the impurities. No loss in activity was observed (Table 4.2).

CONCLUSIONS

Preliminary experiments at the laboratory scale have shown that the *Cephalosporium* xylanase was effective in reducing Kappa content of bagasse pulp at alkaline pH values (pH 8.0 to 10.0).

A noteworthy feature of this enzyme is that substantial reduction in Kappa content at Stage I was obtained as compared to Stage II, indicating that extraction with sodium hydroxide after enzyme treatment could possibly be eliminated. The enzyme was not inhibited by the impurities present in the pulp and there was also no loss in the enzyme activity due to adsorbtion. Based on its properties and performance, the enzyme under study appears to hold promise and potential for developing a enzyme technology for application in pulp industry.

SALIENT FEATURES

The following are the salient features which have emerged out of the present studies from the alkalotolerant *Cephalosporium* sp. (NCL 87.11.9) ATCC No. : 74297

- Production of an extracellular cellulase-free xylanase from an alkalotolerant fungus.
- The first report of a fungal xylanase active at alkaline pH.
- Chemical modification studies of the purified enzyme indicated the involvement of a tryptophan, histidine and 2 carboxyl groups essential for activity
- The enzyme is effective in reducing kappa number of bagasse pulp at alkaline pH and thus appears to have a potential for application in pulp and paper industries.

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