# STUDIES ON ACTINOMYCETE XYLANASE

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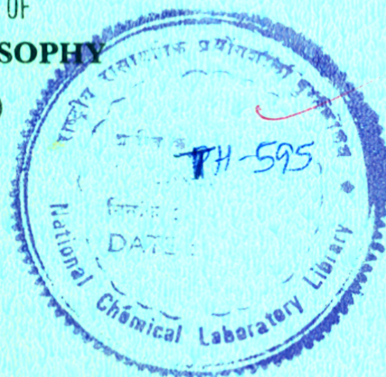


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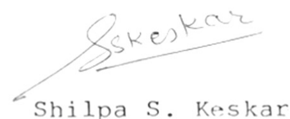


Pune, July, 1990.

Shilpa S. Keskar

# ABBREVIATIONS

| | |
|---|---|
| BrKG | 3-Bromo-2-ketoglutarate |
| BSA | Bovine serum albumin |
| CM-cellulose | Carboxymethyl cellulose |
| DAM | N-(2,4-dinitroanilino)-maleimide |
| DDPM | N-(dimethylamino 3,5-dinitrophenyl maleimide) |
| DEAE-cellulose | Diethylamino ethyl cellulose |
| DEP | Diethylpyrocarbonate |
| DFP | Di-isopropyl fluorophosphate |
| DNS | 3,5-Dinitrosalicylic acid |
| DTNB | 5,5'-Dithiobis-(2-nitrobenzoic acid) |
| EDTA | Ethylene diamine tetra-acetic acid |
| FDNB | 1-Fluoro-2,4-dinitrobenzene |
| HNBB | 2-Hydroxy-5-nitrobenzyl bromide |
| NBS | N-Bromosuccinimide |
| NEM | N-Ethylmaleimide |
| PAGE | Polyacrylamide gel electrophoresis |
| PCMB | *p*-Chloromercuricbenzoic acid |
| PHMB | *p*-Hydroxymercuricbenzoic acid |
| PMSF | Phenylmethylsulfonyl fluoride |
| PNPX | *p*-Nitrophenyl-β-D-xylopyranoside |
| SDS | Sodium dodecyl sulphate |
| TEMED | N,N,N'N'-Tetramethyl ethylene diamine |
| TNBS | 2,4,6-Trinitrobenzene sulfonic acid |
| TNM | Tetranitromethane |
| TPCK | Toleunesulfonylphenylalanine chloromethyl ketone |

# ABSTRACT

In the quest for renewable liquid fuels and chemical feedstocks, plant biomass represent an attractive resource for bioconversion of carbohydrates. Major emphasis has been given to the understanding of the cellulase enzyme systems of diverse microorganisms while relatively less is known about xylanases and their composition.

Xylan is a major component of the hemicellulose in the majority of tropical plant biomass and hence a study of xylanolytic enzymes can play an important role in developing meaningful bioconversion processes with technical as well as economic feasibilities. Xylanases (EC 3.2.1.8) catalyse the random hydrolysis of xylosidic bonds in xylan and related xylo-oligosaccharides. In view of their potential applications in paper and pulp industries in the preparation of dissolving pulps with low hemicellulose content, microbial xylanases have become increasingly the focus of attention in recent years.

Xylanases have been described from diverse microorganisms. Among the prokaryotes, the information on actinomycete xylanase is largely derived from *Streptomyces* sp. and bacterial xylanases have been particularly characterized from *Bacillus* sp. Stable enzymes active at high temperatures are favourable for increasing reaction rates and possibly also for reducing the contamination problems. Microbial xylanases which are not associated with cellulase activity are desirable since the enzyme applied in the crude state

would act on xylan without affecting cellulose pulp.

The present investigation relates to the study of a cellulase free xylanase from a thermotolerant Streptomyces strain $T_7$ and includes the following aspects:

1. Strain isolation through selective enrichment culture.
2. Optimization of fermentation parameters in shake flasks for high xylanase activity and productivity.
3. Purification and characterization of the xylanase.
4. Chemical modification of xylanase and isolation of a reactive cysteinyl peptide.

From humus rich soil collected at Nandesari (Near Baroda) an actinomycete strain was isolated by plating out soil sample on a medium containing wheat bran (5%) and yeast extract (1%). The isolate identified as Streptomyces $T_7$ grew in the temperature range of 37 - 50°C indicating that it is a thermotolerant strain. The production of xylanase was maximum (70 U/ml) at 50°C after 72 h of cultivation in a medium containing 5% wheat bran as a carbon source. Various parameters for xylanase production were studied. The results indicated that xylanase production was optimum at pH 7.0 and at 5% wheat bran concentration. Among the different nitrogen sources tested the strain utilized diammonium hydrogen phosphate, sodium nitrate and ammonium sulphate. The Streptomyces produced xylanase only in the presence of xylan or xylan containing natural materials, such as wheat bran, rice straw etc.

The xylanase was purified by ethanol precipitation, DEAE-cellulose ion exchange chromatography and gel filtration on Sephadex G-50. Streptomyces $T_7$ contained a single protein

component and was purified 41-fold compared to culture filtrate. The purified enzyme showed a single band on gel electrophoresis at pH 4.3.

The molecular weight of purified xylanase determined by SDS gel electrophoresis, by gel filtration on Sephadex G-50 and Bio-Gel P-10 was found to be approximately 20,500. The pH and temperature optima for xylanase activity were 4.5 - 5.5 and 60°C, respectively. The enzyme was highly stable and was found to retain total activity on incubation at pH 5.0 for 6 days at 50°C and after 11 days at 37°C. The $K_m$ and $V_{max}$ values as determined for soluble larch wood xylan were 10 mg/ml and $7.6 \times 10^3$ µmol/min/mg of enzyme, respectively. The isoelectric point was found to be 7.8. Enzyme was a glycoprotein and contained 33 mol of carbohydrate per mol of protein estimated using glucose as standard. Amino acid analysis indicated that the enzyme was rich in glutamic acid and histidine. It also contained substantial amounts of aspartic acid and glycine. DTNB and PHMB titrations showed the presence of 3 mol of -SH per mol of enzyme. Enzyme activity was completely inhibited by $Hg^{2+}$ ($2 \times 10^{-6}$M) and $Ag^+$ ($10 \times 10^{-6}$M). The enzyme degraded xylan producing xylobiose, xylo-oligosaccharides and a small amount of xylose as the end products, indicating that it is an endo-xylanase.

Effect of various chemical modifiers on xylanase activity was investigated. Complete inhibition by N-bromosuccinimide (NBS) (1 mM), 2-hydroxy-5-nitrobenzyl bromide (HNBB) (10 mM) and p-hydroxymercuribenzoate (PHMB) (1 mM) indicated that tryptophan and cysteine were modified. Plots of percentage residual activity as a function of time at various

concentrations of NBS, HNBB and PHMB indicated that the inactivation process exhibited pseudo-first-order kinetics with respect to time at any fixed concentration of the inhibitor. Kinetic analysis indicated that the loss of enzyme activity resulted from reaction of only one tryptophan or cysteine residue per molecule of enzyme. Titration with NBS indicated that for complete inactivation of enzyme 3.6 mol of NBS were required per mol of enzyme. The number of tryptophan residues oxidized per mol of enzyme were found to be 2.2. Protection against inactivation by substrate indicated the presence of these residues at a substrate binding site. Reactivation by cysteine supported the evidence that cysteine is involved in catalysis.

N-Ethylmaleimide is a potent modifier for cysteine. Substituted maleimides have proven useful in locating the active site peptide, since they introduce a chromogenic substituent in the protein. The coloured derivative N-(2,4-dinitroanilino)-malemide was synthesized and used for visualizing and separating the peptides containing cysteine after treatment of protein with the derivative and then digestion with pepsin.

When the mixture of peptides was resolved by high voltage electrophoresis followed by paper chromatography, three coloured peptides were obtained. To discover the position of the active peptide out of these three yellow peptides a similar experiment was carried out in the presence of xylan which protected the reactive cysteine and appeared colourless in the peptide mapping. The active site peptide was isolated and analyzed qualitatively for its amino acid

content. The peptide was composed of six amino acids: Aspartic acid; Glycine; Alanine; Tyrosine/valine; Isoleucine/ phenylalanine and DAM-cysteine.

The results based on studies on the xylanase has been published in Biochemical Journal, **261**, 49-55 (1989).

# CHAPTER 1

# GENERAL INTRODUCTION

# GENERAL INTRODUCTION

Hemicelluloses constitute a group of polysaccharides that are found widespread in association with cellulose in naturally occurring lignocellulosic plant materials to the extent of 40 - 45% [1]. It is estimated that 172 ± 74 x $10^9$ metric tonnes of gaseous carbon is fixed by plants every year through photosynthesis and almost half of it is present in the form of hemicellulose [2]. Energy requirements in the post-fossil fuel era are increasingly expected to be met through bioconversion of renewable carbohydrates including cellulose and hemicellulose [3]. Increased interest in the exploitation of plant biomass has provided an impetus for research on bioconversion of lignocellulose. Flickinger [4] has stated that pentoses derived from hemicelluloses could be an economical source of carbohydrates for conversion to liquid fuels through microbial fermentation. Sufficient attention has not been given so far for the commercial exploitation of hemicellulose in comparison with the other plant polysaccharides viz. cellulose and starch.

Crop residues such as cereal straws, sugarcane bagasse and forestry wastes etc. represent potential hemicellulose rich natural substrates which are available in abundance for bioconversion. Hemicellulose content of diverse plant biomass substrates varies with the source. Table 1.1 summarises the composition of some representative agricultural residues.

**Table 1.1:** Composition of agricultural residues [3,5]

| Type of material | Cellulose (%) | Hemicellulose (%) | Lignin (%) |
|---|---|---|---|
| Bagasse | 45 - 55 | 25 - 27 | 19 - 21 |
| Rice straw | 32 - 53 | 21 - 24 | 12 - 25 |
| Wheat straw | 35 - 40 | 20 - 25 | 15 - 20 |
| Rice husk | 35 - 45 | 25 - 28 | 15 - 20 |
| Jute stick | 41 - 58 | 19 - 22 | 21 - 25 |
| Corn stover | 35 - 40 | 10 - 20 | 15 - 20 |

The name hemicellulose was proposed by Schulze [6] in 1891 to designate those polysaccharides extractable from plants by aqueous alkali. Hemicelluloses are usually extracted from plant tissue after removal of lipid and lignin. Removal of lignin exposes the hemicellulose for easy dissolution in alkali and permits isolation of pure hemicellulose. Although some hemicelluloses are water soluble after isolation, they are generally not water extractable from plants prior to delignification and sometimes not until after the use of strong swelling agents such as alkali or methyl sulfoxide [7]. Various concentrations of alkali from 2 - 18% may be used but 10% is commonly used. When the extracts are neutralised, the fraction that precipitates is called hemicellulose A which is mainly composed of L-arabino-D-xylans with some D-glucuronic acid residues. Hemicellulose B usually contains higher proportion of uronic acid than hemicellulose A [8].

The hemicellulose component of biomass is composed of alkali soluble, linear branched heteropolymers of D-xylose, L-arabinose, D-mannose, D-glucose, D-galactose and D-glucuronic acid. The type of hemicelluloses are often classified according to the sugar residues present. Commonly occurring 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 [9]. L-Arabinans are often associated with the pectic materials but usually are considered to be hemicelluloses [10]. The heteroxylans constitute the major

hemicellulosic component of the Gramineae (grasses and cereals) and angiosperms (hard woods) while β-D-mannans are more abundant in gymnosperms (soft woods). Table 1.2 gives the composition of hemicelluloses in some of the crop residues.

Hemicelluloses from hard woods have an average degree of polymerisation of 150 - 200 while hemicelluloses from other sources have a degree of polymerisation in the range of 50 - 100 which is low enough to make molecular weight measurement difficult [12,13].

The major class of hemicellulose is xylans, which are found in large quantities in annual plants and deciduous trees and in smaller quantities in conifers. β-1-->4 xylans are mainly found in secondary walls as the major component of mature cell walls in woody tissues [14]. Xylan is the major hemicellulose in wood from angiosperms but is less abundant in wood from gymnosperms, it accounts for approximately 15 - 30% and 7 - 12% of the total weight, respectively. The xylan content of corn residues varies from about 17% in the leaves and stalks to 31% in the cobs, but on the average it comprises about 24% of total dry weight of corn stover [9]. Table 1.3 summarises the xylan content of some hard woods and soft woods.

## Extraction of xylan

Among the major hemicelluloses of wood only the hard wood xylans and the larch arabinogalactans can be isolated in representative yields without prior delignification. Direct extraction of hard woods with aqueous alkali makes

Table 1.2: Hemicellulosic carbohydrate content of some common crop residues [11]

| Plant residues | % of total sugars | | | |
|---|---|---|---|---|
| | Xylose | Arabinose | Glucose | Mannose and galactose |
| **Corn** | | | | |
| Cobs | 65.1 | 9.6 | 25.3 | - |
| Leaves | 59.0 | 9.4 | 29.1 | 2.5 |
| Stalks | 70.5 | 9.0 | 14.5 | 5.9 |
| Husks | 53.5 | 12.3 | 32.6 | 1.6 |
| Pith | 71.5 | 9.8 | 15.7 | 3.0 |
| Fibers | 63.8 | 6.6 | 26.8 | 2.8 |
| Wheat straw | 57.9 | 9.1 | 28.1 | 5.0 |
| **Soybean** | | | | |
| Stalks and leaves | 59.9 | 6.6 | 6.1 | 27.4 |
| Hulls | 26.6 | 12.7 | 21.0 | 39.7 |
| **Sunflower** | | | | |
| Stalks | 60.6 | 2.2 | 32.6 | 4.6 |
| Pith | 10.7 | 11.8 | 63.5 | 14.0 |
| Flax straw | 64.6 | 12.8 | 1.2 | 21.4 |
| Sweet clover hays | 49.3 | 21.9 | 8.9 | 9.9 |
| Peanut hulls | 46.3 | 5.0 | 46.6 | 2.1 |
| Sugarcane bagasse | 59.5 | 14.5 | 26.0 | - |

Table 1.3: The carbohydrate composition of representative woods [3,15]

| Wood species | Composition (% of extractive free wood) | | |
|---|---|---|---|
| | Glucan | Xylan | Arabinan |
| **Hard wood** | | | |
| Beech | 47.5 | 17.5 | 0.5 |
| White birch | 44.7 | 24.6 | 0.5 |
| Yellow birch | 46.7 | 20.1 | 0.6 |
| Red maple | 46.6 | 17.3 | 0.5 |
| Sweet gum | 39.4 | 17.5 | 0.3 |
| Mesta | 35.0 | 15.0 | ND |
| Subabul | 30.0 | 20.0 | ND |
| **Soft wood** | | | |
| Balsam fir | 46.8 | 4.8 | 0.5 |
| Eastern hemlock | 45.3 | 4.0 | 0.6 |
| Jack pine | 45.6 | 7.1 | 1.4 |
| White pine | 44.5 | 6.3 | 1.2 |
| White spruce | 46.5 | 6.8 | 1.6 |
| Douglas fir | 43.5 | 2.8 | 2.7 |

ND - Not determined.

it possible to obtain a de-O-acetylated xylan in yields as high as 80 - 95%. This is a great advantage since all delignifying agents cause more or less severe chemical and physical modifications of wood polysaccharides and also often lead to losses of carbohydrate material. Wood from gymnosperms, unlike that from angiosperms, remains practically unchanged on direct treatment with alkali. The reason for this difference is attributable to the lower lignin content of hard woods [16,17]. A highly delignified pulp, the so called holocellulose was used for xylan isolation [9,18,19]. Numerous methods were suggested for the isolation of wood holocellulose involving the use of chlorine [20], chlorine dioxide [21] and peroxyacetic acid [22]. Holocelluloses can also be extracted with dimethyl sulfoxide [23]. Various concentrations of alkali from 2 - 18% may be used but 10% is usual. For exhaustive extraction 24% KOH was used [9]. The best procedure for isolating xylan from soft woods involves the use of barium hydroxide. When barium hydroxide is added to an aqueous or alkaline solutions of hemicellulose mixture the D-mannose containing polymers form a complex and then are precipitated [24].

## Properties of xylan

Xylans are characterised by the following properties:

1. Insolubility in water.
2. Solubility in 10% alkaline solutions.
3. Alkaline solutions of xylans show highly negative optical rotation.
4. Do not reduce Fehling's solution.

## Structural heterogeneity of xylans

Isolated β-1→4 xylans are generally polydispersed (i.e. varying degree of polymerization) and highly branched (with more than one type of substituent) heteropolymers. The prefix "hetero" denotes the presence of sugars other than D-xylose. The frequency and composition of branches in isolated xylans are dependent on their source [25-27] and the method of their isolation [9,14,18,28]. Homoxylans, which consist of xylosyl residues exclusively have been isolated from esparto grass [9] and tobacco [18]. The acetylated xylan of hard woods and the arabinoxylan of soft woods are the two major forms of xylan in wood [14]. Some major structural features are summarized in Fig. 1.1.

**Backbone of xylan:** The main chain of xylan is composed of β-(1→4) linked D-xylopyranose units. The presence of β-(1→4) linkage between two adjacent D-xylose residues has been well established [9,29,30]. The β-linkage is confirmed by indication of high levo rotation $(\alpha)^{20}_{D}$ ranging from -78.2° - -109.5° of alkaline solutions of xylans [9]. Periodate oxidation confirms the presence of a β-(1→4) linkage in xylan [29].

**Nature of side chains:** The first investigation allowing the assignment of a complete structure of wood xylan was carried out by Aspinall and coworkers [31].

<u>Hard wood:</u> Xylans of several hard woods (angiosperms) are acetylated. For example, xylan from Silver birch contains more than 1 mol of acetic acid per 2 mol of D-xylose. Acetylation occurs more frequently at the O-3 than the O-2 position. The presence of acetyl groups makes the xylan signifi-

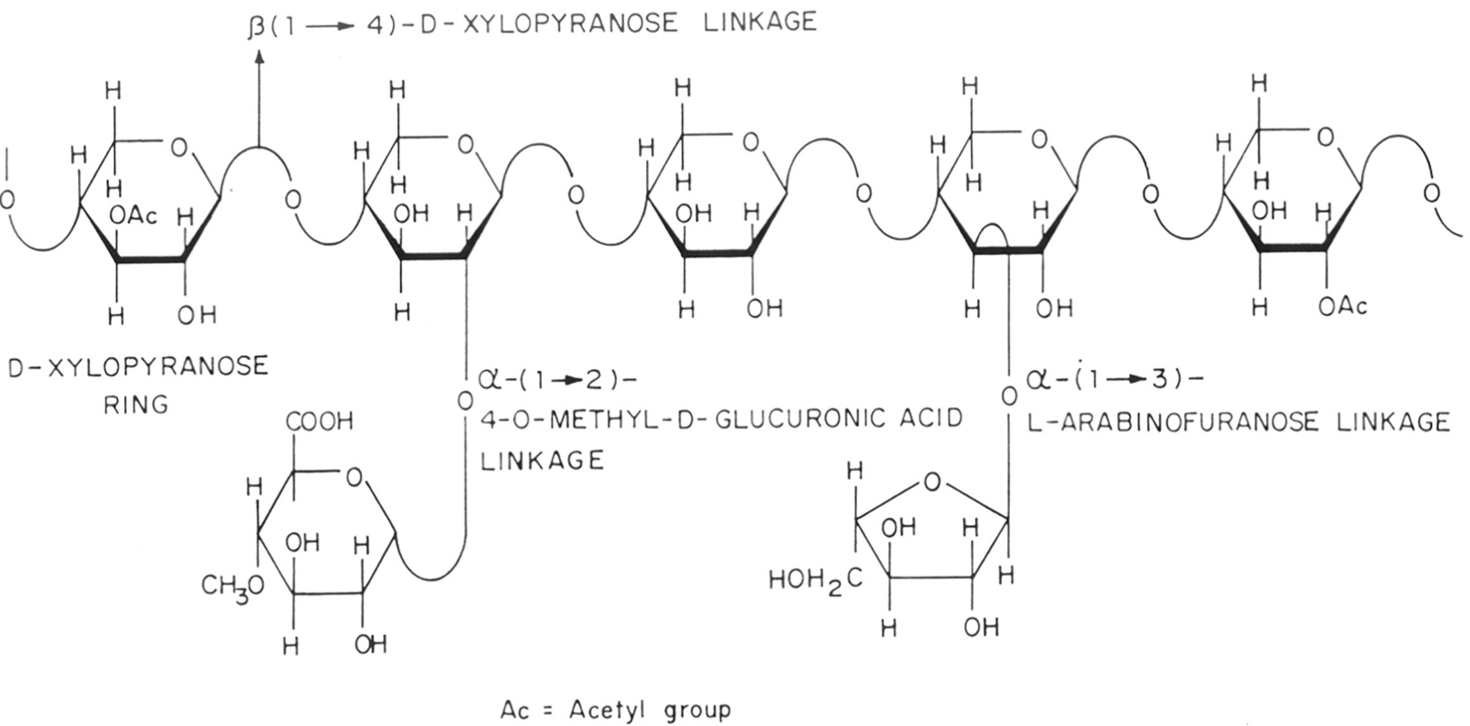



FIG. 1·1 : STRUCTURE OF XYLAN

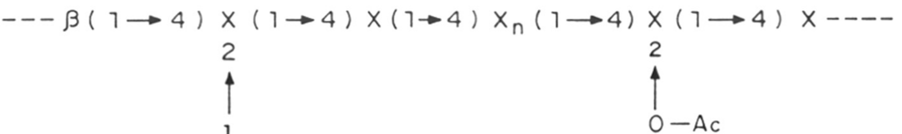
cantly more soluble in water. The polysaccharide is composed of at least 70 (1--> 4) linked β-D-xylopyranose residues with approximately every tenth D-xylose residue carrying through (position 2) single 4-O-methyl-D-glucuronic acid residues attached as side chains [32]. Xylans in hard woods are apparently devoid of arabinose groups [16] (Fig. 1.1A). The glucomannan occuring in the wood of angiosperms is less studied and is present in 3 - 5%. Hard wood glucomannans do not contain any galactose residues.

<u>Soft wood:</u> The xylans in wood of gymnosperms e.g. Western hemlock has the fundamental repeating unit of the 13 β-(1--> 4 ) linked B-D-xylopyranose residues bearing 3 (1--> 2) linked 4-O-methyl-α-D-glucuronic acid residues and 1-(1-> 3) linked L-arabinofuranose residue [32]. Five to six D-xylose residues being present per acid group in the soft wood compared to ten in hard wood. One L-arabinose residue occurring per 7 - 12 D-xylose residues [17] (Fig. 1.1B).

Woods of gymnosperms contain glucomannan. Some of the D-glucose and D-mannose residues carry (1--> 6) linked terminal side chains of α-D-galactopyranose residues. So they are known as galactoglucomannans.

<u>Gramineae:</u> Xylans from cereals and grasses are characterised by the presence of L-arabinofuranose residues linked to the backbone as single unit side chain usually at position 3 of xylose. But in many cases, D-glucuronic or 4-O-methyl-D-glucuronic acid residue or both are also present in smaller proportion [30]. Polysaccharides of greater complexity are found among the hemicelluloses of corncobs, maize fiber maize hulls, wheat bran and barley husks. These xylans

NATURE OF SIDE CHAINS IN REPRESENTATIVE WOOD XYLANS



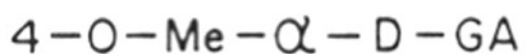
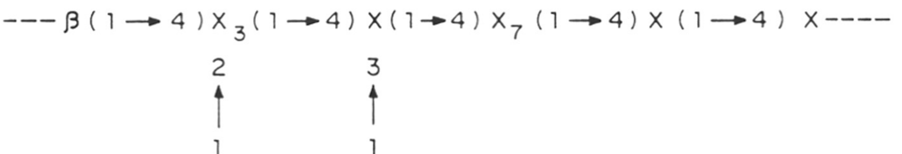




FIG. 1·1 (A) HARD WOOD XYLAN, SILVER BIRCH



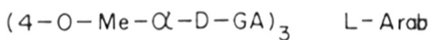




FIG. 1·1 (B) SOFT WOOD XYLAN, WESTERN HEMLOCK

Me — Methyl, GA — Glucuronic Acid, Arab — Arabinose

contain non-terminal L-arabinose residues linked through various positions [33,34].

## Xylanases

Hemicellulose degrading enzymes, referred to as hemicellulases are termed as glycan hydrolases (E.C. 3.2.1). They are also defined and classified according to their substrates on which they act.

L-Arabinanases are capable of hydrolysing only L-arabinans, D-galactanses break down D-galactans and L-arabino-D-galactans. D-mannanases hydrolyse the β-(1-->4)-D-mannopyranosyl linkages of D-mannans and D-xylanases cleave the β-(1--> 4)-D-xylopyranosyl linkages of (1--> 4)-β-D-xylans namely arabinoxylan, arabinoglucuronoxylan, arabino-4-O-methyl-D-glucuronoxylan and glucuronoxylan [35].

## Classification of xylanases

Three different kinds of xylanases are involved in xylan degradation.

1. Endo-β-(1--> 4)-D-xylanase: β-(1--> 4)-D-xylan xylano hydrolase (E.C.3.2.1.8). These enzymes act randomly on xylan to produce large amounts of xylo-oligosaccharides of various chain lengths.
2. Exo-β-(1--> 4)-D-xylanase: β-(1-->4)-D-xylan xylohydrolase, (E.C. 3.2.1.37). These enzymes remove single D-xylose units from the non-reducing end of the xylan chain. These enzymes invert the product configuration.
3. β-xylosidase or xylobiase: These enzymes (E.C. 3.2.1.37) hydrolyse disaccharides like xylobiose and the higher

xylo-oligosaccharides. These enzymes show high transferase activity. They retain the product configuration. Reilly [36] has given an overview of xylanases and categorised them according to their mode of action. An important purpose of such classification is the identification of significant functional contribution by each class of enzymes to the overall hydrolytic system.

Endo-xylanases are further classified into four types:

a. Non-arabinose liberating endo-xylanases which cannot act on L-arabinosyl initiated branch points at β-(1-->4) linkage and produce only xylobiose and xylose as the major end products. These enzymes can break down xylo-oligosaccharides as small as xylobiose.

b. Non-arabinose liberating endo-xylanases which cannot cleave branch points at α-(1-->2) and α-(1-->3) and produce mainly xylo-oligosaccharides larger than xylobiose. These endo-xylanases have no action on xylotriose and xylobiose.

c. Arabinose liberating endo-xylanases which can cleave xylan chain at branch points and produce mainly xylobiose, xylose and arabinose.

d. Arabinose liberating endo-xylanases which can hydrolyse the branch points and produce intermediate size xylo-oligosaccharides and arabinose.

The D-xylanases produced by bacteria and actinomycetes have been shown to be mainly of the endo-type. Fungal xylanases are also of the endo-type but an occasional report has indicated the possibility of exo-type xylanases being present in some of the strains. However, no conclusive

proof has as yet been given for the existence of an exo-xylanase nor has such a xylanase been purified or characterized from any source [36]. Exo-xylanases differ from the β-xylosidases in their ability to attack xylan and long chain xylo-oligosaccharides and their lack of transferase activity.

Relatively little is known about the enzymes which liberate the side chain sugars, α-L-arabinofuranosidase and α-glucuronidase or the enzymes involved in the degradation of acetyl xylans [37]. L-arabinofuranosidase was studied mainly as a component of systems degrading L-arabinans [38]. There are claims that L-arabinofuranosidase activity is an inherent property of some xylanases [35]. α-Glucuronidase was detected in a cellulase preparation of *Trichoderma reesei* [39]. Esterases that deacetylate acetyl xylan occur in fungal cellulolytic systems [40]. The esterases act synergistically with xylanases in the degradation of acetyl xylan [41]. Liberation of acetic acid creates new substrate sites on the polysaccharide backbone for xylanases.

## Occurrence of D-xylanases

D-xylanases are wide spread in nature. Xylanases have been reported from prokaryotes as well as from eukaryotes [35]. They have been reported to occur in bacteria from marine and terrestrial environments, fungi (saprophytes, phytopathogens and mycorrhiza), rumen bacteria and protozoa, ruminant caecal bacteria, insects, snails, crustaceans, marine algae and germinating seeds of terrestrial plants [36]. Most of the bacteria, actinomycetes and fungi show extracellular xylanase secretion. Exceptional intracellular

xylanases have been reported in rumen bacteria and protozoa [42,43].

Structural carbohydrates of plant cells are an important source of energy to ruminant animals. Hemicelluloses, constituting upto 40% of the dietary intake of the ruminants are degraded by xylanases produced by rumen microflora. Williams and Withers [44] have indicated that most of the hemicellulolytic strains in the rumen belong to the genera _Eubacterium_, _Ruminococcus_ or _Bacteroides_. The bacterium _Bacteroides succinogenes_ is the major cellulolytic bacterium in the rumen of cattle which are straw fed [45]. In invertebrate crustaceans _Astacus fluviatilis_ and _Homarus vulgaris_, the hepatopancreas gland has been shown to be a source of D-xylanase in their digestive juices [46]. D-xylanases have also been detected in the digestive juice of the snail [47].

Disruption of plant cell walls is evident during invasion of the plant by pathogenic microorganisms. Anderson [48] has studied the growth of two pathogenic fungi, _Colletotrichum lindemuthianum_ and _Helminthosporium maydis_ which causes blight on corn. Xylanases are responsible for the degradation of the cell wall materials in bean and corn, the products of which are utilised by the pathogens. _Colletotrichum lagenarium_ has been shown to produce xylanase when grown on cell walls of muskmelon [49].

The aleurone layer of cereal grain crops has been shown to consist almost entirely of arabinoxylan (85%) with lesser amount of cellulose (8%) and protein (6%). Xylanases are involved in the degradation of arabinoxylan during germina-

tion of seeds [50].

There are fewer studies on the bacterial xylanases in comparison with xylanases of fungal origin. Among prokaryotes the xylanase production has been reported from *Bacillus* sp. [51-62], *Clostridium* sp. [63-67] and *Cellulomonas* sp. [68-70]. High proportions of xylanolytic strains have been identified in populations of actinomycetes from natural substrates. *Streptomyces* sp. [71-85], *Micromonospora* sp. [78], *Nocardia* [77], *Thermomonospora* [86-88] and *Thermoactinomyces* sp. [85] are some of the reported species for xylanase production. In the case of fungi, xylanases from *Cryptococcus albidus* [89], *Schizophyllum commune* [90], *Trichoderma* sp. [91,92] and *Aspergillus niger* [93,94] are well studied. Recently, the xylanases from thermophilic organisms have become the subject of intensive research. Included in these investigations are bacteria (bacilli and clostridia) and a few actinomycetes. Table 1.4 gives a summary of good producers of xylanases reported from representative prokaryotes as well as thermophilic eukaryotes.

Table 1.4: Xylanases from representative prokaryotes and thermophilic eukaryotes

| Organism | Strain No. | Xylanase U/ml | Reference |
|---|---|---|---|
| **Bacteria** | | | |
| *Bacillus circulans* | WL-12 | 121.6 | 52 |
| *Bacillus* sp* | W1-W4 | 34 - 112 | 61 |
| *Clostridium stercorarium** | | 56.0 | 64 |
| *Clostridium thermolacticum** | DSM 2911 | 26.0 | 65,66 |
| **Actinomycetes** | | | |
| *Streptomyces exfoliatus* | MC 1 | 30.2 | 71 |
| *Streptomyces flavogriseus* | | 46.0 | 72 |
| *Streptomyces lividans** | 1326 | 50.0 | 73 |
| *Streptomyces olivochromogenes* | | 30.7 | 79 |
| *Streptomyces afghaniensis** | | 15.7 | 80 |
| *Streptomyces cyaneus* | | 36.6 | 85 |
| *Streptomyces* sp. | EC 1 | 12.0 | 87 |
| | EC 22 | 9.0 | 87 |
| | 3137 | 35.0 | 83 |
| | VP 5 | 12.0 | 84 |
| *Chainia* sp. | NCL 82-5-1 | 28.0 | 95 |
| *Thermomonospora curvata** | MT 815 | 8.8 | 86 |
| *Actinomadura* sp. | MT 809 | 58.8 | 85 |
| **Fungi*** | | | |
| *Melanocarpus albomyces* | II S | 12.0 | 96 |
| *Thermoascus aurantiacus* | C 436 | 576.0 | 97 |
| *Thielavia terrestris* | ATCC 26917 | 18.8 | 98 |
| *Talaromyces byssochlamydoides* | YH 50 | 5.75 | 99 |

* Thermotolerant or thermophilic organism.

## SCOPE OF THE WORK

Biomass utilization for producing fuel and chemical feed stocks is a major challenge which the world has to face and if possible, work out a satisfactory solution by the turn of the century when fossil fuels would be on the brink of depletion. This obviously necessiates development of new and economically viable processes that will allow utilization of the principal carbohydrate constituents of lignocellulosic materials viz. cellulose and hemicellulose which are the most abundant renewable substrates available for bioconversion [100]. Extensive research has been undertaken on the microbial conversion of cellulose to sugars and/or liquid fuels. Less emphasis has been given to the bioconversion of hemicellulose, principally xylans which are also major constituents of plant biomass. At present, the production of liquid fuels from cellulosic materials has not been shown to be economically feasible on an industrial scale. The successful utilization of hemicellulose in addition to cellulose for bioconversion may contribute positively towards making the biomass fuel programmes economically more attractive [101]. Therefore, the fermentation of pentose sugars and more specifically D-xylose will facilitate the exploitation of plant biomass for the production of ethanol.

Microorganisms matabolising xylan as a carbon source and the enzyme system they employ for its breakdown, would then become important tools in biomass conversion processes for renewable fuels and chemical feed stocks [102]. Biological conversion processes are preferred to chemical con -

version processes since the reactions proceed at moderate temperatures and high yields of products are obtained due to specific reactions. Chemical conversions on the other hand, are often non-specific and also require expensive corrosion proof equipment [103].

Interest in studies of xylanases has increased in recent years because of the possibility of using them for the production of xylose, which can be used as a fermentation substrate. Apart from this, xylanases possess a variety of applications in other areas also.

Xylanases are widely distributed in nature. They are reported from prokaryotes as well as eukaryotes[35]. Bacteria and actinomycetes among prokaryotes are rich in xylan degrading enzymes and have received considerable attention for developing technologies for xylanase production as well as characterization for better understanding of their xylan degrading enzymes. Besides, conventional techniques for strain improvement these are also more amenable to genetic manipulation through gene cloning and similar modern methods. Actinomycetes form an important part of the microbial community responsible for nutrient recycling in natural substrates and several of them occurring in association with compost, manure etc. are capable of bringing about rapid degradation of natural substrates including cellulose and xylan. However, intensive studies on actinomycetes and their associated hydrolytic enzymes are relatively few [104].

Composts are the most likely environments where lignocellulose degradation by actinomycetes could supercede the contributions from fungi. Both the mesophilic and thermo-

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philic growth phases of composting involve the activities of diverse actinomycetes [105]. Thermophilic actinomycetes form a major component of composts prepared for mushroom cultivation [106]. Thermophiles are potentially good sources of enzymes stable to heat and chemical inactivation. Enzymes from thermophilic organisms are more thermostable than their mesophilic counterparts [107]. However, several mesophiles have also been known to produce highly thermostable enzymes [108]. Stable enzymes acting at elevated temperatures are desirable for increasing the reaction rates and minimising the contamination problems [56].

## Applications of xylanases

Xylanases have attracted attention in recent years for their potential application in paper industry [109-111]. Xylanases may yield a range of desirable pulp characteristics by selectively hydrolysing xylan components in the manufacture of low-hemicellulose dissolving pulps, rayon grade pulps etc. [112]. Xylanases can also be used for preparing other lignocellulosic fibers (e.g. retting of flax fibers). Total removal of xylan from fiber is not necessarily desirable because xylan contributes significantly to fiber strength [113]. In the pulping industry dissolving pulp is manufactured from rayon and chemical derivatives. This pulp must be free of hemicellulose and because the sulfite and kraft pulps used for this dissolving grade contain hemicellulose, it is capable of being removed by alkaline extraction [114]. A biochemical alternative involves treating the pulp with

xylanase [115]. The effect is even more pronounced with pulps of higher pentosan content [116]. However, the xylanase preparation is often associated with cellulase resulting in a viscosity drop due to cellulose hydrolysis. So a purer xylanase free of cellulase is preferable, and search for cellulase free xylanase is currently on introducing specific gene by genetic engineering methods [114].

In addition to permitting the introduction of novel genes, cloning techniques would enable amplification of the expression genes already present. For instance, the production of xylanase in Bacillus subtilis was enhanced successfully using a plasmid vector carrying the Bacillus pumilus gene [117]. Promising results include the production of extracellular xylanases by E. coli expressing cloned xylanase genes from Aeromonas sp. 212 [118] and from Bacillus sp. C-125 [119]. Xylanase is also produced by a cellulase negative and xylanase negative double mutant of Streptomyces lividans carrying a xylanase gene cloned from S. lividans 1326 [120].

Xylose, the major product of xylan degradation has many potential applications. The fairly recent discovery that Pachysolen tannophilus can ferment xylose sparked interest in the direct route of xylose fermentation [121-124]. Several bacteria can convert xylose to potentially useful products e.g. Klebsiella pneumoniae can convert all the sugars contained in hemicellulose and cellulose hydrolysate to 2,3-butanediol which is useful in rubber industry [125]. Clostridia sp. are capable of producing acetone, butanol and isopropanol from pentoses [126]. Xylose can also be

used as a substrate to single cell protein, a possible animal feed material, but commercially viable technologies have not emerged [100]. The by-product of pentose utilization is xylitol which can be used as a sweetening agent suitable for diabetes and also in chewing gums [127]. Acetobacter sp. are reported to oxidise xylose to xylonic acid directly [128]. The bioconversion of xylan to triglycerides (lipids) by xylan degrading organisms should not be overlooked. This offers an attractive alternate fuel source [129].

Other identified applications include: Xylanases are used in liquefying coffee mucilage for the manufacture of liquid coffee [130]. They are also used in the isolation of protoplasts from potato for the hybridization studies [131]. The xylanase of Streptomyces E-86 is used to produce xylobiose from commercially available xylan [132]. Xylanases can also be used for the preparation of lower (U-$C^{14}$) labelled (1--> 4)-β-D-xylo-oligosaccharides [133]. In addition, they are used in clarification of juices, the production of fluids and juices from plant materials and the preparation of de-xtrans for use as food thickeners [37,134].

Development of enzyme technology essentially involves the following steps:

1. Screening of microbial cultures to identify potential strains with high activity and productivity.
2. Improving yields of enzymes through strain improvement and/or process optimisation. This can also be inclusive of molecular methods and recombinant DNA technology.
3. Evaluation of the basic properties of the enzyme including identification of unique features to suit specific

applications. This may include characters such as high temperature and pH stability which may be desirable under certain conditions for industrial applications.

## Present Investigation

The present investigation relates to the study of a cellulase free xylanase from a thermotolerant Streptomyces strain $T_7$ and includes the following aspects:

1. Strain isolation through selective enrichment culture.
2. Optimization of fermentation parameters in shake flasks for high xylanase activity and productivity.
3. Purification and characterization of xylanase.
4. Chemical modification of xylanase and identification of essential amino acid residues at the active site.
5. Isolation of a reactive cysteinyl peptide.

## REFERENCES

1. Cowling, E.B. and Kirk, T.K. (1976) Biotechnol. Bioeng. Symp. **6**, 95.
2. Brink, D.L. (1976) Appl. Polymer. Symp. **28**, 1377.
3. Magee, R.J. and Kosaric, N. (1985) Adv. Biochem. Eng. **32**, 61.
4. Flickinger, M.C. (1980) Biotechnol. Bioeng. **22** Suppl. 1, p. 27.
5. Kosaric, N., Duvnjak, Z. and Stewart, G.G. (1981) Adv. Biochem. Eng. **20**, 119.
6. Schulze, E. (1891) Ber. **24**, 2277.
7. Rogers, S.C., Mitchell, R.L. and Ritter, G.J. (1947) Anal. Chem. **19**, 1029.
8. Whistler, R.L. and Bemiller, J.N. (1958) Adv. Carbohydr. Chem. **13**, 289.
9. Whistler, R.L. and Richards, E.L. (1970) In "The Carbohydrates, Chemistry and Biochemistry" (Eds. Pigman, W. and Horton, D.) Academic Press, N.Y. Vol. IIA, p.447.
10. Dannelly, B.J., Helm, J.L. and Lee, H.A. (1973) Cereal Chem. **50**, 548.
11. Gong, C.S., Chen, L.F., Flickinger, M.C. and Tsao, G.T. (1981) Adv. Biochem. Eng. **20**, 93.
12. Greenwood, C.T. (1952) Adv. Carbohydr. Chem. **7**, 289.
13. Greenwood, C.T. (1956) Adv. Carbohydr. Chem. **11**, 335.
14. Timell, T.E. (1967) Wood Sci. Technol. **1**, 45.
15. Lachke, A.H., Srinivasan, M.C., Deshmukh, S.S. and Deshpande, M.V. (1987) Biotechnol. Lett. **9**, 147.
16. Timell, T.E. (1964) Adv. Carbohydr. Chem. **19**, 247.

17. Timell, T.E. (1965) Adv. Carbohydr. Chem. **20**, 410.

18. Wilke, K.C.B. (1979) Adv. Carbohydr. Chem. Biochem. **36**, 215.

19. Timell, T.E. (1965) Methods Carbohydr. Chem. **5**, 135.

20. Kurth, E.T. and Ritter, G.J. (1934) J. Am. Chem. Soc. **56**, 2720.

21. Schmidt, E. and Groumann, E. (1921) Ber. **54**. 1860.

22. Sarkanen, K.V. (1962) Pure Appl. Chem. **5**, 219.

23. Timell, T.E. (1961) Tappi. **48**, 88.

24. Meier, H. (1958) Acta Chem. Scand. **12**, 144.

25. Fincher, G.B. and Stone, B.A. (1981) Encycl. Plant Physiol. New Ser. **13B**, 68.

26. Brice, R.E. and Morrison, I.M. (1982) Carbohydr. Res. **101**, 93.

27. Gorbocheva, I.V. and Rodionova, N.A. (1977) Biochim. Biophys. Acta **484**, 94.

28. Ford, C.W. (1986) Carbohydr. Res. **147**, 101.

29. Whistler, R.L. (1950) Adv. Carbohydr. Chem. **5**, 269.

30. Aspinall, G.O. (1959) Adv. Carbohydr. Chem. **14**, 429.

31. Aspinall, G.O., Hirst, E.L. and Mahomed, R.S. (1954) J. Chem. Soc. 1734.

32. Woodward, J. (1984) In "Topics in Enzyme and Fermentation Biotechnology" (Ed. Wiseman, A.) John Wiley & Sons, N.Y. Vol. 8, p.9.

33. Ehrenthal, I., Montgomery, R. and Smith, F. (1954) J. Am. Chem. Soc. **76**, 5509.

34. Schmorak, J., Bishop, C.T. and Adams, G.A. (1957) Can. J. Chem. **35**, 108.

35. Dekker, R.F.H. and Richard, G.N. (1976) Adv. Carbohydr. Chem. **32**, 277.

36. Reilly, P.J. (1981) In "Trends in the Biology of Fermentations for Fuels and Chemicals" (Ed. Hollaender, A.) Plenum Press, N.Y. Vol. 18, p. 111.

37. Biely, P. (1985) Trends Biotechnol. **3**, 286.

38. Kaji, A. (1984) Adv. Carbohydr. Chem. Biochem. **42**, 384.

39. Dekker, R.F.H. (1983) Biotechnol. Bioeng. **25**, 1127.

40. Biely, P., Puls, J. and Schneider, H. (1985) FEBS Lett. **186**, 80.

41. Johnson, K.G., Mackenzie, C.R. and Fontana, T.O. (1980) Methods Enzymol. **68**, 551.

42. Kistnerand, A. and Ciouws, L. (1964) J. Gen. Microbiol. **34**, 447.

43. Biely, R.W. and Clarke, R.I.J. (1963) Nature, **198**, 787.

44. Williams, A.G. and Withers, S.E. (1981) J. Appl. Bacteriol. **51**, 375.

45. Forsberg, C.W., Beveridge, T.J. and Hellstrom, A. (1981) Appl. Environ. Microbiol. **42**, 886.

46. Kooiman, P. (1964) J. Cellular Comp. Physiol. **63**, 197.

47. Mayers, F.L. and Northcote, D.H. (1958) Biochem. J. **69**, 54P.

48. Anderson, A.J. (1978) Phytopathol. **68**, 1585.

49. Doux-Gayat, A., Auriol, P., Joseleau, J.P. and Touze, A. (1978) Physiol. Plant **42**, 301.

50. Taiz, L. and Honigman, W.A. (1976) Plant Physiol. **58**, 380.

51. Fukui, S. (1958) J. Gen. Appl. Microbiol. **4**, 39.

52. Esteban, R., Villanueva, J.R. and Willa, T.G. (1982) Can. J. Microbiol. **28**, 733.

53. Esteban, R. Chordi, A. and Villa, T.G. (1983) FEMS Microbiol. Lett. **17**, 163.

54. Bernier, R.Jr., Desrochers, M., Jurasek, L. and Paice, M.G. (1983) Appl. Environ. Microbiol. **46**, 511.

55. Simpson, F.J. (1956) Can. J. Microbiol. **2**, 28.

56. Gruninger, H. and Fiechter, A. (1986) Enz. Microb. Technol. **8**, 309.

57. Fogarty, W.M. and Griffin, P.J. (1973) Biochem. Soc. Trans. **1**, 263.

58. Panbangred, W., Shimyo, A., Kinoshita, S. and Okada, H. (1983) Agric. Biol. Chem. **47**, 957.

59. Horikoshi, K. and Atsukawa, Y. (1973) Agric. Biol. Chem. **37**, 2097.

60. Honda, H., Kudo, T., Ikura, Y. and Horikoshi, K. (1985) Can. J. Microbiol. **31**, 538.

61. Okazaki, W., Akiba, T., Horikoshi,. K. and Kahoshi, R. (1984) Appl. Microbiol. Biotechnol. **19**, 335.

62. Uchino, F. and Nakane, T. (1981) Agric. Biol. Chem. **45**, 1121.

63. Berenger, J.F., Frixon, C., Bigliardi, J. and Creuzet, N. (1985) Can. J. Microbiol. **31**, 635.

64. Paul, J. and Verma, A.K. (1990) Biotechnol. Lett. **12**, 61.

65. Brodel, B., Samain, E. and Debeire, P. (1990) Biotechnol. Lett. **12**, 65.

66. Lee, S.F., Forsberg, C.W. and Raltray, J.B. (1987) Appl. Environ. Microbiol. **53**, 644.

67. Donnison, A.M., Brockelsby, C.M., Morgan, H.W. and Daniel, R.M. (1989) Biotechnol. Bioeng. **33**, 1495.

68. Rapp, P. and Wagner, F. (1986) Appl. Environ. Microbiol. **51**, 746.

69. Rajoka, M.I. and Malik, K.A. (1984) Biotechnol. Lett. **6**, 597.

70. Peiris, S.P., Ricard, P.A.D. and Dunn, N.W. (1982) Eur. J. Appl. Microbiol. Biotechnol. **14**, 169.

71. Sreenath, H.K. and Joseph, R. (1982) Folia Microbiol. **27**, 107.

72. Ishaque, M. and Kluepfel, D. (1981) Biotechnol. Lett. **3**, 481.

73. Kluepfel, D., Shareck, F., Mondou, F. and Morosoli, R. (1986) Appl. Microbiol. Biotechnol. **24**, 230.

74. Morosoli, R., Betrand, J.L., Mondou, F., Shareck, F. and Kluepfel, D. (1986) Biochem. J. **239**, 587.

75. Iizuka, H. and Kawaminami, T. (1965) Agric. Biol. Chem. **29**, 520.

76. Ikeda, S., Oguma, T. and Kiyooka, S. (1972) Japanese Patent 6743353.

77. Kusakabe, I., Yasui, T. and Kobayashi, T. (1969) Nippon Nogei Kagaku Kaishi **43**, 145.

78. Sorensen, H. (1957) Acta Agric. Scand. Suppl. **1**, 5.

79. Machenzie, C.R., Bilous, D., Schneider, H. and Johnson, K. G. (1987) Appl. Environ. Microbiol. **53**, 2835.

80. Loginova, L.G., Yusupova, I.Kh. and Tashpulatov, Zh. (1981) Prikl. Biochim. Microbiol. **17**, 133.

81. Sreenath, H.K., Joseph, R. and Murthy, V.S. (1978) Folia Microbiol. **23**, 299.

82. Kusakabe, I., Yasui, T. and Kobayashi, T. (1977) Nippon Nogei Kogaku Kaishi, **51**, 429.

83. Yasui, T., Marui, M., Kusakabe, I. and Nakanishi, K. (1988) Methods Enzymol. **160**, 648.

84. Vyas, P., Chauthaiwale, V., Phadatare, S., Deshpande, V. and Srinivasan, M.C. (1990) Biotechnol. Lett. 12, 225.

85. Zimmermann, W., Winter, B. and Broda, P. (1988) FEMS Microbiol. Lett. **55**, 181.

86. McCarthy, A.J., Peace, E. and Broda, P. (1985) Appl. Microbiol. Biotechnol. **21**, 238.

87. Ball, A.S. and McCarthy, A.J. (1989) J. Appl. Bacteriol. **66**, 439.

88. Ristroph, D.L. and Humphrey, A.E. (1985) Biotechnol. Bioeng. **27**, 832.

89. Biely, P.M., Vrsanska, M. and Kratky, Z. (1980) Eur. J. Biochem. **112**, 375.

90. Paice, M.G., Jurasek, L., Carpenter, M.R. and Smillie, L.B. (1978) Appl. Environ. Microbiol. **36**, 802.

91. Wood, T.M. and McCrae, S.I. (1986) Carbohydr. Res. **148**, 321.

92. Wong, K.K.Y., Tan, L.U.L., Saddler, J.N. and Yaguchi, M. (1987) Can. J. Microbiol. **32**, 570.

93. Frederick, M.M., Frederick, J.R., Fratzke, A.R. and Reilly, P.J. (1981) Carbohydr. Res. **97**, 87.

94. Vrsanska, M., Gorbacheva, I.V., Kratky, Z. and Biely, P. (1982) Biochim. Biophys. Acta, **704**, 114.

95. Srinivasan, M.C., Vartak, H.G., Powar, V.K., Rele, M.V. and Bastawde, K.B. (1984) Biotechnol. Lett. **6**, 715.

96. Maheshwari, R. and Kamalam, P.T. (1985) J. Gen. Microbiol. **131**, 3017.

97. Yu, E.K.C., Tan, L.U.L., Chan, M.K.H., Deschatelets, L. and Saddler, J.N. (1987) Enz. Microb. Technol. **9**, 16.

98. Merchant, R., Merchant, F. and Margaritis, A. (1988) Biotechnol. Lett. **10**, 513.

99. Yoshika, H., Chavanich, S., Nilubol, N. and Hayashida, S. (1981) Agric. Biol. Chem. **45**, 579.

100. Comtat, J. (1983) Carbohydr. Res. **118**, 215.

101. Flickinger, M.C. (1980) Biotechnol. Bioeng. **23**, 27.

102. Fratzke, A.R. and Reilly, P.J. (1976) Enz. Technol. Dig. **5**, 3.

103. Linko, M. (1977) Adv. Biochem. Eng. **5**, 25.

104. McCarthy, A.J. (1987) FEMS Microbiol. Rev. **46**, 145.

105. McCarthy, A.J. and Broda, P. (1976) J. Gen. Microbiol. **130**, 2905.

106. Lacey, J. (1973) In "Actinomycetes: Characteristics and Practical Importance" (Eds. Sykes, G. and Skinner, F.P.) Academic Press, London, p. 231.

107. Sundaram, T.K. (1988) J. Chem. Technol. Biotechnol. **42**, 308.

108. Mitsuishi, Y., Yamanobe, T., Yogisaw, M. and Takasaki, Y. (1987) Agric. Biol. Chem. **51**, 3207.

109. Bar-Leve, S.S., Kirk, T.K. and Chang, H.M. (1982) Tappi J. **65**, 111.

110. Eriksson, K.E. and Vallander, L. (1982) Sven. Papperstidn, **85**:R33.

111. Pilon, L., Barbe, M.C., Desrocher, M., Jurasek, L. and Neumann, P.J. (1982) Biotechnol. Bioeng. **24**, 2063.

112. Sharma, H.S.S. (1987) Appl. Microbiol. Biotechnol. **26**, 358.

113. Tan, L.U.L., Yu, E.K.C., Louis-Seize, G.W. and Saddler, J.N. (1987) Biotechnol. Bioeng. **30**, 96.

114. Jurasek, L. and Paice, M. (1986) Chemtech. **16**, 360.

115. Paice, M.G. and Jurasek, L. (1984) Biotechnol. Bioeng. **26**, 477.

116. Paice, M.G. and Jurasek, L. (1984) J. Wood Chem. Technol. **4**, 187.

117. Panbangred, W., Fukusaki, E., Eipfanio, E.C., Shinmyo, A. and Okada, H. (1985) Appl. Microbiol. Biotechnol. **22**, 259.

118. Kato, C., Kobayashi, T., Kudo, T. and Horikoshi, K. (1986) FEMS Microbiol. Lett. **36**, 31.

119. Honda, H., Kudo, T. and Horikoshi, K. (1985) J. Bacteriol. **161**, 784.

120. Mondou, F., Shareck, F., Morosoli, R. and Kluepfel, D. (1986) Gene, **49**, 323.

121. Schneider, H., Wang, P.Y., Chan, Y.K. and Maleszka, R. (1981) Biotechnol. Lett. **3**, 89.

122. Maleszka, R., Veliky, I.A. and Schneider, H. (1981) Biotechnol. Lett. **3**, 415.

123. Debus, D., Methner, H., Schulze, D. and Dellweg, H. (1983) Eur. J. Appl. Microbiol. **17**, 287.

124. Dekker, R.F.H. (1982) Biotechnol. Lett. **4**, 411.

125. Jansen, N.B. and Tsao, G.T. (1983) Adv. Biochem. Eng./ Biotechnol. **27**, 85.

126. Volesky, B. and Szczesny, T. (1983) Adv. Biochem. Eng. /Biotechnol. **27**, 102.

127. Magee, R.J. and Kosaric, N. (1985) Adv. Biochem. Eng./ Biotechnol. **32**, 1.

128. Bernhauer, K. and Reidl-Tumova, E. (1950) Biochem. J. **321**, 26.

129. Fall, R., Phelps, P. and Spindler, D. (1984) Appl. Environ. Microbiol. **47**, 1130.

130. Wiseman, A. (1975) In "Hand Book of Enzyme Biotechnology" (Ed. Wiseman, A.) Ellis Horwood Ltd., Chichester, p. 243.

131. Butenko, R.G. and Kuchko, A.A. (1979) Fiziol. Rast. **26**, 1110.

132. Kusakabe, I., Yasui, T. and Kobayashi, T. (1975) Agric. Biol. Chem. **39**, 1355.

133. Kratky, Z., Biely, P. and Vrsanska, M. (1981) Carbohydr. Res. **93**, 300.

134. Das, N.N., Das, S.C., Sarkar, A.K. and Mukherjee, A.K. (1984) Carbohydr. Res. **129**, 197.

# CHAPTER 2

# STRAIN ISOLATION AND XYLANASE PRODUCTION

# SUMMARY

From humus rich soil collected at Nandesari, an actinomycete strain was isolated. The isolate was an aerobic Streptomyces designated as $T_7$ which formed clearance zone on xylan - yeast extract - agar indicating secretion of xylanase and hydrolysis of xylan. The strain grew over the temperature range of 37 - 50°C and maximum growth and sporulation was observed within 48 - 72 h at 45 - 50°C. However, the strain did not grow above 50°C, indicating that it was a thermotolerant culture.

The xylanase production was studied over the temperature range of 30 - 50°C. Maximum xylanase production (70 U/ml) was observed at 50°C. When the strain was grown on different sugars, such as arabinose, lactose, sucrose, maltose, glucose, xylose and mannose, low constitutive levels of xylanase ranging from 0.4 - 1.25 U/ml were observed. Wheat bran, wheat and rice straw were found to be good inducers. The induction of xylanase was more with whole wheat bran than with extract or residue. Supplementing the medium with either trace elements or peptone and yeast extract did not enhance the enzyme production. Among the various inorganic nitrogen compounds tested, optimum xylanase activity was observed with ammonium sulphate, diammonium hydrogen phosphate, ammonium chloride or sodium nitrate. 0.7% Ammonium sulphate concentration was optimum for enzyme production. Optimum inoculum concentration was found to be 10 - 15% (v/v) and optimum pH was found to be 7.0 for enzyme production. Substituting diammonium hydrogen phosphate in place of phosphate and nitrogen did not affect the xylanase production.

The xylanase activity markedly increased after 48 h and reached its maximum (75 U/ml) in 72 h when the organism was grown in wheat bran (5%) medium. On the contrary, in xylan medium the activity had a tendency to show steady increase during 48 - 72 h and reached its maximum (28 U/ml) in 96 h. The level of soluble protein was also low in xylan medium compared to wheat bran medium.

# INTRODUCTION

Thermophiles are a fascinating group of microorganisms which have received considerable attention in recent years, because of their potential biotechnological importance. Thermophiles are of interest both from the fundamental and applied point of view [1].

Atinomycetes occur in a wide range of environments in which they have ability to grow on most naturally occuring substrates [2]. Actinomycetes are emerging as an important source of enzymes involved in lignocellulose degradation and activity against xylan is particularly widespread [3]. Isolates representing a diverse range of actinomycete genera and exhibiting degradative enzyme mediated activities have been recovered from soils and composts [4]. Actinomycetes have been extensively exploited for commercial antibiotic production and more recently as a source of industrial enzymes, such as glucose isomerase.

## Isolation

Soil is the primary reservoir of actinomycetes capable of biodegradation of lignocelluloses, which are the most abundant feedstocks available in supporting microbial growth under natural environment. Adaptation of standard procedures for the recovery of actinomycetes by incorporation of ligno-cellulose or related substrates in enrichment and isolation media is the usual approach [2]. Bacterial growth can rarely be excluded but its interference with actinomycete recovery can be minimised in a number of ways. These include heat treatment of samples at temperatures where actinomycete spores can survive [5]. Composts are the most favourable

environments in which extensive lignocellulose degradation by actinomycetes is taking place. Both mesophilic and thermophilic actinomycetes play significant role in composting process which occurs through the development of a diverse actinomycete population [6.7]. Lignocellulose degrading strains have been isolated from a range of composted materials [8-11]. In compost production, for mushroom cultivation a number of thermophilic actinomycetes develop invariably [12-15].

Thermotolerant and extremely thermophilic bacteria, producing xylanases are known. Among them the bacilli and clostridia are well known [16-18]. Xylanase producing thermophilic actinomycetes are reported and include Streptomyces sp Thermomonospora sp, Thermoactinomyces sp and Saccharomonospora sp [19-21].

**Xylanase production**

Xylanases are inducible enzymes and are generally formed when pure xylan or xylan containing natural substrates are present in the medium. Polymeric xylan cannot enter the cells and hence, must be degraded extracellularly. Therefore, xylanases are secreted by cells into the surrounding medium. The oligosaccharides are formed by hydrolysis of xylan in the medium by small amounts of the enzymes produced constitutively [22].

D-xylanases are also produced even in the absence of xylan in the growth medium. Esteban and associates [23] reported that xylanase activity was undetected in glucose grown cultures of Bacillus circulans WL12, but xylose, mannose and cellobiose supported growth and xylanase activity. The

xylanolytic enzymes are induced more effectively by xylose than xylobiose in *Bacillus pumilus* [24].

In the case of *Streptomyces* sp Park and Toma [25] studied induction of xylanases in the presence of xylan. In *Streptomyces flavogriseus* simultaneous production of glucose (xylose) isomerase in addition to cellulases and xylanases has been described [26]. Coproduction of xylanases and cellulases has been observed in other actinomycetes, but in four *Thermomonospora* strains optimum production of either activity was achieved only when grown on the respective substrates [27]. In *Streptomyces* sp induction of xylanase was achieved with synthetic xyloside like methyl-β-D-xyloside [28]. In *Saccharomonospora viridis*, a thermophilic actinomycete which degrades xylan, both extracellular xylanase and cell bound β-xylosidase were induced by growth on xylan but the enzymes were not induced when grown on glucose or xylose. Addition of glucose and/or xylose for enhancing the mycelial growth, depressed xylanase production by 50% [2]. *Streptomyces afghaniensis* showed xylanase induction when it was grown on an agricultural residue, such as wheat bran or straw [19]. Four thermophilic actinomycetes were selected by McCarthy and his coworkers for xylanase production, using xylan as an inducer, although low levels of activity were also detected in glucose grown cultures [20]. Several actinomycetes including a number of mesophilic and thermophilic, were screened for xylanase activity. The effect of growth conditions on the production of enzyme activities was studied with two different hemicellulose preparations, oat spelts xylan and barley straw. The mesophilic strains were grown at 37°C

and the thermophilic at 50°C. Streptomyces cyaneus and Streptomyces viridis produced xylanases on straw while Pseudonocardia thermophila and Thermoactinomyces vulgaris showed higher activities on oat spelt xylan [21].

Reports concerning the production of xylanases in the medium containing sugar are also available. Thus, when glucose was used as a carbon source for the growth of the yeast Cryptococcus albidus var aerius, low levels of primary cell wall associated endo-xylanase was produced [29]. The enzyme was produced constitutively, but it could also be produced inductively during the growth of this yeast on xylan [30]. Xylobiose is also an inducer of xylanase in C. albidus and is taken up by glucose grown cells prior to the appearance of enzyme. The uptake of xylobiose appears to be caused by an induction of active transport system known as β-xyloside permease [31]. In this yeast C. albidus, methyl-β-D-xyloside enabled the production of xylanolytic enzymes in the absence of xylan or xylo-oligosaccharides [32].

Thermophilic microorganisms are potentially good sources of enzymes stable to thermal inactivation [33]. Though several enzymes produced by mesophilic microorganisms exhibit good temperature stability, enzymes from thermophilic organisms are invariably more thermostable than their mesophilic counterparts [34]. Stable enzymes acting at elevated temperatures are advantageous for increasing the hydrolysis efficiencies and also minimising the contamination problems. Xylanases produced from thermophilic organisms are listed in Table 2.1.

Actinomycetes and bacteria exhibit pH optima for growth

Table 2.1: Xylanase production by representative thermophilic microorganisms

| Organism | Temperature °C | Reference |
|---|---|---|
| **Bacteria** | | |
| *Bacillus stereothermophilus* 4125 | 65 | 17 |
| *Bacillus* sp 11-1S | 65 | 36 |
| *Bacillus* sp W1-W4 | 45 - 50 | 35 |
| *Clostridium stercorarium* | 65 | 3 |
| *Clostridium thermocellum* DSM 1237 | 70 | 16 |
| *Clostridium thermolacticum* DSM 2911 | 63 | 38 |
| **Actinomycetes** | | |
| *Streptomyces lividans* 1326 | 40 | 39 |
| *Streptomyces afghaniensis* | 40 | 19 |
| *Thermomonospora chromogena* MT 814 | 50 | 20 |
| *Thermomonospora curvata* MT 815 | 50 | 20 |
| *Thermomonospora fusca* MT 816 | 50 | 20 |
| *Saccharomonospora viridis* NCIB 9602 | 50 | 20 |
| *Pseudonocardia thermophila* | 50 | 21 |
| *Thermoactinomyces sacchari* | 50 | 21 |
| *Thermoactinomyces vulgaris* | 50 | 21 |
| **Fungi** | | |
| *Thielavia terrestris* ATCC 26917 | 48 | 40 |
| *Thermoascus aurantiacus* C 436 | 45 | 41 |
| *Malbranchea pulchella* var. *sulfurea* 48 | 44 | 42 |
| *Talaromyces byssochlamydoides* YH 50 | 50 | 43 |
| *Chaetomium thermophile var coprophile* I 110 | 60 | 44 |

and enzyme production, which are close to neutral in contrast to the generally acidic pH requirements of fungi [3]. However, certain alkalophilic bacilli are known which have pH optima for growth and enzyme production at 9 - 10. In these organisms, no growth occurred below pH 7.0 [35].

## MATERIALS AND METHODS

### Materials

All the chemicals used were of analytical grade. The suppliers of the following chemicals are indicated in parenthesis: Bacto Yeast Extract (Difco Laboratories, USA); 3,5-Dinitrosalicylic acid (DNS). p-Nitrophenyl-B-D-xylopyranoside (PNPX), Carboxymethyl cellulose (CM-cellulose) C-8758, Bovine serum albumin (BSA) (Sigma Chemical Co., USA); Larch wood xylan (Fluka AG, Switzerland); Avicel P.H. 101 (Honeywell and Stein Ltd., London); Tween-80 (Bayer); Cellulose powder CP-100 (Cellulose Products of India, Ahmedabad); Peptone (Biochemical Unit, Delhi, India); Casein (Amul Products, Dist. Khaira, Gujarat).

Rice straw, wheat bran and sugarcane bagasse were purchased locally. Mesta wood (Hibiscus cannabinus) was obtained from the Conservator of Forests, Chandrapur.

### Methods

#### Isolation of the strain

From humus rich soil collected at Nandesari (near Baroda) an actinomycete strain was isolated by plating out on a medium containing wheat bran (5%) and yeast extract (1%). The wheat bran was autoclaved in water, followed by extraction through muslin cloth to remove most of the starch. When incubated

at 50°C for 48 h, several colonies of bacteria and actinomycetes developed which were screened for xylanase activity primarily by scoring for xylan clearance on xylan - yeast extract - agar plates and subsequently in submerged culture.

Maintainance of the strain The strain identified as a thermotolerant Streptomyces and designated as $T_7$ was maintained on wheat bran (5%) and yeast extract (1%) agar slants. Stock cultures were maintained by subculturing at three monthly intervals. After growing at 48 - 50°C for 3 days the slants were preserved at 15°C.

From an actively sporulating stock culture, subcultures were made on fresh slants and after 3 days incubation at 48 - 50°C, were used as the starting material for all fermentation experiments. Cultures occassionally showed nonsporulating sectors which appeared as whitish fuzz among the greish-brown sporulating colonies. Such cultures were plated out to remove colonies with homogeneous sporulation, for stock conservation as well as experimentation. Culture was also lyophilized for long term preservation.

**Fermentation studies**

The xylanase was produced in 250 ml Erlenmeyer flasks containing 50 ml of medium. The flasks were incubated at 50°C on a rotary shaker at 200 rev/min. The cells and solid residues were removed from the culture broth by centrifugation and the clear supernatant was used for determining the xylanase activity.

The medium used for screening the isolates contained in g/l: corn steep liquor, 20; $CoCl_2.6H_2O$, 0.24; $MgSO_4.7H_2O$, 1 and wheat bran 20. The pH of the medium was adjusted to

7.0. The various isolates were initially subcultured on fresh slants and then agar piece (1 x 1 cm) was inoculated in the medium.

The basal medium [26] used for experiments contained in g/l: $KH_2PO_4$. 1.5; $K_2HPO_4$, 2.0; $CaCl_2.2H_2O$, 0.3; $MgSO_4.7H_2O$, 0.3, $(NH_4)_2SO_4$ , 1.4; peptone, 1.0, yeast extract, 2.0; Tween-80, 2 ml; trace elements mg/l: $FeSO_4.7H_2O$, 5.0; $MnSO_4.H_2O$, 1.56; $ZnSO_4.7H_2O$, 1.4; $CoCl_2.6H_2O$, 2.0, pH of the medium was 7.0. Wheat bran was used as a carbon source, the amount of which was varied and is described in the text.

Vegetative inoculum was used to inoculate experimental media. The inoculum was grown for 48 h at 50°C in the same medium described as above and containing 1% wheat bran.

**Preparation of substrate (xylan) (1%)**

A 2 g portion of xylan was suspended in 100 ml of 50 mM sodium acetate buffer, pH 5.0 and was stirred for 12 - 16 h. The insoluble fraction (about 50%) was removed by centrifugation and the soluble fraction was used for xylanase assay.

**Xylanase assay**

Xylanase was assayed by incubating 0.5 ml aliquot of appropriately diluted enzyme with 0.5 ml of 1% xylan at 60°C for 30 min [45]. The reducing sugar formed was determined by the DNS method with D-xylose as standard [46].

**Other enzyme assays**

Activity towards CM-cellulose, starch and filter paper (Whatman No. 1) were determined by incubating 1 ml of a reaction mixture containing suitably diluted enzyme, with 0.5

ml of CM-cellulose (1%) or starch (1%) or filter paper (25 mg) in 50 mM acetate buffer, pH 5.0 at 50°C for 30 or 60 min. The reducing sugar formed was determined as glucose or maltose by the DNS method described above. Proteinase activity was determined by Kunitz's method [47] with casein as substrate.

**β-Xylosidase assay**

β-Xylosidase was estimated as described by Kluepfel and Ishaque [48] by incubating 1 ml of reaction mixture containing suitably diluted enzyme with 0.1% PNPX in 50 mM acetate buffer, pH 5.0 and by determining the p-nitrophenol liberated by the enzyme action at 40°C after 30 min. The same procedure was repeated for the sonicated cell extract and mycelial suspension to assay the intracellular or cell bound β-xylosidase.

**Unit of activity**

The unit of xylanase or xylosidase was defined as that amount of enzyme which produces 1 µmol of xylose or p-nitrophenol per min from xylan or PNPX, respectively under the given assay conditions.

**Determination of protein**

Protein was determined by the method of Lowry et al [49]. BSA was prepared according to the procedure of Cohn et al. [50] ($E_{1\%}^{cm}$ = 6.6 at 280 nm) and used as a standard.

RESULTS

Modifications to the basal medium used in the screening experiments were made to study the effect of varying the

nutrient components for achieving higher enzyme activity as well as productivity. Stepwise amendments to the basal medium were made and used in subsequent experiments, whenever a favourable or positive indication for xylanase production was observed through these modifications.

Screening of isolates The various isolates were grown in submerged culture for 96 h at 50°C. The extracellular xylanase activity was determined at 50°C. The individual isolate was also inoculated on agar medium containing 3% xylan and 1% yeast extract. The plates were incubated at 50°C for 48 h to see the clearance zone.

Table 2.2 shows the xylanase activity of each isolate and the extent of xylan degradation. The isolate (NCL 85-11-$T_7$) showed the highest xylanase activity (7 U/ml). The clearance zone indicated by NCL 85-11-$T_7$ was comparatively larger than that of the other isolates. This isolate was selected for further studies.

Characteristics of the strain The isolate is an aerobic *Streptomyces* designated as $T_7$ which forms greyish sporulating colonies on wheat bran - yeast extract - agar after incubation for 72 h at 50°C. A distinct clearance zone was observed on xylan agar plate, indicating the hydrolysis of xylan and extracellular secretion of xylanase (Fig. 2.1).

Effect of temperature on growth of *Streptomyces* $T_7$ The strain $T_7$ was subcultured on wheat bran - yeast extract slants and incubated at 28, 37, 45, 50 and 52°C.

Figure 2.2 shows that the growth of $T_7$ was very poor at 28°C, while at 37°C the growth was somewhat better.

**Table 2.2:** Screening of the isolates

| Isolate | Isolate No.* | Xylanase (U/ml) | Clearance Zone |
|---|---|---|---|
| *Bacillus* sp | $T_1$ | 0 | - |
| *Bacillus* sp | $T_2$ | 0.50 | - |
| *Bacillus* sp | $T_3$ | 0.70 | - |
| *Bacillus* sp. | $T_4$ | 2.00 | + |
| Actinomycete | $T_5$ | 0.60 | + |
| Actinomycete | $T_6$ | 0.80 | - |
| *Streptomyces* | $T_7$ | 7.00 | + + + |
| Actinomycete | $T_8$ | 0.20 | + |
| Actinomycete | $T_9$ | 0.10 | - |
| Actinomycete | $T_{10}$ | 0.09 | - |

*Isolate No. NCL-85-11.

**Fig. 2.1:** Xylan clearance zone by Streptomyces $T_7$
The strain $T_7$ was inoculated on xylan-yeast extract agar and incubated at 50°C for 48 h.

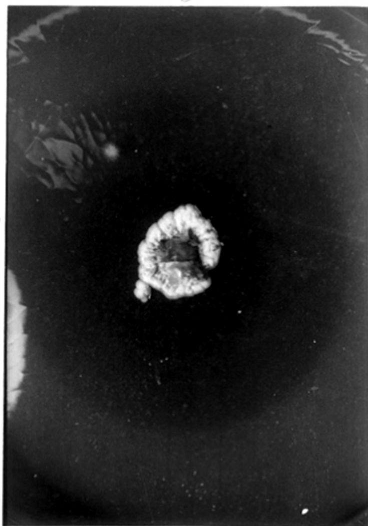
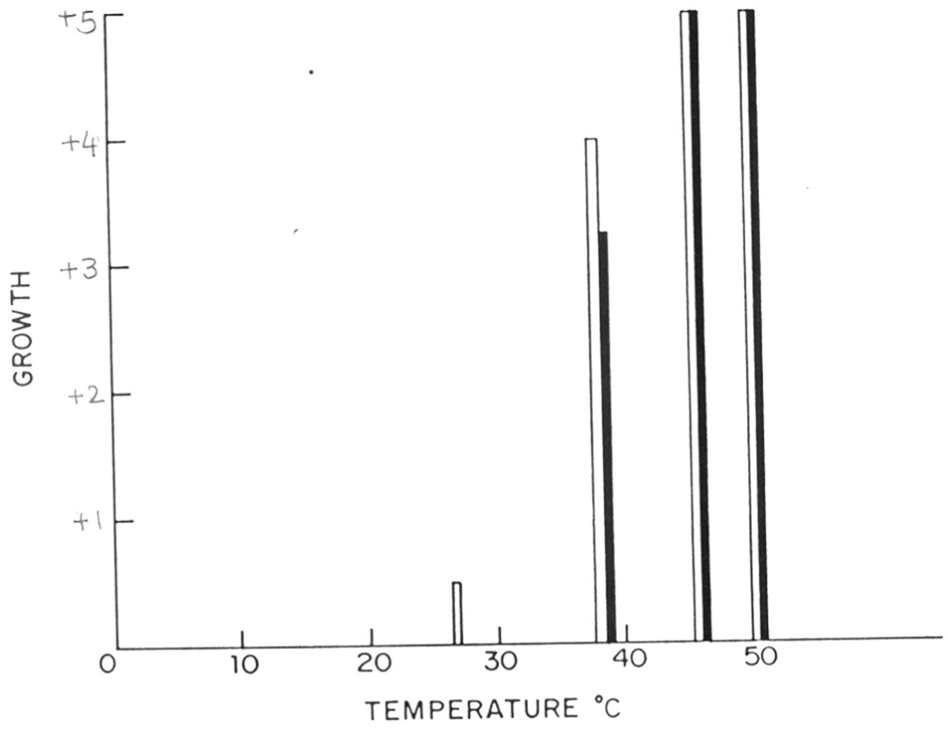


**Fig. 2.2:** Effect of temperature on growth of *Streptomyces* $T_7$

The strain $T_7$ was subcultured on wheat bran, yeast extract slants and incubated at various temperatures (28 - 52°C)

(▭) growth; (▬) sporulation.



+5
+4
+3
+2
+1
GROWTH
0
10
20
30
40
50
TEMPERATURE °C

with the colonies showing sporulation within 4 days. Maximum growth and sporulation was observed within 48 - 72 h, when culture was incubated at 45 - 50°C, while above 50°C the growth was poor. In view of its activity to grow over the temperature range of 37 - 50°C and poor growth beyond 50°C, the strain $T_7$ has been designated "thermotolerant" rather than thermophilic.

Effect of temperature on the growth of $T_7$ in submerged culture

*Streptomyces* $T_7$ was grown in a wheat bran free basal medium containing 1% glucose or xylose as a carbon source. Spore suspension was made by scraping the well sporulated slant and suspending the spores in 10 ml of sterile water. A 2% v/v spore suspension was used as an inoculum. The flasks were incubated at 28, 37, 45 and 50°C for 72 h. The dry weight of mycelium, pH, soluble protein and extracellular xylanase activity were determined.

Table 2.3 shows that the growth of *Streptomyces* $T_7$ on glucose and xylose media was comparable. When the growth was compared at 37 and 45 - 50°C, very little difference in biomass yield was observed, whereas the growth at 28°C resulted in poor yield of biomass. Low basal levels of xylanase activities were observed and the level was increased with the increasing incubation temperature. The soluble protein levels and xylanase activities obtained at all temperatures in the xylose media were slighly higher than those obtained in glucose medium.

Effect of temperature on xylanase production Comparison of xylanase production at various incubation temperatures was studied. The organism was grown on the basal medium

Table 2.3: Growth of *Streptomyces* $T_7$ at various temperatures in submerged culture

| Carbon source | Temperature (°C) | | | |
|---|---|---|---|---|
| | 28 | 37 | 45 | 50 |
| **Glucose** | | | | |
| Dry weight of mycelium (mg/ml) | 2.56 | 3.60 | 4.40 | 4.40 |
| Xylanase (U/ml) | 0.10 | 0.60 | 0.80 | 0.90 |
| Protein (mg/ml) | 0.30 | 0.50 | 0.62 | 0.62 |
| **Xylose** | | | | |
| Dry weight of mycelium (mg/ml) | 2.60 | 4.00 | 4.35 | 4.50 |
| Xylanase (U/ml) | 0.25 | 1.10 | 1.40 | 1.50 |
| Protein (mg/ml) | 0.50 | 0.60 | 1.00 | 1.25 |

containing 2% wheat bran as a carbon source. The flasks were incubated at 28, 37, 45 and 50°C for 120 h. The samples were removed at different time intervals and the culture filtrate was examined for xylanase activity.

Figure 2.3 shows that he xylanase production was considerably more at 50°C than at 30°C. Xylanase activities were maximal (17.7U/ml) after 72 h of incubation. During the fermentation, it was observed that after 48 h, the medium became thick and brown in colour. The fine mycelium started appearing the vigorous growth took place after 60 h and continued upto 72 h. All further fermentation studies were carried out only at 50°C in submerged culture.

Effect of various sugars and sugar alcohols on enzyme production

The organism was grown on wheat bran free basal medium containing various sugars and polyols added at a concentration of 1%. The flasks were incubated at 50°C for 48 h.

Table 2.4 shows that low constitutive levels of xylanase activities ranging from 0.4 - 1.25 U/ml were obtained. Maltose among the sugars gave the highest activity of 4 U/ml.

Effect of various insoluble carbohydrates and agricultural residues on enzyme production The organism was grown in a wheat bran free medium and the effect of various insoluble carbohydrates added in the form of celluloses as well as agricultural and forest residues representing lignocellulosic wastes were tested at 2% level.

Table 2.5 shows that wheat bran and wheat and rice straw were found to be good inducers showing 12 U/ml. Bagasse CP 123 and xylan also showed comparable levels of activity.

**Fig. 2.3:** Effect of temperature on xylanase production

The organism was grown on basal medium containing 2% wheat bran as a carbon source and incubated at various temperatures.

(○) 28°C; (△) 37°C; (●) 45°C; (▭) 50°C.

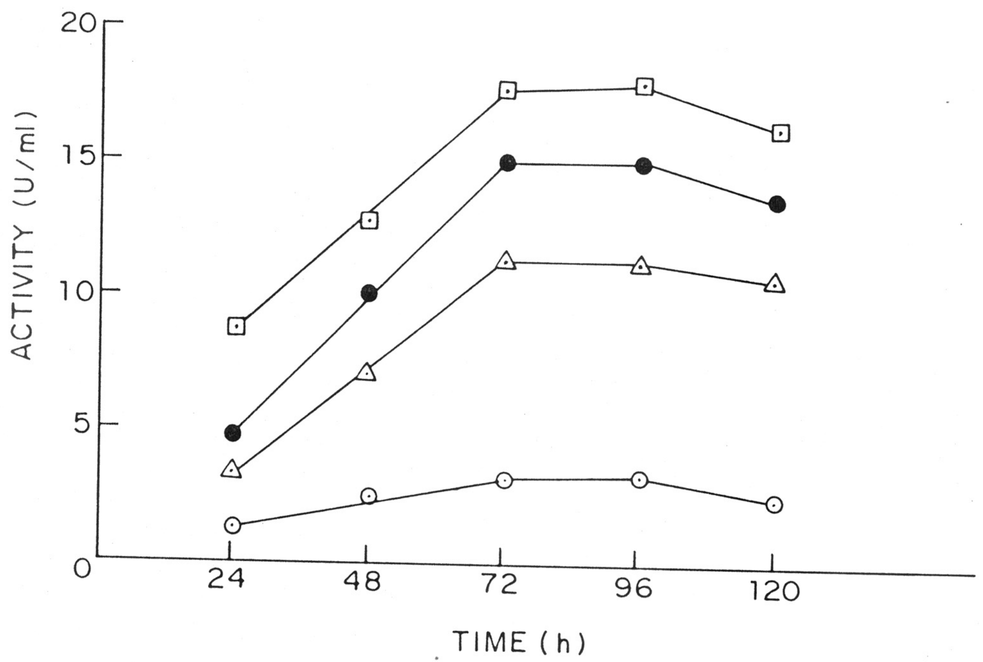

20
15
10
5
0
ACTIVITY (U/ml)
24
48
72
96
120
TIME (h)

Table 2.4: Effect of various sugars and sugar alcohols on enzyme production

| Carbon source (1%) | Xylanase (U/ml) |
|---|---|
| Arabinose | 0.68 |
| Lactose | 0.65 |
| Sucrose | 0.84 |
| Maltose | 4.00 |
| Glucose | 0.80 |
| Xylose | 1.25 |
| Mannose | 1.07 |
| Glycerol | 0.47 |
| Mannitol | 0.60 |
| Sorbitol | 0.82 |

**Table 2.5:** Effect of insoluble carbohydrates and agricultural residues on enzyme production

| Carbon source (2%) | Xylanase (U/ml) |
|---|---|
| Wheat bran | 12.0 |
| Rice straw | 12.0 |
| Wheat straw | 11.5 |
| Xylan | 8.3 |
| CM-cellulose | 0 |
| Cellulose powder | |
| CP-123 | 8.3 |
| CP-100 | 4.0 |
| Solka Floc | 7.0 |
| Bagasse | 11.0 |
| Mesta wood | 6.0 |
| Avicel | 6.0 |

However, CM-cellulose failed to induce xylanase.

Effect of different concentrations of ammonium sulphate

The effect of different concentrations of ammonium sulphate on enzyme production was studied. The basal medium used with various concentrations of ammonium sulphate contained 2% wheat bran as a carbon source.

Table 2.6 shows that with 0.14% ammonium sulphate the xylanase activity was low. But there was not much difference in xylanase activity over a wide range (0.28 - 1.4%) of ammonium sulphate concentration. A concentration of ammonium sulphate (0.7%) was observed to be optimal for growth and enzyme production and was used in further experiments.

Effect of different concentrations of wheat bran The basal medium used contained 0.7% of ammonium sulphate. The organism was grown in the medium, with wheat bran concentrations ranging from 1 - 8%.

Figure 2.4 shows that the xylanase activity increased with increasing amounts of wheat bran. The maximum xylanase activity was obtained at 5 - 6% wheat bran concentration. Above 5 - 6% the growth as well as enzyme production was reduced, possibly due to the high bulk density of the medium.

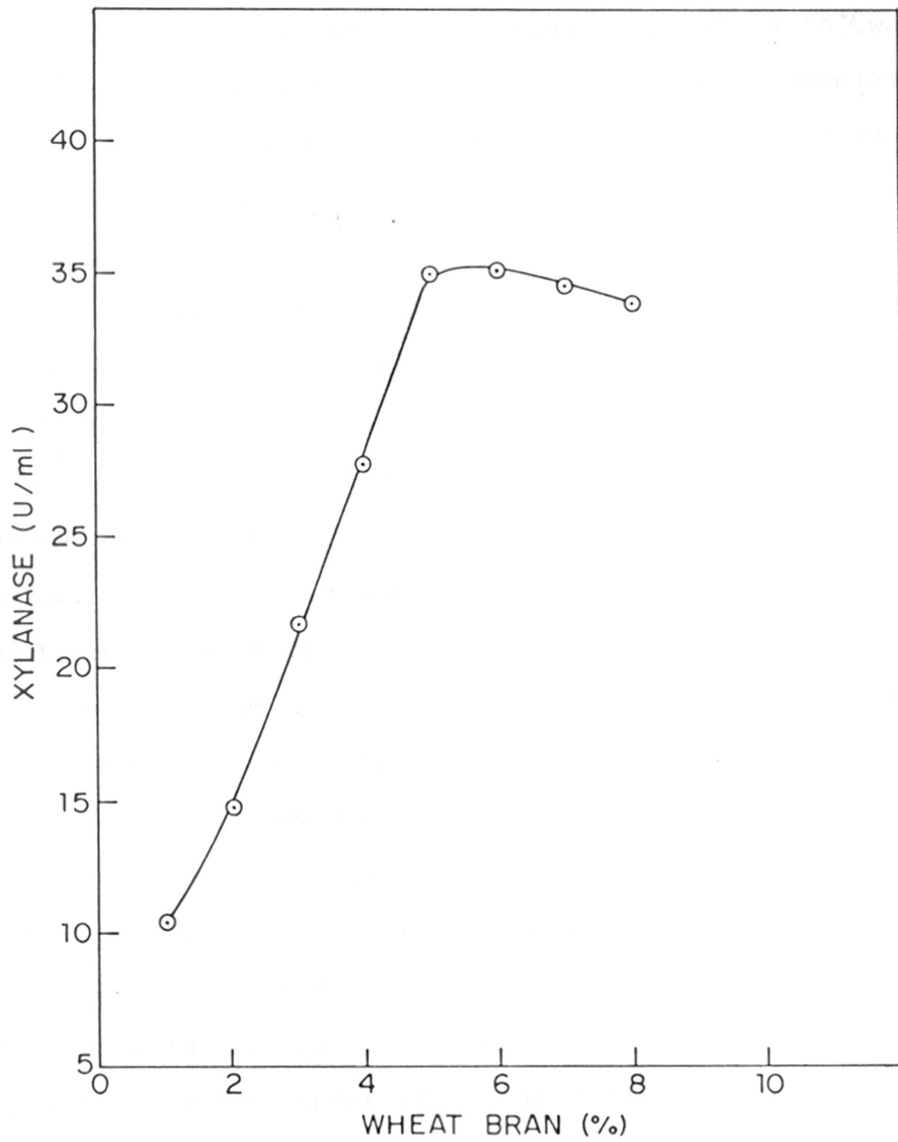
Effect of different fractions of wheat bran To understand which fraction of wheat bran (such as soluble component like extract or residue) induces the xylanase secretion, 2.5 g of wheat bran was suspended in minimum amount of water, steamed and then filtered. The extract and residue were separately added in the wheat bran free basal medium (50 ml). The flasks containing the basal medium with wheat bran (2.5 g) and wheat bran residue plus starch (0.5 g)

**Table 2.6:** Effect of different concentrations of ammonium sulphate on enzyme production

| Ammonium sulphate (%) | Xylanase (U/ml) |
| --- | --- |
| 0.14 | 11.6 |
| 0.28 | 14.3 |
| 0.56 | 15.2 |
| 0.70 | 17.5 |
| 0.84 | 15.0 |
| 1.12 | 14.8 |
| 1.40 | 14.0 |

**Fig. 2.4:** Effect of different concentrations of wheat bran on xylanase production

The organism was grown in the medium, with wheat bran concentrations ranging from 1 - 8%.


XYLANASE (U/ml)
WHEAT BRAN (%)
40
35
30
25
20
15
10
5
0
2
4
6
8
10

were also run under identical conditions.

The induction of xylanase was maximum with whole wheat bran than with the only extract or residue or residue plus starch. The xylanase activity obtained with wheat bran was assumed to be 100%. Xylanase activity of 35% and 55% was obtained with extract or residue, respectively. Supplementing the residue with starch showed only 45% activity (Table 2.7).

Effect of initial pH on enzyme production Enzyme production was studied in media adjusted to pH ranging from 4 - 8.

It is evident from Fig. 2.5 that pH 7.0 is optimum for the production of xylanase. The enzyme activity was low at pH 4.0 and 8.0. Based on this result the pH of the medium in all subsequent experiments was adjusted to 7.0.

Effect of inoculum size on xylanase production The effect of adding various percentages of inoculum (5 - 25) on enzyme production was studied.

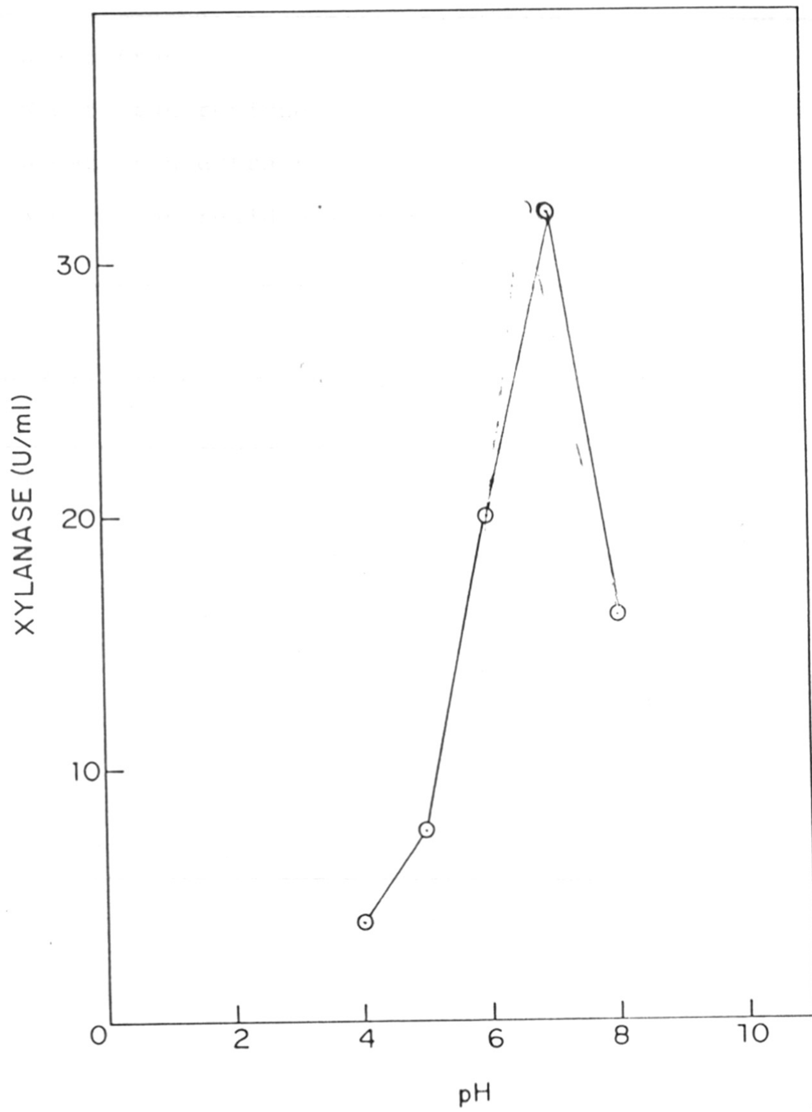
The maximum xylanase activity was obtained when 15% (v/v) inoculum was used. Activities obtained with 10% inoculum were also comparable. Hence, in all the experiments 10 - 15% (v/v) inoculum was used (Table 2.8).

Effect of incubation time on enzyme production The progress of xylanase production was studied in relation to time. The fermentation was carried out for different periods of time (24 - 168 h) under identical conditions.

Figure 2.6 shows that the xylanase activity was maximum when fermentation was carried out for 72 h. After that the xylanase activity did not increase. Beyond 120 h xylanase activity in the culture filtrate began to decrease.

Effect of supplementing the ammonium sulphate with peptone

**Fig. 2.5:** Effect of initial pH on enzyme production
The organism was grown in media adjusted
to pH ranging from 4 - 8.



XYLANASE (U/ml)
30
20
10
0
2
4
6
8
10
pH

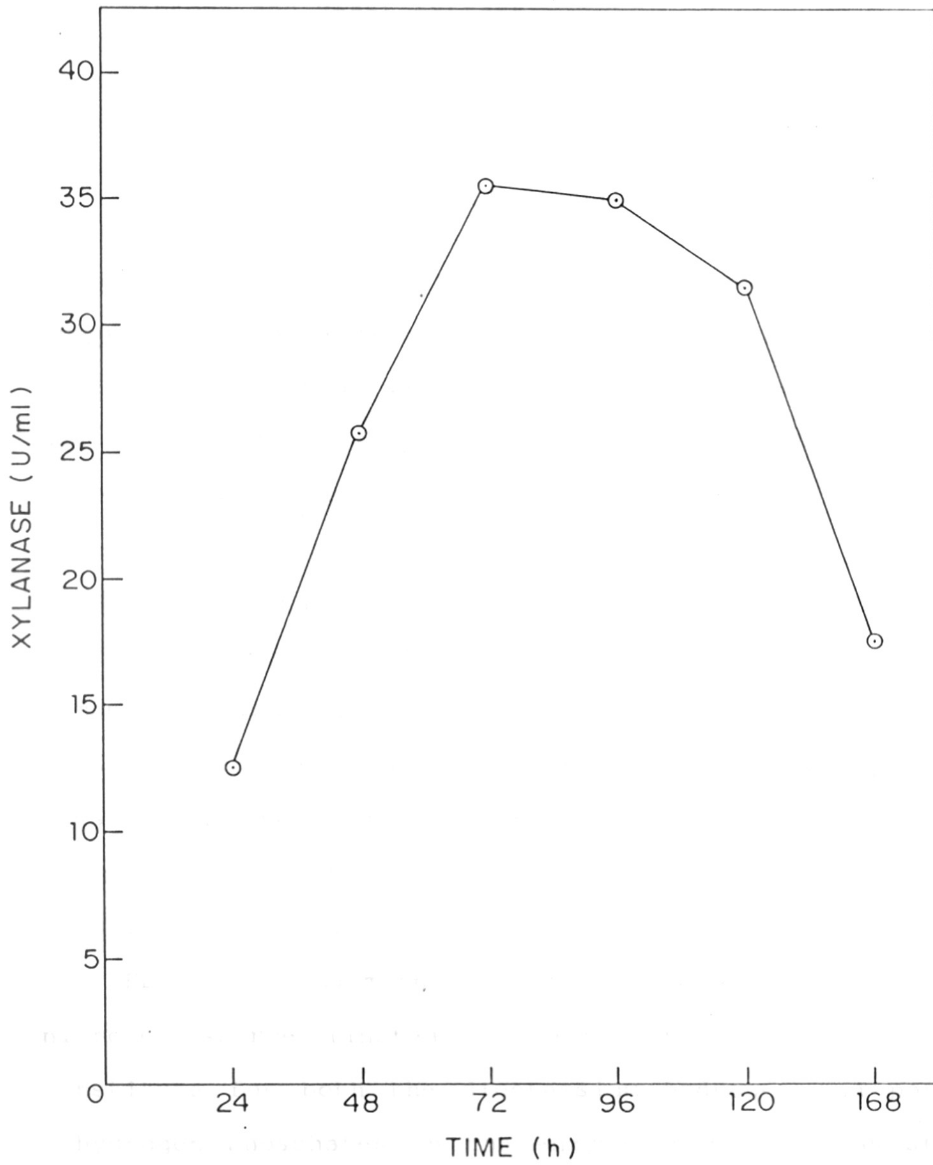
**Table 2.7:** Effect of different fractions of wheat bran on xylanase production

| Carbon source | Xylanase (U/ml) |
|---|---|
| Wheat bran | 34.9 |
| Wheat bran residue | 20.2 |
| Wheat bran extract | 12.3 |
| Wheat bran residue + starch | 16.3 |

**Table 2.8:** Effect of inoculum size on enzyme production

| Inoculum (%) | Xylanase (U/ml) |
|---|---|
| 5 | 25.0 |
| 10 | 33.4 |
| 15 | 35.5 |
| 20 | 32.7 |
| 25 | 31.1 |

**Fig. 2.6:** Effect of incubation time on enzyme production
The organism was grown in the medium for different periods of time (24 - 168 h).



XYLANASE ( U/ml )
40
35
30
25
20
15
10
5
0
24
48
72
96
120
168
TIME (h)

<u>and yeast extract</u> The organism was grown in the medium containing either ammonium sulphate or ammonium sulphate plus peptone and yeast extract.

Supplementation with peptone and yeast extract did not help in enhancement of xylanase activity. Based on this result peptone and yeast extract was not included in the medium.

<u>Effect of various inorganic nitrogen compounds on enzyme production</u> The effect of adding various nitrogen sources on enzyme production was investigated. The amounts of nitrogen source added are shown in Table 2.9. The quantity of nitrogen added was maintained constant at 150 mg/50 ml of medium.

Good xylanase activity were observed with diammonium hydrogen phosphate, ammonium chloride, sodium nitrate and ammonium sulphate. Sodium nitrite and ammonium acetate failed to induce enzyme production. Enzyme production was less with potassium nitrate and ammonium dihydrogen phosphate. In the absence of any added inorganic nitrogen the enzyme production was less than 50% (Table 2.9).

Effect of diammonium hydrogen phosphate, used as a nitrogen source (instead of ammonium sulphate) and as a substitute for both the dipotassium hydrogen and potassium dihydrogen phosphates on the enzyme production was studied. It was observed that in the absence of any phosphates added, the xylanase activity was unaffected. The optimal concentration of diammonium nitrogen phosphate was found to be 0.7%.

<u>Fermentation profile</u> The progress of xylanase production

**Table 2.9:** Effect of various inorganic nitrogen compounds on enzyme production

| Nitrogen source | Amount added (g/50 ml) | Xylanase (U/ml) |
|---|---|---|
| None | - | 17.5 |
| Urea | 0.16 | 29.1 |
| Ammonium sulphate | 0.35 | 36.0 |
| Ammonium nitrate | 0.23 | 31.7 |
| Ammonium chloride | 0.29 | 33.7 |
| Diammonium hydrogen phosphate | 0.35 | 35.5 |
| Ammonium dihydrogen phosphate | 0.62 | 23.2 |
| Potassium nitrate | 0.54 | 27.7 |
| Sodium nitrate | 0.45 | 33.4 |
| Sodium nitrite | 0.37 | 3.7 |
| Ammonium acetate | 0.37 | 12.0 |

was studied in media containing 5% wheat bran as well as 2% xylan. The samples were removed at different time intervals and analysed for activity, protein and pH.

Figure 2.7 shows the xylanase activities, protein and changes of pH in the culture filtrate during the cultivation in wheat bran and xylan medium for 136 h at 50°C. The xylanase activity markedly increased after 48 h and there was sharp increase after that reaching its maximum in 72 h (38 U/ml) when the organism was grown in wheat bran medium. On the contrary, in xylan medium the activity had a tendency to show increase during 48 - 72 h incubation. It reached maximum 14 U/ml in 96 h. The activity is quite low as compared to yield of xylanase in wheat bran medium. Level of soluble protein also increased during the progress of xylanase production. The soluble protein levels were also comparatively lower in xylan media. There was not much change observed in pH during the fermentation in either of the media. Extending the time of fermentation beyond 96 - 120 h did not show enhancement of enzyme activity.

Production of enzyme, on a large scale, for purification purpose was carried out by growing the organism in the basal medium (50°C for 72 h) wherein the concentration of ammonium sulphate was raised 5 times higher (7 g/l) and 5% wheat bran was used as a carbon source.

**Properties of crude xylanase**

Optimum temperature Culture filtrate was assayed under standrd conditions over a temperature range of 30 - 70°C. Optimum temperature for xylanase activity was found to be 60°C, Hence, the xylanase activity was estimated at 60°C.

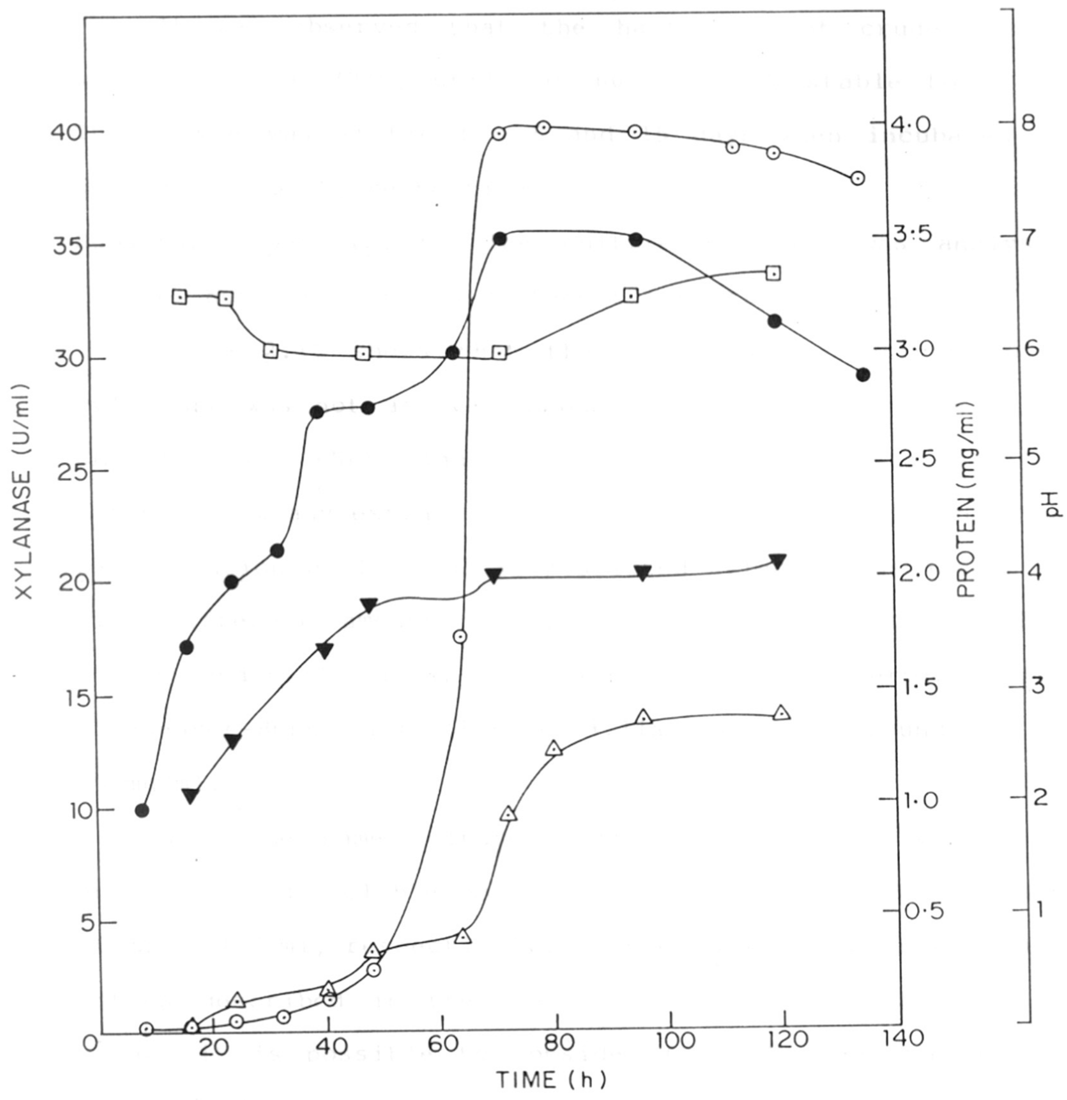
**Fig.2.7:** Fermentation profile

The organism was grown in the medium containing wheat bran (5%) and xylan (2%)

Wheat bran medium - (o) xylanase (●) protein

Xylan medium - (△) xylanase, (▼) protein,

(▭) pH.


XYLANASE (U/ml)
PROTEIN (mg/ml)
pH
TIME (h)
0
20
40
60
80
100
120
140
5
10
15
20
25
30
35
40
0·5
1·0
1·5
2·0
2·5
3·0
3·5
4·0
1
2
3
4
5
6
7
8

Thermostability  Culture filtrate was incubated at various temperatures at pH 5.0 without substrate. The residual activity was determined after different periods of time.

It was observed that the half life of crude enzyme was 30 min at 70°C, while at 60°C it was stable for 2 h. The enzyme was stable for 6 and 15 days when incubated at 50 and 30 - 37°C, respectively.

Substrate specificity  The culture filtrate was analysed for its activity towards different substrates.

Table 2.10 shows that the enzyme showed activity with xylan and was not active towards any other substrates, like CM-cellulose, PNPX, filter paper, casine and starch. It did not show any extra-, intra-- or cell-bound B-xylosidase.

Determination of $K_m$  Suitably diluted xylanase was incubated with different amounts of soluble larch wood xylan (3 - 15 mg) under the assay conditions. $K_m$ was determined from Lineweaver-Burk plot (Figure 4.11a) and was found to be 10 mg/ml.

When the same culture filtrate was assayed with 5 mg and 10 mg of soluble xylan, the activities obtained were 40 and 70 U/ml, respectively. Since, all the xylanase activities described in the text were estimated with 5 mg of xylan, it is possible to consider that the real values are almost double compared with the reported activities.

## DISCUSSION

Mineralization of plant biomass in the environment is mediated by a heterogeneous and interactive microflora,

**Table 2.10:** Activity of culture filtrate towards different substrates

| Substrate | Activity (U/ml) |
|---|---|
| Xylan | 34.90 |
| CM-cellulose | 0.54 |
| Filter paper | 0.21 |
| PNPX | 0 |
| Casein | 0 |
| Starch | 0 |

of which actinomycetes form an integral part. The activity of biodegradative actinomycetes is particularly evident under aerobic and neutral to alkaline pH conditions.

Recently, interest in thermostable enzymes has led to the discovery and isolation of organisms, which can grow at higher temperatures. The thermophilic xylan degrading bacteria especially, bacilli, clostridia and fungi are reported [35,37,42,51]. But investigations on thermophilic streptomycetes have been comparatively few [20,39]. The present study is related to the identification of a thermotolerant Streptomyces sp $T_7$ having high extracellular xylanase activity and much high enzyme productivity, compared to reported activity from other meso- and thermophilic actinomycetes. Table 2.11 represents a comparative xylanase profiles of various organisms with respect to temperature of production, activity and productivity. Productivity of xylanases from bacteria is much higher than the fungal and actinomycete xylanases and this is essentially due to the longer periods of fermentation in the case of latter. However, productivity of xylanase from Streptomyces $T_7$ was much higher (972 U/h/L) than the other reported xylanases from mesophilic as well as thermophilic actinomycetes. The productivities of xylanases from Streptomyces sp EC-1 [3], S. lividans [39] and S. afghaniensis [19] were 166, 694, 95.7 U/h/L, respectively. Among the fungi the xylanase activity produced by thermophilic strain T. aurantiacus is very high (366 U/ml) [41], while T. terrestris produces only 18.8 U/ml [40]. But the productivities are quite comparable, as T. aurantiacus takes 10 days to reach the maximal

**Table 2.11:** Comparison of xylanase productivities of representative microorganisms

| Organism | Temperature of growth (°C) | Optimum time (h) | Xylanase activity (U/ml) | Productivity (U/ml x $10^3$)/h/L | Reference |
|---|---|---|---|---|---|
| Bacillus sp W1 | 45 | 48 | 88.5 | 1843 | 35 |
| W2 | 45 | 48 | 112.0 | 2333 | 35 |
| C. stercorarium | 65 | 60 | 75.0 | 1250 | 37 |
| Chainia sp NCL-82-5-1 | 28 | 96 | 28.0 | 291 | 52 |
| Thermomonospora sp | 50 | 96 | 8.8 | 91.6 | 20 |
| Actinomadura sp MT 809 | 50 | 192 | 58.8 | 306 | 21 |
| S. cyaneus | 30 | 192 | 58.6 | 305 | 21 |
| S. flavogriseus | 30 | 48 | 46.0 | 958 | 26 |
| S. olivochromogenes | 37 | 192 | 30.7 | 161 | 53 |
| S. lividans 1326 | 40 | 72 | 46.0 | 958 | 39 |
| S. afghaniensis | 40 | 168 | 15.7 | 95.7 | 19 |
| Streptomyces $T_7$ | 50 | 72 | 70.0 | 972 | Present work |
| T. aurantiacus C436 | 45 | 240 | 365.8 | 1520 | 41 |
| T. terrestris ATCC 26917 | 48 | 18 | 18.8 | 1044 | 40 |
| T. byssochlamydoides | 50 | 72 | 6.0 | 83.3 | 43 |

activity whereas T. terrestris reaches that within 18 h.

Xylanases are induced enzymes and are best produced in media containing xylan or xylan rich natural substrates such as wheat bran, rice husk, bagasse, rice straw and wheat straw [3,21,53]. Paul and Varma [54] reported that Bacillus sp produced xylanase (5 U/ml) when grown on rice husk. The biosynthesis was enhanced by the addition of 0.2% cellobiose or xylose. The xylanase activities obtained were 8 and 10 U/ml, respectively. Brodel et al [38] reported that in substrate limited continuous or fed batch cultures C. thermolacticum secreted 20 - 26 U/ml of xylanase even when readily metabolisable compounds, such as glucose was used as a substrate and a 10-fold increase in xylanase yield was observed when compared with batch culture on xylan. These results demonstrated that the C. thermolacticum xylanases are constitutive. On the contrary, when C. stereorarium was grown on Walseth cellulose, the xylanase activity reached a value of 20 U/ml after 17 h incubation, but addition of sugars such as xylose, glucose and lactose completely stopped xylanase biosynthesis. Enzyme synthesis was resumed as soon as the sugar was consumed [37].

S. flavogriseus produced considerable amounts of xylanase when grown on xylan containing media. Comparatively lower yields of enzyme were obtained, when hay or Avicel served as the main carbon source [26]. Streptomyces $T_7$ showed higher xylanase production on wheat bran compared with xylan or cellulose. The activities obtained with 1% substrate were 16, 10 and 12 U/ml, respectively. In Chainia sp maximum activity of 28 U/ml was obtained in 72 h on xylan,

while 11 U/ml was reached after 96 h in wheat bran medium [52]. S. cyaneus and S. viridis produced 58 and 24 U/ml of xylanase, when grown on ball milled barley straw, while 37 and 18 U/ml of xylanase activities were produced when grown on oat spelts xylan. The thermophilic strains P. thermophila and T. vulgaris produced 3.9 and 5.0 U/ml when grown on xylan and the activities were higher than those produced on lignocellulose [21]. Similar observation was also reported for S. olivochromogenes and S. flavogriseus which produced 30.7 and 24.2 U/ml on oat spelts xylan whereas 15.0 and 4.9 U/ml were produced on wheat bran, respectively [53]. Streptomyces sp 3137 has been reported to produce xylanase induced in response to non-metabolizable, β-xylosides, which showed even better induction compared to xylan. This study concluded that although uptake of this inducer by the mycelia was observed, the uptake process was of diffusion and did not involve a specific transport system. Repression of xylanase synthesis occurred when α-D-xyloside or glucose was added to the culture medium [56].

The extracellular xylanase of Cryptococcus flavus was induced by β-methyl xyloside more effectively than xylan or xylose [57]. In contrast, the xylanase of M. albomyces was induced by growing this culture on xylose or xylan. But not by alkyl or aryl β-D-xylosides [58], whereas xylose, xylobiose and arabinose were identified as natural or direct inducers of xylanase from a strain of Aureobasidium pullulan and induced xylanase was repressed by glucose [59].

Coproduction of xylanases and cellulases have been described in many organisms [21,26,39]. Extracellular enzyme

preparations from *S. flavogriseus* and *S. olivochromogene* cultures grown on cellulose contained primarily cellulase activities but similar preparations from cultures grown on xylan containing materials possessed high levels of both cellulase and xylanase activities [53]. A thermotolerant *S. lividans* 1326 produced 50 U/ml of xylanase and 1 U/ml of cellulase when grown on xylan. A better production of both enzymes was observed when cellulose was used as a substrate [37]. Xylanase and glucose isomerase production by *Streptomyces* sp has also been reported by Park and Toma [25]. The simultaneous production of cellulase complex and glucose isomerase by *S. flavogriseus* on natural substrates such as hay or straw was reported [60]. When Avicel was used as a substrate, no significant quantities of glucose isomerase were detected, but when xylan was used as a substrate, large amounts of glucose isomerase and xylanase (46 U/ml) were detected [26]. A sclerotial actinomycete *Chainia* sp isolated in our laboratory secreted high levels of extracellular (4 U/ml) and low levels (1.4 U/ml) of cell bound glucose (xylose) isomerase and extracellular xylanase (11 U/ml) when grown in submerged culture on a wheat bran - yeast extract medium [61].

*Streptomyces* $T_7$ did not exhibit any other activities like cellulase, glucose isomerase or amylase when grown on wheat bran. Recently, there has been considerable interest in identifying cellulase-free xylanases for application in paper industry for the manufacture of low hemicellulose dissolving pulps [62]. Among thermotolerant actinomycetes, cellulase-free xylanase production by *Streptomyces* $T_7$ is

probably the first report. A low molecular weight cellulase-free xylanase from Chainia [63] as well as xylanase from alkalophilic Bacillus sp [64] have been investigated and patented from our laboratory.

The optimum temperatures for xylanases from mesophilic organisms were in the range of 50 - 60°C. But the xylanases from some extremely thermophilic strains possessed high temperature optima. For example, the optimum temperature of 78°C was reported for xylanase from B. stereothermophilus, while the optimum temperature for production was 65°C (Table 2.1) [17]. The xylanases from Streptomyces sp did not possess high temperature of optima. The xylanase from Thermomonospora sp possessed an optimum temperature of 80°C, while the cultivation temperature was 55°C [27]. The xylanase of T. aurantiacus has a temperature optimum of 75°C [41].

The temperature relationships of xylanases are better described in their thermostability properties. Table 2.12 shows the comparison of thermostability of xylanases (culture filtrate) from various microorganisms. Comparison of thermostability of various xylanases showed that xylanase from B. stereothermophilus was the most thermostable enzyme reported so far. Among the Streptomyces sp the xylanase from Streptomyces $T_7$ (present work) showed excellent thermostability (6 days at 50°C). The xylanase from Thermomonospora sp was the highly thermostable enzyme (half life 24 h at 65°C) among actinomycete xylanases [27]. Table 2.12 shows that the xylanases produced from thermophilic organisms are generally more stable than those from mesophilic strains.

**Table 2.12:** Thermostability of xylanases from various organisms

| Organism | Thermostability | Reference |
|---|---|---|
| B. stereothermophilus* 4125 | Half life 15 h at 75°C and stable for 5 days at 68°C | 17 |
| Thermomonospora sp* | Half life 24 h at 65°C | 27 |
| T. curvata* | Half life > 60 min at 70°C | 20 |
| T. chromogena* MT808 | Half life > 60 min at 70°C | 3 |
| Micromonospora LL23 | Half life 13 - 40 min at 70°C | 3 |
| S. viridis | Half life < 60 min at 70°C | 20 |
| Streptomyces sp EC1 EC3 | Half life 13 - 40 min at 70°C | 3 |
| S. lividans* 1326 | Half life 30 min at 60°C | 39 |
| S. flavogriseus | Half life 24 h at 40°C | 26 |
| Streptomyces sp* $T_7$ | Stable for 6 days at 50°C | Present work |
| T. aurantiacus* C436 | Half life 4 days at 60°C and stable for 3 days at 50°C | 41 |

*Thermotolerant or thermophilic organism.

Bacteria and actinomycetes produce xylanases generally at neutral pH while acidic pHs were favourable for production of fungal xylanases. Exceptions were from alkalophilic bacilli which exhibit pH optima in the range of 9 - 10 [35, 65], while acidophilic Bacillus sp 11-1S had an optimum pH of 3.5 - 4.0 for xylanase production [36]. There are no reports available on the acidophilic actinomycete xylanases whereas alkalophilic Streptomyces sp $VP_5$ producing xylanase at pH 10 was reported recently from our laboratory [66].

Considering the high xylanase activity as well as productivity of the thermotolerant Streptomyces $T_7$ strain, it is possible to speculate on its potential for industrial applications. Its optimal activity around pH 5.0 as well as its exceptional stability for prolonged periods at 50°C, would make it worthwhile for trying its applicability to the brewing industry in which filtration problems are considerably eased by the use of suitable xylanase enzymes. Currently, some of the xylanase preparations from fungal sources such as Aspergillus niger are being tried out effectively for this industrial application. The economic and technical feasibility of the $T_7$ enzyme to this and other applications appears to be worth investigating and would be taken up as a project for the future.

## REFERENCES


1. Brock, T.D. (1986) In "Thermophiles: General Molecular and Applied Microbiology", (Ed. Brock, T.D.) John Wiley and Sons, p.1.
2. McCarthy, A.J. (1987) FEMS Microbiol. Rev. **46**, 145.
3. Ball, A.S. and McCarthy, A.J. (1989) J. Appl. Bacteriol. **66**, 439.
4. Goodfellow, M. and Williams, S.T. (1983) Ann. Rev. Microbiol. **37**, 189.
5. Rowbotham, T.J. and Cross, T. (1977) J. Gen. Microbiol. **100**, 231.
6. McCarthy, A.J. and Broda, P. (1984) J. Gen. Microbiol. **130**, 2905.
7. Crawford, D.L. (1978) Appl. Environ. Microbiol. **35**, 1041.
8. Hankin, L., Poincelot, R.P. and Anagnostakis, S.L. (1976) Microb. Ecol. **2**, 296.
9. Stutzenberger, F.J., Kaufman, A.J. and Lossin, R.D. (1970) Can. J. Microbiol. **16**, 553.
10. Bellamy, W.D. (1977) Dev. Ind. Microbiol. **18**, 249.
11. VanZyl, W.H. (1985) Biotechnol. Bioeng. **27**, 1367.
12. Lacey, J. (1973) In "Actinomycetes: Characteristics and Practical Importance" (Eds. Sykes, G. and Skinner, E.A.) Academic Press, London, p. 231.
13. Fergus, C.L. (1964) Mycologia, **56**, 267.
14. Gregory, P.H. and Lacey, M.E. (1962) Nature, **195**, 95.
15. Cross, T. (1968) J. Appl. Bacteril. **31**, 36.
16. Donnison, A.M., Brockelesby, C.M., Morgan, H.W. and

Daniel, R.M. (1989) Biotechnol. Bioeng. **33**, 1495.

17. Gruninger, H. and Fiechter, A. (1986) Enz. Microb. Technol. **8**, 309.

18. Uchino, F. and Doi, S. (1967) Agric. Biol. Chem. **31**, 817.

19. Loginova, L.G., Yusupova, I. Kh. and Tashpulatov, Zh. (1981) Prikl. Biochim. Microbiol. **17**, 133.

20. McCarthy, A.J., Peace, E. and Broda, P. (1985) Appl. Microbiol. Biotechnol. **21**, 238.

21. Zimmermann, W., Winter, B. and Broda, P. (1988) FEMS Microbiol. Lett. **55**, 181.

22. Biely, P. (1985) Trends Biotechnol. **3**, 286.

23. Esteban, R., Villanueva, Jr. and Villa, T.G. (1982) Can. J. Microbiol. **28**, 733.

24. Panbangred, W., Fukusaki, E., Epifanio, E.C., Shinmyo, A. and Okada, H. (1985) Appl. Microbiol. Biotechnol. **22**, 259.

25. Park, Y.K. and Toma, M. (1974) J. Gen. Appl. Microbiol. **20**, 67.

26. Ishaque, M. and Kluepfel, D. (1981) Biotechnol. Lett. **3**, 481.

27. Ristroph, D.L. and Humphrey, A.E. (1985) Biotechnol. Bioeng. **27**, 832.

28. Nakanishi, K., Yasui, T. and Kobayashi, T. (1976) J. Ferment. Technol. **54**, 801.

29. Notario, V., Villa, T.G. and Villaneuva, J.R. (1979) J. Gen. Microbiol. **114**, 415.

30. Biely, P., Vrsanska, M. and Kratky, Z. (1980) Eur. J. Biochem. **108**, 313.

31. Kratky, Z. and Biely, P. (1980) Eur. J. Biochem. **112**, 367.

32. Biely, P., Petrakova, E. (1984) J. Bacteriol. **160**, 408.

33. Sunderam, T.K. (1988) J. Chem. Tech. Biotechnol. **42**, 308.

34. Sunderam, T.K. (1986) In "Thermophiles: General Molecular and Applied Microbiology (Ed. Brock, T.D.) John Wiley and Sons, N.Y. p. 75.

35. Okazaki, W., Akiba, T., Horikoshi, K. and Akahoshi, R. (1984) Appl. Microbiol. Biotechnol. **19**, 335.

36. Uchino, F. and Nakane, T. (1981) Agric. Biol. Chem. **45**, 1121.

37. Berenger, J.F., Frixon, C., Bigliardi, J. and Creuzet, N. (1985) Can. J. Microbiol. **31**, 635.

38. Brodel, B., Samain, E. and Debeire, P. (1990) Biotechnol. Lett. **12**, 65.

39. Kluepfel, D., Shareck, F., Mondou, F. and Morosoli, R. (1986) Appl. Microbiol. Biotechnol. **24**, 230.

40. Merchant, R., Merchant, F. and Margaritis, A. (1988) Biotechnol. Lett. **10**, 513.

41. Yu, E.K.C., Tan, L.U.L., Chan, M.K.H., Deschatelets, L. and Saddler, J.N. (1987) Enz. Microb. Technol. **9**, 16.

42. Matsuo, M., Yasui, T. and Kobayashi, T. (1977) Agric. Biol. Chem. **41**, 1593.

43. Yoshika, H., Chavanich, S., Nilubol, N. and Hayashida, S. (1981) Agric. Biol. Chem. **45**, 579.

44. Ganju, R.K., Vithayathil, P.J. and Murthy, S.K. (1989) Can. J. Microbiol. **35**, 836.

45. Mandels, M. and Weber, J. (1969) Adv. Chem. Ser. **95**, 391.

46. Miller, G.L. (1959) Anal. Chem. **31**, 426.

47. Kunitz, M. (1947) J. Gen. Physiol. **30**, 291.

48. Kluepfel, D. and Ishaque, M. (1982) Dev. Ind. Microbiol. **23**, 389.

49. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. **193**, 265.

50. Cohn, E.T., Huges, W.L.Jr. and Weare, H.H. (1947) J. Am. Chem. Soc. **69**, 1753.

51. Lee, S.F., Forsberg, C.W. and Rattray, J.B. (1987) Appl. Environ. Microbiol. **53**, 644.

52. Srinivasan, M.C., Vartak, H.G., Power, V.K., Rele, M.V. and Bastawde, K.B. (1984) Biotechnol. Lett. **6**, 715.

53. MaKenzie, C.R., Bilous, D., Schneider, H. and Kenneth, G.J. (1987) Appl. Environ. Microbiol. **53**, 2835.

54. Paul, J. and Varma, A.K. (1990) Biotechnol. Lett. **12**. 61.

55. Nakanishi, K. and Yasui,T. (1980) J. Ferment. Technol. **58**, 171.

56. Nakanishi, K. and Yasui, T. (1980) Agric. Biol. Chem. **44**, 1885.

57. Yasui, T., Nguyen, B.T. and Nakanishi, K. (1984) J. Ferment. Technol. **62**, 353.

58. Maheshwari, R. and Kamalam, P.T. (1985) J. Gen. Microbiol. **131**, 3017.

59. Leathers, T.D., Detroy, R.W. and Bothast, R.J. (1986) Biotechnol. Lett. **8**, 867.

60. Kluepfel, D., Biron, L. and Ishaque, M. (1980) Biotechnol. Lett. **2**, 309.

61. Srinivasan, M.C., Vartak, H.G., Powar, V.K. and Khire, J.M. (1983) Biotechnol. Lett. **5**, 611.

62. Jurasek, L. and Paice, M. (1986) Chemtech. **16**, 360.

63. Srinivasan, M.C., Vartak, H.G., Rele, M.V. and Bastawde, K.B. (1986) Indian Patent No. 1092/DEL/86.

64. Srinivasan, M.C., Rele, M.V., Balkrishnan, H. and Dutta-Choudhary, M. (1988) Indian Patent No. 984/DEL/88.

65. Horikoshi, K. and Atsukawa, Y. (1973) Agric. Biol. Chem. **37**, 2097.

66. Vyas, P., Chauthaiwale, V., Phadatare, S., Deshpande, V. and Srinivasan, M.C. (1990) Biotechnol. Lett. **12**, 225.

# CHAPTER 3

# PURIFICATION OF XYLANASE

# SUMMARY

The conventional purification methods such as preparative PAGE, isoelectric focusing and gel filtration were attempted for purification of *Streptomyces* xylanase. All the three methods yielded an electrophoretically homogeneous preparation of xylanase. The purified xylanase was cationic in nature and contained a single protein component. The gel filtration method was adopted owing to its ease of operation and rapidity. Using this method the xylanase was purified 41-fold over the culture filtrate and the specific activity of 412 was achieved which was higher than that obtained with the other two methods.

# INTRODUCTION

Xylanases have been separated and purified by using conventional protein purification techniques. The culture filtrate which is used as the starting material is usually concentrated by freeze drying or by flash evaporation under reduced pressure prior to the chromatographic purification procedures. Alternatively, the protein component of the filtrate containing xylanase is precipitated with cold ethanol (-18°C) or with ammonium sulphate at 55 - 90% saturation [1].

The purification techniques include:

**1. Ion exchange chromatography**

Generally, two types of ion exchangers are used in the purification of xylanases viz. (i) Anion exchangers, DEAE-cellulose [2-4], DEAE-Sephadex [5] and Duolite A-2 [6] (ii) cation exchangers,Amberlite resins CG-50 [7], IRC-50 [8], CM-cellulose [9,10], cellulose phosphate [11], cellex-CM [12], CM-Sephadex [13], Duolite C-10 [6], SE-Sephadex [14] and hydroxyapatite [15].

**2. Gel-permeation chromatography**

Xylanases can be purified by molecular sieving through Sephadex G-50 [3,16,17], Sephadex G-75 [18], Sephadex G-100 [19], Bio-Gel P-10 [20], Bio-Gel P-100 [21-23]. Further steps used in the purification were isoelectric focusing [24] and preparative polyacrylamide gel electrophoresis (PAGE) [25].

Purification can also be achieved by affinity binding to xylan [7].

## Xylanases from bacteria

### Bacillus sp.

Esteban and coworkers [26] reported the purification of two xylanases from Bacillus circulans WL-12. Xylanase I was separated by adsorbing the culture filtrate on DEAE-Bio-Gel A (pH 7.3). The elution of adsorbed xylanase I was carried out with 2 M NaCl. This fraction was active against both pure xylan and p-nitrophenyl-β-D-xyloside (pNPX). The unadsorbed xylanase II was adjusted to pH 4.0 and filtered through CM-Bio-Gel A, eluted with 2 M NaCl, showed activity on pure xylan but not on pNPX. Fraction I was resolved into two enzymes viz. endo-xylanase and β-xylosidase by isoelectric focusing in the pH range 3.5 - 10.0. Two xylanases designated A and N from alkalophilic Bacillus sp. C-125 were purified by an ammonium sulphate precipitation followed by gel filtration on Bio-Gel P-30, chromatography on DEAE-cellulose and gel filtration on Sephadex G-75 [27]. Xylanases from Bacillus subtilis PAP 115 were purified by using SP-Sephadex C-50 and Sephacryl S-200 columns [28]. An endo-xylanase of Bacillus sp. 11-1S was purified by ammonium sulphate fractionation followed by SE-Sephadex column chromatography. Elution was carried out with 50 mM acetate buffer, pH 3.2 containing 100 mM NaCl [29]. An endo-xylanase was purified from the culture filtrate of Bacillus coagulans 26. Adsorption of culture broth on DEAE-Bio-Gel A (pH 7.5) and elution with 2 M NaCl separated the fraction which was active against pNPX but not on pure xylan. The non-adsorbed enzyme was adjusted to pH 4.5and applied to CM-Bio-Gel A. Elution with 2 M

NaCl separated endo-xylanase active against xylan [30]. Purification of two endo-xylanases from culture filtrate of two alkalophilic and thermophilic _Bacillus_ sp. W1 (JCM 2888) and W2 (JCM 2889) was reported [31,32]. The procedure involved ammonium sulphate fractionation and chromatography on DEAE-Toyopearl 650 M (pH 6.2). Component I was eluted with the same buffer while component II was eluted with a linear gradient of 0.2 - 0.6 M NaCl in the same buffer of pH 6.2. Endo-xylanase II was rechromatographed on the same exchanger. Then both the components were separately applied on Toyopearl HW-55S equilibrated with 50 mM phosphate buffer (pH 7.0) containing 0.2 M NaCl. Panbangred and coworkers [33] purified endo-xylanase produced by _Bacillus pumilus_ IPO. The purification involved precipitation of culture filtrate with ammonium sulphate followed by chromatography on DEAE-Sephadex A-50 (pH 6.5) and CM-Sephadex C-50. Elution was carried out with the same buffer (pH 6.5) and the buffer containing a linear gradient of 0 - 0.3 M NaCl in phosphate buffer, respectively.

**_Clostridium_ sp.**

Three main xylanase components were separated by ion exchange chromatography on DEAE-Trisacryl M from the 0 - 40% saturated ammonium sulphate preparation obtained from _Clostridium stercorarium_ culture supernatant. Two peaks were obtained: IA (xylanase A) and IB (xylanase B and C). Xylanase B and C were separated by repeated chromatography on Bio-Gel P-100 [34]. Two endo-xylanases produced by _Clostridium acetobutylicum_ ATCC 824 were purified to homogeneity by sequential chromatography on CM-

Sepharose gel, hydroxyapatite (pH 6.0), phenyl Sepharose (pH 6.0) and Bio-Gel P-150 [35].

**Xylanases from Streptomyces sp.**

Fractionation of extracellular xylan hydrolases of a strain of Streptomyces exfoliatus MC1 revealed the presence of five components [3]. The protein was first salted out and was subjected to gel filtration on Sephadex G-50. Further, it was resolved into two components: xylanase I and II. Xylanase II was pure but I showed many bands. Fractionation on DEAE-cellulose column resolved xylanase I into five fractions (Ia-e). The major fractions Ib and Id were studied in detail. Marui and coworkers [21] reported three xylanases X-I, X-II-A and X-II-B which were purified from the culture filtrate of Streptomyces sp. 3137. The purification involved ultrafiltration with DIAFLO UM-10, chromatography on DEAE-Sephadex A-25 followed by gel filtration on Bio-Gel P-100 and isoelectric focusing with Servalyt 6 ~ 8 or 9 ~ 11. An endo-xylanase produced by mutant 8-7 of Streptomyces lividans 1326 [36] was purified by anion exchange chromatography on Accell QMA. Elution was carried out with the phosphate buffer (pH 6.0) containing 100 mM NaCl. Final purification was achieved by HPLC on a protein pak DEAE 5PW anion exchange column at pH 8.5. Xylanase from Streptomyces xylophagus nov. sp. [37] was purified of ammonium sulphate fractionation and chromatography on DEAE-cellulose. Nakagima and coworkers [2] purified the xylanase from Streptomyces sp. KT-23 by chromato-

graphy on DEAE-cellulose and Bio-Gel P-100. The xylanases from Streptomyces flavogriseus 45-CD and Streptomyces olivochromogenes were purified by ion exchange chromatography on DEAE-Bio-Gel A followed by HPLC column CM-3SW and TSK-DEAE-3SW respectively [39]. Two endo-xylanases from Chainia sp. were separated and endo-xylanase II was characterized in detail [40]. Purification involved: a negative adsorption on DEAE-cellulose, chromatography on DEAE-fractogel TSK-650S followed by gel filtration on Sephadex G-50 (pH 7.0). The two xylanases were separated only on Sephadex G-50 in the final purification step.

### Xylanases from fungi

The purification of xylanases from certain thermophilic fungi is also reported. Margaritis and coworkers [22] reported the use of Bio-Gel P-4 and Bio-Gel P-100 for the purification of xylanase from Sporotrichum thermophile. Thermostable xylanase from Talaromyces byssochlamydoides YH-50 was fractionated into three components, tentatively named X-a, X-b-I and x-b-II during the purification steps [41]. They were purified by consecutive column chromatographies on Sephadex G-50, DEAE-Sephadex A-50 and Sephadex G-100. An endo-xylanase produced by thermophilic fungus Humicola lanuginosa was purified to homogeneity by gel filtration on Sephadex G-100 and chromatography on DEAE-Sepharose CL-6B [42]. The xylanase from a thermophilic fungus Malbranchea pulchella var. sulfurea 48 was purified from a culture filtrate by ammonium sulphate fractionation and column chromatographies on DEAE-cellulose and CM-Sephadex [43.44]. A thermostable endo-xylanase was

purified from the culture filtrate of a thermophilic fungus *Thermoascus aurantiacus* C436 using a single chromatographic step on SP Sephadex C-50 [45]. Fractionation of xylanolytic enzyme system of the thermophilic fungus *Chaetomium thermophile* var. *coprophile* revealed five different components of extracellular xylanase. Two xylanases (I and II) purified to homogeneity by a combination of ion exchange and gel filtration chromatographic procedures [46].

The xylanases from mesophilic fungal species such as *Aspergillus* [47,48] and *Trichoderma* [49,50] are well studied. Frederick and coworkers [51] purified two endo-xylanases from a crude *Aspergillus niger* preparation. The first fraction was purified by Ultragel ACA 54 and SP-Sephadex G-25 (pH 4.5) while the other fraction by Sephadex G-50 and SP-Sephadex C-25 (pH 5.8) in 0 - 0.2 M NaCl gradient. The crude xylanase from *Schizophyllum commune* was chromatographed on DEAE-Sephadex A-50. Xylanase A was separated from other xylanases and CM-cellulases from the *S. commune* enzyme complex. The xylanase A fraction was further purified on Sephadex G-50 [52]. A single component of xylanase was purified from the culture filtrate of *Sclerotium rolfsii* UV-8 mutant by gel filtration on Sephadex G-75 and chromatography on DEAE-Sephadex A-50. Further separation of xylanase was achieved by isoelectric focusing [53]. The endo-xylanases (I and II) of *Cryptococcus albidus* were separated on DEAE-cellulose column. The xylanase fraction I was not adsorbed but was eluted in the void volume. It was further purified by adsorption on CM-Sephadex C-50 column and elution with 0.45 M

NaCl gradient. Xylanase fraction II which was adsorbed on DEAE-cellulose column was eluted with 0.25 M NaCl gradient [54]. Recently, Morosoli and coworkers [55] purified endo-xylanase of C. albidus by applying the culture filtrate to a DEAE-cellulose column (pH 7.0), coupled in a series with a hydroxylapatite column which was washed with 100 ml of 0.2 M phosphate buffer (pH 7.0) and the adsorbed enzyme was finally eluted with 0.4 M phosphate buffer (pH 7.0). The enzyme preparation was then subjected to isoelectric focusing in 5% polyacrylamide gel rods wherein two protein bands bearing xylanase activity were separated at their isoelectric pH , 5.3 and 5.7, respectively.

### Criteria of purity

The purity of xylanases was reported using cathodic or anodic disc gel electrophoresis, SDS-PAGE, isoelectric focusing and ultracentrifugation [27,35,36].

The homogeneity of xylanases from B. subtilis was confirmed by SDS-PAGE [28]. Marui and coworkers [5] confirmed the purity of three endo-xylanases from Streptomyces sp. 3137 by carrying out PAGE and isoelectric focusing at pH 7.1, 10.06 and 10.26 respectively. The purity of three endo-xylanases of S. exfoliatus was confirmed on PAGE at pH 8.6 [3].

Isoelectric focusing and SDS-PAGE were reported for homogeneity of xylanase from T. aurantiacus C436 [45], while homogeneity of xylanase from T. byssochlamydoides YH50 was examined by disc PAGE at pH 8.3. The purity of five endo-xylanases from A. niger was checked on PAGE and isoelectric focusing [56-58].

# MATERIALS AND METHODS

## Materials

β-Alanine N,N,N'N'-tetramethyl ethylene diamine (TEMED), Coomassie Brilliant Blue G-250, DEAE-cellulose, N,N-methylene-bis-acrylamide were purchased from Sigma Chemical Co., USA. The suppliers of the following chemicals are indicated in parentheses: Ultrafiltration membranes (Amicon Corporation, USA), Sephadex G-50 (Pharmacia, Sweden), Ampholine (LKB, Sweden). All other chemicals used were of analytical grade.

## Methods

**Determination of protein** Protein was determined by one of these following methods:

Lowry's method [59]

Spectrophotometric method [60]: The method was based on the measurement of absorbance at 280 nm. A correction for the nucleic acid and ultraviolet absorbing impurities was made by the following equation:

$$\text{mg of protein/ml} = 4/7[2.3(O.D._{280} - O.D._{340}) - (O.D._{260} - O.D._{340})]$$

Crystalline bovine serum albumin (BSA) was prepared according to the procedure of Cohn *et al.* [61] and used as a standard for both the methods.

**Source of enzyme** Xylanase was produced by growing *Streptomyces* sp. $T_7$ in a medium containing 5% wheat bran as a carbon source. After cultivation for 72 h at 50°C, the cells and solid residues were removed from the culture broth by centri-

fugation (9,000 g, 30 min) and the clear supernatant liquid was used as an enzyme source.

**Alcohol precipitation** The clear culture filtrate was concentrated by alcohol precipitation. Chilled culture filtrate was precipitated with 3 volumes of prechilled distilled ethanol with constant stirring and allowed to stand at 0°C for 3 - 4 h for complete precipitation. The precipitate was recovered by centrifugation (9,000 g, 30 min) and stored at -20°C until further use.

**Ion exchange chromatography (Batchwise)** DEAE-cellulose was regenerated with 1 N NaOH and 1 N HCl [62] and equilibrated with 10 mM sodium phosphate buffer, pH 7.0 or 8.0. This equilibrated DEAE-cellulose (100 mg) was suspended in enzyme solution (5 ml, 2.5 mg) for 30 min and unadsorbed proteins were recovered by filtration through Whatman No. 1 filter paper. The DEAE-cellulose cake was washed atleast twice with the buffer and the washings were pooled with the first filtrate.

**PAGE** PAGE was carried out in 7.5% gels at pH 4.3 according to Maurer [63] using basic fuchsin as a marker and β-alanine acetic acid (pH 4.5) as the bath buffer. The gels were stained with Coomassie Brilliant Blue G-250 for visualizing the protein band and destained with distilled water.

**Location of xylanase band in the gel** The analytical disc gel electrophoresis was carried out in two 7.5% gels (pH 4.3). Fifty µg (each) of protein was loaded on both the gels. After the electrophoresis was over, as monitored by the movement of the marker dye, one gel was kept for staining

in Coomassie Brilliant Blue G-250 and the other was cut into 8 equal parts. Each piece was crushed finely and the enzyme was eluted with 50 mM acetate buffer, pH 5.0. The xylanase activity of each fraction was estimated. The correspondence between protein band and the activity was determined.

## Purification methods

All the purification steps were carried out at 4°C unless otherwise mentioned.

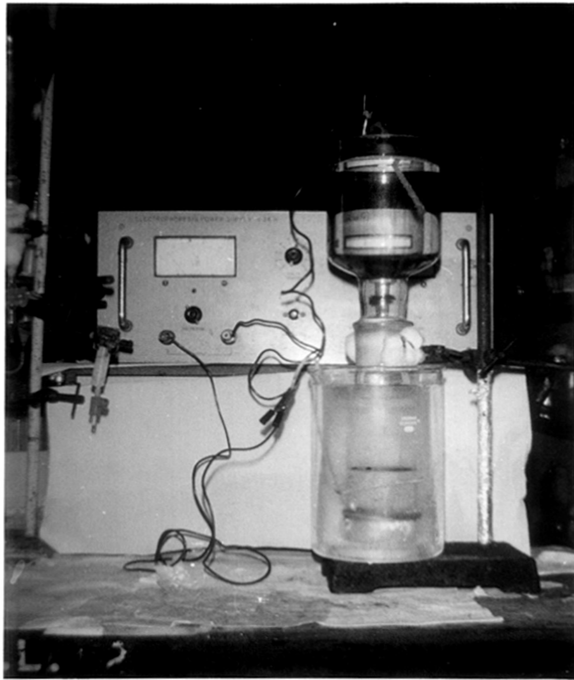
**I. Preparative PAGE**

A 7.5% polyacrylamide gel (pH 4.3) was prepared in a glass column (20 x 5 cm) with gel height of 15 cm. Upper chamber (anodic) was a glass reservoir (23 x 9 cm) with a B-50 male joint which could fit into the glass column having B-50 female joint. A glass beaker of 2 L capacity in which the gel column was immersed, served as the lower chamber (cathodic) (Fig. 3.1). β-alanine acetic acid buffer (pH 4.5) was used as the bath buffer.

The culture filtrate (125 ml) step I was concentrated by ethanol precipitation. The precipitate (1.5 g, 1240 U) was dissolved in 5 ml of 50 mM acetate buffer, pH 5.0. The undissolved solids from the precipitate were separated by centrifugation and the clear supernatant (step II) was loaded on the gel. Electrophoresis was continued till the marker band moved about 10 - 12 cm towards cathode (65 h).

The gel was removed and a thin vertical strip was cut and stained with Coomassie Brilliant Blue G-250 to reveal the protein pattern of the sample. The strip was replaced

**Fig. 3.1:** The preparative PAGE of the xylanase



in the gel and the portion of the gel corresponding (located from the analytical PAGE data) was cut. The gel was homogenised and the xylanase was eluted with 30 - 40 ml of 50 mM acetate buffer, pH 5.0. The traces of xylanase were recovered by eluting twice with 20 ml of the same buffer. The filtrate and washings were pooled and concentrated to 10 ml (step III) by freeze drying. The enzyme was dialysed and further purified on DEAE-cellulose in a batchwise treatment at pH 7.0 and concentrated again by freeze drying to 1.5 ml (step IV).

## II. Preparative isoelectric focusing

Preparative isoelectric focusing was performed as described by LKB (Application note, LKB Produkter AB, Stockholm, Sweden) in the LKB multiphore apparatus using the LKB tray (24.5 x 11 x 0.5 cm) and ampholines in the pH range 3.5 - 10.0. Ultrodex was substituted with 24 strips (5 x 1 x 0.5 cm) of foam (polyurethane strip) [64]. The strips were soaked in ampholine-water mixture (2% w/v) and placed horizontally on the tray. The anodic strip was soaked in 1 M $H_3PO_4$ and cathodic strip in 1 M NaOH.

The alcohol precipitated enzyme (step II) (1.25 g, 1260U) was dissolved in 4 ml of ampholine water solution. The strips were dipped in enzyme-ampholine solution and replaced on the tray. The electrofocusing was carried out with a constant current of 12 mA and a constant power of 8 W for 18 h. After termination of the run, foam strips were squeezed and the eluate was collected in different test tubes and assayed for xylanase activity. The pH of

each fraction was determined. The xylanase positive fractions were pooled and dialysed extensively against distilled water and concentrated by freeze drying to 1.2 ml (step III).

**III. Gel filtration**

The culture filtrate (400 ml) (step I) was concentrated by precipitating the enzyme with ethanol. The precipitate (4 g, 10,600 U) was dissolved in 80 ml of 10 mM sodium phosphate buffer, pH 8.0. The undissolved solid particles from the precipitate were separated by centrifugation and the clear supernatant (step II) was further purified on DEAE-cellulose in a batchwise treatment. The filtrate and washings were pooled and ultrafiltered through an Amicon UM-10 membrane to concentrate the sample to 12 ml (step III). A 1·5ml portion of this enzyme (9 mg, 931 U) was applied to a Sephadex G-50 column (1.5 x 110 cm.) equilibrated with 50 mM potassium phosphate buffer, pH 7.2. The fractions were collected at a rate of 2 ml/10 min and estimated for xylanase activity. The active fractions were pooled, dialysed against water and concentrated by freeze drying (step IV).

Purity of xylanase purified by various methods was checked by analytical PAGE at pH 4.3.

## RESULTS

The preliminary work on purification was directed towards standardization of a purification procedure which is simple, inexpensive, easy to operate and at the same time would give good yields of enzyme together with high specific activity. The conventional purification methods such as PAGE, isoelectric focusing and gel filtration were attempted.

**Preparative PAGE** When the xylanase was subjected to preparative PAGE and a thin strip from the gel was cut into 8 equal parts, the 4th fraction showed xylanase activity corresponding to the middle band of protein (Fig. 3.2a,b).

Table 3.1 shows that the xylanase was purified almost 16-fold over the culture filtrate. The specific activity attainded in the final step was 176. The purified xylanase showed a single band on PAGE at pH 4.3 (Fig. 3.3a).

**Preparative isoelectric focusing** Figure 3.4 shows the fractionation of Streptomyces xylanase by isoelectric focusing. The fractions 10 - 16 showed xylanase activity. The highest activity was obtained at pH 7.8. The purification was 33-fold over the culture filtrate and a specific activity of 331 was obtained (Table 3.2). The analytical disc PAGE showed a single band on 7.5% acrylamide gel at pH 4.3 (Fig. 3.3b).

**Gel filtration** Figure 3.5 shows the elution pattern of xylanase from Sephadex G-50. The fractions 42 - 60 showed the xylanase activity. The fractions 45 - 57 were pooled.

**Fig. 3.2:** PAGE of xylanase

(a) Analytical PAGE of alcohol precipitated xylanase at pH 4.3.

(b) Location of xylanase band

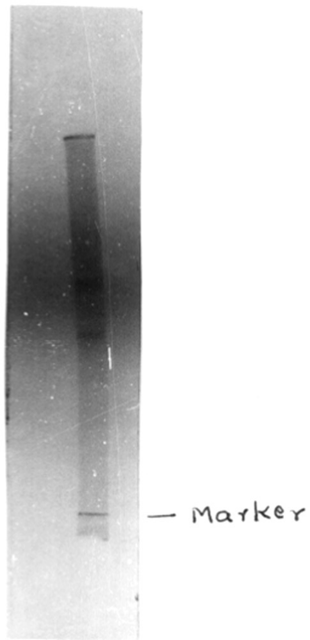



Fig 3.2 a

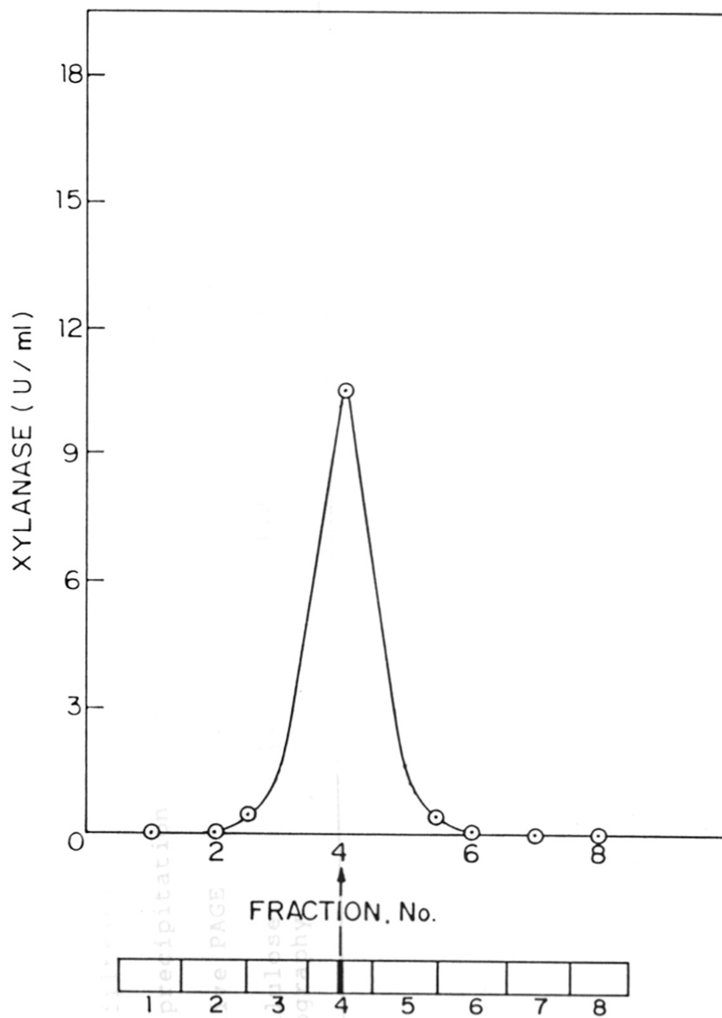



Fig 3.2 b

**Table 3.1:** Purification of *Streptomyces* xylanase by PAGE

| Step | Fraction | Total volume (ml) | Total Protein (mg) | Total activity (U) | Specific activity (U/mg) | Purification (fold) |
|---|---|---|---|---|---|---|
| I | Culture filtrate | 125 | 250 | 2775 | 11.1 | 1.00 |
| II | Ethanol precipitation | 5 | 30 | 1240 | 41.3 | 3.73 |
| III | Preparative PAGE | 10 | 2.2 | 235 | 107 | 9.80 |
| IV | DEAE-Cellulose chromatography | 1.5 | 1.2 | 212 | 176 | 15.80 |

**Fig. 3.3:** Homogeneity of xylanase on PAGE at pH 4.3.

a Preparative PAGE

b Isoelectric focusing

c Gel filtration

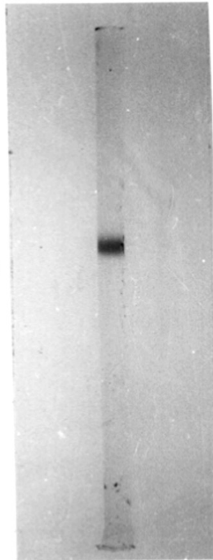

Marker.

**Fig. 3.4:** Fractionation of xylanase by isoelectric focusing

(o) xylanase activity (Δ) pH

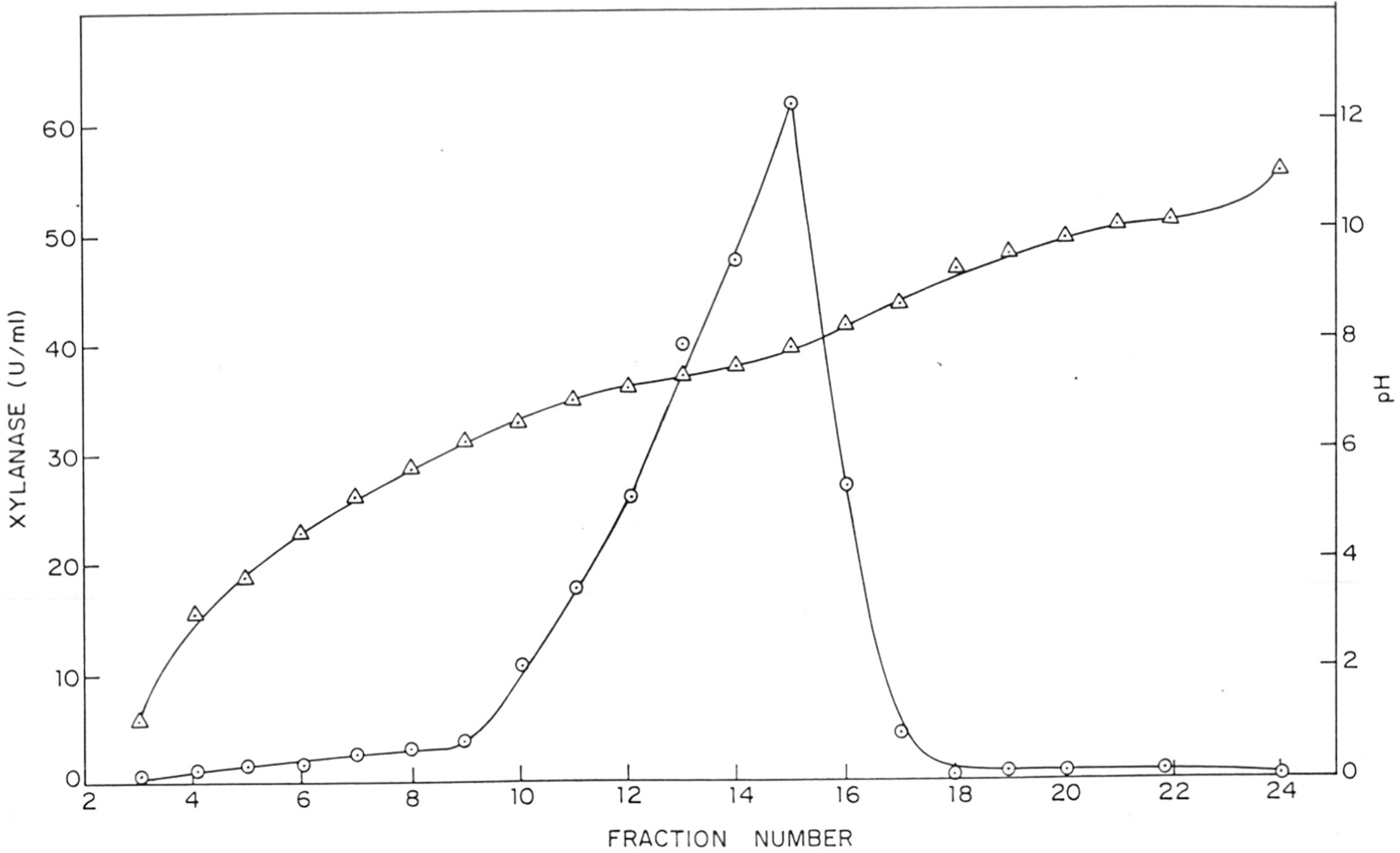
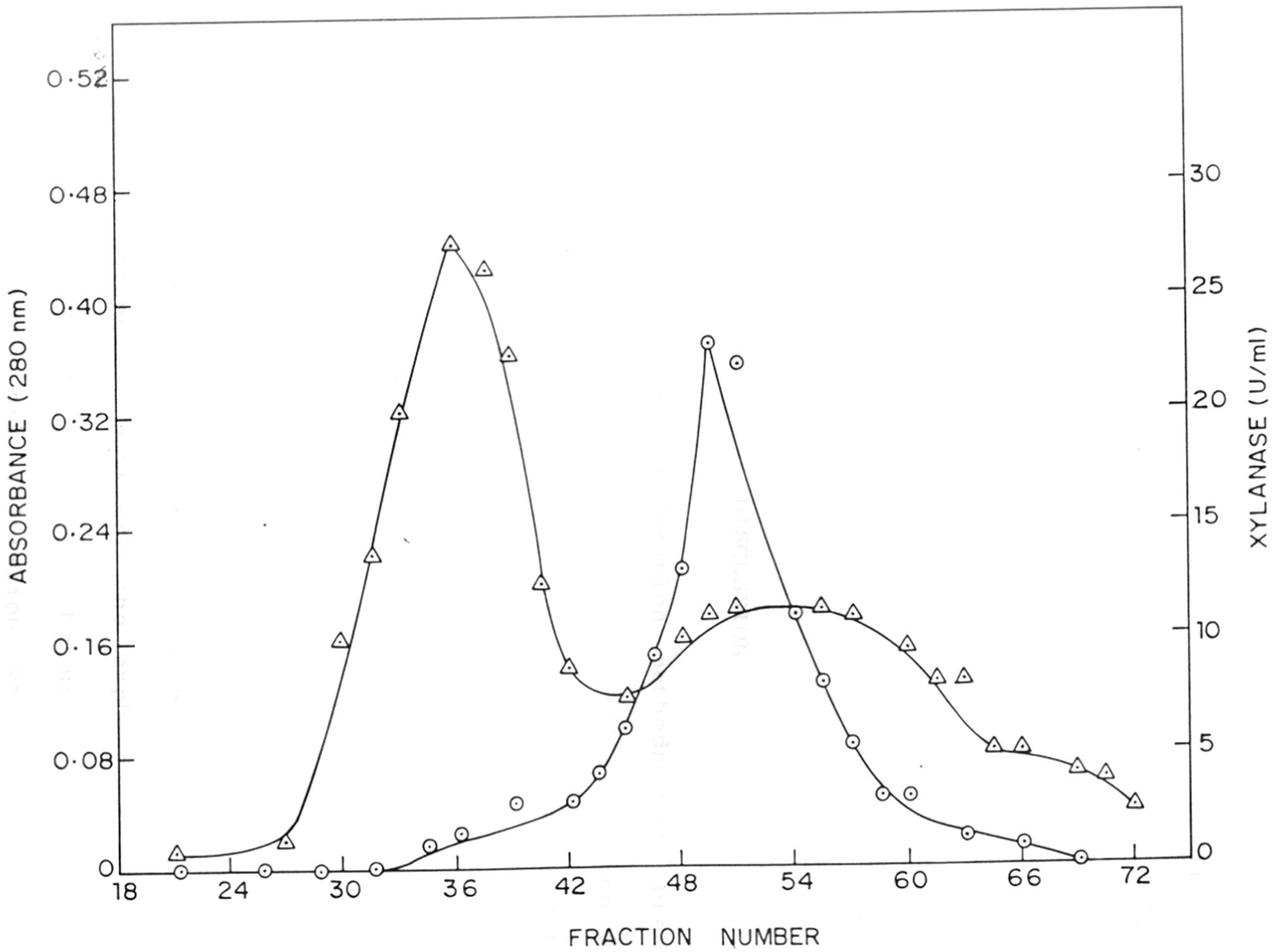

XYLANASE (U/ml)
0
10
20
30
40
50
60
pH
0
2
4
6
8
10
12
2
4
6
8
10
12
14
16
18
20
22
24
FRACTION NUMBER

**Table 3.2:** Purification of _Streptomyces_ xylanase by isoelectric focusing

| Step | Fraction | Total volume (ml) | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Purification (fold) |
|---|---|---|---|---|---|---|
| I | Culture filtrate | 100 | 300 | 3100 | 10.1 | 1.00 |
| II | Ethanol precipitation | 4 | 28.4 | 1260 | 44.3 | 4.38 |
| III | Isoelectric focusing | 1.2 | 1.12 | 371.5 | 331 | 33.00 |

**Fig. 3.5:** Separation of xylanase on Sephadex G-50 (o) xylanase activity (△) absorption at 280 nm.


ABSORBANCE (280 nm)
0·52
0·48
0·40
0·32
0·24
0·16
0·08
0
18
24
30
36
42
48
54
60
66
72
FRACTION NUMBER
XYLANASE (U/ml)
30
25
20
15
10
5
0

The pooled sample showed a single band on analytical PAGE in 7.5% gel at pH 4.3 (Fig.3.3c). It can be seen that Streptomyces $T_7$ xylanase contained a single protein component with enzyme activity. Table 3.3 summarizes the results on purification of xylanase by gel filtration. The enzyme was purified 41-fold over the culture supernatant. The specific activity reached in the final step was 412.

Although the yield of the enzyme obtained by the above three purification procedures was almost same, the specific activity of the enzyme purified by gel filtration was highest. Moreover, Sephadex G-50 column could be reused several times. Owing to its ease of operation and rapidity this method was adopted for purification of Streptomyces xylanase.

## DISCUSSION

All the three purification methods viz. preparative PAGE, isoelectric focusing and gel filtration on Sephadex G-50 yielded an electrophoretically homogeneous preparation of xylanase. However, the enzyme purified by preparative PAGE showed poor yield and low specific activity. Moreover, acrylamide impurities associated with the purified protein could not be removed completely by ion exchange chromatography. The preparative isoelectric focusing was simple and easy to operate. The specific activity of the enzyme obtained through this purification method was also significantly higher. However, the ampholines are expensive and hence are not affordable to use routinely. Against this background gel filtration was inexpensive, simple

**Table 3.3:** Purification of Streptomyces xylanase by gel filtration

| Step | Fraction | Total volume (ml) | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Purification (fold) |
|---|---|---|---|---|---|---|
| I | Culture filtrate | 400 | 1200 | 12,000 | 10.0 | 1.00 |
| II | Ethanol precipitation | 80 | 256 | 10,600 | 41.4 | 4.14 |
| III | DEAE-Cellulose chromatography | 12 | 116 | 7,462 | 64.4 | 6.44 |
| IV | Sephadex G-50 | 3 | 1.95 | 805 | 412.8 | 41.30 |

and the specific activity of the enzyme so purified was higher than the above two methods. In view of its merits this purification method was routinely used in the present work.

The various methods reported for purification of xylanases from prokaryotes or eukaryotes mostly include ion exchange chromatography, gel filtration on Sephadex or molecular sieving through Bio-Gel. Isoelectric focusing was reported where more than one xylanases have to be separated [5]. Preparative PAGE was not the method of choice mainly because of the multiplicity of xylanases and due to certain other disadvantages mentioned earlier.

**Xylanases from bacteria**

The endo-xylanase from an acidophilic and thermophilic Bacillus sp. 11-1S was purified 7-fold using a single purification step viz. chromatography on SE-Sephadex [29]. Horikoshi and Atsukawa [10] have reported 26-fold purification of xylanase from alkalophilic Bacillus sp. C-59-2. B. pumilus xylanase was purified 18-fold with a recovery of 46% using DEAE and CM-Sephadex column chromatography [33]. The above xylanase after purifying further on TSK HW polyvinyl gel gave an overall yield of 22% with a 90-fold purification [65]. Xylanases A and N from alkalophilic Bacillus sp. C-125 were purified 16- and 24-fold by ion exchange chromatography and molecular sieving with 5 and 26% recovery respectively [27]. The xylanase from B. subtilis was purified 25-fold with an overall yield of 21% [28]. Endo-xylanases from the two alkalophilic and thermophilic

Bacillus sp. W1 and W2 were fractionated into two active components (I and II) with 100 - 300-fold purification [31]. Xylanases A, B and C from C. stercorarium were purified about 12-fold over the culture supernatant [34]. Two endo-xylanases A and B from C. acetobutylicum were separated on CM-Sepharose. Xylanase A did not adsorb while adsorbed xylanase B was eluted using NaCl gradient. Hydroxylapatite was used for separating the other enzymes associated with xylanase A. Hydrophobic chromatography with Phenyl Sepharose which could bind xylanase was used and adsorbed xylanase was eluted with Triton X-100 and then freed by rechromatography on hydroxylapatite. Xylanase A and B were purified 19 and 23-fold, respectively [35].

**Xylanases from Streptomyces sp.**

The endo-xylanase from S. lividans 1326 was purified 33-fold over the culture supernatant [36] whereas the xylanase from S. xylophagus was purified 276-fold [4]. The xylanase from mesophilic Streptomyces sp. have also been shown to consist of only one xylanase component [2,38]. The xylanase from Streptomyces sp. KT-23 was purified 10-fold over the culture supernatant and was recovered in 20% yield [2]. The crude xylanase from Streptomyces sp. 3137 was fractionated into three components which were purified approximately 3 - 4-fold [5]. The xylanases (endo I and endo II) were separated from the culture filtrate of Chainia sp. on Sephadex G-50. The endo-xylanase II was purified 31-fold over the culture filtrate and was studied in detail. The xylanase II of Chainia sp. [40]

remained unadsorbed on DEAE-cellulose indicating that it is cationic in nature similar to our Streptomyces $T_7$ xylanase. Enzyme systems of most of the reported streptomycetes were shown to consist of a single xylanase component. However, the enzyme systems of S. ostreogriseus [65] and S. exfoliatus MCl [3] revealed the presence of five species of enzymes.

### Xylanases from fungi

A major xylanase component from crude culture filtrate of T. aurantiacus C436 was found to be well separated from other proteins by purification on SP-Sephadex C-50 at pH 4.4. A yield of 47% with 3-fold purification was obtained [45]. The xylanase from thermophilic fungus T. byssochlamydoides YH-50 was separated from the culture filtrate into three components and the purified xylanase components showed 17-fold increase in specific activity (112 - 117) [41]. The endo-xylanase from thermophilic fungus H. lanuginosa was purified 54-fold with a yield of 68% [42]. An overall 161-fold purification was achieved for the xylanase from Malbranchea pulchella var. sulfurea 48 [43,44]. The endo-xylanase from A. niger strain 14 was purified using five steps. The purification was 5,000-fold with regard to the culture extract [47]. The two xylanases (I and II) from C. thermophile var. coprophile were purified 38 and 5-fold with 1.42 and 0.09% recovery, respectively [46].

In the present investigation, the Streptomyces $T_7$ contained a single component of xylanase which could be purified by ion exchange chromatography on DEAE-cellulose in

a batchwise manner followed by gel filtration on Sephadex G-50. The enzyme was purified 41-fold over the culture supernatant. Specific activity of 412 was reached in the final step.

It is difficult to compare the specific activity of our xylanase with those reported in the literature due to the variation in the substrate (xylan) and the assay method used for xylanase activity.

# REFERENCES

1. Woodward, J. (1984) In "Topics in Enzyme and Fermentation Biotechnology" (Ed. Wiseman, A.) John Wiley & Sons, N.Y. Vol. 8, p. 9.

2. Nakajima, T., Tsukamoto, K.I., Watanabe, T., Kainuma, K. and Matsuda, K. (1984) J. Ferment. Technol. 62, 269.

3. Sreenath, H.K. and Joseph, R. (1982) Folia Microbiol. 27, 107.

4. Iizuka, H. and Kawaminami, T. (1965) Agric. Biol. Chem. 29, 520.

5. Marui, M., Nakanishi, K. and Yasui, T. (1985) Agric. Biol. Chem. 49, 3399.

6. Takahashi, M. and Hashimoto, Y. (1963) Hakko Kogaku Zasshi, 41, 181.

7. Toda, S., Suzuki, H. and Nisizawa, K. (1971) Hakko Kogaku Zasshi, 49, 499.

8. Eriksson, K.E. and Pettersson, B. (1971) Int. Biodeterior. Bull. 7, 115.

9. Fogarty, W.M. and Ward, O.P. (1973) Biochem. Soc. Trans. 1, 260.

10. Horikoshi, K. and Atsukawa, Y. (1973) Agric. Biol. Chem. 37, 2097.

11. McClendon, J.H. and Kreisher, J.H. (1963) Anal. Biochem. 5, 295.

12. Dekker, R.F.H. and Richards, G.N. (1975) Carbohydr. Res. 39, 97.

13. Hashimoto, S., Muramatsu, T. and Funatsu, M. (1971) Agric. Biol. Chem. 35, 501.

14. Claeyssens, M., Loontiens, F.G. Kersters-Hilderson, K. and De Bruyne, C.K. (1971) Enzymologiya, **40**, 177.

15. Hrazdina, G. and Neukom, H. (1966) Biochim. Biophys. Acta, **128**, 402.

16. Paice, M.G., Jurasek, L., Carpenter, M.R. and Smillie, L.B. (1978) Appl. Environ. Microbiol. **36**, 802.

17. Srinivasan, M.C., Vartak, H.G., Power, V.K., Rele, M.V. and Bastawde, K.B. (1984) Biotechnol. Lett. **6**, 715.

18. Kawaminam, T. and Iizuka, H. (1970) Hakko Kogaku Zasshi, **48**, 169.

19. Comtat, J. (1983) Carbohydr. Res. **118**, 215.

20. Lobavitch, J.M. and Greve, L.C. (1983) Plant Physiol. **72**, 668.

21. Marui, M., Nakanishi, K. and Yasui, T. (1985) Agric. Biol. Chem. **49**, 3399.

22. Margaritis, R. Jr., Merchant, R. and Yaguchi, M. (1983) Biotechnol. Lett. **5**, 265.

23. King, N.J. and Fuller, D.B. (1968) Biochem. J. **108**, 571.

24. Eriksson, K.E. and Kzedowski, W. (1969) Arch. Biochem. Biophys. **129**, 689.

25. Fournier, R.A., Frederick, M.M., Frederick, J.R. and Reilly, P.J. (1985) Biotechnol. Bioeng. **27**, 539.

26. Esteban, R., Villanueva, J.R. and Villa, T.G. (1982) Can. J. Microbiol. **28**, 733.

27. Honda, H., Kudo, T., Ikura, Y. and Horikoshi, K. (1985) Can. J. Microbiol. **31**, 538.

28. Bernier, R. Jr., Desrochers, M., Jurasek, L. and Paice,

M.G. (1983) Appl. Environ. Microbiol. **46**, 511.

29. Uchino, F. and Nakane, T. (1981) Agric. Biol. Chem. **45**, 1121.

30. Esteban, R., Chordi, A. and Villa, T.G. (1983) FEMS Microbiol. **17**, 163.

31. Okazaki, W., Akiba, T., Horikoshi, K. and Akahoshi, R. (1985) Agric. Biol. Chem. **49**, 2033.

32. Akiba, T. and Horikoshi, K. (1988) Methods Enzymol. **160**, 655.

33. Panbangred, W., Shinmyo, A., Kinoshita, S. and Okada, H. (1983) Agric. Biol. Chem. **47**, 957.

34. Berenger, J.F., Frixon, C., Bigliardi, J. and Creuzet, N. (1985) Can. J. Microbiol. **31**, 635.

35. Lee, S.F., Forsberg, C.W. and Rattray, J.B. (1987) Appl. Environ. Microbiol. **53**, 644.

36. Morosoli, R., Betrand, J.L., Mondou, F. and Kluepfel, D. (1986) Biochem. J. **239**, 587.

37. Iizuka, H.and Kawaminami, T. (1969) Agric. Biol. Chem. . **33**, 1257.

38. Kusakabe, I., Kawaguchi, M., Yasui, T. and Kobayashi, T. (1977) Nippon Nogei Kagaku Kaishi, **51**, 429.

39. Johnson, K.G., Harrison, B.A., Schneider, H., Mackenzie, C.R. and Fontana, J.D. (1988) Enz. Microb. Technol. **10**, 403.

40. Bastawde, K.B. (1987) Ph.D. Thesis, Poona University.

41. Yoshika, H., Nagato, N., Chavanich, S., Nilubol, N. and Hayashida, S. (1981) Agric. Biol. Chem. **45**, 2425.

42. Kitpreechavanich, V., Hayashi, M. and Nagai, S. (1984)

J. Ferment. Technol. **62**, 415.

43. Matsuo, M. and Yasui, T. (1985) Agric. Biol. Chem. **49**, 839.

44. Matsuo, M. and Yasui, T. (1988) Methods Enzymol. **160**, 671.

45. Tan, L.U.L., Mayers, P. and Saddler, J.N. (1987) Can. J. Microbiol. **33**, 689.

46. Ganju, R.K., Vithayathil, P.J. and Murthy, S.K. (1989) Can. J. Microbiol. **35**, 836.

47. Gorbacheva, I.V. and Rodionova, N.A. (1977) Biochim. Biophys. Acta, **484**, 79.

48. John, M., Schmidt, B. and Schmidt, J. (1979) Can. J. Biochem. **57**, 125.

49. Baker, C.J., Whalen, C.H. and Bateman, D.F. (1977) Phytopathol. **67**, 1250.

50. Matsuo, M., Yasui, T. and Kobayashi, T. (1975) Nippon Nogei Kagaku Kaishi, **49**, 263.

51. Frederick, M.M., Frederick, J.R., Fratzke, A.R. and Reilly, P.J. (1981) Carbohydr. Res. **97**, 87.

52. Paice, M.G., Bourbonnais, R., Desrochers, M., Jurasek, L. and Yaguchi, M. (1986) Arch. Microbiol. **144**, 201.

53. Deshpande, M.V., Sadana, J.C. and Meagher, M.M. (1989) Enz. Microb. Technol. **11**, 678.

54. Biely, P.M., Vrsanska, M. and Kratky, Z. (1980) Eur. J. Biochem. **112**, 375.

55. Morosoli, R., Roy, C. and Yaguchi, M. (1986) Biochim. Biophys. Acta, **870**, 473.

56. Frederick, M.M., Kiang, C.H., Frederick, J.R. and Reilly, P.J. (1985) Biotechnol. Bioeng. **27**, 525.

57. Shei, J.C., Fratzke, A.R., Frederick, M.M., Frederick, J.R. and Reilly, P.J. (1985) Biotechnol. Bioeng. **27**, 533.

58. Tavobilov, I.M., Gorbacheva, I.V., Rodionova, N.A. and Bezborodov, A.M.(1981) Appl. Biochem. Microbiol. **17**, 320.

59. Lowry, O.H., Rosebrough, N.H., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. **193**, 265.

60. Jagannathan, V., Singh, K. and Damodaran, M. (1956) Biochem. J. **63**, 94.

61. Cohn, E.T., Huges, W.L. Jr. and Weave, H.H. (1947) J. Am. Chem. Soc. **69**, 1753.

62. Peterson, E.A. and Sober, H.A. (1956) J. Am. Chem. Soc. **78**, 751.

63. Maurer, H.R. (1971) In "Disc Electrophoresis and Related Techniques of Polyacrylamide Gel Electrophoresis" (Ed. Maurer, H.R.) Walter de Gruyter, N.Y. p. 32.

64. Bodhe, A.M., Deshpande, V.V., Lakshmikantham, B.C. and Vartak, H.G. (1982) Anal. Biochem. **123**, 133.

65. Park, Y.K. and Toma, M. (1982) Korean J. Biochem. **14**, 37.

# CHAPTER 4

# PROPERTIES OF PURIFIED XYLANASE

## SUMMARY

The physicochemical properties of the purified xylanase were studied. The pH and temperature optima for the xylanase activity were 4.5 - 5.5 and 60°C, respectively. The energy of activation was $8.6 \times 10^3$ cals/mol. The enzyme was highly stable and was found to retain total activity on incubation at pH 5.0 for 6 days at 50°C and after 11 days at 37°C. The molecular weight of xylanase as determined by SDS-PAGE by gel filtration on Sephadex G-50 and Bio-Gel P-10 was found to be approximately 20,500. The isoelectric point of xylanase was 7.8. The xylanase was a glycoprotein and contained 33 mol of carbohydrate per mol of protein estimated using glucose as a standard.

The amino acid analysis indicated that the enzyme is rich in glutamic acid and histidine. It also contained substantial amounts of aspartic acid and glycine. DTNB and PHMB titrations showed the presence of 3 mol of -SH per mol of enzyme. The performic acid oxidised sample indicated the presence of 5 mol of cysteic acid, which is in agreement with the DTNB titration of the reduced xylanase.

The xylanase was specific only to xylan and did not act on other substrates such as CM-cellulose, filter paper, Avicel, pNPX, cellobiose, laminarin, pullulan, chitin, Gum Locust. The $K_m$ and $V_{max}$ values as determined for soluble larch wood xylan were 10 mg/ml and $7.6 \times 10^3$ µmol/min/mg of enzyme, respectively. The $K_m$ values for insoluble and total xylan were 33.3 and 25 mg/ml, respectively.

The xylanase was completely inhibited by SDS (1%), $Hg^{2+}$ ($2 \times 10^{-6}$ M) and $Ag^{+}$ ($10 \times 10^{-6}$ M), EDTA and other metals like $Ca^{2+}$, $Mn^{2+}$, $Mg^{2+}$, $Co^{2+}$ and $Mo^{2+}$ did not show any effect on xylanase activity.

The major end products of xylan hydrolysis by the Streptomyces $T_7$ xylanase were $X_2$ and xylo-oligosaccharides ($X_3$ - $X_6$). Very little xylose was produced even after 16 h indicating that it was an endo-xylanase. When the xylan was hydrolysed with a mixture of xylanase and β-xylosidase, the hydrolysis increased from 43 - 60% and the end product was mainly xylose.

## INTRODUCTION

Most of the available information concerning the properties of xylanases has come from studies on bacterial and fungal enzymes. Multiple xylanases have been reported in numerous microorganisms. The nature and relevance of minor xylanases may remain elusive because purification procedures favour the isolation of major xylanases. Individual xylanases have distinct properties that contribute key functions to the overall xylanolytic system of microorganism [1,2].

**Optimum temperature** The optimum temperature for endo-xylanases from bacterial and fungal sources varies between 40 - 60°C. However, the xylanases produced by thermophilic organisms usually have the higher temperature optima (60 - 80°C) than the xylanases produced by mesophiles. The optimum temperature of various xylanases is summarized in Table 4.1.

**Optimum pH** Bacterial D-xylanases usually have pH optima in the range of 5 - 7. D-xylanases of fungal origin are generally most active in the pH range 3.5 - 5.5. Acidophilic strains produce xylanases which generally possess the pH optimum in the acidic range while those produced by alkalophilic organisms may have in the alkaline range. Table 4.1 gives an account of pH optima of xylanases from various organisms.

**Temperature stability** Thermostable enzymes are advantageous for increasing the hydrolysis efficiencies and accelerating the reaction rates. Stable enzymes active at elevated temperatures are reported from thermophilic organisms as well

Table 4.1: Properties of xylanases from different organisms

| Organism | | Xylanase | Optimum | | Molecular weight* X ($10^3$) | pI | Reference |
|---|---|---|---|---|---|---|---|
| | | | Temp. (°C) | pH | | | |
| *Bacillus circulans* | WL-12 | A | 30 | 5.5-7.0 | 85.0 | 4.5 | 3 |
| | | B | 30 | 5.5-7.0 | 15.0 | 9.1 | |
| *Bacillus coagulans* | 26 | | 37 | 6.0 | 22.0 | 10.0 | 4 |
| *Bacillus pumilus* | IPO | | 40 | 6.5 | 24.0 | ND | 5 |
| *Bacillus subtilis* | PAP-115 | | 55 | 5.0 | 32.0 | ND | 6 |
| | Novoban | | 50 | 5.5 | 9.5 | ND | 7 |
| *Bacillus* sp. | C-59-2 | | 60 | 6.0-8.0 | ND | 6.3 | 8 |
| | W1 and W2 | I | 65 | 6.0 | 22.0 | 8.3-8.5 | 9 |
| | | II | 70 | 7.0-8.9 | 49.5 | 3.6-3.7 | |
| | C-125 | A | 70 | 6.0-10.0 | 43.0 | ND | 10 |
| | | N | 70 | 6.0-7.0 | 16.0 | ND | |
| | 11-1S | | 80 | 4.0 | 56.0 | ND | 11 |
| *Clostridium stercorarium* | | A | 65 | 5.5-7.0 | 44.0 | 4.5 | 12 |
| | | B | 65 | 5.5-7.0 | 72.0 | 4.4 | |
| | | C | 65 | 5.5-7.0 | 62.0 | 4.4 | |
| *Clostridium acetobutylicum* ATCC 824 | | A | 50 | 5.0 | 65.0 | 4.4 | 13 |
| | | B | 60 | 5.5-6.0 | 29.0 | 8.5 | |

| Organism | Xylanase | Optimum |  | Molecular weight | pI | Reference |
|---|---|---|---|---|---|---|
|  |  | Temp. (°C) | pH | X ($10^3$) |  |  |
| Clostridium thermolacticum DSM-2911 |  | 80 | 6.0-6.5 |  |  | 14 |
| Streptomyces lividans 1326 Mutant 8-7 |  | 50 | 6.0 | 43.0 | 5.2 | 15 |
| Stemptomyces xylophagus Nov. sp |  | 55-60 | 6.2 | ND | ND | 16 |
| Streptomyces exfoliatus MC-1 | Ib | 50 | 5.5 | ND | ND | 17 |
|  | Id | 55 | 7.0 | ND | ND |  |
|  | II | 55 | 5.5 | ND | ND |  |
| Streptomyces sp. E-86 |  | 60 | 6.0 | 40.5 | 7.3 | 18 |
| 3137 | X-I | 60-65 | 5.5-6.5 | 50.0 | 7.1 | 19 |
|  | X-II-A | 60-65 | 5.0-6.0 | 25.0 | 10.1 |  |
|  | X-II-B | 60-65 | 5.0-6.0 | 25.0 | 10.3 |  |
| KT-23 |  | 55 | 5.5 | 44.0 | 6.9 | 20 |
| Chainia sp NCL 82-5-1 | II | 50 | 5.0 | 5.4 | 8.2 | 21 |
| Humicola lanuginosa |  | 65 | 6.0 | 21.5 | 4.1 | 22 |
| Humicola lanuginosa (Griffon & Maublanc) Bunce |  | 65 | 6.0 | 29.0 | ND | 23 |
| Malbranchea pulchella var. sulfurea 48 |  | 70 | 6.0-6.5 | ND | 8.6 | 24 |
| Talaromyces byssochlamydoides YH-50 | X-a | 75 | 5.5 | 76.0 | 4.3 | 25 |
|  | X-b-I | 70 | 4.5 | 54.0 | 3.8 |  |
|  | X-b-II | 70 | 5.0 | 45.0 | 4.0 |  |

| Organism | Xylanase | Optimum | | Molecular weight | pI | Reference |
|---|---|---|---|---|---|---|
| | | Temp. (°C) | pH | X ($10^3$) | | |
| Thermoascus aurantiacus C-436 | | 80 | 5.1 | 32.0 | 7.1 | 26 |
| Chaetomium thermophile | | | | | | |
| var. coprophile I-110 | I | 70 | 4.8-6.4 | 26.0 | ND | 27 |
| | II | 60 | 5.4-6.0 | 7.0 | ND | |

*Molecular weight estimates were either by SDS-PAGE or by gel filtration.

ND - Not determind.

as from mesophilic organisms. Among the thermophiles Bacillus sp. are well known. Both the endo-xylanases from Bacillus sp. C-125 were reported to be stable upto 50°C for 10 min [10]. Uchino and Nakane [11] reported the xylanase from Bacillus sp. 11-1S to be stable at 70°C for 15 min. Bernier and coworkers [6] reported that xylanase from B. subtilis PAP-115 was stable at 50°C for 20 min, while B. pumilus xylanase had a half life of 30 min at 50°C [5]. The four xylanases purified from alkalophilic and thermophilic Bacillus sp. (W1 and W2) were stable in the temperature range 40 - 60°C for 10 min. The thermal stability of component II from the two strains was higher than that of component I [9]. Berenger and coworkers [12] reported three xylanases A,B and C from C. stercorarium which were stable at 75°C for 1 h. Xylanase A was stable even at 81°C for 90 min.

The mutant 8-7 of thermotolerant S. lividans 1326 produced xylanase which was stable upto 37°C for 24 h [15]. The xylanase from Streptomyces sp. KT 23 reported by Nakajima and coworkers [20] was stable upto 55°C. The three xylanases produced by Streptomyces sp. 3137 were stable upto 55°C for 30 min [19], while an endo-xylanase of S. xylophagus was stable at 40°C for 10 min [16].

Among the fungi, the xylanase from T. aurantiacus was reported to be stable at 70°C for 24 h and its half life was 54 min at 80°C [26]. It is probably the highest thermostability for xylanases reported so far. Some of the fungi which are mesophilic in origin, but can produce thermostable xylanases are Ceratocystis paradoxa, the xylanase of which

was stable at 80°C for 1 h [28]. The xylanases (A and B) of a fungal strain Y-94 were stable upto 70°C for 10 min whereas xylanase C was not stable [29]. Xylanases (I and II) purified from C. thermophile var. coprophile were stable at 50°C for 24 h. But at 60 and 70°C the xylanase I was more stable than II [27].

**pH stability** D-xylanases from different organisms are usually stable over a wide range of pH 3 - 10. Two endo-xylanases A and N from alkalophilic Bacillus sp. C-125 were reported to be stable in the pH range 4 - 12 and 5 - 12, respectively [10]. The xylanase purified from acidophilic Bacillus sp. 11-1S was stable in the pH range 2.0 - 6.0 [11]. Bernier and coworkers [6] reported the xylanase from B. subtilis PAP-115 was stable in the pH range 5 - 7. The four endo-xylanases from two alkalophilic and thermophilic Bacillus sp. (W1 and W2) were stable between pH 4.5 - 10.0 [9]. Three endo-xylanases (A, B and C) from C. stercorarium described by Berenger and coworkers [12] were stable in the pH range 7 - 12. Xylanases A and B reported from C. acetobutylicum ATCC 824 were stable at pH 5.5 - 7.0 and 3.5 - 7.0, respectively [13].

The pH stability reported by Marui and coworkers [19] for xylanase X-I from Streptomyces sp. 3137 was in the pH range 3.0 - 10.5 and that for X-II-A and X-II-B was in the pH range 1.5 - 11.5. Iizuka and Kawaminami [16] reported the pH stability of xylanase from S. xylophagus to be in the range of 5.3 - 7.3 while the pH stability of xylanase from Streptomyces sp. KT-23 was in the range 4 - 10 [19].

Three xylanases (X-a, X-b-I and X-b-II) from *T. byssochlamydoides* YH-50 were stable in the pH range 3 - 9 [25] while the xylanase from *H. lanuginosa* described by Kitpreechavanich *et al.* [22] were stable in the pH range 5 - 8.

**Molecular weight** Endo-xylanases having molecular weights in the range of 5,000 to 90,000 daltons are reported from various organisms (Table 4.1). The conservation of xylanase forms is suggested by xylanases purified from *Aspergillus*, *Bacillus*, *Clostridium*, *Streptomyces* and *Trichoderma* spp, in which xylanase multiplicity has been most extensively examined [1]. These xylanases appear to have a conserved relationship between their molecular weight and their isoelectric point (pI). An examination of xylanase multiplicity in *Bacillus* spp suggest that these bacteria produce two xylanases: one is basic (pI 8.3 - 10.0) with low molecular weight (16,000 - 22,000), and the other is acidic (pI 3.6 - 4.5) with high molecular weight (43,000 - 60,000). Although the data is incomplete, it appears that all xylanases isolated from *Bacillus* spp fit into one of these categories, except for the xylanase from *B. subtilis* PAP-115 which has an apparently intermediate molecular weight of 32,000 [6]. The two forms appears to be conserved in *Bacillus* spp.

There are low molecular weight/basic and high molecular weight/acidic xylanases in *Clostridium* and *Streptomyces* spp. Although the high molecular weight xylanases in *Streptomyces* spp. have relatively neutral pI's (range 5.2 - 7.3) the apparant dichotomy in multiple xylanases appears to be retained. The dichotomy is suggested in *Aspergillus*

and Trichoderma spp (Table 4.1), in which low molecular weight/basic xylanases are common. There are, however, several exceptional cases in which acidic xylanases have apparently low molecular weights. [1,9].

**Isoelectric point (pI)** The isoelectric points for endo-xylanases from various sources ranged from 3.75 - 10.30 [2,30]. The xylanase reported in Cryptococcus albidus CCY-17-4-4 has a pI of 5.3 and thus appears to belong to the high molecular weight/acidic category. On the other hand, xylanases that belong to the low molecular weight/basic category have isolated from C. albidus CCY-17-4-1 (molecular weight 26,000) [31] and Cryptoccus flavus IFO-047 (molecular weight 25,000, pI 10.0) [32]. The pI's of xylanases from different sources is shown in Table 4.1.

**Carbohydrate content** Xylanase heterogeneity may arise from post-translational modifications such as differential glycosylation or proteolysis or both [1]. The xylanases A and B from B. circulans WL-12 [3] and that from Streptomyces sp E-86 [18] were not glycoproteins while the xylanase isolated from Chainia sp [21] was reported to be a glycoprotein.

The three xylanases of T. byssochlamydoides YH-50 were reported to be glycoproteins. The carbohydrate residues were mannose, glucose and fucose. The total contents of carbohydrate in X-a, X-b-I and X-b-II were 36.6%, 31.5% and 14.2%,respectively [25]. The xylanases were also reported which are glycoproteins from mesophilic fungal sources such as A. niger and Trichoderma viride [33,34].

Both differential glycosylation and proteolysis also

appear to contribute to multiplicity with partial proteolysis having been reported to alter functional characteristics of some cellulases [35,36].

**Amino acid composition** Amino acid analysis gives the information about the amount of each and every amino acid present in the protein. It also helps to study the homology between the same proteins derived from different sources. It is interesting to study the amino acid composition of proteins which are derived from organisms adapted to extremes of pH or temperature, such as the acidophilic, alkalophilic or thermophilic strains. The amino acid composition of xylanases reported from various sources is shown in Table 4.2.

**Substrate specificity** It would be inappropriate to consider certain xylanases if their xylanolytic activity is a secondary and redundant function. Cross specificity has been reported in numerous lignocellulolytic enzymes. There can be always arguments concerning the purity of both the enzyme and substrate preparation in these cases.

The purified xylanases are reported to have a broad specificity that can attack xylan as well as a number of other related and unrelated polysaccharides [13,38,39].

The three xylanases from _T. byssochlamydoides_ show weak activity towards CM-cellulose, Avicel and starch but no activity towards xylobiose, cellobiose or maltose [25]. The endo-xylanase from _H. lanuginosa_ had no activity on Avicel, CM-cellulose, starch _p_-nitrophenyl-β-D-glucoside (pNPG) and pNPX suggesting that the enzyme is a true xylanase [22]. The xylanase from _C. paradoxa_ was reported to be capable of hydrolysing Avicel [26]. Endo-xylanase from _S. rolfsii_

Table 4.2: Amino acid composition of xylanases from different organisms

| Amino acid (% mol) | *B. pumilus* IPO [5] | *B. subtilis* PAP-115 [6] | *Streptomyces* sp [37] 3137 | | | | | *C. thermophile* var *coprophile* [27] |
|---|---|---|---|---|---|---|---|---|
| | | | X-I | X-II-A | X-II-B | E-86 | KT-23 | |
| Aspartic acid | 11.3 | 10.7 | 13.1 | 13.7 | 11.5 | 12.4 | 9.4 | 7.8 |
| Cysteine | 0.5 | 1.4 | 2.7 | 0.9 | 1.3 | 1.9 | 0.6 | 2.0 |
| Threonine | 8.6 | 9.0 | 7.7 | 14.0 | 11.6 | 7.8 | 5.4 | 17.5 |
| Serine | 9.3 | 12.1 | 7.9 | 13.9 | 10.4 | 8.2 | 17.5 | 8.7 |
| Glutamic acid | 7.1 | 6.2 | 8.8 | 4.5 | 5.7 | 8.1 | 14.9 | 9.1 |
| Proline | 3.7 | 6.0 | 2.5 | 2.1 | 3.3 | 4.2 | 2.8 | 4.2 |
| Glycine | 11.3 | 18.6 | 12.4 | 13.3 | 15.9 | 11.7 | 16.3 | 14.9 |
| Alanine | 7.0 | 7.6 | 9.4 | 3.3 | 3.8 | 9.4 | 9.0 | 8.0 |
| Valine | 3.8 | 4.8 | 6.0 | 5.2 | 6.1 | 5.4 | 4.8 | 7.7 |
| Isoleucine | 4.3 | 3.1 | 3.5 | 2.5 | 2.2 | 4.0 | 2.8 | 3.3 |
| Leucine | 4.3 | 3.5 | 5.9 | 4.3 | 4.0 | 5.6 | 4.3 | 4.7 |
| Tyrosine | 4.9 | 4.5 | 3.4 | 8.6 | 8.2 | 3.9 | 1.2 | 3.3 |
| Phenylalanine | 3.8 | 1.03 | 2.8 | 3.1 | 3.3 | 4.1 | 2.0 | 1.2 |
| Histidine | 2.2 | 2.07 | 1.9 | 1.0 | 1.5 | 2.2 | 3.1 | 1.3 |
| Lysine | 4.3 | 4.8 | 3.1 | 2.3 | 2.7 | 3.8 | 3.1 | 2.8 |
| Arginine | 3.8 | 3.5 | 5.1 | 3.4 | 3.5 | 5.8 | 2.5 | 2.4 |
| Methionine | 2.2 | 1.03 | 1.3 | 1.2 | 1.6 | 1.5 | ND | 0.6 |
| Tryptophan | 7.6 | ND | 2.6 | 2.9 | 3.1 | ND | ND | ND |

ND - Not determined.

UV-8 mutant had no action on cellulose, CM-cellulose or starch [40].

**Kinetic studies** From a practical point of view, the $K_m$ of an enzyme is very important. The $K_m$ of xylanase is generally measured using soluble xylan. Larch wood xylan is partially soluble in water, the water soluble portion represents about 1/3rd of the total weight at 30°C [41]. The $K_m$ values are dependent on the structure of xylan used. The $K_m$ values vary because xylan is heterogeneous in nature.

The enzyme from *Bacillus* sp. 11-1S has potential for practical use based on its high thermal stability and its low $K_m$ (1.68 mg/ml) [11]. Xylanases A and B from *B. circulans* WL-12 possess $K_m$ of 8 mg/ml and 4 mg/ml, respectively, when the purified xylan was used as a substrate [3]. A $K_m$ of 10 mg/ml was reported for xylanase from *B. coagulans* 26 [4]. A $K_m$ of 4.5 and 4 mg/ml with larch wood xylan was reported for component I from alkalophilic and thermophilic *Bacillus* spp. W1 and W2 respectively, while component II had lower $K_m$ than I viz. 0.95 and 0.57 mg/ml respectively [9]. A $K_m$ of 3.2, 2.9 and 3.7 mg/ml using insoluble portion of xylan suspension was reported for three xylanases (A, B and C) from *C. stercorarium*. The corresponding $V_{max}$ values were 5.5, 3.5 and 4.0 µmol/min/µg of enzyme, respectively [12].

Sreenath and Joseph [17] reported $K_m$ of 12.5, 16.6 and 2.5 mg/ml with xylan for xylanases II, Ib and Id from *S. exfoliatus*, while a $K_m$ 0.78 mg/ml with soluble oat spelts xylan was reported for xylanase from mutant 8-7 of *S. lividans* 1326 [15]. Nakajima and coworkers [20] used purified xylan from straw to determine $K_m$. A $K_m$ of 0.2 mg/ml was reported

for xylanase from Streptomyces sp. KT-23. Kusakabe and co-workers [42] and Meagher [43] used pure xylo-oligosaccharides for the determination of $K_m$ and $V_{max}$ of endo-xylanases from A. niger and Streptomyces sp E-86. The maximum $K_m$ of 22.5 mM for Streptomyces E-86 xylanase was reported with xylohexaose ($X_6$) while a lower $K_m$ of 6.9 mM was reported for xylotetraose ($X_4$). The $V_{max}$ values increased from $X_3$ - $X_7$ [43].

**Effect of metal ions and other compounds** The effect of various metal ions such as $Hg^{2+}$, $Cu^{2+}$, $Mg^{2+}$, $Co^{2+}$, $Fe^{2+}$, $Ba^{2+}$, and $Ca^{2+}$ on xylanse activity was reported by many workers. Xylanase of B. subtilis was reported to be inhibited by $Hg^{2+}$, $Ag^{+}$, $Cu^{2+}$, $Fe^{2+}$ and $Fe^{3+}$ [6]. Xylanase of C. stercorarium was inhibited by $Hg^{2+}$ and $Fe^{2+}$. Moreover, $Ca^{2+}$ and $Cu^{2+}$ also inhibited the xylanase to a certain extent [12]. A and B xylanases of C. acetobutylicum were also inhibited by $Hg^{2+}$, $Cu^{2+}$ and $Pb^{2+}$ [13].

Response of xylan hydrolases II, Ib and Id from S. exfoliatus towards different metals differed, however, $Hg^{2+}$ inhibited all the three enzymes [17]. The xylanase from Streptomyces sp KT-23 was inhibited 58% by $Hg^{2+}$ and 51% by $Mn^{2+}$, while other metals did not show any effect. SDS showed inhibition while EDTA had no effect [20]. $Hg^{2+}$ was found to be a potent inhibitor for many xylanases [9,12,25].

Yoshika and coworkers [25] reported the inhibition of three xylanases X-a, X-b-I and X-b-II by $HgCl_2$ and $KMnO_4$. Tan and coworkers [26] reported that the thermostable xylanase from T. aurantiacus was inhibited by $Hg^{2+}$. Two endo-xylanases from A. niger were found to be inhibited with various metal ions like $Hg^{2+}$, $Cu^{2+}$, $Fe^{3+}$, $Co^{2+}$, $Cr^{3+}$ and $Al^{3+}$ while $Li^{+}$,

$K^+$, $Na^+$, $Zn^{2+}$, $Mn^{2+}$ and $Ca^{2+}$ did not show any inhibition [44].

**Analysis of hydrolysis products and mode of action** The analysis of hydrolysis products gives an information about the mode of action of xylanases. D-Xylanases are either the exo- or endo- type [45]. The two types (exo-xylanase and β-xylosidase which attack xylo-oligosaccharides can be clearly distinguished from one another by nuclear magnetic resonance and spectroscopic analysis which determined the configuration of the xylose residue released. Retention of configuration indicates a xylosidase and inversion of configuration an exo-xylanase [46]. D-Xylanases having action patterns of the exo-type have been claimed to be produced extracellularly by Aspergillus batatae [47], A. niger [48] and Coniphora cerebella [49] but they have not been extensively purified as the endo-D-xylanase preparation.

Xylanases A and B from B. circulans WL-12 were reported to be endo-xylanases. Xylanase A produced xylotriose ($X_3$) initially and after prolonged incubation (48 h) produced xylobiose ($X_2$), $X_3$, $X_4$ and xylose, whereas xylanase B produced $X_2$, $X_3$ and $X_4$ as the end products [3]. Two xylanases from alkalophilic Bacillus sp C-125 hydrolysed xylan giving $X_2$, $X_3$, $X_4$ and higher oligosaccharides but no xylose [10]. The xylanase secreted by B. coagulans 26 is considered as an endosplitting enzyme, as it released $X_4$ as major product in short incubation time whereas, longer incubation resulted in the formation of $X_2$ and xylose plus small quantities of xylo-oligosaccharides [4]. Patterns of hydrolysis demonstrated that the three xylanases A, B and C from C. stercorarium

were endosplitting and able to break down xylan at random giving $X_2$ and $X_3$ as main products [12]. Degradation products obtained by the action of xylanases A and B from C. acetobutylicum were $X_2$, $X_3$, $X_4$, $X_5$, $X_6$ and $X_2$, $X_3$, respectively [13].

The xylanase from a mutant strain 8-7 of S. lividans 1326 degraded xylan producing $X_2$, a mixture of xylo-oligosaccharides and a small amount of xylose as end products [15]. Nakajima and coworkers [20] reported that the endo-xylanase from Streptomyces sp. KT-23 produced $X_2$, $X_3$ and $X_4$ without the accumulation of larger intermediates. After 24 h, xylose and $X_2$ were the final products. Sreenath and Joseph [17] studied the products of hydrolysis of xylan by the three hydrolases from S. exfoliatus MC-1. Xylan hydrolase Ib indicated the presence of xylose, while the other two xylan hydrolases II and Id indicated the presence of only xylo-oligosaccharides. Kusakabe and coworkers [37,42] reported the xylan hydrolysis with xylanase from Streptomyces sp. The end products produced were xylose and $X_2$ indicating the endo-type action. The endo action of three xylanases from Streptomyces sp. 3137 was reported. Hydrolysis of xylan or xylo-oligosaccharides ($X_3$ - $X_5$) resulted in the formation of xylose and $X_2$ but was not able to attack $X_2$ [19].

The bonds most likely to be broken are determined by the length of the substrate, its degree of branching, the presence of substituents and subsite pattern of hydrolase. Obviously, many enzymes with different specificities must take part in the breakdown of a substrate as chemically and structurally heterogeneous as xylan [50]. Much less is known about the xylanases that can release L-arabinose from

xylan than those which cannot. The fungal strains reported for debranching endo-xylanases were *C. paradoxa* [28], *Cephalosporium sacchari* [51] and *A. niger* 14 [52].

## MATERIALS AND METHODS

### Materials

Molecular mass markers, Coomassie Brilliant Blue R-250, CM-cellulose C-8758, *p*-hydroxymercuribenzoate (pHMB), 5,5'-dithiobis-(2-nitrobenzoic acid (DTNB), chitin, lichenan, pullulan, β-mercaptoethanol, ninhydrin and standard amino acid mixture were purchased from Sigma Chemical Co., U.S.A. The suppliers of the following chemicals are indicated in parentheses: SDS (Koch-Light Laboratories, U.K.), Bio-Gel P-10 (Bio-Rad Laboratories, U.S.A.), Sephadex G-50 (Pharmacia, Sweden). All other chemicals used were of analytical grade. Xylo-oligosaccharides were a kind gift from Professor P.J. Reilly, Department of Chemical Engineering, Iowa State University, Ames IA 50011, U.S.A.

### Methods

#### Determination of molecular weight

(a) Gel filtration The molecular weight of the xylanase was determined by gel filtration on Sephadex G-50 and Bio-Gel P-10 according to the method of Andrews [53].

Sephadex G-50 or Bio-Gel P-10 was suspended in water and was allowed to swell by heating in a boiling water bath for 2 - 3 h. After cooling, the gel was deaerated under mild vacuum before packing in a column (1.5 x 110 cm) and equilibrated with 50 mM potassium phospate buffer, pH 7.2.

The void volume ($V_o$) of the column was determined with Blue Dextran 2000 (molecular weight $2 \times 10^6$ daltons). Marker proteins and xylanase (2 mg/ml) were separately loaded on the column and eluted with the same buffer. Fractions (2 ml each) were collected at a flow rate of 12 ml/h. The elution volume ($V_e$) for each protein was determined by measuring the absorbance at 280 nm. The elution volume for xylanase was determined by estimating the xylanase activity of the fractions. The molecular weight of the enzyme was determined by plotting the graph of $V_e/V_o$ versus logarithm of molecular weight and by extrapolating $V_e/V_o$ for the enzyme sample.

(b) SDS-PAGE It is based on the principle that SDS treatment minimises the native charge differences between the proteins and all proteins migrate as anions. The migration rate of SDS treated proteins is proportional to their molecular weights. SDS-PAGE was carried out according to the method of Laemmli [54].

Gels containing 3% (stacking gel) and 10% acrylamide were prepared from a stock solution of 30% by weight of acrylamide and 0.8% by weight of N,N'-methylene bis acrylamide. The final concentrations in the separation gel were as follows: 0.375 M Tris-HCl (pH 8.8) and 0.1% SDS. The gel was polymerized by the addition of 0.025% by volume of TEMED and ammonium persulphate.. The stacking gel contained 0.125 M Tris-HCl (pH 6.8) and 0.1% SDS, was polymerized in the same way as for the separation gel. The electrode buffer (pH 8.3) contained 0.025 M Tris, 0.192 M glycine and 0.1% SDS.

The protein (50 µg) in sample buffer [0.125 M Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 0.002% bromophenol blue (BPB)]

containing 5% β-mercaptoethanol was boiled for 5 min at 100°C. Reference proteins were also loaded for comparison. Electrophoresis was carried out initially with a current of 10 mA and then increased to 20 mA (at 50 V). The electrophoresis was discontinued when the marker (BPB) reached the bottom of the gel (about 4 - 5 h). The gel was fixed in the destaining solution (ethanol:acetic acid:water 4:1:5) for 20 min and kept overnight in the 0.2% Coomassie Brilliant Blue R-250 made in destaining solution. The gel was destained till the bands were visualised. Migration of BPB was used as a reference point. The electrophoretic mobility ($R_f$) i.e. the ratio of distance of protein migration to the distance of dye migration were plotted against the logarithm of molecular weights of reference proteins and the molecular weight of the enzyme was determined.

**Determination of isoelectric pH**

The isoelectric pH of the enzyme was determined by isoelectric focusing in PAGE, according to the method of Vesterberg [55] in the pH range 3.5 - 10.0. Two identical cylindrical gels were made in tubes (0.5 x 10 cm). The composition of the gel was as follows: Acrylamide:Bis (30:0.8), 10%; glycerol, 12%; Ampholines, 2%, pH range 3.5 - 10.0, 0.001%; 0.4% Riboflavin, 13.2%.

The enzyme was loaded in 50% glycerol and above that it was layered with 20% glycerol. Upper compartment containing 1 N $H_3PO_4$ was used as an anode and lower compartment containing 1N NaOH served as a cathode. Electrophoresis was carried out at 4°C for 16 - 18 h at constant voltage (100 V). After the termination of the run one gel was used for

protein staining and the other was used for activity and pH determination.

The gel was cut into 10 equal fractions. The gel pieces were crushed and water (1 ml) was added to homogenised gel. pH of each fraction was determined and the fractions were assayed for xylanase activity. The other gel was kept in 20% trichloroacetic acid for 20 - 30 min for fixing before transferring to Coomassie Brilliant Blue R-250 dye (0.15%) in destaining solution (ethanol:acetic acid:water 2.5:0.8:10.0) heated for 10 min at 62°C with shaking. The gel was destained till the band was visible. The pH and activity of the fraction was corresponded with the protein band and isoelectric pH of the enzyme was determined.

**Carbohydrate content**

The glycoprotein nature of xylanase was determined by thymol $H_2SO_4$ method [56]. The carbohydrate content was determined by phenol $H_2SO_4$ method [57]. Fifty µg of xylanase was used to determine the carbohydrate content using glucose as a standard.

**Amino acid analysis**

For amino acid analysis, hydrolysis of protein samples (1 mg in 1 ml of 6 M HCl) was carried out in nitrogen atmosphere in sealed test tubes at 110°C for 24, 48 and 72 h. Excess acid was removed by evaporation in vacuum at room temperature (25 - 28°C) followed by dissolving in 2 ml water and evaporating to dryness. It was finally dissolved in citrate buffer, pH 2.2, and was analysed in Spinco model 120-B automatic amino acid analyser by the method of Spackman <u>et al</u>. [58].

Determination of tyrosine and tryptophan:

(a) Goodwin and Morton's method [59] The method is based on the principle that proteins show selective absorption in the ultraviolet region and the position of absorption maximum varies with the pH. 0.3 mg of purified xylanase ($1.65 \times 10^{-2}$ M) was taken in 1 ml of 0.1 N NaOH and the absorbance of the enzyme at 257 nm, 294.4 nm and 280 nm was noted.

(b) Bencze and Schmid's method [60] The absorbance of the purified enzyme 0.3 mg in 1 ml of 0.1 N NaOH was measured between 278 and 294 nm at 2 nm intervals. A graph of absorbance versus wavelength was plotted and a line was drawn tangentially to the two characteristic peaks. From the slope, and the maximum absorption between 270 and 290 nm and the molecular weight of the protein, tyrosine and tryptophan content can be determined.

Determination of free -SH groups:

Free thiol groups in the enzyme were determined by titrating the enzyme against DTNB and PHMB.

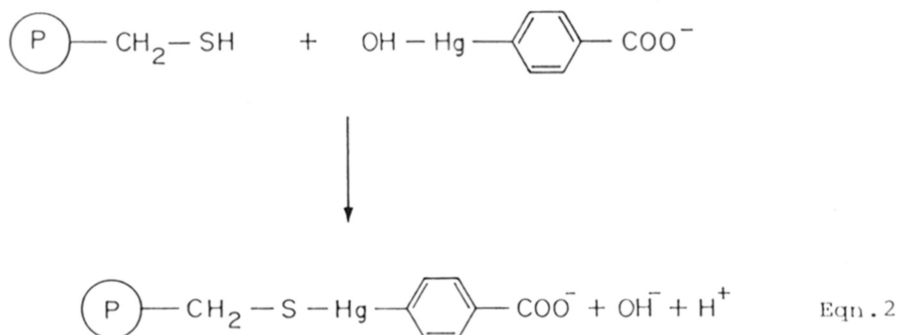
(a) Tritration with DTNB Free thiol groups were determined according to the procedure of Ellman [61]. Xylanase ($5 \times 10^{-6}$ M) in 1 ml of 50 mM sodium phosphate buffer, pH 7.5 was treated with 20 µl of DTNB ($1 \times 10^{-2}$ M) and the net absorption at 412 nm was employed to calculate the sulfhydryl content. A molar extinction coefficient of $1.36 \times 10^4$ $cm^2$ per mol was assumed for the free thiol ion of the reagent. DTNB reacts with free thiol groups of proteins, forming thionitrobenzoate protein and liberating for each -SH group 1 mol of thionitrobenzoate anion (Eqn. 1). This strongly coloured anion can be determined from its absorption at 412 nm [62].

$$\text{(P)}-SH + NO_2-C_6H_3(COO^-)-S-S-C_6H_3(COO^-)-NO_2 \underset{}{\overset{PH \leq 6.8}{\rightleftharpoons}} \text{(P)}-S-S-C_6H_3(COO^-)-NO_2 + {}^-S-C_6H_3(COO^-)-NO_2 + H^+$$

Eqn.1

(b) <u>Titration with PHMB</u> The solution of PHMB was prepared by dissolving 8 - 9 mg of the compound in 1 ml of 0.04 N NaOH and was diluted further with buffer. The xylanase ($2.5 \times 10^{-4}$ M) in 50 mM acetate buffer, pH 6.0 was placed in 1 ml cuvette. Ten µl aliquots of PHMB solution ($1 \times 10^{-3}$ M) were added to the enzyme and the blank which contained equal volume of the buffer. The contents of the cuvettes were mixed and absorbance recorded at 250 nm after each addition. The addition of PHMB solution was continued till there was no change in the absorbance. The observed absorbance values were corrected for dilution and plotted against the volume of PHMB added. The end point was obtained from intersection of two lines. The extinction coefficient of $2.2 \times 10^4$ $cm^2$ per mol was used to calculate the total number of free thiol groups per mol of enzyme [63].

Organic mercurials absorb strongly in the ultraviolet region. They combine very strongly with sulfhydryl groups and mercaptides are formed (eqn. 2):




PHMB exhibits an absorption maximum at 233 nm with a molar absorptivity of $1.69 \times 10^4$. On formation of a mercaptide the molar absorptivity increases to $2.2 \times 10^4$. However, this change is small in comparison with the maximal difference in absorbance which occurs between PHMB and its mercaptide in the region 250 - 255 nm and directly reflects the amount of reagent reacted with protein [64 ].

Determination of total sulfhydryl and disulfide groups:

Total -SH and -S-S-linkages in xylanase were determined by two different procedures -

(a) Reduction of enzyme and DTNB titration Reduction of -S-S-linkages using sodium borohydride in 8 M urea followed by DTNB titration after removal of excess of sodium borohydride according to the method of Cavallini *et al.* [65].0.17 mg of xylanase ($8.5 \times 10^{-6}$ M) was used.

(b) Performic acid oxidation Total content of cysteine and cystine in the protein was determined as cysteic acid after oxidation with performic acid [66]. One mg of xylanase was used. Standard amino acid mixture containing known quantity

of half cystine was treated in a similar way with performic acid and run as a control.

**Substrate specificity**

Xylanase (0.5 U) was incubated with different substrates (10 mg each) at 50°C for 24 h. The reducing sugars released were determined by DNS method. The following substrates were used. Xylan, CM-cellulose, filter paper (Whatman No. 1), Avicel (PH 101), pNPX, cellobiose, laminarin, pullulan, chitin, Gum Locust.

**Kinetics**

Determination of $K_m$ A suitably diluted xylanase (1.3 µg) was incubated with different amounts of xylan (3 - 18 mg ) under the assay conditions. $K_m$ and $V_{max}$ were determined from Lineweaver-Burk plots. $K_m$ was determined by using three different kinds of xylan viz. soluble xylan, insoluble xylan (the insoluble residue remained after removing the soluble portion) and total xylan (the xylan as such containing both soluble and insoluble fractions).

**Xylan degradation products**

Xylanase (1 U) was incubated with xylan (10 mg) in 20 mM acetate buffer, pH 5.0 for different periods of time at 50°C. The end products formed were analysed by paper chromatography in the solvent system (butanol:acetic acid: water 3:1:1) by the method of Trevelyn et al.[67].

**Synergism with β-xylosidase**

Xylanase (1 U) and β-xylosidase (0.07 U) from A. niger were mixed with 10 mg of xylan in 50 mM acetate buffer, pH 5.0. The reaction was carried out at 37°C for 20 h.

The control with only xylanase was also run under identical conditions. The extent of hydrolysis was determined by measuring the reducing sugar.

## RESULTS AND DISCUSSION

While studying the effects of temperature and pH on the xylanase activity, the highest activity was assumed as 100% and the other activities were expressed relative to the highest activity.

**Optimum temperature**

High temperature optima are reported for endo-xylanases from bacteria and fungi [5,6,8,10,22-24]. The xylanases from Streptomyces sp like E-86, KT-23 and 3137 were reported to have temperature optimum in the range of 55 - 65°C [37]. The xylanase from a mutant 8-7 of S. lividans 1326 has a temperature optimum of 60°C [15].

Our present investigation shows that the xylanase from Streptomyces $T_7$ has also a temperature of 60°C which is comparable to xylanases from other Streptomyces sp. (Fig. 4.1a). The logarithm of velocity was plotted versus the reciprocal of absolute temperature (Arrhenius plot) (Fig. 4.1b). The energy of activation for xylanase was calculated to be 8.6 x $10^3$ cals/mol).

**Optimum pH**

The pH optima reported for endo-xylanases from bacteria (bacilli and clostridia) are in the range of pH 5.5 - 7.0 [3-6,12,13]. The optimum pH reported for endo-xylanases from various Streptomyces sp were in the range of 5.5 - 7.0 [17,19,20,37]. However, the xylanase from alkalophilic

**Fig. 4.1:** Effect of temperature on xylanase activity

a. The xylanase (1.1 μg) was assayed over a temperature range of 30 - 70°C.

b. Arrhenius plot

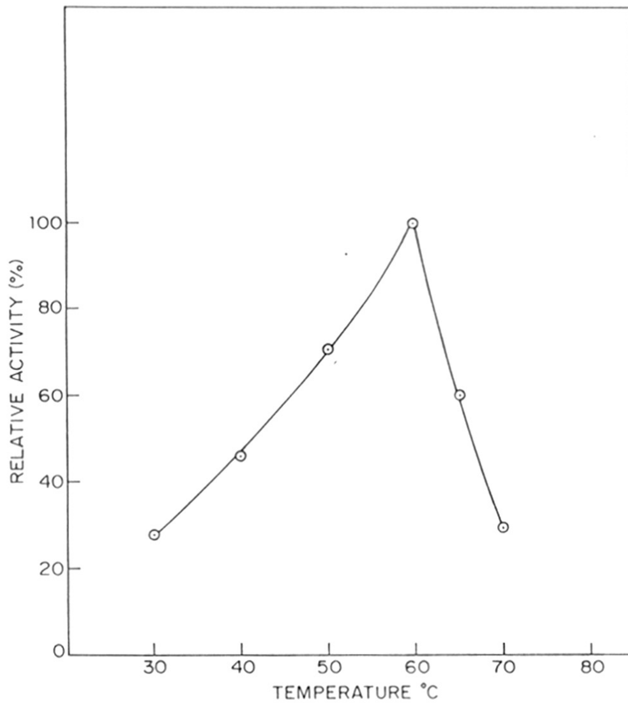



Fig. 4.1a

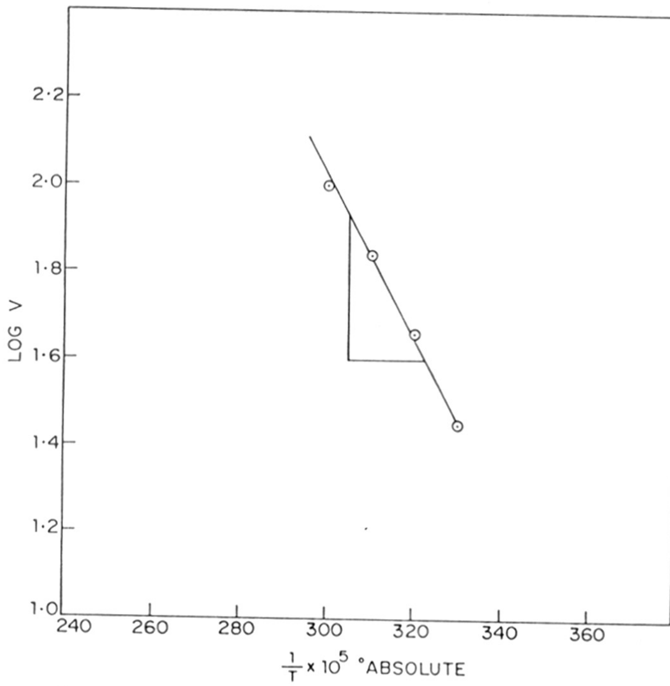



Fig. 4.1b

Streptomyces sp. VP-5 was reported to have a broad pH optimum of 4.8 - 10.0 [68].

The xylanase of Streptomyces $T_7$ (1.1 µg) was assayed under standard conditions at 60°C over a pH range of 3 - 9. The buffers used were 50 mM citrate (pH 3.0 - 4.0),acetate (pH 4.5 - 5.5), phosphate (pH 6.0 - 7.0) and Tris-HCl (pH 8.0 - 9.0). The optimum pH for xylanase activity was found in the pH range of 4.5 - 5.5 (Fig. 4.2).

**Temperature stability**

The thermostability of an enzyme is a function of the various stabilizing forces, such as hydrogen bonding, hydrophobic interactions, ionic interactions, metal binding and disulfide bridges [69]. The xylanases from Streptomyces sp were reported to be stable in the temperature range of 40 - 55°C for 10 - 30 min [16,19,20]. The xylanase from cellulase negative mutant 8-7 of S. lividans 1326 was stable for 24 h at 37°C and for 8 h at 43°C [15].

The xylanase (1 µg) was heated at various temperatures (30 - 70°C) for 30 min in 50 mM acetate buffer, pH 5.0. After rapid cooling the residual activity was assayed under standard conditions. The enzyme was stable upto 50°C. At 60°C, the half life of the enzyme was 30 min (Fig. 4.3). It was observed that at 70°C the enzyme was stable only for 10 min. There was no loss in activity after incubating the enzyme at 50°C for 6 days, at 37°C for 11 days and at 30°C for 15 days.

**pH stability**

Xylanases isolated and purified from prokaryotes and eukaryotes are stable over a wide range of pH. Xylanases

**Fig. 4.2:** Effect of pH on xylanase activity

The xylanase (1.1 μg) was assayed under standard conditions at 60°C over a pH range of 3 - 9. The buffers used were 50 mM, citrate (pH 3.0 - 4.0) acetate (pH 4.5 - 5.5), phosphate (pH 6.0 - 7.0) and Tris-HCl (pH 8.0 - 9.0).

**Fig. 4.3:** Effect of temperature on stability of xylanase

The xylanase (1.0 μg) was heated for 30 min in 50 mM acetate buffer over a temperature range of 30 - 70°C.

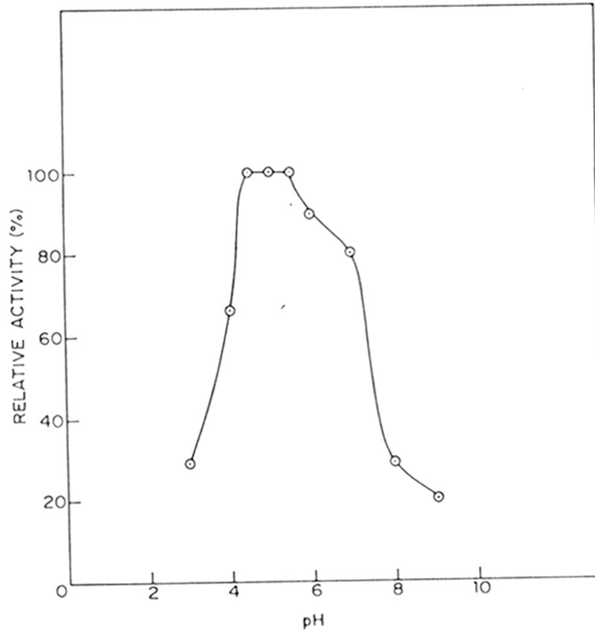



Fig. 4.2

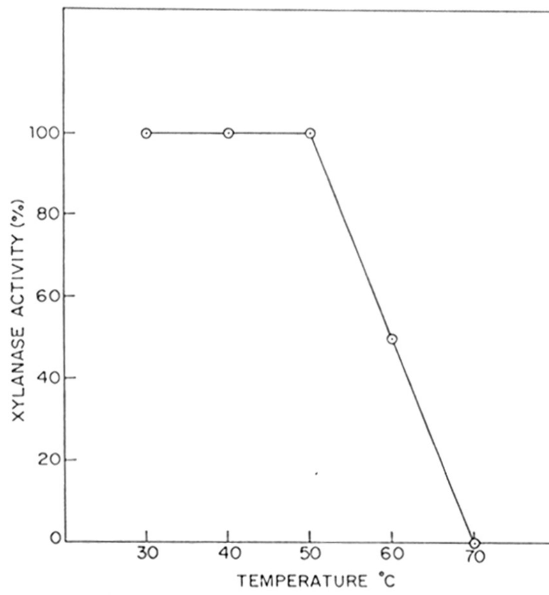



Fig. 4.3

are mostly stable in the pH range of 4 - 10. The endo-xylanase from Streptomyces sp. E-86 was stable in the pH range of 4.5 - 10.5 [37]. The endo-xylanase II from Chainia sp. was stable over a pH range of 4 - 9 [21].

The Streptomyces $T_7$ xylanase (1.1 µg) was incubated at 50°C for 24 h in 50 mM buffer solutions of various pHs. After the incubation the enzyme was readjusted to its optimum pH (5.0) and residual activity was determined. Figure 4.4 shows that the enzyme was stable in the pH range of 5 - 6 and retained 100% of its activity. There was a slight decrease in activity at alkaline pH (7 - 9) whereas 57% of its activity was **lost** at pH 4.0.

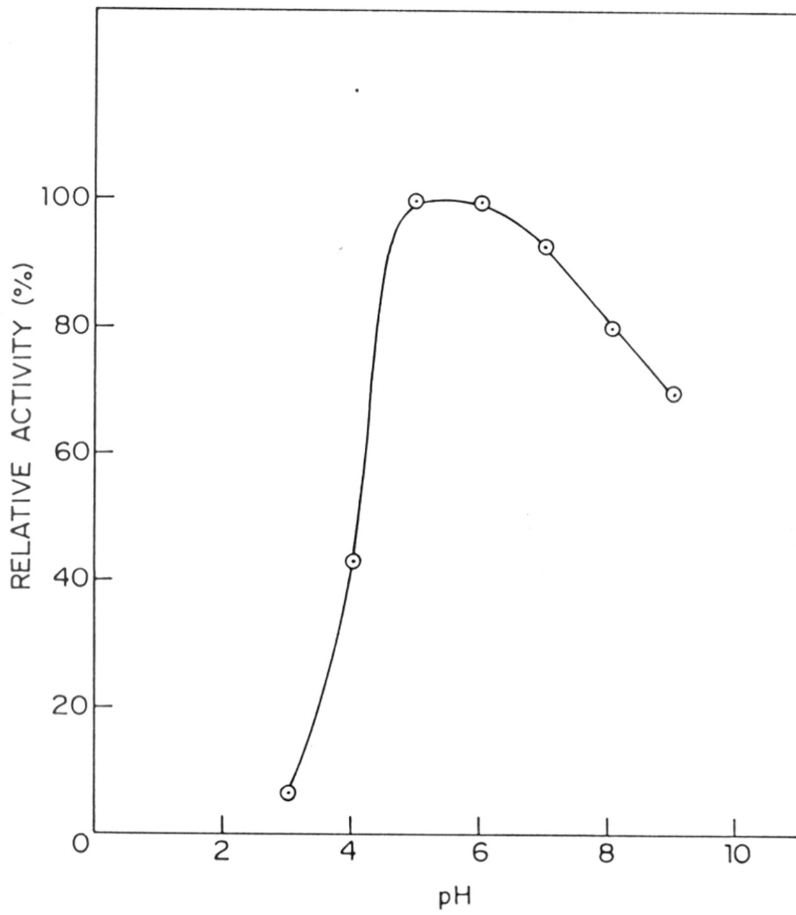
**Molecular weight**

The molecular weights of endo-xylanases reported from different microorganisms are in the range of 5,500 - 80,000 daltons (Table 4.1) [3-6,10,21,27,51]. The molecular weights of three endo-xylanases from Streptomyces sp 3137 ranged between 25,000 - 50,000 daltons [19]. The molecular weight of 43,000 was reported for xylanase from cellulase negative mutant 8-7 of S. lividans 1326 [15] and molecular weights of 40,500 and 43,000 daltons were reported for xylanases from Streptomyces E-86 and Streptomyces KT-23, respectively [37]. So far there are few reports on low molecular weight endo-xylanases viz. the endo-xylanase II isolated from Chainia sp. which has the lowest molecular weight of 5,500 daltons [21]. The enzyme was found to be very effective in paper and pulp industry and has been patented [70].

Two different methods were used for the determination of molecular weight of Streptomyces $T_7$ xylanase.

**Fig. 4.4:** Effect of pH on stability of xylanase

The xylanase (1.0 µg) was incubated for 24 h in 50 mM buffer solutions over a pH range of 3 - 9.



RELATIVE ACTIVITY (%)
100
80
60
40
20
0
2
4
6
8
10
pH

Gel filtration Results of the molecular sieving of the purified xylanase through Sephadex G-50 and Bio-Gel P-10 are shown in Fig. 4.5 and Fig. 4.6, respectively. A plot of $V_e/V_o$ versus logarithm of molecular weight indicated that molecular weight of the enzyme was 20,180 (Fig. 4.5) and 19,230 daltons (Fig. 4.6).

SDS-PAGE A plot of logarithm of molecular weight versus relative mobility yielded a straight line. The molecular weight estimated by this method was 21,880 daltons (Fig. 4.7a). SDS-PAGE showed a single band indicating absence of subunits and isoenzymes (Fig. 4.7b).

**Isoelectric pH**

The pI values reported for xylanases from *Streptomyces* sp. E-86 [37], KT-23 [20] and mutant 8-7 of *S. lividans* 1326 [15] were 7.3, 6.9 and 5.2, respectively. A pI value of 7.1 was reported for endo-xylanase X-I of *Streptomyces* sp. 3137 and high pI values of 10.1 and 10.3 for the two endo-xylanases X-II-A and X-II-B from the same source [19].

Fifty µg of *Streptomyces* $T_7$ xylanase was subjected to electrofocusing in the pH range 3.5 - 10.0. A plot of xylanase activity versus pH indicated that the maximum xylanase activity was obtained at pH 7.8, in the fraction No. 8 (Fig. 4.8a). A single protein band was obtained corresponding to the 8th fraction on the gel (Fig. 4.8b).

The xylanases appear to have a conserved relationship between their molecular weight and pI. It was observed that the xylanases with basic or high pI values (8.0 - 10.0) have low molecular weights while the xylanases with acidic or low pI values have high molecular weights. This hypothesis

**Fig. 4.5:** Determination of molecular weight of xylanase by gel filtration on Sephadex G-50

| Marker protein | Molecular weight (daltons) |
|---|---|
| 1. Ovalbumin | 43,000 |
| 2. Chymotrypsinogen A | 25,000 |
| 3. Ribonuclease | 13,700 |

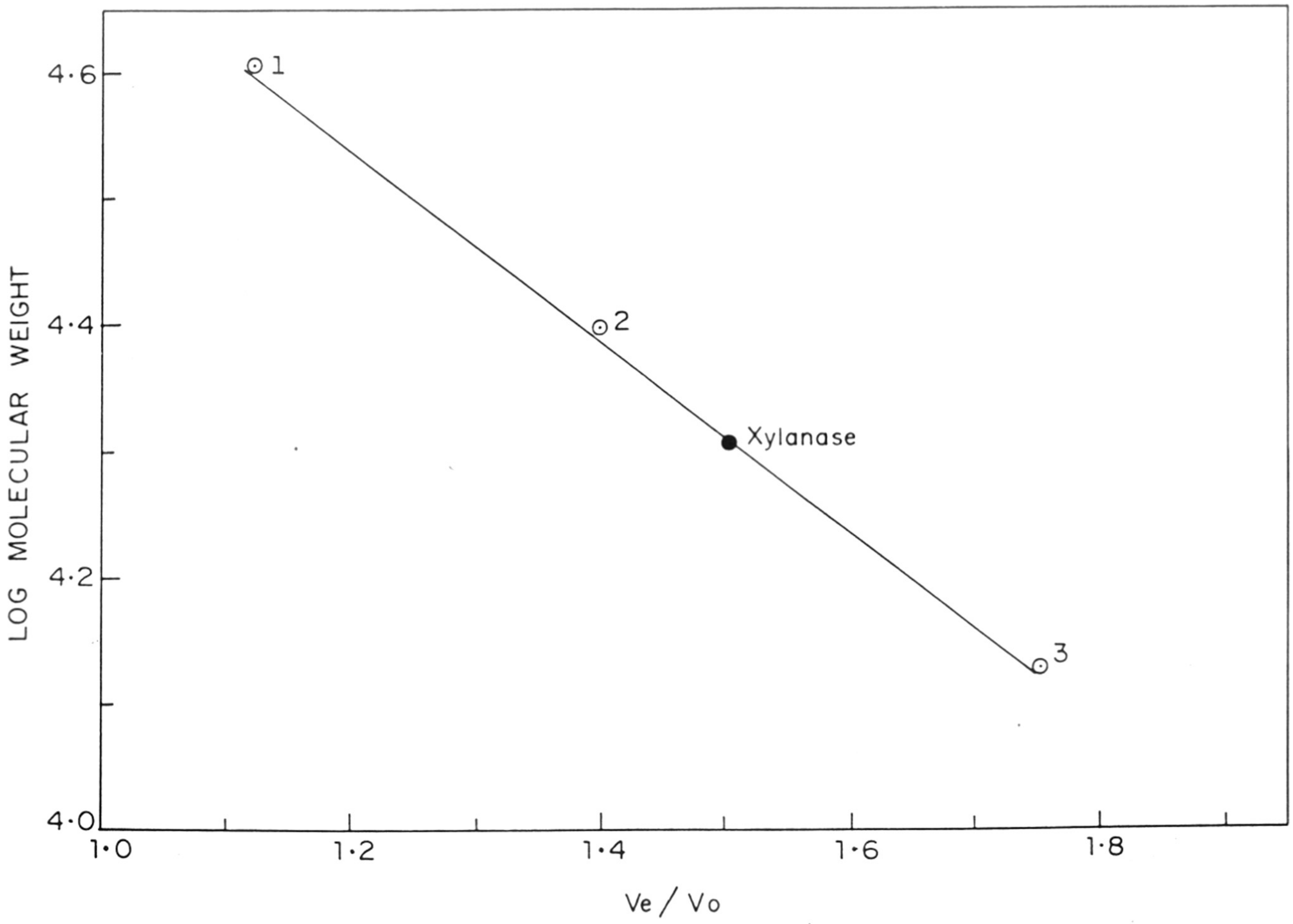

LOG MOLECULAR WEIGHT
4·6
4·4
4·2
4·0
1·0
1·2
1·4
1·6
1·8
Ve / Vo
1
2
3
Xylanase

**Fig. 4.6:** Determination of molecular weight of xylanase by gel filtration on Bio-Gel P-10

| Marker protein | Molecular weight (daltons) |
|---|---|
| 1. Soybean trypsin inhibitor | 21,130 |
| 2. Myoglobin | 17,000 |
| 3. Cytochrome c | 12,500 |

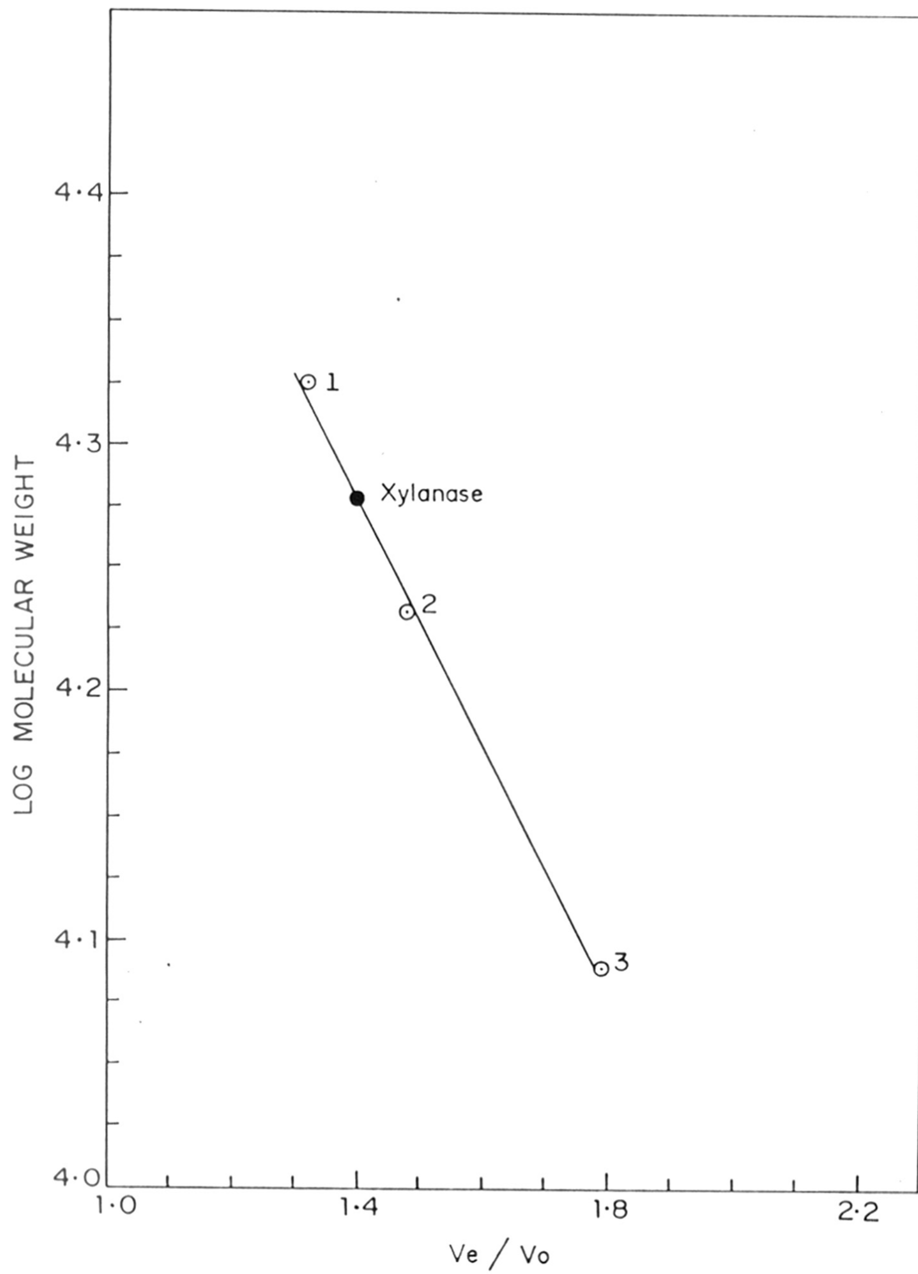
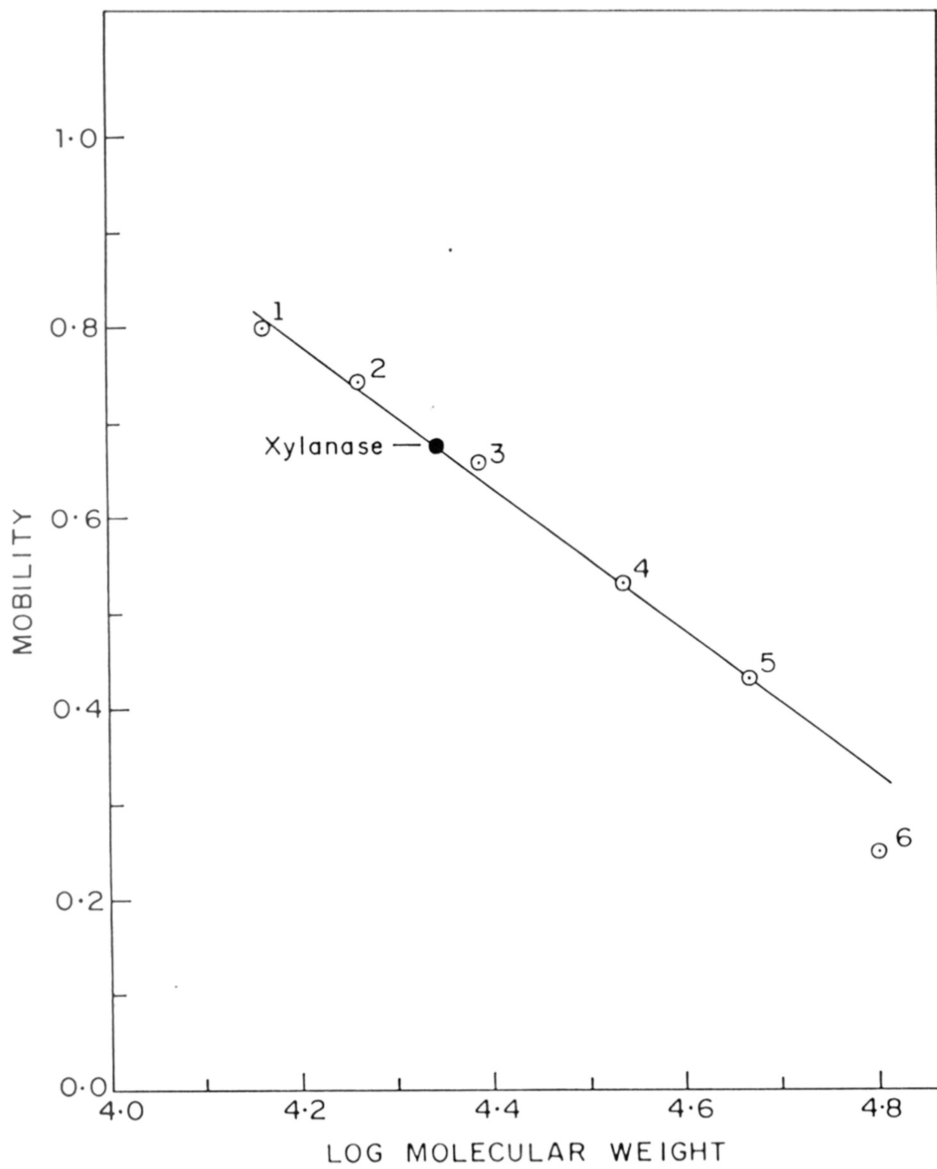

4·4
4·3
4·2
4·1
4·0
LOG MOLECULAR WEIGHT
1
Xylanase
2
3
1·0
1·4
1·8
2·2
Ve / Vo

**Fig. 4.7a:** Determination of molecular weight of xylanase by SDS-PAGE

| Marker protein | Molecular weight (daltons) |
|---|---|
| 1. Lysozyme | 14,300 |
| 2. β-Lactoglobulin | 18,400 |
| 3. Trypsinogen | 24,000 |
| 4. Pepsin | 34,700 |
| 5. Ovalbumin | 45,000 |
| 6. Albumin | 66,000 |



1·0
0·8
0·6
0·4
0·2
0·0
MOBILITY
Xylanase
1
2
3
4
5
6
4·0
4·2
4·4
4·6
4·8
LOG MOLECULAR WEIGHT

**Fig. 4.7b:** SDS-PAGE of xylanase

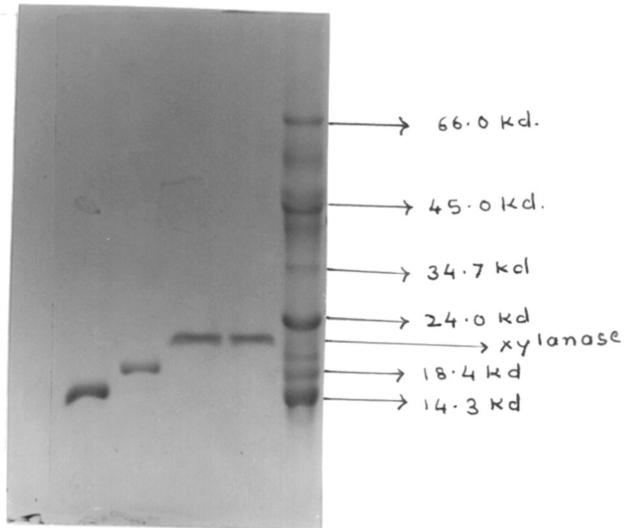

66.0 kd.
45.0 kd.
34.7 kd
24.0 kd
xylanase
18.4 kd
14.3 kd

**Fig. 4.8a:** Determination of isoelectric pH of xylanase

(o) xylanase activity (●) pH

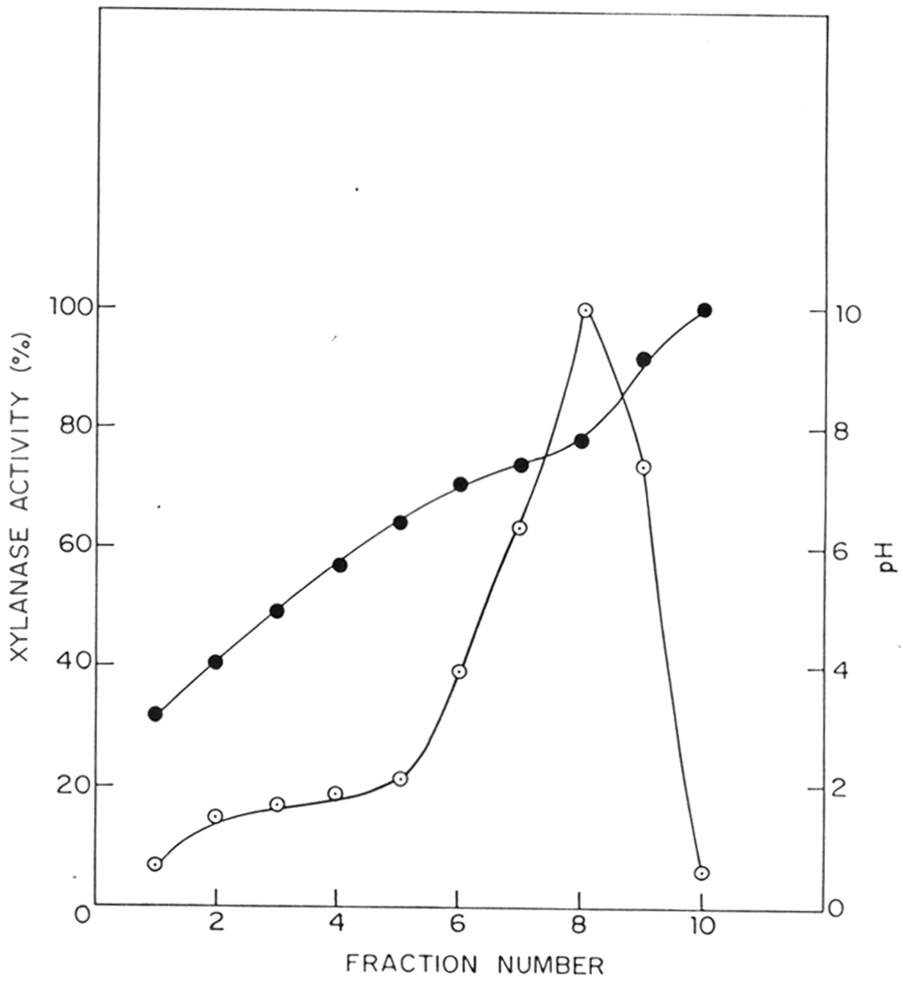
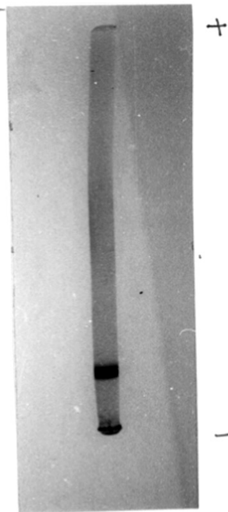

XYLANASE ACTIVITY (%)
100
80
60
40
20
0
10
8
6
4
2
0
pH
2
4
6
8
10
FRACTION NUMBER

**Fig. 4.8b:** Isoelectric focusing of the xylanase in the pH range 3.0 - 10.0.



was supported by evidences of xylanases from Bacillus, clostridium and fungal species, although the high molecular weight xylanases in Streptomyces sp have relatively neutral pIs (range 5.2 - 7.3) [1]. But in the case of Streptomyces $T_7$ for pI of 7.8, molecular weight of 20,000 was observed.

**Carbohydrate content**

The carbohydrate content for three endo-xylanases reported from C. stercorarium was 19, 3 and 4%, respectively [12]. The endo-xylanases of B. circulans WL-12 [3] and C. thermolacticum [14] were not glycoproteins. The carbohydrate content in endo-xylanases X-I, X-II-A and X-II-B of Streptomyces 3137 was less than 1% [19]. The endo-xylanase from mutant 8-7 of S. lividans 1326 was not glycosylated [15]. The endo-xylanase II of Chainia sp. was a glycoprotein [21]. Fungal xylanases are mostly glycosylated, but the endo-xylanase reported from S. rolfsii [40] is not a glycoprotein.

In case of $T_7$ xylanase, when the gel was stained by thymol $H_2SO_4$ reagent, the red band corresponding to the protein band was visible indicating the glycoprotein nature of the xylanase. The xylanase contained 33 mol of carbohydrate per mol of protein determined by phenol $H_2SO_4$ method using glucose as a standard.

**Amino acid composition**

Very few reports are available on the amino acid composition of xylanases from different sources. Among the three endo-xylanases of Streptomyces sp 3137, the molar ratio of tyrosine in X-II-A and X-II-B was more than double than that of X-I. Amino acid analysis indicated that X-I was a different protein from X-II-A and X-II-B in structure which

are very similar proteins [19]. Xylanases from Streptomyces sp. E-86 and KT-23 were reported to be rich in aspartic acid and glycine. KT-23 xylanase was rich in glutamic acid while tyrosine and cysteine content was low as compared to xylanases from Streptomyces sp 3137 and E-86 [37].

Present investigation shows that the xylanase from Streptomyces $T_7$ has high content of aspartic acid, glutamic acid, glycine and histidine. Histidine content is higher than any mesophilic Streptomyces xylanases. It contained four methionine and three cysteine residues (Table 4.3).

Tyrosine and tryptophan content

(a) Goodwin and Morton's method From the data in Table 4.4, it was calculated that xylanase contained 5.45 mol of tyrosine and 5.0 mol of tryptophan per mol of enzyme.

(b) Bencze and Schmid's method The absorbance of xylanase in 0.1 N NaOH showed two maxima viz. at 284 nm and 292 nm. From the slope of the graph S value was -8.6. From Fig. 4.9, the molar ratio of tyrosine:tryptophan was found to be 0.5. The tyrosine and tryptophan content was calculated to be approximately 5.6 mol each per mol of enzyme. This is in agreement with the value calculated by Goodwin and Morton's method.

Determination of free -SH groups

(a) DTNB titration When the xylanase was titrated with DTNB, there was no change in O.D. after 2 h. However, after keeping the enzyme with DTNB overnight colour developed. The increase in O.D. at 412 nm was 0.230. There was no change in O.D. when the same reaction was carried out in the presence of 1% SDS. The free thiol groups calculated from extinction

Table 4.3: Amino acid composition of xylanase from Streptomyces $T_7$

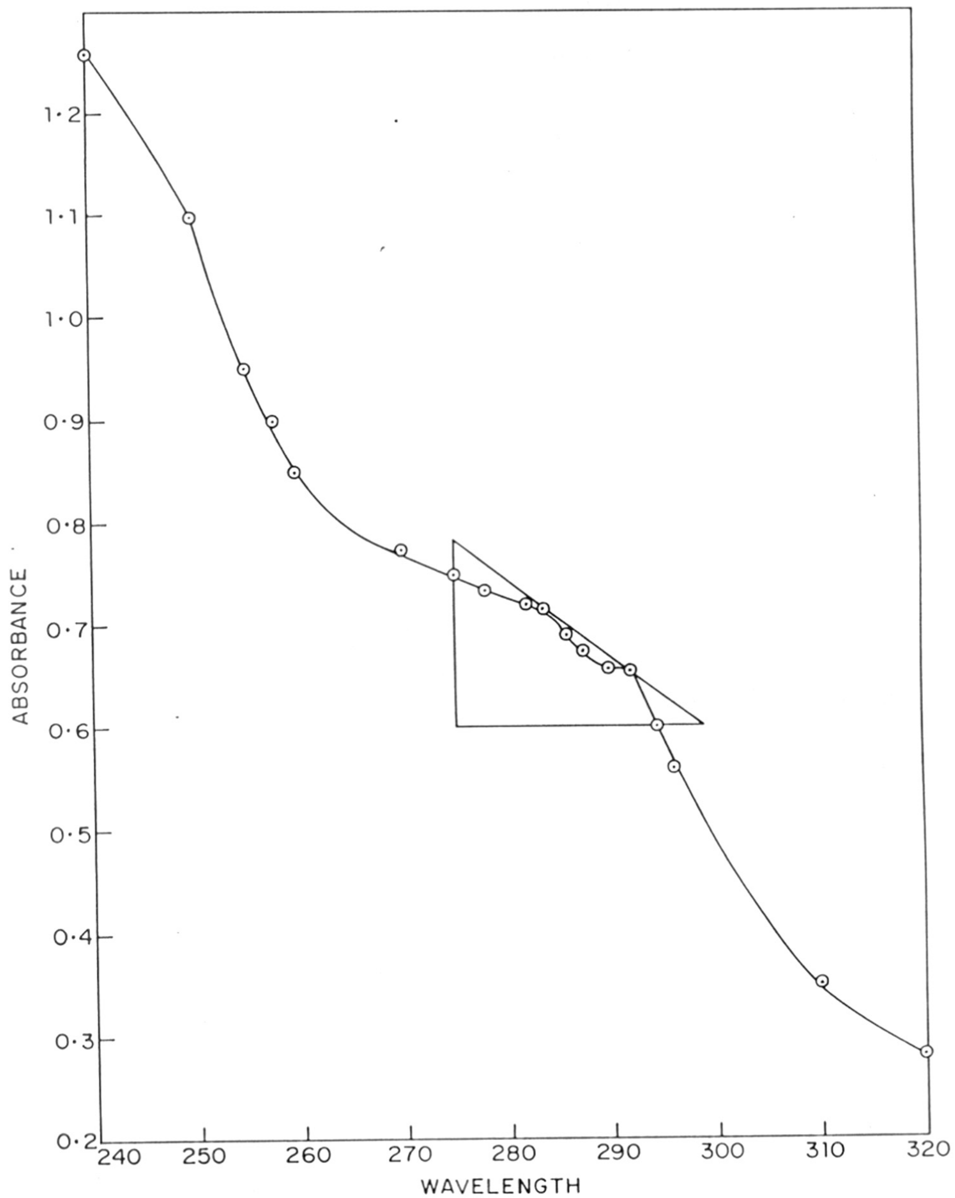
| Amino acid | Mol/mol of enzyme | % Mol |
|---|---|---|
| Aspartic acid | 20 | 10.9 |
| Threonine | 10 | 5.5 |
| Serine | 11 | 6.0 |
| Glutamic acid | 31 | 16.9 |
| Proline | 4 | 2.1 |
| Glycine | 28 | 15.3 |
| Alanine | 5 | 2.7 |
| Methionine | 4 | 2.1 |
| Isoleucine | 2 | 1.0 |
| Leucine | 2 | 1.0 |
| Tyrosine | 5 | 2.7 |
| Phenylalanine | 2 | 1.0 |
| Lysine | 5 | 2.7 |
| Histidine | 37 | 20.2 |
| Arginine | 2 | 1.0 |
| Tryptophan | 5 | 2.7 |
| Valine | 5 | 2.7 |
| Cysteine* | 5 | 2.7 |
| | ----- | |
| | 183 | |

*Determined as cysteic acid after performic acid oxidation.

**Table 4.4:** Absorbance of xylanase ($1.65 \times 10^{-2}$ M) in 0.1 N NaOH.

| Wavelength (nm) | Absorbance (O.D.) |
|---|---|
| 257.0 | 0.677 |
| 280.0 | 0.504 |
| 294.4 | 0.365 |

**Fig. 4.9:** Determination of tyrosine and tryptophan by Bencze and Schmid method.



ABSORBANCE
1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
240
250
260
270
280
290
300
310
320
WAVELENGTH

coefficient were 3.38 mol per mol of enzyme.

(b) PHMB titration When the xylanase was treated with PHMB, the O.D. at the intersection was taken as the end point of reaction (Fig. 4.10). The free thiol groups calculated from extinction coefficient were found to be 2.72 mol per mol of enzyme.

Total -SH and -S-S- groups

(a) When the reduced xylanase was treated with DTNB, the change in O.D. was 0.090 after 16 h. This gave a value of 5.2 mol of -SH per mol of enzyme.

(b) Performic acid oxidation indicated 5 mol of cysteic acid per mol of enzyme.

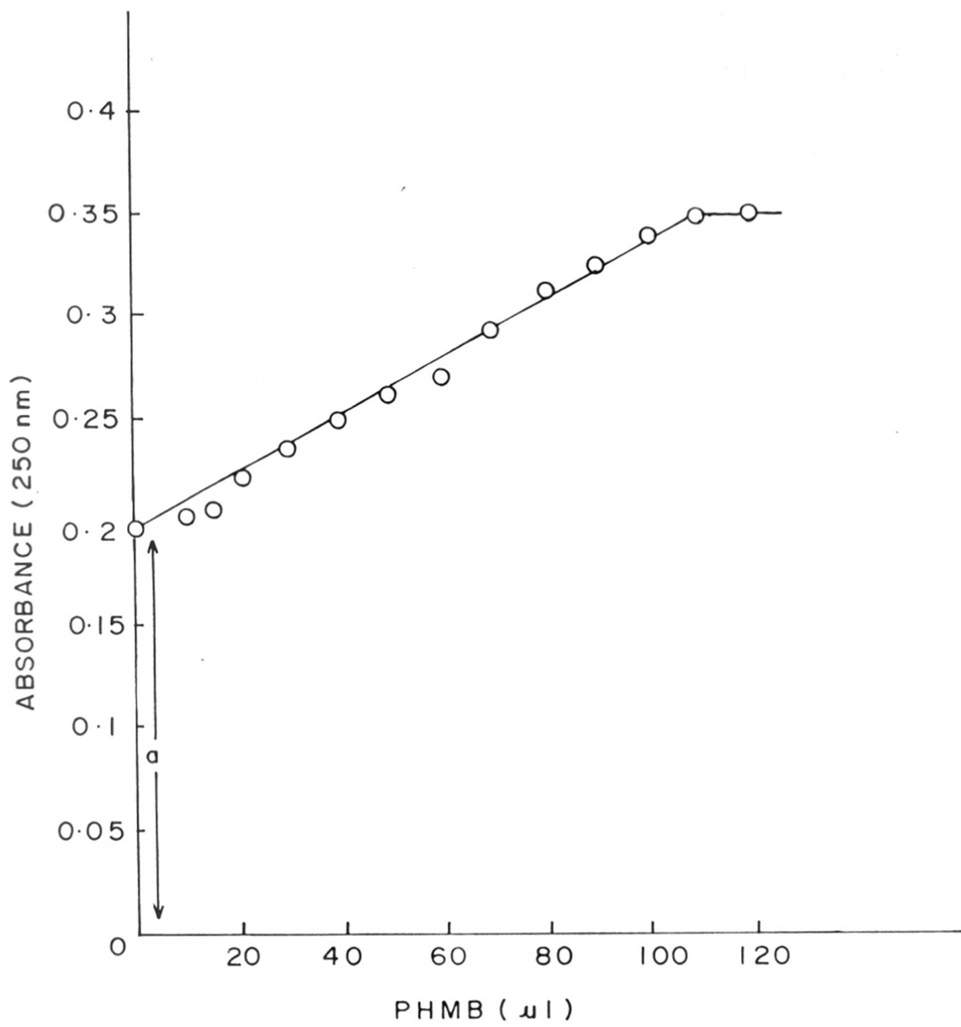
From the above experiments, it was deduced that there are 3 - 3.5 mol of sulfhydryl and one disulfide residue per mol of xylanase.

**Substrate specificity**

Xylanase A from C. acetobutylicum hydrolysed xylan, CM-cellulose and acid swollen cellulose and lichenan but was unable to degrade laminarin, mannan and galactan etc. while xylanase B hydrolysed xylan and lichenan but none of the other substrates [13]. The xylanase from C. thermolacticum was inactive towards arabino-galactan, CM-cellulose and pNPX [14]. Xylan hydrolase II from S. exfoliatus was specific to xylan while Ib was specific to xylan and cellulose. Xylan hydrolase Id attacked inulin, pectin and cellulose [17]. The xylanase from Streptomyces sp KT-23 was active towards only xylan and did not hydrolyse CM-cellulose, dextran and pNPX etc. [20]. Similarly, the xylanases from Streptomyces sp 3137 did not attack $X_2$, cellobiose, maltose, starch, CM-

**Fig. 4.10:** Determination of free thiol groups of xylanase by spectrophotometric titration with PHMB

a - Refers to the absorbance of protein at 250 nm before addition of PHMB.


0·4
0·35
0·3
0·25
0·2
0·15
0·1
0·05
0
ABSORBANCE ( 250 nm)
a
20
40
60
80
100
120
PHMB ( µl )

cellulose but xylan was hydrolysed easily [37].

The Streptomyces $T_7$ xylanase was found to be specific only for xylan and produced 4.3 mg of reducing sugar per ml in 24 h and it did not produce any reducing sugar from other substrates such as CM-cellulose, filter paper (Whatman No. 1). Avicel, pNPX, cellobiose, laminarin, pullulan, chitin and Gum Locust.

**Kinetics**

The $K_m$ values for xylanases of bacilli and clostridia ranged between 1.5 - 10.0 mg/ml. The affinity of Streptomyces sp. KT-23 xylanase increased with increasing molecular weight of the substrate [20], whereas Streptomyces E-86 xylanase had higher affinity for the short chain oligosaccharides ($X_3$ and $X_5$) [18].

The kinetic parameters viz. $K_m$ and $V_{max}$ were evaluated for Streptomyces $T_7$ xylanase using soluble, insoluble and total xylan from the respective Lineweaver-Burk plots (Fig. 4.11a,b and c) and the results are listed in Table 4.5. The Streptomyces $T_7$ xylanase has a $K_m$ of 10 mg/ml when soluble larch wood xylan was used as a substrate. The affinity of xylanase decreased with more resistant substrate yielding $K_m$ values of 25 mg/ml for total xylan and 33.3 mg/ml for insoluble xylan. The $K_m$ values are apparent since xylan is a heterogeneous substrate and new oligosaccharides are formed during the course of the reaction.

**Effect of metal ions and other compounds**

Among the various metal ions $Hg^{2+}$ was found to be the potent inhibitor for many xylanases [8,9,12,13,22,25,26].

**Fig. 4.11:** Lineweaver-Burk plot for xylanase

a) Xylanase (1 µg) was incubated with 3 - 17 mg of soluble xylan under the assay conditions.

b) Xylanase (1 µg) was incubated with 4 - 18 mg of insoluble xylan under the assay conditions.

c) Xylanase (1 µg) was incubated with 3 - 15 mg of total xylan under the assay conditions.

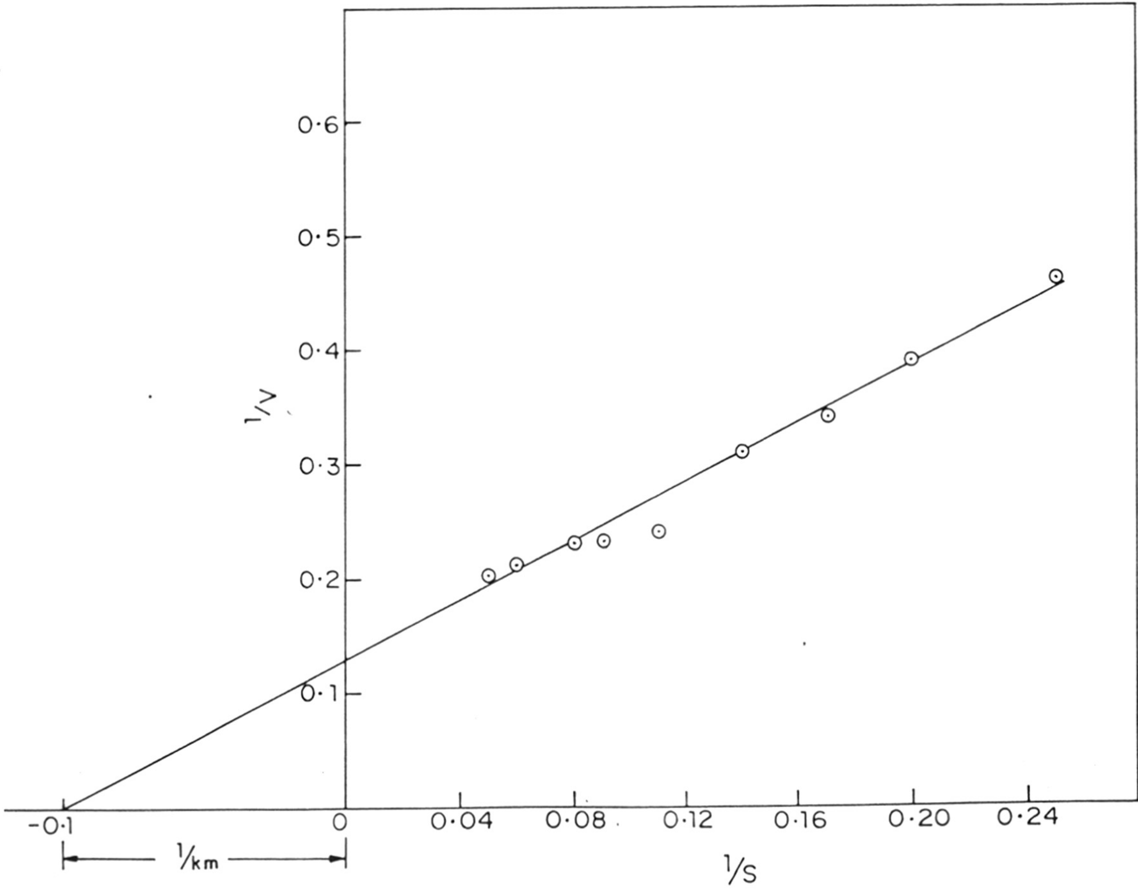



Fig. 4.11a

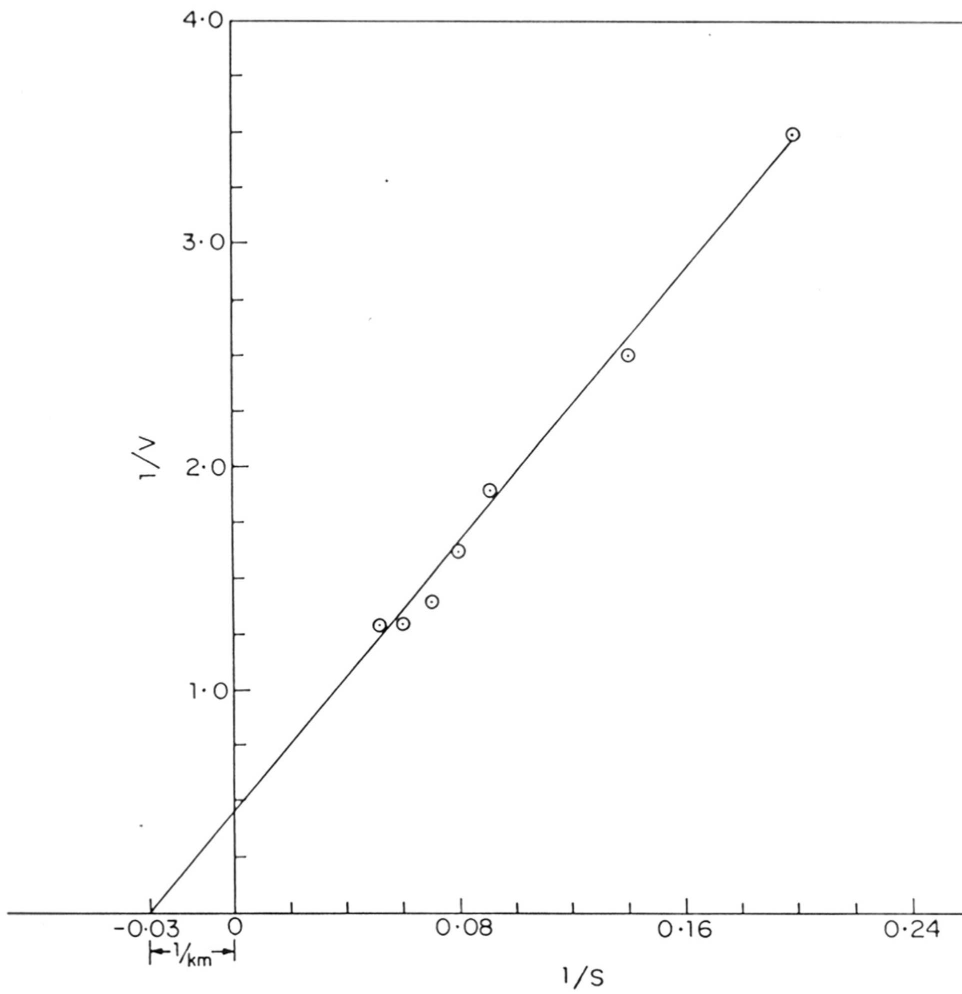



Fig. 4.11b

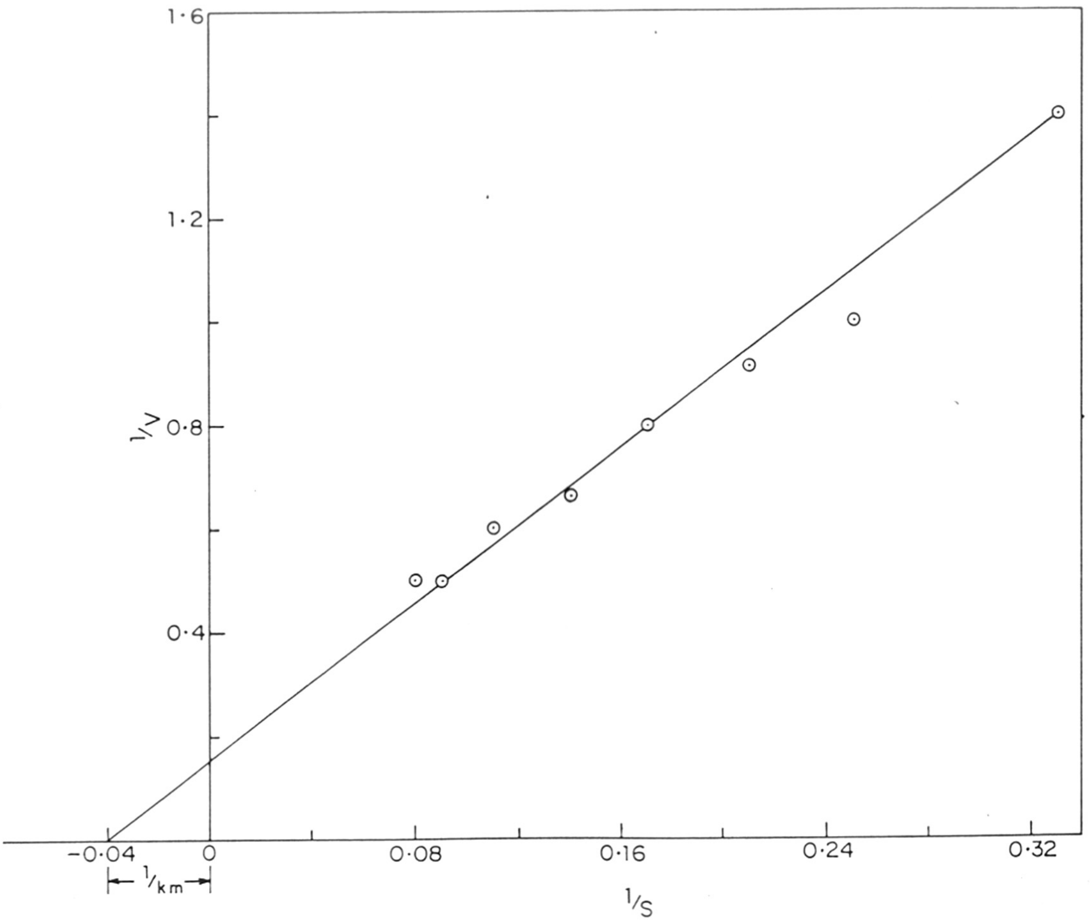



Fig. 4.11c

**Table 4.5:** Kinetic parameters of xylanase from *Streptomyces*-$T_7$

| Substrate (Xylan) | $K_m$ (mg/ml) | $V_{max}$ (m Mol/min/mg) |
|---|---|---|
| Soluble | 10.0 | 7.6 |
| Insoluble | 33.3 | 2.2 |
| Total | 25.0 | 6.6 |

exception was from Bacillus sp 11-1S which was not inhibited by $Hg^{2+}$ as well as by any other metal ion [11]. Three xylanases from C. stercorarium were completely inhibited by $Hg^{2+}$ (1 mM) and $Fe^{3+}$ (10 mM). Significant inhibition was also observed with $Ca^{2+}$ (10 mM) and $Cu^{2+}$ (10 mM) [12]. The common inhibitor for xylanases from Streptomyces sp were $Hg^{2+}$, $Fe^{3+}$ and SDS [19,20,51]. However, Streptomyces KT-23 xylanase was also inhibited (51%) by $Mn^{2+}$ [20]. $Ca^{2+}$ stimulated both xylanases from S. exfoliatus, Ib (24%) and II (15%) but inhibited Id (11%) [17].

The Streptomyces $T_7$ xylanase (1 µg) was incubated with various metal ions and compounds in the presence of 50 mM acetate buffer, pH 5.0 at 25°C for 10 min and then assayed at standard conditions. Inhibitory effect of $Hg^{2+}$ and $Ag^{+}$ on xylanase activity is summarized in Table 4.6. $Ca^{2+}$, $Mn^{2+}$, $Mg^{2+}$, $Co^{2+}$, $Mo^{6+}$ each ($1 \times 10^{-3}$ M) did not show any effect on the xylanase. The enzyme activity was unaffected in the presence of 10 mM EDTA, indicating that metal ions are not required for the activity. Xylitol (5 mM) showed 50% inhibition. 68% inhibition was observed in the presence of 6 M urea while xylanase was completely inhibited by 1% SDS (Table 4.7).

**Xylan degradation products**

Xylan degradation products from the action of xylanases from various organisms indicate: Endo-xylanases reported by several authors showed that the hydrolysis products include $X_2$, $X_3$ and higher xylo-oligosaccharides in early periods of time whereas longer incubation resulted in small amount of xylose as end product [4,15,16,19,20] or xylose was not

**Table 4.6:** Effect of $Hg^{2+}$ and $Ag^{+}$ on xylanase activity

| Compound | Concentration ($M \times 10^{-6}$) | Inhibition (%) |
|---|---|---|
| $HgCl_2$ | 0.2 | 20.0 |
| | 0.5 | 35.0 |
| | 1.0 | 45.6 |
| | 2.0 | 100.0 |
| $AgNO_3$ | 1.0 | 12.7 |
| | 2.0 | 19.2 |
| | 4.0 | 52.4 |
| | 10.0 | 100.0 |

**Table 4.7:** Effect of various compounds on xylanase

| Compound | Concentration | Inhibition (%) |
|---|---|---|
| EDTA | 10 mM | 0.0 |
| Xylitol | 5 mM | 50.0 |
| Mannitol | 5 mM | 18.6 |
| Sorbitol | 5 mM | 0.0 |
| Urea | 6 M | 68.0 |
| SDS | 1 % | 100.0 |

formed [5,8,10]. The end products obtained for two components from same Bacillus sp. W1 and W2 differed. Component I did not produce any xylose while componant II produced xylose. β-xylosidase was not detected in the culture filtrate of both the strains. So production of xylose was considered to be due to xylanase activity. [9]. Xylan hydrolase II and Id of S. exfoliatus showed other xylo-oligosaccharides except xylose, while Ib xylanase showed the xylose in the hydrolysis product at short incubation period but at present it is controversial to assert that it is an exo-xylanase [17].

The major end products of xylan hydrolysis of the Streptomyces $T_7$ xylanase are $X_2$ and $X_3$ - $X_6$ (Fig. 4.12a,b). Very little xylose was produced even after 16 h indicating that it is an endo-xylanase.

**Synergism with β-xylosidase**

Our investigation shows that hydrolysis of xylan was 43% when xylan was incubated with Streptomyces $T_7$ xylanase alone. But when xylan was incubated with a mixture of xylanase and β-xylosidase, hydrolysis increased to 60% with an increased amount of xylose (Fig. 4.12b). This may be due to the action of β-xylosidase of $X_2$ as well as short chain xylo-oligomers. The efficiency on hydrolysis of xylan by Streptomyces $T_7$ xylanase could be increased by supplementing it with β-xylosidase resulting in obtaining xylose as the major product of hydrolysis, which has a high commercial value.

**Fig. 4.12:** Paper chromatogram showing the hydrolysis products from xylan

a) Xylanase (1 U) was incubated with xylan (10 mg) in 20 mM acetate buffer, pH 5.0 for different periods of time.

b) Xylanase (1 U) and β-xylosidase (0.07 U) were mixed and incubated with xylan (10 mg) in 20 mM acetate buffer, pH 5.0 at 37°C for 20 h.

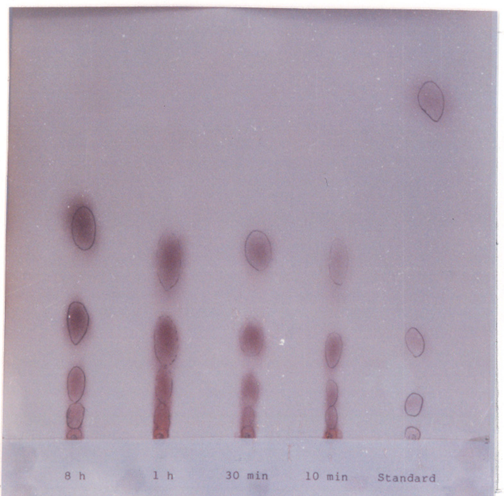



Fig. 4·12a

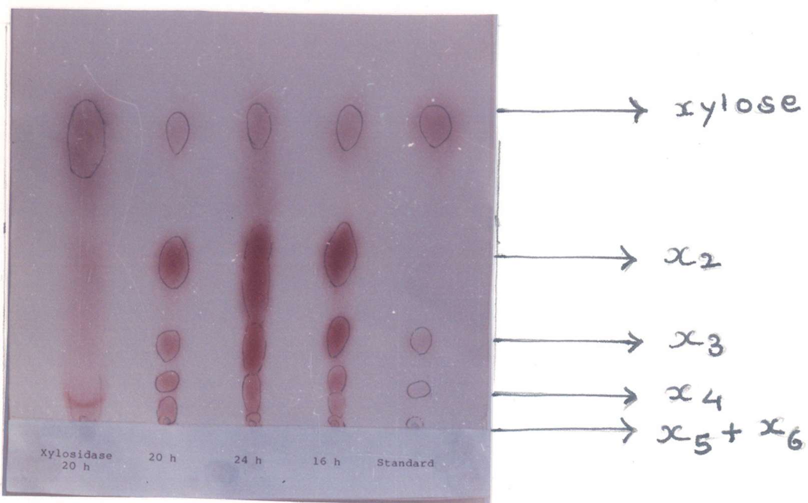



Fig. 4·12b

REFERENCES

1. Wong, K.K.Y., Tan, L.U.L. and Saddler, J.N. (1988) Microbi l. Rev **52**, 305.

2. Dekker, R.F.H. (1985) In "Biosynthesis and biodegradation of wood components (Ed. Higuchi, T.) Academic Press Inc., Orlando Fla, p. 505.

3. Esteban, R., Villanueva, J.R. and Villa, T.G. (1982) Can. J. Microbiol. **28**, 733.

4. Esteban, R., Chordi, A. and Villa, T.G. (1983) FEMS Microbiol. Lett. **17**, 163.

5. Panbangred, W., Shinmyo, A., Kinoshita, S. and Okada, H. (1983) Agric. Biol. Chem. **47**, 957.

6. Bernier, R.Jr., Desrochers, M., Jurasek, L. and Paice, M.G. (1983) Appl. Environ. Microbiol. **46**, 511.

7. Kato, Y. and Nevins, D.J. (1984) Plant Physiol. **75**, 753.

8. Horikoshi, K. and Atsukawa, Y. (1973) Agric. Biol. Chem. **37**, 2097.

9. Okazaki, W., Akiba, T., Horikoshi, K. and Akahoshi, R. (1985) Agric. Biol. Chem. **49**, 2033.

10. Honda, H., Kudo, T., Kura, Y. and Horikoshi, K. (1985) Can. J. Microbiol. **31**, 538.

11. Uchino, F. and Nakane, T. (1981) Agric. Biol. Chem. **45**, 1121.

12. Berenger, J.F., Frixon, C., Bigliardi, J. and Creuzet, N. (1985) Can. J. Microbiol. **31**, 635.

13. Lee, S.F., Forsberg, C.W. and Rattray, J.B. (1987) Appl. Environ. Microbiol. **53**, 644.

14. Debeire, P., Priem, B., Strecker, G.and Vignon, M. (1990)

Eur. J. Biochem. **187**, 573.

15. Morosoli, R., Betrand, J.L., Mondou, F., Shareck, F. and Kluepfel, D. (1986) Biochem. J. **239**, 587.

16. Iizuka, H. and Kawaminami, T. (1965) Agric. Biol. Chem. **29**, 520.

17. Sreenath, H.K. and Joseph, R. (1982) Folia Microbiol. **27**, 107.

18. Kusakabe, I., Kawaguchi, M., Yasui, T. and Kobayashi, T. (1977) Nippon Nogei Kagaku Kaishi, **51**, 429.

19. Marui, M., Nakanishi, K. and Yasui, T. (1985) Agric. Biol. Chem. **49**, 3399.

20. Nakajima, T., Tsukamoto, K.I., Watanabe, T., Kainuma, K. and Matsuda, K. (1984) J. Ferment. Technol. **62**, 269.

21. Bastawde, K.B. (1987) Ph.D. Thesis, Poona University.

22. Kitpreechavanich, V., Hayashi, M. and Nagai, S. (1984) J. Ferment. Technol. **62**, 63.

23. Anand, L., Krishnamurthy, S. and Vithayathil, P.J. (1990) Arch. Biochem. Biophys. **276**, 546.

24. Matsuo, M., Yasui, T. and Kobayashi, T. (1977) Agric. Biol. Chem. **41**, 1593.

25. Yoshiko, H., Chavanich, S., Nilubol, N. and Hayashida, S. (1981) Agric. Biol. Chem. **45**, 579.

26. Tan, L.U.L., Mayers, P. and Saddler, J.N. (1987) Can. J. Microbiol. **33**, 689.

27. Ganju, R.K., Vithayathil, P.J. and Murthy, S.K. (1989) Can. J. Microbiol. **35**, 836.

28. Dekker, R.F.H. and Richards, G.N. (1975) Carbohydr. Res. **39**, 97.

29. Mitsuishi, Y., Yamanabe, T., Yagisawa, M. amd Takasaki, Y. (1987) Agric. Biol. Chem. **51**, 3207.

30. Woodward, J. (1984) In "Topics in Enzyme and Fermentation Biotechnology" (Ed. Wiseman, A.) John Wiley and Sons, N.Y. vol. 8, p. 9.

31. Morosoli, R., Roy, C. and Yaguchi, M. (1986) Biochim. Biophys. Acta, **870**, 473.

32. Nakanishi, K., Arai, H. and Yasui, T. (1984) J. Ferment. Technol. **62**, 361.

33. Fournier, A.R., Frederick, M.M., Frederick, J.R. and Reilly, P.J. (1985) Biotechnol. Bioeng. **27**, 539.

34. Frederick, M.M., Frederick, J.R., Fratzke, A.R. and Reilly, P.J. (1981) Carbohydr. Res. **97**, 87.

35. Eriksson, K.E. and Wood, T.M. (1985) In "Biosynthesis and Biodegradation of Wood Components" (Ed. Higuchi, T.) Academic Press Inc., Orlando, Fla.p. 469.

36. Wood, T.M. (1985) Biochem. Soc. Trans. **13**, 407.

37. Yasui, T., Marui, M., Kusakabe, I. and Nakanishi, K. (1988) Methods Enzymol. **160**, 648.

38. Shei, J.C., Fratzke, A.R., Frederick, M.M., Frederick, J.R. and Reilly, P.J. (1985) Biotechnol. Bioeng. **27**, 533.

39. Toda, S., Suzuki, H. and Nisizawa, K. (1971) J. Ferment. Technol. **49**, 499.

40. Deshpande, M.V., Sadana, J.C. and Meagher, M.M. (1989) Enz. Microb. Technol. **11**, 678.

41. Paice, M.G., Jurasek, L., Carpenter, M.R. and Smillie, L.B. (1978) Appl. Environ. Microbiol. **36**, 802.

42. Kusakabe, I., Yasui, T. and Kobayashi, T. (1977) Nippon Nogei Kogaku Kaishi, **51**, 669.

43. Meagher, M.M. (1984) M.S. Thesis, Iowa State University, Ames, Iowa, USA.

44. Frederick, M.M., Kiang, C.H., Frederick, J.R. and Reilly, P.J. (1985) Biotechnol. Bioeng. **27**, 525.

45. Dekker, R.F.H. and Richards, G.N. (1976) Adv. Carbohydr. Chem. Biochem. **32**, 278.

46. Gorin, P.A.J., Spencer, J.F.T. and Eveleigh, D.E. (1969) Carbohydr. Res. **11**, 387.

47. Fukui, S. and Sato, M. (1957) Bull. Agric. Chem. Soc. (Japan) **21**, 392.

48. Claeyssens, M., Loontiens, F.G., Kersters-Hilderson H, and De Bruyne, C.K. (1971) Enzymologiya, **40**, 177.

49. King, N.J. and Fuller, D.B. (1968) Biochem. J. **108**, 571.

50. Reilly, P.J. (1981) In "Trends in the Biology of Fermentations for Fuels and Chemicals" (Ed. Hollaender, A.) Plenum Press, N.Y. vol. 18, p. 111.

51. Richards, G.N. and Shambe, T. (1976) Carbohydr. Res. **49**, 371.

52. Gorbacheva, I.V. and Rodionova, N.A. (1977) Biochim. Biophys. Acta, **484**, 94.

53. Andrews, P. (1965) Biochem. J. **96**, 595.

54. Laemmli, U.K. (1970) Nature (London) **227**, 680.

55. Vesterberg, O. (1972) Biochim. Biophys. Acta, **257**, 11.

56. Gander, J.E. (1984) Methods Enzymol. **104**, 447.

57. Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956) Anal. Chem. **28**, 350.

58. Spackman, D.H., Stein, W.H. and Moore, S. (1958) Anal. Chem. **30**, 1190.

59. Goodwin, T.W. and Morton, R.A. (1946) Biochem. J. **40**, 628.

60. Bencze, W.L. and Schmid, K. (1957) Anal. Chem. **29**, 1193.

61. Ellman, G.L. (1959) Arch. Biochem. Biophys. **82**, 72.

62. Means, G.E. and Feeney, R.E. (1971) In "Chemical Modification of Proteins" (Eds. Means, G.E. and Feeney, R.E.) Holden-Day, Inc., U.S.A. p. 149.

63. Boyer, P.D. (1954) J. Am. Chem. Soc. **76**, 4331.

64. Riordan, J.F. and Vallee, B.L. (1967) Methods Enzymol. **11**, 541.

65. Cavallini, D., Graziani, M.T. and Dupre, S. (1966) Nature (London), **212**, 294.

66. Hirs, C.H.W. (1967) Methods Enzymol. **11**, 59.

67. Trevelyn, W.E., Procter, P.D. and Harrison, J.S. (1950) Nature (London), **166**, 444.

68. Vyas, P., Chauthaiwale, V., Phadatare, S., Deshpande, V. and Srinivasan, M.C. (1990) Biotechnol. Lett. **12**, 225.

69. Brock, T.D. (1986) In "Termophiles: General Molecular and Applied Microbiology" (Ed. Brock, T.D.) John Wiley and Sons, p. 17.

70. Vartak, H.G. and Bastawde, K.B. (1988) U.S. Patent Application Ser. No. 224,590.

# CHAPTER 5

# CHEMICAL MODIFICATION OF XYLANASE

## SUMMARY

Effect of various chemical modifiers on xylanase activity was investigated. Complete inhibition by N-bromosuccinimide (NBS) (1 mM), 2-hydroxy-5-nitrobenzylbromide (HNBB) (10 mM) and *p*-hydroxymercuribenzoate (PHMB) (1 mM) indicated that tryptophan and cysteine were modified. Plots of percentage residual activity as a function of time at various concentrations of NBS, HNBB and PHMB indicated that the inactivation process exhibited pseudo-first-order kinetics with respect to time at any fixed concentration of the inhibitor. Kinetic analysis indicated that the loss of enzyme activity resulted from reaction of only one tryptophan or cysteine residue per molecule of enzyme. The second order rate constants for inactivation were determined and found to be $3.84 \times 10^3$, 13.8 and $12 \times 10^3$ per min/mol for NBS, HNBB and PHMB, respectively.

Titration with NBS indicated that for complete inactivation of enzyme 3.6 mol of NBS were required per mol of enzyme. The number of tryptophan residues oxidized per molecule of enzyme were found to be 2.2. Protection against inactivation by substrate (xylan) indicated the presence of these residues (tryptophan and cysteine) at the substrate binding site. Reactivation by cysteine supported the finding that cysteine is involved in catalysis.

# INTRODUCTION

## Chemical modification - General aspects

Proteins are extremely complex molecules. Their complexity is due to their relatively large size and unique three-dimensional arrangement of their many functional groups. The three-dimensional structure of a protein is governed by its primary structure (sequence of amino acids) and its environment. Changes in either of these can have important effect on its properties. Modifying any of its amino acid residues necessarily changes the primary structure of protein [1].

Enzymes represent a most remarkable set of biomolecules, owing both to their high catalytic activity and their ligand specificity. The study of enzyme mechanisms can follow several different approaches. One of them is to analyse the kinetics of the reaction. This analysis does not tell, however, the detailed molecular mechanism by which these transformations occur and how the structural elements of the enzyme contribute to them. Enzyme and substrate come in close contact in a limitated area of the enzyme surface called the "active site" at which the catalytic process takes place. To learn about the catalytic mechanism of an enzyme, it is therefore, essential to study the structural elements of this site (amino acid side chains and prosthetic groups) and the three-dimensional conformation of the site [2].

Chemical modification is of special importance in probing active site structure. A chemical reagent is placed in contact with the enzyme and chemical reaction is allowed to occur. This reagent binds covalently to the specific amino

acid side chains in the enzyme and produces changes in some measurable property of the enzyme. Ideally, the reagent should be of sufficient selectivity to combine with only one residue and cause minimal alterations in the conformation of the enzyme [2]. Essential groups are those involved in, or in some way required for a particular property of the enzyme. Active site is composed of one or more such groups. When modification of a group in protein effects loss of biological activity, it is usually considered essential to that activity [1]. Of the 20 natural amino acids, only those possessing a polar side chain are normally the object of chemical modification. The chemical reactivity of these groups is basically a function of their nucleophilicity. Two main types of approaches to chemical modification can be recognised. One is based on the use of group specific reagent, and the second utilises affinity labels.

Most reagents react with more than one side chain. The enzyme may possess many residues of a certain kind, which can potentially react with group specific reagents. The reactivity of an amino acid side chain is greatly influenced, however, by its microenvironment. At the active site of enzymes, which is in a less polar microenvironment, amino acid residues often show a markedly different $pK_a$ than that of the same free amino acid. Microenvironment around a specific residue is a key factor in determining its reactivity. Several factors such as polarity, hydrogen bonding forces, electrostatic forces, pH and temperature influence the microenvironment [3].

The method used to detect the identity and the number

of essential amino acid residues is the kinetic approach of Ray and Koshland [4]. This method is based on the relationship between the rate of loss of biological activity and the rate of modification of amino acid residues.

Essential amino acid residues in the active site can participate in substrate binding or in catalysis. The protection by substrates or related compounds have been used to selectively prevent the modification of active centre residues in many enzymes. If enzymatic activity is retained, following modification in the presence of substrate but is lost in the absence, it is usually assumed that a group is present at the substrate binding site. Correlation between the reversal of the modification and recovery of the altered biological function is additional useful evidence to establish the possible function of the residue. Several workers [5-7] have reviewed the modification of amino acid residues and are listed in Table 5.1.

**Chemical modification of xylanases**

Reports on the inhibition of xylanases by different chemical reagents, which are specific to certain amino acids are available [30,31]. However, the number of amino acid residues essential for activity and their role in the catalytic site have not been investigated. Large active centres containing several subsites appear to be characteristic of fungal xylanases in general [32-35]. However, there is little data correlating the structure and function of the binding-site region of xylanase.

The three xylanases viz. A, B and C from Clostridium stercorarium were inhibited by PHMB but showed no inhibition

Table 5.1: Reagents used in the modification of amino acid residues

| Amino acid | Chemical reagent | Type of reaction | Group involved | pH of the reaction | Special feature | Reference |
|---|---|---|---|---|---|---|
| Cysteine | N-ethylmaleimide (NEM) | Thiol-ether formation | Sulfhydryl | 6.0 - 7.0 | Decrease in O.D. at 305 nm | 8,9 |
| | p-hydroxymercuri-benzoate (PHMB) | Mercaptide formation | Sulfhydryl | 5.0 - 7.0 | Change in O.D. at 250 - 255 nm | 10,11 |
| | 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) | Disulfide formation | Sulfhydryl | 7.0 - 8.0 | Increase in O.D. at 412 nm | 12,13 |
| | Iodoacetamide | Carboxym-methy-lation | Sulfhydryl | 8.0 - 9.0 | | 14 |
| Methio-nine | Iodoacetamide | Alkyla-tion | Thioether | below 4.0 | | 7,15 |
| Aspartic, glutamic acid | Carbodimide | N- acyl urea formation | Carboxyl | 5.0 - 7.0 | | 16 |
| | Woodward's K | Ketoket-enimine formation | Carboxyl | 6.0 | | 2 |
| Arginine | Phenyl glyoxal | | Guanidino | 8.0 - 9.0 | | 17-19 |
| | Butane-2,3-dione | | | | | |

| Amino acid | Chemical reagent | Type of reaction | Group involved | pH of the reaction | Special feature | Reference |
|---|---|---|---|---|---|---|
| Lysine | 2,4,6-trinitro benzene sulfonic acid (TNBS) | Displacement of sulfite from TNBS | Amino | 9.5 | Increase in O.D. at 420 nm | 7 |
| Histidine | Diethylpyrocarbonate (DEP) | Carbethoxylation | Imidazole | 6.0 - 7.5 | Increase in O.D. having maximum between 230 nm and 250 nm | 20,21 |
| Tryptophan | N-bromosuccinimide (NBS) | Oxidation | Indole | 4.0 | Decrease in O.D. at 280 nm | 22 |
| | 2-hydroxy-5-nitro benzyl bromide (HNBB) | Alkylation | Indole | 4.0 - 7.0 | | 23,24 |
| Tyrosine | Tetranitromethane (TNM) | Nitration | Phenolic | 8.0 | Increase in O.D. at 428 nm | 25 |
| | N-acetylimidazole | Acylation | Phenolic | 7.5 or less | | 26 |
| Serine | Phenylmethylsulfonyl fluoride (PMSF) | Sulfonylation | Hydroxy | 7.0 - 8.0 | | 27,28 |
| | Di-isopropyl fluorophosphate | Phosphorylation | Hydroxy | 7.5 | | 29 |

with thiol specific reagents such as DTNB or NEM [36]. Effect of PCMB, iodine and iodoacetic acid showed that the D-xylanase from Bacillus subtilis requires certain -SH groups for activity. [37]. The involvement of thiol groups at the active site of endo-xylanase from Bacillus sp. was investigated by $HgCl_2$ treatment [38].

Involvement of -SH groups at the catalytic site of two endo-xylanases from Streptomyces exfoliatus was determined with iodoacetamide and PCMB [39]. The three xylanases X-I, X-II-A and X-II-B from Streptomyces sp were inhibited by NBS suggesting the presence of tryptophan at the active centre [30].

Zalewska-Sobezak and Urbanek [40] reported the inactivation of xylanase by NBS and HNBB suggesting the presence of tryptophan residue in the active centre. Moreover, inactivation by tetranitromethane suggested that tyrosine may also be involved in the catalytic mechanism of xylanase from Fusarium avenecum. Similar reports for xylanase from Trametes hirsuta were obtained. So it was assumed that tryptophan and tyrosine residues may influence enzyme catalysis [41].

In this chapter, on the basis of chemical modification we have provided the first evidence (kinetic data) for the involvement of tryptophan and cysteine residues at the active site of xylanase.

# MATERIALS AND METHODS

## Materials

NBS, NEM, DEP, HNBB, PHMB, DTNB, phenyl glyoxal, butane-2,3-dione, iodoacetamide, N-acetylimidazole, PMSF, citraconic anhydride and Woodward's Reagent K were purchased from Sigma Chemical Co., U.S.A. Cysteine was obtained from Fluka AG, Switzerland. All the other chemicals used were of analytical grade.

## Methods

### Reaction with different chemical modifiers

A 0.5 ml portion of enzyme (10 $\mu g/ml$) was incubated in a total volume of 2.5 ml with various concentrations of modifier in appropriate buffer. Control tubes containing only enzyme or inhibitor only were incubated under identical conditions. Aliquots (0.5 ml) were removed at 10, 20, 30 and 40 min for measurement of residual enzyme activity.

The apparent first order rate constant of inactivation depends on the concentration of modifier and can be expressed by the following equation:

$$K_{app} = K(M)^n$$

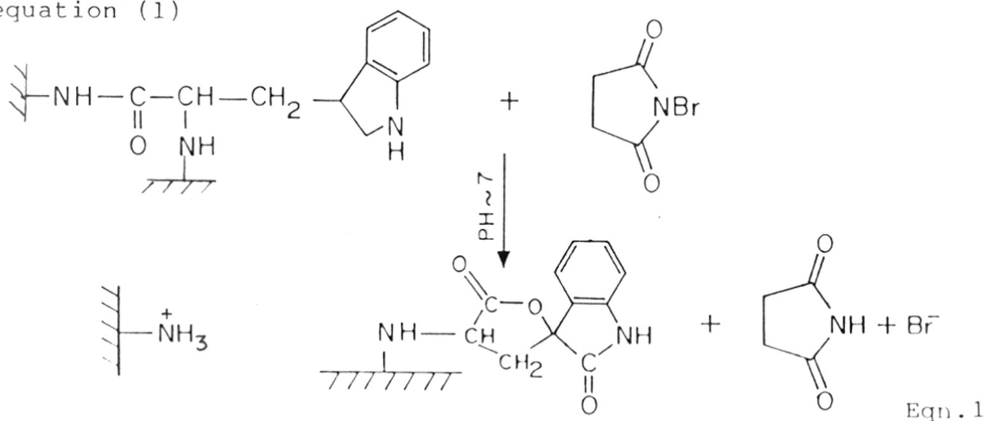
where $K'_{app}$ is the apparent first order rate constant for the inactivation, K is the second order rate constant, M signifies the concentration of modifier and n is the number equal to the average order of the reaction with respect to the concentration of modifier. Taking the logarithm of both sides.

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

$K_{app}$ can be calculated from a semi logarithm plot of residual enzyme activity as a function of time. The second order rate constant for inactivation was determined from the slope of the plot of pseudo-first order rate constant against inhibitor concentration. The order of the reaction (n) can be experimentally estimated by determining $K_{app}$ at a number of different concentrations of modifier. A plot of log $K_{app}$ against log (M) should give a straight line with a slope equal to n, where n is the number of molecules of modifier reacting with each active unit of the enzyme to produce an enzyme inhibitor complex [42-44].

**Modification of tryptophan**

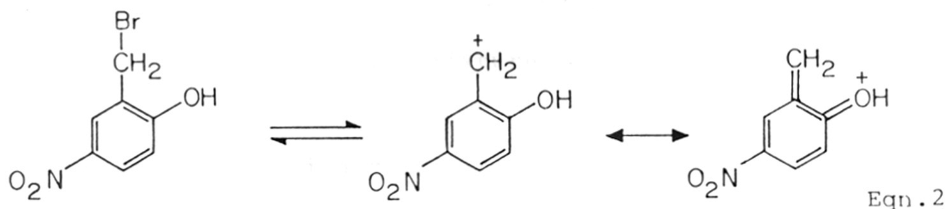
Reaction with NBS can be used to probe the accessibility and reactivity of tryptophan residues in proteins. NBS is a potent oxidizing agent which oxidises the indole residue to oxyindole derivative [2]. The reaction can be monitored by following the decrease in absorbance at 280 nm. This method is based on the principle that the indole chromophore of tryptophan absorbing strongly at 280 nm is converted on oxidation with NBS, to oxyindole a much weaker chromophore at this wavelength. The reaction occurring is shown in equation (1)



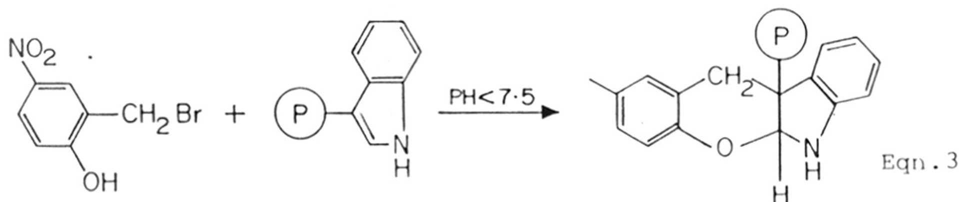

Eqn.1

The reaction is best performed in acidic media, usually in acetate or formate buffer at pH 4.0 [1].

Koshland and coworkers [45] have introduced the reagent HNBB for modification of tryptophan residues. It reacts in neutral and acidic solutions with cysteine less than 1/5th as rapidly as with tryptophan. In alkaline solutions it can also react with tyrosine. The reactivity of HNBB appears due to resonance stabilization of its incipient carbonium ion (eqn. 2). Only tryptophan and to a lesser extent cysteine are able to effectively compete with water for this reactive intermediate.




The reaction between protein and HNBB is shown in equation 3. This reagent forms a hydroxybenzyl derivative.




<u>Titration with NBS</u> Oxidation of tryptophan residues by NBS was carried out in two cuvettes: One containing xylanase ($2.72 \times 10^{-5}$ M) in 50 mM acetate buffer, pH 4.5 and another

containing buffer. Successive 10 $\mu l$ aliquots of NBS (1 x $10^{-4}$ M) were added to the sample as well as to the reference cuvette and absorbance at 280 nm was measured. After each addition of NBS, the number of tryptophan residues oxidized ($\Delta n$) per mol of enzyme was calculated from the following equation [22,46].

$$\Delta n = \frac{1.31 \times \Delta A_{280}}{5500 \times \text{molarity of enzyme}}$$

where $\Delta A_{280}$ is the decrease in absorbance at 280 nm, 1.31 is an empirical factor based upon oxidation of model tryptophan peptides by NBS [47] and 5500 is the molar absorption coefficient for tryptophan at 280 nm. Simultaneously, portions of the reaction mixture were assayed for xylanase activity.

**Substrate protection studies**

A 0.1 ml portion of enzyme (1 $\mu g$) in buffer was added to different amounts of xylan (0.1 - 5.0 mg) in a total volume of 0.5 ml before the addition of NBS (4 $\mu M$) or PHMB (5 $\mu M$). The reaction mixture was incubated at 25°C for 10 min. Control tubes without the inhibitor were also run under identical conditions. The activity of the enzyme was determined as usual by adding the remaining amount of xylan.

For different concentrations of HNBB (2.5, 4 and 8 mM), percentage inhibition of xylanase was determined. For every HNBB concentration the amount of xylan needed to give 100% protection was determined by the above procedure.

**Reactivation of xylanase after modification by PHMB**

The enzyme (1 µg) was incubated with PHMB (2 µM) in 50 mM acetate buffer, pH 6.0 in a volume of 0.25 ml at 25°C. At different time intervals, the residual activity was determined. Simultaneously, the incubation mixtures (0.25 ml) were also transferred to 0.25 ml of 50 mM cysteine and incubated at 25°C for 20 min. The enzyme activity was determined in the usual way by adding the substrate and incubating at 60°C for 30 min. Control enzyme without inhibitor was also kept under identical conditions and percentage activity during reactivation was calculated by comparing with the activity in control tube.

Alternatively, regain of xylanase activity completely inactivated by PHMB (5 µM) was determined by transferring aliquots of inactivated enzyme in different concentrations of cysteine and incubating at 25°C for 20 min.

## RESULTS

**Effect of chemical modifiers**

Table 5.2 indicates the effect of various modifiers on xylanase activity. Complete inhibition by NBS, HNBB and PHMB showed that tryptophan and cysteine residues are modified. The enzyme was not inhibited by DEP, phenyl glyoxal, PMSF and Woodward's Reagent indicating that histidine, arginine, serine and aspartate, glutamate residues are not involved in the active site of enzyme.

**Involvement of tryptophan at the active site of xylanase**

Kinetics of inactivation of xylanase:

(a) NBS: The xylanase activity progressively decreased as

Table 5.2: Effect of chemical inhibitors on xylanase

| Chemical reagent | Inhibitor (mM) | Inhibition (%) | Incubation buffer (50 mM) |
|---|---|---|---|
| NBS | 1.0 | 100 | Sodium acetate buffer, pH 4.5 |
| HNBB | 10.0 | 100 | Sodium acetate buffer, pH 4.5 |
| DEP | 10.0 | 0 | Potassium phosphate buffer, pH 7.0 |
| NEM | 2.0 | 50 | Sodium acetate buffer, pH 6.0 |
| Iodoacetamide | 5.0 | 30 | Sodium acetate buffer, pH 6.0 |
| Phenylglyoxal | 10.0 | 0 | Tris-HCl, pH 8.0 |
| PHMB | 1.0 | 100 | Sodium acetate buffer, pH 6.0 |
| PMSF | 10.0 | 0 | Potassium phosphate buffer, pH 7.0 |
| Woodward's K | 10.0 | 0 | Sodium acetate buffer, pH 5.0 |
| N-acetylimidazole | 10.0 | 0 | Potassium phosphate buffer, pH 7.0 |
| Butane-2,3-dione | 10.0 | 0 | Sodium borate buffer, pH 8.0 |
| Citraconic anhydride | 10.0 | 0 | Sodium acetate buffer, pH 5.0 |

The enzyme was incubated with the inhibitor at 25°C for 10 min before the addition of the substrate.

the concentration of NBS was increased from 0.6 - 2.5 µM. Residual activity of 95% and 74% was observed within 10 min incubation for 0.6 µM and 2.5 µM NBS concentration, respectively. But the extent of inhibition increased with time and residual activity of 40% was observed within 40 min incubation at 2.5 µM NBS concentration. Complete loss in enzyme activity was observed at 4 uM NBS concentration within 10 min of incubation time.

The plot of percentage residual activity as a function of time at various concentrations of NBS (Fig. 5.1) indicates that the inactivation process exhibits pseudo-first order kinetics with respect to time at any fixed concentration of the inhibitor. Applying the analysis described by Levy *et al*. [42] the pseudo-first order rate constants were calculated from the slope of the plots of logarithm of residual activity against the time of reaction. The order of the reaction was estimated from the slopes of the plots of log (pseudo-first order rate constant) against log (inhibitor concentration). This graph (inset to Fig. 5.1) indicated that the loss of enzyme activity resulted from reaction of only one tryptophan residue per molecule of enzyme.

The second order rate constant was determined from the slope of plot of pseudo-first order rate constant against inhibitor (NBS) concentration (Fig. 5.2). The second order rate constant for inactivation by NBS was $3.84 \times 10^3$ per min/mol.

(b) HNBB The progressive inactivation of xylanase was observed with increase in concentration of HNBB. Residual activity of 89.13% and 56.23% was observed within 10 min incu-

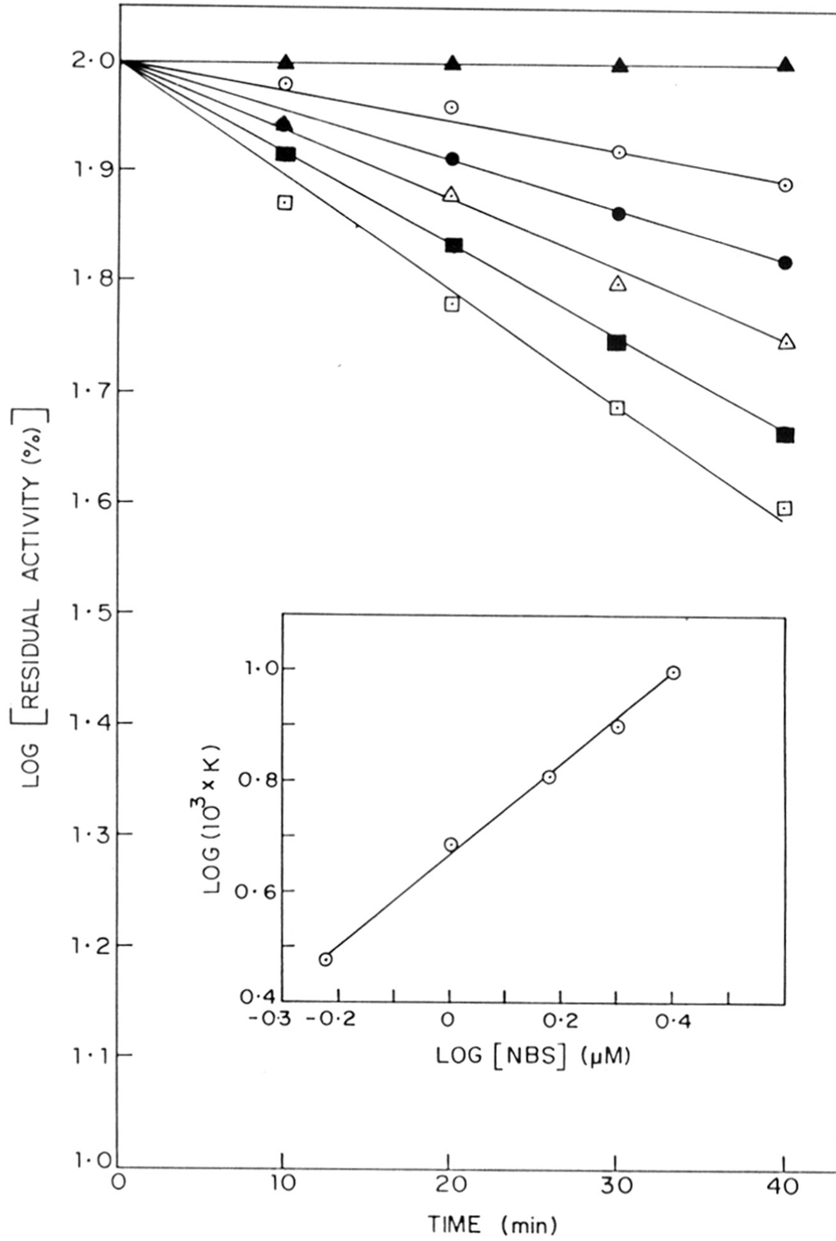
**Fig. 5.1:** Kinetics of inactivation of xylanase by NBS

The enzyme (5 μg) was incubated with NBS

(▲) 0 μM, (o) 0.6 μM, (●) 1.0 μM,

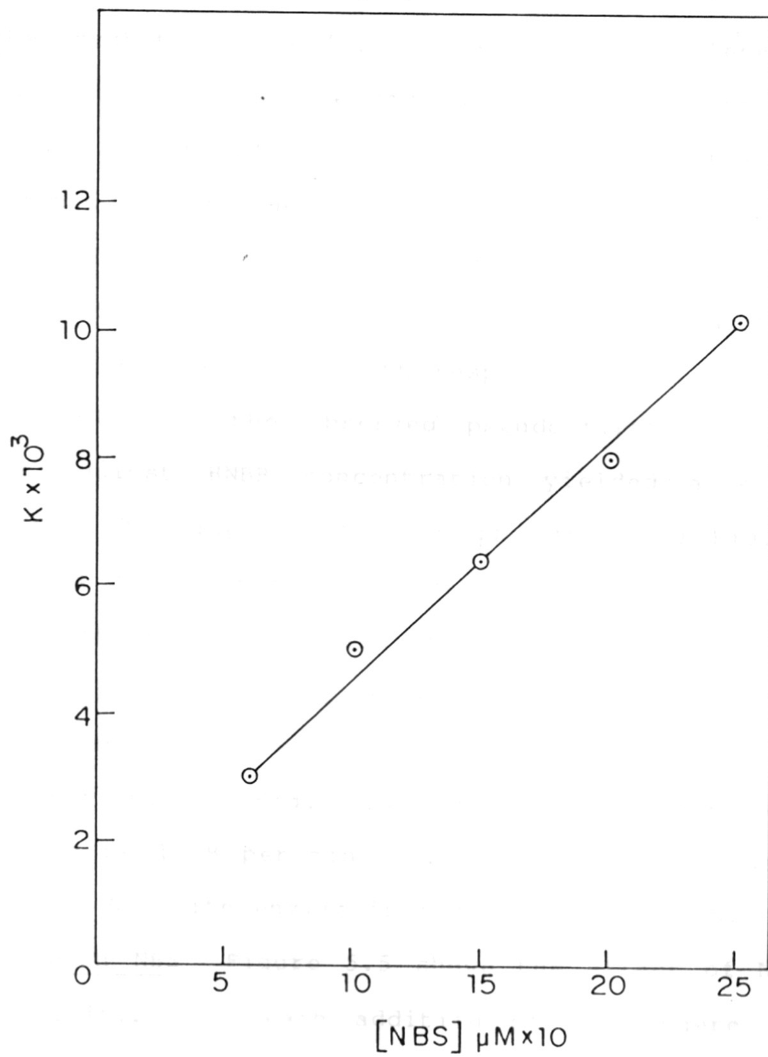
(△) 1.5 μM, (■) 2.0 μM, (□) 2.5 μM

The inset shows the apparent order of reaction with respect to reagent concentration.



2·0
1·9
1·8
1·7
1·6
1·5
1·4
1·3
1·2
1·1
1·0
LOG [RESIDUAL ACTIVITY (%)]
0
10
20
30
40
TIME (min)
1·0
0·8
0·6
0·4
LOG (10³ x K)
-0·3
-0·2
0
0·2
0·4
LOG [NBS] (μM)

**Fig. 5.2:** Determination of second order rate constant for inactivation by NBS.



K x 10$^3$
12
10
8
6
4
2
0
5
10
15
20
25
[NBS] μM x 10

bation time at 0.25 mM and 2.0 mM HNBB concentration ,respectively. Very little residual activity (14.1%) was remained after incubation of xylanase with 2 mM HNBB, for 40 min. However, with higher concentration of HNBB (3, 4 and 6 mM) and in 10 min incubation time the residual activity was 35.4, 32.6 and 29.0%, respectively. But prolonging the incubation time (from 10 - 40 min) complete inhibition was observed for the same above HNBB concentration. So there was a very slow reaction between the xylanase and inhibitor and marginal decrease in inactivation was observed with increase in the concentration of HNBB.

Figure 5.3 indicates that the inactivation follows pseudo-first order kinetics with respect to time. A double logarithmic plot of the observed pseudo-first order rate constants against HNBB concentration yielded a straight line. From the slope of this graph (inset to Fig. 5.3) the order of the reaction was estimated to be 1.0. This indicated that the modification of a single tryptophan residue results in the loss of enzyme activity.

The second order rate constant for inactivation by HNBB was determined (Fig. 5.4) as described earlier and was found to be 13.8 per min/mol. This indicates that the reactivity of NBS with enzyme is much more than HNBB.

<u>Titration with NBS</u> Figure 5.5 shows the effect of NBS on enzyme activity. After each addition of NBS, there was a progressive decrease in absorption at 280 nm, as well as increase in the extent of inactivation. For complete inactivation of enzyme, 3.6 mol of NBS were required per mol of enzyme.

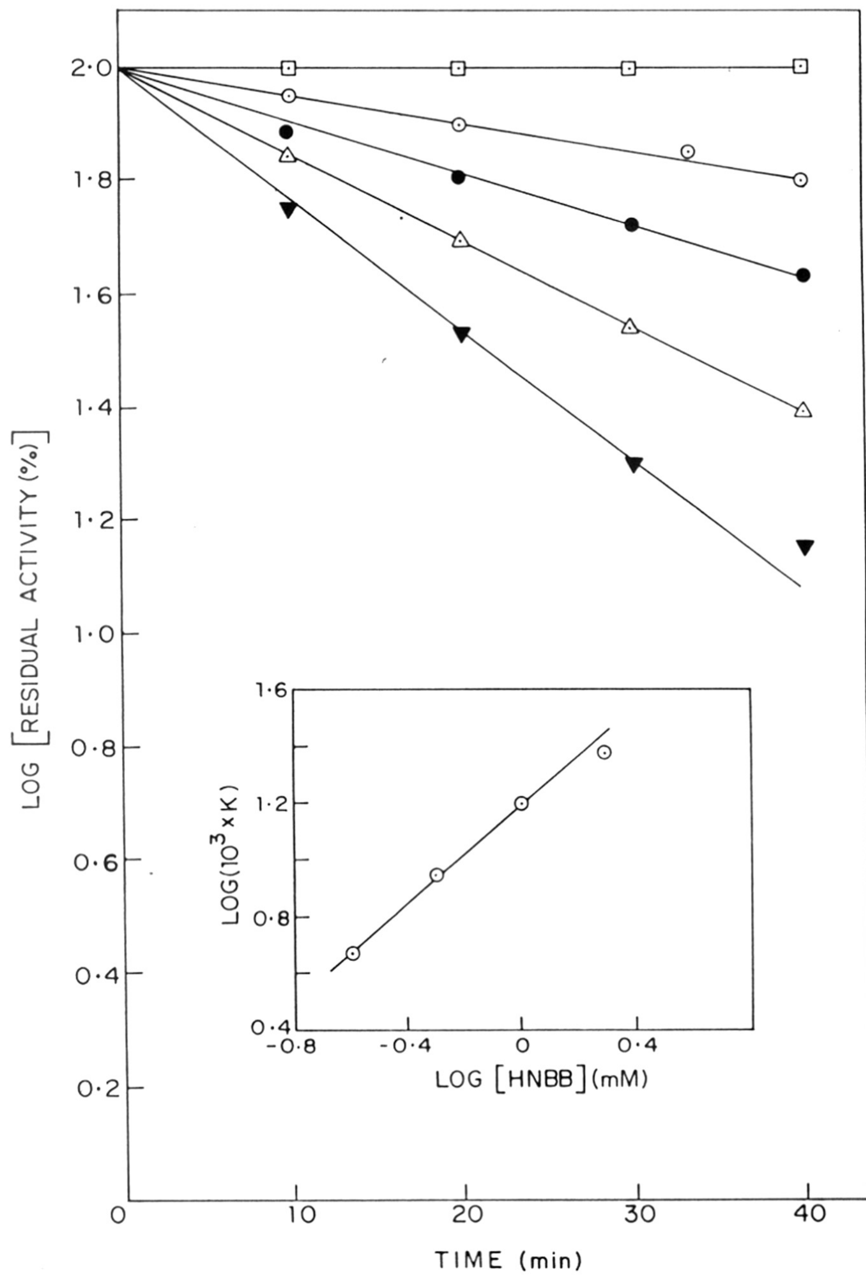
**Fig. 5.3:** Inactivation of xylanase by HNBB

Enzyme (5 μg) was incubated with HNBB

(□) 0 mM, (o) 0.25 mM, (●) 0.5 mM,

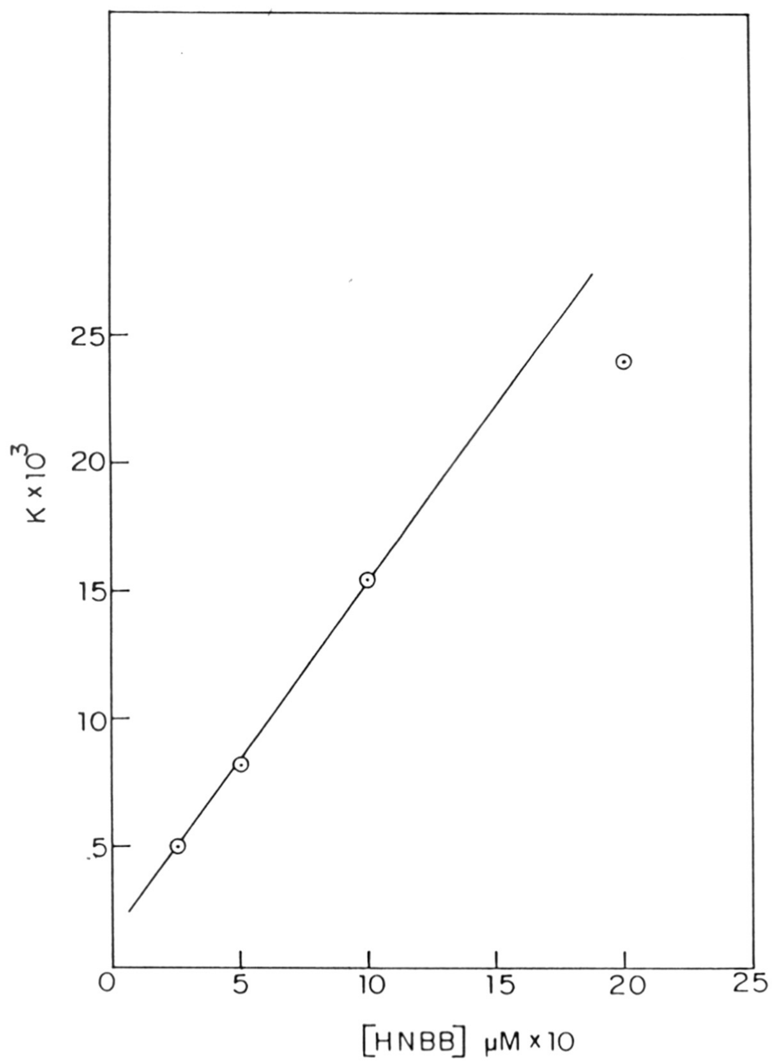
(△) 1.0 mM, (▼) 2.0 mM

The inset shows a plot of the logarithm of pseudo-first-order rate constant against the logarithm of HNBB concentration.



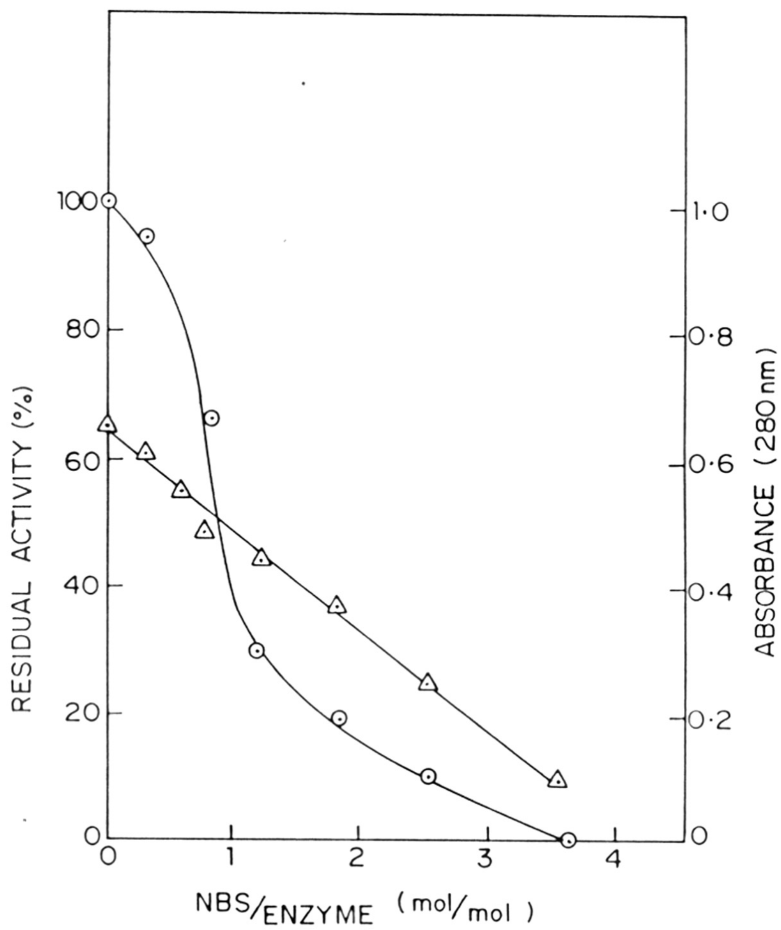
LOG [RESIDUAL ACTIVITY (%)]
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
10
20
30
40
TIME (min)
LOG(10³ x K)
1·6
1·2
0·8
0·4
-0·8
-0·4
0
0·4
LOG [HNBB] (mM)

**Fig. 5.4:** Determination of the second order rate constant for inactivation by HNBB.



K x 10^3
25
20
15
10
.5
0
5
10
15
20
25
[HNBB] μM x 10

**Fig. 5.5:** Activity and absorbance changes of xylanase as a function of molar excess of NBS Aliquots (10 µl) of NBS ($1 \times 10^{-4}$ M) were added successively to the enzyme ($2.72 \times 10^{-5}$ M). After each addition the residual activity (o) and decrease in absorption at 280 nm (Δ) were measured.



RESIDUAL ACTIVITY (%)
100
80
60
40
20
0
ABSORBANCE (280 nm)
1·0
0·8
0·6
0·4
0·2
0
0
1
2
3
4
NBS/ENZYME (mol/mol)

Oxidation of tryptophan residues in xylanase was carried out by stepwise addition of NBS and were calculated as described in Materials and Methods. By extrapolating the initial portion of the plot to zero activity, the number of tryptophan oxidised per molecule of enzyme were found to be 2.2 (Fig. 5.6). This usually gives the number of residues modified, when the enzyme is completely inactivated, but not the number of residues essential for activity.

Protection by substrate (xylan) against inactivation The xylanase was completely inactivated by NBS (4 $\mu$M). The effect of different amounts of xylan (0.1 - 1.0 mg) on inactivation was studied. Figure 5.7 shows that there was progressive increase in the extent of protection as the substrate concentration was increased and 1 mg of substrate was needed to give 100% protection against inactivation by NBS. Protection of substrate indicated the presence of essential tryptophan residue at the substrate binding site.

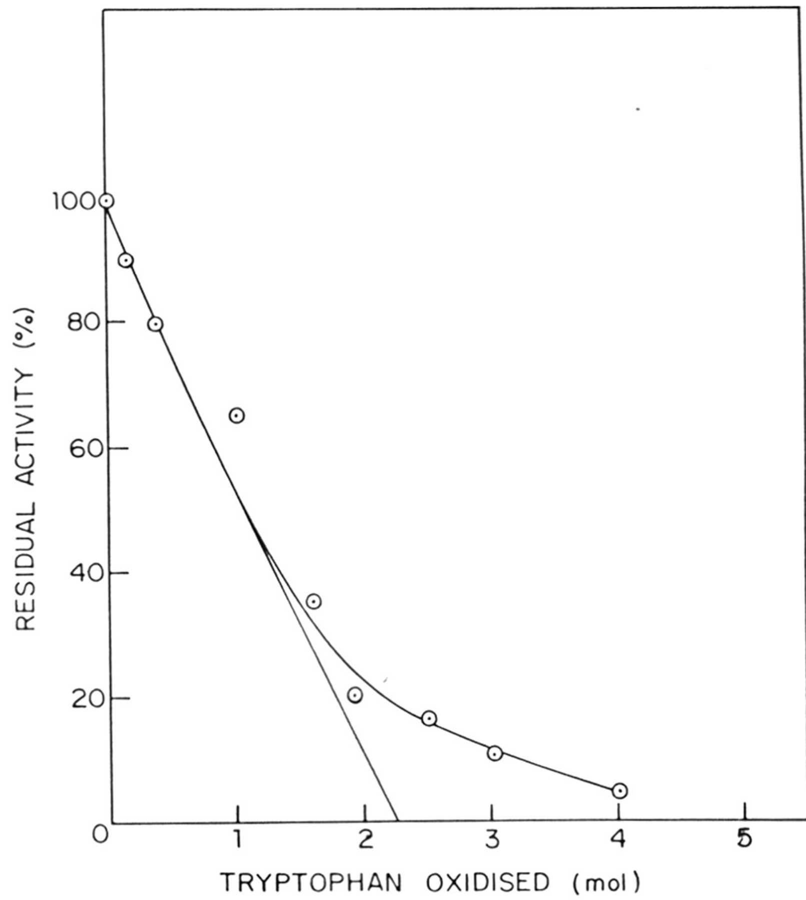
The xylanase was incubated with different concentrations of HNBB (2.5 - 8.0 mM). The amount of xylan needed to give 100% protection increased from 2.5 - 7.5 mg as the extent of inhibition increased (Table 5.3). This again confirmed the presence of essential tryptophan residue at the substrate binding region of xylanase.

**Involvement of cysteine at the active site of xylanase**

Kinetics of inactivation of xylanase by PHMB

The xylanase was progressively inactivated with increase in concentration of PHMB. A residual activity of 95% was observed within 10 min of incubation time at 0.25 $\mu$M PHMB

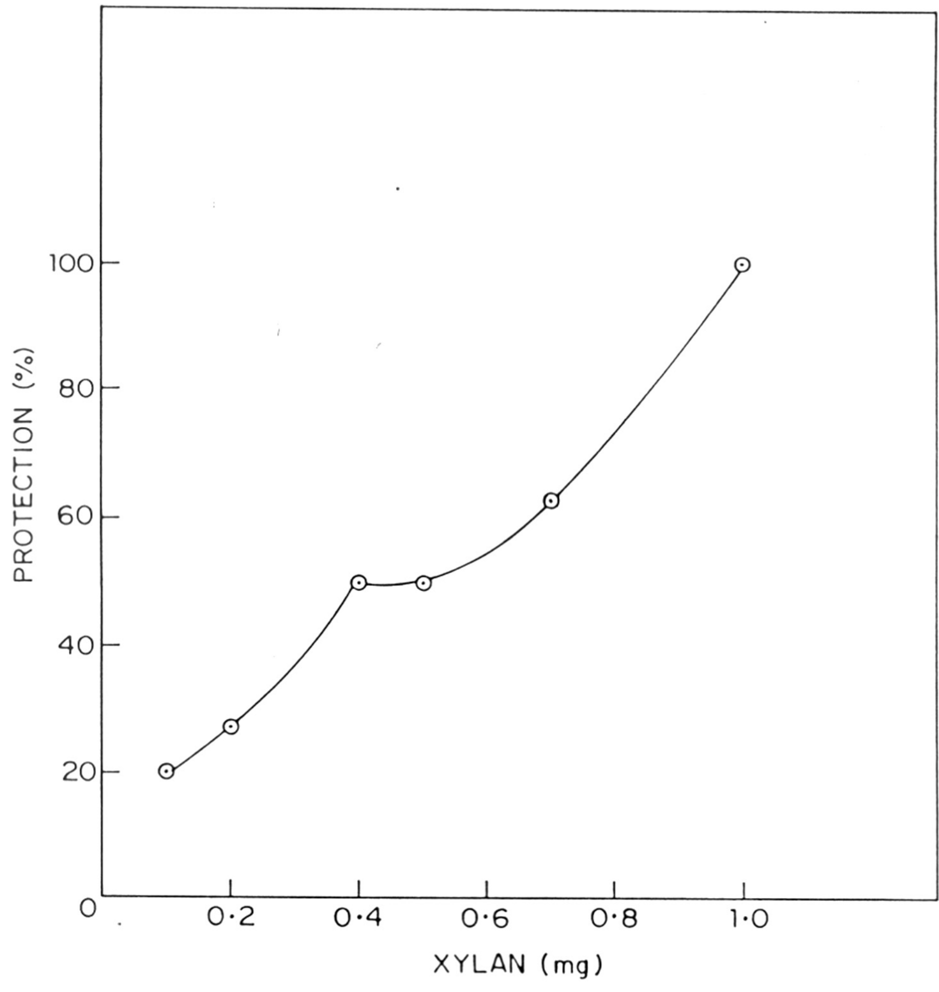
**Fig. 5.6:** Titration of NBS with xylanase
Oxidation of tryptophan residues in xylanase was carried out with stepwise addition of NBS to the enzyme. The number of tryptophan residues oxidized was determined as described in Materials and Methods.



100
80
60
40
20
0
RESIDUAL ACTIVITY (%)
1
2
3
4
5
TRYPTOPHAN OXIDISED (mol)

**Fig. 5.7:** Protection by substrate (xylan) against inactivation by NBS

Different amounts of xylan (0.1 - 1 mg) was added to 1 $\mu g$ of xylanase before the addition of NBS.



100
80
60
40
20
0
PROTECTION (%)
0·2
0·4
0·6
0·8
1·0
XYLAN (mg)

**Table 5.3** Substrate protection against inactivation by HNBB

| HNBB (mM) | Inhibition (%) | Xylan* (mg) |
|---|---|---|
| 2.5 | 30 | 2.5 |
| 4.0 | 70 | 5.0 |
| 8.0 | 100 | 7.5 |

* Required to give 100% protection.

concentration. At 3.5 $\mu$M PHMB concentration and in 10 min incubation time, the activity was reduced to 40% and showed 100% inhibition within 40 min. Hundred percent inactivation was achieved within 10 min at a PHMB concentration of 5 $\mu$M.

Figure 5.8 indicates that the inactivation shows pseudo-first order kinetics with respect to time at a fixed concentration of PHMB. The order of the reaction was estimated from the slopes of the plots of log (pseudo-first order rate constant) against log (PHMB concentration). This graph (inset to Fig. 5.8) indicated that loss of enzyme activity resulted from reaction on only one cysteine residue per molecule of enzyme. It also indicated that one mol of PHMB is required to produce inactivation.

The second order rate constant for inactivation by PHMB was determined (Fig. 5.9) as described earlier and was found to be 12 x $10^3$ per min/mol indicating the strong reactivity of PHMB with enzyme.

Protection by substrate (xylan) against inactivation The xylanase was completely inhibited by PHMB (5 $\mu$M). The effect of different amounts of xylan (0.1 - 1.0 mg) on this inactivation was determined. Figure 5.10 shows that there was progressive increase in percent protection as the substrate concentration was increased. A 1 mg portion of substrate was needed to give 100% protection against inactivation by PHMB. Protection by the substrate indicated the presence of essential cysteine residue at the substrate binding site of xylanase.

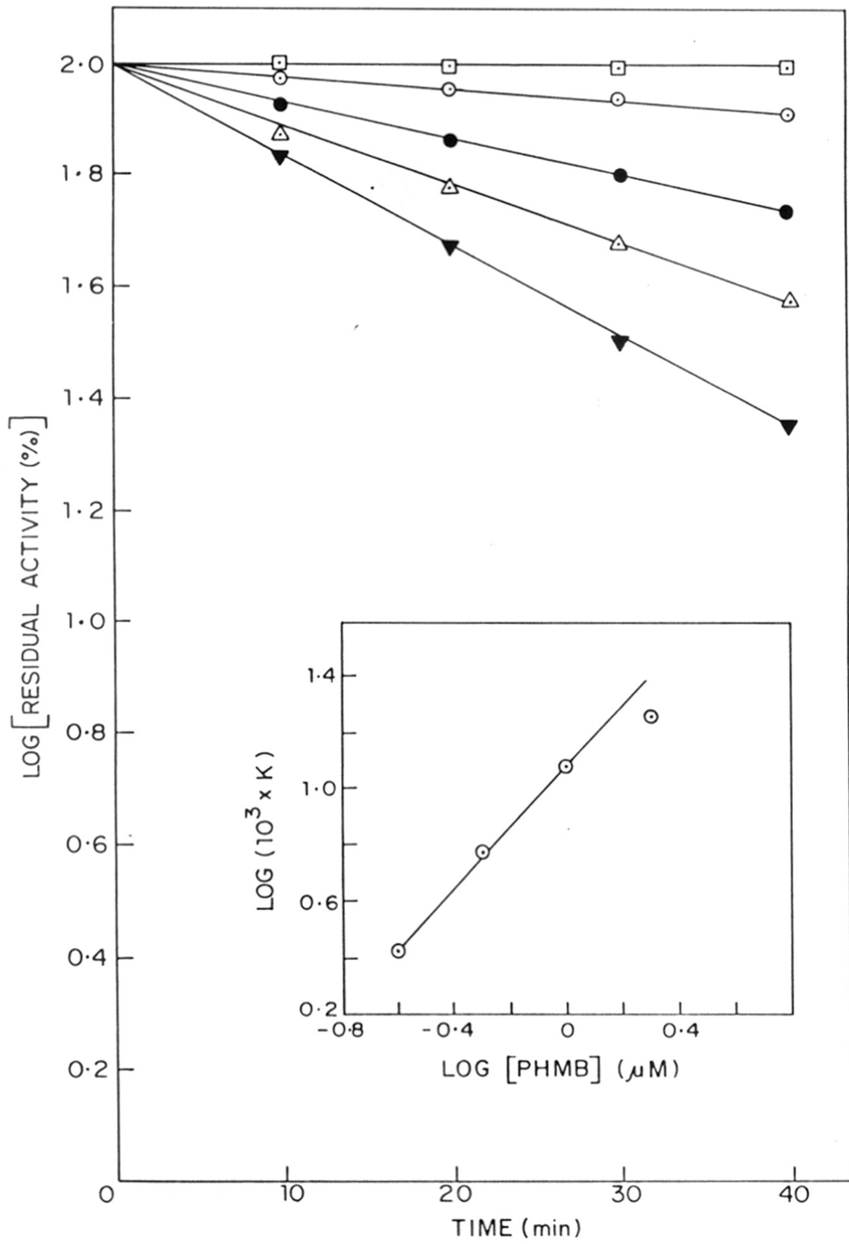
**Fig. 5.8:** Effect of PHMB on xylanase activity

Enzyme (5 μg) was incubated with PHMB

(■) 0 μM, (o) 0.2 μM, (●) 0.5 μM,

(Δ) 1,0 μM, (▼) 2.0 μM

The inset shows a plot of the logarithm of the pseudo first-order rate constant of PHMB inactivation against the logarithm of PHMB concentration.



LOG [RESIDUAL ACTIVITY (%)]
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
10
20
30
40
TIME (min)
LOG ($10^3$ x K)
1·4
1·0
0·6
0·2
−0·8
−0·4
0
0·4
LOG [PHMB] (μM)

**Fig. 5.9:** Determination of second order rate constant for inactivation by PHMB

**Fig. 5.10.** Protection by substrate (xylan) against inactivation by PHMB.
Different amounts of xylan (0.1 - 1.0 mg) were added to 1 µg of xylanase before the addition of PHMB.

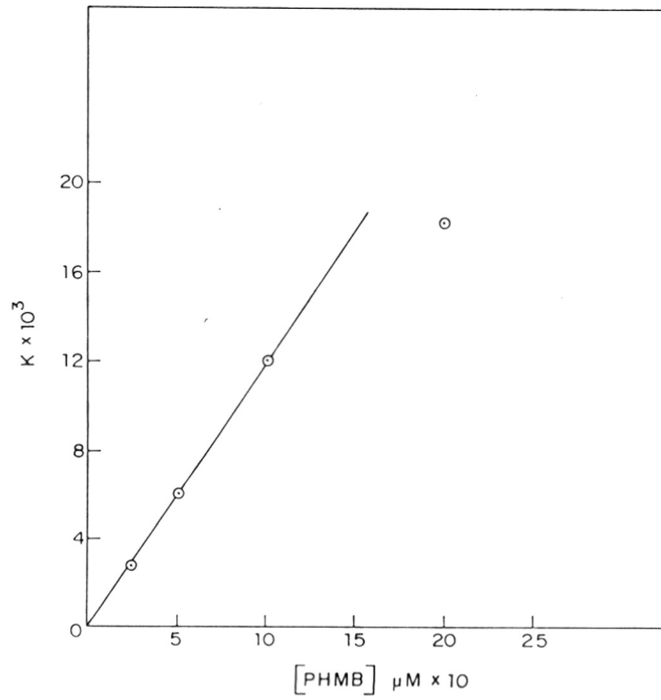



Fig. 5·9

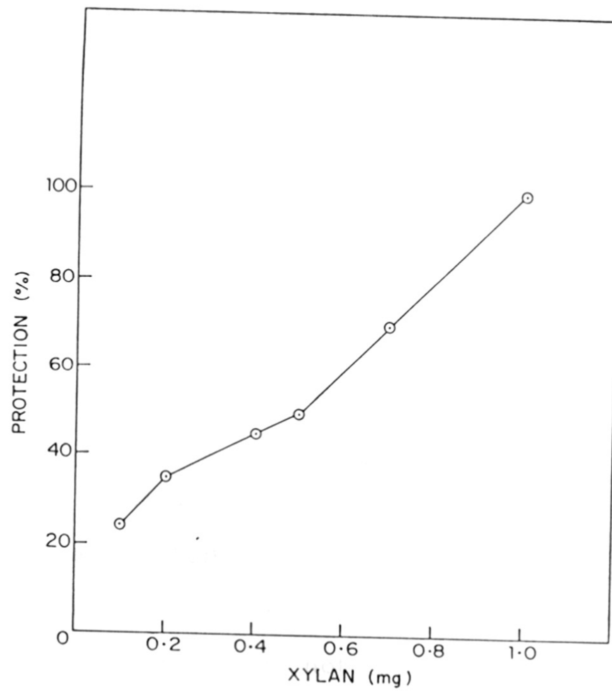



Fig. 5.10

Reactivation of xylanase after modification by PHMB

The progressive loss of activity as a function of time when the enzyme was treated with PHMB and its reactivation by cysteine were studied. Figure 5.11 shows that the xylanase inactivated by PHMB (2 µM) and having 68% of its original activity, could recover nearly 100% of its native activity in the presence of 25 mM cysteine. However, the extent of reactivation became less with the enzyme samples that were inactivated to a greater extent. So a reactivation of only 45% was obtained for inactivated xylanase having residual activity of 18%. Similarly, reactivation of 68% was obtained for inactivated xylanase having residual activity of 35%.

The reactivation by different concentrations of cysteine on completely inactivated xylanase was observed. Figure 5.12 shows that the enzyme which was completely inactivated by PHMB, was reactivated fully with 50 mM cysteine indicating a competitive displacement of PHMB by the high concentration of thiol [1].

## DISCUSSION

It is seen from the majority of the available reports that the possibility of involvement of tryptophan and cysteine in the active site of xylanase cannot be ruled out. The 34 and 23% inhibition of xylanase I by p-chloromercuricbenzoic acid (PCMB) (5 mM) from two Bacillus strains W1 and W2 was reported. However, the xylanase component II of both the strains was not affected. Iodoacetamide showed only 43% inhibition for xylanase component I of

**Fig. 5.11:** Reactivation after 20 min of xylanase by cysteine after modification by PHMB (o) inactivation of enzyme, (△) reactivation of xylanase.

**Fig. 5.12:** Reactivation of xylanase by different concentrations of cysteine.

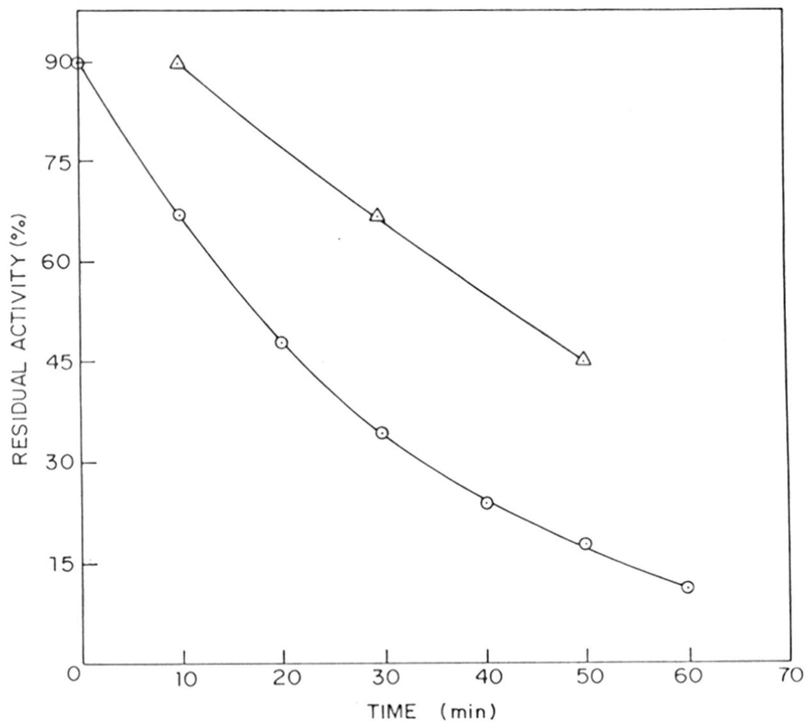



Fig. 5.11

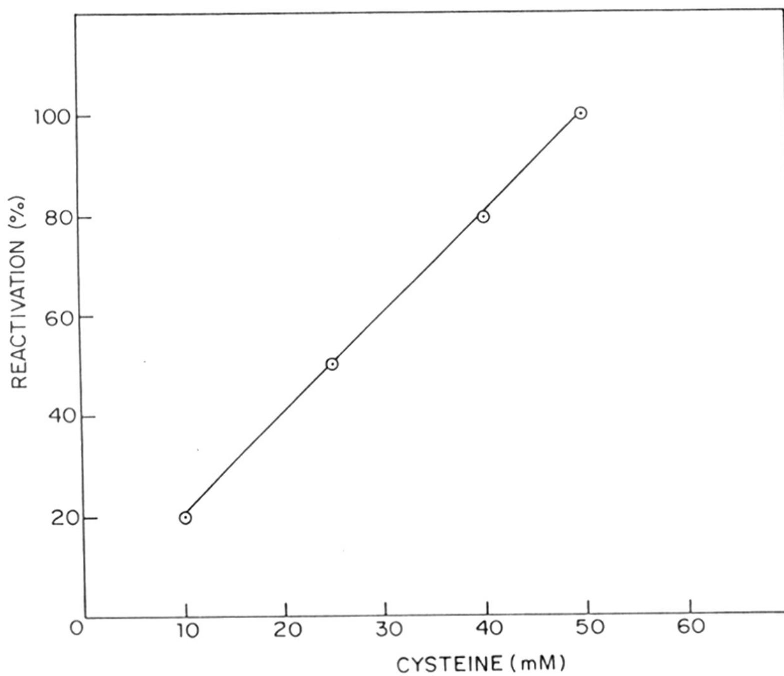



Fig. 5.12

strain W2. Strong inhibition in both the cases was not observed to conclude anything [48]. Xylanases A, B and C from C. stercorarium were inhibited by 1 mM PHMB and the inhibition was partially reversed by the addition of 30 mM cysteine giving 64, 49 and 96% regain in the activity. However, treatment of xylanase with NEM and DTNB did not result in any significant inhibition [36].

The endo-xylanase II of Chainia sp was inhibited by PCMB and NBS suggesting the presence of tryptophan and cysteine at the active site [49]. The inhibition of xylanase Ib and II from S. exfoliatus and stimulation of Id by iodo-acetamide and PCMB at 5 mM concentration was reported [39]. The three xylanases X-I, X-II-A and X-II-B from Streptomyces sp 3137 were inhibited by NBS at 5 mM concentration indicating the probable presence of tryptophan at the active site of xylanase [30]. Strong inhibition by NBS, HNBB and tetranitromethane indicated the presence of tryptophan and tyrosine at the active centre of endo-xylanase from F. avenaceum [40]. The xylanase from Aspergillus ochraceus was inactivated by PHMB, DTNB and NEM suggesting the presence of thiol groups [41].

Our results agree well with the reports cited in the literature. The xylanase from Streptomyces sp $T_7$ was strongly inhibited by NBS, HNBB and PHMB suggesting the presence of tryptophan and cysteine at the active site. Kinetic analysis and the other evidences confirm the presence of tryptophan and cysteine at the active site. Protection by substrate against inactivation suggests the presence of these residues at the substrate binding site. Complete

inhibition by PHMB but less inhibition by iodoacetamide may be due to the fact that at pH 6.0, there is very small proportion of the ionised form of cysteine ($-CH_2-\bar{S}$) which is the reactive nucleophile [50]. As the optimum pH for enzyme activity was found to be in the pH range of 4.5 - 5.5, the effect of iodoacetamide on xylanase activity was not determined at pH values above 6.0. Ideally, it should have been determined at pH values above 8.0 which is an appropriate $pK_a$ of the side chain of cysteine. Hence at pH 6.0 iodoacetamide may be causing only 30% inhibition while PHMB causes 100% inhibition.

The differences in the reactivity partly depends on the hydrophobic character of sulfhydryl groups, their presence and the varied ability of modifying reagents to penetrate such hydrophobic regions. Thus, it is frequently observed that apolar organo-mercurials like PHMB are much more effective than hydrophilic reagents like NEM and iodo-acetamide [1]. Hence, NEM may be causing 50% inhibition while PHMB causes 100% inhibition.

It is surprising that 100% protection by substrate is offered against inhibition at a substrate concentration less than $K_m$. This observed deviation from Michaelis Menten kinetics, in the present studies may be attributed to the heterogeneity of high molecular weight substrate viz. xylan. Although a homogeneous soluble portion of xylan was used for the xylanase assay, it may in reality be consisting of xylo-oligomers of various chain lengths and the value of $K_m$ so obtained may be an apparent $K_m$ value which may account for the observed discrepancy.

Limited information concerning the mechanism of action of xylanases is available in spite of its great biotechnological importance. In contrast, lysozyme which is functionally related to cellulases and xylanases, is one of the most thoroughly studied enzymes so far [51] and has been delineated in structural detail at the molecular level [52]. It has been shown for lysozyme that tryptophyl residues are involved in the binding of the substrate to the enzyme molecule [53].

While the generality of a lysozyme type mechanism has yet to be established for the other hydrolytic enzymes. Carboxyl groups are thought to participate as the general acid catalyst in the active site of enzymes from Aspergillus and Oxysporum species [54,55]. On the other hand, Pettersson [56] has implicated the involvement of both histidine and tryptophan residues in the mechanism of action of cellulase from Penicillium notatum. The participation of carboxyl groups in the catalytic mechanism of the cellulase of Schizophyllum commune was first proposed by Yaguchi et al. [57], on the basis of amino acid sequence homology between the active site region of lysozyme and cellulase and it was confirmed that cellulase and lysozyme follow the same mechanism [58]. Tryptophan residues have been shown to be involved in the binding of the substrate to these enzymes [54.59]. The present results add further support to these earlier findings and suggest a structural relationship between xylanase, lysozyme and cellulase.

# REFERENCES


1. Means, G.E. and Feeney, R.E. (1971) In "Chemical Modification of Proteins" (Eds. Means, G.E. and Feeney, R.E.) Holden-Day, USA.
2. Eyzaguirre, J. (1986) In "Chemical Modification of Enzymes", Active site studies, (Ed. Eyzaguirre, J.) John Wiley & Sons, N.Y. p. 9.
3. Cohen, L.A. (1970) In "The Enzymes" (Ed. Boyer, P.D.) 3rd Edition, Academic Press, N.Y. Vol. 1, p. 147.
4. Ray, W.J.Jr. and Koshland, D.E.Jr. (1961) J. Biol. Chem. **236**, 1973.
5. Glazer, A.N. (1970) Ann. Rev. Biochem. **39**, 101.
6. Glazer, A.N. (1976) In "The Proteins" (Eds. Neurath, H. and Hill, R.L.) 3rd Edition, Academic Press, N.Y. Vol. 2, p.1.
7. Lundblad, R.L. and Noyes, C.M. (1984) In "Chemical Reagents for Protein Modification", Vol. 1 and 2, CRC Press, Boca Raton, Florida.
8. Benesch, R., Benesch, R.E., Gutcho, M. and Laufer, L. (1956) Science, **123**, 98.
9. Ambrose, M.C. and Perham, R.N. (1976) Biochem. J. **155**, 429.
10. Boyer, P.D. (1954) J. Am. Chem. Soc. **76**, 4331.
11. Bai, Y. and Hayashi, R. (1979) J. Biol. Chem. **254**, 8473.
12. Ellman, G.L. (1959) Arch. Biochem. Biophys. **82**, 70.
13. Brocklehurst, K. (1982) Methods Enzymol. **87**, 427.
14. Crestfield, A.M., Moore, S. and Stein, W.H. (1963) J. Biol. Chem. **238**, 622.

15. Gundlach, H.G., Moore, S. and Stein, W.H. (1959) J. Biol. Chem. **234**, 1761.

16. Hoare, D.G. and Koshland, D.E.Jr. (1966) J. Am. Chem. Soc. **88**, 2057.

17. Takahashi, K. (1968) J. Biol. Chem. 243, 6171.

18. Yankeelov, J.A.Jr., Kochert, M., Page, J., and Westphal, A. (1966) Fed. Proc. **25**, 590.

19. Riordan, J.F. (1979) Mol. Cell Biochem. **26**, 71.

20. Miles, E.W. (1977) Methods Enzymol. **47**, 431.

21. Horike, K., Tsuge, H. and McCormick, D.B. (1979) J. Biol. Chem. **254**, 6638.

22. Spande, T.F. and Witkop. B. (1967) Methods Enzymol. **11**, 498.

23. Horton, H.R. and Koshland, D.E.Jr. (1965) J. Am. Chem. Soc. **87**, 1126.

24. Barman, T.E. and Koshland, D.E. (1967) J. Biol. Chem. **242**, 5771.

25. Riordan, J.F., Sokolovsky, M. and Vallee, B.L. (1967) Biochemistry, **6**, 358.

26. Riordan, J.F., Wacker, W.E.C. and Vallee, B. L. (1965) Biochemisry, **4**, 1758.

27. Whitaker, J.R. and Perez-Villasemor, J. (1968) Arch. Biochem. Biophys. **124**, 70.

28. Fahrney, D.E. and Gold, A.M. (1963) J. Am. Chem. Soc. **85**, 997.

29. Kraut, J. (1977) Ann. Rev. Biochem. **46**, 331.

30. Marui, M., Nakanishi, K. and Yasui, T. (1985) Agric. Biol. Chem. **49**, 3399.

31. Nakajima, T., Tsukamota, K.I., Watanabe, T., Kainuma,

K. and Matsuda, K. (1984) J. Ferment. Technol. **62**,269.

32. Biely, P., Kratky, Z. and Vrsanska, M. (1981) Eur. J. Biochem. **119**, 559.

33. Vrsanska, M., Gorbacheva, I.V., Kratky, Z. and Biely, P. (1982) Biochim. Biophys. Acta, **704**, 114.

34. Meagher, M.M. (1984) M.S. Thesis, Iowa State University, Ames.

35. Meagher, M.M., Tsao, B.Y., Chow, J.M. and Reilly, P.J. (1988) Carbohydr. Res. **173**, 273.

36. Bernger, J.F., Frixon, C., Bigliardi, J. and Creuzet, N. (1985) Can. J. Microbiol. **31**, 635.

37. Takahashi, M. and Hashimoto, Y. (1963) Hakko Kagaku Zasshi, **41**, 116.

38. Simpson, R.B. (1961) J. Am. Chem. Soc. **83**, 4711.

39. Sreenath, H.K., Joseph, R. and Murthy, V.S. (1978) Folia Microbiol. **23**, 299.

40. Zalewska-Sobezak, J. and Urbanek, H. (1981) Arch. Microbiol. **129**, 247.

41. Kubackova, M., Karacsonyi, S., Billisics, L. and Toman, R. (1978) Folia Microbiol. **23**, 202.

42. Levy, H.M., Leber, P.D. and Ryan, E.M. (1963) J. Biol. Chem. **238**, 3654.

43. Ramakrishna, S. and Benzamin, W.B. (1981) Biochem. J. **195**, 519.

44. Marcus, F., Schuster, S.M. and Lardy, H.A. (1976) J. Biol. Chem. **251**, 1775.

45. Koshland, D.E., Karkhanis, Y.D. and Latham, H.G. (1964) J. Am. Chem. Soc. **86**, 1448.

46. Witkop, B. (1961) Adv. Protein Chem.. **16**, 221.

47. Patchornik, B., Lawson, W.B. and Witkop, B. (1958) J. Am. Chem. Soc. **80**, 4747.

48. Okazaki, W., Akiba, T., Horikoshi, K. and Akahoshi, R. (1985) Agric. Biol. Chem. **49**, 2033.

49. Bastawde, K.B. (1987) Ph.D. Thesis, Poona University.

50. Price, N.C. and Stevens, L. (1982) In "Fundamentals of Enzymology" (Eds. Price, N.C. and Stevens, L.) Oxford University Press, N.Y.

51. Perutz, M.G. (1967) Proc. Roy. Soc. (London) Ser. B. **167**, 348.

52. Imoto, T., Johnson, L.N., North, A.C.T., Phillips, D.C. and Rupley, J.A. (1972) In "The Enzymes" (Ed. Boyer, P.D.), 3rd Edition, Academic Press, N.Y., Vol. 7, p. 666.

53. Blake, C.C.F., Mair, G.A., North, A.C.T., Phillips, D.C. and Sarma, V.R. (1967) Proc. Roy. Soc. (London) Ser. B. **167**, 365.

54. Hurst, P.L., Sullivan, P.A. and Shepherd, M.G. (1977) Biochem. J. **167**, 549.

55. Legler, G. and Bause, E. (1973) Carbohydr. Res. **28**, 45.

56. Pettersson, G. (1968) Arch. Biochem. Biophys. **126**, 776.

57. Yaguchi, M., Roy, C., Rollin, C.F., Paice, M.G. and Jurasek, L. (1983) Biochem. Biophys. Res. Commun. **116**, 408.

58. Clarke, A.J. and Yaguchi, M. (1986) Eur. J. Biochem. **149**, 233.

59. Clarke, A.J. (1987) Biochim. Biophys. Acta, **912**, 424.

# CHAPTER 6

# ISOLATION OF A REACTIVE CYSTEINYL PEPTIDE

## SUMMARY

N-Ethylmaleimide (NEM) is a potent modifier for cysteine. The yellow derivative of NEM viz. N-(2,4-dinitroanilino)-maleimide (DAM) was synthesized and used to selectively label the thiol groups of the xylanase.

Since, the Streptomyces $T_7$ xylanase contained three -SH groups, the active site peptide was distinguished from the other thiol peptides by selective protection of the active site cysteine by the substrate (xylan). Differential peptide mapping between the peptic digest of xylanase which has previously been treated with DAM in the presence or absence of xylan allowed specific isolation of reactive cysteinyl peptide. The active site peptide was isolated and analyzed qualitatively for its amino acid content by acid hydrolysis followed by paper chromatography. It was found to contain: Aspartic acid; Glycine; Alanine, Tyrosine/valine; Isoleucine/phenylalanine and DAM-cysteine (X).

# INTRODUCTION

Information regarding the amino acid involved in the active site is a prerequisite for manipulating the protein by site directed mutagenesis which can lead to the synthesis of a new protein with desirable properties. Various kinds of approaches have been used for identifying the active centre fragments of proteins. Radioactive label can be used to isolate and identify the active peptide resulting from chemical or enzymatic degradation. Cohen and Warringa [1] treated choline esterase with di-isopropylfluorophosphate (DFP) in the presence of butyryl choline which masked the active site. Then the modified protein was treated with $^{32}P$-labeled DFP in the absence of butyryl choline. A combination of peptide mapping with radioactive tracer methodology facilitated studies of protein synthesis, particularly with a protein of known amino acid sequence. Dintzis [2] in his studies on hemoglobin demonstrated that the protein synthesis proceeds from the N-terminal end. The use of $^{14}C$-iodoacetic acid for labelling -SH group in the active centre of sulfhydryl enzymes has focussed attention on the isolation of peptide containing $^{14}C$-carboxymethylcysteine for subsequent sequence study [3]. NEM is commercially available in $^{3}H$ and $^{14}C$ labeled form. In isolating sulfhydryl peptides, the modification of sulfhydryl groups prior to electrophoresis, has additional advantages. The initial radioactive lebeling allowed prepurification of cysteinyl peptides and making them available in higher amounts for electrophoresis [4].

Affinity labels have proved to be powerful tools in the study of relationship between structure and function of proteins. These reagents are characterised by being structurally similar to the substrates. The peptide containing the radioactive analog was isolated and sequenced from chymotrypsin [5]. Utilising toluenesulfonylphenylalaninechloromethyl ketone (TPCK) Schoellmann and Shaw [6] were able to effect a highly specific alkylation of an active centre histidine in the protein. The reagent was designed to possess substrate like features in order to localize it at the active centre of $\alpha$-chymotrypsin, where the reactive chloromethyl moiety could irreversibly combine with an active group. Affinity labeling takes the advantage of the normal enzyme-substrate interactions to ensure that a large local concentration of reagent exists at the active site. It depends upon the ability of a reactive group in the affinity label to form a stable linkage with the enzyme [7].

The introduction of fluorescent labels into proteins has proved to be a valuable technique in the study of ligand binding to enzymes. The method can provide information concerning the active site of enzyme, provided a fluorescent chromophore has reacted with specific amino acid residues located at the active centre. The fluorescence group should have absorption and fluorescence properties distinct from that of tyrosyl and tryptophanyl residues of the protein [9].

Procedures have also been developed to bind certain fluorescent dyes to proteins, as a means to increase their

fluorescence. Changes in the environment of fluorescent chromophores are reflected by changes in the intensity of fluorescence. These changes in the fluorescence are sensitive criteria of conformational changes [7].

The proteins are fluorescently labeled by bromobimanes. these heterocyclic molecules reacted preferably with thiols and hence can be useful for labeling of both small and large molecules in biological systems [9]. The compound itself is only weakly fluorescent but selectively reacts with thiols to yield highly fluorescent and stable thioethers which can be easily detected. The biomane derivatives can be separated by HPLC [10].

Active site protection by the ligand combination of xylitol plus $Mg^{2+}$ completely blocked diethylpyrocarbonate (DEP) derivatization of histidine in the active site of D-xylose isomerase. Differntial peptide mapping between D-xylose isomerase which has previously been treated with DEP in the presence or absence of xylitol plus $Mg^{2+}$ allowed specific isolation and sequencing of a peptide containing this active site histidine [11]. Cysteinyl peptide labeled by 3-bromo-2-ketoglutarate (BrKG) was isolated in the active site of pig heart $NAD^{+}$-dependent isocitrate dehydrogenase. Protection against inactivation was provided by isocitrate and $Mn^{2+}$. A modified peptide was identified by comparison of the peptides labeled by BrKG in the absence and presence of isocitrate and $Mn^{2+}$. Modified enzyme isolated from unreacted BrKG was incubated with $[^{3}H]NaBH_4$ to reduce the keto-group of protein-bound 2-ketoglutarate and thereby introduce a radioactive tracer into the modified amino

acid. Following carboxymethylation and digestion with trypsin, the specific modified peptide was isolated by HPLC [12].

The well known activity of cysteine or cysteinyl peptides with alkylating agents has resulted in their wide application for the detection of cysteinyl peptides in proteins [13]. The wide spread use of 1-fluoro-2,4-dinitrobenzene (FDNB) for labeling amino groups with the yellow DNP residue and for characterising the N-terminal amino acids in polypeptide chains indicated the value of coloured reagents in protein chemistry [14]. It was felt that it could be desirable to find a colour label which could be applied to protein sulfhydryl groups and used for the identification of cysteine residues, especially those which are involved in the active site of enzyme [15].

NEM is a potent modifier for cysteine residues. NEM reacts rapidly with thiols forming a stable thio-ether link and had frequent application as a reagent for thiol groups of proteins [16,17]. However, as a marker of such groups it is of little value, as it forms colourless derivatives. A number of other substituted maleimides have proven useful, since they introduce a chromogenic substituent into the protein. These include N-(dimethylamino-3,5-dinitrophenylmaleimide) (DDPM) [15], N-(2,4-dinitroanilne)-maleimide (DAM) [18] and N-(4-hydroxy-L-naphthyl) maleimide [19]. Such reagents allow modification of reactive sulfhydryl groups in the protein and ready detection of the appropriate peptide, after proteolytic degradation and separation [14,21]. The use of DDPM has been developed

for the labeling of sulfhydryl groups and for the isolation of sulfhydryl containing peptides from serum albumin and tobacco mosaic virus protein [15,21]. The yellow colour of this reagent greatly facilitated the purification of labeled peptides. The reactions of DDPM with cysteine and with the free -SH groups of human and bovine serum albumin have been studied [15]. DDPM has also been utilized for the isolation of peptides containing sulfhydryl groups from the rabbit muscle glyceraldehyde 3-phosphate dehydrogenase [20].

Reports regarding the presence of cysteine at the active site of xylanases are available [21-25]. Based on the chemical modification and kinetic studies, we have shown the presence of tryptophan and cysteine residues at the active site of the xylanase from Streptomyces $T_7$. However, there are no reports so far for the isolation of reactive peptides from xylanases. This chapter deals with the isolation and identification of amino acid involved in the cysteinyl active site peptide using a chromophoric reagent.

## MATERIALS AND METHODS

### Materials

N-2,4-Dinitroanilino maleimide (DAM) was synthesized in our laboratory. Sephadex G-25 was obtained from Pharmacia, Sweden, pepsin and standard amino acid mixture were purchased from Sigma Chemical Co., USA. All the other chemicals used were of analytical grade.

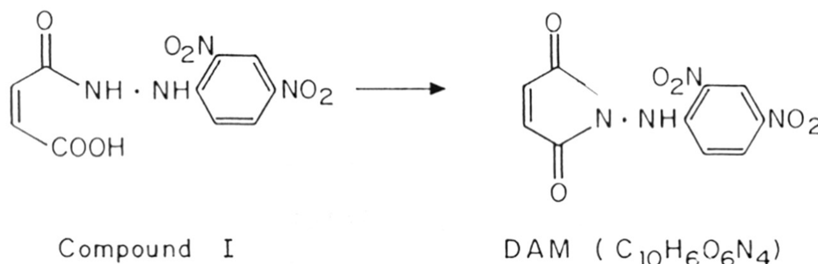
Methods

Synthesis of DAM

DAM was prepared in two steps according to the procedure of Clark-Walker and Robinson [18]:

N-2,4-Dinitroanilino-maleamic acid Maleic anhydride (16 g) and 2,4-dinitrophenyl hydrazine (30 g) were refluxed in chloroform (350 ml) with occasional shaking for 1/2 h. After cooling, the orange solid was collected. Four crystallizations from acetone-benzene gave the maleamic acid as yellow needles (Compound I).

DAM Compound I (5 g) was refluxed in 50 ml acetic acid for 10 min. The yellow imide which crystallized (4 g) was further crystallized from acetone followed by benzene. The final product was pale yellow needles (M.P. 237°C, $M_r$ 278).




Compound I DAM ($C_{10}H_6O_6N_4$)

Reaction of xylanase with DAM

The alcohol precipitated and DEAE treated (partially purified xylanase (2 µg) was incubated with various concentrations of DAM (dissolved in methyl cellosolve) in 50 mM acetate buffer, pH 5.0 in a volume of 0.05 and 0.5 ml at 25°C for 10 min and the residual activity was determined.

The control tubes containing the enzyme alone and enzyme plus DAM were also run under identical conditions.

**Isolation and purification of the cysteinyl peptides**

(a) Alkylation of the xylanase with DAM 40 ml of DAM (50 mM) was added to 10 mg of partially purified enzyme in a total volume of 10 ml in 50 mM acetate buffer, pH 5.0. The reaction was allowed to proceed for 2.5 h at 25°C. The xylanase activity was estimated before and after alkylation.

(b) Precipitation of DAM treated enzyme DAM treated enzyme was precipitated with three volumes of chilled ethanol. The precipitate was recovered by centrifugation at 9,000 x g for 30 min and dried under vacuum. The absorption spectra of native and DAM treated enzymes were recorded.

(c) Proteolysis of DAM treated xylanase The yellow pellet was dissolved in 1.5 ml of 0.01 N HCl and pH was adjusted to 2.0. Proteolytic digestion was carried out at 37°C for 24 h at pH 2.0 with pepsin (in 0.01 N HCl) using the protein to protease ratio of 10:1. 30 µl of this digest was used for peptide mapping.

(d) Separation and purification of the peptides

(i) Sephadex G-25 chromatography The peptic digest of DAM treated xylanase was applied to a Sephadex G-25 column (3 x 26 cm) equilibrated with water. Fractions were collected at the rate of 36 ml/h. Fractions were analyzed for absorption at 280 and 340 nm. Fractions showing maximum absorption at 280 nm (Peak I) and 340 nm (Peak II) were pooled separately. Peak II was further concentrated by freeze-drying.

(ii) <u>Peptide mapping</u> Separation of the Peak II was obtained by a two dimensional combination of electrophoresis and chromatography [26].

(1) <u>Paper electrophoresis</u>

Paper electrophoresis was carried out on Whatman No. 3 MM paper cut to T shape (30 x 30 cm) with pair of sleeves of 14 x 6 cm. The paper was soaked in MICHL's buffer [27], which contained pyridine:acetic acid:water (10:0.4:90), pH 6.4. Excess liquid was removed carefully by blotting firmly between two sheets of blotting papers. The spot where the sample was to be loaded (24 cm from the edge of the sleeve and 3 cm below the upper edge of the paper) was dried, while the rest of the paper was kept moist. The peptic digest (30 $\mu$l) was loaded on the spot. The paper was then placed on a myelin sheet resting on a metal plate. A glass plate was kept over the moist filter paper. The sleeves on either sides of paper were dipped in glass containers with MICHL's buffer and the respective electrodes. The chamber was closed and the electrophoresis was carried out at 1,000 volts for 2.5 h. The sleeves were cut off and the paper was dried at room temperature in a current of air for 2 h.

(2) <u>Paper chromatography</u>

The dried paper was saturated in chromatographic chamber containing pyridine:n-butanol:acetic acid:water (20:30:6:24) for 2 h. Ascending chromatography was performed for 15 - 16 h. The paper was then dried in a current of air at room temperature for 1 h. The peptide spots were revealed by spraying with 0.3% ninhydrin in acetone.

In order to intensify the spots, the paper was dried in oven at 60°C for 10 min. The paper was sprayed with 1% cupric nitrate in acetone containing 20% nitric acid to fix the spots.

**Substrate protection and modification of the xylanase**

The partially purified xylanase (2 µg) was incubated with different amounts of xylan (0.12 - 1.2 mg) prior to addition of 10 µl DAM (50 mM) in a total volume of 50 µl. The reaction mixture was incubated at 25°C for 10 min. The xylanase activity was estimated as usual by adding the remaining amount of xylan.

5 mg of partially purified xylanase was mixed with 150 ml of 3% xylan and allowed to stand for 1 h at 25°C. 20 ml DAM (50 mM) was added and the mixture was incubated at 25°C for 10 min. An aliquot of 50 µl was removed and checked for xylanase activity. The above mixture was concentrated to 55 ml by freeze drying and then precipitated with chilled ethanol (165 ml). The precipitate recovered after centrifugation was dried.

**Location and isolation of reactive peptide**

The dried precipitate was digested with pepsin and the separation and purification of peptides was carried out as described earlier.

For the isolation of the reactive peptide, the treatment with DAM and subsequent peptide mapping was carried out with a purified preparation of xylanase (10 mg) in the absence of substrate. The active peptide was isolated by extracting the corresponding region of the chromatogram with 10% acetic acid. The extract was concentrated by

freeze-drying. The peptide mapping of Peak II was repeated five times to obtain sufficient quantity of reactive peptide.

**Amino acid analysis of the peptide**

An aliquot of the peptide extract was hydrolysed in 6 N HCl for 24 h at 110°C and analysed qualitatively for its composition by paper chromatography.

## RESULTS AND DISCUSSION

The yellow derivative of NEM, DAM was used to selectively label the thiol groups of the xylanase. Due to the yellow colour of the reagent, the labeled peptides were readily identifiable visually during the steps of purification. Since the <u>Streptomyces</u> $T_7$ xylanase contains three -SH groups, the active site peptide was distinguished from the other thiol peptides by selective protection of the active site cysteine by the substrate (xylan). The preliminary experiments for locating the active site peptide were carried out with a partially purified xylanase preparation. The isolation of the active peptide was performed using an electrophoretically homogeneous enzyme preparation.

**Reaction of xylanase with DAM**

Complete inhibition of xylanase activity was observed at 1.0 mM DAM concentration when the reaction was performed in 0.5 ml volume (Fig. 6.1a). When the same reaction was performed in a volume of 0.05 ml, complete inhibition of xylanase was observed at 10 mM DAM concentration (Fig. 6.1b).

These experiments suggest that the molar ratio of the enzyme to the specific inhibitor rather than the inhibitor concentration was important for the inactivation of

**Fig. 6.1:** Inactivation of xylanase with DAM

a) The reaction was performed with DAM (0.2 - 1.2 mM) in a volume of 0.5 ml.

b) The reaction was performed with DAM (2 - 12 mM) in a volume of 0.05 ml.

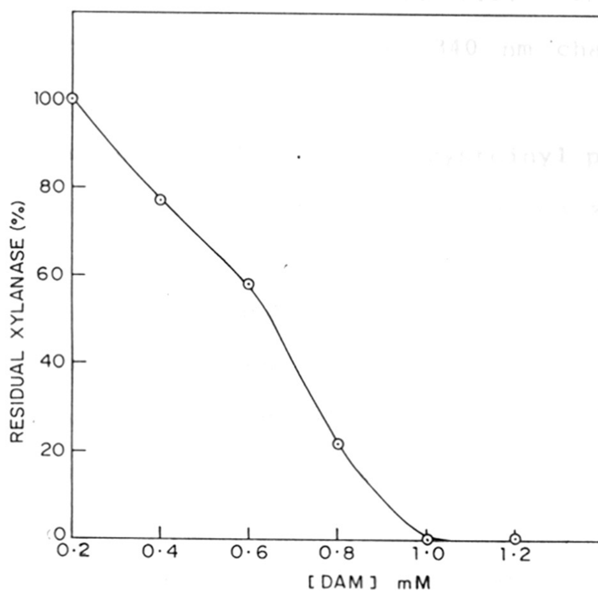



Fig. 6.1a

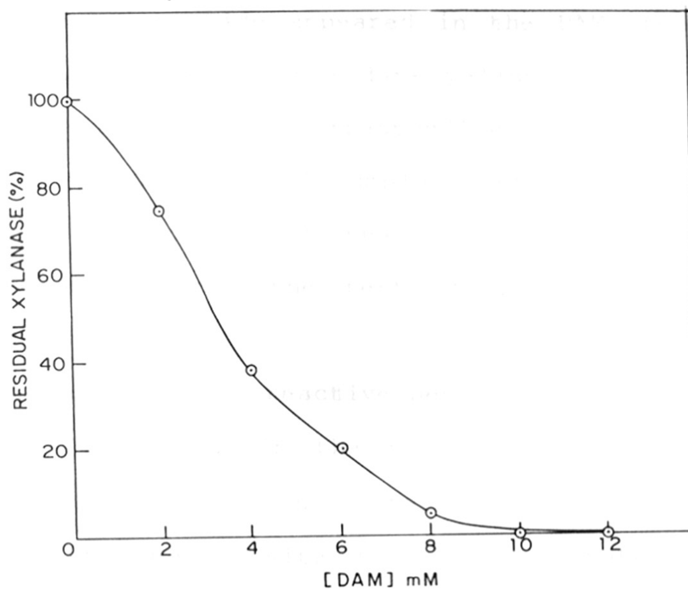



Fig. 6.1b

enzyme. The absorption spectra of the native and alkylated enzyme are presented in Fig. 6.2. The alkylated enzyme shows an additional peak at 340 nm characteristic of DAM -cysteine.

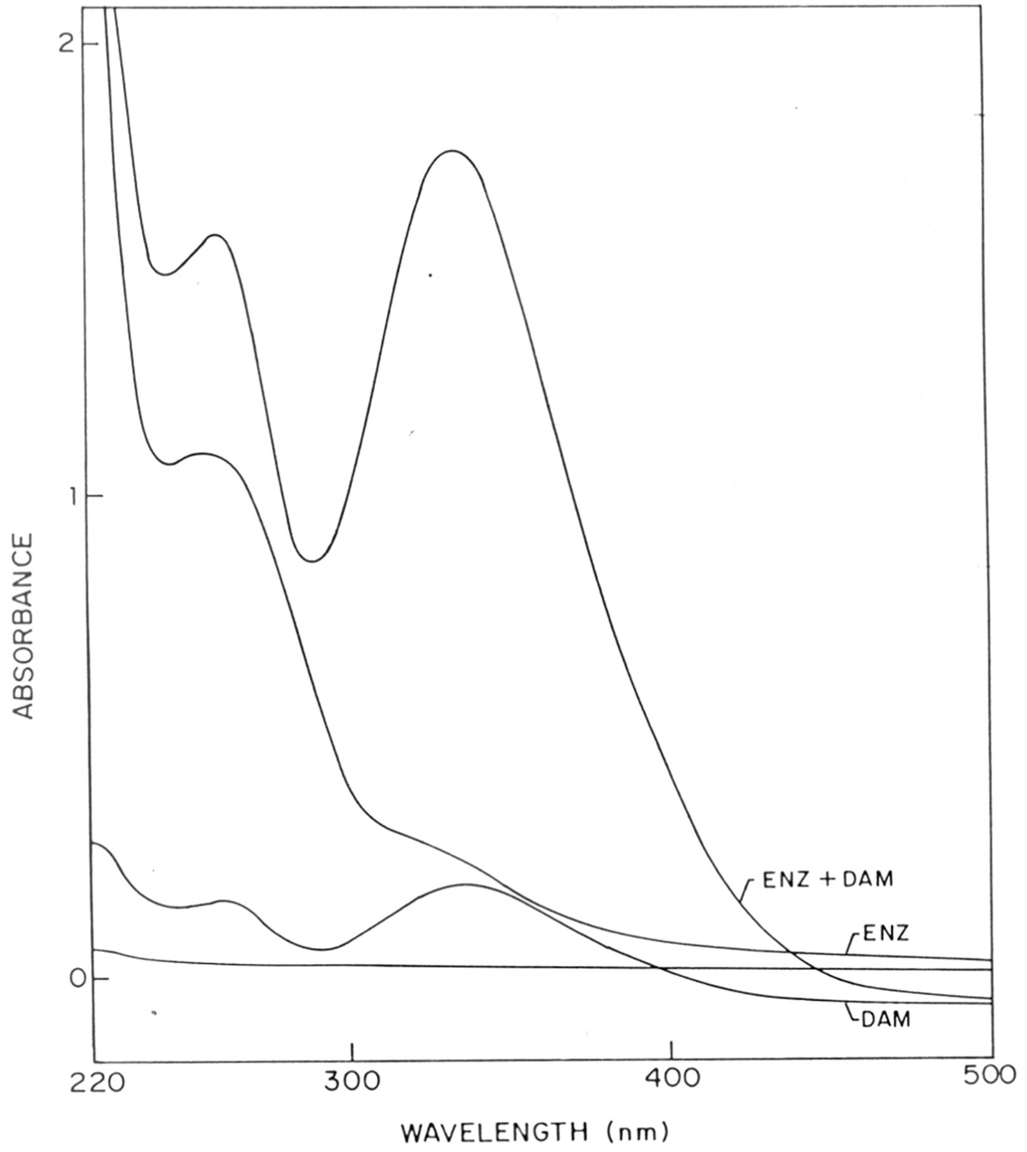
**Isolation and purification of cysteinyl peptides**

The peptic digest of DAM treated xylanase was fractionated on Sephadex G-25 (Fig. 6.3). One peak each at 280 nm (Peak I) and at 340 nm (Peak II) was observed. Peak I was colourless while Peak II was yellow, indicating the presence of cysteine containing peptides. Further purification of Peak II was achieved by paper electrophoresis followed by chromatography. Figure 6.4a,b show the peptide maps of the native and DAM treated xylanase. The peptide maps were identical except that the faint yellow coloured peptide appeared in the DAM treated sample. Peak II showed only three dark yellow coloured peptides (1,2and 3) (Fig. 6.5a), corresponding to the faint yellow peptide in the DAM treated sample. Absence of ninhydrin positive peptides indicated that yellow cysteinyl peptides were separated from the rest of the peptides during the gel filtration.

**Location of the reactive peptide**

To ascertain the position of the reactive cysteinyl peptide among the three yellow peptides, the property of the active site to bind substrate and get protected against the inactivation by cysteine-specific reagent was utilized. When the modification of xylanase was carried out in the presence of substrate, the peptide containing the reactive cysteine will appear colourless in contrast

**Fig. 6.2:** The absorption spectra of the native and DAM treated enzyme



2
1
0
ABSORBANCE
ENZ + DAM
ENZ
DAM
220
300
400
500
WAVELENGTH (nm)

**Fig. 6.3:** Sephadex G-25 chromatography of peptic digest of DAM treated xylanase.

(o) O.D. at 280 nm (●) O.D. at 340 nm

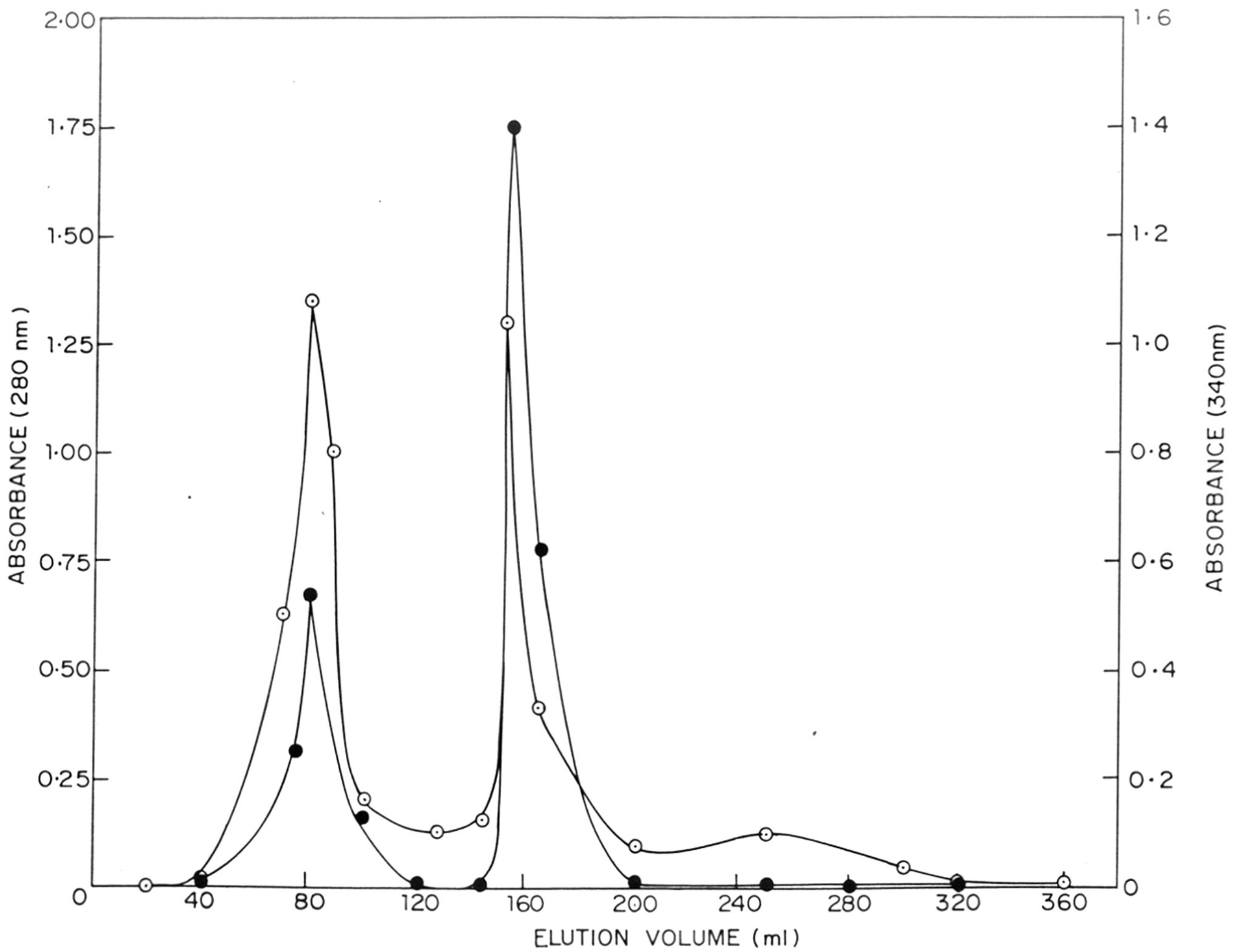

ABSORBANCE (280 nm)
2·00
1·75
1·50
1·25
1·00
0·75
0·50
0·25
0
ABSORBANCE (340nm)
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
40
80
120
160
200
240
280
320
360
ELUTION VOLUME (ml)

**Fig. 6.4:** Peptide maps for xylanase

a) Peptic digest of native xylanase

b) Peptic digest of DAM treated xylanase

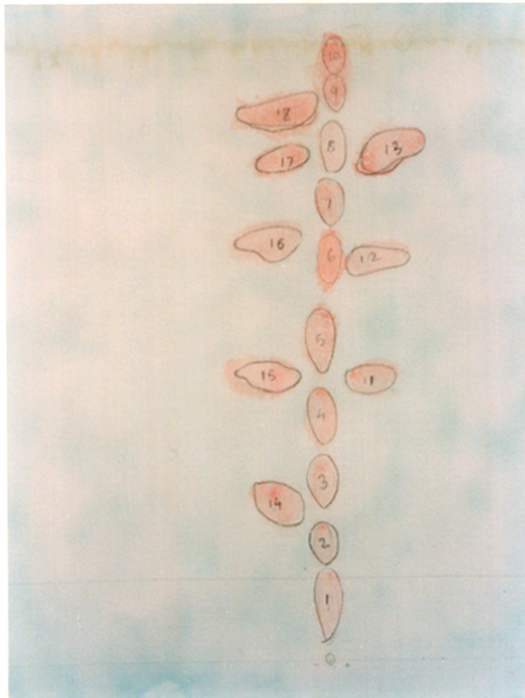



Fig. 6·4 a

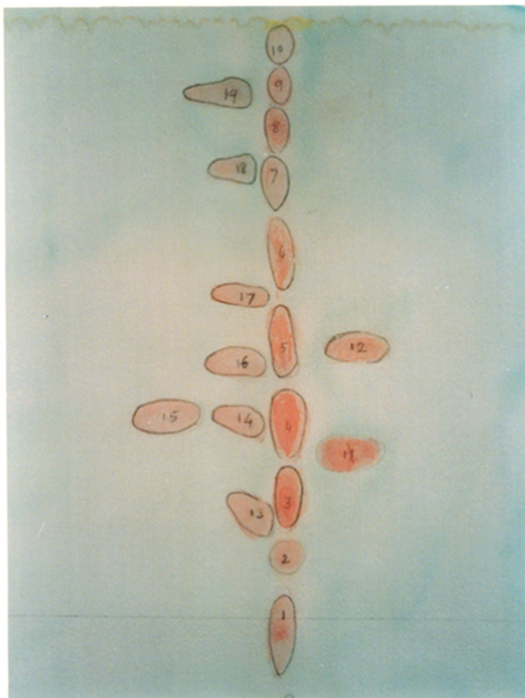



Fig. 6·4 b

**Fig. 6.5:** Peptide maps for xylanase (Peak II)

a) Peptic digest of DAM treated xylanase in the absence of substrate (xylan)

b) Peptic digest of DAM treated xylanase in the presence of substrate (xylan)

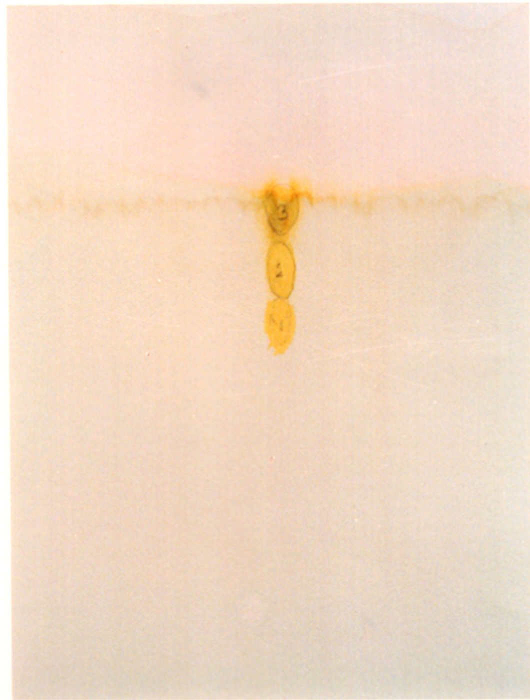


Fig. 6·5a

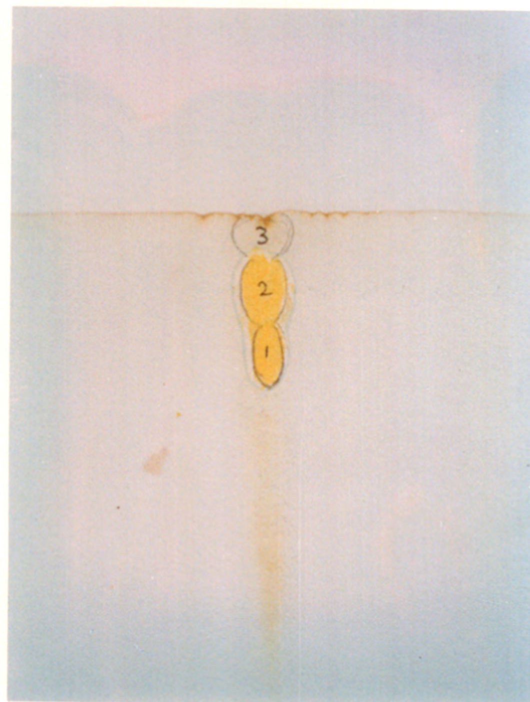



Fig. 6·5 b

to the other yellow cysteinyl peptides.

Figure 6.6 shows the protection of the xylanase by substrate against inactivation by DAM. 100% protection was obtained by 1 mg of xylan for 2 μg of enzyme.

The comparison between the peptide maps obtained in the absence (Fig. 6.5a) and presence (Fig. 6.5b) of xylan shows that the peptide 3 from Fig. 6.5b failed to indicate the characteristic yellow colour and turned pink on spraying with ninhydrin. After locating the position of active site peptide, a similar experiment with the purified xylanase was carried in the absence of xylan and the active peptide (No. 3) was isolated. Figure 6.7 shows the absorption spectrum of the purified peptide.

**Amino acid analysis of the active peptide**

Figure 6.8 shows the paper chromatogram for the amino acid analysis of isolated peptide. The $R_f$ values for amino acids generated by the peptide were calculated and compared with those of standard amino acids. The data suggested that the peptide is composed of six amino acids: Aspartic acid; Glycine; Alanine; Tyrosine/valine; Isoleucine/phenylalanine and DAM cysteine (X).

**Fig. 6.6:** Protection of xylanase by substrate against inactivation by DAM

Different concentrations of xylan (0.1 - 1.2 mg) were added to 2 µg of xylanase before the addition of DAM.

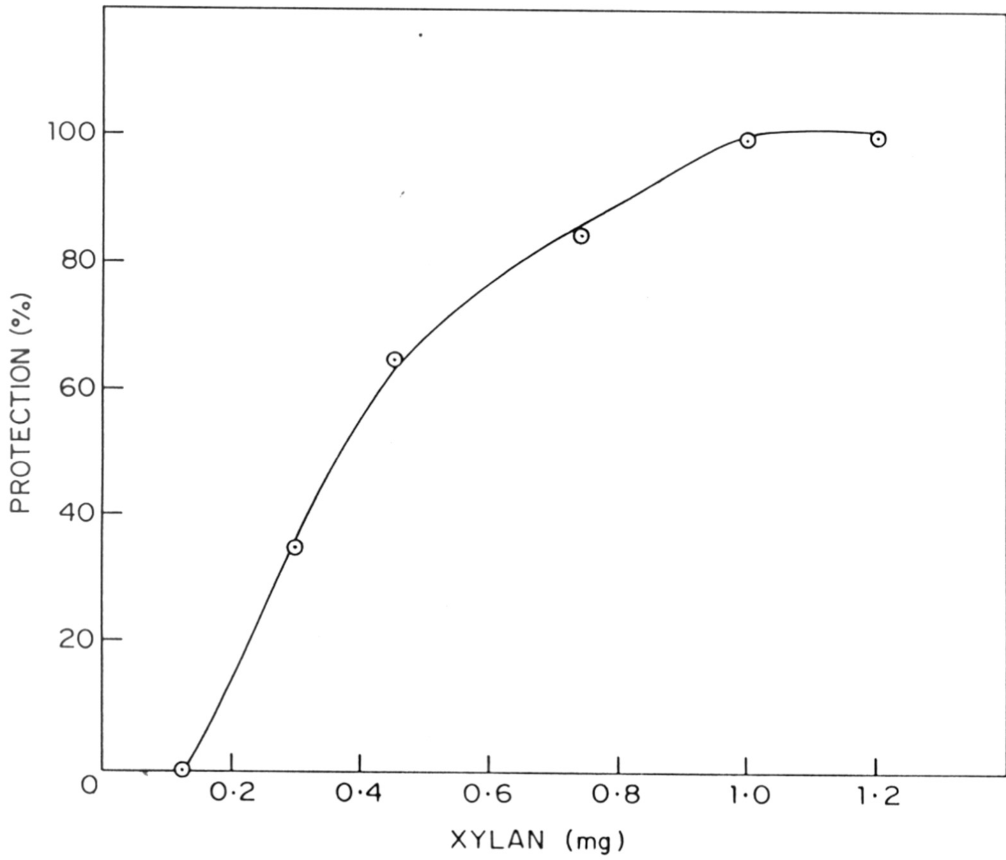
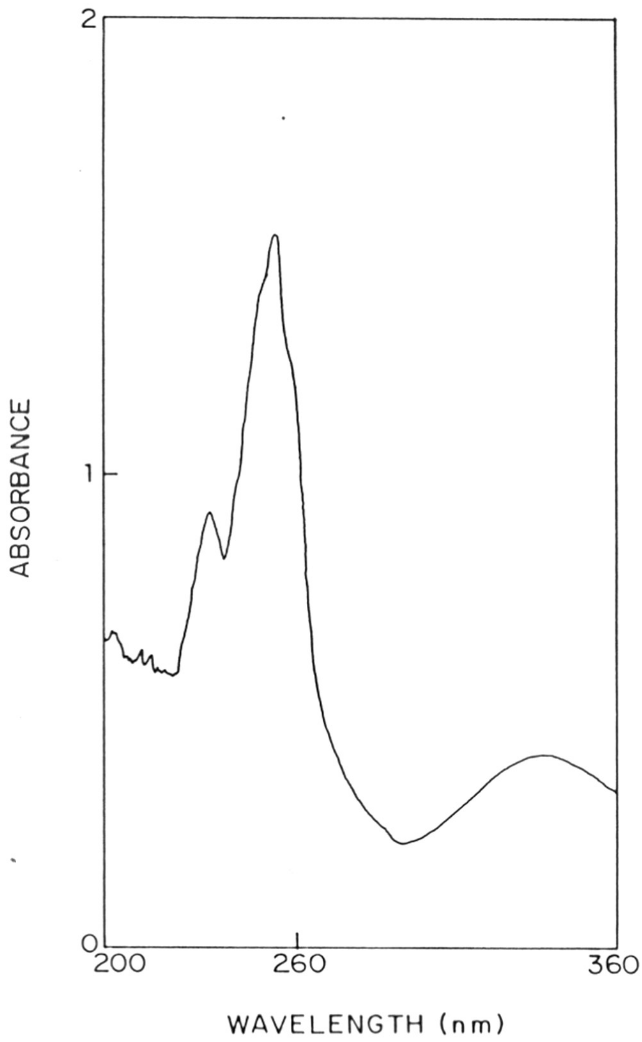

100
80
60
40
20
0
PROTECTION (%)
0·2
0·4
0·6
0·8
1·0
1·2
XYLAN (mg)

**Fig. 6.7:** Absorption spectrum of purified peptide



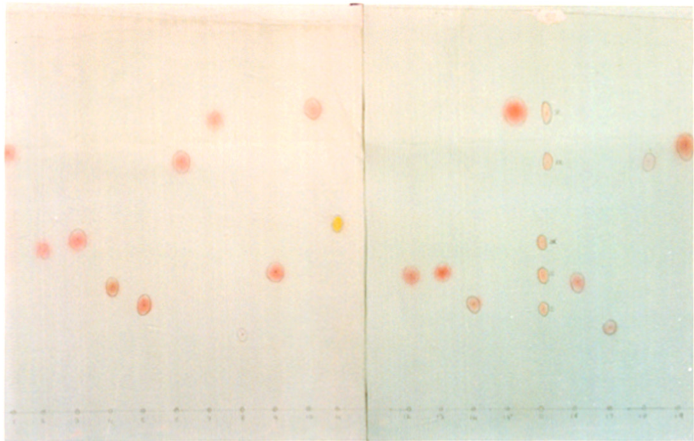
ABSORBANCE
2
1
0
200
260
360
WAVELENGTH (nm)

**Fig. 6.8:** Amino acid analysis of the purified peptide

| | | |
|---|---|---|
| 1. Methionine | 2. Threonine | 3. Alanine |
| 4. Histidine | 5. Lysine | 6. Valine |
| 7. Phenylalanine | 8. Cystine | 9. Serine |
| 10. Leucine | 11. Proline | 12. Glycine |
| 13. Glutamic acid | 14. Aspartic acid | 15. Isoleucine |

S. Hydrolysed cysteinyl peptide

| | | |
|---|---|---|
| 16. Arginine | 17. Cysteine | 18. Tyrosine |
| 19. Tryptophan | | |



1 S 19

## REFERENCES


1. Cohen, J.A. and Warringa, M.G.P.J. (1953) Biochim. Biophys. Acta, **11**, 52.
2. Dintzis, H.M. (1961) Proc. Natl. Acad. Sci. **47**, 247.
3. Li, T.K. and Vallee, B.L. (1964) Biochemistry, **3**, 869.
4. Gehring, H. and Christen, P. (1983) Methods Enzymol. **91**, 392.
5. Colman, R.F. (1983) Ann. Rev. Biochem. **52**, 67.
6. Schoellmann, G. and Shaw, E. (1963) Biochemistry, **2**, 252.
7. Means, G.E. and Feeney, R.E. (1971) In "Chemical Modification of Proteins" (Eds. Means, G.E. and Feeney, R.E.) Holden-Day, USA, p. 25.
8. Churchich, J.E. (1985) In "Chemical Modification of Enzymes" (Ed. Eyzaguirre, J.) John Wiley & Sons, N.Y., p. 75.
9. Fahey, R.C. and Newton, G.L. (1987) Methods Enzymol. **143**, 85.
10. Fahey, R.C., Newton, G.L., Dorian, R. and Kosower, E.M. (1981) Anal. Biochem. **111**, 357.
11. Vangrysperre, W., Ampe, C., Kersters-Hilderson, H. and Tempst, P. (1989) Biochem. J. **263**, 195.
12. Saha, A., Huang, U.C. and Colman, R.F. (1989) Biochemistry, **28**, 8425.
13. Walsh, K.A., Sampath Kumar, K.S.V., Bargetzi, J.P. and Neurath, H. (1962) Proc. Natl. Acad. Sci. **48**, 443.
14. Sanger, F. (1952) Adv. Protein Chem. **7**, 1.
15. Witter, A. and Tuppy, H. (1960) Biochim. Biophys. Acta

**45**, 429.

16. Alexander, N.H. (1958) Anal. Chem. **30**, 1292.
17. Tsao, T.C. and Bailey, K. (1953) Biochim. Biophys. Acta, **11**, 102.
18. Clark-Walker, G.D. and Robinson,H.G. (1961) J. Chem. Soc. **10**, 547.
19. Tsou, K.C., Barnett, R.J. and Seligman, A.M. (1955) J. Am. Chem. Soc. **77**, 4613.
20. Gold, A.H. and Segal, H.L. (1964) Biochemistry, **3**, 778.
21. Tuppy, H. (1961) Monatsh Chem. **92**, 992.
22. Sreenath, H.K. and Joseph, R. (1982) Folia Microbiol. **27**, 107.
23. Nakajima, T., Tsukamota, K.I., Watanabe, T., Kainuma, K. and Matsuda, K. (1984) J. Ferment. Technol. **62**, 269.
24. Marui, M., Nakanishi, K. and Yasui, T. (1985) Agric. Biol. Chem. **49**, 3399.
25. Biswas, S.R., Jana, S.C., Mishra, A.K. and Nanda, G. (1990) Biotechnol. Bioeng. **35**, 244.
26. Ingram, V.M. (1958) Biochim. Biophys. Acta, **28**, 539.
27. MICHL, H. (1951) Monatsh, Chem. **82**, 489.

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# Chemical modification of a xylanase from a thermotolerant *Streptomyces*

## Evidence for essential tryptophan and cysteine residues at the active site

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Extracellular xylanase produced in submerged culture by a thermotolerant *Streptomyces* $T_7$ growing at 37–50 °C was purified to homogeneity by chromatography on DEAE-cellulose and gel filtration on Sephadex G-50. The purified enzyme has an $M_r$ of 20463 and a pI of 7.8. The pH and temperature optima for the activity were 4.5–5.5 and 60 °C respectively. The enzyme retained 100 % of its original activity on incubation at pH 5.0 for 6 days at 50 °C and for 11 days at 37 °C. The $K_m$ and $V_{max.}$ values, as determined with soluble larch-wood xylan, were 10 mg/ml and $7.6 \times 10^3$ μmol/min per mg of enzyme respectively. The xylanase was devoid of cellulase activity. It was completely inhibited by $Hg^{2+}$ ($2 \times 10^{-6}$ M). The enzyme degraded xylan, producing xylobiose, xylo-oligosaccharides and a small amount of xylose as end products, indicating that it is an endoxylanase. Chemical modification of xylanase with *N*-bromosuccinimide, 2-hydroxy-5-nitrobenzyl bromide and *p*-hydroxymercuribenzoate (PHMB) revealed that 1 mol each of tryptophan and cysteine per mol of enzyme were essential for the activity. Xylan completely protected the enzyme from inactivation by the above reagents, suggesting the presence of tryptophan and cysteine at the substrate-binding site. Inactivation of xylanase by PHMB could be restored by cysteine.

## INTRODUCTION

Hemicellulose is one of the major components of ligno-cellulosic biomass and consists largely of xylan. Xylanases (EC 3.2.1.8) catalyse the random hydrolysis of xylosidic bonds in xylan and related xylo-oligosaccharides. In view of their possible application in the paper and pulp industries, information on microbial xylanases has been increasingly forthcoming in recent years (Jurasek & Paice, 1986). Xylanases have been described from a wide range of micro-organisms, especially fungi (Dekker & Richards, 1976). Among the prokaryotes, the information on actinomycete xylanase is largely derived from *Streptomyces* spp. (Kusakabe *et al.*, 1977; Sreenath *et al.*, 1978; Ishaque & Kluepfel, 1981). There are comparatively fewer reports on production and purification of xylanases from thermophilic micro-organisms (McCarthy *et al.*, 1985; Kluepfel *et al.*, 1986; Morosoli *et al.*, 1986). Stable enzymes active at high temperatures are favourable for increasing reaction rates and possibly for lessening contamination problems. Generally the xylanases are also associated with other activities, such as cellulase and glucose isomerase (Ishaque & Kluepfel, 1981; Kluepfel *et al.*, 1986). There are no published reports so far on microbial xylanases which are not associated with cellulase activity.

Reports on the inhibition of xylanases by different chemical compounds which are specific to certain amino acids are available (Sreenath & Joseph, 1982; Nakajima *et al.*, 1984; Marui *et al.*, 1985). However, the number of amino acid residues essential for activity and their role in the catalytic site have not been investigated. Large active centres containing several subsites appear to be characteristic of the fungal xylanases in general (Biely *et al.*, 1981; Vrsanska *et al.*, 1982; Meagher, 1984; Meagher *et al.*, 1988). However, there is little data correlating the structure and function of the binding-site region of xylanase.

In the present paper we report the isolation of a high-xylanase-producing thermotolerant *Streptomyces* $T_7$ that is free from cellulase activity. On the basis of the chemical modification of the purified enzyme, our results have provided the first evidence for the involvement of tryptophan and cysteine residues at the active site of xylanase.

## MATERIALS AND METHODS

### Materials

*N*-Bromosuccinimide (NBS), *N*-ethylmaleimide, diethyl pyrocarbonate, 2-hydroxy-5-nitrobenzyl bromide (HNBB), *p*-hydroxymercuribenzoate (PHMB), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), phenylglyoxal, butane-2,3-dione, iodoacetamide, *N*-acetylimidazole, β-alanine, 3,5-dinitrosalicylic acid (DNS), *p*-nitrophenyl β-D-xyloside, molecular-mass markers, *NNN′N′*-tetramethylethylenediamine (TEMED), Coomassie Blue G-250 and R-250, DEAE-cellulose, *NN′*-methylenebisacrylamide and CM-cellulose C8758 were purchased from Sigma. The suppliers of the following chemicals are indicated in parentheses: SDS (Koch–Light Labora-

Abbreviations used: NBS, *N*-bromosuccinimide; HNBB, 2-hydroxy-5-nitrobenzyl bromide; PHMB, *p*-hydroxymercuribenzoate; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DNS, 3,5-dinitrosalicylic acid; TEMED, *NNN′N′*-tetramethylethylenediamine.

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tories); yeast extract (Difco); peptone (Biochemicals Unit, New Delhi, India); larch-wood xylan and cysteine (Fluka); ultrafiltration membranes (Amicon); Sephadex G-50 (Pharmacia); Ampholines (LKB). All the other chemicals used were of analytical grade. Xylo-oligosaccharides were kindly given by Professor P. J. Reilly, Department of Chemical Engineering, Iowa State University, Ames, IA 50011, U.S.A. Wheat bran was purchased locally.

### Isolation of the strain

From humus-rich soil collected at Nandesari (near Baroda, India) an actinomycete strain was isolated by plating out a soil sample on a medium containing wheat bran (5 %) and yeast extract (1 %). The wheat bran was autoclaved in water, followed by extraction through muslin cloth to remove most of the starch. When incubated at 50 °C for 48 h, several colonies of bacteria and actinomycetes developed which were screened for xylanase activity in submerged culture. One of them, which produced relatively high xylanase activity and a larger clearance zone on xylan/yeast extract/agar plate, was designated as '*Streptomyces* $T_7$' and selected for further studies.

### Enzyme production

Enzyme was produced in 250 ml Erlenmeyer flasks containing 50 ml of medium (Ishaque & Kluepfel, 1981), except that the amount of $(NH_4)_2SO_4$ was increased five times and 5 % (w/v) wheat bran was used as a carbon source. Vegetative inoculum was used to inoculate experimental media. The inoculum was grown in the same medium but containing 1 % wheat bran. The flasks were incubated at 50 °C on a rotary shaker at 200 rev./min. The experimental flasks contained 5 % wheat bran as carbon source and were cultivated for 72 h. The cells and solid residues were removed from the culture broth by centrifugation and the clear supernatant used as an enzyme source.

### Enzyme purification

All steps were carried out at 4 °C unless otherwise mentioned.

The culture filtrate (400 ml) (Step I) was concentrated by precipitating the enzyme with 3 vol. of chilled distilled ethanol. The precipitate was recovered by centrifugation (9000 *g*; 30 min) and dried under vacuum and stored at −10 °C until further use. The precipitate (4 g, 10600 units) was dissolved in 80 ml of 10 mM-sodium phosphate buffer, pH 8.0. The undissolved solid particles from the precipitate were separated by centrifugation and the clear supernatant (Step II) was further purified on DEAE-cellulose in a batchwise treatment. A 260 ml portion of DEAE-cellulose (40 mg/ml, equilibrated with 10 mM-sodium phosphate buffer, pH 8.0) was suspended in 80 ml of enzyme solution (Step II) for 30 min. The slurry was filtered through Whatman no. 1 filter paper. The filtrate was collected and the DEAE-cellulose 'cake' was resuspended twice, each time with 20 ml of the same buffer. The filtrate and washings were pooled and ultrafiltered through an Amicon UM 10 membrane to concentrate the sample to 12 ml (Step III). A 1 ml portion of this enzyme (9 mg, 621 units) was applied to a Sephadex G-50 column (1.5 cm × 110 cm) equilibrated with 50 mM-potassium phosphate buffer, pH 7.2. The fractions were collected at a rate of 2 ml/10 min and estimated for xylanase activity. The active fractions were pooled, dialysed against water and concentrated by freeze-drying (Step IV).

### Analytical methods

A 2 g portion of xylan was suspended in 100 ml of 50 mM-sodium acetate buffer, pH 5.0, and was stirred for 12–16 h. The insoluble fraction (about 50 %) was removed by centrifugation and the soluble fraction was used for xylanase assay.

Xylanase was assayed by mixing a 0.5 ml aliquot of appropriately diluted enzyme with 0.5 ml of 1 % xylan and incubating at 60 °C for 30 min (Mandels & Weber, 1969). The reducing sugar released was determined by the DNS method with D-xylose as standard (Miller, 1959).

$\beta$-Xylosidase was estimated as described by Kluepfel & Ishaque (1982) with *p*-nitrophenyl $\beta$-D-xyloside as substrate by determining the *p*-nitrophenol liberated by the enzyme action at 40 °C after 30 min.

The unit of xylanase or xylosidase was defined as that amount of enzyme which produces 1 $\mu$mol of xylose or *p*-nitrophenol/min from xylan or *p*-nitrophenyl $\beta$-D-xyloside respectively under the given assay conditions. Activity towards CM-cellulose and filter paper (Whatman no. 1) were determined by incubating 1 ml of reaction mixture, containing suitably diluted enzyme, with 0.5 ml of CM-cellulose (1 %) or filter paper (25 mg) in 50 mM-acetate buffer, pH 5.0, at 50 °C for 30 or 60 min respectively. The reducing sugar formed was determined by the DNS method described above. Proteinase activity was determined by Kunitz's (1947) method.

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Electrophoresis at pH 4.3 was performed as described by Maurer (1971), with 7.5 % (w/v) acrylamide gel.

SDS/polyacrylamide-gel electrophoresis was carried out as described by Laemmli (1970), with albumin ($M_r$ 66000), ovalbumin (45000), pepsin (34700), trypsinogen (24000), $\beta$-lactoglobulin (18400) and lysozyme (14300) as reference proteins.

$M_r$ values were also determined by gel filtration on Sephadex G-50 and Bio-Gel P-10.

Suitably diluted xylanase was incubated with different amounts (3–15 mg) of xylan under the assay conditions given. $K_m$ and $V_{max.}$ were determined from Lineweaver–Burk plots.

Isoelectric focusing in thin polyacrylamide gels was carried out by the method of Vesterberg (1972) over the pH range 3.5–10.0.

Free thiol groups in the enzyme were determined by titrating enzyme against PHMB (Boyer, 1954) and DTNB (Ellman, 1959).

### Xylan degradation products

Xylanase (1.0 unit) was incubated with xylan (10 mg) in 20 mM-acetate buffer, pH 5.0, for different periods of time. The end products formed were analysed by paper chromatography in the solvent system butanol/acetic acid/water (3:1:1, by vol.) by the method of Trevelyn *et al.* (1950).

### Synergism with $\beta$-xylosidase

Xylanase (1.0 unit) and $\beta$-xylosidase from *Aspergillus niger* (0.07 unit) were mixed with 10 mg of xylan in

0.5 ml of 50 mM-acetate buffer, pH 5.0. The reaction was carried out at 37 °C for 20 h. The control with only xylanase was also run under identical conditions. The percentage hydrolysis was determined by measuring the reducing sugar.

#### Reaction with different chemical modifiers

A 0.5 ml portion of enzyme (10 $\mu$g/ml) was incubated in a total volume of 2.5 ml with various concentrations of modifier in appropriate buffer. Control tubes containing enzyme only or inhibitor only were incubated under identical conditions. Aliquots (0.5 ml) were removed at 10, 20, 30 and 40 min for measurement of residual enzyme activity.

#### Titration with NBS

Oxidation of tryptophan residues by NBS was carried out in two cuvettes: one containing xylanase ($2.72 \times 10^{-5}$ M) in 50 mM-acetate buffer, pH 4.5, and another containing buffer. Successive 10 $\mu$l aliquots of NBS ($1 \times 10^{-4}$ M) were added to the sample as well as to the reference cuvette and absorbance at 280 nm was measured. After each addition of NBS, the number of tryptophan residues oxidized ($\Delta n$) per mol of enzyme was calculated from the equation (Witkop, 1961; Spande & Witkop, 1967):

$$\Delta n = \frac{1.31\,\Delta A_{280}}{5500 \times \text{molarity of enzyme}}$$

where $\Delta A_{280}$ is the decrease in absorbance at 280 nm, 1.31 is an empirical factor based upon oxidation of model tryptophan peptides by NBS (Patchornik *et al.*, 1958) and 5500 is the molar absorption coefficient for tryptophan at 280 nm. Simultaneously portions of the reaction mixture were assayed for xylanase activity.

#### Substrate-protection studies

A 0.1 ml portion of enzyme (1 $\mu$g) in buffer was added to different amounts of xylan (0.1–5 mg) in a total volume of 0.5 ml before the addition of NBS (4 $\mu$M) or PHMB (5 $\mu$M). The reaction mixture was incubated at 25 °C for 10 min. The activity of the enzyme was determined as usual by adding the remaining amount of xylan.

For different concentrations of HNBB (2.5 mM, 4 mM and 8 mM), percentage inhibition of xylanase was determined. For every HNBB concentration the amount of xylan needed to give 100% protection was determined by the above procedure.

#### Re-activation of xylanase after modification by PHMB

The enzyme (1 $\mu$g) was incubated with PHMB (2 $\mu$M) in 50 mM-acetate buffer, pH 6.0, in a volume of 0.25 ml at 25 °C. At different time intervals, residual activity was determined. Simultaneously the incubation mixtures (0.25 ml) were also transferred to 0.25 ml of 50 mM-cysteine and incubated at 25 °C for 20 min. The enzyme activity was determined in the usual way by adding the substrate and incubating at 60 °C for 30 min.

Alternatively, regain of xylanase activity completely inactivated by PHMB (5 $\mu$M) was determined by transferring aliquots of inactivated enzyme in different concentrations of cysteine and incubating at 25 °C for 20 min.

## RESULTS AND DISCUSSION

#### Characteristics of strain $T_7$

The isolate is an aerobic *Streptomyces* which forms greyish sporulating colonies on wheat bran/yeast extract/agar after incubation for 3 days at 50 °C. When grown on agar plates containing 1% xylan, a distinct clearance was observed, indicating the hydrolysis of xylan and extracellular secretion of xylanase. The optimum temperature for the growth of the *Streptomyces* $T_7$ was over the range 45–50 °C; the strain was also able to grow at 37 °C, but it did not grow above 50 °C, indicating that it is a thermotolerant culture. Fig. 1 shows the xylanase production at different incubation temperatures. The production of enzyme was maximum (70 units/ml) at 50 °C after 72 h of cultivation when 5% wheat bran was used. The strain did not show detectable intra- or extra-cellular $\beta$-xylosidase activity, extracellular activity against CM-cellulose or filter paper, or proteinase activity at pH 7.0 or 10.0.

The *Streptomyces* $T_7$ is similar to *Streptomyces lividans*, which was reported by Kluepfel *et al.* (1986) to grow over the temperature range 18–46 °C. The optimum temperatures for growth and enzyme production were 29 and 40 °C respectively. Xylanases from *S. lividans* (Kluepfel *et al.*, 1986) and *S. flavogriseus* (Ishaque & Kluepfel, 1981) were reported to be associated with cellulase and glucose isomerase activities respectively.

#### Enzyme purification

Table 1 summarizes the results for the purification of xylanase. The enzyme was purified 41-fold over the culture supernatant. The xylanase from *S. lividans* has been purified 33-fold over the culture supernatant (Morosoli *et al.*, 1986), whereas that from *S. xylophagus* was purified 276-fold (specific activity 581 units/mg of protein) (Iizuka & Kawaminami, 1965). The *Streptomyces* $T_7$ contains a single component of xylanase. The purified enzyme showed a single band on gel electrophoresis in the presence or absence of SDS. The xylanases from mesophilic *Streptomyces* have also been

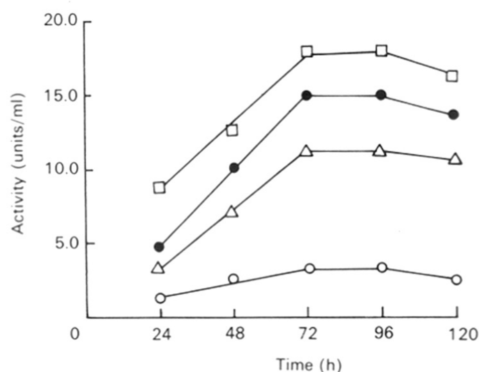


**Fig. 1. Effect of temperature on xylanase production**

The organism was grown on the basal medium containing 2% wheat bran. The samples were removed periodically and the culture filtrate was examined for xylanase activity. ○, 28 °C; △, 37 °C; ●, 45 °C; □, 50 °C.

**Table 1. Purification of *Streptomyces* xylanase**

| Step | Fraction | Total volume (ml) | Total protein (mg) | Total activity (units) | Specific activity (units/mg) | Purification (fold) |
|---|---|---|---|---|---|---|
| I | Culture filtrate | 400 | 1200 | 12000 | 10.0 | 1.00 |
| II | Ethanol precipitation | 80 | 256 | 10600 | 41.4 | 4.14 |
| III | DEAE-cellulose chromatography | 12 | 116 | 7462 | 64.4 | 6.44 |
| IV | Sephadex G-50 | 3 | 1.95 | 805 | 412.8 | 41.30 |

**Table 2. Properties of purified xylanase**

| Parameter | Value |
|---|---|
| Optimum pH | 4.5–5.5 |
| Optimum temperature | 60 °C |
| $K_m$ | 10 mg/ml |
| $V_{max.}$ | 7.6 mmol/min per mg |
| pI | 7.8 |
| $M_r$ by: | |
| SDS/PAGE* | 21880 |
| Bio-Gel P-10 | 19230 |
| Sephadex G-50 | 20180 |

* Abbreviation: PAGE, polyacrylamide-gel electrophoresis.

shown to consist of only one xylanase component (Kusakabe *et al.*, 1977; Nakajima *et al.*, 1984).

## Properties

Table 2 shows the physico-chemical properties of the purified enzyme. The activity was maximum at 60 °C over the pH range 4.5–5.5. At 60 °C the half-life of the enzyme was 30 min. The enzyme retained full activity on incubation at 50 °C for 6 days and at 37 °C for 11 days. Morosoli *et al.* (1986) reported a xylanase from *S. lividans* which was stable at pH 6.0 for 24 h at 37 °C.

Titration with PHMB and DTNB showed the presence of 3.5 thiol groups per molecule of enzyme. Among the metal ions tested, $Hg^{2+}$ completely inhibited the enzyme activity, indicating that thiol-containing amino acids may be involved in the activity.

## Degradation of xylan

The major end products of xylan hydrolysis by the *Streptomyces* $T_7$ xylanase were xylobiose and xylo-oligosaccharides ($Xyl_3$–$Xyl_6$). Very little xylose was produced even after 16 h, indicating that it is an endoxylanase. When xylanase was mixed with $\beta$-xylosidase, the end product was mainly xylose, and the hydrolysis increased from 43 to 60%, owing to the synergistic action of xylanase and $\beta$-xylosidase.

## Effect of chemical modifiers

Table 3 indicates the effect of various modifiers on xylanase activity. Complete inhibition by NBS, HNBB and PHMB showed that tryptophan and cysteine residues are modified. Complete inhibition by PHMB but less

**Table 3. Effect of chemical inhibitors on xylanase**

The enzyme was incubated with the inhibitor at 25 °C for 10 min before the addition of substrate.

| Chemical | [Inhibitor] (mM) | Inhibition (%) | Incubation buffer (50 mM) |
|---|---|---|---|
| NBS | 1.0 | 100 | Sodium acetate buffer, pH 4.5 |
| HNBB | 10.0 | 100 | Sodium acetate buffer, pH 4.5 |
| Diethyl pyrocarbonate | 10.0 | 0 | Potassium phosphate buffer, pH 7.0 |
| *N*-Ethylmaleimide | 2.0 | 50 | Sodium acetate buffer, pH 6.0 |
| Iodoacetamide | 5.0 | 30 | Sodium acetate buffer, pH 6.0 |
| Phenylglyoxal | 10.0 | 0 | Tris/HCl, pH 8.0 |
| PHMB | 1.0 | 100 | Sodium acetate buffer, pH 6.0 |
| Phenylmethanesulphonyl fluoride | 10.0 | 0 | Potassium phosphate buffer, pH 7.0 |
| *N*-Acetylimidazole | 10.0 | 0 | Potassium phosphate buffer, pH 7.0 |
| Butane-2,3-dione | 10.0 | 0 | Sodium borate buffer, pH 8.0 |

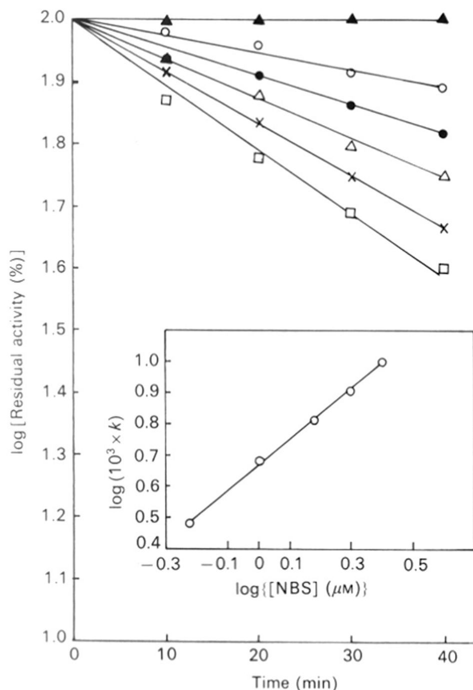


**Fig. 2. Kinetics of inactivation of xylanase by NBS**

The enzyme (5 μg) was incubated with NBS (▲, 0 μM; ○, 0.6 μM; ●, 1.0 μM; △, 1.5 μM; ×, 2.0 μM; □, 2.5 μM). The inset shows the apparent order of reaction with respect to reagent concentration. The logarithm of observed pseudo-first-order rate constants ($k$) calculated from Fig. 2 was plotted against the logarithm of the inhibitor concentration.

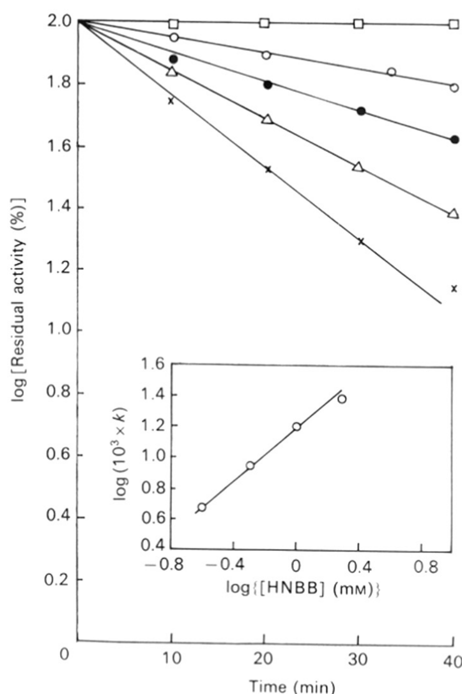


**Fig. 3. Inactivation of xylanase by HNBB**

Enzyme (5 μg) was incubated with HNBB (□, 0 mM; ○, 0.25 mM; ●, 0.5 mM; △, 1.0 mM; ×, 2.0 mM). The inset shows a plot of the logarithm of pseudo-first-order rate constant against the logarithm of the HNBB concentration.

inhibition by iodoacetamide may be due to the fact that, at pH 6.0, there is a very small proportion of the ionized form of cysteine ($-CH_2-S^-$), which is the reactive nucleophile. Inhibition by $Hg^{2+}$, NBS and PHMB has been reported for xylanases from several *Streptomyces* species (Sreenath & Joseph, 1982; Nakajima *et al.*, 1984; Marui *et al.*, 1985).

Plots of percentage residual activity as a function of time at various concentrations of NBS (Fig. 2), HNBB (Fig. 3) and PHMB (Fig. 4) indicate that the inactivation process exhibits pseudo-first-order kinetics with respect to time at any fixed concentration of the inhibitor. Applying the analysis described by Levy *et al.* (1963), the pseudo-first-order rate constants were calculated from the slope of the plots of logarithm of the residual activity against the time of reaction. The order of the reaction was estimated from the slopes of the plots of log(pseudo-first-order rate constant) against log(inhibitor concentration). These graphs (insets to Figs. 2, 3 and 4) indicated that the loss of enzyme activity resulted from reaction of only one tryptophan (Figs. 2 and 3) or cysteine residue (Fig. 4) per molecule of enzyme.

### Titration with NBS

Fig. 5 shows the effect of NBS on enzyme activity. After each addition of NBS, there was a progressive decrease in absorption at 280 nm. For complete inactivation of the enzyme, 3.6 mol of NBS were required per mol of enzyme. The number of tryptophan residues oxidized per molecule of enzyme were found to be 2.2 (Fig. 6). This usually gives the number of residues modified but not the number of residues essential for activity.

### Protection by substrate against inactivation

A 1 mg portion of substrate was needed to give 100% protection against inactivation by NBS and PHMB. Protection by the substrate indicated the presence of essential tryptophan and cysteine residues at the substrate-binding site.

The xylanase was incubated with different concentrations of HNBB (2.5–8 mM). The amount of xylan needed to give 100% protection increased from 2.5 to 7.5 mg as the extent of inhibition increased. This again confirmed the presence of essential tryptophan residue at the substrate-binding region of the xylanase. It is surprising that 100% protection by substrate is obtained at a concentration less than the $K_m$. This may be attributed to the heterogeneity of xylan, which results in an 'apparent' value for the $K_m$.

The catalytic mechanism of two other hydrolytic

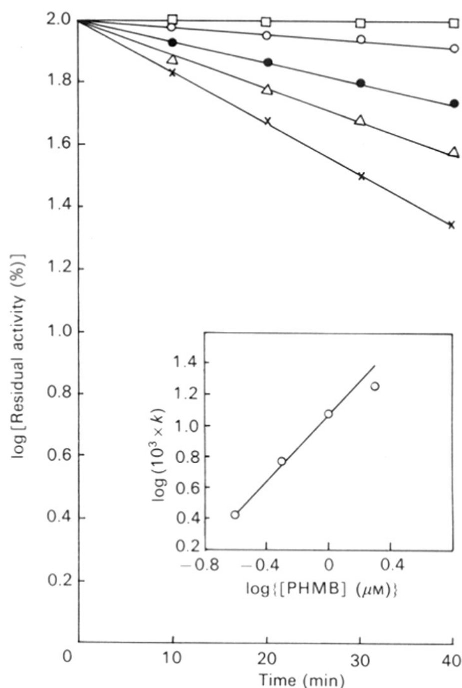


**Fig. 4. Effect of PHMB on xylanase activity**

Enzyme (5 μg) was incubated with PHMB (□, 0 μM; ○, 0.25 μM; ●, 0.5 μM; △, 1.0 μM; ×, 2.0 μM). The inset shows a plot of the logarithm of the psuedo-first-order rate constant of PHMB inactivation against the logarithm of PHMB concentration.

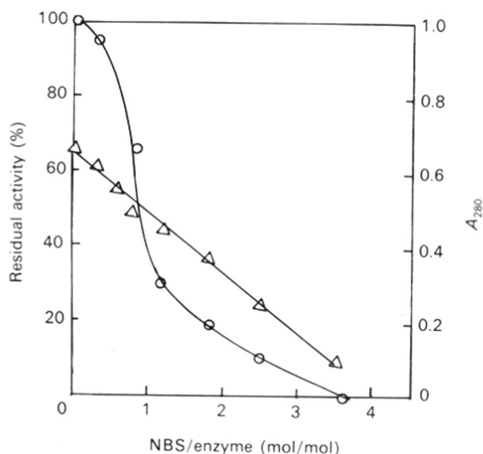


**Fig. 5. Activity and absorbance changes of xylanase as a function of molar excess of NBS**

Aliquots (10 μl) of NBS ($1 \times 10^{-4}$ M) were added successively to the enzyme ($2.72 \times 10^{-5}$ M). After each addition the residual activity (○) and the decrease in absorption at 280 nm (△) were measured.

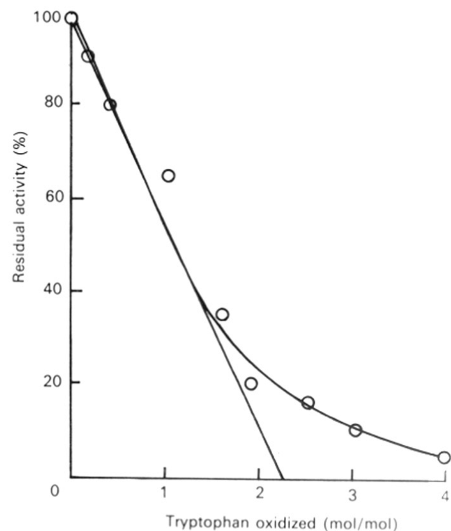


**Fig. 6. Titration of NBS with xylanase**

Oxidation of tryptophan residues in xylanase was carried out with stepwise addition of NBS to the enzyme, as described in Fig. 5. The number of tryptophan residues oxidized was determined as described in the Materials and methods section.

enzymes, namely lysozyme and cellulase, which are functionally related to xylanase, has been delineated in structural detail at the molecular level (Imoto *et al.*, 1972; Yaguchi *et al.*, 1983). Tryptophan residues have been shown to be involved in the binding of substrate to these enzymes (Hurst *et al.*, 1977; Clarke, 1987). The present results add further support to these earlier findings and suggest a relationship between xylanase, lysozyme and cellulase.

### Re-activation of xylanase after modification by PHMB

The progressive loss of activity as a function of time when the enzyme was treated with PHMB and its re-activation by cysteine were studied. The enzyme, which was completely inactivated by PHMB, was re-activated fully with 50 mM-cysteine, indicating a competitive displacement of PHMB by the high concentration of thiol (Means & Feeney, 1971).

Involvement of cysteine residues at the active site of cellulase or lysozyme has not hitherto been reported.

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## REFERENCES


Biely, P., Kratky, Z. & Vrsanska, M. (1981) Eur. J. Biochem. **119**, 559–564

Boyer, P. D. (1954) J. Am. Chem. Soc. **76**, 4331–4337

Clarke, A. J. (1987) Biochim. Biophys. Acta **912**, 424–431

Dekker, R. F. H. & Richards, G. N. (1976) Adv. Carbohydr. Chem. Biochem. **32**, 278–352

Ellman, G. L. (1959) Arch. Biochem. Biophys. **82**, 72–77

Hurst, P. L., Sullivan, P. A. & Shepherd, M. G. (1977) Biochem. J. **167**, 549–556
Iizuka, H. & Kawaminami, T. (1965) Agric. Biol. Chem. **29**, 520–524
Imoto, T., Johnson, L. N., North, A. C. T., Phillips, D. C. & Ruplex, J. A. (1972) Enzymes 3rd Ed. **7**, 666–868
Ishaque, M. & Kluepfel, D. (1981) Biotechnol. Lett. **3**, 481–486
Jurasek, L. & Paice, M. (1986) Chemtech **16**, 360–365
Kluepfel, D. & Ishaque, M. (1982) Dev. Ind. Microbiol. **23**, 389–395
Kluepfel, D., Shareck, F., Mondou, F. & Morosoli, R. (1986) Appl. Microbiol. Biotechnol. **24**, 230–234
Kunitz, M. (1947) J. Gen. Physiol. **30**, 291–310
Kusakabe, I., Yasui, T. & Kobayashi, T. (1977) Nippon Nogei Kagaku Kaishi **51**, 669–679
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Levy, H. M., Leber, P. D. & Ryan, E. M. (1963) J. Biol. Chem. **238**, 3654–3659
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
Mandels, M. & Weber, J. (1969) Adv. Chem. Ser. **95**, 391–414
Marui, M., Nakanishi, K. & Yasui, T. (1985) Agric. Biol. Chem. **49**, 3399–3407
Maurer, H. R. (1971) in Disc Electrophoresis and Related Techniques of Polyacrylamide Gel Electrophoresis (Maurer, H. R., ed.), pp. 32–110, Walter de Gruyter, Berlin and New York
McCarthy, A. J., Peace, E. & Broda, P. (1985) Appl. Microbiol. Biotechnol. **21**, 238–244
Meagher, M. M. (1984) M.S. Thesis, Iowa State University, Ames
Meagher, M. M., Tao, B. Y., Chow, J. M. & Reilly, P. J. (1988) Carbohydr. Res. **173**, 273–283
Means, G. E. & Feeney, R. E. (1971) in Chemical Modification of Proteins (Means, G. E. & Feeney, R. E., eds.), pp. 198–204, Holden–Day, San Francisco
Miller, G. L. (1959) Anal. Chem. **31**, 426–428
Morosoli, R., Betrand, J. L., Mondou, F., Shareck, F. & Kluepfel, D. (1986) Biochem. J. **239**, 587–592
Nakajima, T., Tsukamota, K. I., Watanabe, T., Kainuma, K. & Matsuda, K. (1984) J. Ferment. Technol. **62**, 269–276
Patchornik, A., Lawson, W. B. & Witkop, B. (1958) J. Am. Chem. Soc. **80**, 4747–4748
Spande, T. F. & Witkop, B. (1967) Methods Enzymol. **11**, 498–506
Sreenath, H. K. & Joseph, R. (1982) Folia Microbiol. (Prague) **27**, 107–115
Sreenath, H. K., Joseph, R. & Murthy, V. S. (1978) Folia Microbiol. (Prague) **23**, 299–303
Trevelyn, W. E., Procter, P. D. & Harrison, J. S. (1950) Nature (London) **166**, 444–445
Vesterberg, O. (1972) Biochim. Biophys. Acta **257**, 11–19
Vrsanska, M., Gorbacheva, I. V., Kratky, Z. & Biely, P. (1982) Biochim. Biophys. Acta **704**, 114–122
Witkop, B. (1961) Adv. Protein Chem. **16**, 221–314
Yaguchi, M., Roy, C., Rollin, C. F., Paice, M. G. & Jurasek, L. (1983) Biochem. Biophys. Res. Commun. **116**, 408–411

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