STUDIES ON NEUTROPHILIC *BACILLUS* sp. XYLANASE

A THESIS
SUBMITTED TO THE
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MASTER OF SCIENCE
(PARTLY BY PAPER AND PARTLY BY RESEARCH)

by

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DEDICATED TO

MY PARENTS

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DECLARATION

Certified that the work incorporated in the thesis entitled: "Studies on Neutrophilic *Bacillus* sp. Xylanase" submitted by Mrs. Snehal V. More was carried out under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

M.C. Srinivasan

Research Guide

ABSTRACT

Xylan is the major component of plant hemicelluloses and after cellulose, it is the most abundant polysaccharide in nature. Hence a study of xylanolytic enzymes can play an important role in development of meaningful bioconversion processes with economical feasibilities. In view of their potential application in paper and pulp industry, and others like food industry, microbial xylanases have become important in recent years.

The present investigation relates to:

a. Isolation of an organism capable of producing high levels of xylanase.

b. Identification of the organism

c. Optimization of the cultural and fermentation conditions for maximum enzyme production.

d. Preliminary characterization of the enzyme with regard to pH and temperature stability and analysis of end products of xylan hydrolysis.

The work is presented in four chapters and a brief outline of the contents is given below:

CHAPTER I – INTRODUCTION

This chapter presents a comprehensive literature survey on the natural occurrence and structure of xylan, xylanolytic microbial strains and characteristics of their xylanases and potential industrial applications of xylanolytic enzymes.

CHAPTER II – MATERIALS AND METHODS

In this section details of microbiological and biochemical techniques employed in the study of a high activity xylanase secreting *Bacillus* strain are described which includes isolation, *in vitro* culture, conservation, fermentation, assay and preliminary enzyme characterization.

CHAPTER III– ISOLATION AND FERMENTATION STUDIES

This chapter describes the results obtained in regard to isolation and identification of the xylanolytic organism and standardization of fermentation conditions for optimum xylanase production.

The organism isolated (NCL 90-10-50) was identified as *Bacillus pumilus*. It was aerobic, gram positive rod and formed median endospores. Colonies of the organism were with reticulate surface, off-white in colour and gave

prominent clearance zone of xylan hydrolysis when grown on an agar medium containing xylan. The organism was able to grow over a wide pH and temperature range. The survival and biochemical performance of the culture under laboratory conditions was found to be very good and the culture revived within 24 hours even after 6 years of storage at 4 - 10°C and showed no decline in xylanase production capacity.

The *Bacillus* produced significant xylanase activity in shake flasks with xylan or xylan-rich residues added to culture media. Low levels of activity were obtained only with xylose, while no enzyme was induced by other soluble sugars and sugar alcohols. Optimization of fermentation conditions showed that the maximum xylanase levels (300 – 350 IU/ml) were obtained in 72 hours when the culture was grown in a medium containing wheat bran (3%), yeast extract (1%) and Tween 80 (0.1%). The organism was able to utilize both organic and inorganic nitrogen sources for growth. Comparable amount of xylanase was produced with most inorganic or organic nitrogen sources. Urea produced low levels of xylanase, while yeast extract at 1% concentration gave maximum enzyme activity.

CHAPTER IV – PROPERTIES OF XYLANASE

Biochemical properties were studied with the cell-free filtrates rich in extracellular xylanase. The enzyme had optimum activity at pH of 7.0 and 50°C. At pH 8.0 it showed about 50% of the activity. The enzyme broth was free of associated cellulase and amylase activity and showed only small amount of protease activity. The Km determined with oatspelts xylan, were 11.1 mg/ml for total and 12.5 mg/ml for soluble xylan, respectively. The Vmax values were found to be 1.66 mmol/ml/min and 4.76 mmol/ml/min for total and soluble xylan respectively. The major end products of xylan hydrolysis were xylobiose and higher xylo oligosaccharides suggesting the enzyme to be an endoxylanase. The enzyme is a neutral xylanase having application prospects in industries such as baking and brewing which need further detailed investigation.

CHAPTER I

INTRODUCTION

OCCURANCE AND STRUCTURE OF XYLAN

Hemicelluloses are extensively present in plant cell walls. Along with cellulose it represents major renewable feed stock in plant biomass produced through photosynthesis. Xylans the major component of hemicellulose are heteropolysaccharides having β-1-4 linked xylopyranosyl residues. Different sources of xylans carry o-acetyl, α-L-arabinofuranosyl, α-D-glucopyranosyl, uronic acid substituents. β-1-4-Xylans are abundantly present in the secondary walls of plants and particularly woody tissues of tropical forests. Wide variation in the xylan content among different plant biomass has been recorded; for example birchwood is reported to contain 35% xylan, while some gymnosperms may contain as little as 7% (Whistler and Richards, 1970). The xylan content of different plant biomass is presented in Table 1.1 and structure of xylan in Figure 1.1 (Biely, 1991).

Xylans being renewable resources and easily subjected to hydrolysis either by acid or by microbial enzymes, have received world wide attention for possible exploitation in the generation of useful products. Enzymatic hydrolysis of xylan compared to chemical hydrolysis is advantageous by virtue of high specificity, mild reaction conditions and no substrate loss due to chemical

Table 1.1: The carbohydrate composition of representative wood (Lachke *et al.*, 1987)

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	0.5
Softwood			
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

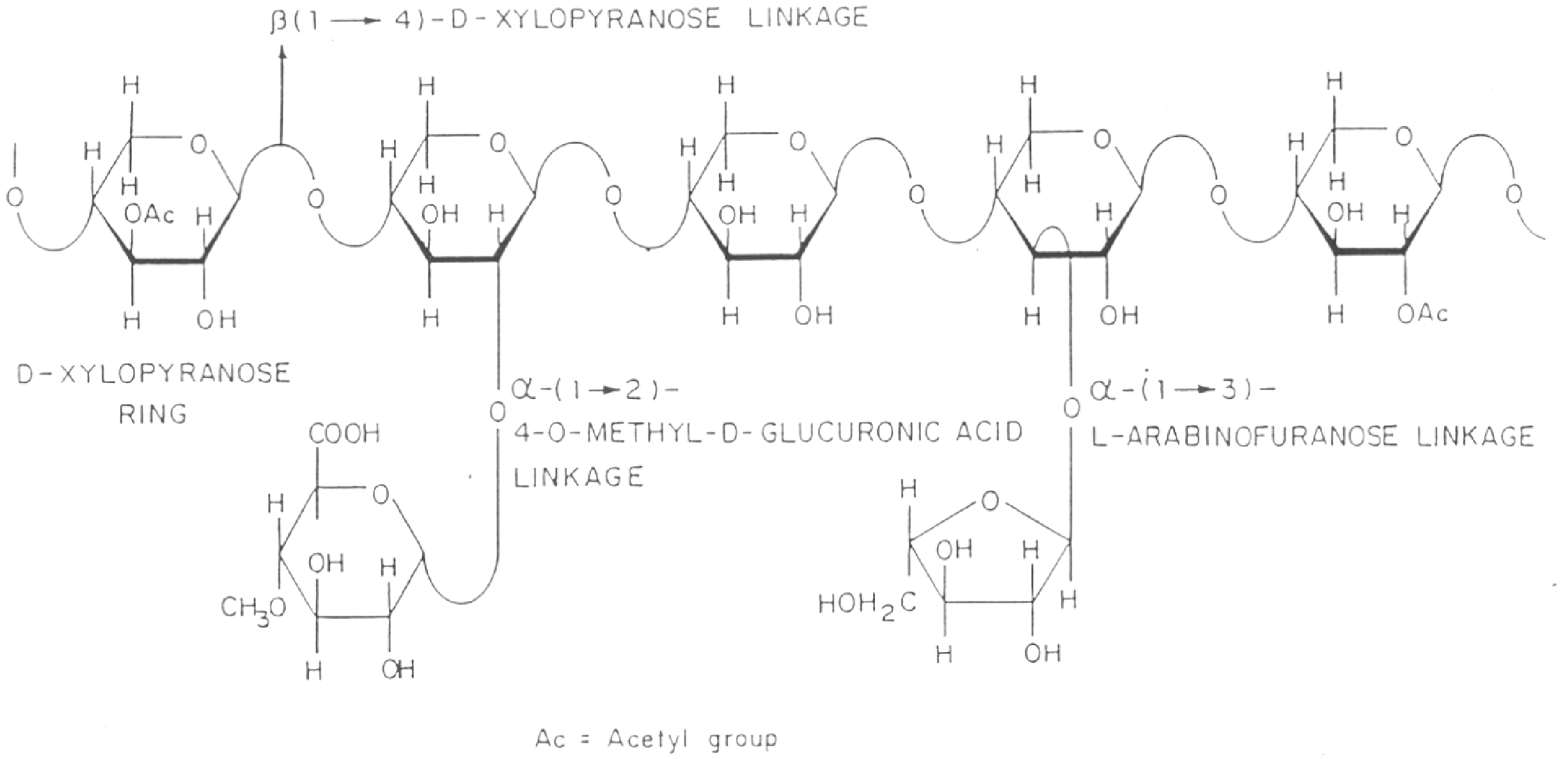


FIG. 1·1 : STRUCTURE OF XYLAN

modification. Products of xylan hydrolysis are mainly xylooligosaccharides and the sugar xylose is capable of microbial bioconversion into liquid fuels, solvents, single cell proteins or even chemical intermediates through the application of specific microorganisms (Biely, 1985). The abundant agricultural wastes that are generated during crop cultivation could thus be effectively and economically utilized through bioconversion. Several useful biochemical products can be manufactured through bioconversion of both cellulose and xylan.

Occurance of xylanases

In recent years xylanases have become highly significant for their potential application in the paper and pulp industry and in particular for biobleaching and production of cellulose pulp. Extensive studies on microbial degradation of xylan through enzyme systems secreted by a variety of microorganisms have been published. The literature contains several authoritative review articles as well as symposia proceedings on xylanases and their applications (Gong *et al.*, 1981; Biely, 1985; Wong *et al.*, 1988; Biely, 1991; Bastawde, 1992; Thomson, 1993; Sunna and Antranikian, 1997, Bajpai, 1997).

Bacteria, fungi and actinomycetes isolated from different ecosystems have been studied for xylanase production (Sadana *et al.*, 1980; Panbangred *et al.*, 1983; Steiner *et al.*, 1987; Paul

and Verma, 1990; Kluepfel *et al.*, 1990). Bacteria produce high levels of xylanase which could be effectively used for various industrial applications such as baking, biobleaching and textile industries (Matt *et al.*, 1992; Nissen *et al.*, 1992). The characteristics of individual xylanases suited for various applications are highly different. In the baking industry the xylanases are required to be active at neutral or slightly acidic pH whereas for pulping applications the enzyme should be active under alkaline conditions and also free from associated cellulase activity (Viikari *et al.*, 1990; Srinivasan and Rele, 1995).

Temperature stability of the xylanases is a crucial factor and in recent years research on microbial xylanases active at and stable to high temperature have received particular attention. In the pulping industry the application of xylanase is increasingly becoming relevant in the developing world since the use of toxic chlorine compounds is being prohibited for protection of environment from hazardous pollutants. The search for high activity microbial xylanases free of associated cellulase activity has become an important area of development and in this regard, National Chemical Laboratory (NCL), Pune has been among the earliest to discover *Chainia*, a sclerotial actinomycete secreting high levels of cellulase free xylanase (Srinivasan *et al, 1984).* Several additional microbial sources of cellulase free

xylanase with different pH and temperature optima have been identified at NCL, Pune (Balakrishnan *et al.*, 1992; Keskar, 1992; Dey *et al.*, 1992; Bansod *et al.*, 1993) and in several other laboratories abroad (Roberts *et al.*, 1990; Grabski and Jeffries, 1991; Olama, 1998). The subject of cellulase-free xylanases and their application to paper biotechnology has been comprehensively reviewed (Vikari *et al.*, 1994; Srinivasan and Rele, 1995).

Species of *Bacillus* have received extensive attention for their high growth rates and capability for producing a variety of extracellular xylanases. A comprehensive statement of the earlier literature on xylanase production by neutrophilic sp. as well as alkalophilic *Bacillus* species has been in presented in Table 1.2.

Table 1.2: Xylanase production from neutrophilic and alkalophilic bacteria

Organism	C-Source	Temp. (°C)	pH	Incubation (h)	Activity (IU/ml)	References
Bacillus circulans WL-12	Xylan	30	6.5	70	121.6	Esteban *et al.*, 1982
Bacillus coagulans	Xylan	37	6.0	70	40.2	Esteban *et al.*, 1983
Bacillus stereothermophilus	Xylose	60	7.0	18	1.45	Khassin *et al.*, 1993
Bacillus sp.	Rice husk	38	7.2	42	5.0	Paul and Verma 1990
Bacillus subtilus	-	50	5.0	-	2.0	Bernier *et al.*, 1983
Cellulomonas flavogena	Kaller grass straw	30	7.3	72	16.0	Mohomad *et al.*, 1984
Alkalophiles						
Aeromonas sps.	Xylan	37	10.0	48	1.95	Ohkoshi *et al.*, 1985
Bacillus C-59-2	Wheat bran	37	10.2	72	3.50	Horikoshi and Atsuka

Table 1.2 cont.

Organism	C-Source	Temp. (°C)	pH	Incubation (h)	Activity (IU/ml)	References
Bacillus NCL 87-6-10	Wheat bran	28	10.0	48	150.00	Balakrishnan *et al.*,1992
Bacillus sp. DSNC 101	Xylan	40	8.0	-	305.00	Cho, 1997
Bacillus sp. 41-M	Xylan	27	10.0	120	20.00	Nakamura *et al.*, 1992
Bacillus sp.	*Xylan*	27	10.5	48	10.00	Nakamura *et al.*, 1994
Bacillus YL 335	Xylan	37	10.0	-	1.20	Park *et al.*, 1992
Bacillus sp. W	Xylan	45	10.0	48	112.00	Okazaki *et al.*, 1984
AT Bacillus sp.	*Bagasse*	60	9.0	24	57.00	Rajaram and Verma 1990
AT Bacillus sp. NCIM 59	*Wheat bran*	48	10.0	48	50.00	Dey et al., 1992
Bacillus circulans	Xylan	-	8.0	48	400.00	Ratoo *et al.*, 1992

- denotes information not available
- AT - Alkalophilic thermophilic

PRESENT INVESTIGATION

The present investigation is based on identifying a *Bacillus* strain isolated from soil sample collected from Pune in the course of our extensive ongoing programme on screening of microbial cultures for xylanases with novel properties. A *Bacillus* strain secreting very high extracellular xylanase activity in submerged culture has led to the present investigation. It was found to grow and produce xylanase on inexpensive media containing commercial grade raw materials. The strain was fast growing over a wide range of pH and temperature. The present investigation has focussed on the following aspects.

(a) Isolation and characteristization of the organism.

(b) Its growth behaviour in pure culture

(c) Methods for long term conservation to ensure strain stability and xylanase productivity

(d) Fermentation variables in submerged culture for maximizing enzyme production.

(e) Preliminary enzyme characterization with regard to pH and temperature optima, stability and identification of end products of enzymatic hydrolysis of xylan.

CHAPTER II

MATERIALS AND METHODS

Materials

Pure or analytical grade chemicals were used in all the experiments including media preparation for growth and enzyme production. Inorganic salts used were either from BDH (AR) or Sara-Merck (GR). Peptone, yeast extract, malt extract, beef extract, Tween-40, Tween-80 and Triton X-100 were supplied by Hi-Media, India. 3,5-dinitrosalicylic acid (DNSA), oatspelts xylan and carboxymethyl cellulose (CMC) used were obtained from Sigma Chemical Co., USA. Filter paper used for paper chromatography was Whatman No. 1 grade from Whatman Ltd., England. Hammerstein casein was purchased from Sisco Research Laboratories, Mumbai, India. Wheat bran used was purchased locally.

Isolation of the culture

Soil was collected from the local area of Pune. After appropriate dilutions it was plated on a nutrient medium containing oatspelts xylan. The colony having the largest xylan clearance zone was selected for detailed investigation.

The *Bacillus* sp. was maintained by periodic subculture on the following media (Table 2.1). The slants were incubated for 7-15 days at 28°C and transferred to cold.

Table 2.1 : Composition of media used for maintanance

Constituent	MGYP	MYP	MXYP	NA
	g/l			
Malt extract	3	3	3	-
Yeast extract	3	3	3	2
Beef extract	-	-	-	1
Peptone	5	5	5	5
Glucose	10	-	-	-
Xylan	-	-	10	-
Sodium chloride	-	-	-	5
Agar	20	20	20	20

Identification

Characterization and identification of the organism was carried out according to Bergy's Manual of Determinative Bacteriology (1974).

Fermentation

All the glassware used for fermentation experiments were washed, dried, plugged with cotton and sterilized at 121°C (15 lbs pressure) for I h. Culture media containing wheat bran were sterilized for 40 minutes at 15 lbs pressure. Fermentations were generally carried out in 250 ml. Erlenmeyer flasks containing 50 ml of medium. Flasks were incubated at 28°C on a rotary shaker

at 220 revolutions per minute (rpm). Fermentations were carried for 72 h unless otherwise mentioned.

All the buffers and reagents were prepared in double distilled water.

Substrate Preparation

Two grams of oatspelt xylan were suspended in 100 ml of 50 mM phosphate buffer of required pH and stirred for 16 h at 5 -10°C. The insoluble material was removed by centrifugation (9226 g, 20 min) and soluble fraction (approximately 0.9 - 1%) was used as the substrate.

Enzyme assays

Xylanase : The total reaction mixture of I ml contained 0.5 ml of suitably diluted enzyme in phosphate buffer (50 mM pH 7.0 unless otherwise mentioned) and 0.5 ml of xylan solution. The reaction mixture was incubated at 50°C for 30 min followed by the addition of I ml DNSA to terminate the reaction. The resultant reaction mixture was kept in a boiling water bath for 5 min and the colour intensity was read at 540 nm after dilution with 10 ml of distilled water (Bernfeld, 1955). One unit of xylanase activity is defined as the amount of enzyme that produced one μmole of xylose equivalent/min under the assay conditions.

CMCase (carboxy methyl cellulase) : An aliquot of 0.5 ml of suitably diluted enzyme solution was mixed with 0.5 ml of l% CMC in 50 mM phosphate buffer, pH 7.0 and incubated at 50°C for 30 min. The reducing sugar liberated was measured as glucose equivalent. One unit of CMCase activity is defined as the amount of enzyme that produced one μmole of glucose equivalent/min under the assay conditions.

Protease: Extracellular protease activity was determined according to Kunitz (1947). The reaction mixture contained an aliquot of suitably diluted enzyme solution and hammerstein type casein (10 mg) in 100 mM potassium phosphate buffer, pH 7.0, in a total volume of 2 ml. After incubation at 40°C for 10 min; the reaction was terminated by the addition of 5% trichloroacetic acid. The precipitate formed was filtered through Whatman No. 1 filter paper after standing for 30 min at room temperature. The absorbance of trichloroacetic acid soluble fraction was measured at 280 nm. One unit of protease activity was defined as the amount of enzyme required to bring about an increase in absorbance of 1.0 unit per ml of reaction mixture per min under the assay conditions.

Amylase : Amylase activity was determined according to Bernfeld (1955). The reducing sugars liberated following the hydrolysis of 1% (w/v) soluble starch at pH 7.0 and 50°C were

estimated by DNSA method. One unit was defined as the amount of enzyme required to liberate I μmol of reducing sugar/min under the assay conditions.

Hydrolysis of xylan

50 mg of oatspelts xylan was hydrolysed with xylanase by incubating at 50°C, pH 7.0 for different intervals of time. The reducing sugar liberated was estimated by DNSA and percentage hydrolysis was calculated. For end product analysis the hydrolysate was concentrated and used for spotting on paper chromatogram.

Paper chromatography for end product analysis

To identify the products of hydrolysis of oatspelts xylan, descending chromatography was performed by using the solvent system of butanol:acetic acid:water (3:1:1) for 18 h. A mixture of xylo-oligosaccharides were spotted as standard and run along with the samples. The paper was removed and dried at 100°C for 30 min and it was sprayed with mixture of pthalic acid (0.332 g) and p-anisidine (0.246 g) in 20 ml of absolute ethanol as described by Gunther and Sherma (1972).

CHAPTER III

ISOLATION AND FERMENTATION STUDIES

SECTION I

ISOLATION AND CHARACTERIZATION OF THE ORGANISM

In a screening programme undertaken to isolate microbial cultures capable of xylanase production, natural materials such as soil and decomposing plant detritus were plated out after appropriate dilutions on nutrient agar supplemented with 1% oat spelt xylan (pH 7 to 7.5, temperature 28 – 30^{o}C). Colonies exhibiting clearance zones around them were selected and transferred individually on slants of the same composition and similarly incubated. After preliminary comparison of the various isolates for the extent of xylan clearance, a gram positive bacterial strain designated NCL 90-10-50 was taken up for detailed investigation (Fig. 3.1). Production of extracellular xylanase was confirmed in shake flask by growing the culture on a medium containing xylan or wheat bran and yeast extract. Details of the morphological and physiological studies of the strain as well as cultural conditions to optimize xylanase secretion and some preliminary characterisation of xylanase enzyme are presented in this dissertation.

Characteristics of the bacterial isolate (NCL 90-10-50)

Growing colonies after 72 hours appear flat with a dry reticulate surface which on further incubation tend to become

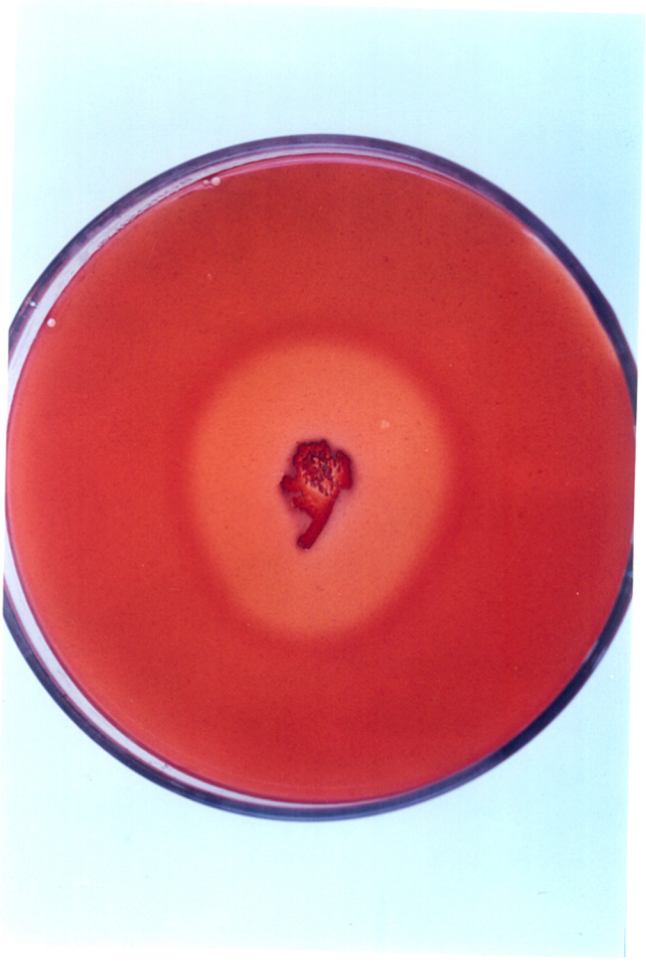


FIG. 3.1 : XYLAN CLEARANCE ZONE BY BACILLUS NCL 90-10-50

mucoid due to development of endospores. The colony is somewhat off-white in colour. The culture was routinely maintained through serial subcultures on MGYP agar every three months and refrigerated after incubation at 28°C for 7-10 days. Vegetative cells were rod shaped, motile and gram positive and differentiated median endospores on the aforesaid incubation temperature after 5-7 days. (Fig. 3.2). The physiological and biochemical properties of the organism were studied. The organism was found to be catalase positive and Vogues Prosker positive, it was able to produce acid from glucose, xylose and mannitol (no gas production). Citrate was utilized by the *Bacillus* sp. and casein was hydrolysed (Fig. 3.2a). The organism could not hydrolyse the starch and it was not able to reduce nitrate to nitrite. According to Bergy's Manual and Gorden *et al.* (1973) key for identification of *Bacillus* the organism appears to be referable to *Bacillus pumilus*.

PHYSIOLOGICAL PARAMETERS FOR GROWTH

Effect of pH

In order to study the optimum pH for growth *Bacillus* sp. was grown in MGYP shake cultures at initial pH values ranging from 5.0-8.0. 24 h grown inoculum (10% v/v) was used to inoculate the experimental flasks. Optical density after (10-fold dilution) of 24 h grown broth was measured at 600 nm. As seen

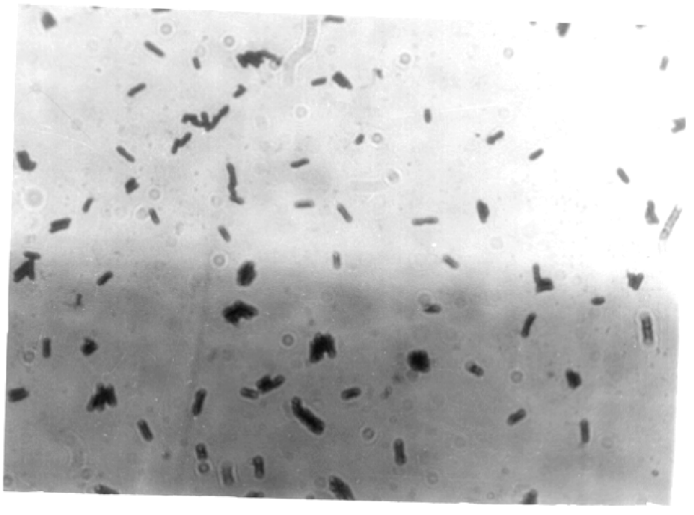
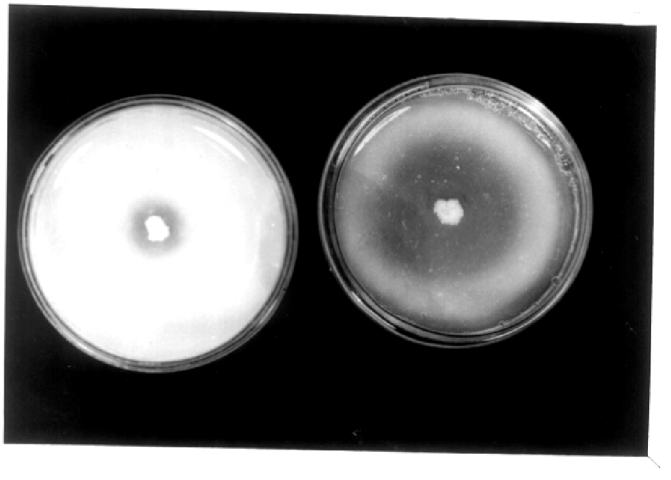


FIG. 3.2: MICROSCOPIC PICTURE OF BACILLUS sp. SHOWING ROD SHAPE



24 h 96 h

FIG. 3.2a: ZONE OF HYDROLYSIS OF CASEIN BY BACILLUS sp.

Bacillus sp. grown on casein agar (Bergy's manual, 1974) for 24 h and 72 h and flooded with TCA to visualize the clear zone of casein hydrolysis.

in (Fig. 3.3) the *Bacillus* sp. was able to grow over a wide pH range of 5-8 with an optimum at 7.0.

Effect of Temperature

When incubated at temperatures ranging from 15-50°C the bacterium showed comparable growth over a wide temperature range of 28-50°C (Fig. 3.4). However, the growth at 15°C was about 60-65% of that at 28°C which is the optimum temperature for growth.

Effect of Sugars and Sugar Alcohols on Growth

Broth cultures were grown in shake flasks with different carbon sources and 1% yeast extract as nitrogen source. Optical density was measured at 600 nm after 24 h and 48 h of growth. The results presented in Table 3.1 indicate that rapid growth occurred in all the sugars and utilization of glycerol was poor. Decline in optical density was observed after 48 hours.

Table 3.1: Effect of sugars and sugar alcohols

Sugars and sugar alcohol (1.0%)	OD at 660 nm	
	24 h	48 h
Glucose	0.550	0.760
Xylose	0.347	1.400
Sucrose	0.737	0.906
Lactose	0.352	0.665
Glycerol	0.185	0.217

Inoculum was developed on the same medium containing respective sugars for 24 h

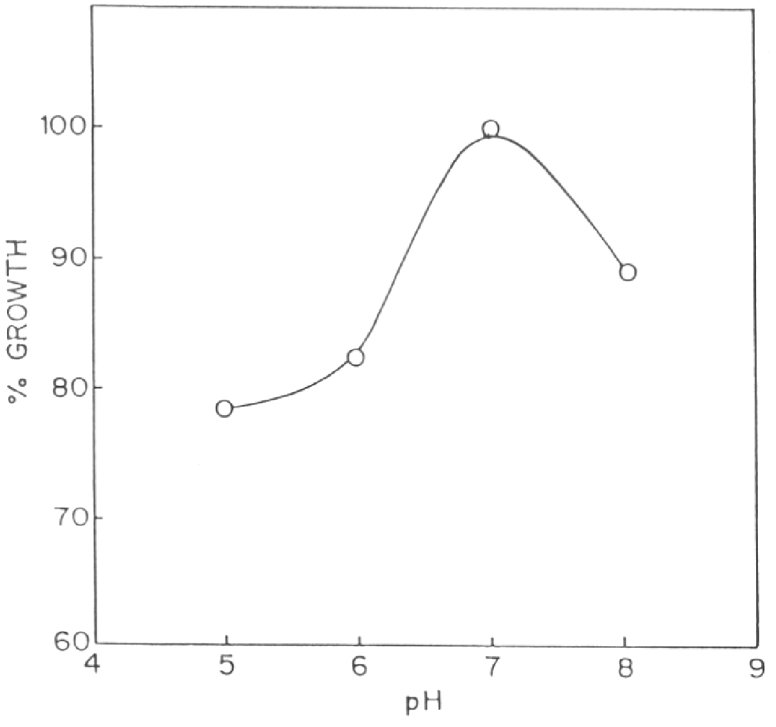


FIG. 3.3: EFFECT OF INITIAL pH ON GROWTH

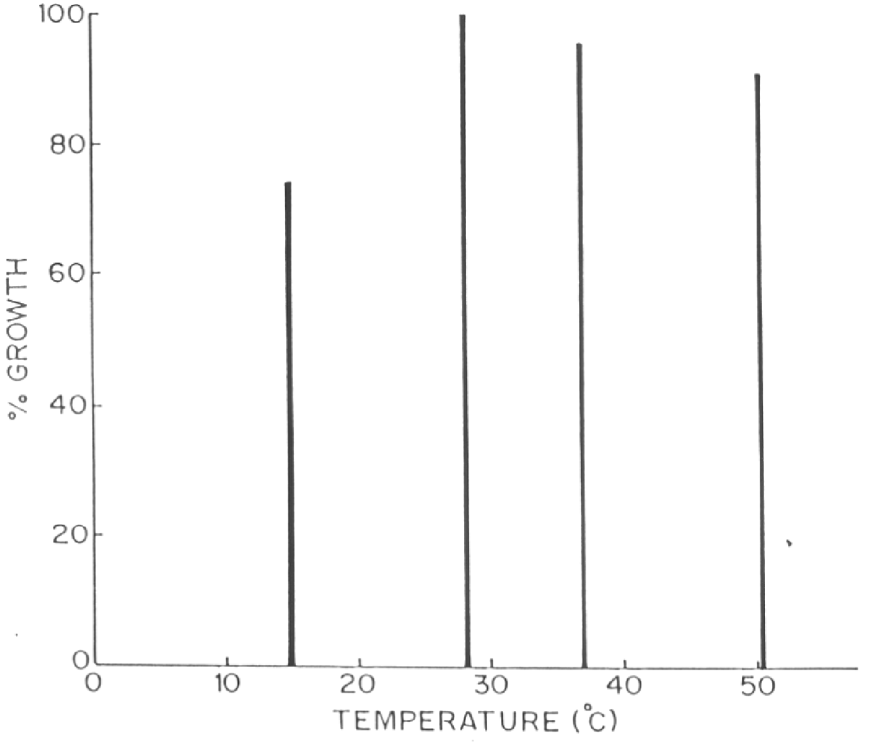


FIG. 3.4: EFFECT OF TEMPERATURE ON GROWTH

Effect of medium used for maintenance

In order to make a comparative evaluation of the best medium for long term conservation of the stock cultures, with reference to enzyme productivity, the following additional media were used and xylanase production was studied after 12 months storage at 4°C in a sporulated condition.

Stock cultures maintained on different media were subcultured on MGYP slants. Seven days old slants were used for developing the inoculum. It was observed that enzyme was produced in wheat bran medium only after two serial subcultures on MGYP slants. The results in Table 3.2 indicate that the strain gave highest xylanase yield when grown and conserved on MGYP agar hence it was routinely maintained on MGYP agar slants.

Table 3.2: Effect of medium for maintainance

Medium	Activity IU/ml
NA	277
MXYP	198
MYP	163
MGYP	300

Inoculum was grown on MGYP for 24 h
Production medium contained 3% wheat bran and 1% yeast extract
Xylanase production was tested after 72 hours

Effect of period of preservation on xylanase production

The *Bacillus* sp. was maintained on MGYP slants and preserved at 4°C for different periods of time. Revival rate and biochemical performance of the organism was tested. Even after preservation for 6 years at 4°C the organism was able to revive within 24 h. Xylanase production was investigated only after two serial subcultures as it was found in our earlier studies. It was found that over different periods of storage on slants the activities were comparable, indicating good genetic stability of the strain. The comparative data is presented in Table 3.3.

Table 3.3: Effect of period of preservation on xylanase production

Time for preservation (Years)	Activity (IU/ml) 72 h
1	341
2	325
3	315
5	311
6	323

The fermentation medium contained 3% wheat bran, 1% yeast extract and 0.1% Tween 80

SECTION II

ENZYME PRODUCTION

In this section results of shake flask studies to optimize the fermentation conditions for xylanase production by the *Bacillus* sp. (NCL 90-10-50) are discussed. Results presented are mean of three independent experiments based on duplicate shake flasks. Stock culture grown on MGYP slant for seven days was used for initiating the studies and developing the inoculum. 10% (v/v) inoculum grown in MGYP liquid for 24 hours was used unless otherwise mentioned. Enzyme production was studied in 250 ml Erlenmeyer flasks containing 50 ml medium adjusted to pH 7.0 and incubated at 28°C on a rotary shaker (220 rpm).

Since the *Bacillus* sp. exhibited a large clearance zone of hydrolysis on xylan agar plate, shake flask studies with xylan and wheat bran as substrate were performed. High yields of xylanase were produced in both wheat bran and xylan medium in 72 h fermentation batches (upto 300 IU/ml). Slight variation in activity and productivity was observed with different commercial batches of wheat bran used in these experiments.

Effect of xylan concentration

Effect of xylan concentration on enzyme production was studied. As seen from the Table 3.4, the enzyme activity did not show appreciable difference when the xylan concentration in fermentation media varied from 0.5% to 2%.

Table 3.4: Effect of xylan concentration on xylanase production

Xylan (%)	Activity (IU/ml)
0.5	253
1.0	295
1.5	277
2.0	281

1% yeast extract was used as nitrogen source
Activity was estimated after 72 h

Optimization of wheat bran concentration

Xylan is expensive and is not a practical substrate for large scale enzyme production. Hence wheat bran which is rich in xylan, readily available and an inexpensive commercial raw material was used as the substrate of choice for all subsequent experiments. Different concentrations of commercial wheat bran were tested in medium containing 1% yeast extract. Xylanase activity comparable to activities obtained with pure xylan was obtained using 3% wheat bran and hence in all further experiments 3% wheat bran was used. Fig. 3.5 gives the data

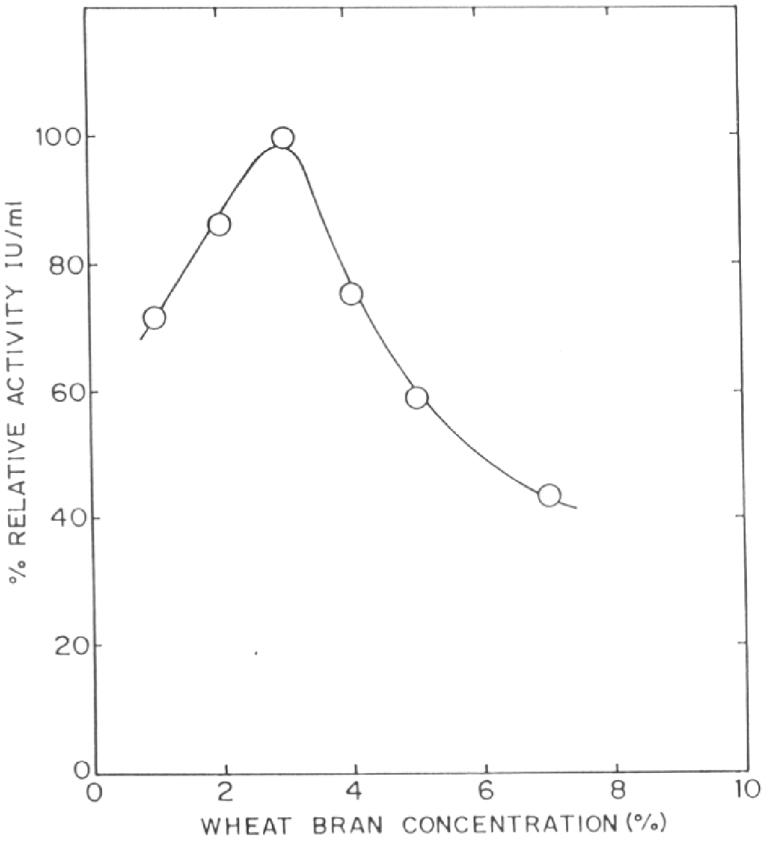


FIG. 3.5: EFFECT OF WHEAT BRAN CONCENTRATION ON XYLANASE PRODUCTION

obtained on xylanase production in relation to wheat bran concentration.

Effect of different fraction of wheat bran on xylanase production

To understand which fraction of wheat bran (soluble component versus insoluble residue) is most favourable for xylanase production, 3 g of wheat bran was suspended in 30 ml water and autoclaved for 15 lb 20 min followed by filteration and washing. Filtrate and washings were pooled and made volume upto 100 ml and I g of yeast extract and 0.1 ml of Tween-80 were added. For comparision 3% wheat bran residue as well as residue plus 0.5% starch were used in parallel experimental flasks. The results are presented in Table 3.5. Wheat bran extract as well as residue with or without starch supplementation gave less activity than natural wheat bran.

Table 3.5 : Effect of different fractions of wheat bran on xylanase production

Carbon Source	Xylanase (IU/ml)
Wheat bran	334.4
Wheat bran extract	223.3
Wheat bran residue	260.0
Wheat bran residue +starch	284.6

Activity was estimated after 72 h

In both xylan and wheat bran media adjusted to initial pH of 7.0, xylanase secretion was accompanied by a pH rise upto 8.5 within 72 h.

Effect of yeast extract concentration

As yeast extract was used as the nitrogen source for xylanase production effect of yeast extract concentration on xylanase production was studied in media containing 3% wheat bran as substrate. Concentrations above 1% did not significantly increase xylanase production and therefore 1% yeast extract concentration appeared to be optimal for enzyme production (Fig. 3.6).

Effect of different temperatures

As the *Bacillus* sp. was found to have wide temperature range for growth, xylanase production was investigated at temperature between 28 – 50°C. Though the growth was comparable at all the temperatures, xylanase production was optimum at 28°C, showing poor production at 50°C (Table 3.6).

Table 3.6: Effect of different temperatures on xylanase production

Temperature (°C)	Activity (IU/ml)
28	280.8
37	160.3
50	1.3

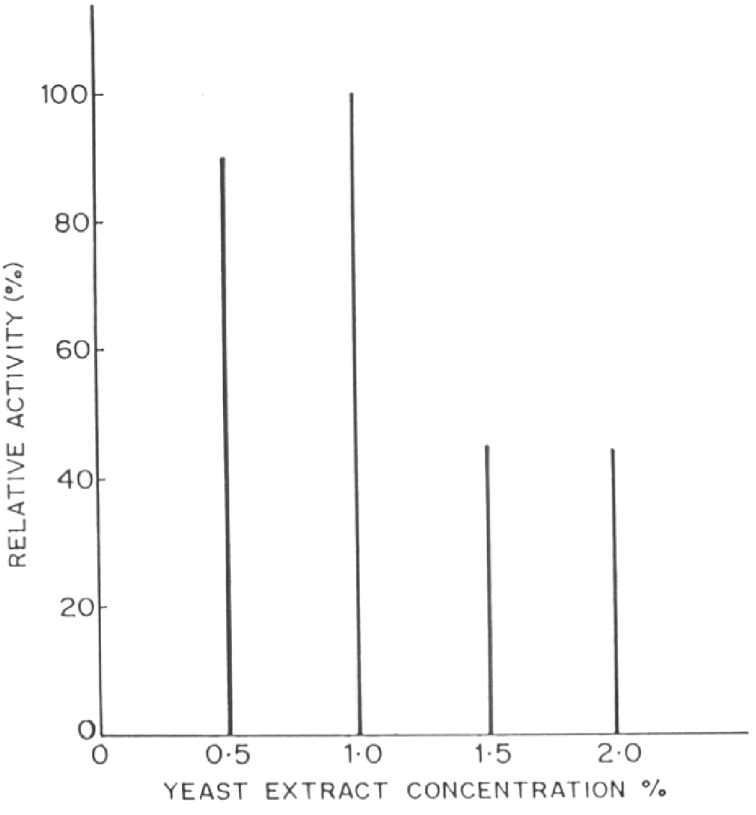


FIG. 3.6: EFFECT OF YEAST EXTRACT CONCENTRATION ON XYLANASE PRODUCTION

Effect of inoculum size

24 h old MGYP broth was used for studying the effect of inoculum size (5-20%) in wheat bran (3%) and yeast extract (1%) medium. 5-10% inoculum was found to be optimum and higher inoculum concentrations were not beneficial for enhancing xylanase production.

During the course of investigation it was found that *Bacillus* sp. was able to utilize both inorganic and organic nitrogen sources for growth. The effect of various inorganic nitrogen sources (nitrates and ammonium salts) as well as organic nitrogen sources in the form of complex substrates such as peptone and tryptone were studied for comparative xylanase production.

Effect of different inorganic nitrogen sources

Effect of various inorganic nitrogen sources on production was investigated in medium containing 3% wheat bran as carbon source. Yeast extract at 1% concentration was used as control. Xylanase activity was highest in media containing yeast extract, however activity comparable to yeast extract were obtained in media containing either ammonium salts or nitrates. Urea however showed poor enzyme activity (Table 3.7).

Table 3.7: Effect of different inorganic nitrogen sources on xylanase production

Nitrogen source* (%)	Relative activity (%)
Yeast extract (1.0)	100
Ammonium sulphate (0.40)	91
Diammonium hydrogen phosphate (0.40)	89
Sodium nitrate (0.50)	86
Urea (0.18)	31

*Nitrogen used was equivalent to 1% yeast extract
Adjusted the pH of medium before autoclaving to 6.5 – 7.0

Effect of organic nitrogen sources

Organic nitrogen sources such as peptone, tryptone and casein hydrolysate were used as alternative to yeast extract in medium containing 3% wheat bran as carbon source, 1% yeast extract was however found to be best followed by peptone and tryptone (Table 3.8).

Table 3.8 : Effect of different organic nitrogen sources on xylanase production

Nitrogen source* (%)	Relative activity (%)
Yeast extract (1.0)	100
Casein hydrolysate (0.84)	69
Peptone (0.52)	73
Tryptone (0.64)	70

*Nitrogen source was added equivalent as in 1% yeast extract
Adjust the pH of medium before autoclaving to 6.5 – 7.0

Effect of various surfactants

As reported in the literature surface active agents increase the secretion of enzymes. Hence different surface active agents were tested at 0.1% concentration for their influence on xylanase production by the *Bacillus* sp. Tween 80 enhanced while Triton X-100 markedly inhibited growth as well as xylanase production (Table 3.9).

Table 3.9: Effect of various surfactants on xylanase production

Surfactant (0.1%)	Relative activity (%)
Control	100.0
Tween 40	91.6
Tween 80	125.7
Triton X 100	11.7

Fermentation Profile

The optimized medium containing wheat bran (3%), yeast extract (1%) and 0.1% (v/v) Tween 80 was used to monitor the xylanase production by the *Bacillus* sp. Fig. 3.7 shows that the highest activity was attained at 72 h.

Effect of different sugars and sugar alcohols on xylanase production

As the *Bacillus* sp. was able to grow well on a variety of sugars and sugar alcohols, constitutive xylanase production was investigated in the absence of xylan inducer. Sugars and sugar

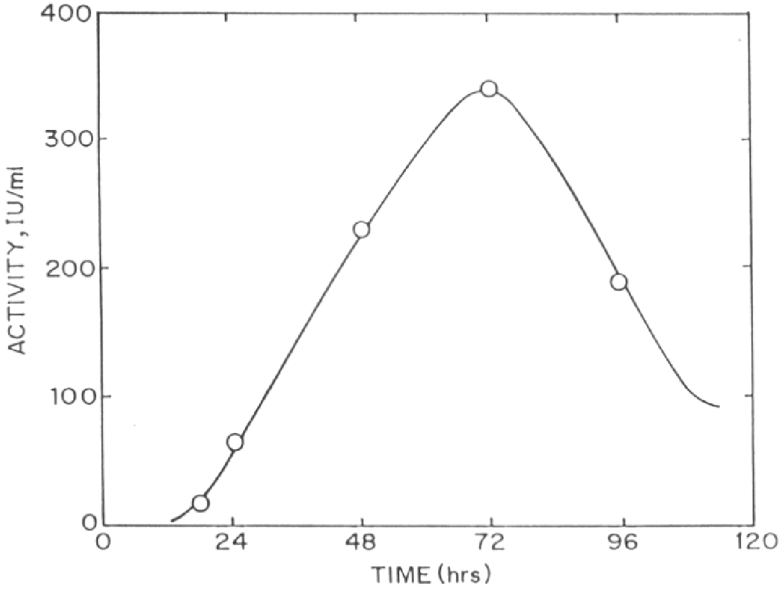


FIG. 3.7: FERMENTATION PROFILE

alcohols were used at 1% concentration in medium containing 1% yeast extract and 0.1% Tween 80. Samples were removed aseptically at periodic intervals and xylanase activity was estimated. Results are presented in Table 3.10. Low constitutive xylanase activity was only found in xylose medium, while in other sugars activity was absent or negligible.

Table 3.10: Effect of different sugars and sugar alcohols on xylanase production

Sugar/sugar alcohol	Xylanase IU/ml	
	24 h	48 h
Glucose	NA*	0.73
Xylose	11.0	20.3
Sucrose	0.71	0.57
Lactose	0.41	0.65
Glycerol	NA*	NA*

* No activity

Effect of Xylose Repression

Effect of repression of xylanase by xylose was studied in medium containing xylan as an inducer. Basal medium contained 0.5% xylan, 1% yeast extract and 0.1% Tween 80. Xylose was separately autoclaved (10 lb, 10 min) and added aseptically before inoculation. 1 ml bacterial spore suspension in sterile water was used to inoculate the fermentation medium. As shown in Table 3.11 the repressive effect of xylose increased with increasing xylose concentrations.

Table 3.11: Effect of xylose repression

Xylose concentration (%)	Xylanase IU/ml	Repression (%)
Control	114.4	0
0.05	75.7	33.83
0.1	63.1	44.84
0.2	45.6	60.14
0.5	35.9	68.68

Xylanase production was tested after 72 h
Control was without xylose

CHAPTER IV

PROPERTIES OF XYLANASE

BIOCHEMICAL PROPERTIES

Biochemical properties of the crude xylanase enzyme was studied with the enzyme prepared as described in Chapter III.

Activities of crude culture filtrate towards different substrates

Various substrates like casein, carboxymethyl cellulose, starch were checked for the activity of the culture filtrate. It was observed that cellulase and amylase activites were negligible and 1.0 KU/ml of protease activity was detected in the culture filtrate.

Optimum pH

The xylanase activity was estimated at 50°C for 30 min and at different pH values ranging from 3 to 11. It was observed that the highest activity was at pH 7.0. The activities were 70% and 55% at pH 6 and 8, respectively and were considerably lower above pH 8.5 (Fig. 4.1).

Optimum temperature

Xylanase was assayed at pH 7.0 (with 50 mM phosphate buffer) and temperatures ranging from 30 to 70°C (Fig. 4.2). Maximum activity was obtained at 50°C with 75% activity at 40°C and 50% activity at 60°C. The logarithm of velocity was plotted versus the reciprocal of absolute temperature (Fig. 4.3). The energy of activation was calculated to be 15.98×10^3 cals/mol.

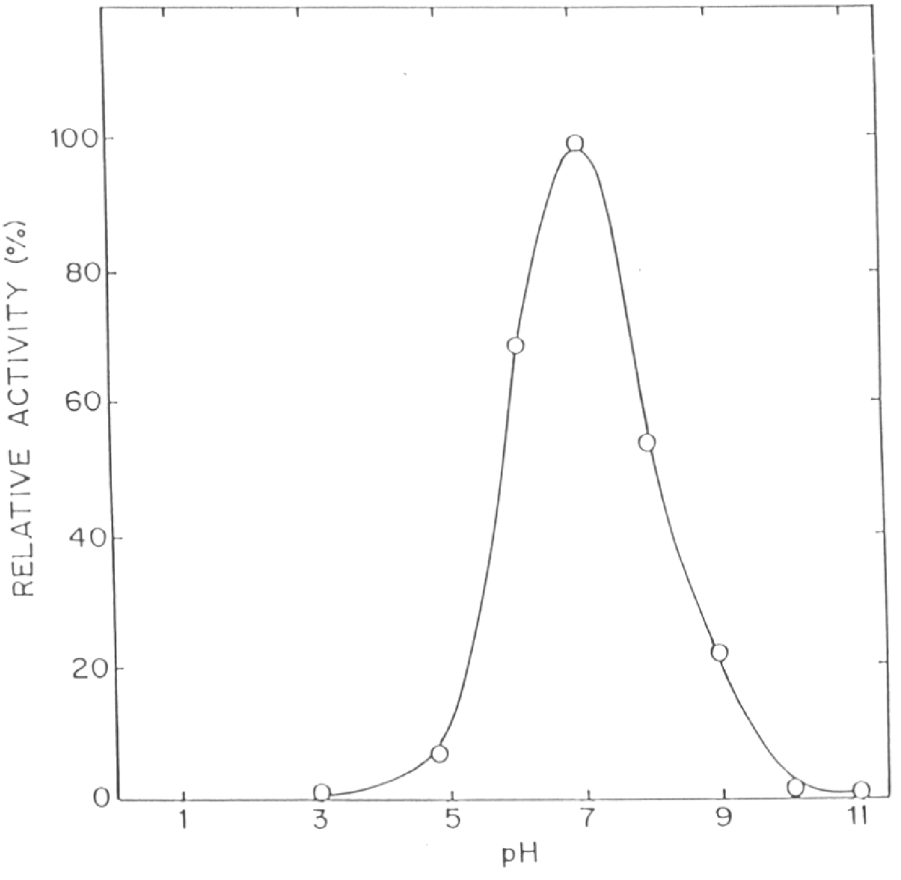


FIG. 4.1: EFFECT OF pH ON XYLANASE ACTIVITY

Buffers used were 50 mM, over the range of (3 to 11) citrate (pH 3.0 to 4.0) acetate (pH 5.0) phosphate pH (6.0 to 8.0) and carbonate - bicarbonate (pH 9.0 - 11.0)

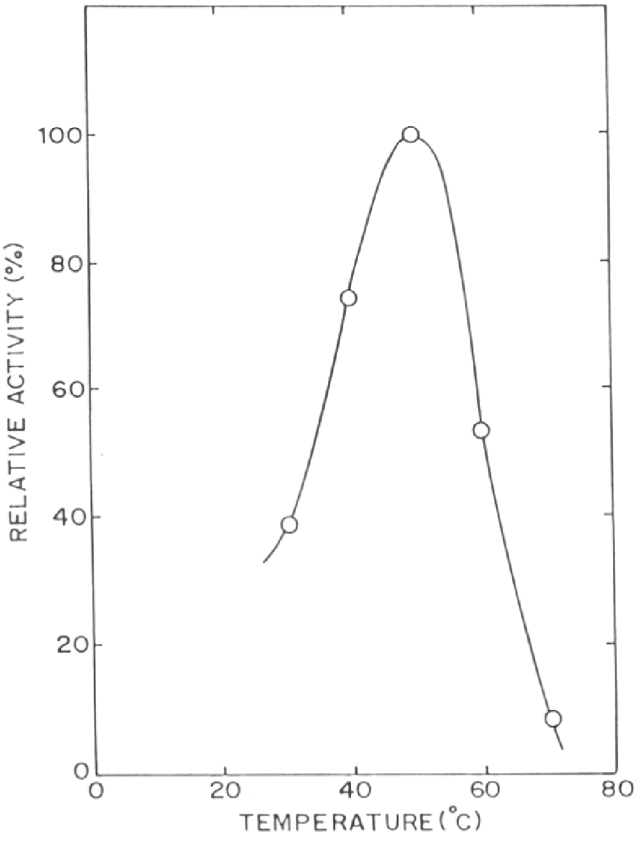


FIG. 4.2: EFFECT OF TEMPERATURE ON XYLANASE ACTIVITY

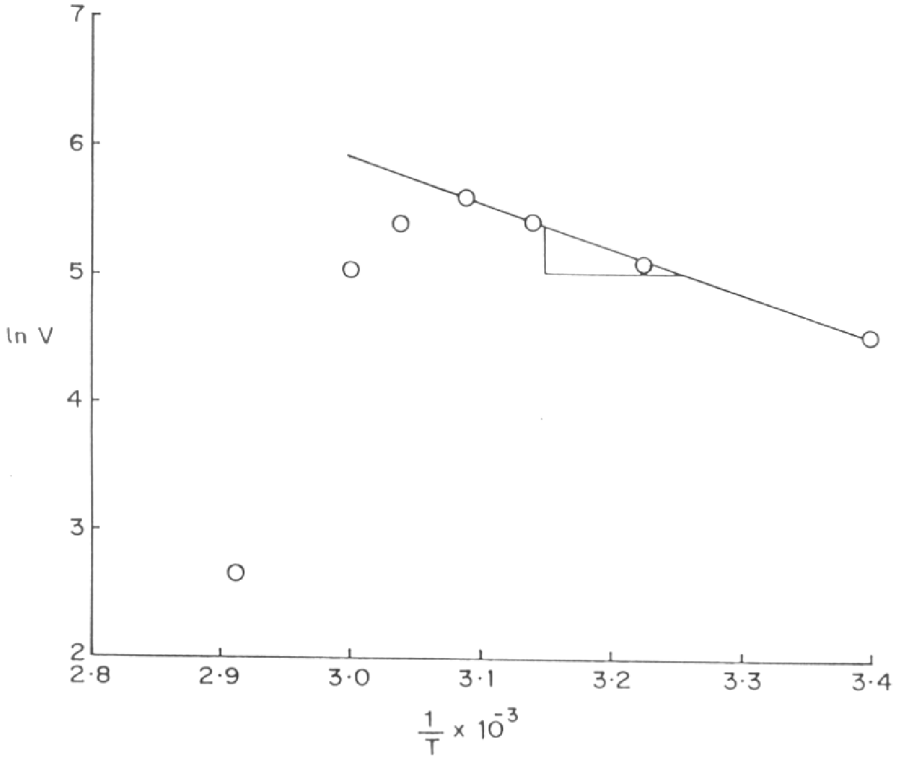


FIG. 4.3: ARRHENIUS PLOT

pH Stability

The culture filtrate was adjusted to different pH values ranging from 6-8 and was incubated at 50°C for I h. pH was readjusted to the original pH (8 – 8.3) and assayed with 50 mM phosphate buffer, pH 7.0. The results are presented in Fig. 4.4. After 90 min around 50% of the enzyme activity was retained at all the pHs tested. This intrinsic instability could be due to the presence of a protease.

Temperature Stability

The enzyme was incubated at temperatures ranging from 40 – 70°C for 30 min. The residual xylanase activity was tested at 50°C, pH 7.0. The residual activities after incubation at 40, 50 and 60°C were 85, 80 and 60% respectively (Fig. 4.5).

Stability of xylanase at a fixed temperature of 50°C was also studied for different time intervals upto 8 hours. After 4 hours at 50°C, 50% of the activity was detected. The results are shown in Fig. 4.6.

Effect of metals on xylanase

To study the effect of various metal ions on xylanase activity, metal ions at 1.0 mM concentration were added in the reaction mixture. With Ba^{+2} and Ca^{+2}, marginal increase in xylanase activity was observed, Cu^{+2} Pb^{+2} and Hg^{+2} completely

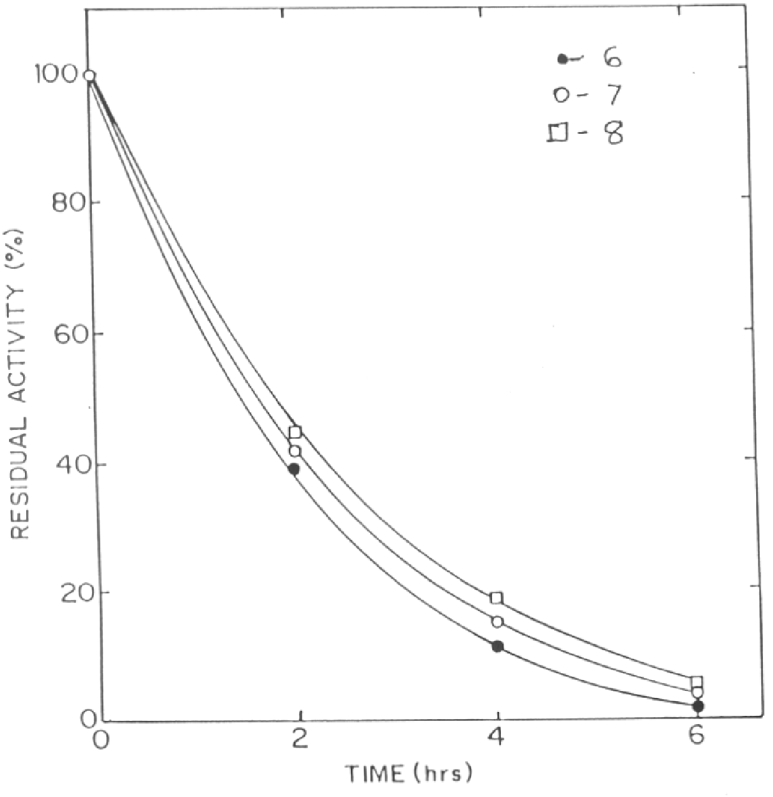


FIG. 4.4: EFFECT OF pH ON STABILITY OF XYLANASE

Buffer used was phosphate buffer (50 mM)

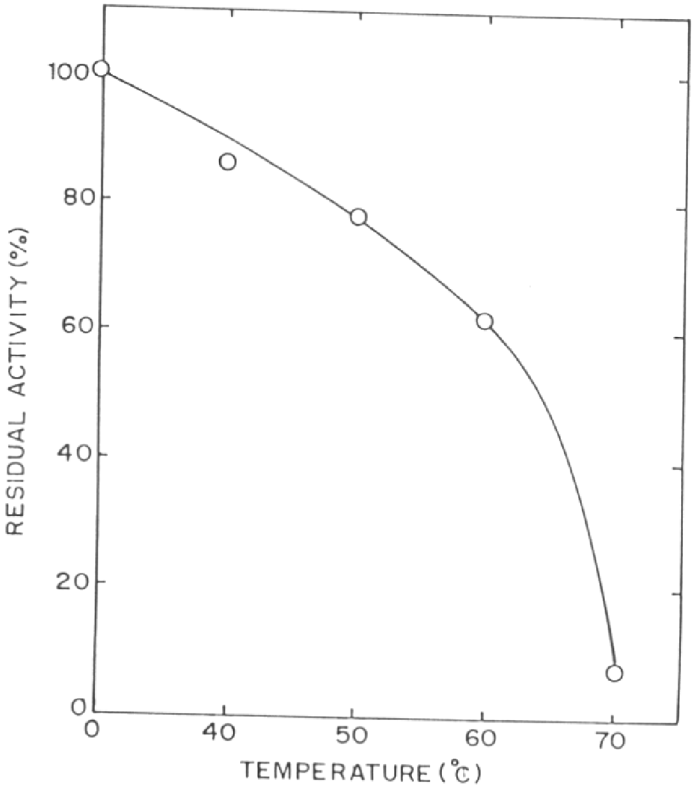


FIG. 4.5: EFFECT OF TEMPERATURE ON STABILITY OF XYLANASE AT FIXED TIME (30 min)

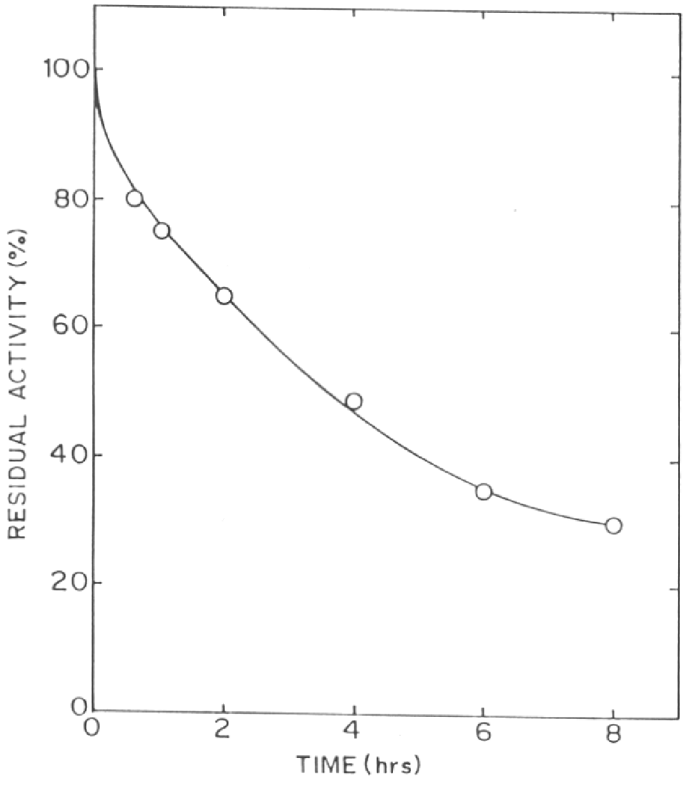


FIG. 4.6: EFFECT OF TEMPERATURE ON STABILITY OF XYLANASE AT FIXED TEMPERATURE (50°C)

inhibited the activity while Co^{+2} and Cr^{+2} showed approximately 12 and 70% inhibition (Table 4.1).

Table 4.1: Effect of different metals on xylanase activity

Metal (1.0 mM)	Relative activity (%)
Control (None)	100.0
Ba^{+2}	127.5
Ca^{+2}	124.9
Co^{+2}	88.1
Cr^{+2}	29.4
Cu^{+2}	ND
Pb^{+2}	ND
Hg^{+2}	ND

*ND – Activity not detected

Determination of Km

Km was determined with both soluble and total xylan. A suitably diluted culture filtrate was incubated with solid oatspelts xylan (1-10 mg) at pH 7.0 and 50°C. Soluble xylan was prepared as described in Materials and Methods. A suitably diluted culture filtrate was incubated under the same conditions as mentioned above. Km and Vmax were determined from Lineweaver Burks plots (Figs. 4.7 and 4.8). Km was found to be approximately the same for both soluble xylan (12.5 mg/ml)as well as total xylan (11.1 mg/ml) and the Vmax values were found to be 4.76 mmol/ml/min and 1.66 mmol/ml/min for soluble and total xylan respectively.

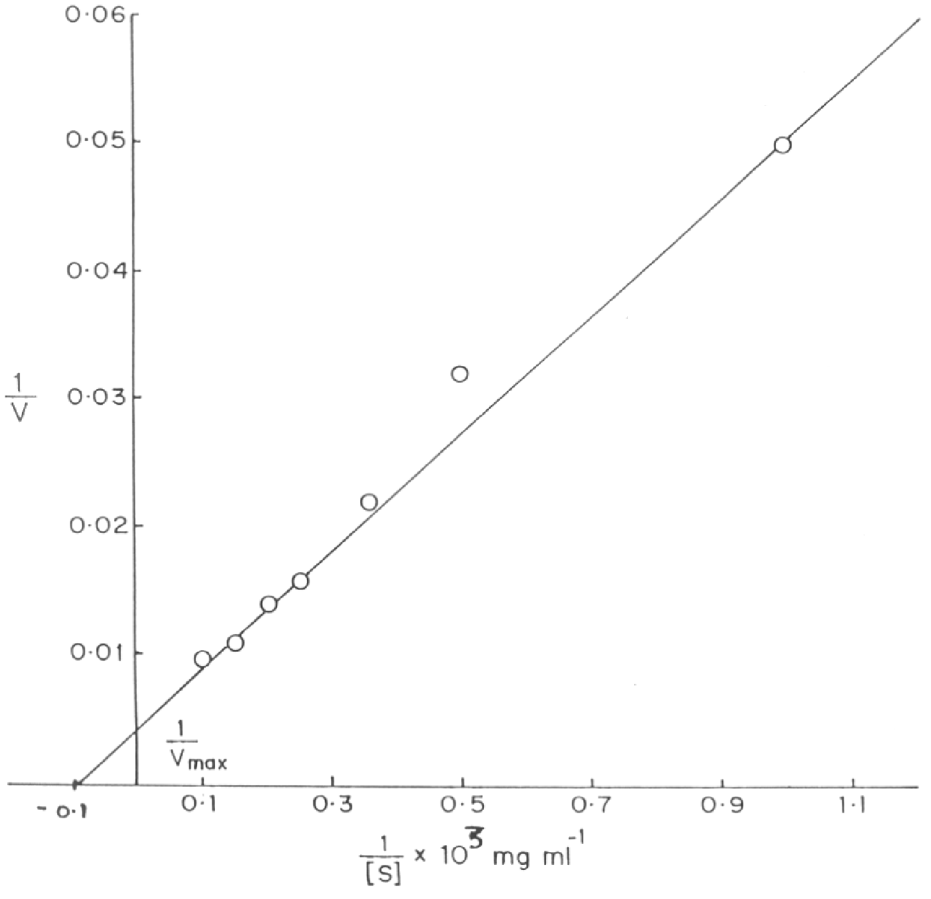


FIG. 4.7: LINEWEAVER-BURK PLOT FOR XYLANASE USING TOTAL XYLAN

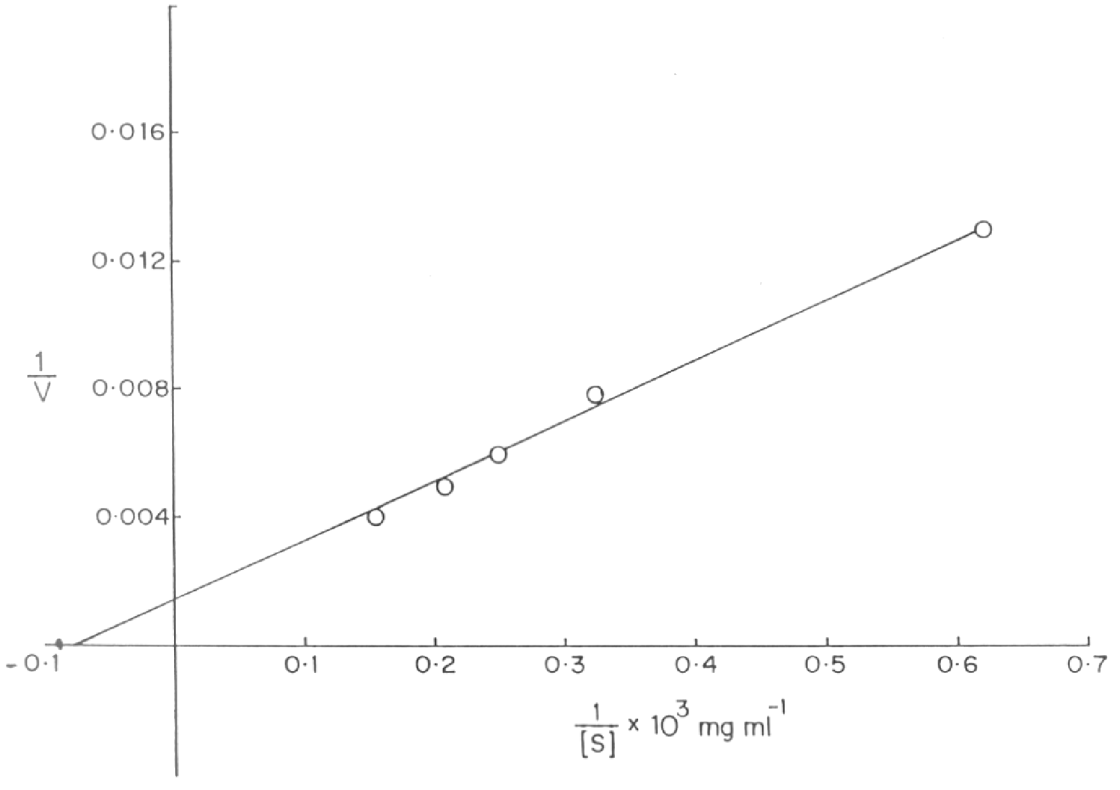


FIG. 4.8: LINEWEAVER-BURK PLOT FOR XYLANASE USING SOLUBLE XYLAN

End product analysis

In order to study the mode of action of the enzyme on oatspelt xylan, 50mg of xylan was incubated with 50 IU of crude broth for various periods of time. The hydrolysed products were analysed by paper chromatography as described in Materials and Methods (Fig. 4.9).

Hydrolysis studies

Hydrolysis of oatspelts xylan was carried out at 50°C and pH. 7.0, xylanase produced on wheat bran (72 h) was used unless otherwise mentioned.

Hydrolysis at 50°C

Hydrolysis was carried out at 50°C (for I to 8 hours), using 32 IU of xylanase per g of xylan. Results are shown in Figure 4.10. A maximum hydrolysis of 15% was obtained in 6-8hrs.

Effect of enzyme concentration on hydrolysis

The effect of enzyme concentration on hydrolysis was studied with different enzyme concentrations varying from 6 IU to 50 IU per gram of xylan for 8 h. Samples were removed at the interval of 2 h upto 8 h. The hydrolysis increased with increase in the enzyme concentration though the increase was not significant beyond 32 IU of xylanase per g of xylan (Fig. 4.11).

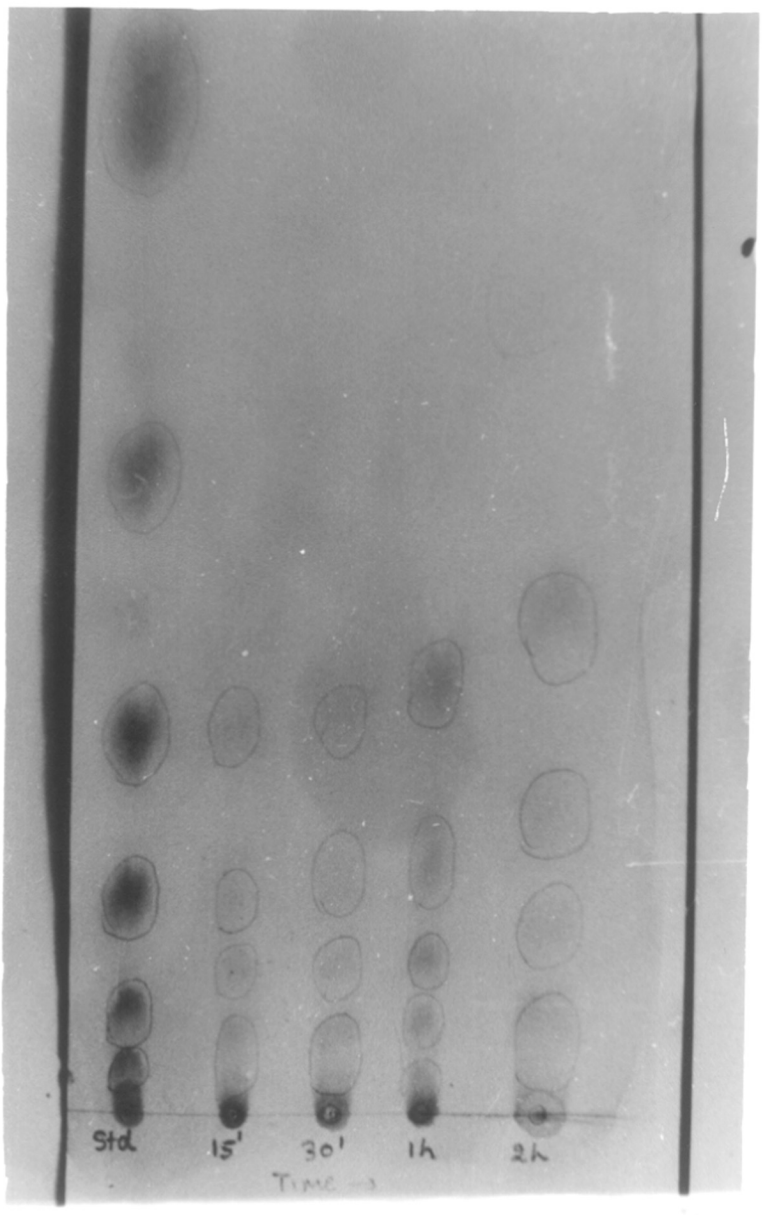


FIG. 4.9: PAPER CHROMATOGRAM OF THE HYDROLYTIC PRODUCTS OF XYLAN

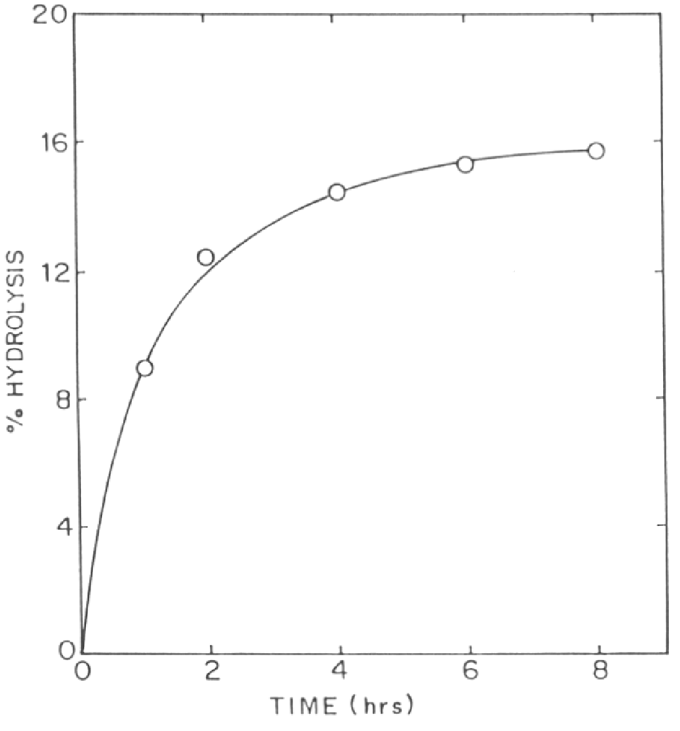


FIG. 4.10: HYDROLYSIS OF OATSPELTS XYLAN AT 50°C

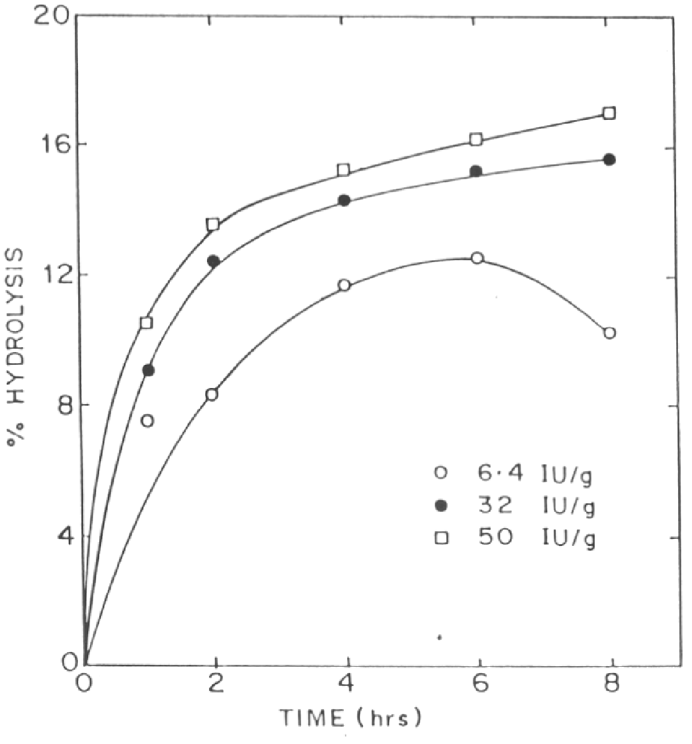


FIG. 4.11: EFFECT OF ENZYME CONCENTRATION ON HYDROLYSIS OF XYLAN

Effect of different enzyme preparations on hydrolysis of xylan

Hydrolysis with the enzyme grown on wheat bran medium, xylan medium and xylose medium was studied at enzyme to substrate ratio of 50 IU/g of xylan. Hydrolysis was carried out from 4 h to 48 h. No singificant difference in the hydrolysis pattern was observed with the differently grown enzymes (Table 4.2).

Table 4.2: Effect of medium of production of xylanase on xylan hydrolysis

Time	Enzyme produced on		
(h)	Wheat bran	Xylan	Xylose
4	15.0	11.5	16.2
8	17.0	16.8	18.3
24	24.0	18.8	19.8
48	25.6	22.7	17.8

CHAPTER V

DISCUSSION

The present isolate showing high activity xylanase is a mesophilic soil bacterium which has been identified as a *Bacillus* sp. on the basis of its ability to produce endospores by the transformation of the vegetative cells. The strain when cultivated on nutrient agar supplemented with xylan exhibited a clear zone of hydrolysis of xylan around the growing colony, the optimum temperature and pH for growth as well as xylan hydrolysis being 28°C and 7.0 respectively. The *Bacillus* sp. however exhibited a wide pH and temperature tolerance exhibiting growth on media adjusted to pH 5 – 8 as well as when incubated at temperatures upto 50°C.

Physiological and biochemical tests carried out according to established bacteriological studies indicated that the strain is referable to *Bacillus pumilus*. Slant cultures conserved at temperatures ranging from 4 - 10°C retained high level of viability as well as consistency in enzyme production and it is remarkable that cultures so conserved upto six years revived within 24 hours and did not show any decline in both enzyme activity and productivity when tested under standard conditions.

The *Bacillus* strain has consistently produced upto 300 IU/ml of extracellular xylanase activity in submerged culture within short periods of fermentation cycle ranging from 48 to 72h. Xylanase production was maximum in the presence of xylan

added at concentrations ranging from 0.5 – 2%. Activities comparable to pure xylan were also obtained in media where the xylan was replaced by various concentrations of xylan rich residue such as wheat bran. A survey of literature on xylanase production by bacilli as well as other xylanolytic actinomycetes and fungi has clearly indicated that high activity xylanase is always obtained as an induced enzyme in presence of either pure xylan or xylan rich residues, while xylanase activities in absence of xylan inducer were comparatively very poor (Esteban *et al.*, 1982; Panbangred *et al.*, 1983; Morosoli *et al.*, 1986; Yoshida *et al.*, 1989, Chandra and Chandra, 1995 and Cho 1997). Okazaki *et al.* (1984) reported high xylanase with a strain of *Bacillus* W-3 on pure xylan medium but got very less activity when wheat bran was substituted in place of pure xylan.

In the present study among the various soluble sugars tested for possible extracellular xylanase production, only xylose at 1% concentration gave positive xylanase activity which was however less than 10% of the activities obtained with xylan or xylan rich substrates. Supplmenting xylose at 0.05 to 0.5% in a xylan containing medium did not enhance the activity but showed repression especially at the higher levels of addition. Our results are similar to those reported by Nakamura *et al.* (1993).

In our experiments, yeast extract at 1% concentration added to the wheat bran medium produced the highest xylanase activity. Supplmenting such a medium or replacing the yeast extract with either inorganic nitrogen salts such as ammonium sulphate or sodium nitrate or nitrogen rich organic substrates such as peptone or casamino acids did not significantly improve the level of enzyme production. It is apparent that the *Bacillus* sp. is able to metabolise the bound nitrogen component of wheat bran along with yeast extract and to synthesize the enzyme in high yields and secrete it into the medium. In case of *Bacillus polymyxa*, Pinaga *et al.* (1993) reported low yields of xylanase production in media containing inorganic salts while reporting high activity where yeast extract was added.

Supplementing surface active agents especially Tween 80 and related compounds has been known to increase extracellular enzyme secretion and has been widely reported in the literature for enzymes such as cellulase (Reese and Magurie, 1969) and also alkalophilic *Bacillius* sp. xylanase (Balkrishnan *et. al.*, 1992) . In the present study 0.1 % Tween 80 enhanced extracellular enzyme yields by 25-30% over the activity in control flasks in which Tween 80 was not added. Other surfactants including Tween-40 tended to lower the enzyme

titres while Triton X-100 was positively inhibitory to both bacterial growth and enzyme secretion.

As already indicated the optimum pH for xylanase production is around pH 7.0 and it is also most active at around neutral pH. Several bacterial and actinomycete xylanases reported in literature exhibit similar characteristics of pH optima for both production and activities (Esteban *et al.,* 1982, Mohammad *et al.*, 1984; Srinivasan *et al.*, 1984, Grabski and Jeffries, 1991, Tripathi *et al.*, 1992). Fungi generally tend to produce xylanases with maximal activites at pH 5-6 (Sadana *et al.*, 1980, Mishra *et al.*, 1985, Bailey and Poutanen, 1989; Yoshida *et al.*, 1989, Ghosh and Nanda, 1993). However, an alkalophilic *Cephalosporium* sp. secreting high activity xylanase which is cellulase free and active under high alkaline conditions has been reported for the first time from this laboratory (Bansod *et al.*, 1993; Rele *et al.,* 1996) .

The *Bacillus* sp. xylanase in the present study exhibited optimum activity at 50°C while at 60°C, 60% of the activity was retained. The enzyme appeared to be stable for 1-2 h when incubated upto 50°C while at higher temperatures the stability was less.

The Km values of xylanases from different sources vary between 0.2 and 15 mg/ml (Bastawde, 1992; Olama, 1998). Km

values of the xylanase from *Bacillus* sp. calculated from Lineweaver Burk plot were 12.5 mg/ml of soluble xylan and 11.1 mg/ml for total xylan. respectively. Vmax values were 4.76 mmol/ml/min for soluble xylan and 1.66 mmol/ml/min for total xylan.

Mode of action studies of the enzyme indicate that it is an endoxylanase with the major products of hydrolysis being xylo oligosaccharides ranging from X2 to X4. In literature several endoxylanases producing similar mixture of xylooligosaccharides as products of hydrolysis are similarly reported from a variety of organisms (Estaban *et al.*, 1982, Berniar *et al.*, 1983; Keskar *et al, 1989; Bansod et al.,* 1993; Lopez *et al, 1998; Breccia et al.*, 1998).

The enzyme by virtue of its high activity and productivity on commercial media and its optimal performance at neutral pH coupled with reasonable stability upto 50°C holds promise in commercial applications where a xylanase active at or around neutral pH are in demand such as in baking and brewing industries. The alkalotolerant nature of the enzyme being active upto pH 8.0 and also absence of associated cellulase activity could possibly make this enzyme useful for industries such as biobleaching, textile and detergent industries. In the recent study evaluation of the enzyme for practical applications have

not been carried out but based on the characteristics that have been observed, the high activity xylanase from *Bacillus* 90-10-50 could hold promise and potential for future technological developments and industrial usefulness.

CHAPTER VI

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