STUDIES ON MICROBIAL XYLANASE

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CONTENTS

DECLARATION

This is to certify that the work incorporated in this thesis: **"Studies on microbial xylanase"** submitted by **Mr. Himadeep Balakrishnan** was carried out by the candidate under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

Dr. M.C. Srinivasan
Research Guide

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H. Balakrishnan

ABSTRACT

Xylan is the major component of plant hemicellulose and after cellulose, it is the most abundant renewable polysaccharide in nature. The main component of xylan is D-xylose, a five carbon sugar, which can be converted to single cell protein and chemical fuels by fermentation. Appreciable quanitites of xylan are present in materials released from wood during pulping and pulp processing. It is presently regarded as waste and is often deposited in streams and rivers where it is ecologically harmful. Considerable amounts of xylan are also present in agricultural residues. The conversion of xylan to useful products therefore represents an effort to strengthen the overall economics of the processing of hemicellulose biomass and also to develop new ways of energy production from renewable resources.

Recent years have witnessed a growing interest in cellulase free xylanases and in particular, such enzymes from alkalophilic organisms due to their potential application in paper and pulp industries. Alkalophilic organisms produce xylanases which are active and stable at alkaline pH and hence they can be readily applied to pulp without prior pH adjustment. Since most of the cellulase free xylanases, reported so far, are from bacterial sources, studies were carried to (a) isolate a bacterial culture capable of producing high levels of xylanase at alkaline pH (b) optimize

the culture conditions for enzyme production and (c) purify and characterize the enzyme to assess its potential applications.

The work is presented in three Chapters and a brief outline of the contents are given below:

Chapter I - General Introduction

This part comprises of literature survey, pertaining to xylan structure, xylan degradation, microbial xylanases and their applications.

Chapter II - Isolation of an alkalophilic *Bacillus* (NCL 87-6-10) and optimization of xylanase production

This chapter describes the isolation, identification and preliminary characterization of alkalophilic *Bacillus* NCL 87-6-10 and the media optimization for xylanase production.

Bacillus (NCL 87-6-10) was an aerobic, Gram positive rod and formed endospores after 2-3 days. Colonies of the organism were rhizoidal and gave a prominant clearance zone, when grown on nutrient agar medium containing xylan, suggesting a growth associated extracellular secretion of the enzyme. Microscopic examination of the culture indicated chains of Gram positive bacilli in the young culture whereas, the older cultures showed terminally-borne endospores. The culture grew optimally above pH 9.0 and showed an obligate requirement of sodium.

Optimization of the fermentation conditions showed that the maximum xylanase levels (100 - 120 IU/ml) were obtained, in 48 h, when the culture was grown in a medium containing wheat bran (3% w/v), yeast extract (1% w/v) and Tween-80 (0.5% v/v) adjusted to pH 9.5 - 10.0. The extracellular broth was free from cellulase, amylase and ß-xylosidase activities.

The alkalophilic Bacillus could produce high levels of extracellular xylanase in a medium containing soluble sugars like glucose, xylose, sucrose and sorbitol (3% w/v). However, enzyme production in presence of soluble sugars was observed only when the medium was supplemented with Baker's yeast as the nitrogen source. It was also observed that 0.5% (w/v) DL-norvaline could enhance the enzyme production, approximately two fold, when added to glucose-yeast extract medium.

Chapter III - Purification and characterization of an extracellular xylanase from alkalophilic *Bacillus* (NCL 87-6-10)

A simple procedure, involving ammonium sulfate precipitation followed by ion exchange chromatography on CM-Sephadex, was developed for the purification of xylanase, with an overall yield of approximately 24%. The purified enzyme showed a pH and temperature optima of 8.0 and 60°C, respectively. Moreover, the purified enzyme showed high pH and temperature stabilities. The molecular weight of the enzyme by gel filtration and SDS-PAGE was 7 kDa and 18.5 kDa,

respectively. Moreover, the purified enzyme showed high pH and temperature stabilities. The molecular weight of the enzyme by gel filtration and SDS-PAGE was 7 kDa and 18.5 kDa, respectively. The purified xylanase was a basic protein with a pI of 8.9. The Km and Vmax determined with oatspelts xylan, were 3.77 $mg.ml^{-1}$ and 1.43 x 10^3 µmol/min/mg protein, respectively. The enzyme was completely inhibited by low concentrations (1 mM) of Hg^{2+} but a significant inhibition was also observed in presence of 5 mM Co^{2+}, Zn^{2+}, Cu^{2+} and Mn^{2+}. Pure xylanase showed absolute specificity for xylan and did not show any activity on other polysaccharide substrates like cellulose, starch and laminarin. In addition, no ß-xylosidase activity was observed when assayed on *p*-nitrophenyl-ß-D-xyloside. The major end products of xylan hydrolysis were xylose, xylobiose and higher xylo-oligosaccharides suggesting the enzyme to be an endoxylanase.

Publications

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CHAPTER 1

GENERAL INTRODUCTION

Plant cell walls, the reservoirs of fixed carbon in nature, consist of three major structural components namely cellulose, hemicellulose and lignin in a ratio 4:3:3. However, the exact composition of these constituents varies from source to source. After cellulose, hemicellulose is the most abundant fraction available in nature. They are predominantly xylans in angiosperms (Aspinall and McGrath, 1966; Siddiqui and Wood, 1977) and gluco- and galacto-mannans in gymnosperms (Timell, 1964; 1965).

The term hemicellulose was first introduced by Schulze (1891) for the low molecular weight polysaccharides from plant tissues extractable with dilute alkaline solutions (Timell, 1964). The hemicellulose fractions thus isolated are heterogenous in nature and the main constituents are xylan, mannan, galactan and arabinan. Xylan is made up of D-xylose and trace amounts of L-arabinose while galactan, mannan and arabinan are composed of D-galactose, D-Mannose, and L-arabinose units respectively.

The major source of hemicelluloses are sugarcane, sorghum, corn, sweet potatoes, casava and other agricultural and forest residues. These sources make up approximately 40% hemicelluloses in terms of pentose sugars (Magee and Kosaric, 1985). The products obtained by hydrolysis of hemicelluloses are of commercial importance, as they have several applications in food, pharmaceutical and chemical industries.

STRUCTURE OF XYLAN

Among the hemicellulosic components, xylans are the most predominant and their basic structure consits of ß-1,4 linked D-xylose residues with attached side chains of other sugar residues. However, the presence of xylans devoid of other sugar residues have been reported in esparto grass (Chanda *et al.*, 1950) and in *Tamarindus indica* seeds (Savur, 1956).

After extensive investigations on xylan from European beechwood, Aspinall *et al.* (1954) demonstrated that it is made up of a linear frame work of at least seventy ß-1,4-linked D-xylopyranose units and on an average every tenth residue has a terminal 4-O-methyl ∝ -D-glucuronic acid attached to its second position. Subsequently, Timell (1964) showed that though all wood xylans, in general, have the same structure, slight differences exist in the average moleculear size and proportion of hexuronic acid side chains.

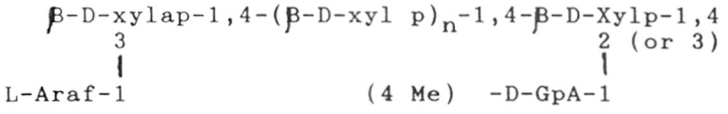
The xylan content of hard wood lies in the range of 20 - 25% while that of soft wood is in the range of 7-12% (Whistler, 1950). Moreover, soft wood xylans are characterized by the presence of a higher proportion of 4-O-methyl-∝-D-glucuronic acid (15-20%) than in hard wood xylans (8-15%) (Aspinall, 1959). The other difference between these two xylans is the absence of L-arabinofuranose side chains in hard wood xylans (Timell, 1964). However, members of the family *Rosaceae*, namely apple and cherry, are known to contain xylan with a higher frequency of acid side chains.

Additionally, most of the wood xylans though appear to be linear with one acid side chain per ten xylose residues (Aspinall, 1959), the ones from loblolly pine (Jones and Painter, 1959) and North American beechwood (Adams, 1957) have been shown to contain branching in the backbone.

The xylan content of cereals and grasses are in the range of 15-30% (Aspinall, 1959) and they are characterized by the presence of L-arabinofuranose residues linked to the C-3 of D-xylose in the xylan backbone. In addition, either D-glucuronic acid or 4-O-∝-methyl D-glucuronic acid or both can be present in small amounts. Though the main structural features of wheat straw, hemicelluloses are in agreement, significant differences exist in the proportions of D-glucuronic acid residues (Aspinall, 1959). For example, in one sample of wheat straw hemicellulose, D-glucuronic acid and 4-O-∝-methyl D-glucuronic acid residues are attached to D-xylose through an ∝-1,3 linkage (Bishop, 1953) whereas another sample showed the presence of ∝-1,2-linkage (Aspinall and Meek, 1956). Interestingly, another hemicellulose fraction did not contain glucuronic acid residues (Ehrenthal *et al.*, 1954) but contained a small amount of glucose, which in turn was an integral part of xylan. The presence of xylan devoid of arabinose has also been shown in wheat straw (Aspinall and Mahomed, 1954).

The basic structure of non-endospermic xylans from other species of grasses and cereals show considerable similarity

and can be represented by the following structure:



However, hemicellulose from corn cob, maize fiber, wheat bran and barley husks are more complex and show variations from the aforementioned general structure. For example, a sample of corn cob xylan, apart from L-arabinofuranose side chains, showed the presence of small amounts of glucose residues linked through positions 1,3 and 4. Barley husk hemicellulose, on the other hand, has side chains terminating in 2-O-ß-D xylopyranosyl-L-abinofuranose apart from a single unit of L-arabinofuranose and D-glucuonic acid side chains joined to xylose by 1,3 and 1,2 linkages respectively (Aspinall and Ferrier, 1957). While maize fibre hemicellulose contains L-galactose in addition to D-galctose (Whistler and Corbett, 1955), wheat bran hemicellulose contains a higher proportion of L-arabinose and these residues not only occur as non-reducing end groups, but also in non-terminal positions. On the contrary, xylans from endosperm show double substitutions of L-arabinofuranosyl groups on 0-2 and 0-3 positions of xylose residues, in addition to common single substitutions. Apart from the sugars mentioned above, Roudier (1958) demonstrated the presence of uronic acid residues in maritime pine xylan.

Moreover, maritime plants contained xylans with 1,3 linkages. Growth and maturation can also affect the hemicellulosic composition in plants.

ENZYMATIC HYDROLYSIS OF XYLAN

Xylans can be hydrolyzed to xylose either by acid or enzymes. Acid hydrolysis is carried out at high temperature and requires the use of corrosion resistant vessels. It also gives rise to undesirable side products. On the other hand, enzymes being very specific catalysts, their use not only gives rise to higher yields of sugars, but also eliminates the use of corrosion resistant vessels.

Enzymes involved in xylan hydrolysis

The degradation of xylan is catalysed by endo- and exo-xylanases and ß-xylosidases. Since xylans are known to contain arabinose and its 4-O-methyl derivatives, the complete hydrolysis of xylan also requires the side chain cleaving enzymes such as ∝-arabinofuranosidase and ∝-glucuronidase. In short, the complete hydrolysis of xylan requires the synergistic action of all the aforementioned enzymes.

Occurrence of xylanases

The ß-1,4 xylanases are produced by a large number bacteria and fungi such as *Bacillus subtilis* (Bernier *et al.*, 1983), *Bacillus coagulans* (Esteben *et al.*, 1983), *Streptomyces lividans* (Morosoli *et al.*, 1986), *Streptomyces*

T7 (Keskar, 1992), *Sclerotium rolfsii* UV-8 (Sadana *et al.*, 1980), *Schizophyllum commune* (Steiner *et al.*, 1987) & *Trichoderma longibrachiatum* (Royer and Nakas, 1989). They are also known to occur in rumen bacteria and protozoa, ruminant caecal bacteria, insects, small crustaceans, marine algae and germinating seeds of terresterial plants (Dekker and Richards, 1976).

Xylanase production

Most of the bacteria, yeast and fungi produce D-xylanases that are secreted extracellularly. However, some microorganisms like rumen bacteria (Walker, 1961; Kock and Kristner, 1969; Kristner and Gouws, 1964; Bailey and Clarke, 1963), *Sporocytophaga myxococcoides* (Clermont *et al.*, 1970) & *Aspergillus niger* (Iwamoto *et al.*, 1973a) produce D-xylanases intracellularly. There is considerable debate regarding the manner in which xylanases are produced by bacteria and fungi. Some reports claim that these enzymes are produced constitutively (Lyr, 1960; Strobel, 1963; Hulme and Stranks, 1971; Dekker and Richards, 1974; Berenger *et al.*, 1985; Esteban *et al.*, 1983; Smith and Wood, 1991), while others have shown them to be inducible (Kawaminami and Iizuka, 1969; Kitpreechavanich *et al.*, 1984; Leathers, 1986).

Xylan has been the most favoured and natural substrate for the production of xylanases (Kelly *et al.*,1989; Yoshida *et al.*, 1989; Thaker *et al.*, 1986; Yu *et al.*, 1987; Esteban

et al., 1982; Ishaque and Kluepfel, 1981; Morosoli *et al.*, 1986). However, pure xylan being very costly cannot be used for commercial production of xylanases. Therefore, a variety of other cheaper substrates rich in hemicellulosic content such as wheat bran, rice bran, rice straw, corn cob, corn stalk, bagasse, canola meal , sugar beet pulp, aspen wood, saw dust, etc. have been tested as substrates for xylanase production in various microorganisms (Panbangred *et al.*, 1983; Paul and Varma, 1990; Keskar, 1992; Brown *et al.*, 1987; Grajek, 1987; Gattinger *et al.*, 1990) (Table 1.1).

Esteban *et al.*, (1982) reported that xylanase was undetected in glucose grown cultures of *Bacillus circulans* WL-12, but xylose, mannose and cellobiose supported growth and xylanase production. In *Bacillus pumilus* (Panbangred *et al.*, 1985) xylanolytic enzymes are induced more effectively by xylose than xylobiose. In *S. lividans* 66 (Kluepfel *et al.*, 1990) xylose gave higher enzyme yields probably due to the absence of enzyme-substrate formation which was observed with xylan. Xylose did not repress enzyme synthesis as in the case of *S. lividans* 1326 (Bertrand, 1989). Similarly xylose was reported to stimulate xylanase synthesis in *Aureobasidium pullulans* (Leathers, 1986). In fact, the highest amount of D-xylanase productivity (300 U/h) could be obtained when *A. pullulans* (Priem *et al.*, 1991) was cultured in a medium containing xylose, glucose, yeast extract and corn steep liquor. On the other hand, in the yeast *Cryptococcus albidus*

Table 1.1 : Cultural conditions for the production of xylanase in shake flasks

Source	Carbon source	Temperature °C	pH	Incubation (h)	Activity (IU/ml)	References
I. BACTERIA						
Bacillus circulans WL12	Xylan	30	6.5	70	121.6	Esteban *et al.*, 1982
Thermophilic acidophilic *Bacillus* sp.	Xylan	65	3.5	96	29.5	Uchino & Nakane, 1981
Alkalophilic *Bacillus* No. C-59-2	Wheat bran	37	10.2	72	3.5	Horikoshi & Atsukawa, 1973
Alkalophilic thermophilic *Bacillus* sp. W-3	Xylan	45	10.0	48	111.8	Okazaki *et al.*, 1984
Bacillus coagulans strain 26	Xylan	37	-	20	40.2	Esteben *et al.*, 1983
Bacillus sp.	rice husk	38	7.2	48	5.0	Paul & Varma, 1990
Alkali tolerant *Bacillus Circulans*	Xylan	-	8.0	48	400.0	Ratoo *et al.*, 1992
Alkalophilic thermophilic *Bacillus*	wheat bran	48	10.0	48	50.0	Dey *et al.*, 1992
Bacillus thermoalkalophilus	Bagasse	60	9.0	24	56.9	Rajaram & Varma,1990
Cellumonas flavogena	Kaller grass straw	30	7.3	72	16.0	Mahomad *et al.*, 1984

Table I.1 cont.

Source	Carbon source	Temp-erature	pH	Incubation (h)	Activity (IU/ml)	References
Alkalophilic *Streptomyces* VP5	Wheat bran	37	10.0	120	12.0	Vyas *et al.*, 1990
Streptomyces lividans 1326	Xylan	40	7.0	72	50.0	Kluepfel *et al.*, 1986
Streptomyces olivochromogenes	Xylan	37	7.0	96	30.7	Mackenzie *et al.*, 1987
Streptomyces T7	Wheat	50	7.0	72	71.0	Keskar, 1992
Streptomyces wedmorensis	Xylan	37	7.0	56	41.0	Tripathi *et al.*, 1992
Actinomadura sp.	Xylan	55	7.4	192	58.8	Zimmerman *et al.*, 1988
Chainia (NCL 82-5-1)	Xylan	28	6.8	72	28.0	Srinivasan *et al.*, 1984
Thermomonospora fusca	Xylan	50	8.0	80	20.0	Bachmann & McCarthy, 1991
II. FUNGI						
Sclerotium rolfsii UV-8	Cellulose -123	30	6.5	336	200.0	Sadana *et al.*, 1980
Fusarium oxysporium SuF 850	Xylan	27	-	96	42.0	Yoshida *et al.*, 1989
Aureobasidium pullulans	Xylose	28	-	72	40.0	Leathers *et al.*, 1986
Pullularia pullulans	Xylan	-	5.0	24	167.0	Pou-Llinas & Driguez, 1987

Table 1.1 cont...

Source	Carbon source	Temperature	pH	Incubation (h)	Activity (IU/ml)	References
Schizophyllum commune	Avicel	-	-	276	1244.0	Steiner *et al.*, 1987
Penicillium pinophilum	Barley straw	28	-	240	27.0	Brown *et al.*, 1987
Humicola lanuginosa	sugar beet pulp	45	6.5	72	18.7	Grajek, 1987
Humicola grisea var. Thermoidea	Xylan	70	6.0	24	79.2	Monti *et al.*, 1991
Trichoderma longibrachiatum	Solka Floc	28	7.0	144	272.4	Røyer & Nakas, 1989
Thermoascus auranticus	Xylan	45	-	240	576.0	Yu *et al.*, 1987
Trichoderma reesei	Canola	27	-	216	210.0	Gattinger *et al.*, 1990

(Biely, 1985) xylose repressed enzyme production. However, in some thermophilic actinomycetes xylose did not influence the regulation of xylanase expression (McCarthy *et al.*, 1985).

Biswas *et al.* (1988) used non-metabolizable inducers such as ß-methyl xyloside for the production of xylanase from *Aspergillus ochraceus*. It was observed that the xylanase induction by ß-methyl xyloside was 4.2 times more than that using xylan. In *Cryptococcus flavus* (Yasui *et al.*, 1984) addition of ß-methyl xyloside to the medium increased xylanase production 15-20 fold as compared to xylan or xylose medium.

In *Streptomyces wedmornesis* (Tripathi *et al.*, 1992) higher xylanase activities were obtained on xylan than on cellulose. Kluepfel and Ishaque (1982) also reported very low xylanase activities when *Streptomyces flavogriseus* was grown on microcrystalline cellulose and much higher activities when grown on xylan. However, in *Streptomyces* sp. (Okeke and Paterson, 1992) xylanase activities were the highest when the organism was grown on microcrystalline cellulose and the enzyme activities appeared to increase with the crystallanity of the cellulosic substrate. This regulation was different from that of other *Streptomyces*.

Fungal systems are excellent xylanase producers, but these xylanases are normally co-secreted with cellulases. In *T. longibrachiatum*, growth on xylan resulted in very low

levels of cellulase activity (Royer and Nakas, 1989) and was similar to *Trichoderma harzianum* but different from *T. reesei* Rut C-30, which produced high levels of xylanase activity from cellulosic substrates rather than on purified xylan (Saddler *et al.*, 1985).

Bacterial xylanases are generally not co-secreted with cellulases and *Bacillus* species are used more extensively than other bacteria in industrial fermentations.

XYLANASE (ß-1,4-xylan xylanohydrolase, EC 3.2.1.8)

It is an endo-enzyme and cleaves the ß-1,4 xylosidic bonds in xylan yielding xylo-oligosaccharides of varying chain length. Due to its random action, endo-xylanase causes a rapid decrease in the viscosity of xylan and this can form a basis for monitoring its activity.

Purification

Xylanases from microbial sources are in general extracellular enzymes. However, irrespective of the source, most of the purification procedures involve concentration of the crude extract with salt or solvent precipitation followed by conventional procedures like ion exchange chromatography and gel filtration. Though in most of the cases, ammonium sulphate, alcohol and/or acetone are used for concentration, polyethylene glycol (PEG), freeze-drying, evaporation under low pressure and ultra filtration have also been used (Dekker and Richards, 1976).

The glycoprotein nature of some of these enzymes has been exploited for their purification on concanavalin A-Sepharose (Paul and Varma, 1992). Holden and Walson (1992) purified xylanase from a fungal maize pathogen *Cochliobolus carbonum* by hydrophobic interaction chromatography. Lappalainen (1986) purified *Trichoderma reesei* xylanase using immunoaffinity chromatography. Apart from the above methods, other techniques such as isoelectric focussing, (Dobozi *et al.*, 1992) chromatofocussing (Wong *et al.*, 1986), preparative PAGE (Dey *et al.*, 1992) and FPLC (Simpson *et al.*, 1991) have also proved extremely beneficial for the purification of xylanase from various sources.

Physico-chemical properties

The pH optima of bacterial xylanases are generally in the range of 4.0 - 7.0 (Table 1.2). However, the enzyme from alkalophilic *Bacillus* sp. showed a pH optima of 7-10 (Horikoshi and Atsukawa, 1973; Okazaki *et al.*, 1985; Honda *et al.*, 1985). Fungal xylanases on the other hand, exhibit an optimum pH on the acid side and are in the range of 4.0 - 6.0 (Table 1.2). The low molecular weight xylanase from *Penicillium herque Bainier* and *saroty* (Funaguma *et al.*, 1991) and the xylanase from *Aspergillus kawachii* (Ito *et al.*, 1992) showed an optimum pH of 3.0 and 2.0 respectively whereas, the enzyme from *Paecilomyces varioti Bainier* (Krishnamurthy and Vithayathil, 1989) exhibited a broad

Table 1.2: Properties of xylanases from different sources

Source		Molecular weight kDa	Optimum pH	Optimum Temp. °C	pI	Carbohydrate (%)	Reference
I. BACTERIA							
Aeromonas sp. alkalophilic Y-20	L	145	7.0-8.0	50			Ohkoshi *et al.*, 1985
	M	37	6.0-8.0	50			
	S	23	5.0-7.0	60			
Aeromonas caviae W61		22	7.0	55	9.2		Viet *et al.*, 1991
Bacillus circulans	A	85	5.5-7.0	-	4.5		Esteban *et al.*, 1982
	B	15	5.5-7.0	-	9.1		
Bacillus sp. 11 IS		56	4.0	80			Uchino and Nakane, 1981
Bacillus subtilis		32	5.0	50		35	Bernier *et al.*, 1983
Bacillus sp.Alkalophilic			6.0-8.0	60	6.3		Horikoshi & Atsukawa 1973
Bacillus sp. Alkalophilic thermophilic	WI-1	21.5	6.0	65	8.5		Okazaki *et al.*, 1985
	WI-2	49.5	7.0-9.0	70	3.6		
	WII-1	22.5	6.0	65	8.3		
	WII-2	50	7.0-9.5	70	3.7		
Bacillus coagulans		22	6.0	37	10.0		Esteban *et al.*, 1983
Bacillus pumilus		24	6.5	40			Panbangred *et al.*, 1983
Bacillus sp. alkalophilic C-125	A	43	6.0-10.0	70			Honda *et al.*, 1985
	N	16	6.0-7.0	70			

Table 1.2 cont

Source		Molecular weight kDa	Optimum pH	Optimum Temp. °C	pI	Carbohydrate (%)	Reference
Bacillus sp. alkalophilic		40	6.0	55			SeoPark *et al.*, 1992
Bacillus sp. alkalophilic thermophilic	I	35.0	6.0	50-60	4.0	15	Dey *et al.*, 1992
	II	15.8	6.0	50-60	8.0	30	
Chainia NCL 82-5-1		5.5	6.0	60	8.5-9.0		Bastawde, 1987
Clostridium acetobutylicum ATCC 824	A	65	5.0	50	4.4		Lee *et al.*, 1987
	B	29	5.5-6.0	60	8.5		
Clostridium stercorarium	A	44	6.0	75	4.53	19	Berenger *et al.*, 1985
	B	72	6.5	75	4.43	3	
	C	62	6.0	75	4.39	4	
	D	53	6.5	75	4.5		Sakka *et al.*, 1991
Clostridium thermolacticum (thermophilic anaerobe)		39	6.5	80			Debeire *et al.*, 1990
Cryptococcus flavus		25	4.5	55	10.0		Nakanishi *et al.*, 1984
Eubacterium thermotoga thermophilic		31	5.5	80			Simpson *et al.*, 1991
Streptomyces sp. KT-23		42	5.5	55	6.9		Nakajima *et al.*, 1984

Table 1.2 cont.

Source		Molecular weight kDa	Optimum pH	Optimum Temp. °C	pI	Carbohydrate (%)	Reference
Streptomyces lividans		43	6.0	60	5.2		Morosoli *et al.*, 1986
Streptomyces T7		20	4.5-5.5	60	7.8		Keskar *et al.*, 1989
Streptomyces sp.No. 3137	XI	50	5.5-6.5	60-65	7.1	≤ 1	Marui *et al.*, 1985
	XIIA	25	5.0-6.0	60-65	10.0	≤ 1	
	XIIB	25	5.0-6.0	60-65	10.2	≤ 1	
Streptomyces lividans 66		31	6.5	55	8.4		Kluepfel, 1990
Streptomyces roseiscleroticus		22.6	6.5-7.0	60	9.5		Grabaski & Jeffries 1991
Streptomyces thermoviolaceus OPC-520	STX1	54	7.0	70	4.2		Tsujibo *et al.*, 1992
	STXII	33	7.0	60	8.0		
II. FUNGI							
Aspergillus kawachi	1A	35	5.5	60	6.7	1.5	Ito *et al.*, 1992
	1B	26	4.5	55	4.4	17.6	
	1C	29	2.0	50	3.5	8.7	
Aspergillus ochraceus NG13		48	6.0	50			Biswas *et al.*, 1990
Aspergillus niger		28	5.0	40-45	3.65		Fournier *et al.*, 1985
Asgergillus niger str.14		33	4.0	50	4.2	20	Gorbacheva & Rodinova 1977

Table 1.2 cont.

Source	Molecular weight kDa	Optimum pH	Optimum Temp. °C	pI	Carbohydrate (%)	Reference
Aspergillus niger	14	4.7	45	4.5		Shei *et al.*, 1985
Aspergillus niger I	13	6.0	45	8.6		Frederick *et al.*, 1985
II	13	5.5	45	9.0		
Basidiomycetes lentinula	41	4.5-5.0	60	3.6		Mishra *et al.*, 1990
Basidiomycetes Irpex lacteus	38	4.6-5.2	60	7.6	23.0	Hoebler & Brillouet, 1984
Chaetomium thermophile var 1	26	4.8-6.4	70			Ganju *et al.*, 1989
Coprophile 2	7	5.4-6.0	60			
Cochliobolus carbonum 1	24	5.0	45	≥9.3		Holden & Walton, 1992
Humicola grisea var *Thermoidia*	23	5.5	70		45.0	Monti *et al.*, 1991
Humicola lanuginosa	22	6.0	65		1.18	Anand *et al.*, 1990
Humicola lanuginosa	21	6.0	65		4.1	Kitpreechavanich *et al.*, 1984
Melanocarpus albomyces 1	104		60			Chaudhari *et al.*, 1988
2	18		50			
Mesophilic fungus Y-94 A	51	4.9	80			Mitsuishi *et al.*, 1987
B	48	4.9	80			
C	35	4.9	80			
Paecilomyces varioti Bainier	25	5.5-7.0	6	3.9		Krishnamurthy & Vithayathil, 1989

Table 1.2 cont.

Source	Molecular weight kDa	Optimum pH	Optimum Temp. °C	pI	Carbohydrate (%)	Reference
Penicillium herquei Bainier & Sartory	11	3.0	50			Funaguma *et al.*, 1991
Robillarda sp. Y-20 1	17.6	4.5-6.0	50	9.7		Koyama *et al.*, 1990
2	59	4.5-6.0	50	3.5		
Schizophyllum commune	33	5.0	55			Paice *et al.*, 1978
Schizophyllum radiatum	25.7	4.9	55			Cvazzoni *et al.*, 1989
Sclerotium rolfsii UV-8		4.5	65	7.1		Deshpande *et al.*, 1989
Talaromyces byssochlamyoides Xa	76	5.5	75	4.3	36.6	Yoshoika *et al.*, 1981
Xb1	54	4.5	70	3.8	31.5	
Xb2	45	5.0	70	4.0	14.2	
Thermoascus aurantiacus Thermophilic	32	5.1	80	7.1		Tan *et al.*, 1987
Trichoderma harzianum	22	4.5-5.0	45-50	8.5		Wong *et al.*, 1986
Trichoderma harzianum 1	20	5.0	50	9.4		Tan *et al.*, 1985
2	29	5.0	60	9.5		
Trichoderma koningii G-39	21	5.5	60	8.9	4.4	Huang *et al.*, 1991
Trichoderma koningii 1	29		60-65	7.24		Wood and McCrae, 1986
2	18		60-65	7.3		

Table 1.2 cont.

Source		Molecular weight kDa	Optimum pH	Optimum Temp. °C	pI	Carbohydrate (%)	Reference
Trichoderma longibrachiatum	A	21.5	5.0	55	9.45		Royer & Nakas, 1991
	B	33.0	5.0	60	9.25		
Trichoderma reesei	1	32	4.0-5.0		4.1	14.0	Lappalainen, 1986
	2	23	4.0-5.0		6.4	8.0	
Trichoderma reesei	1	19	4.0-4.5	45	5.5	1.0	Tenkanen *et al.*, 1992
	2	20	5.0-5.5	55	9.0	1.0	
Trichoderma viride		22	5.0	53	9.3		Ujiie *et al.*, 1991
Trichosporon cutaneum		45	5.0	50			Stuettgen & Sahm, 1982

optimum pH of 5.5 - 7.0.

The isoelectric point (pI) of both bacterial and fungal xylanases show a wide variation and they are in the range of 3-10 (Table 1.2).

Most of the fungal xylanases show very high stability over a very wide range of pH. The pH stability of bacterial xylanases are comparable with those of fungal xylanases except those from *S. lividans* (for 24 h at pH 6.0 and 37°C) and *Streptomyces T-7* (for 11 days at pH 5.0 and 37°C). (Morosoli *et al.*, 1986; Keskar, 1990).

In general, the temperature optima of bacterial xylanases are in the range of 50 - 65°C. However, the enzyme from *B. coagulans* had a very low optimum temperature of 37°C (Esteban *et al.*, 1983). On the contrary, xylanases from several *Bacillus* sp. exhibit very high optimum temperature i.e. 70-80°C. Fungal xylanases too have an optimum temperature in the range of 45 - 60°C. The enzymes from *Talaromyces byssochlamyoides* YH-50 (Yoshoika *et al.*, 1981) showed an optimum temperature of 70-75°C, whereas those from the mesophilic fungus Y-94 (Mitsuishi *et al.*, 1987) and the xylanase from the thermophilic fungus, *Thermoascus aurantaicus* (Tan *et al.*, 1987) exhibited an optimum temperature of 80°C.

Most of the bacterial xylanases are stable upto 55°C. The two enzymes, B and D, from *Clostridium stercorarium* were

stable upto 70°C whereas the enzymes, A and C, from the same organism, were stable upto 75°C (Sakka *et al.*, 1991). Fungal xylanases on the other hand generally show lower temperature stability i.e. upto 40°C. However, the enzymes from *Humicola lanuginosa* and *Paecilomyces* are stable upto 60°C (Kitpreechavanich *et al.*, 1984; Krishnamurthy and Vithayathil, 1989).

The molecular weight of xylanases are in the range of 20 kDa - 145 kDa but the majority of them, fall in the range of 20 kDa - 50 kDa (Table 1.2). However, the enzymes from *Melanocarpus albomyces* and *Aeromonas* sp. Y-20 are very high molecular weight proteins with molecular weights of 104 kDa and 145 kDa, respectively (Chaudhari *et al.*, 1988; Ohkoshi *et al.*, 1985). On the contrary, the enzymes from *P. herquei Bainier* and *saroty* (Funaguma *et al.*, 1991) is comparatively a low molecular weight protein with a molecular weight of 11 kDa. Two xylanases with unusually low molecular weights have also been reported. While the enzyme from *Chaetomium thermophile* var. *Corpophile* (Ganju *et al.*, 1989) and an alkalophilic thermophilic *Bacillus* (Dey *et al.*, 1992) had a molecular weight of 7 kDa and 8.8 kDa, respectively, the one from *Chainia* sp. (Bastawde, 1992) showed a molecular weight of 5.5 kDa. These are probably one of the smallest enzymes reported so far.

Some of the well studied xylanases are glycoproteins and their carbohydrate content varies from 1 - 45% (Table 1.2).

The carbohydrate moieties are either covalently linked to the protein or present as dissociable complexes. Moreover, the carbohydrate moiety is believed to have a role in imparting greater stability to these enzymes (Woodward, 1984) and in their multiplicity (Wong *et al.*, 1988).

In most of the xylanases, tryptophan and cysteine have been implicated in the catalytic activity. Berenger *et al.*, (1985) noted that xylanases (A,B and C) from *C. stercorarium* were inhibited by pHMB and the inhibition could be reversed to a great extent by the addition of cysteine, suggesting the probable involvement of cysteine in the catalytic activity. On the contrary, other cysteine modifying reagents like NEM and DTNB did not have any significant effect. By chemical modification studies, the involvement of cysteine and tryptophan have been clearly shown in the case of xylanases from *Chainia* sp. and an alkalophilic thermophilic *Bacillus* (Deshpande *et al.*, 1990) and a thermotolerant *Streptomyces* sp. T7 (Keskar *et al.*, 1989). Bray and Clarke (1990) demonstrated the involvement of carboxyl groups in the catalytic activity of *Schizophyllum commune* xylanase by selective modification of the carboxylate groups by carbodiimide.

Mode of action

The D-xylanases could be either exo- or endo-acting liberating only xylose or xylobiose in case of the former and

xylo-oligosaccharides in case of the latter. Exo-xylanases have not been as extensively studied as the endo-xylanases, although there have been occasional reports (Fukui and Sato, 1957; Sasaki and Inaoka, 1967; King and Fuller, 1968).

Xylanases from bacteria like *Bacillus*, *Streptomyces* etc. are, in general, endo-enzymes. Esteban *et al.* (1982) isolated two endo-xylanases from *B. circulans* exhibiting different modes of action. Endo-xylanase I could degrade xylan to xylose, xylobiose and xylotriose whereas xylanase II predominantly yielded xylobiose, xylotriose and xylotetraose, but not xylose. An endo-xylanase from acidophilic *Bacillus* sp. 11-15 (Uchino and Nakane, 1981) showed a similar mode of action as that of the *B. circulans* endo-xylanase I. Horikoshi and Atsukawa (1973) while studying the action of an endo-xylanase from alkalophilic *Bacillus* sp., on rice straw arabinoxylan, noted that the principal products of hydrolysis were xylobiose and xylotriose with trace amounts of higher oligosaccharides containing arabinose. However, no xylose was detected during the initial stages of hydrolysis. On the contrary, the enzymes from an alkalophilic thermophilic *Bacillus* sp. yielded mainly xylobiose, xylotriose and higher xylo-oligosaccharides and traces of xylose from xylans (Dey *et al.*, 1992).

The major end products of xylan hydrolysis by *Streptomyces* T7 endo-xylanase were xylobiose and xylo-oligosaccharides. However, xylose could be detected after 16

h (Keskar *et al.*, 1989). Action pattern of endo-xylanases from *Streptomyces* sp. (Kusakabe *et al.*, 1977) were similar in that they yielded xylose and xylobiose. On the other hand, the enzymes from *Streptomyces* sp. KT-23 initially produced large amounts of xylobiose which was subsequently degraded to xylose (Nakajima *et al.*, 1984). The low molecular weight xylanase from *Chainia* sp. mainly produced xylotriose and xylohexaose, during the initial stages of the hydrolysis of xylan though, other low and high molecular weight oligosaccharides could also be detected. However, extensive hydrolysis did not show the presence of xylose (Bastawde, 1992).

Endo-xylanases from fungal sources can be classified into two types namely, debranching and non-debranching enzymes, depending on their products of hydrolysis. While the non-debranching enzymes do not liberate arabinose from arabinoxylans and arabinoglucuronoxylans, the debranching enzymes liberate arabinose from the above substrates. Enzymes from both these groups can degrade glucuronoxylans and D-xylans. Though arabinose liberating endo-xylanases hydrolyze (1→4) ß-D-xylopyranosyl linkages in xylans, using highly purified enzyme preparations from different strains of *Aspergillus niger* (Sasaki, 1971; Iwamoto *et al.*, 1973a) and its different isolates (Iwamoto *et al.*, 1973b; Tsujisaka *et al.*, 1971; Takenishi and Tsujisaka, 1973), *Agaricus bisporus* (Hashimoto and Takahashi, 1970), *Ceratocystis paradoxa*

(Dekker and Richards, 1975a) and *Diplodia viticola* (Strobel, 1963), it has been demonstrated that these enzymes can also cleave (1→3) ∝-L-arabinofuranosyl branch points in arabino-xylans. Though the crude enzyme preparations from *D. viticola* could liberate L-arabinose from corn cob and grape arabino-xylans, the purified preparations degraded xylan to xylo-oligosaccharides with the liberation of L-arabinose. Based on this data, it was speculated that the crude enzyme preparation contains two xylanases viz. one liberating arabinose or a xylanase and an ∝-arabinofuranosidase. Xylanase system from *A. niger* consisted of two different xylanases namely, xylanase I and xylanase II. While xylanase I hydrolyzed rice straw arabinoxylan to D-xylose, L-arabinose and a mixture of oligosaccharides, xylobiose and xylotriose were resistant. However, arabinoxylo-oligosaccharide was hydrolyzed to xylotriose and L-arabinose (Sasaki, 1971; Iwamoto *et al.*, 1973a,b). Moreover, purification of the crude extract showed the presence of two xylanases and one of them always liberated L-arabinose from arabinoxylan (Iwamoto *et al.*, 1973a). *A. niger* var Tieghem (Tsujisaka *et al*, 1971; Takenishi and Tsujisaka, 1973) produced three xylanases (I, II and III) and two of them i.e. xylanase II and III, degraded rice straw arabinoxylan to D-xylose, L-arabinose and a mixture of arabinoxylo-oligosaccharides. The mode of action of xylanase II was similar to that of xylanase I from *A. niger* except that the former could liberate arabinose from

arabinoxylobiose. Moreover, xylotriose obtained following the hydrolysis of arabinoxylotriose was further degraded to xylose and xylobiose. This enzyme also hydrolyzed phenyl ∝-L-arabinofuranoside yielding L-arabinose.

One of the xylanases (HC-I) from *C. paradoxa* (Dekker and Richards, 1975a) liberated L-arabinose from spear grass, hemicellulose B from sugarcane bagasse (arabino-4-O-methyl glucuronoxylan) and wheat endosperm arabino-xylan. Action of HC(I) on xylo- and arabinoxylo-oligosaccharides showed that xylotetraose is the smallest oligosaccharide attacked. Moreover, the ability of this enzyme to rapidly cleave xylopentaose to xylotriose and xylobiose probably suggests that arabinose substituents may not interfere with the binding of ß-D-(1→4) linked D-xylose chain to the binding site. The degree of substitution of xylans is known to influence the hydrolytic pattern of xylanases (McNeil *et al.*, 1975). Xylanase HC(II) from *C. paradoxa* was reported to attack selectively, those portions in the hemicellulose molecules which are most substituted with arabinose and uronic acid residues and leaving the less substituted low molecular weight xylan unhydrolyzed (Dekker and Richards, 1975b).

Non arabinose liberating endo-xylanases, which form the major part of known fungal xylanases, degrade arabinoxylans and other xylans to xylose and oligosaccharides containing arabinose and xylose. These enzymes have been purified from different strains of *A. niger* (Sasaki, 1971; Iwamoto *et al.*,

1973a,b; Tsujisaka *et al.*, 1971), *C. paradoxa* (Dekker and Richards, 1975), *D. viticola* (Strobel, 1963), *Stereum sanguinolentum* (Eriksson and Petterson, 1971) and *Trichoderma viride* (Toda *et al.*, 1971). Action pattern of most of the above mentioned xylanases showed that, they degraded xylan in a random manner yielding xylose and xylo-oligosaccharides. However, the absence of xylose in the hydrolysates of *D. viticola* xylanase was correlated to the assaying of the samples during the initial stages of hydrolysis. Fungal xylanases are known to attack ß-D(1→3) and ß-D(1→4) linked xylose residues from marine algal xylan, obtained from *Rhodymenia palmata* (Bjorndal *et al.*, 1965; Barry *et al.*, 1954). Purified xylanase preparation from *S. sanguinolentum* hydrolyzed xylan from *R. palmata* to xylobiose, xylotriose and higher oligosaccharides of mixed ß-D-(1→3) and ß-D-(1→4) linkages in a ratio 1:2. The enzymatic attack probably occurred at the ß-D(1→4) linkage only when it was flanked on both sides by similar linkages. While xylobiose and xylotriose were resistant, higher xylo-oligosaccharides were hydrolysed to di- and tri-saccharides. This enzyme showed transferase activity (transglycosylation) and could synthesize higher oligosaccharides from xylotetraose and xylopentaose (Eriksson and Petterson, 1971). The mode of action of *T. viride* xylanase (Toda *et al.*, 1971) was similar to that of one of the enzymes from *S. sanguinolentum*.

Interestingly, *C. paradoxa* xylanase (HC-II) could hydrolyze carboxymethylcellulose yielding glucose and cellobiose as the main products. When hemicellulose was subjected to hydrolysis, D-xylose and arabinoxylobiose appeared only after 4 h whereas, arabinose could not be detected.

Xylanases from rumen bacteria are endo enzymes. They attack D-xylan, arabinoxylan and hemicellulose B and yield xylose and xylobiose. The latter does not undergo further hydrolysis. These enzymes (Howard *et al.*, 1960; Bailey and MacRae, 1970) show a higher affinity towards linear xylans compared to branched substrates. Moreover, substituents like L-arabinose, D-galactose and uronic acid inhibit their activity.

Applications

The past decade has witnessed a growing interest in microbial xylanolytic systems free of cellulases. The reason being, such systems can be applied in processes where xylan is to be removed from cellulose without affecting the fibre structure of cellulose. These include the improvement of the quality of pulp and modification of its paper making properties. Paice and Jurasek (1984) suggested the application of cellulase-free xylanases for the production of high quality dissolving pulp. The ecologically harmful alkaline extraction of hemicellulose can be replaced with an enzymic treatment, leading to a mixture of fermentable sugars

instead of polluting alkaline waste liquors. Xylanolytic enzymes attracted further attention after Viikari *et al.*, (1990) demonstrated that they facilitate the chemical extractibility of lignin from crude pulp, by their ablity to hydrolyse xylan in ligninocellulosic complexes, leading to a significant saving of chemicals used for bleaching.

In addition to the pulp and paper industry, xylanases free of cellulases can be applied in the textile and food industries. They can be used in combination with pectinases in the processing of plant fibre sources such as flax and hemp. Classical fiber liberation is caused by the natural retting *in situ* by microorganisms, leading to the removal of pectin and hemicellulosic binding material. Pectinases are believed to play an important role in this process, but xylanases may also be involved.

The application of xylanases in the food industry does not strictly require the absence of cellulases. Xylanases can be used for the modification of bakery products by affecting dough development (McCleary *et al.*, 1986) and by changing the rheological properties of cooked rice. Together with pectinases, xylan degrading enzymes can be used to dissolve the precipitates in fruit juices.

Pure xylanases can be useful in studying xylan structure and to prepare defined xylo-oligosaccharides by the hydrolysis of xylan (Kusakabe *et al.*, 1977)

PRESENT INVESTIGATION

Recently, there has been a growing interest in cellulase free xylanases, due to their potential applications in paper and pulp industries viz, the removal of hemicelluloses in the manufacture of dissolving pulp (Jurasek and Paice, 1986), improvement of filterability of pulp (Fuentes and Robert, 1986; Karsila *et al.*, 1990) and biobleaching of pulp. The prior enzyme treatment of the pulp offers additional advantages like decreased consumption of chlorine and chlorine dioxide (Viikari *et al.*, 1990; Kruss and Koljonen, 1991, Kruss *et al.*, 1991).

Alkalophilic microorganisms and their cellulase free xylanases are of importance in paper industry because of their stability at alkaline pH. This property has the added advantage in that, such enzymes can be readily applied to the pulp without prior pH adjustment (Zamost *et al.*, 1991). In view of the high commercial potential of alkali stable, cellulase free xylanases, the present investigation was carried out to (a) isolate alkalophilic bacterial cultures capable of producing high levels of cellulase free xylanase (b) optimize the production conditions and (c) purify and characterize the enzyme to assess its potential application.

CHAPTER II

ISOLATION OF AN ALKALOPHILIC BACILLUS (NCL 87-6-10) AND OPTIMIZATION OF XYLANASE PRODUCTION

SUMMARY

From soil, rich in decomposing coconut detritus collected at Calicut (Kerala), a *Bacillus* strain (NCL 87-6-10) was isolated, which showed a clearance zone on alkaline nutrient agar xylan plates indicating the extracellular secretion of xylanase. The strain grew over a pH range of 8.0 - 10.0 at 28 - 37°C, but could not grow below pH 8.0, indicating its alkalophilic nature.

Media optimization studies for xylanase production, in shake flasks, revealed that in a medium containing wheat bran, 3% (w/v); yeast extract, 1% (w/v) and Tween-80, 0.5% (w/v), the *Bacillus* strain produced 100-120 IU of xylanase/ml in 48 h.

High levels of xylanase (> 120 IU/ml in 48 h) from the *Bacillus* could also be obtained with soluble sugars and sugar alcohols and in the total absence of xylan rich substrates normally required for xylanase production. However, Baker's yeast supplied as the sole nitrogen source was essential for enzyme production with soluble sugars, and sugar alcohols.

Several amino acids were tested for their effect on xylanase production. Among them, DL-norvaline could enhance the production, approximately 2-fold, in a glucose-yeast extract medium.

INTRODUCTION

Xylanases are important industrial enzymes and have found application in paper, textile and food industries. Recently, considerable attention has been directed towards cellulase free xylanases since they can be used for the production of high quality dissolving pulp. In this respect, naturally occurring microbial strains capable of secreting xylanases, free of cellulolytic activity, would have considerable industrial potential. Moreover, xylanases from alkalophilic organisms offer considerable advantages as the enzymes from such organisms show high activity and stability at alkaline pH and they can be readily applied without prior pH adjustment of the pulp. Though several fungi and bacteria are capable of producing high levels of xylanase, most of the reports on cellulase free xylanases are from bacteria. This prompted us to look for an alkalophilic bacterium, capable of producing high levels of xylanase, suitable for various commercial applications. This chapter describes the isolation, identification and preliminary characterization of an alkalophilic *Bacillus* (NCL 87-6-10) and the media optimization for xylanase production.

MATERIALS AND METHODS

Materials

Bacto yeast extract, bacto peptone, malt extract and soybean meal (Hi Media, India); sodium dodecyl sulphate (SDS), 3,5-dinitrosalicylic acid (DNSA), carboxymethyl cellulose (CMC) and oatspelts xylan (Sigma Chemical Co., USA); Tween-20 (CSIR Centre for Biochemicals, India); Tween-80, Hammarsten caesin (Sisco Research Laboratories, India) and cellulose powder CP-100 (Cellulose Products, India) were used. Wheat bran, rice bran, corn cobs and sugar cane bagasse were purchased locally. All the chemicals used were of analytical grade.

Soil sample

The soil sample was collected at Calicut (Kerala, India) from the base of a coconut tree where the soil was rich in decomposing coconut detritus. The soil was ash coloured and not particularly alkaline.

Methods

Isolation of the culture

A small amount of soil was suspended in sterile water and spread on a medium containing wheat bran (2% w/v, autoclaved and washed), yeast extract (0.1%) and Na_2CO_3 (1%) adjusted to pH 10.0. Plates were incubated at 28°C and the cultural characteristics were studied after 72 h. For the preliminary screening of xylanase producers, clearance on alkaline nutrient agar supplemented with xylan was used.

Following preliminary evaluation of the isolates, in shake flasks, one of the alkalophilic *Bacillus* strains, designated as NCL 87-6-10, was selected for further studies.

Determination of DNA base composition

Chromosomal DNA was isolated according to the method of Chater *et al.* (1982). The DNA base composition (G+C content) was calculated from thermal denaturation temperature as described by Marmur and Doty (1962).

Culture preservation methods

For long term preservation of the *Bacillus*, it was stored in the following systems, at 10°C, for one year.

In plain distilled water, Na_2CO_3 (0.1%), NaCl (1%), glycerol (10%), soil, paraffin oil and by freeze drying.

Substrate preparation

Two g of oatspelts xylan was suspended in 100 ml of 50 mM potassium phosphate buffer, pH 8.0 and stirred for 16 h at 10°C. The insoluble material was removed by centrifugation (9226 g, 20 min) and the soluble fraction corresponding to 0.7% (w/v) was used as the substrate.

Enzyme assays

Xylanase assay: The total reaction mixture of 1 ml contained 0.5 ml of suitably diluted enzyme in phosphate buffer, (50 mM, pH 8.0) and 0.5 ml of xylan solution. The reaction mixture was incubated at 50°C for 30 min and then terminated

by the addition of 1.0 ml DNSA. The 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 (Miller, 1959). One unit of xylanase activity is defined as the amount of enzyme that produced 1 µmol of xylose equivalent/min under the assay conditions.

CMCase (Carboxy methyl cellulase) assay: An aliquot of 0.5 ml of suitably diluted enzyme solution was mixed with 0.5 ml of 1% carboxymethyl cellulose (CMC) in 50 mM phosphate buffer, pH 8.0 or citrate buffer, pH 5.0 and incubated for 30 min at 50°C. The reducing sugars liberated were measured as glucose equivalents by the DNSA method. One unit of enzyme corresponds to one µmol of reducing sugars produced/min under the assay conditions.

Filter paper activity (FPase): The reaction mixture contained 50 mg (rolled 1 x 6 cm strip) of Whatman No. 1 filter paper, in 1.0 ml of 50 mM phosphate buffer, pH 8.0 or citrate buffer, pH 5.0 and 1.0 ml of the suitably diluted enzyme solution. The mixture was incubated for 1 h at 50°C and the reducing sugars liberated were measured as glucose equivalents by the DNSA method. One unit of enzyme corresponds to one µmol of reducing sugars produced/min under the assay conditions.

Alkaline protease assay: The protease activity was determined according to Kunitz (1947). The reaction mixture contained

an aliquot of suitably diluted enzyme solution and Hammarsten casein (10 mg) in 10 mM sodium bicarbonate buffer, pH 9.7, in a total volume of 2 ml. After incubation at 37°C for 10 min, the reaction was terminated by the addition of 3 ml of 5% (v/v) trichloroacetic acid. The precipitate formed was filtered through Whatman No. 1 filter paper, after standing for 30 min at room temperature. The absorbance of the trichloroacetic acid soluble fraction was measured at 280 nm. One unit of protease activity is defined as the amount of enzyme required to bring about an increase of 1.0 absorbance/ml of reaction mixture/min under the assay conditions.

Amylase assay : Amylase activity was determined according to Bernfeld (1955). The reducing sugars liberated following the hydrolysis of 1% (w/v) soluble starch at pH 5.0 and 8.0 and 50°C were estimated by the DNSA method. One unit of the enzyme activity is defined as the amount of enzyme required to liberate 1 µmol of reducing sugars/min under the assay conditions.

ß-Xylosidase assay: ß-xylosidase assay was carried out according to Berghem and Petterson (1973) at 50°C in 50 mM citrate buffer, pH 5.0, using *p*-nitrophenyl ß-D-xylopyranoside (pNPX) as the substrate. One unit is defined as the amount of enzyme which could release one µmol of *p*-nitrophenol/ min under the assay conditions.

Inoculum and enzyme production

Unless otherwise mentioned, the initial pH of all the fermentation media was adjusted to 9.5-10.0. The organism was maintained on nutrient agar-xylan (1%) slants (pH 9.5-10) for routine subculturing and a well sporulating culture was used to build up the inoculum. The inoculum tubes contained 5.0 ml of the medium containing 1% wheat bran, 1% yeast extract and 1% Na_2CO_3 and after inoculation was incubated for 24 h at 28°C, on a rotary shaker (150 rpm).

Xylanase production studies were carried out in 250 ml Erlenmeyer flasks containing wheat bran (3%), yeast extract (1%), Tween-80 (0.5%) in 50 ml medium, supplemented with Na_2CO_3 (5 ml of 10% solution), for 48 - 72 h at 28°C on a rotary shaker (200 rpm). Na_2CO_3 solution was autoclaved separately and added to the fermentation medium aseptically prior to inoculation.

RESULTS

Isolation of alkalophilic microorganisms

Four different types of bacterial colonies were isolated from the agar plates containing 2% wheat bran, at 28°C,after 48 h. These colonies were further screened for extracellular xylanase production on alkaline nutrient agar-xylan plates. All the colonies gave good xylan clearance zone, on the agar plates, within a period of 48 - 72 h at 28°C (Fig. 2.1).

Xylanase production under submerged conditions, by these individual colonies, was carried out in the medium containing wheat bran (1%) and yeast extract (1%). The initial pH of the medium was adjusted to 9.5 - 10.0 using 1% Na_2CO_3. Table 2.1 shows the xylanase production by the four isolates after 72 h.

Table 2.1: Xylanase production by the isolates

Isolate	Xylanase (IU/ml) (72 h)
1	1.1
2	3.9
3	1.7
4	3.5

The isolate No. 2, designated as NCL 87-6-10 showed the highest xylanase production and was selected for further studies.

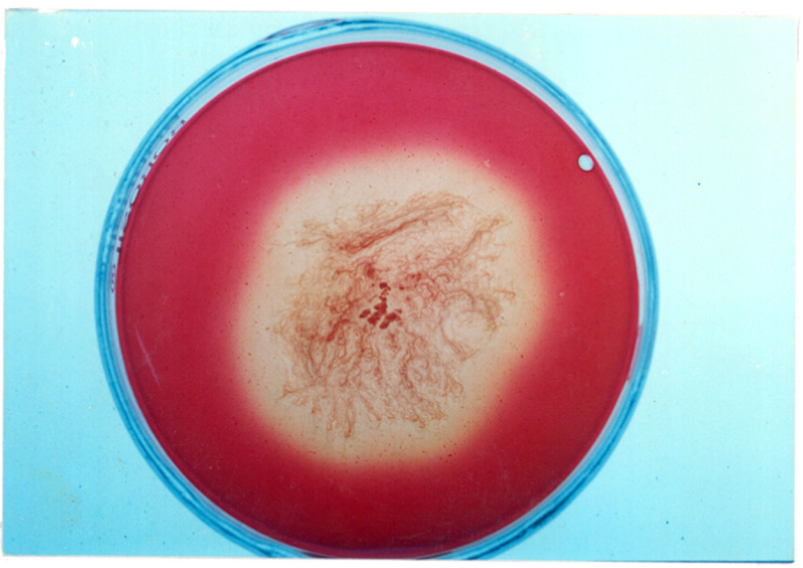


Fig. 2.1 : Xylan clearance zone by alkalophilic Bacillus NCL 87-6-10.

The organism was inoculated on nutrient agar xylan plate pH 9.5-10.0 and incubated at 28°C for 48 h. Plates were stained with congo red (Mackenzie, C.R. and Williams, R.E. 1984, Can. J. Microbiol. **30**: 1522).

Identification of the isolate

The isolated strain was an aerobic, spore forming, motile, Gram positive bacterium identified as *Bacillus* species (Gordon *et al.*, 1973). Colonies on nutrient agar appeared rhizoidal and spread to form a filamentous growth. The vegetative cells showed terminal spore formation after 72-120 h.

Morphological, cultural and biochemical characteristics of the alkalophilic *Bacillus* NCL 87-6-10 are as follows:

1. **Morphological characteristics**

Form	:	Rod
Motility	:	Motile
Gram stain	:	Positive
Spore	:	Terminal/Sub-terminal.

2. **Cultural characteristics**

Medium	Growth at pH 10.0 24 h
Nutrient broth	+++
Nutrient agar slant	+++
Xylan agar slant	+++
Glucose agar slant	+++
MGYP liquid	+++
Nutrient broth with $\leq$ 10% NaCl	+++

+++ denotes luxuriant growth.
At pH 7.0, no growth was observed.

3. Biochemical characteristics

Sugar and sugar alcohols tested (0.5%)	pH after 24 h	Growth
Mannitol	9.5	+
Sorbitol	9.3	++
Xylose	9.3	+++
Mannose	9.3	++
Raffinose	9.3	++
Glucose	9.4	+++

Initial pH of the medium was adjusted to 10.0

\+ *Poor Growth*
++ *Good growth*
+++ *Luxuriant growth*

Range of pH and temperature for growth

Figure 2.2 indicates that the *Bacillus* strain (NCL 87-6-10) grows in an alkaline pH range of 8.0 - 11.0 and the maximum growth is between pH 9.0 - 10.0.

G+C content of the *Bacillus*

High molecular weight DNA was isolated from the *Bacillus* as described under Materials and Methods. The Tm and G+C contents were determined by thermal denaturation of the DNA at 1°C/min with *Escherichia coli (E. coli)* and calf thymus DNA as standards (Table 2.2).

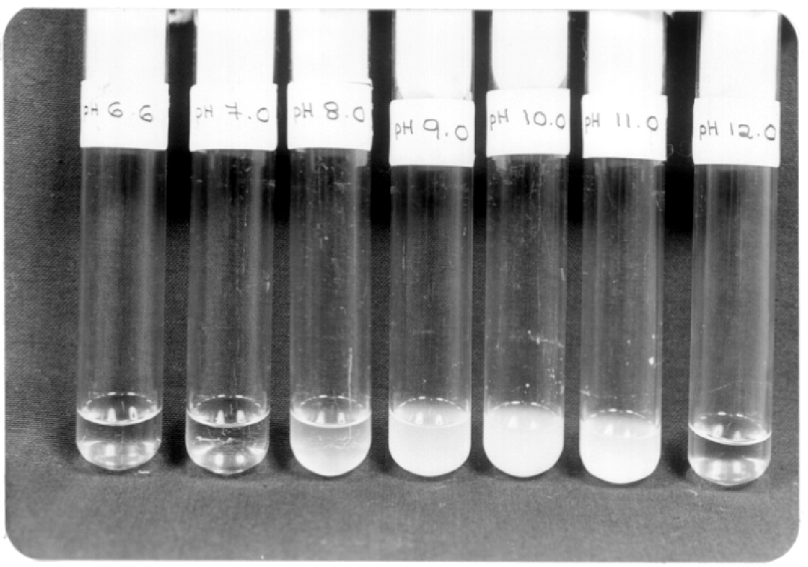


Fig. 2.2 : Growth of Bacillus 87-6-10 at different pH

The Bacillus was inoculated in nutrient broth of different pH and incubated at 28°C for 48 h.

Table 2.2: G+C content of the *Bacillus*

Source	Tm °C	G+C %
Alkalophilic *Bacillus* NCL 87-6-10	85.6	39.77
Calf thymus	85.8	40.82
E. coli	91.0	52.06
Bacillus subtilis[a]	-	43.6

[a] *CRC Handbook of Biochemistry and Molecular Biology. Nucleic Acids, Volume II (1976)*

Maintenance and preservation of *Bacillus* NCL 87-6-10

The selection of the microorganisms in fermentation processes and the methods used for the maintenance of these organisms are among the most important factors that have to be considered in designing an industrial fermentation process (Oniens,1971).

Hesseltine and Haynes (1973) have listed the general attributes that microbial cultures must have if the processes they generate are to be operable. They are: (i) the strains must be genetically viable (2) they can be readily maintained for reasonably long periods of time (3) they must readily produce many vegetative cells, spores or other reproductive units and (4) they should be pure and rapidly growing.

Various methods of maintenance employed are designed to minimize the hazards to which the cultures are exposed. The

repeated transfer is usually avoided.

There are three methods for maintenance which seem to be generally used (Perlman and Kikuchi, 1977). These include: (a) storing organisms on agar slants (b) drying organisms on soil or some other solid materials and (c) lyophilization.

Storage

1. **Room temperature:** The simplest way of storage is to keep the culture at room temperature (Dade, 1960). They are best protected from dust or aerial contamination by placing in a wooden box. Wood being a poor conductor of heat, the cultures are not likely to be subjected to rapid changes in temperature. However, the cultures maintained at room temperature tend to dry out rapidly depending on the climate. It is therefore necessary to make frequent transfers to fresh medium every two months (Fennel, 1960).

2. **Refrigeration or cold storage** : An easy method of storing cultures is keeping them at 5 - 8°C, in a refrigerator. Under these conditions drying is considerably reduced. The intervals between transfers would be longer (6-8 months) than that of room temperature (Smith, 1969). There have been several reports (McDonald, 1972; Hamilton and Weaver, 1949; Meyer, 1955; Carmichael, 1962) on cultures being successfully preserved at sub-zero temperatures (-10 to -20°C) with prolonged life. But one of the main objections to deep freeze storing is rupturing of the microbial cell walls due

to freezing and thawing.

3. **Mineral oil**: Healthy cultures can be covered with mineral oil and they will survive for longer periods by growing at a very reduced rate. This method of preservation is cheap, easy and requires no special skill or apparatus. It was extensively used by Buell and Weston (1947). The paraffin oil must be autoclaved or heated in an oil bath at 170°C for 1 h. The depth of the oil from the top of the slant should be 1 cm and is fairly critical (Fennel, 1960), as the oxygen transmission by layers of mineral oil in excess of 1 cm is unfavourable. If less oil is used then the cultures may get dried. The method depends on reduced rates of metabolism and prevention of drying. The oil cultures can be stored at room temperature or in a refrigerator (Hesseltine *et al.*, 1960).

4. **Soil**: This method is usually used for sporulating bacteria. The period of survival is greater than on agar and the strains remain viable. However, care has to be taken to see that the soil used for preservation is completely free of other microorganisms. This is assured by autoclaving the soil, for 1 h, for three consecutive days.

5. **Lyophilization or freeze drying**: Lyophilization or freeze drying, as a method for the preservation of microorganisms consists of drying the cultures or the spore suspension in the frozen state under reduced pressure (Haynes *et al.*, 1955). When cells are dried under these conditions they

remain dormant for long periods and on reconstitution and return to normal media, usually grow well. The process of lyophilization was first applied to fungi on a large scale by Raper and Alexander (1945).

The pre-grown cells are distributed in small quantities in ampoules using sterile techniques. The ampoules are frozen by immersing in a freezing mixture and then dried in a vacuum desiccator containing a desiccant, such as P_2O_5 or silica gel. When the material is completely dry, the ampoules are sealed. The dried and sealed ampoules can be stored at room temperature, 4-8°C (refrigeration) or even at -15°C in a deep freeze (Lany *et al.*, 1969).

Among the various methods of preservation and maintenance of microorganisms (Perlman and Kikuchi, 1977) some methods were tested for the maintenance of *Bacillus* 87-6-10.

Both short term preservation (on agar slants of different composition) as well as long term (in soil, paraffin oil, lyophilization, in plain distilled water, 10% glycerol, etc.) preservation methods were tried. The viability, morphological and physiological (xylanase production) characteristics were studied at different time intervals.

Long term preservation of *Bacillus* NCL 87-6-10

The *Bacillus* was preserved using some of the methods mentioned above and then revived on nutrient agar-xylan slants, at regular intervals and the xylanase production studied (Table 2.3).

Table 2.3: Long term preservation of *Bacillus* 87-6-10

Method of preservation	Xylanase production (IU/ml) After 6 months	After one year
Plain distilled water	105.0	99.0
Na_2CO_3 (0.1%)	101.0	82.0
NaCl 1.0%	100.0	100.0
Glycerol (10.0%)	98.0	98.0
Soil cultures	103.0	103.0
Paraffin oil cultures	99.0	103.0
Lyophilized cultures	106.0	105.0

Standardization of culture conditions for optimum xylanase production

Since wheat bran is an easily available substrate, it was used as the carbon source for optimization studies. The organism was initially grown in a wheat bran, 1% (w/v) yeast extract 1% (w/v) medium, at pH 9.5 - 10.0 for 72 h at 28°C, under shaking conditions. During optimization studies, aliquots of culture filtrate were withdrawn asceptically at an interval of 24 h, centrifuged to sediment the cells and the supernatant was assayed for xylanase

activity. The results presented are the average values of two sets of experiments carried out in duplicate.

Effect of initial pH of the culture medium

The organism was grown in a medium adjusted to different pH values ranging from 8.0 -10.0 and the xylanase production was monitored.

Table 2.4 : Effect of initial pH of medium on xylanase production.

Initial pH	24 h		48 h		72 h	
	pH	Xylanase (IU/ml)	pH	Xylanase (IU/ml)	pH	Xylanase (IU/ml)
8.0	6.3	ND	6.4	ND	6.8	ND
9.0	6.9	ND	7.2	ND	7.4	ND
9.5	8.4	12.6	8.7	31.7	9.2	27.6
10.0	8.7	23.1	9.0	39.1	9.3	31.6

ND = Not detected

All flasks contained wheat bran (1%) yeast extract (1%) medium.

There was no growth or xylanase production when the *Bacillus* was grown in a medium of pH 9.0. The pH range for optimum xylanase production was 9.5 - 10.0 and the maximum enzyme production was observed within 48 h. The initial pH of the fermentation medium was adjusted to 10.0 in the subsequent experiments.

Effect of wheat bran

The effect of increasing concentrations of wheat bran (1-7%) on xylanase production was studied using 1% yeast extract as the nitrogen source (Table 2.5).

Table 2.5: Effect of different concentrations of wheat bran on xylanase production

Wheat bran (% w/v)	Xylanase (IU/ml) 24 h	48 h	72 h
1.0	23.0	39.0	31.6
3.0	42.8	74.6	69.1
5.0	32.5	74.3	66.4
7.0	28.7	56.5	42.3

Xylanase production increased when the wheat bran concentration was increased from 1-3%. However, further increase did not show any appreciable effect on enzyme production. The pH of the fermentation medium was routinely adjusted to 10.0 using 1% sodium carbonate. However, if the pH was adjusted with potassium carbonate, the addition of sodium chloride was necessary for the growth of the culture as well as enzyme production (Fig. 2.3). Similar observations of sodium requirements have been reported in case of alkalophilic microorganisms by Kitada *et al.*, (1987).

Effect of yeast extract

The effect of different concentrations of yeast extract (0.5 - 2.0%) was studied using 3% wheat bran as a sole carbon

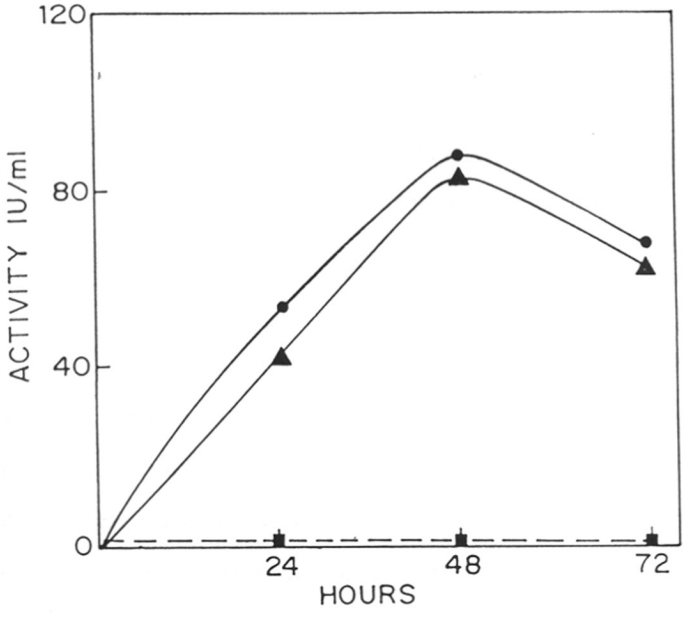


Fig. 2.3: Effect of Na^+ on the growth and xylanase production by the Bacillus (NCL 87-6-10) in wheat bran-yeast extract medium.

(●) Na_2CO_3, 1%; (▲) K_2CO_3, 1% + NaCl, 0.5%; (■) K_2CO_3, 1%.

source. (Table 2.6).

Table 2.6: Effect of different concentrations of yeast extract on xylanase production

Yeast extract %	Xylanase (IU/ml) 24 h	48 h	72 h
0.5	21.5	52.8	30.6
1.0	32.7	74.5	62.0
1.5	39.3	68.6	53.4
2.0	37.2	59.6	48.0

The results indicated that 1% yeast extract yielded maximum xylanase production by alkalophilic *Bacillus*. Further increase in yeast extract concentrations did not effect the enzyme production. The concentration of yeast extract used in further experiments was 1%.

Effect of agricultural residues

Different xylan rich agricultural residues as the carbon source (3%) with 1% yeast extract as the only nitrogen source was tried for xylanase production (Table 2.7).

Table 2.7: Effect of different agricultural residues on xylanase production

Agricultural residues (3%)	Xylanase (IU/ml) 48 h
Wheat bran	76.0
Rice bran	51.0
Corn cob	45.0
Bagasse	15.0
Oatspelt xylan	100.0

Maximum xylanase activity was observed when pure xylan (oatspelts) was used as a carbon source. However in the presence of wheat bran, as the main carbon source, comparable xylanase levels were observed.

Effect of organic nitrogen sources

The *Bacillus* strain was grown in a medium containing 3% wheat bran as the sole carbon source with different nitrogen sources and the xylanase production was monitored (Table 2.8)

Table 2.8: Effect of different organic nitrogen sources on xylanase production*

Nitrogen source	Xylanase (IU/ml)		
	24 h	48 h	72 h
Yeast extract (Difco)	67.5	90.3	77.4
Peptone	38.6	74.9	60.9
Casein	23.8	51.0	53.6
Pro-flo (Traders protein)	2.0	25.7	39.6
Pharma-media	4.3	47.6	57.3
Malt extract	1.4	9.1	6.8
Soy bean meal	18.2	33.3	34.3

* *Nitrogen source added on equivalent nitrogen to 1% (w/v) yeast extract.*

Bacillus strain when grown in the presence of peptone or yeast extract as the nitrogen source produced high xylanase activity (75-90 IU/ml), whereas low xylanase levels were observed in presence of other nitrogen sources.

Effect of surface active agents

Different concentrations of surface active agents such as, Triton X-100, SDS, Tween-20 and Tween-80, were used in in wheat bran (3%) yeast extract (1%) medium and the extracellular xylanase production was studied in shake flasks (Table 2.9).

Table 2.9 : Effect of different surface active agents on xylanase production

Surfactant %		Xylanase (IU/ml) 24 h	48 h	72 h
Control		37.7	75.6	67.8
Triton X-100	(0.01)	29.1	37.8	31.5
SDS	(0.01)	36.1	51.8	38.9
Tween-20	(0.01)	11.9	31.0	27.9
Tween-80	(0.1)	80.9	84.3	70.1
Tween-80	(0.5)	119.0	119.0	117.0
Tween-80	(1.0)	116.0	131.3	125.3

Among the various surfactants used, Tween-80 (at 0.5% and 1.0%) enhanced xylanase production while SDS, Tween-20 and Triton X-100 depressed enzyme production. The ability of Tween-80 to enhance enzyme production can be due to the release of the adsorbed enzyme from wheat bran or it may be increasing the cell permeability for the proper oligomer as an inducer.

Optimized medium for enzyme production

In shake flasks, optimum xylanase production (100-120 IU/ml) by alkalophilic *Bacillus* NCL 87-6-10 was observed in 48 h, when the culture was grown in 50 ml medium containing 3% wheat bran, 1% yeast extract and 0.5% Tween-80.

Inoculum size

Influence of inoculum size (5-20%) in the optimized medium showed that the maximum xylanase activity is obtained with 10% (w/v) inoculum.

Table 2.10: Enzymes detected in alkalophilic *Bacillus* NCL 87-6-10

Enzyme	Assay pH	Activity IU/ml
Xylanase	8.0	120
Carboxymethyl cellulase (CMCase)	5.0 8.0	0.024 0.074
Filter paper degrading activity (FPase)	5.0 & 8.0	ND
Amylase	5.0 & 8.0	ND
β-Xylosidase	5.0 & 8.0	ND

ND = not detected

Specificity of the culture broth

In order to ensure that the *Bacillus* does not produce any cellulase, inducers such as CMC, cellulose CP 123, Solka floc and cellobiose, at a concentration of 1%, were added in the wheat bran-yeast extract medium and CMCase and FPase activities were monitored. Although comparable levels of xylanase (100 IU/ml) were obtained in the presence of these substrates, the culture filtrate was virtually free of any cellulase activity (data not shown).

Xylanase production in presence of soluble sugars and sugar alcohols

Xylanases are inducible enzymes, produced mainly in media containing either pure xylan or xylan rich substrates, although there are reports of constitutive production of the enzyme (Chapter 1). However, in the presence of readily metabolizable soluble sugars, xylanase production was reported to be low (Esteban *et al.*, 1982).

Studies on the production of xylanase from *Bacillus* 87-6-10 was carried out in a medium containing glucose, xylose, sorbitol, etc. with yeast extract as both the carbon and the nitrogen source. In this media, *Bacillus* 87-6-10 produced very little or no xylanase even after 72 h. However, when 1% wheat bran grown inoculum was used in place of nutrient broth inoculum, significant amounts of xylanase activity (50 IU/ml) was observed in 48 h. Interestingly, when yeast extract was replaced by whole Baker's yeast, as the organic nitrogen source, it was observed that the organism could produce high levels of xylanase activity even though the inoculum was grown in nutrient broth i.e. in the total absence of xylan rich substrates. When the concentration of glucose and Baker's yeast in the shake flask medium was varied, it was found that a concentration of 3% glucose and 2.4% Baker's yeast was optimum and the extracellular enzyme activity was 152 IU/ml, in 48 h. Xylanase production in the presence of other sugars and sugar alcohols along with

Baker's yeast (2.4%) was also studied (Table 2.11).

Table 2.11 : Effect of different sugars and sugar alcohols (with Baker's yeast) on xylanase production

Sugars and sugar alcohols tested 3%	Xylanase (IU/ml) 48 h
Glucose	152.0
Xylose	142.6
Glycerol	98.9
Sorbitol	148.6
Lactose	6.8
Starch	104.0
Sucrose	101.3

Table 2.11 shows that xylose, glycerol, sorbitol, maltose and sucrose gave comparable levels of xylanase, while very low enzyme levels were observed in the presence of lactose.

The effect of DL-norvaline on xylanase production

Ikura and Horikoshi (1987) reported that the addition of glycine, DL-norvaline and D-alanine enhanced xylanase production in an alkalophilic *Bacillus* sp. The high xylanase levels in presence of D-alanine was attributed to enhanced growth of this culture. On the contrary, the increased enzyme levels in presence of glycine and DL-norvaline was correlated to the depression of protease production.

The effect of different concentrations of DL-norvaline on growth and xylanase production by *Bacillus* NCL 87-6-10, in glucose yeast extract medium, was studied and the results are shown in Table 2.12.

Table 2.12: The effect of DL-norvaline on growth and xylanase production by *Bacillus* NCL 87-6-10*.

DL-Norvaline %	Optical density at 600 nm (18 hours)	Xylanase (IU/ml) 48 h
None	0.348	41.3
0.1	0.308	52.9
0.25	0.198	53.9
0.5	0.158	75.6
1.0	0.133	19.0

**The inoculum was built up in 1% wheat bran*

From Table 2.12 it is evident that the growth of *Bacillus* is inhibited in presence of increasing concentrations of DL-norvaline (Fig. 2.4). However, DL-norvaline at a concentration of 0.5% could enhance xylanase production approximately two times the control value, after 48 h incubation, even though the growth of the *Bacillus* is reduced by about 50%.

In order to determine the effect of DL-norvaline, the xylanase, alkaline protease and total protein in the culture filtrate were estimated (Table 2.13).

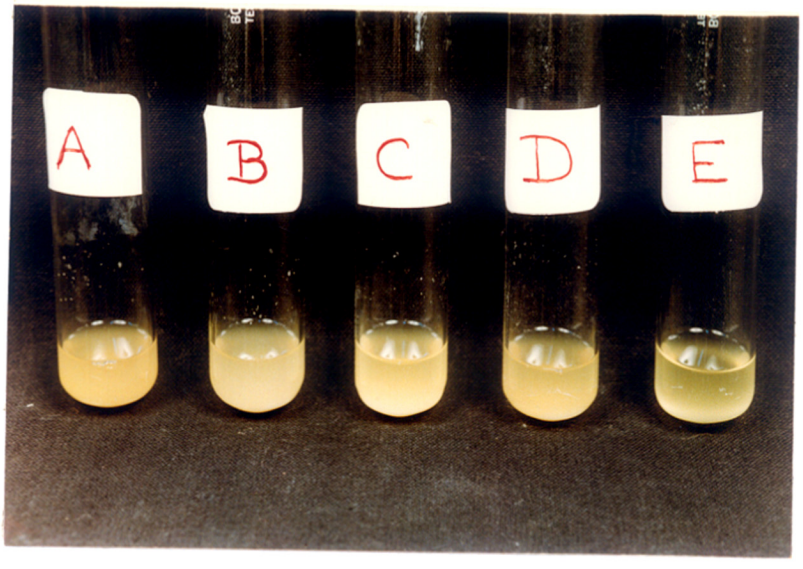


Fig. 2.4: Growth of the Bacillus NCL 87-6-10 in glucose-yeast extract medium containing different concentrations of DL-norvaline (w/v).

A - None; B - 0.1%; C - 0.25%; D - 0.5%; and E - 1.0%.

Table 2.13: Effect of DL-norvaline on xylanase and alkaline protease production in 48 h

DL-Norvaline %	Alkaline protease IU/ml	Xylanase IU/ml	Total protein mg/ml
None	229	43.8	0.193
0.1	126	48.8	0.279
0.25	99.3	56.9	0.393
0.5	32.0	70.9	0.582

The results show that alkaline protease production is inhibited in presence of DL-norvaline.

DISCUSSION

Most of the alkalophilic bacilli, like the one under investigation, have been isolated from soil that are not particularly alkaline (Krulwich and Guffanti, 1989). *Bacillus* (87-6-10) could grow in the pH range of 8 - 11, and the optimum growth was between pH 9.0 - 10.0. This is similar to the pH optimum reported for the growth of other alkalophilic bacilli (Horikoshi and Atsukawa, 1973; Okazaki *et al.*, 1984; Honda *et al.*, 1985; Dey *et al.*, 1992). The G+C content of our isolate was lower (39.77%) than that of other alkalophilic bacilli reported. For instance, Okazaki *et al.* (1984) reported G+C contents of 42.2, 41.7, 42.9 and 41.9% for four different alkalophilic thermophilic bacilli W1, W2, W3 and W4, respectively. The value reported for alkalophilic *Bacillus* sp. C-125 was 44.2 (Honda *et al.*, 1985) while that

of alkalophilic thermophilic *Bacillus* was 46.7 (Dey *et al.*, 1992).

Generally, *Bacillus* strains require higher temperature (40°C) for their optimum growth in liquid medium. For example, alkalophilic *Bacillus* C-59-2 (Horikoshi and Atsukawa, 1973) could grow upto a temperature of 42°C while another alkalophilic *Bacillus* sp. 125 (Honda *et al.*, 1985) could grow upto a temperature of 55°C. However,*Bacillus* NCL 87-6-10 could grow only at or below 40°C, suggesting that it is a mesophilic organism.

The cost of xylanases is one of the factors which determine the economics of their use in paper and pulp industry and this can be overcome, to some extent, by optimizing the production conditions, or by the isolation of hyper producing mutants and/or by constructing efficient producers using genetic engineering (Biely, 1985). At present, xylanases are produced from processed and refined substrates such as sugars, cellulose and xylan. These substrates are relatively expensive for industrial scale production of this enzyme. Consequently, to lower the production costs, cheaper substrates for the manufacture of these enzymes are being sought (Warzywoda *et al* 1983; Tangnu *et al*, 1981; Sarkar and Prabhu, 1983; Gattinger *et al.* 1990).

Horikoshi and Atsukawa (1973) reported xylanase activity of the order of 5.1 IU/ml in 48 h on 5% wheat bran from

alkalophilic *Bacillus* C-59-2. The highest xylanase activity of 400 IU/ml was reported from an alkali tolerant *Bacillus circulans* like strain, on xylan and corn steep liquor (Ratoo *et al.*, 1992). The alkalophilic *Bacillus* sp. NCL 87-6-10 produced significant xylanase activity (100-120 IU/ml) when grown on agricultural residues like wheat bran. Okazaki *et al.* (1984) reported an activity of 111.8 IU/ml from the alkalophilic thermophilic *Bacillus W3*, when grown on xylan while the corresponding activity on wheat bran was only 35.6 IU/ml.

Generally, bacteria and actinomycetes produce xylanase at neutral pH, while fungi prefer acidic pH (Kelly *et al.*, 1989; Ganju *et al.*, 1989; Thaker *et al.*, 1986). However, alkalophilic bacilli produce xylanase in the pH range of 9.0-10.0 (Okazaki *et al.*, 1984; Horikoshi and Atsukawa, 1973). The pH required for optimum xylanase production for the alkalophilic *Bacillus* NCL 87-6-10 was 9.5-10.0 The presence of sodium (added as Na_2CO_3) was essential for growth and xylanase production. This observation is consistent with that of Kitada *et al.* (1987) who suggested the role of Na^+ in maintaining the internal pH balance as well as for nutrient uptake in alkalophilic organisms.

The nature and amount of nitrogen source also affects the enzyme production. Alkalophilic *Bacillus* NCL 87-6-10 produced optimal amount of xylanase in the presence of 1% yeast extract. Inorganic nitrogen sources did not support

growth and enzyme production (data not shown).

Reese and Maguire (1969) have demonostrated the importance of surfactants in increasing the enzyme yield. Surfactants are known to act by increasing the permeability of the cells, by allowing the higher oligosaccharides to pass through the cell wall. Among the various surfactants tried, Tween-80 (0.5 - 1.0%) significantly increased the xylanase levels. Dubeau *et al.* (1987) reported a 1.6 fold increase in *Chaetomium cellulolyticum* xylanase activity in presence of 0.05% Tween-80. In comparison, *Bacillus* NCL 87-6-10 required much higher concentration of the surfactant (0.5%) to achieve a significant increase in the enzyme production.

Xylanase production in some *Bacillus* sp. has been reported to be constitutive (Esteban *et al.*, 1983) or to be induced by xylose (Okazaki *et al.*, 1984). In the case of *Bacillus* (NCL 87-6-10) although high xylanase production could be induced by hemicellulosic substrates, such as wheat bran, high levels of enzyme production could also be obtained with soluble sugars when yeast extract was replaced by Baker's yeast as the nitrogen source. However, further studies are required to understand the role of Baker's yeast in the enhancement of xylanase production. Since Baker's yeast is cheap and readily available, it can be used for the large scale production of xylanases.

The stimulatory effect of DL-norvaline and glycine on xylanase production has been reported for alkalophilic

Bacillus No. C-125 by Ikura and Horikoshi (1987). A similar effect was observed in case of alkalophilic *Bacillus* NCL 87-6-10 in presence of DL-norvaline. However, when DL-norvaline was added in a wheat bran-yeast extract medium it did not show any stimulatory effect on xylanase production.

The ability of alkalophilic *Bacillus* 87-6-10 to produce high levels of extracellular cellulase free xylanase suggests that it has a potential for commercial application.

CHAPTER III

PURIFICATION AND CHARACTERIZATION OF AN EXTRACELLULAR XYLANASE FROM ALKALOPHILIC *BACILLUS* (NCL 87-6-10)

SUMMARY

A simple procedure for the purification of xylanase from the alkalophilic *Bacillus* (NCL 87-6-10) is described. The steps involved were ammonium sulphate precipitation and chromatography on CM-Sephadex C-50 at pH 7.0. The enzyme was purified approximately 9 fold with an overall yield of 24%. The pH and temperature optima of purified xylanase were 8 and 60°C, respectively. The enzyme exhibited high pH and temperature stability. The purified xylanase showed a molecular weight of 7 kDa but its subunit molecular weight was 18.5 kDa. It is a basic protein with a pI of 8.9. The enzyme was specific only to xylan and it did not act on other related polysaccharides. The Km and Vmax on oatspelts xylan were 3.77 mg/ml and 1.4 x 10^3 µmol/min/ mg protein respectively. The purified xylanase was completely inhibited by Hg^{2+} and to a significant extent by Co^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+} and Fe^{2+}. The major end products of xylan hydrolysis were xylose, xylobiose and xylo-oligosaccharides suggesting it to be an endoxylanase.

INTRODUCTION

Xylan degrading enzymes have been known and studied for over 40 years, but it is only in recent years that reports on homogenous xylanases and their mechanism of xylan breakdown have started appearing. Xylan is hydrolyzed to monosaccharides by the concerted action of exo-xylanases, endo-xylanases, (1,4-ß-D-xylan xylanohydrolase, EC 3.2.1.8) and ß-D-xylosidase (1,4-ß-D-xylan xylohydrolase, EC 3.2.1.37) (Dekker and Richards, 1976; Reilly, 1981; Woodward, 1984). Several xylanases from fungal and bacterial sources have been purified and biochemically characterized. Multiple forms of xylanases have been found to occur in many microbial systems (Dekker, 1985; Frederick *et al.*, 1985; Wong *et al.*, 1988). Wong *et al.* (1988) suggested that the multiplicity of xylanases produced by microorganisms could be a strategy to achieve superior xylan hydrolysis.

Although a number of xylanases have been purified and their enzymatic properties investigated, very little is known about their structure-function relationship. Recently, several low molecular weight xylanases have been purified and these appear to be suitable not only for protein structure-function studies (Okada, 1988) but also in paper industry. This chapter describes the purification and characterization of a low molecular weight xylanase from alkalophilic *Bacillus* (NCL 87-6-10).

MATERIALS AND METHODS

Materials

CM-Sephadex C-50, N,N,N',N'-tetramethylethylenediamine (TEMED), Coomassie Brilliant Blue G-250, acrylamide, bis acrylamide, Tris and molecular weight markers were obtained from Sigma Chemical Co. USA. Ampholine (3-10) was from Biorad, USA. All other chemicals used were of analytical grade.

Methods

Enzyme production

The enzyme production was carried out as described in Chapter 2.

Xylanase assay

Xylanase was assayed at pH 8.0 and 50°C, by measuring the reducing sugars liberated, at 540 nm, following the hydrolysis of oatspelts xylan as described in Chapter 2.

Protein determination

Protein in the crude culture filtrate was first precipitated with 10% trichloroacetic acid (TCA). The precipitate was collected by centrifugation and after the removal of TCA, was dissolved in 0.1 N NaOH. The protein concentration was then determined according to Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard. The blue colour developed after the addition of Folin phenol reagent was measured at 660 nm.

In the column effluents, the protein was measured by monitoring the absorbance at 280 nm.

PURIFICATION OF XYLANASE

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

Ammonium sulphate precipitation: The culture filtrate (500 ml) was brought to 0.6 saturation by the addition of 180 g of solid ammonium sulphate and the mixture was stirred for 3 h. The precipitated protein was collected by centrifugation (9226 g, 30 min), dissolved in minimum amount of 20 mM potassium phosphate buffer, pH 7.0 and dialysed extensively against the same buffer to remove the ammonium sulphate. The dialysed enzyme solution was concentrated to approximately 5.0 ml using an Amicon ultrafiltration unit having a YM-5 membrane.

CM-Sephadex C-50 chromatography: The concentrated enzyme solution was adsorbed on to a CM-Sephadex column (25 x 4 cm) pre-equilibriated with 20 mM potassium phosphate buffer, pH 7.0, at a rate of 17.0 ml/h. The column was then washed with the same buffer, till the flow through fractions showed no xylanase activity. The bound enzyme was then eluted with a linear gradient, 500 ml total volume, of KCl (0 - 0.8 M) in the above buffer. Fractions of 4.0 ml were collected at a rate of 30 ml/h and fractions having activity greater than 4 IU/ml were pooled, concentrated by ultrafiltration using YM-

5 membrane and stored at -20°C. No loss of activity was observed over a period of 4-6 months when the enzyme was stored under these conditions.

Gel electrophoresis

Analytical disc gel electrophoresis, at pH 4.5, was performed using 7.5% (w/v) polyacrylamide gels according to Davis (1964). After the electrophoresis, the gels were stained with Coomassie Brilliant Blue G-250 as described by Blakesley and Boezi (1977).

SDS-PAGE was carried out using 12% (w/v) polyacrylamide gels, at pH 8.3, according to Laemmli (1970). The gels were stained with Coomassie Brilliant Blue R-250 and destained with methanol, acetic acid, water (25:10:65).

Isoelectric focussing (IEF) was carried out in a glycerol gradient according to Pawar *et al.* (1986) over the pH range of 3-10. The pH of each fraction was measured using a surface electrode and the individual fractions were assayed for enzyme activity.

Molecular weight determination

Molecular weight determination was carried out by High Performance Liquid Chromatography (HPLC) on LKB (Uppsala, Sweden) model 2150 solvent delivery system. The column (TSK 2000, LKB) was calibrated with carbonic anhydrase (29,000), myoglobin (17,000) cytochrome C (13,000) and aprotinin

(6,500). The mobile phase comprising of 60 mM potassium phosphate buffer, pH 7.0, containing 100 mM NaCl was used at ambient temperature (28 ± 1°C) and at a flow rate of 0.5 ml/min. 100 µl (500 µg) of the protein sample and the marker proteins were injected on to the column and the protein was monitored using an LKB model 2158 UV Cord SD detecter at 280 nm.

The samples for analysis were prepared by dissolving the marker proteins and the enzyme sample in glass distilled water and clarified on a millipore filter.

Subunit molecular weight was determined on 12% (w/v) SDS-polyacrylamide gels at pH 8.0 (Laemmli, 1970) with bovine serum albumin (66,000), ovalbumin (45,000), glyceraldehyde 3 phosphate dehydrogenase (36,000) carbonic anhydrase (29,000) soybean trypsin inhibitor (20,100) and lactalbumin (14,200) as reference proteins.

Paper chromatography

The seperation of xylo-oligosaccharides obtained following the hydrolysis of oatspelt xylan was carried out on Whatman 3, in an ascending manner, using pyridine, acetic acid, water (36:46:19) as the mobile phase. After the chromatographic run, the paper was air dried and the products were visualized by spraying the chromatogram with a solution of silver nitrate in acetone (0.1 ml of saturated aqueous silver nitrate solution in 20 ml of acetone). The

chromatogram was then sprayed with 0.5 N NaOH, when the dark brown spots of reducing sugars gradually appeared (Trevelyan *et al.*, 1950). Subsequently, the chromatogram was soaked in 5% (w/v) sodium thiosulphate for 10-15 min and after removing the excess thiosulfate with water, was air dried.

RESULTS

The results of a typical procedure for the purification of xylanase to homogeneity are summarized in Table 3.1.

Table 3.1:Purification of xylanase

Step	Total protein mg	Xylanase activity IU	Specific activity U/mg protein	Recovery %	Fold purification
Culture filtrate	281	26,160	93.1	100	-
Ammonium sulphate fraction	105.2	14,898	141.6	56.9	1.52
CM Sephadex C-50	7.68	6,280	817.0	24.0	8.78

The enzyme was purified approximately 9 fold with an overall yield of 24.0%. The purified enzyme moved as a single band in native and SDS gels indicating its homogeneity (Fig. 3.1 and inset Fig. 3.3). The molecular weight of the pure enzyme determined by gel filtration and SDS-PAGE were 7 kDa and 18.5 kDa, respectively. The pI of the purified

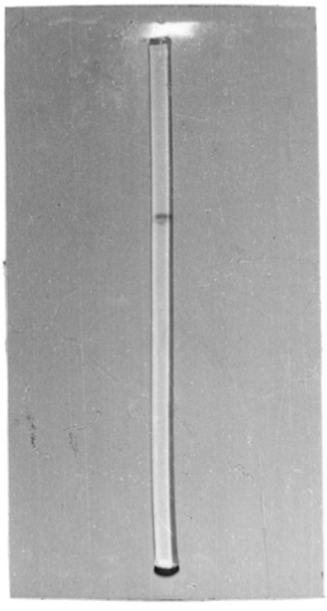


Fig. 3.1: Polyacrylamide gel electrophoresis (PAGE) of purified xylanase.

7.5% (w/v) polyacrylamide gel; β-alanine-acetic acid buffer, pH 4.3; 4 mA/tube; protein loaded 50 µg.

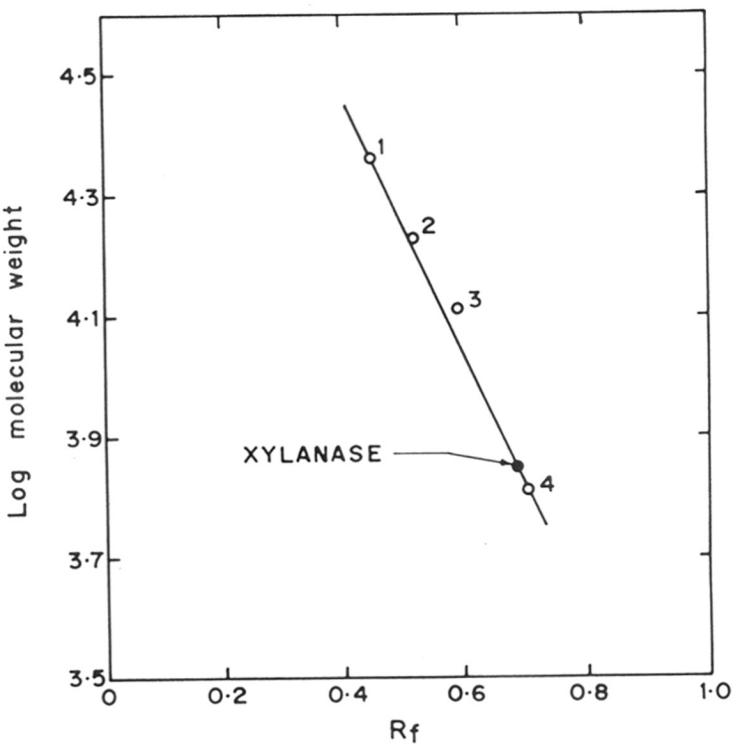


Fig. 3.2 : **Molecular weight determination of xylanase.**

Gel filtration (LKB TSK-G 2000 7.5 x 600 nm) column was equilibriated with 60 mM potassium phosphate buffer, pH 7.0, containing 100 mM sodium chloride. The column was calibrated with (1) carbonic anhydrase (29,000); (2) myoglobin (17,000); (3) cytochrome c (13,000) and (4) aprotinin (6,500).

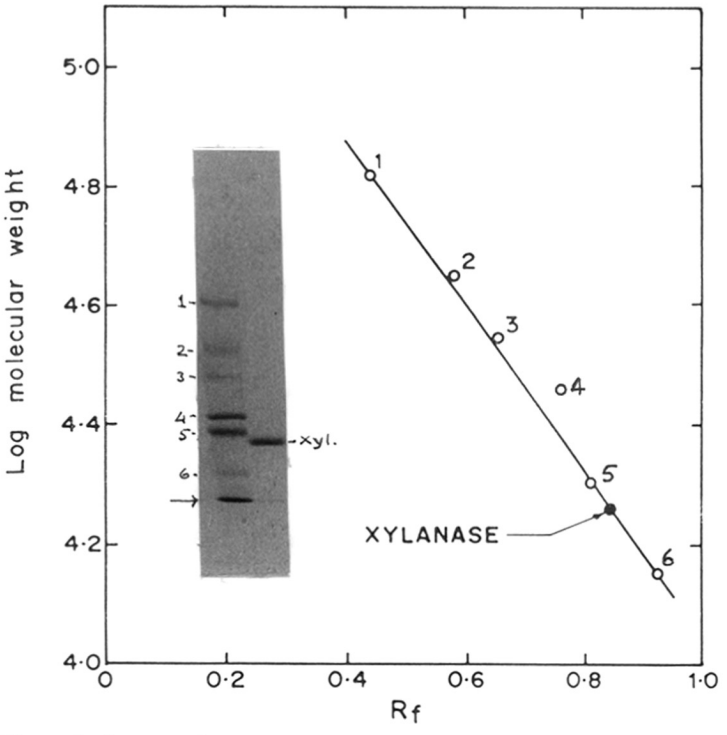


Fig. 3.3 : **Molecular weight determination of xylanase.**

SDS-Polyacrylamide gel electrophoresis (12%). Relative mobilities of the reference proteins were plotted against their log molecular weights. Reference proteins were (1) BSA (66,000); (2) ovalbumin (45,000); (3) Gly-3-phosphate dehydrogenase (36,000); (4) carbonic anhydrase (29,000); (5) soybean trypsin inhibitor (20,100) and (6) ∝-lactalbumin (14,200).

enzyme was 8.9. Determination of the carbohydrate content by phenol:sulphuric acid method revealed that the enzyme is not a glycoprotein.

The purified xylanase showed an optimum pH of 8.0 and the enzyme exhibited approximately 45% of its activity at pH 6.0 and 9.0 (Fig. 3.4). Determination of the pH stability of the enzyme showed it to be stable over a wide range of pH i.e. from 7.0 - 10.0 (Fig. 3.5).

The purified xylanase was found to be optimally active at 60°C and approximately 75% increase in enzyme activity was observed when the temperature was raised from 40 - 60°C (Fig. 3.6). The determination of the temperature stability showed that the enzyme retained its full activity at 60°C and pH 8.0 for 90 min whereas, at 65°C it retained approximately 40% of its activity. When the temperature was increased to 70°C, the enzyme showed a gradual decrease in its activity and retained approximately 35% of its initial activity after 90 minutes. However, at 75°C, the enzyme showed a rapid loss of activity and lost its activity completely in 20 min (Fig. 3.7).

The Km and Vmax of the purified enzyme determined using oat spelts xylan were 3.77 mg/ml and 1.43 x 10^3 µmol/min/mg protein respectively (3.8).

Effect of metal ions on the activity of purified enzyme revealed that Co^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+} and Fe^{2+} at 5 mM,

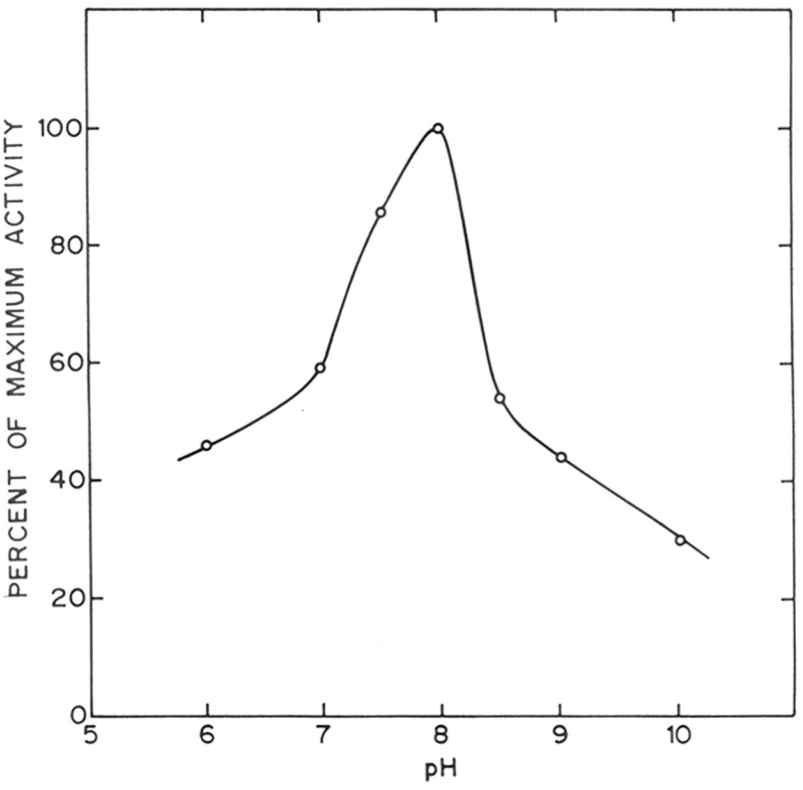


Fig. 3.4 : pH activity profile of xylanase.

Appropriate amounts of purified xylanase (0.5 - 1.0 U) were assayed in a series of pH (6-10) at 50°C as described under Methods. The buffers used were 50 mM potassium phosphate (pH 6-8) and 50 mM sodium bicarbonate (pH 9.0-10.0).

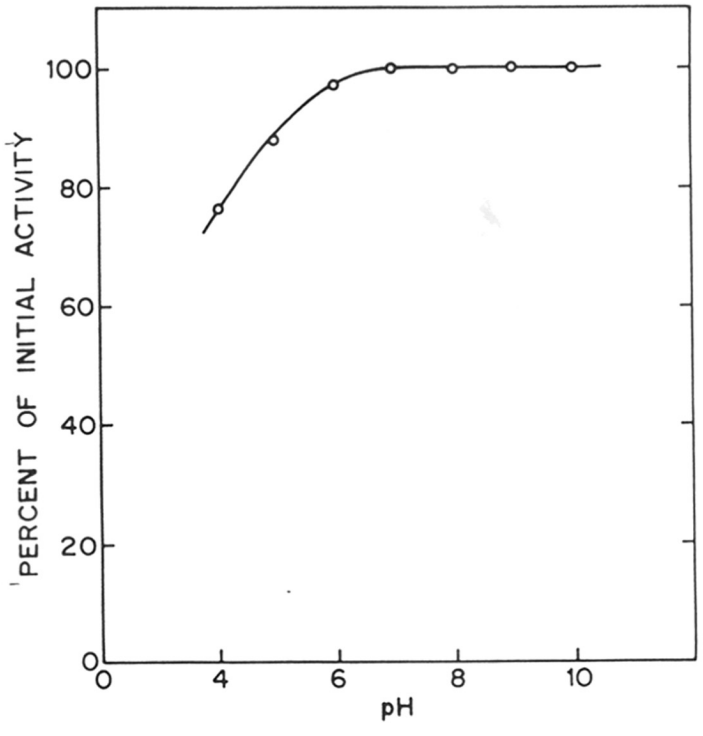


Fig. 3.5: pH stability of xylanase.

Purified xylanase (80 U) was pre-incubated at different pH (4-10) for 1 h at 37°C and their activities were determined under standard assay conditions. The buffers used were 50 mM sodium acetate (pH 4-5), 50 mM potassium phosphate (pH 6-8) and 50 mM sodium bicarbonate (pH 9-10).

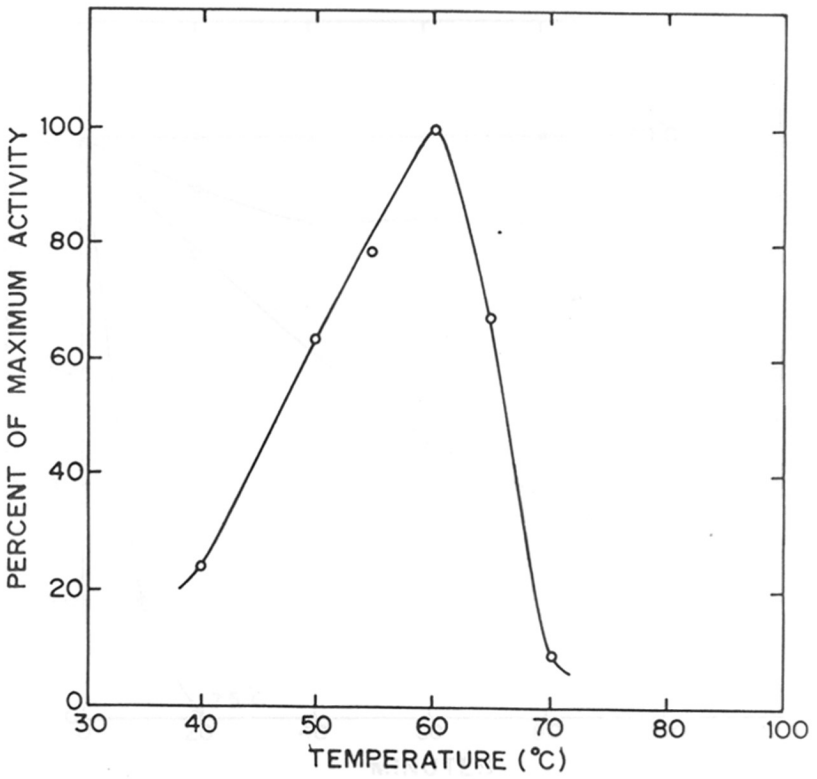


Fig. 3.6 : **Temperature activity profile of xylanase.**

Appropriate amounts of purified xylanase (0.5 - 1.0 U) were incubated in a series of temperatures (40-70°C) at pH 8.0 and their activities were determined as described under Methods.

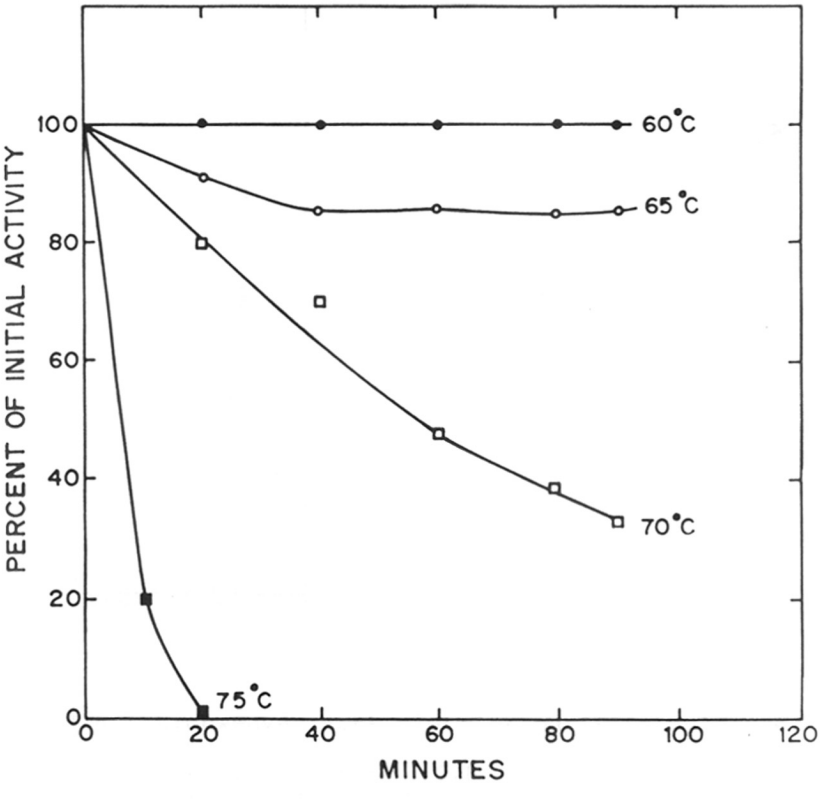


Fig. 3.7: Temperature stability of xylanase.

Purified xylanase (100 U) was pre-incubated at different temperatures varying from 60-75°C for 90 min at pH 8.0. Aliquots were removed at 20 min intervals and assayed for residual enzyme activity.

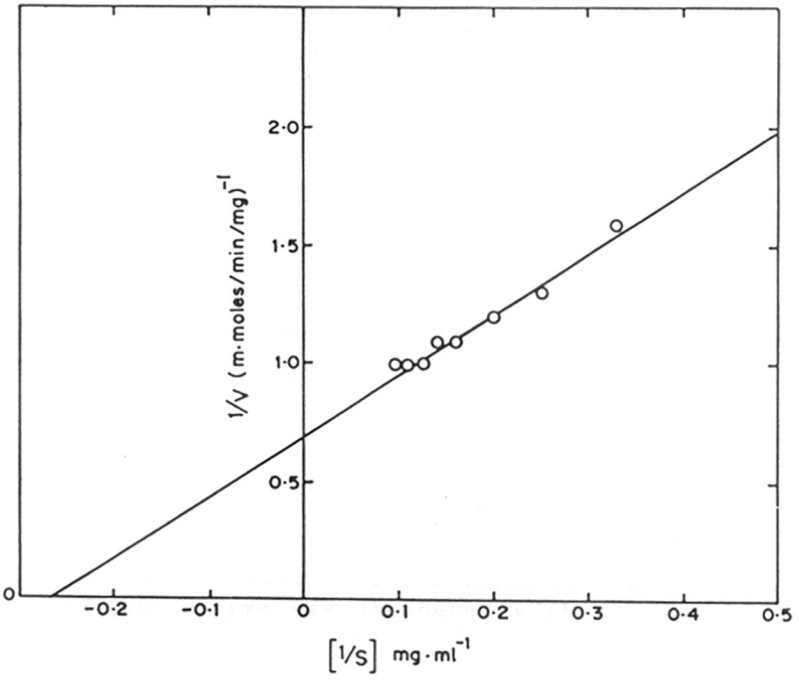


Fig. 3.8 : **Lineweaver Burk plot for purified xylanase.**

Purified enzyme (0.8 U) was assayed in a series of substrate concentrations (1-10 mg/ml) at pH 8.0 and 50°C.

significantly inhibited the enzyme activity, whereas, Ca^{2+} and Mg^{2+} had no effect. Hg^{2+} was the most potent inhibitor since low concentration (1 mM) of the metal ion could completely inhibit the enzyme (Table 3.2).

Table 3.2: Effect of metal ions

Metal	Concentration mM	Relative activity (%)
None	-	100
Co^{2+}	0.5	15
Zn^{2+}	0.5	10
Mn^{2+}	0.5	10
Cu^{2+}	0.5	25
Ca^{2+}	0.5	100
Mg^{2+}	0.5	100
Fe^{2+}	0.5	40
Hg^{2+}	0.1	0

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

The specificity of the enzyme for various substrates with different types of linkages was studied. The purified enzyme showed no activity against microcrystalline cellulose, CM-cellulose, Avicel, cellobiose, *p*-nitrophenyl ß-D-xyloside, starch and laminarin (Table 3.3).

Table 3.3: Substrate specificity of xylanase

Substrate	Linkage	Activity (%)
Xylan		
Oatspelts	ß-1,4	100
Larchwood	ß-1,4	69.45
Birchwood	ß-1,4	61.7
Cellulose		
Crystalline	ß-1,4	Nil
Carboxymethyl	ß-1,4	Nil
Avicel	ß-1,4	Nil
Cellobiose	ß-1,4	Nil
p-Nitrophenyl-B-D-xyloside	ß-1,4	Nil
Soluble starch	∝-1,4 ∝-1,6	Nil
Laminarin	ß-1,3	Nil

In all cases, 100 ng protein was used and the reaction was carried out in 50 mM potassium phosphate buffer, pH 8.0 for 30 min at 50° C.

The products of time course hydrolysis of oatspelts xylan by purified xylanase were anlysed by paper chromatography (Fig. 3.9). From the initial stage of hydrolysis, the reaction products contained low molecular weight xylo-oligosaccharides and xylose. With increase in the duration of hydrolysis, the concentration of low molecular weight xylo-oligosaccharides and xylose gradually increased.

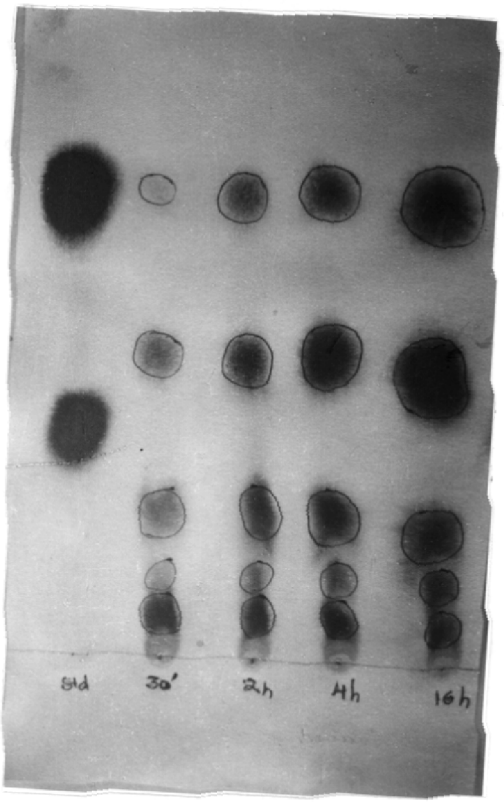


Fig. 3.9: Paper chromatogram of the hydrolytic products of xylan

10 mg oatspelts xylan was incubated with 100 U of purified xylanase at pH 8.0 and 50°C. Aliquots were withdrawn at specified time intervals and products were separated on Whatman No. 3 as described in Methods.

DISCUSSION

In the present studies, a homogenous xylanase preparation could be obtained by a two step procedure involving ammonium sulphate precipitation and CM Sephadex chromatography. Honda *et al.* (1985) purified xylanases (A and N) from an alkalophilic *Bacillus* sp. in three steps with an overall yield of 25% and 4.0% respectively. Dey *et al.* (1992) purified two xylanases (I and II) from alkalophilic thermophilic *Bacillus* sp. in four steps with a recovery of 8.0 and 20.0%, respectively. Similarly, Horikoshi and Atsukawa (1973) purified a xylanase from alkalophilic *Bacillus* sp. in three steps with 35% recovery. In the present case, we could purify the enzyme to homogeneity in two steps with comparable yields (Table 3.1). The specific activity of purified xylanases from different *Bacillus* species are in the range of 5 - 300 (Bernier *et al.*, 1983; Dey *et al.*, 1992; Honda *et al.*, 1985; Horikoshi and Atsukawa, 1973; Kato and Nevins, 1984; Panbangred *et al.*, 1983; Uchino and Nakane, 1981). In comparison, the purified enzyme from alkalophilic *Bacillus* 87-6-10 showed significantly high specific activity (Table 3.1).

The purified enzyme was a low molecular weight protein with a molecular weight of 7 kDa. However, the subunit molecular weight was 18,500. Such descripancies in the native and subunit molecular weights have been reported in case of several low molecular weight xylanases. Grabaski and

Jeffries (1991) noted that the purified xylanase from *S. roseisclerticus* showed a molecular weight of 5.5 kDa and 22.6 kDa on gel filtration and SDS-PAGE respectively. Similar anomalies were observed in case of purified xylanase from *Cochliobolus carbonum* (8.5 kDa and 24 kDa) (Holden and Walton, 1991) and xylanase II from alkalophilic thermophilic *Bacillus* sp. (7.58 kDa and 15.8 kDa), (Dey *et al.*, 1992) respectively. Such discrepancies between the native and subunit molecular weights were correlated to non-specific adsorption to agarose and polyacrylamide based matrices and to the shape and surface properties of the protein. Since the molecular weight of the native enzyme was carried out on silica based matrix, the observed discrepancy between the native and subunit molecular weights might be due to the shape and surface property of the protein.

Xylanases from alkalophilic organisms exhibit pH optima in the alkaline region. Similarly, the purified enzyme showed an optimum pH of 8.0 (Fig. 3.4). Like other xylanases from alkalophilic microorganisms, the pure enzyme showed high stability between pH 7.0 and 10.0 (Fig. 3.5). However, it was comparatively unstable below pH 7.0 and lost approximately 25% of its initial activity at pH 4.0. Honda *et al.* (1985) described two xylanases, A and N, from alkalophilic *Bacillus* sp. 125, stable in the pH range of 4-12 and 5-12 respectively when incubated at 4°C for 24 h. While xylanase A was more stable in the pH range of 4-12, it became

gradually unstable below pH 4.0 and above pH 12.5. However, xylanase N rapidly lost its activity below pH 5.0 and above pH 12.0. Similarly, endoxylanases from an alkalophilic thermophilic *Bacillus* sp. WI-1, WI-2, WII-1 and WII-2 were stable between pH 4.5 - 10.0 for 1 h at 45°C. WI-1 and WII-1 retained more than 90% of its initial activity at pH 4.0, whereas WI-2 and WII-2 were inactive at this pH. In this respect, the pH stability of *Bacillus* NCL 87-6-10 xylanase is comparable to xylanase WI-1 and WII-1 from alkalophilic thermophilic *Bacillus* sp. (Okazaki *et al.*, 1985).

The purified xylanase from alkalophilic *Bacillus* (NCL 87-6-10) exhibited an optimum temperature of 60°C. A similar observation was made by Horikoshi and Atsukawa (1973) with a xylanase from alkalophilic *Bacillus*. However, it is lower than the one (70°C) observed in case of xylanases from alkalophilic *Bacillus* sp. C-125 (Honda *et al.*, 1985). Temperature stability experiments showed the enzyme to be highly stable at 60°C. Similar observations were made in case of xylanase WI-2 and WII-2 from alkalophilic thermophilic *Bacillus* (Okazaki *et al.*, 1985).

Determination of kinetic parameters revealed that the Km of purified xylanase with oatspelts xylan is high (3.77 mg/ml) compared to the values reported for alkalophilic *Bacillus* sp. YC-35 xylanase (1.56 mg/ml) (SeoPark *et al.*, 1992). However, it is lower than the values (8.5 and 8.3 mg/ml) reported for xylanases from alkalophilic thermophilic

Bacilli W1 and W2 respectively (Okazaki *et al.*, 1985).

Purified xylanase from alkalophilic *Bacillus* 87-6-10 showed significant inhibition in presence of Co^{2+}, Zn^{2+}, and Mn^{2+}. Hg^{2+} was the most potent inhibitor since very low concentrations of the metal ion could completely inhibit the enzyme (Table 3.2). In this respect, our enzyme is similar to xylanases from alkalophilic thermophilic *Bacillus* (Okazaki *et al.*, 1985).

Specificity of the purified enzyme for substrates with different linkages showed that it acts only on xylan and not on related ß-1,4, ⍺-1,4, ⍺-1,6 and ß-1,3 linked polysaccharides, suggesting that it is a true xylanase as observed in several bacterial (Panbangred *et al.*, 1983; Nakajima *et al.*, 1984; Keskar, 1990) and fungal (Kitpreechavanich *et al.*, 1984; Deshpande *et al.*, 1989) cultures.

Xylanases from Bacilli are of two types namely, a xylose liberating (Uchino and Nakane, 1981; Bernier *et al.*, 1983; Dey *et al.*, 1992) and a non-xylose liberating (Estaben *et al.*, 1982; Horikoshi and Atsukawa, 1973; Panbangred *et al.*, 1983; Honda *et al.*, 1985) endo-enzymes. In the present studies, the paper chromatographic analysis of the products, following hydrolysis of oatspelts xylan by pure xylanase, revealed the presence of low molecular weight xylo-oligosaccharides including xylose, from the initial stages of

hydrolysis. With the increase in the duration of hydrolysis, the amount of low molecular weight products increased, clearly demonstrating an endo type of action. This observation suggests that the enzyme from alkalophilic *Bacillus* (NCL 87-6-10) is an endo-xylanase.

In conclusion, the xylanase from alkalophilic *Bacillus* sp. (NCL 87-6-10) is a low molecular weight endo-enzyme, highly specific for xylan. The high temperature and pH stability coupled with its optimum pH in the alkaline side makes it a potentially useful enzyme in paper and pulp industry.

REFERENCES

Adams, G.A. (1957) Can. J. Chem. 35: 556.

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

Aspinall, G.O. (1959) Adv. Carbohydr. Chem. Biochem. 14: 429.

Aspinall, G.O. and Ferrier, R.J. (1957) J. Chem. Soc. 4188.

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

Aspinall, G.O. and McGrath, D. (1966) J. Chem. Soc. 2133.

Aspinall, G.O. and Meek, E.G. (1956) J. Chem. Soc. 3830.

Aspinall, G.O. and Mahomed, R.S. (1954) J. Chem. Soc. 1731.

Bachmann, S. and McCarthy, A.J. (1991) Appl. Environ. Microbiol. 57: 2121.

Bailey, R.W. and Clarke, R.T.J. (1963) Nature 198: 787.

Bailey, R.W. and MacRae, J. (1970) J. Agr. Sci. 75: 321.

Barry, J.E., MeCornick, J.E., Mitchell, P.W.D. (1954) J. Chem. Soc. 3692.

Bastawde, K.B. (1987) Ph.D. thesis, University of Poona.

Bastawde, K.B. (1992) World J. of Microbiol. Biotechnol. 8: 353.

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

Berghem, E.R. and Pettersson, L.G. (1973) Eur. J. Biochem. 37: 21-30.

Bernfeld (1955) Methods in Enzymol. 1: 155.

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

Bertrand, J.L., Morosoli, R., Shareck, F. and Kluepfel, D. (1989) Biotechnol. Bioeng. 33: 791.

Biely, P. (1985) Trends in Biotechnol. 3: 286.

Bishop, C.T. (1953) Can. J. Chem. 31: 135.

Biswas, S.R., Mishra, A.K. and Nanda, G. (1988) Folia Microbiol. 33: 355.

Biswas, S.R., Jana, S.C., Mishra, A.K. and Nanda, G. (1990) Biotechnol. Bioeng. 35: 244.

Bjorndal, H., Eriksson, K.E., Garegg, P.J., Lindberg, B. and Swan, B. (1965) Acta Chem. Scand. 19: 2309.

Blakesley, R.W. and Boezi, J.A. (1977) Anal. Biochem. 82: 580.

Bray, M.R. and Clarke, A.J. (1990) Biochem. J. 270: 91.

Brown, J.A., Collin, S.A. and Wood, T.M. (1987) Enzyme Microb. Technol. 9: 355.

Buell, C.B. and Weston, W.H. (1947) Amer. J. Bot. 34: 555.

Carmichael, J.W. (1962) Mycologia 47: 664.

Chanda, S.K., Hirst, E.L., Jones, J.K.N. and Percival, E.G.V. (1950) J. Chem. Soc. 1289.

Chater, K.F., Hopwood, D.A., Kieser, T. and Thompson, C.J. (1982) Curr. Top. Microbiol. Immunol. 96: 69-95.

Chaudhari, S., Thakur, I.S., Goel, R. and Johri, B.N. (1988) Biochem. Int. 17: 563.

Clermont, B.S., Charpentier, M. and Fercheron, F. (1970) Bull. Soc. Chim. Biol. 52: 1481.

Cvazzoni, V., Manzoni, M., Parini, C. and Bonferoni, M.C. (1989) Appl. Microbiol. Biotechnol. 30: 247.

Dade, H.A. (1960) in "Herb" I.M.J. Handbook, p. 40, CMI, Kew England.

Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121: 404.

Debeire, P., Priem, B., Strecker, G. and Vigonon (1990) Eur. J. Biochem. 187: 573.

Dekker, R.F.H. and Richards, G.N. (1974) Carbohydr. Res. 38: 257.

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

Dekker, R.F.H. and Richards, G.N. (1975b) Carbohydr. Res. 42: 107.

Dekker, R.F.H. and Richards, G.N. (1976) Adv. Carbohydr. Chem. Biochem. 32: 277.

Dekker, R.F.H. (1985) In Biosynthesis and Biodegradation of wood components, ed. Higuchi, T., pp. 505, Academic Press, Tokyo.

Deshpande, M.V., Sadana, J.C. and Meagher, M.M. (1989) Enzyme Microb. Technol. 11: 678.

Deshpande, V., Hinge, J. and Rao, M. (1990) Biochim. Biophys. Acta. 1041: 172.

Dey, D., Hinge, J., Shendye, A. and Rao, M. (1992) Can. J. Microbiol. 38: 436.

Dobozi, M.R., Szakacs, G. and Brushi, C.V. (1992) Appl. Environ. Microbiol. 58: 3466.

Dubeau, H., Chahal, D.S. and Ishaque, M. (1987) Biotechnol.

Letts. 9: 275.

Ehrenthal, I., Montgomery, R. and Smith, F. (1954) J. Amer. Chem. Soc. 76: 5509.

Eriksson, K.E. and Petterson, B. (1971) Int. Biodeterior. Bull. 7: 115.

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

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

Fennel, D.I. (1960) Bot. Rev. 26: 79.

Fournier, A.R., Frederick, M.M., Frederick, J.R. and Reilly, P.J. (1985) Biotech. Bioeng. 27: 539.

Frederick, M.M., Kiang, C.H., Frederick, J.R. and Reilly, P.J. (1985) Biotech. Bioeng. 27: 525.

Fukui, S. and Sato, M. (1957) Bull. Agri. Chem. Soc. Japan, 21: 392.

Fuentes, J.L. and Robert, M. (1986) French Patent 8613208.

Funaguma, T., Naito, S., Morita, M., Okumura, M., Sugiura, M. and Hara, A. (1991) Agri. Biol. Chem. 55: 1163.

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

Gattinger, L.D., Duvnjak, Z. and Khan, A.W. (1990) Appl. Microbiol. Biotechnol. 33: 21.

Grabski, A.C. and Jeffries, T.W. (1991) Appl. Environ. Microbiol. 57: 987.

Grajek, W. (1987) Biotechnol. Letts. 9: 353.

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

Gordon, R.E., Haynes, W.C. and Pang, C.H.N. (1973) The Genus *Bacillus*, U.S. Dept. Agric. Handbook 427.

Hamilton, J.M. and Weaver, L.O. (1949) Phytopathol. **33**: 612

Hashimoto, K. and Takahashi, Z. (1970) Toyo Shokuhin Kogyo Tanki Diagaku **9**: 320.

Haynes, W.C., Wickerham, L.J. and Hesseltine (1955) Appl. Microbiol. **3**: 36.

Hesseltine, C.W., Bradle, B.J. and Benjamin (1960) Mycologia 82: 762.

Hesseltine, C.W. and Haynes, W.C. (1973) Prog. Ind. Microbiol. **12**: 1.

Hoebler, C. and Brillouet, J.M. (1984) Carbohydr. Res. **128**: 141.

Holden, F.R. and Walton, J.D. (1992) Physiol. Molec. Plant Pathol. **40**: 39.

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

Horikoshi, K. and Atsukawa, Y. (1973) Agri. Biol. Chem. 37: 2097.

Howard, B.H., Jones, G. and Purdom, M.R. (1960) Biochem. J. 74: 173.

Huang, L., Hseu, T.H. and Wey, T.T. (1991) Biochem. J. 278: 329.

Hulme, M.A. and Stranks, D.W. (1971) J. Gen. Microbiol. **69**: 145.

Ikura Y. and Horikoshi, K. (1987) Agric. Biol. Chem. 51: 3143.

Ishaque, M. and Kluepfel, D. (1981) Biotechnol. Letts. 3: 481.

Ito, K., Ogasawara, H., Sugimoto, T. and Ishikawa, T. (1992) Biosci. Biotechnol. Biochem. 56: 547.

Iwamoto, T., Sasaki, T. and Inaoku, H. (1973a) Mem. Ehime Univ. 17: 13.

Iwamoto, T., Sasaki, T. and Inaoku, H. (1973b) Mem. Ehime Univ. 17: 185.

Jones, J.K.N. and Painter, T.J. (1959) J. Chem. Soc. 573.

Jurasek, L. and Paice, M.G. (1986) Chemtech. 16: 360.

Karsila, K., Kruss, I. and Puppo (1990) Eur. Pat. Appl. 351655.

Kato, Y. and Nevins, D.. (1984) Plant Physiol. 75: 753.

Kawaminami, T. and Iizuka, H. (1969) Agric. Biol. Chem. 33: 1787.

Kelly, C.T., O'Mahony, M.R. and Fogarty, W.M. (1989) Biotechnol. Letts. 11: 885.

Keskar, S.S. (1990) Ph.D. thesis, University of Poona.

Keskar, S.S. (1992) Biotechnol. Letts. 14: 481

Keskar, S.S., Srinivasan, M.C. and Deshpande, V.V. (1989) Biochem. J. 261: 49.

King, M.J. and Fuller, D.B. (1968) Biochem. J. 108: 571.

Kitada, M., Wijayanti, L. and Horikoshi, K. (1987) Agric. Biol. Chem. 51: 2429.

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

Ferment. Technol. 62, 415.

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

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

Kluepfel, D., Mehta, S.V., Aumont, F., Shareck, F. and Morosoli, R. (1990) Biochem. J. 267: 45.

Kock, S.G. and Kristner, A (1969) J. Gen. Microbiol. 55: 459.

Koyama, H., Ujiie, M., Taniguchi, H. and Sasaki, T. (1990) Enzyme Microb. Technol. 12: 218.

Krishnamurthy, S. and Vithayathil, P.J. (1989) J. Ferment. Bioeng. 67: 77.

Kristner, A. and Gouws, L. (1964) J. Gen. Microbiol. 34: 447.

Krulwich, T.A. and Guffanti, A.A. (1989) Ann. Rev. Microbiol. 43: 435.

Kruss, I., Laine, J. and Koljonen, M. (1991) Int. Pat. Appl. No. W091/02840.

Kruss, I. and Koljonen, M. (1991) Int. Pat. Appl. No. W091/02791.

Kunitz (1947) J. Gen. Physiol. 30: 291.

Kusakabe, T., Kanaguchi, M., Yasiu, T. and Kubayashi, T. (1977) Nippon Nogei Kaishi 51: 429.

Laemmli, U.K. (1970) Nature 227: 680.

Lany, F.T., Price, D., Dey, D.W. and May, E.M. (1969) Trans. Br. Mycol. Soc. 53: 328.

Lappalainen, A. (1986) Biotechnol. Appl. Biochem. 8: 644.

Leathers, T.D. (1986) Appl. Environ. Microbiol. 52: 1026

Leathers, T.D., Detroy, R.W. and Bothast, R.J. (1986) Biotechnol. Letts. 8: 867.

Lee, S.F., Forseberg, C.W. and Rattray, J.B. (1987) Appl. Environ. Microbiol. 53: 644.

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

Lyr, H. (1960) Arch. Microbiol. 35: 258.

Magee, R.J. and Kosaric, M. (1985) Adv. Biochem. Eng. Biotechnol. 32: 61.

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

Marmur, J. and Doty, P. (1962) J. Mol. Biol. 5: 109.

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

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

McCleary, B.V., Gibson, T.S., Allen, H. and Gams, T.S. (1986) Die Starke 38: 433.

McDonald, K.D. (1972) Appl. Microbiol. 23: 990.

McNeil, M., Albersheim, P., Taiz, L. and Jones, R.L. (1975) Plant Physiol. 55: 64.

Meyer, E. (1955) Mycologia 47: 664.

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

Mishra, C., Forrester, I.T., Kelley, B.D., Burgess, R.R. and Leatham, G.F. (1990) Appl. Microbiol. Biotechnol. 33: 226.

Mitsuishi, Y., Yamanobo, T., Yagisawa, M. and Takasaki, Y.

(1987) Agric. Biol. Chem. 51: 3207.

Mohammad, I., Rajoka and Malik, K.A. (1984) Biotechnol. Letts. 6: 597.

Monti, R., Terenzi, H.F. and Jorge, J.A. (1991) Can. J. Microbiol. 37: 675.

Morosoli, R., Bertrand, J.L., Mondou, F., Shareck, F. and Kluepfel, D. (1986) Biochem. J. 239: 587.

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

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

Ohkoshi, A., Kudo, T., Mase, T. and Horikoshi, K. (1985) Agric. Biol. Chem. 49: 3037.

Okada, H. (1988) Advances in Protein Design International Workshop 12: 81.

Okazaki, W., Teruhiko, A., Horikoshi, A. and Akahoshi, R. (1984) Appl. Microbiol. Biotechnol. 19: 335.

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

Okeke, B.C. and Paterson, A. (1992) World J. Microbiol. Biotechnol. 8: 483

Oniens, A.H.S. (1971) Meth. in Microbiol. 4: 113.

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

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

Panbangred, W., Shinmyo, A., Kinoshita, S. and Okada, H.

(1983) Agric. Biol. Chem. 47: 957.

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

Paul, J. and Varma, A.K. (1990) Biotechnol. Letts. 12: 61.

Paul, J. and Varma, A.K. (1992) Biotechnol. Letts. 14: 207.

Pawar, H.S., Bodhe, A.M., Rele, M.V. and Vartak, H.G. (1986) J. Biosci. 13: 43.

Perlman, D. and Kikuchi, M. (1977) in "Annual Reports on Ferm. Processes", Vol. 1 ed., Pearlman, p. 41 in "Herb", I.M.J. Handbook.

Priem, B., Dobberstein, J. and Emeis, C.C. (1991) Biotechnol. Lett. 13 149.

Pou-Llinas, J. and Driguez, H. (1987) Appl. Microbiol. Biotechnol. 27: 134.

Rajaram, S. and Varma, A. (1990) Appl. Microbiol. Biotechnol. 34: 141.

Raper, K.B. and Alexander, D.J. (1945) Mycologia 37: 499

Ratoo, M., Poutanen, K. and Vikari, L. (1992) Appl. Microbiol. Biotechnol. 37: 470.

Reese, E.T. and Maguire, A. (1969) Appl. Microbiol. 17: 242.

Reilly, P.J. (1981) Basci Life Sciences 18: 111.

Roudier, A. (1958) Assoc. Techni. Indust. Papetiere 4: 99.

Royer, J.C. and Nakas, J.P. (1989) Enzyme Microbiol. Technol. 11: 405.

Royer, J.C. and Nakas, J.P. (1991) Eur. J. Biochem. 202: 521.

Sadana, J.C., Shewale, J.G. and Deshpande, M.V. (1980) Appl. Environ. Microbiol. 39: 935.

Saddler, J.N., Hogan, C.M. and Louis, S.G. (1985) Appl. Microbiol. Biotechnol. 22: 139.

Sakka, K., Maeda, Y., Hakamada, Y., Takanishi, N. and Shimada, K. (1991) Agric. Biol. Chem. 55: 247.

Sarkar, C. and Prabhu, K. (1983) Agric. Waste 6: 99.

Sasaki, T. and Inaoka, M. (1967) Mem. Ehime Univ. 12: 149.

Sasaki, T.(1971) Ehime Daigaku Nagakubu Kiyo 15: 71.

Savur, G.R. (1956) J. Chem. Soc. 2600.

Schulze, E. (1891) Ber. 24: 2277.

SeoPark, Y., Yum, D.Y., Bai, D.H. and Yu, J.H. (1992) Biosci. Biotechnol. Biochem. 56: 1355.

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

Siddiqui, I.R. and Wood, P.J. (1977) Carbohydr. Res. 54: 231.

Simpson, H.D., Haufler, U.R. and Daniel, R.M. (1991) Biochem. J. 277: 413.

Smith, G. (1969) in "An introduction to Industrial Mycology, Edward Arnold Ltd., London, p. 233.

Smith, D.C. and Wood, T.C. (1991) World J. Microbiol. Biotechnol. 7: 343.

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

Steiner, W., Lafferty, R.M., Gomes, I. and Esterbauer, H. (1987) Biotechnol. Bioeng. 30: 169.

Strobel, G.A. (1963) Phytopathology 53: 592.

Stuettgen, E. and Sahm, H. (1982) Eur. J. Appl. Microbiol. Biotechnol. **15**: 93.

Takenishi, S., and Tsujisaka, Y. (1973) Agric. Biol. Chem. **39**: 2315.

Tan, L.U.L., Wong, K.K.Y., Yu, E.K.C. and Saddler, J.N. (1985) Enzyme Microbiol. Technol. **7** :425.

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

Tangnu, S.K., Blanch, H.W. and Wilke, C.R. (1981) Biotechnol. Bioeng. **23**: 1837.

Tenkanen, M., Puls, J. and Poutanen, K. (1992) Enzyme Microb. Technol. **14**: 566.

Thaker, A.J., Ray, R.M. and Patel, H.C. (1986) Ind. J. Exp. Biol. **24**: 659.

Timell, T.E. (1964) Adv. Carbohydr. Chem. Biochem. **19**: 247.

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

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

Trevelyan, W.E., Procter, D.P. and Harrison, J.S. (1950) Nature **166**: 444.

Tripathi, C.K.M., Ali, S. and Srivastava, R. (1992) Ind. J. Exp. Biol. **30**: 741.

Tsujibo, H., Miyamoto, K., Kuda, T., Minami, K., Sakamoto, T., Hasegawa. T. and Inamori, Y. (1992) Appl. Environ. Microbiol. **58**: 371.

Tsujisaka, S., Takenishi, S. and Fukumoto, J. (1971) Nippon

Nogei Kagaku Kaishi **45**: 253.

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

Ujiie, M., Roy, C. and Yaguchi, M. (1991) Appl. Environ. Microbiol. **57**: 1860.

Viikari, L., Kantelinen, A., Ratoo, M. and Sundquist, J. (1990) Amer. Chem. Soc. Symp. **460**: 12.

Viet, D.N., Kamio, Y., Abe, N., Kaneko, J. and Izaki, K. (1991) Appl. Environ. Microbiol. **57**: 445.

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

Walker, D.J. (1961) Aust. J. Agric. Res. **12**: 171.

Warzywoda, M., Ferre, V., Pourquie, J. (1983) Biotechnol. Bioeng. **23**: 3005.

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

Whistler, R.J. and Corbett, W.M. (1955) J. Amer. Chem. Soc. **77**: 6327.

Wong, K.K.Y., Tan, L.U.L. and Saddler, J.N. (1986) Can. J. Microbiol. **32**: 570.

Wong, K.K.Y., Tan, L.U.L. and Saddler, J.N. (1988) Microbiol. Rev. **52**: 305.

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

Woodward, J. (1984) "Topics in enzyme and fermentation biotechnology" (Ed. Wiseman, H.) John Wiley and Sons, NY **8**: 9.

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

Yoshida, N., Fukushima, T., Saito, H., Shimosaka, M. and Okazaki, M. (1989) Agric. Biol. Chem. **53**: 1829.

Yoshoika, H.. Nagata, N., Chavanich, S., Nilubol, N. and Hayashida, S. (1981) Agric. Biol. Chem. 45: 2425

Yu, E.K.C., Tan, L.U.L. and Chan, M.K.H., Deschatelets, L. and Saddler, J.N. (1987) Enzyme Microbiol. Technol. 9: 16.

Zamost, B.L., Nielsen, H.K. and Starnes, R.L. (1991) J. Ind. Microbiol. 8: 71-82.

Zimmermann, W., Winter, B. and Broda, P. (1988) FEMS Microbiol. Letts. 55: 181.