THE ENZYMIC UPGRADATION OF JUTE CUTTINGS

A DISSERTATION SUBMITTED
TO THE UNIVERSITY OF DHAKA

IN PARTIAL FULFILLMENT OF THE
REQUIREMENT FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

BY

MD. SERAJUL HAQUE TALUKDER

Department of Biochemistry
University of Dhaka, Dhaka
December, 1997

Principal Scientific Officer
Bangladesh Jute Research Instt.
Manik Mia Avenue, Dhaka-1207

DEDICATED

TO

MY LATE PARENTS

BELOVED WIFE

AND AFFECTIONATE SONS

(PAVEL & BONNY)

ACKNOWLEDGEMENTS

I would like to express my heartfelt gratitude to my reverend teacher **Prof. K. Ahmed** for his invaluable suggestions and interest throughout this study.

I am grateful to my supervisor **Dr. G. Mohiuddin, C.S.O, Biotechnology Division, BJRI**, Dhaka for suggesting me the topic of this dissertation and for his constant and sincere guidance throughout this study. Without his bountiful support and patience it would not have been possible to complete this work.

I am greatly indebted to **Prof. Khalilur Rahman, Chairman,** Dept. of Biochemistry, University of Dhaka for his inspiration and valuable comments about this study. I am also grateful to Prof. Rafiqul Islam, Dept. of Biochemistry, University of Dhaka, for his encouragement and constructive suggestion whenever sought.

I must acknowledge the help and sincere cooperation of Sk. A. Hasib and Miss Afsana Marzina of BJRI throughout this study.

I am greatly obliged to Mr. Md. Siddiqur Rahman, P.S.O. of BJRI (Technology) for going through the manuscript and making necessary corrections.

382473

I would like to acknowledge the help and co-operation extended by the members of Biotechnology Division of BJRI (Technology), friends and well wishers of BJRI, Manik Mia Avenue, Dhaka.

Finally, I am grateful to **IDRC** (Canada), **IJO** and **Director General, Director (Tech)** of BJRI for providing me assistance in the form of chemicals and laboratory facilities.

The Author

ABSTRACT

Jute is a lignocellulosic bast fibre. It is obtained from two cultivated species viz. C.capsularis and C.olitorius by a biological process known as retting. The quality of jute is mostly influenced by the retting process. But due to improper retting and over maturity the bottom portion of jute plant remains under retted and results in a bulk production of low grade jute with barks and specks. The stiff bottom portions of most of the fibres are cut down in the mills and are known as "cuttings". The low grade jute and cuttings together constitute almost 40-50% of total fibre production. These fibres can not be processed as such and an additional mill softening treatment is required to make them suitable for processing. But softening in this conventional process is inadequate and thus limits the use of these fibres to the production of sacking only.

In order to upgrade the present mill softening process a detailed study of temperature rise, microbial load and pH changes during piling process were recorded. Highest temperature of 65°C and thermophilic microbes 4.8×10^6/gm of fibre were recorded on 5th days of piling process. pH of

water extract was 6.5 at the beginning of the piling process which was changed to pH 5.47 at the end. From these study it was revealed that a number of microbes and their enzymes were implicated in the softening process. Hence the present softening process can be improved either by application of suitable microbes or by addition of a crude enzyme preparation during the piling process. The enzymatic upgradation process was chosen for its suitability and effectiveness.

Microbes, specially the mesophilic fungi, were isolated from their natural habitats. After screening the CMCase, PGase and xylanase activities the most potential one was chosen from 20 isolates. A pectinolytic fungus Aspergillus sojae from ATCC and a commercial enzyme preparation Flaxzyme (NOVO) were also collected for comparative study.

The simplicity and inexpensiveness of solid state fermentation makes it the most suitable and attractive process for production of jute softening enzymes. Among the indigenous substrates, wheat bran with 50% added moisture was found to be the most suitable medium for growth and maximum enzyme production by both the fungi. Aspergillus niger

produced maximum 2.62 IU/ml and Aspergillus sojae produced 2.16 IU/ml of enzymes with optimum moisture level of 50% at 32°C in 3 days fermentation.

Enzymes were extracted from fermented bran by adding 5 volumes of (W/V) 1% NaCl solution. The milipore filtered culture filtrate was used for assay of enzymes following the DNS method. Enzymes were found to be quite stable for 28 days at room temperature with 0.1% (W/V) sodium azide as preservative. Enzyme activities were optimised in respect of temperature and pH. Temperature 50°C, 48°C and 45°C were found to be optimum temperature for CMCase, Xylanase and PGase components respectively of the enzyme extracts. And pH range 5.5 - 6.5 was found to be optimum for the three enzyme components. Effect of various ion on enzyme activities were studied and some of them have enhancing effect and the others have inhibitory effects. Enzyme components were tested for their thermal stability. Enzymes component CMCase and Xylanase were found to be more heat stable than PGase. Both the enzyme extracts retained almost 50% of their original activities after storage at 40-60°C for 72 hours. pH stabilities of the enzyme extracts were carried out and the were found to be stable at the pH range 5-7. Kinetic study c

xylanase component of culture filtrate of A.niger was carried out. The K_m value of xylanase component was calculated as 2.3. mg/ml Enzyme extract of A.niger was run through gel electrophoresis for the determination of molecular weights of its enzyme components. And accordingly 14,000, 66,000, 36,000, 24,000 were found to be the molecular weights of CMCase, Exo-PGase, endo-PGase and xylanase respectively.

In order to compete with commercial enzyme preparation attempts were made to develop hyper enzyme producing mutants by Gamma radiation from the potential fungi. A few mutants were found to be promising and their real development and establishment as hyper enzyme producing mutants need further endeavour in respect of their cultural exploitation.

Enzyme extracts were applied on low grade jute and cuttings to assess their effectiveness in softening and upgrading the fibres. Enzyme treated fibre showed improved physico-mechanical behaviours in respect of separation index, fibre fineness and stiffness values than untreated ones. To evaluate the enzymatic hydrolytic removal of non-cellulosic materials different constituent of enzyme treated fibre were estimated. Enzyme action removed almost 30% hemicellulose and

40% pectin from the low grade fibres but cellulose fraction was least removed.

Large scale enzyme production was carried out in flat aluminum trays and enzyme extracts were mixed well with jute softening emulsion and applied to the fibres through a fibre softening machine. Enzyme action softened the fibres with reduction of maturation time to half of the normal time. Pilot scale trials were carried out in Bangladesh Jute Research Institute (BJRI) experimental mill and the improved spinning performances of enzyme treated fibres were evaluated. A marked improvement in spinning behaviour of enzyme treated low grade fibres was recorded. Enzyme treated low grade jute could be spun into yarns of higher quality ratio and lower C.V. value without impairing the production efficiency. The enzyme action transformed the coarse and stiff fibres into finer, smoother and more pliable ones and consequently their spinning behaviour was improved. For improved spinning performances the enzyme treated low grade fibres could easily be blended with superior/higher grade fibres/batches for production of quality jute goods and thereby the production cost of jute goods could be reduced appreciably.

LIST OF ABBREVIATION

et al	=	and others
gm	=	gram
l	=	litre
ml	=	millilitre
°C	=	Degree celsius
r.p.m	=	Rotation per minute
W/V	=	Weight/volume
V/V	=	Volume/volume
nm	=	Nano meter
%	=	Percent
pH	=	Negative logarithm of hydrogen ion (H^+) concentration
DNS	=	Dinitrosalicylic acid
i.e	=	that is
M	=	Molar
Sp.	=	Species
min.	=	Minute
BSA	=	Bovine Serum Albumin
SSF	=	Solid State Fermentation
SMR	=	Straight Morah Rejection

CONTENTS

produced maximum 2.62 IU/ml and Aspergillus sojae produced 2.16 IU/ml of enzymes with optimum moisture level of 50% at 32°C in 3 days fermentation.

Enzymes were extracted from fermented bran by adding 5 volumes of (W/V) 1% NaCl solution. The milipore filtered culture filtrate was used for assay of enzymes following the DNS method. Enzymes were found to be quite stable for 28 days at room temperature with 0.1% (W/V) sodium azide as preservative. Enzyme activities were optimised in respect of temperature and pH. Temperature 50°C, 48°C and 45°C were found to be optimum temperature for CMCase, Xylanase and PGase components respectively of the enzyme extracts. And pH range 5.5 - 6.5 was found to be optimum for the three enzyme components. Effect of various ion on enzyme activities were studied and some of them have enhancing effect and the others have inhibitory effects. Enzyme components were tested for their thermal stability. Enzymes component CMCase and Xylanase were found to be more heat stable than PGase. Both the enzyme extracts retained almost 50% of their original activities after storage at 40-60°C for 72 hours. pH stabilities of the enzyme extracts were carried out and they were found to be stable at the pH range 5-7. Kinetic study of

List of Tables

List of Figures

List of Plates

1. Introduction :

Jute is a lignocellulosic bast fibre. The fibres of commercial importance are obtained from the bark of two cultivated species, Corchorus capsularis and Corchorus olitorius. of the family Tiliaceae. Among the fibre crops, jute occupies a position next only to cotton and is used extensively in the manufacture of different types of packing materials.

1.1 Production, export and importance of jute :

Soil and climatic conditions of Bangladesh make it an ideal site for jute cultivation. Jute is the most important commercial crop of Bangladesh and it plays a vital role in her national economy. Jute is still the larger export earner of the country. About 40-60 lakh bales (0.9-1.2 million tons) of raw jute are being produced annually in Bangladesh alone and this is about 30% of total world production (Table-1). The importance of jute has been revealed by the fact that Bangladesh is the premier exporting country of jute and jute goods and her economy is related with the jute and its future. Jute sector is the largest employer in the form of farm families, industrial worker and persons engaged in trade aspects of jute.

Table-1

World Production and Exports of Jute

PRODUCTION AREAS	PRODUCTION (1993/94)	EXPORT* (1993)
	(000 m.t.)	
WORLD	2,968.50 (100%)	1,273.80 (100%)
BANGLADESH	783.00 (26.37%)	799.70 (62.78%)
CHINA	650.00 (21.89%)	80.10 (6.28%)
INDIA	1,224.00 (41.23%)	217.90 (17.10%)
NEPAL	10.00 (0.33%)	10.70 (0.84%)
THAILAND	134.00 (4.51%)	65.00 (5.10%)
REST OF WORLD	167.50 (5.64%)	100.40 (7.88%)

* Quantity by weight, expressed in raw jute equivalents.
Source : FAO, Jute, Kenaf and allied fibres, June 1994.
CCP: JU/ST/94/1

1.2 The chemistry of jute fibre :

Jute is a composite fibre and the main constituents of jute fibre are alpha cellulose (60-65%), hemicellulose (15-23%) and lignin (12-14%). The minor constituents are fats., waxes, pectins, etc. Chemical constituents of jute along with other natural fibres are shown below (Table-2):

Table-2

Chemical constituents of jute fibre with other natural fibres

Fibre	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Pectin (%)	Fats (%)	Waxes (%)
Jute (Good quality)	64.00	20.00	14.00	0.50	1.00	0.30
Jute (Low grade)	61.00	20-21	14.00	2-4	1.00	0.30
Cotton	98.20	---	---	---	1.10	0.70
Hemp	77.50	10.10	6.80	2.90	1.80	0.90
Flax	71.30	18.50	2.20	2.00	4.30	1.70
Ramie	76.60	8.00	5.60	3.80	5.60	0.40

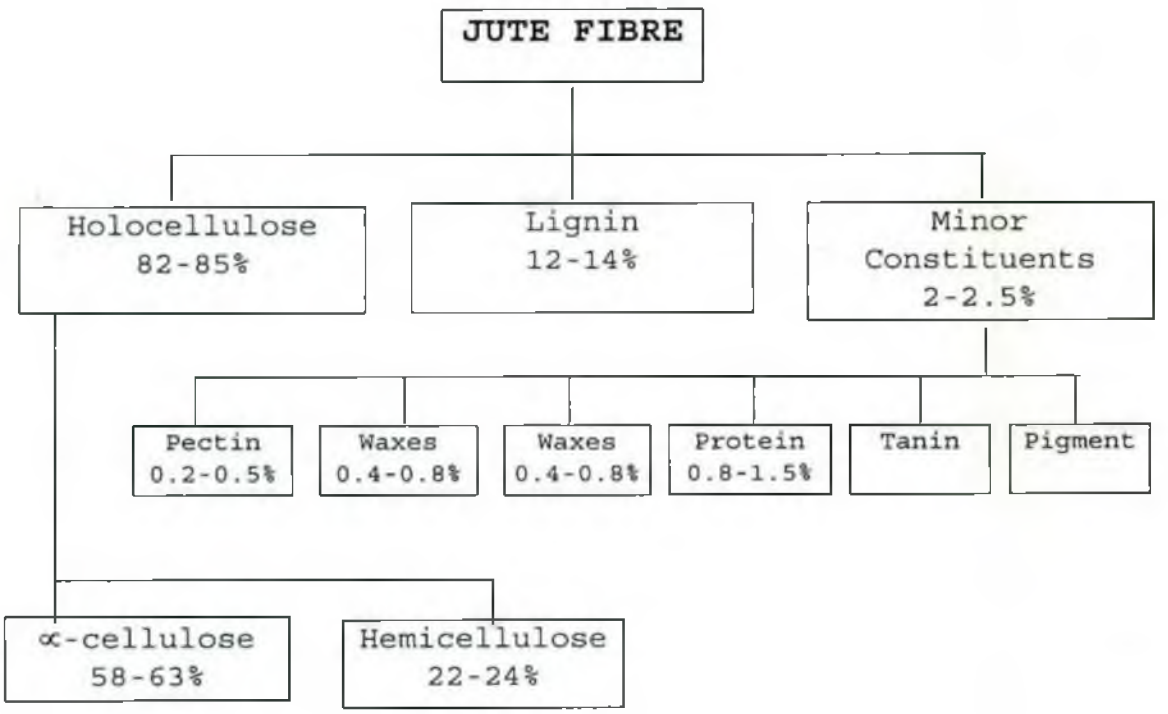
Source: M.Azizur Rahman et al.B.J.Jute Fib.Res.15 (1&2) 23-34, 1990

Chemically jute is a lignocellulosic fibre and cellulose, hemicellulose and lignin are its major constituents. But the low grade jute and cuttings contain higher percentage of non-cellulosic materials specially pectin (2-4%) than the upper and middle portion of better quality jute fibres (Mohiuddin et al. 1978).

The quality of jute is usually judged by its suitability for the production of various types of yarns and its behaviour in the manufacturing process. Jute with its major components constitute a three dimensional configuration. The physico-mechanical behaviour of jute fibre depends on the shape and size of cellulose molecule, fibrillar arrangement, various bonds and interaction of non-cellulosic constituents of the fibre.

The x-ray diffraction study revealed the crystalline and amorphous region in jute fibre. Macromolecular polymeric structure is built of cellulose, hemicellulose and lignin in a three dimensional pattern where cellulose is mainly in the crystalline part. Hemicellulose, lignin, protein, fat, wax are present in the noncrystalline amorphous and para crystalline part. The physical properties such as elasticity, lustre, softness, etc. depend on the amount of crystalline orderly matter present in fibre. On the other hand chemical behaviours such as swelling, dye acceptability, etc. depend on the disordered amorphous portion of the fibre.

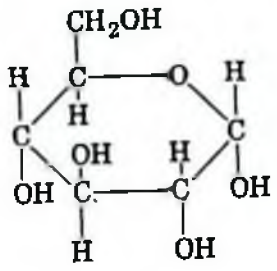
1.2.1 Chemical Composition of Jute at a glance :



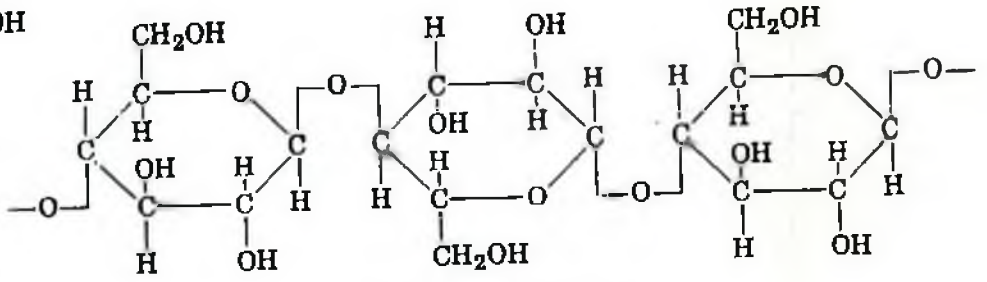
1.2.2 Cellulose of Jute :

Cellulose is a prominent constituent of higher plants and abundant in nature. It is the major component of jute (60-65%). Jute cellulose is the cellulose obtained either by Cross and Bevan method or Norman and Jenkin's sodium chlorite method. The yield and composition of cellulose vary considerably according to the extraction methods. In jute a number of other polysaccharides like hemicellulose, pectin and lignin are associated with the cellulose (Jute in India-1958). The chemical identity of jute cellulose was established from the yield of crystalline glucose and cellulose acetate (Chowdhury and Basu - 1932). It was also established that cellulose from jute had the same crystalline structure as those from cotton, flax, ramie, etc. (Herzog and Jancke - 1920). The size of cellulose molecule i.e. degree of polymerisation (DP) was established using cellulose from jute through the measurement of its viscosity in cuprammonium solution (Chowdhury and Bardhan - 1936). Different workers have found variable D.P. values for jute cellulose and the variation was possibly due to partial degradation during isolation. DP value of jute cellulose was established to be 1150-1400 (Pal and Sarker - 1954). Efforts were made to

establish a direct relationship between spinning quality of jute and its cellulose content but no such correlation could be established (Barker - 1939).



glucose



β-1,4-glucoside structure

In structure, cellulose is a polymer of glucose units bound together in long chain of b-linkages at carbon atoms 1 and 4 of the sugar molecule.

Jute cellulose exists in an organized state known as the fibrous crystal. Structurally, the smallest fibre unit of cellulose is the elementary fibril. These fibrils are, in fact, made of sequences of the crystallite unit. There are two representative structural models - fringed fibrillar model and folding chain model regarding the molecular orientation in the crystallite. Among the fibrils, there are

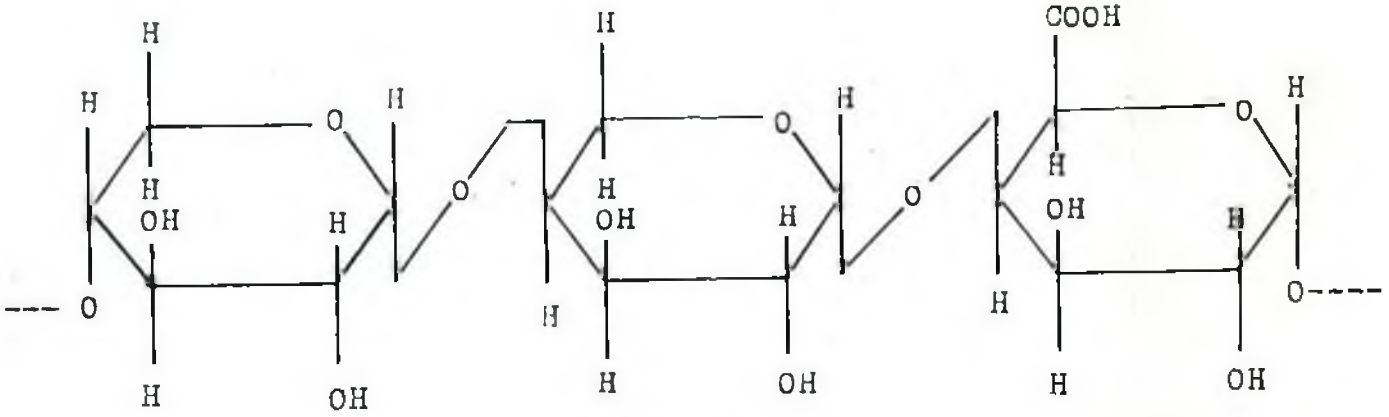
intermittent highly ordered areas of crystalline regions separated by less ordered amorphous regions.

1.2.3 Hemicellulose of Jute fibre :

Hemicellulose ranks next to cellulose in quantity and is present in all layers of plant cell where they are closely associated with cellulose and lignin. The hemicellulose content in jute was found to be 15-18% (Norman - 1936). The fibres with high xylan content is characteristic of a fibre of low quality (Barker - 1939). Hemicelluloses are a class of water insoluble polysaccharides extracted from plants by dilute alkali. They yield hexoses, pentoses and uronic acid upon hydrolysis with hot dilute mineral acid. This is often named xylan on the basis of its main sugar component xylose. Thus xylan serves as an integral part of the cellulosic structure of plants and natural fibres besides cotton. The polyuronide hemicellulose is present in plant materials and the predominant unit in the molecule is xylose. In nature xylose polymers occur in two forms, one is associated with glucuronic acid in the polyuronide hemicellulose and the other is found as the simple polymerised chain of pentose units, the xylan. Estimates of the molecular size indicate

the presence of 50-150 pentose units in the xylan chain. Most of the hemicelluloses are in the from of branched heteropolymers consisting of 2 to 4 and occasionally 5 to 6 different sugar residues. Xylose, mannose, glucose, galactose, arabinose, glucuronic acid and 4-6 methyl-D-glucuronic acid residues comprises the majority of hemicellulose sugar (Reilly - 1977).

Xylan is a major component of hemicellulose. Xylan consists of a structure of D-xylose residues and often has a-L- arabinofuranose and glucuronic acid side chains. Although overall structures of most xylans have not been determined the predominant linkages and common structural features are known. In both hard and soft woods hemicelluloses are 0-acetylgacto-glucomannan and arabian (4-0-menthyl glucurono) xylan.



Chemical structure of a part of hemicellulose .

With the exception of the galactose-based hemicellulose, which are linked b-1, 3, all plant hemicelluloses share a main chain structure based on b-1, 4 linked glycopyranosal polymers.

Thus the main chain structure if not the conformation, the xylans and glucomannan is quite similar to that of cellulose. Unlike cellulose, however, plant hemicelluloses have monosaccharide units ranging from 50-200 units (whistler and Richards - 1970); nearly all show some degree of branching.

The structural characteristics of hemicellulose as well as the number and proportions of different sugar residues present (degree of hetero-polymerization) largely determine the observed physical properties of various hemicelluloses.

In nature decomposition of hemicellulose takes place initially at a rapid rate, but later at a slower rate. This variation in decomposition is probably due to chemical heterogeneity of the hemicellulose fraction. Hemicelluloses of more mature plants are degraded more slowly than those in younger tissue (Martin et al., 1961). Various fungi, bacteria and actinomycetes can decompose hemicelluloses when they are

used as the sole sources of carbon and energy. The fungal groups - the imperfect fungi, the Phycomycetes, Ascomycetes and Basidiomycetes have the capacity to degrade hemicelluloses. The active micro-flora first hydrolyse the hemicellulose by means of their extracellular enzymes to shorter fragments which the cell can assimilate.

1.2.4 Lignin in Jute :

The third major constituent of jute is lignin. It seems to be united with the uronic acid of the hemicellulose in an ester linkage. Undegraded native lignin, as it occurs in jute, can hardly be obtained by the existing methods. However, a number of workers came to the conclusion that jute lignin is not different from other lignins. Structurally lignin is an aromatic polymer rather than being of carbohydrate type. The molecule is a polymer of aromatic nuclei with either a single repeating unit or several similar substances as the basic building blocks. The basic unit in lignin seems to be a phenyl - propane (C_6-C_3) type of structure containing one methoxyl - carbon, C_6 representing the benzene ring linked to a C_3 propyl- type side chain. The benzene nucleus contains a hydroxyl group in addition to the

methoxyl. Methoxyl disappearance has some times been used to indicate lignin break down. In plants lignin is formed by the initiation of enzyme through dehydrogenative polymerization of three primary lignin precursors: trans-p-cumaryl alcohol, trans-coniferyl alcohol and trans-sinapyl alcohol (Sarkanen et al., 1971). Molecular weight of lignin molecule itself varies from 300-11,000.

Sarkar 1971 studied jute lignin extensively and came to the conclusion that jute lignin is not different from other lignins. Lignin does not appear to serve as the cementing material for the cellulose ultimates in bast or leaf fibres in as much as the fibre structure remains intact.

Little work has been reported about the natural lignin degradation by enzymes. Yet a number of workers have reported lignolytic activity of fungi specially the white rot fungi and wood degradation fungi (Ulla Westermark and Karl-Erik Eriksson - 1974). They reported that polypous Versicolor. a white-rot fungi, produced an extra-cellular enzyme polyphenol oxidase which reduced the stable quinone. The enzymes which are used by microbes for the degradation of lignin type compounds have been found to be extracellular and highly

substrate specific and are unlikely to attack lignin. From this findings it is assumed that the enzymes which degrade lignin macromolecules may be very different from those that attack lignin model compounds. Oxidase of Fusarium solanin has been reported to non-specifically oxidise a wide range of lignin model compounds. The enzyme cellobiose-quinone oxidoreductase has been reported to take part in both lignin and cellulose degradation. It is established that the phenol oxidases are absolutely required in lignin degradation through its exact role is yet to be elucidated. (Bisaria and Ghose - 1981).

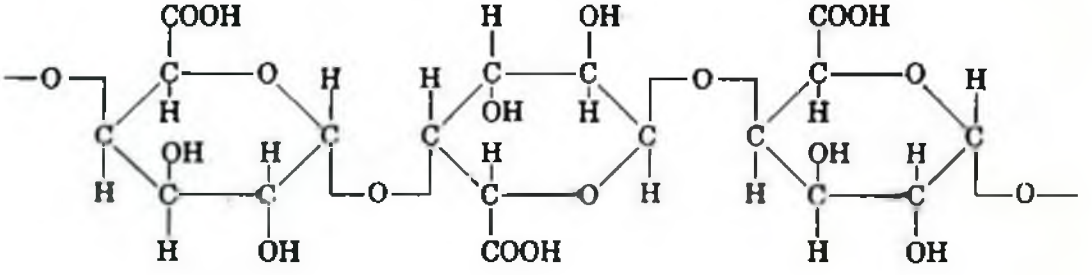
1.2.5 Pectin of Jute :

Pectin substances occur in the jute plant (2-5%) and their importance lies to the physical structure of the plant. Fairly well retted good quality fibre contains less than 0.5% pectin. But in low grade jute the pectin substances do not decompose fully during retting, (Mohiuddin et al, 1978). That is why the low grade fibres contain more pectic materials (2-4%) than good quality fibres. Thus the high content of pectic substances hold the fibre filaments together making them hard and compact. A number of workers have suggested that if this

pectic materials of low grade jute can be decomposed by some methods the resultant fibres would be more softer, separable and easy to spin (Shaha et al., 1952).

The pectin materials are found as calcium and magnesium pectates in the middle lamella, a tissue constituent located between individual cells. The pectic carbohydrates are complex polysaccharides and composed of galacturonic acid units bound to one another in a long chain.

The pectic carbohydrates are complex polysaccharides composed of galacturonic acid units bound to one another in a long chain.



PECTIN STRUCTURE

There are three types of pectic substances: (a) protopectin, a water insoluble constituent of the cell wall, (b) pectin, a water soluble polymer of galacturonic acid

which contains methyl ester linkages and (c) pectic acids, the water soluble galacturonic acid polymers that are devoid of methyl ester linkages. Each of these three classes represents a group of closely related pectic substances.

1.3 Inherent structural drawbacks of jute :

A fibre having good length and strength, bright colour, lustre, being free from any defects and faults and with smallest percentage of cuttings is called a good quality fibre. Further, the quality of jute is judged by its suitability for production of various types of yarns and its behaviour in the manufacturing process. Jute with major components constitute a three dimensional configuration. The physico-mechanical behaviour of jute fibre depends on the shape and size of cellulose molecule, fibrillar arrangement, various bonds, and interaction of non-cellulosic components of the fibre.

The individual fibre filaments of jute are composed of a number of ultimate cells cemented together by an isotropic, non-cellulosic intercellular substance (hemicellulose, lignin and pectin) which forms a layer of middle lamella in between the fibre cell walls. The walls of the fibre cells are thick

and lignified and except for original cracks, are relatively smooth and unmarked. The ultimate fibre cells are elongated in the direction of the stem axis with pointed or tapering ends and appear more or less polygonal with well defined angles in a cross section.

Each fibre cell is known as ultimate fibre. The number of fibre cells in a bundle is variable in both the species of C.capsularis and C.olitorius. The fibre cells are 500-6500 μm in length and 10-30 μm in diameter. The fibre cells of jute are much shorter than those of cotton and other natural fibres. For spinning by ordinary methods the ultimate fibres should normally be fairly long and the length-breath ratio should be of the order of 1000 to 2000. In jute this ratio is on the average only about 98 to 118, thus limiting its use to coarse fabrics. (Kundu, 1942, 1954).

To find out fibre structure in relation to quality it was interesting to note that the ultimate fibres are much longer in the best quality fibres than those in the medium or worst quality of fibres of both the varieties. So, naturally, it is obvious that length breadth ratio is comparatively higher in best quality jute which makes them more suitable

for spinning fine yarns for quality products. It has been reported that the average quality ratio is higher in olitorius than in capsularis. (Annual Report, jute Agricultural Research Institute, 1950-51, 1951-1952). Further it was also reported that quality of jute does not depend only on the fibre length because there is little or no difference in fibre length between medium or worst quality fibres. The amount of adherent tissues also appears to affect the fibre quality. It has been found that the periderm and other unretted tissues are particularly absent in the best quality while their proportion is more in the worst than in the medium quality of fibres. So it is revealed and may be concluded that the length of ultimate fibres and the amount of periderm tissues seem to be the only structural features that govern the fibre quality (Annual Report, Jute Agricultural Research Laboratories 1942-43 to 1944-45).

In order to study the effect of plant maturity on the dimensions of ultimate fibre the length breadth, lumen size and thickness of the cell wall were studied in different stages of plant maturity. Fibres undergo some thickening and change in shape with maturity and such changes are mostly confined to a few outer most layers of the fibres. The

breadth of the fibre and lumen size attain their maximum dimensions at the bud stage, when the best quality fibre is obtained. After the bud stage the quality is reduced due to lesser moisture content of the plant which effect the retting process.

1.4 X-ray study of jute fibre :

The x-ray diffraction study gives an idea about the atomic arrangements in the crystalline areas in the fibre, average size, shape and distribution of the crystalline regions, orientation of the crystallites in relation to the fibre axis and also the degree of crystallinity.

From the positions and intensities of x-ray detraction spots on photographic film, the crystal structure of the crystallites can be determined. It was concluded from the observation of different workers that the atomic arrangement in the crystalline organisation of the jute fibre is very similar to that of other cellulosic fibres. The size of crystallites may be estimated from the width of the x-ray diffraction spots. The width increases with the decrease of crystal size. The average crystallites in jute was found to be 62A° and the dimension normal to the length were 40A° and

24A°. It may be concluded that crystalline portions in jute are small and lamellar in shape and further the length of the crystallites is correlated to the intrinsic strength of the fibre.

The degree of crystallinity i.e. the amount of crystalline cellulose in cellulosic fibre can not be exactly defined, as neither the crystalline portions are perfect crystals nor the non-crystalline portions are completely disordered. From x-ray diffraction spots of crystalline and non-crystalline portions the percent of crystallinity can be determined. The percent of crystallinity on this basis of alpha-cellulose content was 77 percent. The physical properties such as strength, elasticity etc. depend on the amount of crystalline matter present in a fibre but the chemical behavior such as dye absorption, inter crystalline swelling etc. depend on the amorphous portion of the fibre. Thus we can assume the structure of the jute fibre from the x-ray diffraction study that the crystalline areas have a high degree of order in the molecular arrangement. These crystalline areas gradually merge into highly disordered areas in the inter crystalline areas passing though varying degrees of order. The crystalline regions gave a uniaxial

orientation with the length of the crystallites making a small angle with the fibre axis. During chemical reaction, the reagents enter first into the inter-crystalline regions and gradually reach the crystallite areas.

1.5 Formation and structure of fibres in the jute plant:

The jute fibre is developed in the bark of the jute plant stem. The fibres that are commercially important are the secondary phloem or phloic fibres developed by the activity of the cambium. The fibre layers which are generally four to six cells thick which constitute only 6-7 percent of whole plant. These fibre layers are made up of a varying number of fibre bundles separated by thin walled ray cells. All the fibres of an entire plant form a fibre strand or reed which is more compact and firmer towards the base and finer towards the top. The number and size of fibre bundles depend on the activity of the cambium and also on the growth of the plant. The fibre bundles vary greatly in size and shape. The oldest fibre bundles are always towards the periphery and the youngest one next to the cambium.

The number of fibre cells in a bundle is variable and each fibre cell is known as ultimate fibre. These small

units of ultimate fibres overlap length wise and are cemented together laterally. The ultimate cells are elongated in the direction of the stem axis with pointing ends and appear more or less polygonal in outline. Bhattacharja (1961) reported that the cementing materials are mainly hemicellulose, lignin and pectin etc. The walls of the fibre cells are thick and lignified and except for occasional transverse cracks, are relatively smooth. The lumen or cell cavity is as wide as cell walls, but it has characteristic construction at irregular intervals. Sometimes the fibre cell cavity is completely closed due to the uneven thickening of the cell wall.

1.6 Retting process, extraction of fibre and its impact on quality :

Traditionally jute plant is retted biologically, preferably in clean slow moving water, after which fibre is extracted, washed and dried in the sun. All the fibres of an entire plant, when properly retted, together form a fibre strand or reed.

Retting is a process through which the fibre embedding materials need to be disintegrated and removed to make the

fibre bundles loose for extraction as commercial fibre. This process of distegration of fibre cementing materials is accomplished by the combined action of water and micro-organisms.

The structural changes that took place during retting process are due to the disintegration of tissues caused by microorganisms. The breakdown of tissues follow a definite sequence, the cambium being the first to be attacked. The microorganisms enter through the stomata and cut ends of the stem. They first disintegrate the top, then the middle and finally the basal region of the stem. As a result of their action the thin walled ray cells and soft tissues of the phloem disintegrate first and finally the cortex. When all the soft tissues are dissolved and the fibre bundles get separated, retting becomes complete. Neither the middle lamella nor the secondary wall of the fibre is attacked and the fibre bundles remain intact.

Allen (1967) reported that during retting the pectic materials binding the fibre was broken and the fibres got loosened from the stem. A fibre strand or reed, which is more compact and stiffer towards the base and finer towards the

upper end. Each fibre strand consists of a loose network comprising of many smaller strands which overlap length wise and are cemented together laterally.

The most important factor on which quality of jute mainly depends is retting and also the retting technique followed. Retting is a process by which the fibres in the bark get loosened and separated from the woody stalk due to the removal of pectin, gum and other mucilaginous cementing materials. But in over matured stems the lignified and suberized tissue resists distegration and the fibre bundles instead of being separated, come off along with the cortex and periderm, yielding an inferior quality of jute. Thus the amount of adherent tissue affect the quality of fibres to a great extent. Incomplete disintegration of fibre embedding materials results in production of hard and stiff fibres with barks and specks. As a result of incomplete retting a dark strip of dried up periderm and gummy materials remain sticking on to the fibres - which make them hard and stiff through binding the fibre filaments in a compact way. Microscopic examination of such samples showed that the periderm persists as such and portion of the cortex remained undissolved (M.K.Basak et al., 1987). These defects in low grade fibres are primarily caused by an extensive development of the periderm combined with incomplete or defective retting. In normal biological retting the percentage of loss of non-cellulosic materials from the bark are 45.6% in C.capsularis and 38.5% in C.olitorius whereas this value is

lessened by chemical retting and is 39.6% and 28.7% respectably. Kundu (1964) reported that the separation of fibre bundle was ultimately related to the removal of sugars, tannins, proteins, pectins and hemicelluloses. It was also reported that pectin and hemicellulose are the major components that are removed during retting and hemicellulose being the maximum. The loss of pectin was almost the same in both the species, while the loss of hemicellulose in C.capsularis is almost double of that of C.olitorius. This quantitative variations has a profound effect not only on the quality and quantity of fibres but also on the retting period. Roy and Mondol (1967) indicated that good retting was a precondition for production of quality fibres.

Thus quality of the fibre produced and the ease with which it is spun into yarns and fabrics depend to a great extent on the proper control of the process of retting.

1.6.1 Causes of inferior quality of jute :

The quality of jute is influenced by several factors of which the method of retting is number one. In usual retting practice, by the time the top portions of the stems are retted, the over mature thick lower portions still remain underretted. If the lower portions are allowed to ret properly, the top portions get over retted. So the cultivators have to extract fibre when the middle portion of

the stem is properly retted. As a result of usual retting practice the thick over matured bottom portion remain under retted and the fibre bundles, instead of being separated come off along with the cortex and periderm yielding an inferior hard and stiff fibre commonly termed as low grade jute or SMR (Straight Morah Rejection). The production of low grade jute is further attributed to the scarcity of retting water in northern region. Thus due to improper retting practice and scarcity of retting water the bulk of jute produce in Bangladesh (almost 30-40%) is of low grade. The year-wise production of low grade jute and cuttings are as follows:

1.7 Physical features of low grade jute :

The quality of jute is usually judged by its performances for the production of various types of yarns and its behavior in the manufacturing process. A fibre having good length, strength, bright colour and lustre, free from any fault and with smallest percentage of cuttings is called a good quality fibre. It has been found that besides rettings a number of factors have much influence on the quality of jute fibre. These factors may be categorised as

non-controllable and controllable factors. Defects in the fibre may be summarised as follows:

Rooty- This is known as barky or hard bottom of jute. It is one of the commonest defects especially in the low-lying jute growing areas. Root is developed in the lower part of the plant by the flood water and is resistant to retting. Due to this dark strips of dried up periderm and gummy materials remain sticked on the fibre near the root ends and make it hard and stiff. This is due to incomplete retting of periderm and cortex.

Hard centres- This type of fibres contain dark strips of periderm in the middle portion which make the middle region of the fibre hard and stiff.

Runners- This defect is caused by incomplete retting, adherence of periderm to the fibre and careless washing.

Stiff fibre- This type of fibre has dark strips of periderm and dried up gum is found to be sticking especially near the basal region that makes them very hard, rough and stiff.

All the above defects are primarily caused by an extensive development of the periderm combined with incomplete or defective retting.

Specking and Knotty- This type of fibres contain small patches of barky matters (Specks) agglutinated here and there. Barky matters are found to stick to the areas infected by insects and branching at the nodes.

Sticky- Fibre with broken pieces of wood or stick. This is not due to incomplete retting but for disorganization of cambium, the ray cells and the soft tissues.

The above defects are more prevalent in capsularis, whereas olitorius fibres are comparatively free from such defects.

1.8 Production of low grade jute in Bangladesh :

Table - 3

Year wise low grade jute production in Bangladesh during the period 1976-1986 (figures in million metric ton)

Year	Total production	White		Tossa		Total L.G	% L.G.
		Cross	SMR	Cross	SMR		
1976-77	0.847	0.227	0.050	0.085	0.018	0.380	44.86
1977-78	0.991	0.268	0.059	0.099	0.020	0.446	45.00
1978-79	1.207	0.327	0.073	0.121	0.024	0.545	45.15
1979-80	1.297	0.352	0.078	0.130	0.026	0.587	45.25
1980-81	0.865	0.234	0.052	0.087	0.018	0.391	45.20
1981-82	0.775	0.176	0.039	0.098	0.018	0.391	42.70
1982-83	0.991	0.223	0.050	0.124	0.025	0.421	42.58
1983-84	0.955	0.215	0.048	0.119	0.024	0.406	42.51
1984-85	0.923	0.208	0.046	0.115	0.023	0.392	42.36
1985-86	1.530	0.351	0.078	0.195	0.039	0.636	42.50

Source : BJMC, **SMR** Straight March Regection

In table-3 total production of both cross and SMR are shown. It is observed that 42%-45% of the total jute fibres are cross and SMR. These cross and SMR are low grade jute. It is also necessary to mention that the percentage of low grade jute is quite substantial and may go up in future due to gradual increase in the scarcity of retting water.

1.9 Enzymatic Maceration of plant tissue :

It has been revealed by Mitscherlich (1850) for the first time that in the retting process pectic substances were decomposed by the pectinolytic enzymes secreted by the micro organisms. Kundu (1964) reported that factors like pH, temperature, volume of water, maturity of plant at harvest, fertilizers received by plants and natural activators were found to influence the retting period of jute. Kundu (1960) also reported that presence of pectinolytic and hemicellulolytic organisms were necessary for retting to take place. Rahman (1963) reported the degradative product of pectin in the retting liquors of flax. Kertsen and Mecolloch (1951) indicated that the retting bacteria were effective as they possessed pectinolytic enzymes which included protopectinase, pectinesterase (PE) polygalacturonase (PG) and pectin depolymerase. Protopectinase liberated the insoluble pectin from plant materials and PE demethylated pectin molecules which were then attacked by PG to form a soluble product, galacturonic acid. Ali and Islam (1965) reported that enzymes PG and PME of Penicillium frequentans macerated potato dices, jute bark and jute stems. Baruah and Baruah (1944) indicated that a mixture of

lamellae, pectinase and protopectinase retted jute in much shorter time without affecting the fibres. Stussain (1958) reported that Sclerotium rolfsii produced pectic enzymes causing stem rot and blight disease. Joslyn (1962) and Wood (1960) predicted the role of hemicellulase in the maceration of plant tissue. Han Cock (1976) reported that sunflower hypocotyls infected with Sclerotinia sclerotiorum contained an enzyme xylanase that degraded sunflower xylan and a commercial xylan into xylose and xylo-oligomer. Hancock (1964, 1965) observed the combined action of hemicellulolytic and pectinolytic enzymes on the break down of the cell walls. Ishii and Kiho (1976) isolated pectolytic enzymes from culture filtrates of Aspergillus japanicus. Bateman (1963) indicated that PGase was primarily responsible for tissue maceration, whereas cellulase was assumed to play a supporting role in the process. Hancock (1965) reported the association of high PGase activity with sunflower or tomato stems infected with Sclerotia sclerotiorum. Fischer (1965) reported the presence of pectinolytic enzymes, PGase, PME and CMCase in the cultural filtrates of sweet potato surface rot and wilt fusaria growth in identical cultural conditions. Bateman (1968) reported high enzymatic activities in recoverable extracts

of infected plant tissues by Sclerotium rolfsii and the fungus appeared to be a source of enzymes for studying invitro enzymatic degradation of plant cell wall materials. Meclendon et al. (1960) reported that certain plant pathogenic fungi produced a variety of hydrolytic enzymes potentially capable of destroying various components of plant cell walls. Bateman (1964) also reported that cell maceration by these enzymes were inhibited in the presence of Ba^{++} or Ca^{++} and less by Mg^{++} and monovalent ions had no influence. Jensen et al. (1960) reported that increased ionic concentration influenced the release of PME in the infected host. Edgintom et al. (1961) indicated that calcium deficiency increased the susceptibility to hydrolysis by pectinol 100-D, a commercial pectinase preparation.

1.10 Lignocellulotytic fungi and their enzymes :

Many fungi and bacteria for their heterophilic metabolism degrade lignocellulosic materials. The QM collection at the university of Massachusetts, U.S.A, numbered over 14,000 fungi which can degrade materials like cellulose, hemicellulose and other polysaccharides.

Although three major enzyme systems take part in cellulose degradation, existence of these enzymes in multiple form have also been reported by different workers. The reason for this multiplicity is still obscure.

It is supposed that the multiple form of cellulases may result due to the action of concomitantly secreted proteases and amylases which might alter the initially secreted original cellulases, in particular in old culture mediums (Esterbauer et al, 1983).

1.10.1 Cellulase, Hemicellulase and Pectinase :

Cellulases :

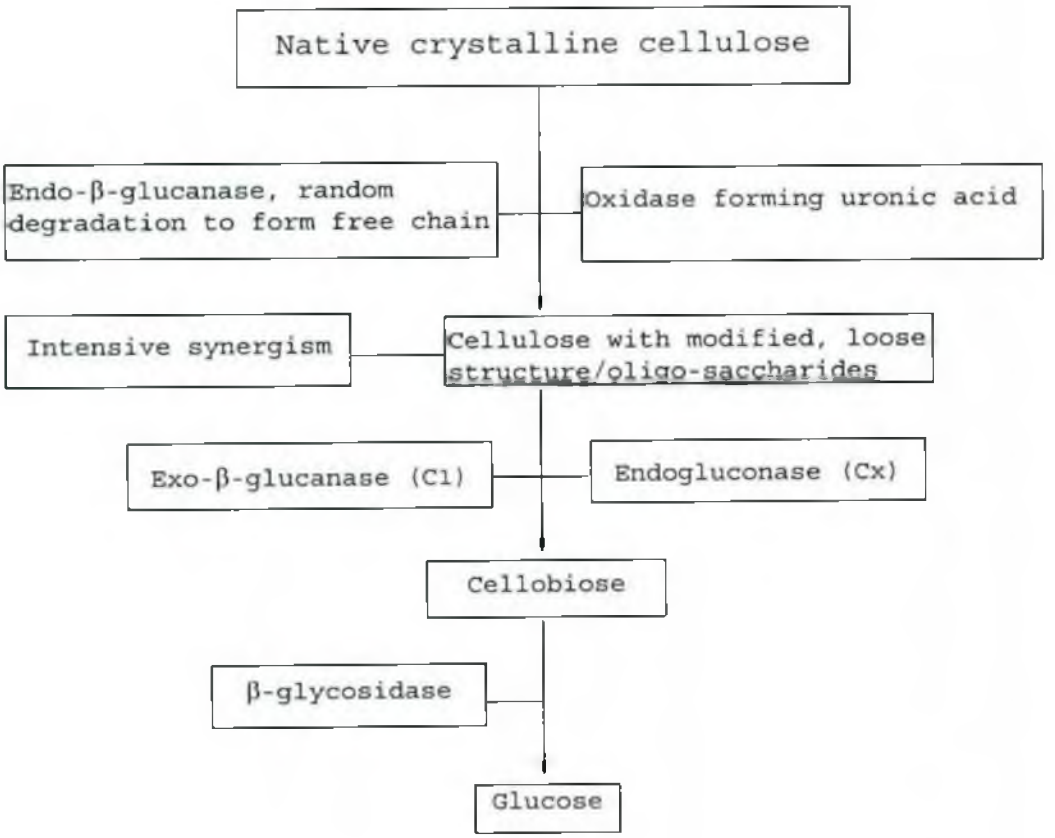
Celluose is more resistant to microbiological and enzymatic breakdown (Martin el al., 1961). The initial step in cellulose degradation is the enzymatic hydrolysis of the polymer. The enzyme or enzyme complex has been given the name of cellulase. Cellulase catalyses the conversion of insoluble cellulose into simple water soluble products. The hydrolysis is affected by cellulase ultimately leading to the liberation of mono or disaccharides, a reaction characteristic of the entire cellulolytic flora.

The microbial cell is impermeable to the cellulose molecule. So the organism must excrete extracellular enzymes in order to make the carbon source available. Once the soluble sugars penetrate the cell membrane they are utilised for biosynthetic reactions. The cellulase activity can be characterised in agar media containing cellulose as the carbon source by the formation of clear zone. Cellulase is adaptative in most microorganisms i.e. it is synthesised only in the presence of its specific substrates, cellulose or cellulose derivatives. For example, Trichoderma viridi can secrete cellulase only with cellulose but not with other compounds (M. Mandel et al., 1957).

The C_1-C_x hypothesis underwent several changes and a number of modified hypothesis have been proposed. (Wood, 1975, Paterson, 1975, Esikson, 1975 and Reese, 1977).

Recent fractionation studies have shown that cellulase complex consists of three main types of enzymes b-1,4-endoglucanases (C_x), b-1, 4- exoglucanase (C_1) and b-glycosidase. It is now widely accepted that there is a strong synergism between endow and exoglucanases. The endoglucanases act randomly over the cellulose chain and

make new chain ends. Then exoglucanases act on exposed chain ends producing cellobiose units. The final splitting of cellobiose to end product glucose occurs by the action of b-glucosidase. It is assumed that first stage of degradation reaction starts with the adsorption of the enzymes on the surface of cellulose fibre (Lee, et al., 1982, Lee and Fan, 1982 and Lee et al.,1983), and the endo cellulases are accompanied by the oxidases or some other unidentified enzyme components (Eriksson, 1975) according to following stages :



Mechanism of the enzymatic degradation of cellulose (based on Erikson, 1975).

There are two possible mechanisms for the cellulose cleavage. Cellulase may cause a random splitting of the long cellulose molecule or alternately, cellobiose units may be removed from the terminal ends of the chain. Whitaker (1956) suggested a random cleavage of cellulose molecule to be the likeliest hydrolytic degradation through C_{14} poly-saccharides. On the basis of studies with cellulose derivatives Levison et al., (1951) suggested two- steps way in cellulose degradation. One is a reaction catalysed by C_1, which yield long linear chain composed of many glucose units. The 2nd step C_x hydrolyses the cellulose derivatives formed by C_1 step.

$$\text{Native cellulose} \xrightarrow{C_1} \text{long glucose chain} \xrightarrow{C_x} \text{cellobiose.}$$

The degradation of crystalline cellulose is a complex process, mechanism of which is not fully established. Reese et al. (1950) put forward their classic hypothesis popularly known as C_1-C_x concept. According to this concept C_1

component convert the cellulose to a reactive state that was subsequently acted on by C_x component to form oligomers and dimers, which are in turn hydrolysed by b-glycosidase to glucose as the end product.

Hemicellulase :

The next group of enzymes which degraded hemicelluloses of lignocellulosic materials are collectively known as the hemicellulases. Since many hemicellulosic constituents are found in nature, there are undoubtedly several hemicellulose decomposing enzymes. The hemicellulases act upon respective substrates with the liberation of sugar or sugars which are the building blocks of the hemicellulose molecule. The hemicellulolytic activity is assayed by the measurement of released reducing sugars. Since these enzymes are extracellular, their activity is affected by a number of external factors. Although many of the hemicellulase enzyme components have been characterized and classified according to their being substrate specific, relatively very little knowledge of the structures, mechanisms and the biosynthesis and regulation of the hemicellulases have been known. This lacking was mainly due to incomplete knowledge of

hemicellulose structure. Like cellulase, hemicellulases occur in two basic forms i.e. exo- and endow-type, where as endow type hemicellulases are more common.

Since xylans are the chief constituents of hemicelluloses, hemicellulase is generally expressed as the xylanase activity. As the xylans are structurally of mixed constitution of pentose sugar xylose, the complete hydrolysis is possible through the combined action of a series of xylan enzymes (Esterbauer et al., 1983) which act on different glycosidic linkages.

The endo 1,-4-β xylanase (EC 3.2.18) acts on xylan to form small fragments of xylo-oligosaccharides and β-D-glysisidase (EC 3.2.1.37) which hydrolyses dimers and trimers of xylose to monomeric sugars (Biely, 1985). There are at least six types of xylanases which differ from one another in their affinity towards particular bonds to release different kinds of sugars. These enzymes are as follows:

a) **β-xylosidase,** the enzyme that acts on xylo-oligosaccharides to produce xylose.

b) **Exo-xylanases**, which produce xylose at high rates from long chain xylan and at low rates from short chain xylan.

c) **Endo-xylanases**, which produce primarily xylobiose and xylose from xylans, but cannot cleavage L-arabinosyl branch points.

d) **Endo-xylanases**, which produce mainly xylo-oligosaccharides and are inactive on xylotetranose and smaller substrates.

e) **Endo-xylanases**, which can act on side chain α, 1-3-arbinosyl and α-1, 2-glucuronic acid and produce mainly xylobiose and xylose.

f) **Endo-xylanase**, which produce mainly xylo-oligosaccharides of intermediate size and can act on branch points (Reilly -1981).

More recently, studies on the mode of action of endoxylanases have been carried out with fungi like Aspergillus niger (Groba Cheva and Rodionova, 1977; John et al., 1979).

Ligninase :

Lignin is more resistant to enzymatic degradation than cellulose and hemicellulose. Thus it protect the associated substances like cellulose and hemicellulose from destruction. The lignin encrustation over cellulose and hemicellulose act as mechanical barrier for microbial attack. Many of the Basidiomycetes are capable of degrading lignin, but invariably at slower rate. Most fungi which attack lignin also utilize cellulose and due to more availability of cellulose growth is sparse in lignin containing medium. Fungi which attack both lignin and cellulosic constituents is grouped as white retting fungi. The biochemistry of lignin degradation, the mechanism of the enzymatic cleavage and intermediates in the process have still to be established. The enzymes concerned as a single catalyst termed as ligninase. Degradative products like vallinin, vanillic acid and ferulic acid etc. have been reported by Henderson et al., (1952). During the fungus rotting of spruce sawdust. Thus enzyme system functioning converts the lignin molecule to give simple aromatic substance for further growth. The enzyme system is undoubtedly extracellular. (Mandels and stern berg (1979).

But only very few of these fungi are able to produce considerable level of extracellular enzyme which can degrade the cellulosic materials invitro and are found mostly in higher fungi.

Some of the well known fungi which produce cellulases, hemicellulases and pectinases are Trichoderma reesei. T Koningii M anxdel's, Hontzs and Nystrom, 1971). Sclerotium rolfsii (Sadana, Shewale and Despande, 1980) Sporticum Pulvaranticulum (Eriksson et al., 1983) Schizophyllum commune (Paiee et al., 1978), Phoma hibernica (Urbanek et al., 1978) and the members of the genus Aspergillus, Penicillium and Fusarium (Fahnrich et al., 1981).

However, a great variations exist among the lignocellulolytic organisms in respect of production, composition and abilities to degrade lignocellulosic materials. Some of them produce all the essential components for cellulose hydrolysis, while others produce incomplete cellulose system. For instance T. reesii which is known to be the best of all cellulose producing fungi, has a complete cellulose system rich in C_1 and C_x activities but with a suboptimal β-glycosidase activity for rapid and complete

degradation of cellulose to glucose. (Sternberg et al., 1977).

Schurz et al. (1985) put forwod a reaction mechanism for cellulose hydrolysis, calling it as "All or nothing". They reported that during the enzymatic degradation of cellulose 1) the degree of polymerization decreased very little, 2) the index of crystalinity increases quite little, 3) volume of the hollow-surface increased slightly so that the surface enlarged a little. Based on this findings they postulated that, a cellulose chain will be either totally degraded to cellobiose, specially to glucose or it will not, at all, be degraded. This findings correlates with previous hypothesis of (Chang and Tsao, 1981, Schurz, 1984) and according to their postulation endo-cellulases attack any where on the surface of the cellulose chain. Then the exo-cellulases attack this chain in both directions.

Pectinase :

Pectin matter occur in the jute plant and it holds the fibre bundles together. Fairly well retted good quality fibres contain less than 0.5% of pectin. But in low grade jute and cuttings this pectin is not decomposed fully during retting (Mohiuddin et al. 1978). That is why low grade jute

and cuttings contain more pectin materials (3-4%) than that of good quality fibres. If this can be decomposed by some methods the resultant fibre would be more softer and more separable.

A number of workers have suggested that this pectin can be decomposed by microbial action promoted by the addition of ammonium phosphate with the emulsion during conventional softening process (Shaha, P.K., Paul, S.S. and Sarker, P.B. 1952).

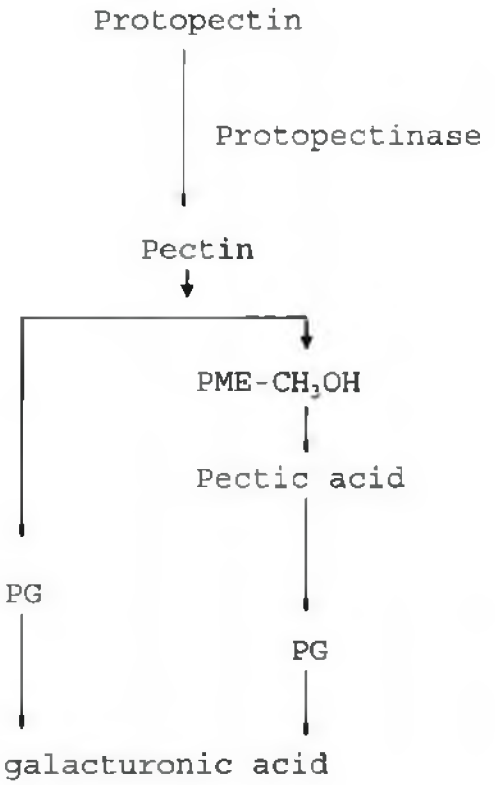
Bacteria, fungi and actinomycetes are capable of acting upon pectin substances through hydrolyzing them as carbon and energy sources to support their proliferation. Pectin degrading microflora can be screened by growing them in culture media having pectin as a sole carbon course. Species of *Bacillus, Clostridium* and *Pseudomonas* are among the dominant pectin hydrolyzers (V.A. Lambina - 1957).

Acidity affects the growth of pectolytic microfloras, streptomycetes is favoured by high pH while the fungi become prominent with a fall in pH. In forest litter and compost heaps, fungi and Pseudomonas spp. are particularly vigorous (K.T.Wieringa - 1955).

In biological retting of fibre plants relies on microbial enzymes to digest the pectic substances of the middle lamella. The results of hydrolyzing of pectic polysaccharides leads to a loosening of the cellulose fibre filaments from the residual tissues. Among the organisms that cause natural retting are Bacillus and Clostridium spp. They solubilise the pectic materials of middle lamella by means of their pectic enzymes and thereby separate individual fibre cell from one another.

Three enzymes are concerned in the degradation of pectin substances: (a) Protopectinase, which decomposes protopectin with the formation of soluble pectin, (b) pectin methyl esterase (PME), which hydrolyses the methyl ester linkage of pectin to yield pectic acid and methanol, (c) Polygalacturonase (PG), which hydrolyses the linkages between galacturonic acid units of either pectin or pectic acid with the release of smaller fragments and ultimately free galacturonic acid.

The interrelationships between the various reactions are summarised in the following figure.



Substrate specificities of pectic enzymes.

Polygalacturonase and pectin methylesterase are formed and released into the surroundings as extracellular enzymes. These catalysts do not seem to be affected by the presence of clays, which frequently lower the activity of extracellular enzymes (D.L.Lynch, et al., 1956). PME and PG are apparently adaptive in most microorganisms, synthesized only in the presence of the specific polygalacturonic acid by PG, only small amounts of free galacturonic acid

accumulate during the early stages of decomposition; di-, tri-, tetra-, and pentagalacturonic acids are produced by the enzyme. In the later stages of hydrolysis, the longer molecules disappear under the catalytic action of PG, and the concentration of free galacturonic acid rises (A.L.Demain, et al., 1954).

1.11 Solid state fermentation process for production of enzymes :

The term "solid state fermentation" refers to the processes with microorganisms on solid substrates without the presence of free liquid. While the presence of moisture is necessary in solid state fermentation, it exists in an absorbed form within the solid matrix. Thus the solid state fermentation does not refer to the fermentation of solid substrates in liquid media. The efficiency of solid state fermentation mainly depends on the function of absorbency rather than moisture content of substrates. There is a minimum level of moisture for solid state fermentation (about 12%) since all biological activity ceases below this level.

Fungi are most suitable micro-organisms for solid state fermentation as they are capable of growing in a low level of available water during solid state fermentation process (E. Cannel-1980). Men used the solid state fermentation process without knowing about micro-organisms and biochemical reactions. Three traditional S.S.F. forms, food fermentations, mould-ripened cheese and composting are still in use today. Because of the following facts SSF. is more advantageous.

(a) In SSF no free water is present, thus the volume of the `media' per gram of substrate is drastically reduced, which leads to the following benefit in relation to the yield of the product. There is no enormous amounts of liquid waste to deal with. In many cases there is no need for filtration as the product is concentrated in the substrate and can be used directly.

(b) SSF reduces greatly the problem of bacterial contamination as they can not survive in such low level of moisture.

(c) The `media' is relatively simple usually a natural media is used instead of costly synthetic media.

(d) Aeration is easily achieved as there are air spaces between the substrate particles.

(e) In solid waste management the volume is reduced.

In addition to the above advantages there are some disadvantages too in solid state fermentations.

(a) Reduced level of moisture in SSF limits the use of wide range of microbes and permits only to fungi, yeast and streptomyces.

(b) In large scale operation, the heat generated is more difficult to manage than LSF.

(c) Measurement of moisture levels, pH, Oxygen levels, Carbon dioxide levels and product is more difficult in SSF.

Japan has the most advanced SSF industry for preparation of popular food items. Koji process is basically an enzyme preparation process by growing a mould A.oryzae or A.sojae on steamed rice that bring important flavour changes. In Japan koji processes are also used for production of 45 tons of cellulase per year by *T.viride* (K. Yamada, 1977).

In spite of some drawback of the SSF fungi are the source of enzymes of commercial interest including pectinase, ligninase, xylanase etc. The ability of the fungi to produce enzymes is widely distributed but only a few species are used commercially for this purpose. (Water steam, L. 1939).

1.12 Aim of the present work :

Due to improper retting about 30-40% of total jute produced in Bangladesh is of low grade. Processing of these fibres is one of the major problems, of jute industries.

It is urgently needed to develop a suitable methodology for proper processing of low grade jute and cuttings. In the light of the findings of various workers. (M.K. Basak et al 1987: B.L. Ghose, 1983 and Mohiuddin et al 1992) it was learnt that the softening process of fibres may be achieved by the application of suitable enzyme during piling.

In view of the above the following work plan has been undertaken:

1. A detailed study of the conventional mill softening practices to assess the microbial growth, temperature, rise and pH changes during piling process.

2. Isolation and collection of suitable microorganisms for the production of extra cellular fungal enzymes for the softening and improvement of low quality jute a cuttings.

3. Optimization of cultural condition for production of enzymes by the potential fungus.

4. Kinetic study and molecular weight of the xylanase components of extracted enzymes.

5. Production of mutants of potential fungal strain by Gamma radiation

6. Assessment of improvement of fibre quality by the application of crude enzymes.

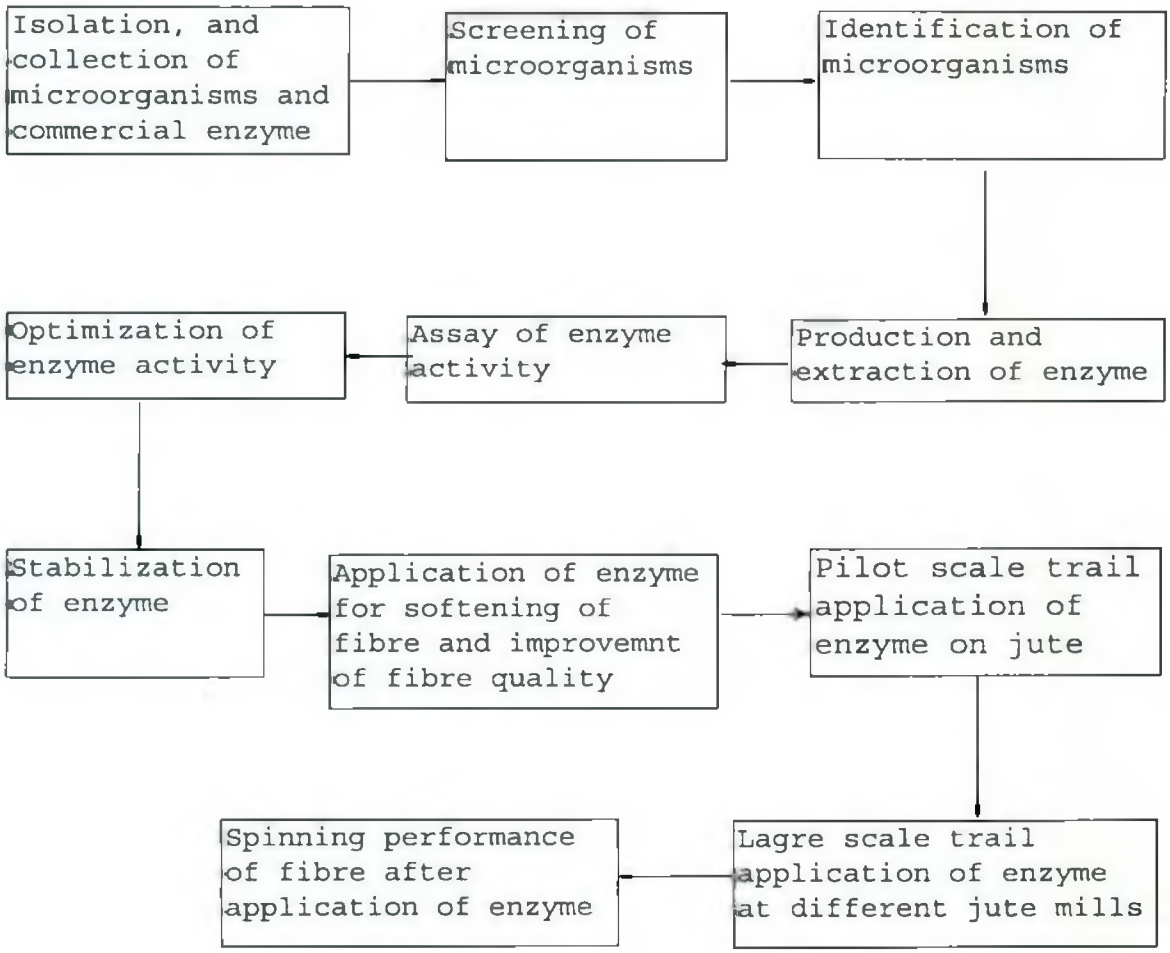
7. Study the changes of chemical constituents of jute by the application of enzymes.

8. Large scale trail application of enzymes at Bangladesh Jute Research Institute (BJRI) experimental mills and another jute mills.

9 Evaluation of improvement of quality of fibres through assessment of spinning performance after large scale enzyme application.

1.13 A flow sheet of production and application of Jute softening enzymes

Production and application of jute softening enzyme



MATERIALS AND METHOD

2.1 Study of conventional mill softening process :

The low grade jute and cuttings can not be processed as such. An additional mill softening treatment is required to make them suitable for processing. The conventional method as practiced in the jute mills involves the application of an oil-in-water emulsion (@ 30-35%) through the softener machine followed by piling of the fibres in a concrete bin for 6-10 days.

In order to make a detailed study of the present mill softening process, jute samples were collected and temperature rise in the piling bin were recorded daily. Jute samples were collected from the three positions of the bin (top, middle and bottom) with a long iron rod and put into the sterile polythene bags or cotton plugged conical flasks.

2.1(a) Temperature recording of piling bin :

Daily rise of temperature was recorded from three positions; top, middle and bottom position of the jute piling bin. Usually a hole was made with a long sharp rod and temperature was recorded by inserting a long thermometer into it. P^H of water extract of fibres were measured by shaking a little of fibre into distilled water.

2.1(b) Counting of microbial growth :

For counting the microbial growth 1 gram of the collected samples were mixed well with 100 ml of sterile water in stoppered conical flasks. Before inoculation a suitable dilution of the liquid (fibre extracts) was made so that the microbial colony could easily be counted. For microbial growth (fungi and bacteria) agar plates of Potato-Dextrose, and Nutrient media of pH 5.5 and 7 respectably were used. For counting the thermophilic micro-organisms samples were boiled in a conical flask at 50°C for 30 minutes to eliminate the mesophilic organisms. The inoculation was made on agar plates with suitably diluted fibre extracts. The inoculated plates were incubated at 35°C and 55°C for overnight. The individual microbial spot of both mesophilic and thermophilic nature were counted from the samples collected during mill softening process and expressed as microbial load per gram of jute samples. A mean result from three plates of each samples were taken as standard. pH changes during the piling process were recorded from the pH value of water extract of fibre samples collected daily.

2.1.1 Isolation of microorganisms, collection of fungi and commercial enzyme preparation :

In order to isolate microorganisms for the upgradation (i.e. softening) of low grade jute and cuttings, samples were collected from natural habitats i.e. jute cuttings maturation piling bins, caddies, jute dust, damaged jute goods and retting water etc.

Samples were collected in sterile polythene bags and cotton plugged conical flasks. For initial isolation, agar plates of potato-Dextrose, Czapex-dox and Nutrient media of pH 5-6 were used. A little portion (1gm) of collected samples were taken in a sterile stoppered conical flax containing 100ml of sterile water and were mixed well. For the isolation of thermophilic microorganism jute samples in the conical flasks were heated at 50°C for thirty minutes to eliminate the mesophilic microbes. The inoculation was done on agar plates by spread culture method with serial dilution according to necessity for counting and separating the individual viable spores. The inoculated agar plates were incubated at 32°C and 55°C for mesophilic and thermophilic microbes respectively. Individual colonies were counted after 24 hours of incubation and transferred at their early stage

of growth. Single strain was isolated through gradual transfer from one plate to another and maintained in PDA, Czapex-dox and nutrient agar slants in the refrigerator for screening of their enzyme activities.

Collection of fungus : A fungus named Aspergillus sojae having PGase and Xylanase activity was collected from ATCC through computer search.

Collection of commercial enzymes: In order to make a comparative study regarding the effectiveness of enzymes extracted from potential fungi and collected strain, a commercial enzyme preparation Flaxzyme (NOVO) used in falx retting was collected. The enzyme preparation was a highly concentrated one and was applied during mill trials to the fibre in similar proportion as extracted one after proper dilution with distilled water. The enzyme extract was stored at 20°C prior to use in mill trials.

2.1.2 Screening of microorganisms for xylanase and CMCase activity :

Principle :

Enzyme activity is demonstrated by the hydrolysis of substrate incorporated, as the main carbon source, in a solid agar medium distributed in petri dishes. The medium contains all the necessary ingredients (minimal medium) to afford sufficient initial growth of the inoculum. After an appropriate incubation period, the activity can be detected around the colonies by the appearance of zones revealed either by substrate clearances, coloration and decoloration.

Procedure :

Step 1: Petri-plates of following media composition were prepared for the respective enzymes

	CMCase	Xylanase
Carbon source:	filter paper 2.0 g/L	xylan (larchwood) 2.0 g/L
Yeast-N-base :	6.7 g/L	6.7 g/L
Oxgall :	10.0 g/L	10.0 g/L
Yeast extract :	2.0 g/L	2.0 g/L
Agar :	15.0 g/L	15.0 g/L

Step 2: Media of above composition were prepared in distilled water and autoclaved at 120°C for 15 minutes. The sterile liquified medium was cooled to 45°C - 50°C and distributed to petri-dishes.

Step 3: The plates were inoculated with microorganisms to be screened for enzyme activity.

Step 4: The plates were incubated at optimum temperature for 3-10 days.

Step 5: Hydrolytic zones around the growing colonies were recorded for CMCase activity and xylan hydrolysis can only be observed after staining the plates.

Step 6: To enhance the visibility of hydrolytic zones around the growing colonies, the plates were treated as described below.

a) For CMCase activity: the plates were flooded with 10 ml Congo red solution. The coloration was terminated by pouring off the dye after 20 minutes and replacing with 10 ml of 5 mol/L NaCl solution. After an additional 20 minutes the salt solution was

discarded and CMCase activity was revealed by a pale orange zone around the colonies producing CMCase.

b) For Xylanase activity: the plates were flooded with 96% ethanol. Although clear zones were visible within 3-4 hours of incubation but prolonged incubation enhanced the contrast.

Materials :

1. Yeast-N-base, oxgall, yeast extract agar, CMC were obtained from Difco & Sigma Chemical Co.

2. Congo red solution: 0.1 g Congo red was to be dissolved in 100 ml distilled water.

3. 5M NaCl solution: 29.2 g NaCl was dissolved in 100 ml distilled water.

4. Citrate buffer: 0.05 mol/L Na-citrate solution was prepared dissolving 14.7 g Na-citrate in 1 L distilled water. 0.05 mol/L solution of citric acid was prepared by dissolving 10.5 g citric acid in 1 L distilled water. Citric acid was added in Na-citrate solution until the pH reached 5.3

2.1.3 Screening of fungi for pectinase activity :

Principle :

Most of the pectinolytic fungi produce polygalacturonase (PG) and pectinlyase (PL), PG attacks polygalacturonic acid and PL attacks pectin. Enzymes are secreted by fungi when grown on minimal media supplemented with polygalacturonic acid for detecting PG activity and pectin for detecting PL activity. After a period of incubation, hydrolytic zones were revealed by staining the plates with cetyltrimethyl ammonium bromide which precipitates the undegraded substrate, leaving a clear zone.

Step 1: The following media composition for detecting PG and PL enzyme activities were prepared.

	PL	PG
Pectin (from apple)	10 g/L	0 g/L
Polygalacturonic acid (Na salt)	0 g/L	10 g/L
$NaNO_3$	2.0 g/L	2.0 g/L
KH_2PO_4	1.0 g/L	1.0 g/L
$MgSO_4.\ 7H_2O$	0.5 g/L	0.5 g/L
Yeast extract	0.2 g/L	0.2 g/L
Agar	20.0 g/L	20.0 g/L
Buffer HEPES		Na-citrate
buffer 0.1	buffer 0.1 mol/L	
mol/L (pH 8.0)	(pH 4.8)	

(a) pH of the pectin solution in water should be 4.0 which is natural pH of pectin. This prevents unusual hydrolysis of pectin during autoclaving.

(b) HEPES: N-2 hydroxyethyl piperazine-N_2-ethane sulphonic acid. It is non-metabolizable buffer. Therefore, the pH of the medium dose not change significantly.

Step 2: Polysaccharide was dissolved in 500 ml water and rest other chemicals in their respective buffers (500 ml). Polysaccharide solutions and mineral solutions were autoclaved separately at 120°C for 15 minutes.

Step 3: Plates containing PG or PL medium were inoculated with microorganisms to be screened for enzyme activities.

Step 4: Plates were incubated at optimum temperature for 2-7 days.

Step 5: Plates were flooded with 1% (W/V) cetyltrimethyl ammonium bromide solution and the Zones of hydrolysis appeared after 5-10 minutes of incubation.

Reagents :

a) Polygalacturonic acid-(Na salt), pectin (from apple), HEPES, and cetyltrimethyl ammonium bromide used were from Sigma Chemical Co.

b) Citrate buffer: 29.4 g Na-citrate were dissolved in 1 L distilled water and 21.01g citric acid in 1 L distilled water to prepare 0.1 mol/L solutions. Citric acid solution is to be mixed in Na-citrate solution until the pH reaches 4.8 (approx 45 ml of 0.1 mol/L citric acid was required for 100 ml of mol/L Na-citrate to give a buffer solution of 0.1 mol/L, pH 4.8).

c) HEPES Buffer: 23.83 g of HEPES were dissolved in 1 L distilled water. Concentrated NaOH (saturated solution) was added to increase pH to 8.0.

d) Cetyltrimethyl ammonium bromide: 1% (W/V) solution was prepared dissolving 1g cetyltrimethyl ammonium bromide in 100 ml distilled water.

2.2 Selection of potential fungi :

Out of 20 isolates potential fungal strains were selected through screening tests and softening effect on jute

cuttings. Enzyme activities of CMCase, polygalacturonase and xylanase were demonstrated by the appearance of clear zones around the fungal colonies. The three most potential fungi were characterized and selected for enzyme production on the basis of their formation of clear zones.

2.2.1 Morphological features and microscopic of potential fungus :

From the results of screening tests the potential fungal strain was chosen and identified upto species level according to the cultural and morphological characters. Gross colonial morphology gave a preliminary idea about identification (Koneman et al., 1978).

A small amount of inoculum was placed on the centre of PDA plates and incubated at 32°C. The development of the colony over a period of several days was observed, considering the growth rate, texture, size of colony, color of the spore, reverse color. The young vegetative mycelial growth turned to dark black with the age of the culture (Plate 1).

a) Microscopic study of fungi :

Recognition of characteristic microscopic feature of the filamentous fungi provides a reliable criteria for their identification.

Following techniques were used to prepare slide mounts for the microscopic study of the fungal colony. A small portion of fungal colony was taken from the growth medium and transferred to a drop of mounting medium (lactophenol solution) on a glass microscope slide. Then the colony was teased apart with two sharp needles, polished to remove any barbs and covered with a cover glass for observation under microscope.

b) Microculture study :

In order to avoid interference to the fungal arrangement of the fungal structures, microculture slide technique was used (Banek and Rogers). 1cm square PDA media was placed on a sterile glass slide and the agar block was inoculated with spore or hyphal fragments. A flamed cover slip was placed centrally upon the agar block. The slide was placed inside of a petri-dish with water and incubated at 32°C. After

sporulation, the cover slip was removed from the agar block. A drop of mounting reagent was placed on the slide. The agar block was removed and lactophenol regent was placed on it and covered with cover slip.

The excess mounting reagent was blotted from the slide and sealed with wax. The slide mounts were examined microscopically for the specialized fungal structure, hyphae, fruiting body spore etc.

The fungus has profused mycelial growth and conidiophore arose directly from the substrum, mycelium are hyaline, (glass like) septated and multinucleated. Conidia arise from sterigma and were borne with chains. Conidial head focus carbon black. (Plate 3) On the basis of the above structural features the fungus was characterised as Aspergillus niger.

Organisms :

The wild strain Aspergillus niger and ATCC strain Aspergillus sojae were used in this work. The fungal strain Aspergillus niger was isolated from jute softening piling bin of a local jute mill. The other strain Aspergillus sojae 20235 was purchased from ATCC considering its pectinolytic and xylanolytic ability through computer search.

Culture and maintenance of the fungi :

The isolated fungi (Aspergillus niger) could grow well on jute waste under sufficient moist condition (35-40%) and does not require any extra nutrient. In the laboratory, the fungal culture was maintained on potato-Dextrose-Agar (PDA) slants at pH 5.6 in the refrigerator and subcultured after one month. The young vegetative whitish compact mycelial growth turned to dark along with the age of the culture after four days of growth.

The other fungi, Aspergillus sojae (ATCC-20235) was also maintained on potato-Dextrose-Agar (PDA) slants after sufficient growth at 32°C in the refrigerator. The fungi was subcultured after one month. The young vegetative white mycelial growth turned to greenish after four days of growth.

The isolated fungi *Aspergillus niger* was used in this study along with known pectinolytic fungi *Aspergillus sojae*-20235 collected from ATCC to make a comparative study. The 3rd potential strain (BJX-13) though produced considerable jute softening enzymes was discarded due to the instability of its enzymes for considerable period.

Aspergillus niger started with whitish mycelial growth and turned into dark black with the age of culture (after 3-5 days).

Aspergillus sojae also started its initial growth with whitish mycelial mass which turned into greenish with the age of the culture (3-4 days).

2.3 Softening test with potential fungi on jute cuttings :

Jute cuttings (about 8-10 cm) of uniform quality were moistened with czapex-dox solution and jute extract in petri-dishes. These petri-dishes were sterilized keeping the moistened cuttings into the petri-dishes. The cuttings inside petri-dishes were inoculated with the fungi which were chosen as potential from screening tests. The inoculated petri-dishes were placed in the incubator for fermentation at 32°C for 4 days. The cuttings were washed and dried for testing the softening effect, Control experiments were carried out by keeping the cuttings to similar conditions soaking with sterile water only. Softening activity was tested by touch and feel method. The three potential fungi showed sufficient softening effect on jute cuttings.

2.4 Solid state fermentation with different substrates:

Wheat bran being the cheap source and locally available indigenous material was used as substrate for enzyme production by solid state fermentation. But in order to find out the suitability of different indigenous materials as substrate for solid state fermentations a number of experiments were carried out. Indigenous materials like saw dust, and rice bran as carbon source were used along with wheat bran in the ratio of 1:1 and solid state fermentations were carried out following the optimum conditions of growth and enzyme productions by the fungi. Enzyme productions with these substrates were extracted and assayed following the usual procedures.

It was found that initial fungal growth was slower with the substrate wheat bran mixed with saw dust and enzyme out put was also lower compared with that of wheat bran alone. On the other hand, the fungal growth was profuse with the substrate wheat bran and rice bran but the enzyme out put was less and the enzyme solution was also curdy. This might be due to production of amylase. Results are presented in chapter-3.

2.4.1 Optimization of Solid state fermentation by the fungi on wheat bran :

Fungi are most suitable for solid state fermentation as they can grow in low level of moisture and secrete extracellular enzymes into the substrates on which they grow. Wheat bran being the cheap source and locally available indigenous material was chosen as a substrate for SSF. Moreover, the wheat bran is enriched enough as such no other media ingredients are necessary for the fungal growth. Just the addition of water is sufficient enough to start fungal growth. In order to optimize the cultural conditions for growth and maximum enzyme production, the addition of water level to the bran and ambient fermentation temperature for the potential fungus were optimized.

2.4.1 (a) Optimization of added moisture of SSF :

Moisture level in substrate is an important factor for the culture of solid state fermentations. To find out the optimum moisture level for maximum growth and enzyme production fermentations were carried out by varied moisture level (20-100%). Equal amounts (50 gm) of wheat bran were

taken in cotton plugged conical flask of one litre capacity. Water was added to each flask at the rate of 20%-100% weight by volume at natural pH of around 6. The flasks with water soaked bran were autoclaved at 120°C for 15 minutes. After cooling each flask was inoculated with 5ml spore suspension (viable spore 10^{-7}/ml). This spore suspension was prepared by pouring 5ml sterile water into fungal PDA slant. The spore suspension was added to the sterile bran and after mixing thoroughly the flasks were allowed to ferment at 32°C for 4 days. Thus the added moisture level was optimized after evaluating the growth and enzyme production by the fungus.

2.4.2 Temperature optimization for fermentation :

In order to find out the optimum temperature for the fungus SSF were carried out in a temperature range of 20°C to 45°C with optimum moisture level of 50% (W/V). Wheat bran with 50% added moisture was inoculated with equal volume of spore suspension and was allowed to ferment at different temperatures (20°C to 45°C) for four days. The optimum temperature for maximum growth and enzyme production was chosen after assaying the enzyme preparation from the fermented bran at different temperatures.

2.4.3 Effect of extra aeration on fungal growth and enzyme production :

Though the production of enzyme by solid state fermentation is simple and inexpensive yet it was quite necessary to know whether a forced aeration of air stream has got any profound effect on enzyme production. The study was conducted to find out the effect of aeration on the production of enzymes by the fungus through solid state fermentation.

The experiments were conducted on flat aluminium trays containing 300 gms of 50% (W/V) moisture added wheat bran under controlled temperature of 32°C and moisture added saturated air from modular fermentor. Three types of aeration were studied and they were (a) natural aeration i.e no forced aeration, the oxygen requirement for the fermentation was provided by the free air head space of the trays (b) intermittent aeration of one hour on and one hour off at the air flow rate of 0.20 L/minute and (c) continuous aeration at the air flow rate of 0.20 L/minute. The experiments were conducted in sterile conditions with the potential fungal strain A.niger. The enzymes produced during the three fermentations were assayed to summarize the effect of aeration on the production of enzymes. The detailed results are shown in chapter-3.

2.4.4 Effect of mineral salt on enzyme production by the fungus on W/B :

Wheat bran medium was found to be suitable for supporting the growth and enzyme production by the fungi (for both isolated and collected strains). It is known that wheat bran contains a high percentage of carbohydrate along with some vitamins and several minerals. In order to enrich the wheat bran medium different mineral salts were added with the hope that addition of salt might have profound effect on fungal growth and their enzyme productivity. In order to ascertain their effect 10M solution of different salts were prepared. Wheat bran was moistened with each of these mineral solutions separately and each flask was inoculated with the respective fungus. Fungal growth and enzyme production were recorded following the set cultural and extraction procedures. The mineral salts used were $MgSO_4$, $7H_2O$ K_2HPO_4, $FeSO_4$, $7H_2O$, $CaCl_2$ and $MnCl_2$, $4H_2O$.

2.4.5 Effect of antibiotics on enzyme production by the fungus :

It is known that bacterial contamination often interfere in growth and enzyme production during solid state fermentation process. In order to suppress the bacterial growth during solid state fermentation process a number of bactericide like penicillin, zentamycin. Crystapen-v, pen-v and streptomycin were used. These bactericides were used at the rate of 30 mg/L of liquid added to the bran. The antibiotics were first added with sterile water and the antibiotics added water was used for preparation of spore suspension which was later used as inoculum to the bran. After inoculation fermentation, extraction, etc. were carried out as usual and variation in growth, enzyme, production with added antibiotics are shown in chapter-3.

2.5 Genetic Improvement of fungi by Gamma radiation :

The fungal isolates-BJN/5, and *A. Sojae* were used for the production of jute softening enzyme. But their enzyme productivity is comparatively low incomparison to commercial enzyme preparations of NOVO and others. So far we

have exploited the maximum ability of potential fungus for enzyme production through culture medium and growth conditions. The potential productivity of these fungal isolates is controlled by their respective genome and genome must be modified by mutagenic agents to increase their enzyme productivity. Thus the process of strain improvement involves the genetic modification by mutagenic agents, followed by reappraisals of its cultural requirements.

It is known that gamma radiation has lethal effect on fungi. Fungal spore suspension surviving low doses of gamma radiation produces mutants. Generally the dose which gives 20% survival rate in particular fungal spore suspension produces higher number of mutants with varying degrees of changes in the genetic makeup.

Preparation of spore suspension and exposure dose for mutation :

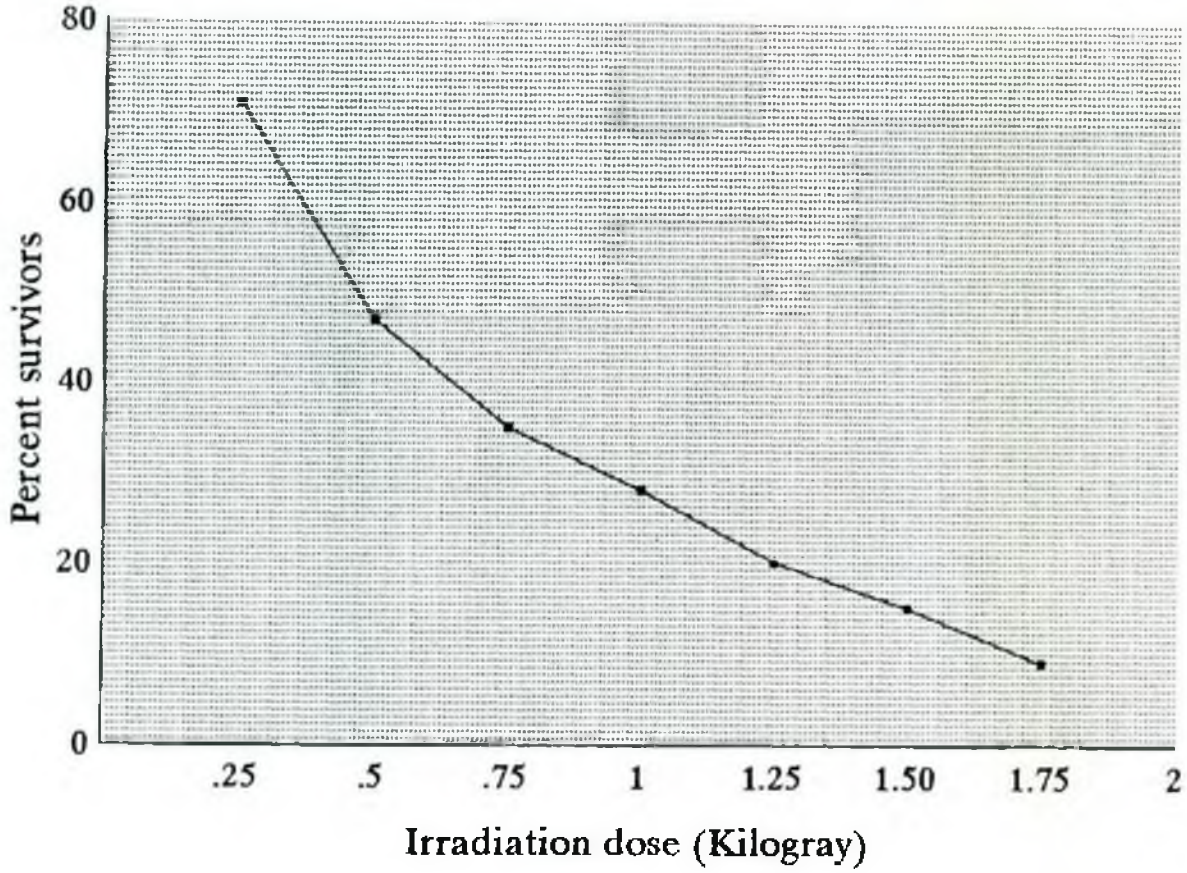
A uniform spore suspension was prepared by adding 5 ml sterile water and 0.5 ml tween 80 in 4 days old fungal PDA slants. After shaking thoroughly 1 ml of the spore suspension was taken in a small test tube for exposure by Gamma radiation.

Fungal spore suspended in sterile distilled water were exposed to radiation doses ranging from 0.52 to 2.0 KGy to construct the survival curve. From the survival curve a dose of 1.20 KGy was found to give a 20% survival.

Calculating the radiation decay state time period for exposure of 1.20 KGy was found out to be 8 minutes. After exposure for 8 minutes by the 1.20 KGy resultant dose, the spore suspension was suitably diluted. one ml from each dilution spore solution was spread over PDA plates and incubated at 32°C.

After a period of incubation for two days the fungal colonies found on PDA plates were transferred to fresh PDA plates. Three such successive transfers were made to get stable mutants which are necessary for their stability. The unstable mutant by this time would revert back to its original state. After the 3rd successive transfer each colony was further transferred into individual PDA slants for preservation and screening tests for the mutants showing higher enzyme producing capacity.

Effect of different doses of gamma ray on survival of Aspergillus niger



Screening of fungal mutants by formation of plate clearing zone :

A plate clearing assay method was used for rapid screening of CMCase, xylanase and pectinase activities of isolated fungal mutants. About fifty mutants from the fungal strain of A.niger. were tested for enzyme activity using the Potential Index (PI) method.

The isolates were grown on respective plates for forming clearing zones around their colonies. The diameter of both colony and clearing zone was measured with the help of a meter scale. Potency index was determined by dividing the clearing zone diameter with colony diameter.

Results of rapid screening of parent strain along with their mutants strains for CMCase, xylanase and pectinase as Potency index (PI) method are shown in chapter-3.

2.6 Extraction of enzymes from solid state fermentation :

Enzymes were extracted from fermented bran by adding 5 volumes of (W/V) 1% saline water. 250ml of 1% sodium chloride water was added to each flask containing fifty grams of fermented bran. The fermented bran was macerated with clear

stick and the slurry was left for two hours with occasional stirring. The slurry was then filtered through a nylon cloth, centrifuged at 3000 r.p.m. for 10 minutes. The clear brown culture extract containing the enzymes was preserved for jute softening tests and assays of enzymes.

2.6.1 Optimization of enzyme extraction time :

At the end of solid state fermentation extra cellular jute softening enzymes can be easily extracted with saline water and they were filtered to remove the solid substrate. In order to find out the optimum extraction time of enzymes solid state fermentations were carried out in a number of flasks in identical conditions. Enzymes were extracted after two days of fermentation upto six days of fermentation. It was found that maximum amount of enzymes can be extracted from the bran fermented for 3-4 days and the ratio of xylanase, polygalacturonase and carboxy-methylcellulase were at the relative highest values.

2.7 Estimation of soluble protein of culture filtrate :

Soluble protein in culture filtrate was estimated according to the method of Lowry et al. (1951) by using bovine serum albumin as the standard.

a) Preparation of standard curve for the estimation of protein.

To prepare a standard curve for protein estimation 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 ml of the standard stock BSA solution (10 mg in 100 ml is 100 m g/ml) was taken in each test tube separately. The volume of each tube was made upto 1 ml with distilled water. To all the tubes 4 ml of alkaline copper sulphate reagent was added and the mixture was incubated at 40°C for 15 minutes. Then 0.5 ml of the FCR working solution was added and shacked vigorously. It was then incubated at room temperature to develop colour. The absorbance was measured against a reagent blank at 690 nm.

A calibration curve was plotted by taking absorbance as ordinate and respective protein concentration as abscissa. This curve was used for the estimation of protein in culture filtrate as mg/ml.

Standard calibration curve data for BSA (stock solution 10mg/100ml)

BSA concn. g/L	Absorbance at 690 nm
0.0	0.0
0.10 µg	0.025
0.20 µg	0.045
0.30 µg	0.08
0.40 µg	0.09
0.50 µg	0.125
0.60 µg	0.162
0.70 µg	0.185
0.90 µg	0.248
100 µg	0.275

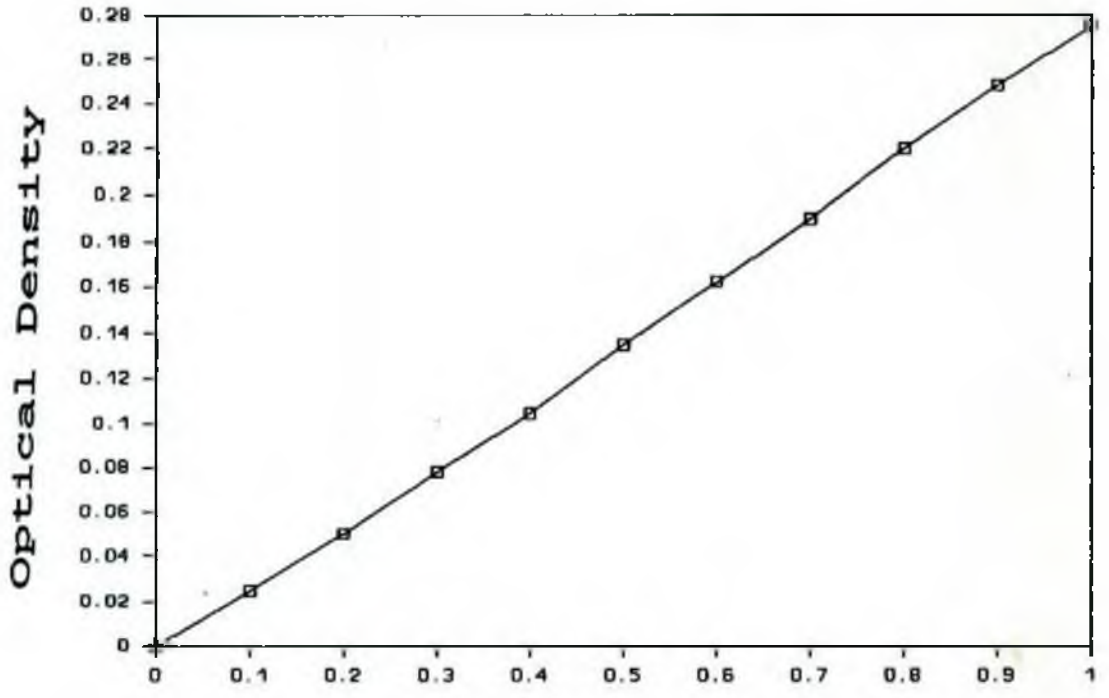
Determination of protein content in culture filtrate :

Soluble protein of culture filtrate was estimated according to the method of Lawry et al (1951) using bovine serum albumin as the standard. Absorbance of color was measured at a length of 690 nm. To avoid interference of impurities in culture it is necessary to purify the enzyme samples to some extent. Culture filtrate was precipitated

with 3 volume of ethanol and kept overnight in the refrigerator. The solution was filtered and the precipitate was dissolved in distilled water to bring its original volume of culture filtrate and centrifuged to remove the undissolved particles. The clear solution was used for determination of soluble protein.

5ml of reagent mixture was added to 0.25 ml of suitably diluted clear enzyme solution. Then 0.5 ml of working Folin-Ciocalteu phenol (FCR) reagent was added and shaken vigorously. In order to develop color the reaction mixture was incubated at room temperature for 30 minutes. Absorbance was measured at wavelength of 690 nm against a blank prepared with 0.25 ml of distilled water.

Standard curve calibration for BSA (stock soln.10 mg/100mL)



Reagents :

1. FCR- Working solution was made by mixing 2 volume of the original stock with 1 volume of distilled water before use.

2. Alkaline copper sulphate reagent:

   a) 2% Nacl in 0.1 N NaOH.
   b) 1% $CuSO_4$ solution was mixed with an equal volume of 2% Na-K tartrate.

   100 ml of alkaline copper sulphate reagent was prepared by mixing 99ml of (a) with 1 ml of (b) immediately before use.

Standard Bovine Serum Albumin Solution : Standard bovine serum albumin of Sigma Chemical Co. was used and 10 mg of BSA was dissolved in 100 ml of distilled water.

2.8 Assay of enzymes (CMCase, PGase and Xylanase) in culture filtrate :

Principle :

The assay is based upon the increase of reducing groups following the incubation of substrate with the enzyme solution (Khan et al.,1981). The enzyme is incubated with carboxymethyl cellulose (CMC) for assaying CMCase activity, xylan for assaying xylanase activity and polygalacturonic acid for assaying polygalacturonase (PG) activity. The reducing sugar released into solution, following hydrolysis by the enzyme, is quantified by dinitrosalicylic (DNS) method (Miller, G.L.1954)

Procedure :

The following tubes were taken for each sample of enzyme solution.

Tube No.	1	2	3	4
used for	Zeroing spectrophotometer	Enzyme control	Substrate control	Test sample
Symbol	Z	EC	SC	TS
Content 1 ml	Glucose buffer	Glucose buffer	Glucose buffer	Glucose buffer
0.5 ml	Acetate buffer	Acetate buffer	Substrate solution	Substrate solution
0.5 ml	Distilled water	Enzyme solution	Distilled water	Enzyme solution

The DNS method for detection of reducing sugar is not sensitive below 100 mg glucose. For accurate determination of low concentration of reducing sugar, glucose should be added to each test tube.

Substrate solutions (1% W/V) were prepared in an acetate buffer (pH 4.8) as follows: Na-CMC for essaying CMCase activity, xylan (from larch wood) for assaying xylanase activity and Na-polygalacturonic acid for assaying polygalacturonase (PG) activity. The above three substrates were from Sigma Chemical Co. st. Lious, USA.

The enzyme solution was filtered through filter paper (milipore, pore size 5 mm) to remove spore and other particles.

The enzyme control (EC) and the substrate control (SC) measures the reducing sugar present in the enzyme solution and substrate solutions respectively. A standard glucose solution along with the test sample should be included to spot check the procedure. The tubes were incubated at 40°C for 20 minutes.

2 ml of DNS reagent was added to each tube to stop the enzyme reaction and to develop the color reaction for

detecting the released reducing sugars in the test tubes. The tubes were heated at 90°C for 15 minutes and cooled by tap water.

6 ml of distilled water was added to each tube. The absorbance of the resultant colored solution was read at 575 nm in the spectrophotometer. Reducing sugar released was then determined from absorbance reading (OD) using the standard calibration curve for CMCase, Xylanase and PGase activity measurement.

Actual absorbance was equal to absorbance of test samples minus absorbance of enzyme control (EC) and absorbance of substrate control (SC). Standard curves for glucose, xylose and galacturonic acid were made by dissolving 4 gm D-Glucose for glucose curve, 4 gm xylan for xylose curve and 4 gm polygalacturonic acid for galacturonic curve in 1 L of distilled water.

Calculation of enzyme activity :

Enzyme activity was calculated by puting O.D. values of enzymes to the corresponding sugar values in the respective graph sheet and expressed as international unit (I.U). One I.U of enzyme is the amount of enzyme activity which

liberates 1 m mol of reducing sugars per minute under assay conditions. Accordingly CMCase activity = m mol of glucose equivalent released/ml/minute.

a. **CMCase activity I.U** = $\frac{\text{Glucose concn. released (g/L) x 1000}}{\text{incubation time (min) x 180}}$

b. **Xylanase activity I.U** = $\frac{\text{Xylose concn. released (g/L) x 1000}}{\text{incubation time (min) x 150}}$

c. **PGase activity I.U** = $\frac{\text{Galacturonic acid concn. released (g/L)x1000}}{\text{incubation time (min) x 194}}$

Reagents :

1. Carboxymethyl cellulose-Na (low viscosity), xylan (larch wood), polygalacturonic acid-Na, D-xylose, D-galacturonic acid monohydrate, and D-glucose were purchased from Sigma Chemical Co.

2. Acetate buffer pH 4.8 - Glacial acetic acid (8.66 ml) was mixed with distilled water to 1 L of 0.15 mol/L acetic acid solution. 12.34 gm of sodium acetate was added in distilled water to 1 L of 0.15 mol/L sodium acetate solution. 0.15 mol/L sodium acetate solution was

added into 0.15 mol/L acetic acid solution until the pH of the mixture reaches 4.8.

3. Substrate solutions: Dissolve 1 g of CMC-Na or 1 g of xylan or 1 g of polygalacturonic acid-Na salt in 100 ml acetate buffer at pH 4.8 for solutions of respective substrates.

4. Glucose buffer: Dissolve 0.13 g of glucose 1 L of acetate buffer at pH 4.8.

5. Standard solutions: Dissolve 4 g of D-glucose or 4 of D-xylose or 4 g of galacturonic acid-monohydrate in 1 L of distilled water.

6. Dinitrosalicylic acid reagent: dissolve the following chemicals in 2 L distilled water: NaOH 40 g; dinitrosalicylic and 20 g; sodium sulfite 1 g; phenol 4 g. This regent should be kept in dark. It is stable for 2-3 weeks in refrigerator.

2.8.1 Assay of pectinlyase activity of culture filtrate (acidic and alkaline) :

Principle : (absorbance change method)

The principle for assaying the pectinlyase (PL) activity is based upon the formation of 4,5 unsaturated reaction products (Albershein, P. 1966 and Collmer, A, J.L. Read and M.S. Mount 1988). The increase of absorbance at 235 nm of the reaction products after incubation was monitored with the spectrophotometer.

Procedure: Enzyme extract was passed through a filter paper (milipore of pore size 5μ m) to make it spore free. The enzyme solution was appropriately diluted with distilled water so that absorbance readings remained within the spectrophotometer's scale. Enzyme solution was diluted for other reason so as to avoid the substrate limiting factor during hydrolysis with high concentration of enzymes. Diluted enzyme solution 0.5 ml was mixed well with 4.5 ml of 0.1% pectin buffer solution into screw stopper test tube. For control 0.5 ml of distilled water was mixed well with 4.5 ml of 0.1% pectin buffer solution into another test tube. The absorbance of above sets were made at 235 nm at time zero.

The tubes were then incubated at 40°C for 20 minutes. Again their absorbance were taken at 235 nm and the actual increase in absorbance of the samples were made as follows.

Actual increase in absorbance in 20 minutes.

=(Absorbance of test samples in 20 minutes - Initial absorbance of the test samples) - (Absorbance of control in 20 minutes - Initial absorbance of the control)

Units: One unit of PL is defined as an increase in absorbance at 235 nm by 1.0 in the reaction mixture per minute under assay conditions.

$$\text{PL activity (units/ml)} = \frac{\text{Actual increase in absorbance } \mathbf{x} \text{ DF}}{\text{20 minutes}}$$

Materials and Regents :

1. Pectin (from apple) was purchased from Sigma Chemical Co.
2. Tris-Hcl buffer of pH 8.5: 15.76g Tris-Hcl was dissolved in 1L distilled water to make 0.1 mol/L solution. Standard solution of NaOH was added to it to increase the pH to 8.5.

3. Phosphate -citric acid buffer of pH 5.5: 17.41g K_2HPO_4 was dissolved in 1L distilled water, 21.01g citric acid in 1L distilled water to prepare 0.1 mol/L solution. Citric acid solution was mixed with phosphate solution until the pH reached 5.5.

4. Pectin solution pH 8.5: 0.1% (W/V) pectin buffer solution at pH 8.5 was prepared by dissolving 0.1gm pectin in 100 ml Tris-Hcl buffer solution (0.1 mol/L,pH 8.5).

5. Pectin solution pH 5.5 0.1% pectin (W/V) solution was prepared by dissolving 0.1gm pectin in 100 ml phosphate-citric acid buffer (0.1 mol/L, pH 5.5).

2.8.2 Preparation of standard curve of D-glucose, D-xylose and D-galacturonic acid :

To prepare a standard curve D-glucose, D-xylose and D-galacturonic acid 0.2, 0.4, 0.6, 0.8, 1 ml of each stock (4 g/l) was taken in each test tube separately. The volume of each tube was made upto 2 ml with distilled water. To all the tubes 2 ml of DNS solution were added and the mixture was heated at 90°C for 15 minutes to develop colour. A blank was prepared with 2 ml acetate buffer instead of sugar solution. Then 6 ml of distilled water was added to each tube and mixed

well. The absorbance was measured at 575 nm against the blank. Calibration curves of each sugars were plotted by taking absorbance (O.D) as ordinate and corresponding sugar concentration as abscissa (Fig.1, 2 and 3). These curves were used for the estimation of reducing sugars released during incubation by the action of enzymes.

Standard calibration curve data for D-glucose (solution 4 g/L)

Glucose concentration, g/L	Absorbance at 575 nm (O.D)
0.8	0.33
1.6	0.60
2.4	0.91
3.2	1.08
4.0	1.34

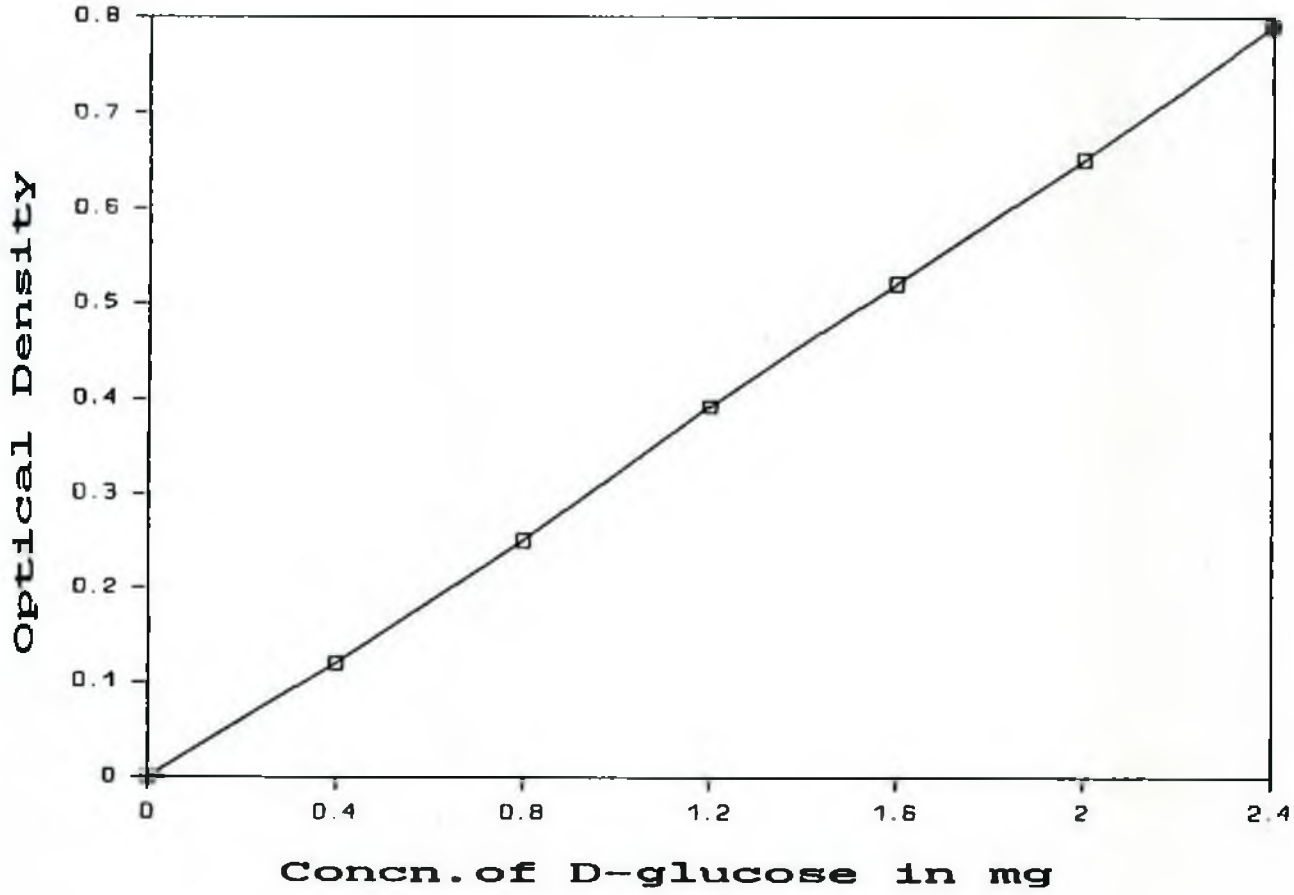
Standard calibration curve data for D-xylose (stock solution 4 g/L)

Xylose concentration, g/L	Absorbance at 575 nm (O.D)
0.8	0.32
1.6	0.62
2.4	0.86
3.2	1.14
4.0	1.36

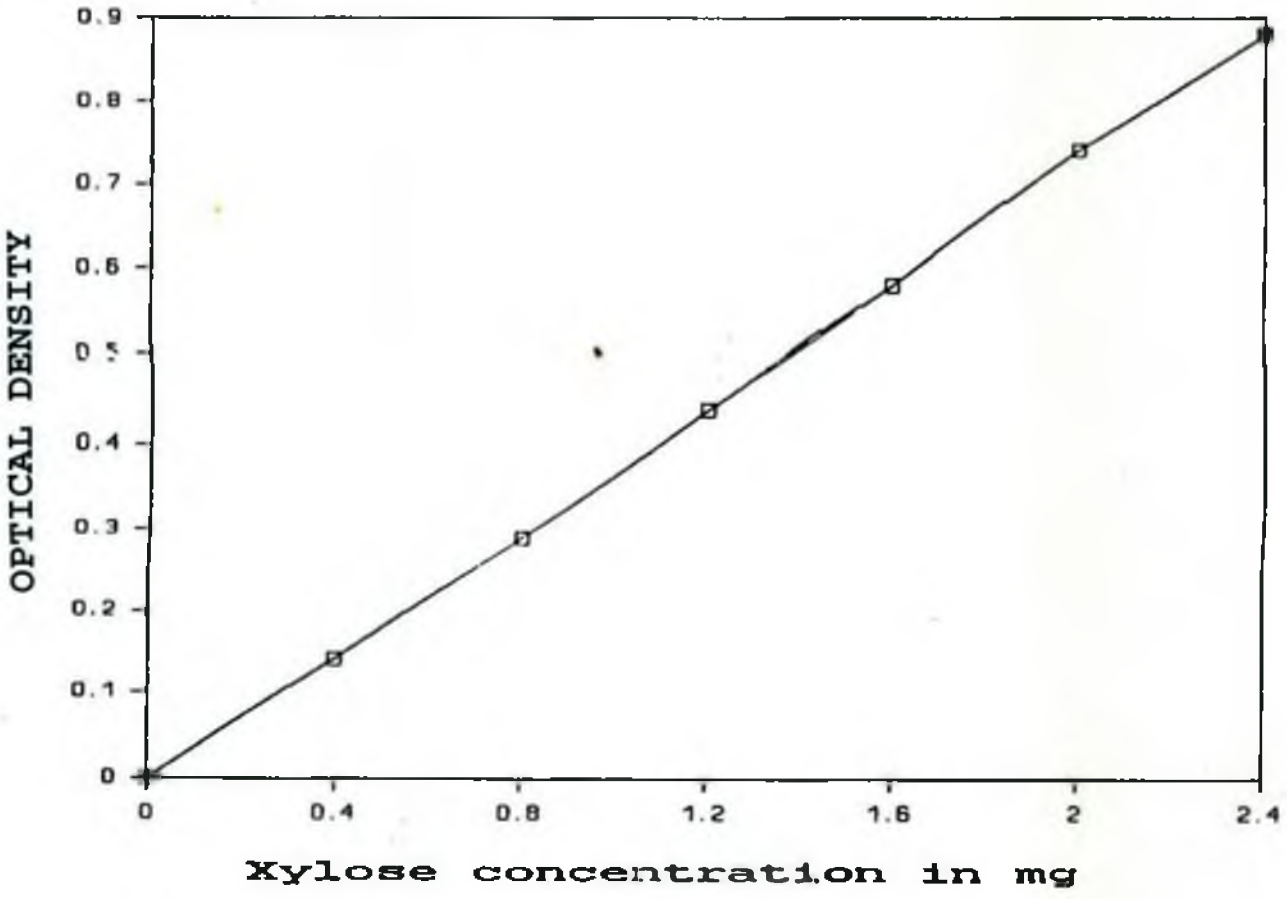
Standard calibration curve data for D-galacturonic acid (stock solution 4 g/L)

Xylose concentration, g/L	Absorbance at 575 nm (O.D)
0.8	0.33
1.6	0.60
2.4	0.91
3.2	1.08
4.0	1.34

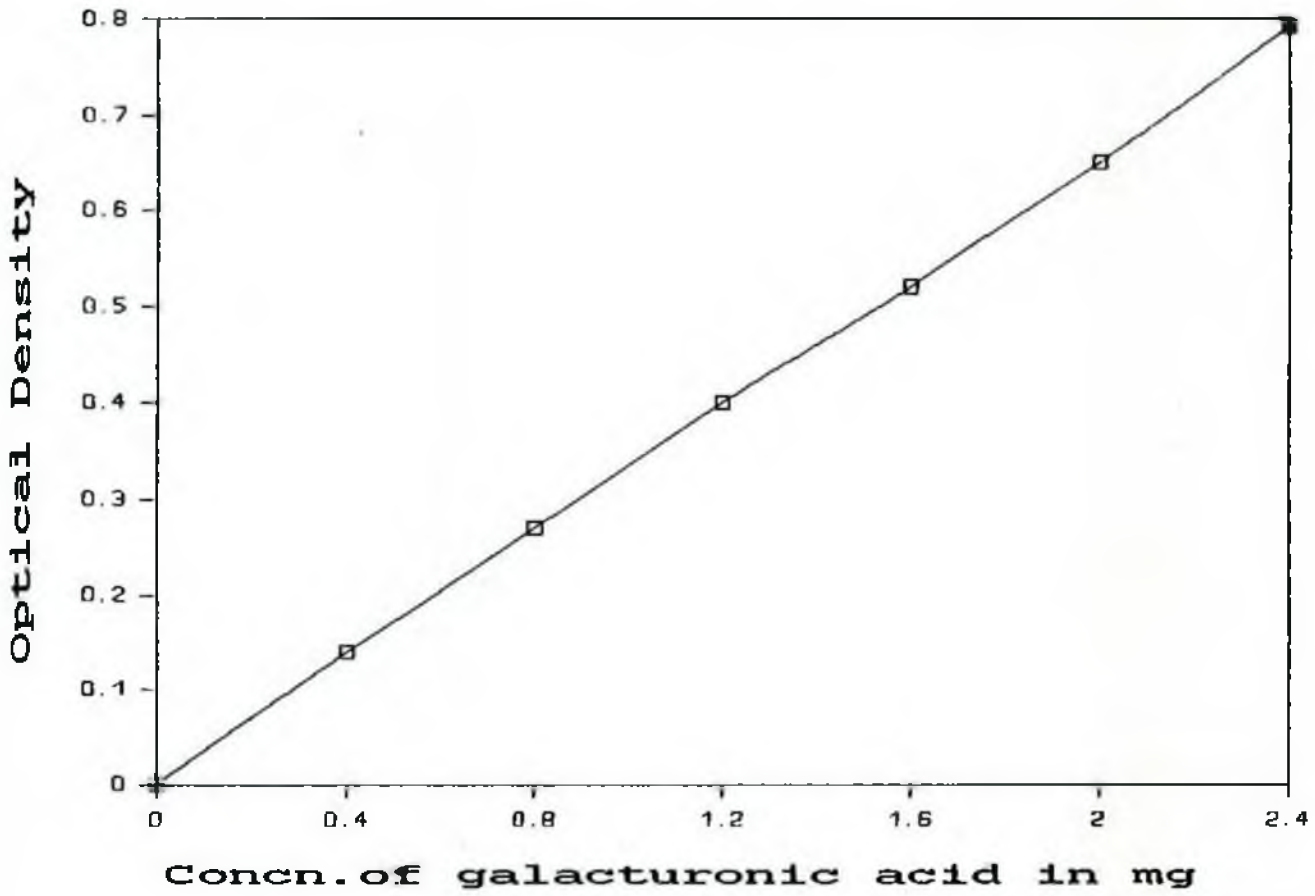
Standard calibration curve for D-glucose (stock 4 g/L)



Standard calibration curve for xylose (stock 4 g/L)



Standard curve calibration for galacturonic acid (stock soln.4 g/L)



2.9 Storage stabilities of enzyme extracts at different storage temperatures :

As the enzymes are protein they are susceptible to temperature and destruction of enzymes increases with the rise of temperature. The average daily temperature in Bangladesh is around 32°C and long stability of enzyme extracts at this temperature is required before use in jute mills. In order to ascertain the storage stabilities of enzyme extracts, the enzyme extracts were kept at different storage conditions with preservatives. A part of the spore free enzyme extracts was kept at room temperature (32°C) and the other part was kept in the refrigerator (at 4°C). Residual enzyme activity of both types of enzyme extracts stored ones were measured every week upto four weeks. For long storage study the enzyme activity was measured after every one month at this conditions and the results are shown in chapter-3.

2.9.1 Thermal stability of enzyme extracts :

It is known that most enzymes are inactivated by heat well above 50°C due to the denaturation of the enzyme protein. The rate of inactivation of enzymes is in most cases

greatly dependent on the pH of the solution. In general, there is a zone of maximum stability and the inactivation increases beyond this zone.

The enzyme preparations were used to improve the conventional mill softening practices. Enzyme treatment has successfully softened and upgraded the low grade jute and cuttings and also reduced the maturation time to almost half of normal time. During the mill softening process the temperature of the piling bin gradually rises to around 60°C. In this context, the thermal stability of enzyme component is of utmost importance. To ascertain the thermal stability, the enzyme extracts were incubated at a temperature almost similar to the temperatures of piling bin conditions and the residual enzyme activity was measured after each incubation period.

First the enzyme extracts were kept in the incubator at 40°C for 24 hours, and their activity were measured. Then the enzyme extracts were incubated at 50°C for another 24 hours and their activity were measured. Finally the enzyme extracts were incubated at 60°C for 24 hours and their activity were measured.

Enzyme assays were carried out after each storage period by incubating for 20 minutes at 40°C in acetate buffer of pH 4.8. Enzyme components of both the extracts were found to lose their activity gradually with the rise of temperature and storage period. But both extracts retained almost 50% of their original activity after 72 hours of storage in conditions similar to piling temperatures. Results of thermal stability of enzyme extracts of A.niger and A.sojae are placed in chapter-3.

2.9.2 pH stabilities of enzyme components of A.niger and A.sojae :

The pH activity profiles of enzymes reflect the pH at which the catalytic site of an enzyme are in their required state of ionization. Since enzymes are proteins, containing many ionizable groups, they exist in a whole series of different state of ionization depending on the surrounding pH. In general, there is a zone of maximum ionization and the ionization decreases either on the acid or alkaline sides beyond this zone.

pH stabilities of enzyme components of culture filtrates were carried out by incubating the enzyme solution at different pH for 24 hours at 4°C. The assay of enzymes were carried out following the standard procedures.

2.10 Test of different chemicals as preservatives :

The enzyme extracted need to be stored as it may not be used immediately after extraction. At room temperature it get pungent smell due to bacterial growth. Moreover it may not be possible for jute industry to carry them in refrigerated van. So in order to keep the enzyme extract free from microbial contamination a number of chemicals butanol, sodium azide, toluene, sorbitol etc. were added as preservatives and their performance as preservatives is shown in chapter-3.

2.11 Optimization of enzyme activity in respect of pH and temperature :

Temperature affects the velocity of enzyme reacting in various ways. It may have effect on the stability of enzyme, velocity of breakdown of the complex, on the enzyme substrate affinity, on the pH functions of any or all components and these suggest that the effects of temperature are extremely complex. Heat stability and suitable temperature for the

enzyme itself can be studied by exposing the enzyme to various temperatures for a definite period, before measuring its activity at a temperature at which it is stable. The effect of temperature on the affinity was eliminated by using sufficiently high concentrations of substrate to saturate the enzyme. The limiting effects of other enzymes in multi-enzyme system of both fungal extracts were studied separately.

It is known that as the temperature is raised the initial velocity increases together with a destruction of the enzyme at the higher temperature, producing a continuous destruction of enzyme until the velocity falls to zero. Thus velocity of enzyme reaction at any finite time first increases and then falls, giving an apparent optimum temperature. The optimum temperature is determined by the balance between the effect of temperature on the rate of enzyme reaction and rate of enzyme destruction.

The optimum temperatures for the enzyme components of A.niger and A.sojae were determined by measuring the enzyme activity after incubations at temperatures ranging from 30°C t2o 60°C for 20 minutes at pH 4.8. Results of optimization of temperatures for the enzyme components of A.niger and A.sojae are shown in chapter-3.

2.12 Effect of different ions on enzyme activity :

Mechanism of metal ion activation is greatly influenced by the nature and concentration of ions present. Certain ions are absolutely necessary for the activity of some enzymes, while others are highly toxic (Ag^+, Hg^+, Pb^+) Generally enzyme is active without electrolytes and almost any anion will have an effect. One of the important factors which determine which metal ions will activate enzymes is the size of the ions. The ionic effect may vary with substrate, pH, Purity and age of the enzyme.

The mechanisms of metal ion activation is that it forms an essential part of the centre of the enzyme. In other way, metal ion may act as a binding link between enzyme and substrate, combining with both and thus holding the substrate at the active centre of the enzyme. Other metal ions may have apparent activation effect on the enzyme by changing the equilibrium constant of the enzyme reaction. The metal ions may accelerate the enzyme reactions by removing the inhibitor present by forming a complex or precipitating with it.

To determine the effect of different chemicals on enzyme activities the enzyme solutions were pre-incubated with each

chemical solutions (10 mM/L) at room temperature for one hour. The enzyme activities were measured in each solution following the standard procedures. The ionic effects on enzyme activities were recorded and compared with the control as the percentage of the original without chemicals as 100 percent. Results of ionic activation on the enzyme activities are shown in chapter-3.

2.13 Kinetic study of xylanase :

The initial velocity of an enzymatic reaction according to the Michaelis and Menton equation is determined by the following equation.

$$V_0 = \frac{V_{max}\ [S]}{S] + Km}$$

Where V_0 = initial rate at substrate concentration [S]
V_{max}= maximum rate

Km = Michaelis-Menton constant of particular substrate. This is the Michaelis-Menton equation, the rate equation for a one substrate enzyme catalyzed reaction. It is a statement of the equation relationship between the initial velocity V_0, the maximum velocity V_{max}, and the initial substrate

concentration, all related through the Michaelis-Menton constant Km.

An important numerical relationship for enzyme activity may be derived from the Michaelis-Maintain equation in the special case when the initial reaction rate is exactly one-half the maximum velocity, i.e.

When V_0 = 1/2 Vmax then

$$\frac{Vmax}{2} = \frac{Vmax\ [S]}{Km + [S]}$$

If we divide by V_{max}, we get

$$\frac{1}{2} = \frac{[S]}{Km + [S]}$$

Solving for Km, we get Km + [S] = 2[S]

. . Km =[S] when V_o is exactly 0.5 V_{max}

The Michaelis-Menton equation is the basic to all aspects of kinetics of enzyme reaction. Thus if Km and V_{max} are known, we can find out the reaction rate of an enzyme at any given concentration of its substrate.

Effect of substrate (xylan) concentration on xylanase activity and determination of Km value.

The reaction was carried out at 40°C for 20 minutes incubation, using acetate buffer (pH 4.8) with different concentration of xylan from 0.5% to 5% and expressed as m mol/ml/min.

Effect of substrate (xylan) concentration on xylanase activity and Km value have been given in chapter-3.

Transformation of Michaelis-Menton equation

The Michaelis-Menton relationship equation can be algebraically transformed into other forms that are more useful in plotting experimental data. One common transformation is derived simply by taking the reciprocal of both sides of Michaelis-Menton equation

$$V_o = \frac{V_{max}}{S} \quad \text{After rearrangement we have} \quad \frac{1}{V_o} = \frac{S}{V_m}$$

This is line weaver- Burk equation. When $1/V_o$ is plotted against 1/[S], a straight line is obtained. This line will have a slope of Km/V_{max}, an intercept of $1/V_{max}$ on the $1/V_o$

axis and an intercept of -1/km on the 1/[S] axis (Fig. No.). Such as double- reciprocal plot has the advantage of allowing a much more accurate determination of V_{max}. The double reciprocal plot can also give valuable information regarding enzyme inhibition reaction pattern.

Effect of inhibitor on enzyme activity :

An enzyme inhibitor is a substrate that reduces or blocks an enzymatic catalysis. They are classified as competitive, uncompetitive and noncompetitive depending on the type of effects they exert on the reaction kinetics of enzymes.

A competitive inhibitor is that which competes with the substrate for binding at the active site. A competitive inhibitor reacts reversibly with the enzyme to form an enzyme inhibitor complex (EI), analogous to the enzyme substrate complex E+I _ EI.

The inhibitor is not changed chemically by the enzyme. Thus the inhibitor constant Ki, as the dissociation of

enzyme-inhibitor complex can be formulated through Michaelis-Menton formulation

$$K_1 = \frac{[E]\ [I]}{[EI]}$$

The inhibitor constant K_1 is comparable to Km, the dissociation constant of the enzyme substrate complex.

Competitive inhibitor is easily recognized experimentally because the percent inhibition at a fixed inhibitor concentration is decreased by increasing the substrate concentration. For quantitative kinetic analysis, the effect of varying the substrate concentration (S) on the initial velocity v_0 is determined at a fixed concentration of inhibitor. This experiment was repeated with a different concentration of inhibitor, often several series of such experiments were carried out, each at a different concentration of inhibitor. Plots of $1/V_0$ verses 1/(S) were prepared one for each concentration of inhibitor. These plots characteristically gave a family of straight lines intersecting at common intercept on the $1/v_0$ axis (Fig. No.). The presence of a competitive inhibitor thus increases the apparent km of the enzyme substrate i.e. causes it to require a higher substrate concentration to achieve maximum velocity.

The apparent km for the substrate will be greater than the true km by the increase in the intercept on the 1/(s) axis. Since the slope of the plot of the inhibited reaction is km/v_{max} and the slope for the inhibited reaction is km/v_{max} [1+(I)/ki], the slope is increased by a factor of 1+(I)/ki. From this relationship Km value can be calculated. On the other hand, a competitive inhibitor characteristically does not affect v_{max} indicating that it does not interfere with the rate of break down of the enzyme substrate complex.

2.14 Gel Electrophoresis for the determination of molecular weight :

Enzyme extract from four days fermented bran was passed through milipore filter to make it spore free and this crude solution was subjected to SDS-Polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (1970).

Preparation of separating gel :

3.55 ml of 44% (W/V) acrylamide solution, 6.25 ml of 0.75 Tris-Hcl buffer, 125 ml of 20% SDS, 12.5 ml of TEMED were mixed gently with 2.25 ml of distilled water. Finally 350 ml of 40 mg/ml ammonium persulphate (APS) was added and mixed homogeneously by gently shaking so that bubbles can not

be formed. The solution was poured immediately into glass slab gel apparatus so that upper 3 cm remained vacant. The vacant space was filled with water saturated isobutanol and was left for 30 minutes for solidification. After solidification iso-butanol was removed and washed with distilled water.

Preparation of stacking gel :

2 ml of 30% (W/V) acrylamide solution, 5 ml 0.5 M Tris-Hcl (pH-6.8) 200 µL SDS (20% W/V), 25 µL TEMED were mixed gently with 12.5 ml distilled water.

480 µL of ammonium persulphate (40 mg/ml) was added with it and mixed homogeneously by gentle shaking so that no bubble was formed. Immediately, the solution was poured into the top of the solidified separating gel. A suitable comb was set into the stacking solution and allowed to solidify. After solidification the comb was removed carefully.

Preparation of sample and application :

Sample (protein) was mixed with 3 µL of sample buffer. The sample buffer was prepared by mixing 1 ml each of 100 mm phosphate buffer (pH 7), 3% (W/V) SDS, 40% (V/V) glycerol, 6%

(V/V) βb-mercapto ethanol (W/V) bromo-phenol blue. The prepared samples were boiled for 2-5 minutes and applied to the top whole of the solid electrophoretic gel.

Running of electrophoresis :

Electrophoresis was carried out at a constant voltage, first at 50 volt for 1 hour and then at 100 volt until the marker bromophenol blue migrated at the end of the gel (just 1 cm above the last end).

When the run was completed, the gel was taken out from the glass chamber and was submerged in staining solution for 3 hours at 37°C. After 3 hours the slab was submerged in destaining solution (10% acetic acid) for 30 minutes. Then gel was washed with distilled water and the photograph was taken.

2.15 Estimation of different chemical constituents of enzyme treated fibres :

Low grade jute and cuttings are hard and stiff. The enzyme extracts were applied to soften and defibrate them through hydrolytic degradation of non-cellulosic materials - pectin and hemicellulose. The softening effect of enzyme extract was carried out by incubating the uniform pieces of

jute cuttings in sporefree enzyme extract for 24 hours at 35°C. For control uniform pieces of fibres were soaked in sterile water at similar conditions. After incubation the fibres were washed and dried carefully. The dried fibres were grounded for the estimation of following constituents.

Estimation of cellulose :

Cellulose content of enzyme treated fibres was estimated by Cross and Bevan method (C.Doree. 1950). One gm powder of enzyme treated fibre was boiled with 1% NaOH solution in ground bottom flask. The content of the flask was then cooled, filtered and washed well with water in previously cleaned and weighed sintered crucible of porosity 2. Residue of each crucible was then transferred in conical flask placed in an ice bath and a slow stream of washed chlorine gas was passed through the materials for half an hour and allowed to stand for sixty minutes to ensure completion of reaction. Chlorinated fibres were then filtered, washed well with water to remove HCl, transferred to a beaker containing 50 ml of 2% sodium sulphite solution. Content of each beaker was then heated to boiling. Later 2.5 ml of 4% NaOH solution was added and boiled for another five minutes. The resulting mixture of

the beaker was then filtered, washed repeatedly with hot water and finally bleached by immersion in 1% K_2MnO_4 solution followed by 5% oxalic acid. Cellulose content was then digested for thirty minutes with hot distilled water, filtered and dried to a constant weight and the results are shown in chapter-3.

Estimation of Hemicellulose :

Hemicellulose was estimated by the method of Callow (1952). Jute powder was first defatted and then depectinized by refluxing with 0.5% ammonium oxalate solution for about four hours at 80-85°C. For delignification, the residue was first heated to boiling for about 30 minutes with 0.7% sodium chlorite solution. The resulting mixture was then filtered in a sintered crucible of porosity 2, washed several times with water and finally with sodium bisulphite (pH 5.5). The process was repeated thrice for each sample. The above process was again repeated thrice by using 2.8% sodium chlorite solution instead of 0.7% sodium chlorite solution. The residue on sintered crucible was then transferred to a beaker and extracted by mechanical stirring with 9.3% NaOH solution for 2 hours. Extract of each sample was taken in 100

ml volumetric flask and made up to the volume by using the same extractor. 25 ml of the aliquot of each sample was first neutralized with acetic acid and then saturated with absolute alcohol to precipitate out the hemicellulose. The resulting mixture was then centrifuged in a previously cleaned and weighed centrifuged tube, precipitate of hemicellulose was first washed with different grades of alcohol and then with acetone to remove the last trace of water. Content of each tube was then dried to a constant weight and the results are shown in chapter-3.

Estimation of Pectin :

Pectin content of enzyme treated fibres was estimated by calcium pectase method, a modified method of Carre and Hayness (Kertesz, Z.I. 1957). Two gm of fibres (Wax free by petroleum ether) was extracted with 0.05 N in a distillation flask. Extractions were repeated 3-4 times with 50 ml extractor each time after half an hour interval. Extract was then collected in a 250 ml volumetric flask and made up to the volume by using the same extractor. 25 ml of this extraction was used to estimate the pectic substances in the sample. 25 ml aliquot of extraction was first neutralized in

a 500 ml beaker. 5 ml of 10% HCl was added and the mixture was made to a volume of 100 ml. 200 ml of 95% ethanol was added with vigorous stirring and it was allowed to stand overnight. The content of the beaker was filtered and the residue was first washed thrice with a water-ethanol (1:2) mixture and then with 95% ethanol. The precipitate was then dissolved in hot water.

The filtrate was made up to a volume of 200 ml, 150 ml of 0.2 N NaOH was added and the resultant mixture was allowed to stand overnight. 60 ml of 1N CH_3COOH was added with stirring, the mixture was allowed to stand for a few minutes. First 25 ml of 0.1M then 25 ml of 0.2N $CaCl_2$ were added drop wise with constant stirring. The mixture was boiled for exactly 2 minutes, filtered and washed three times with boiling water. The precipitate was washed back with 100 ml of water and boiled for 2 minutes. Finally it was then filtered through previously cleaned dried and weighed sintered crucible of porosity 4 and the results are shown in chapter-3.

2.16 Effect of enzyme extracts on physico-mechanical properties of jute cuttings :

Culture filtrate from 4 days fermented bran by A.niger. A.sojae and Flaxzyme (commercial enzyme) were used as enzyme source for softening of jute cuttings. Softening activity of culture filtrates on jute cuttings were performed in big glass jars. Jute cuttings of uniform quality (30 cm by 2.5 cm) were incubated with culture filtrates for 24 hours at 35°C, washed with distilled water and dried. Air dried samples were used for the study of physico-mechanical properties. Control experiments were performed with samples treated with distilled water instead of enzyme solution. The culture filtrates were made spore free by filtration (milipore) before application to fibres.

Fibre separation index- This is one of the most important characteristics used in the jute industry for fibre analysis. This index indicates the ability of fibres to sperate from barks and speaks. In measuring this index, iron combs were used for separating fibres from barks. The number of combings was kept same for each samples (20 times). The fibre bundle left after combing was weight to be WS and was

sorted out manually to sperate the specks. The clean fibres (without specks) were weighed again to be WC. The Separation Index (SI) was found out by the following equation:

$$S.I. = \frac{WC}{WS} \times 100$$

The higher the values of separation index the more separable the fibres are and the ultimate objective of any softening treatment is to improve this attribute of fibres.

Fibre Fineness: For textile raw materials in general, the transverse fibre dimensions are of utmost importance for spinning. The mean diameter of a fibre was taken as a measure of fineness. The individual fibre filament was sorted out and cleaned by hand. Ten such individual fibre filaments were placed on mounting slide of projectina microscope by scotch tape. Ten fibre filaments were taken for each type of sample and ten readings were taken from ten different positions of a fibre filament. Mean value of each filament readings was taken as a measure of fibre fineness.

Stiffness: It is a measure of degree of softening of fibres and was measured as described by V.Ramamurty et al. (1987). A.fibre bundle of 30 cm long (mass 0.55- 0.58 g) was hung

over a glass rod (3.8 mm in diameter) and a constant mass of 1 gm was attached to each end of the fibre bundle. The distance (cm) between the two hanging ends was measured and taken as the stiffness index. Higher values mean that the fibre is more stiff.

Fibre strength (presley index) It is the quality of fibres by which the strength of different fibres are assessed and compared. Here a small flat bundle of parallel fibres gripped between special clamps was tested by the presley strength tester. The fibres were manipulated into a parallel ribbon about 1/4 inch wide. The ribbon was placed across two clamps which were tightened to a predetermined limit. A heavy rolling weight was held by a clutch and when the clutch is lifted the weight rolls down a bar. As soon as the break occurs the weight stops instantly. The fibres were collected and weight in a balance. The breaking load was obtained in pounds and the weight is in milligrams. The presley index is expressed as follows :

$$\text{Pressly index} = \frac{\text{Breaking load in pounds}}{\text{Bundle weight in milligrams}}$$

2.17 Large scale enzyme productions for mill trials :

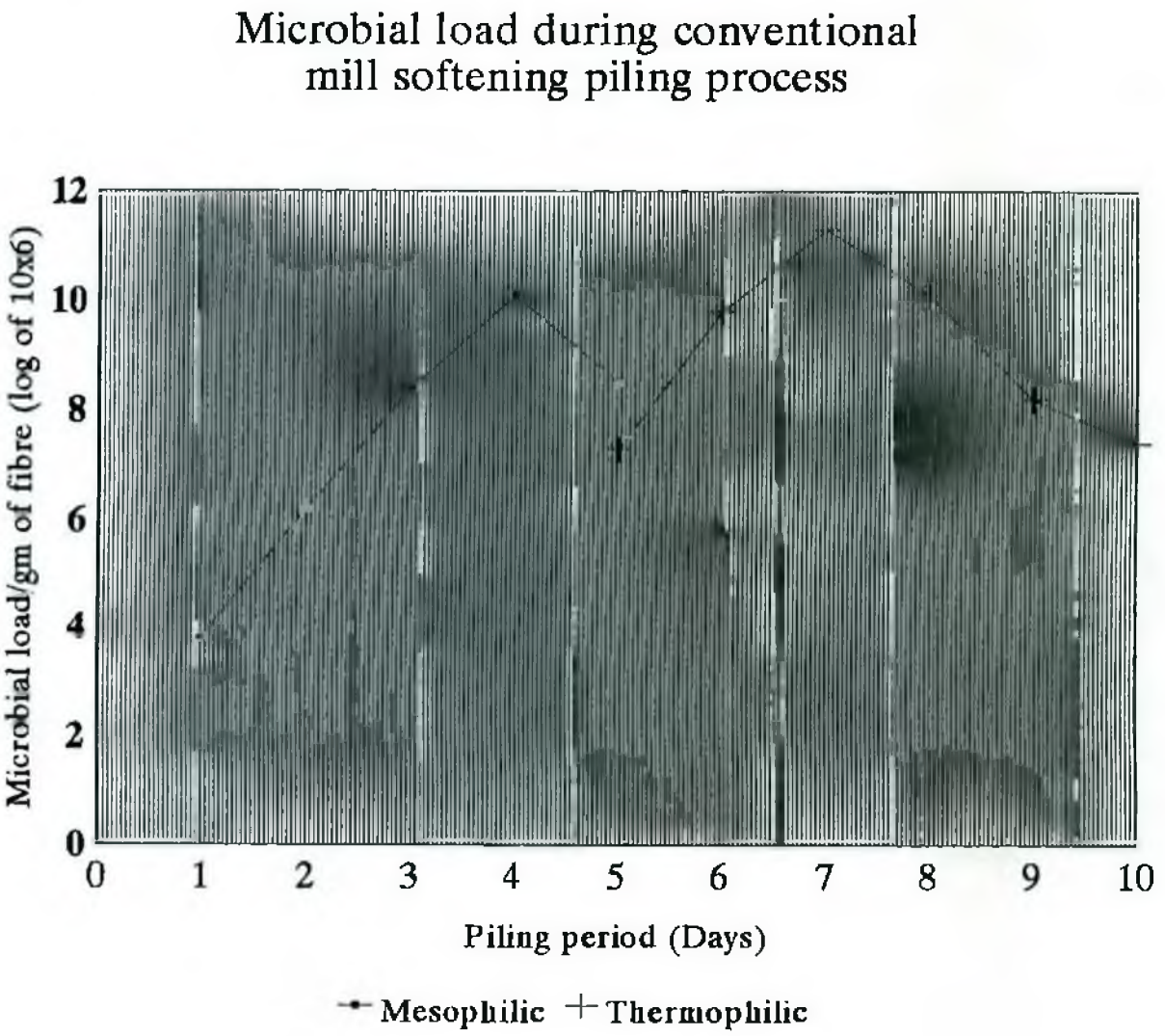
For large scale mill trials enzyme extracts were prepared in flat aluminum trays with coverings. Wheat bran was moistened and sterilized in trays and inoculated with inoculum (50 ml) prepared earlier. The inoculum was prepared in yeast malt extract containing 0.3% yeast, 0.3% malt, 0.5% peptone and 1% dextrase at pH 6 in a shaker at 100 r.p.m at 35°C for 24 hours. The inoculated bran was allowed to ferment for 4 days. Enzymes were extracted from fermented bran by adding 5 volumes (W/V) of 1% sodium chloride solution. The water soaked bran was macerated for two hours and the slurry was first filtered with nylon cloth and then centrifuged at 3000 r.p.m for 10 minutes. The clear supernatant filtrate was preserved with preservatives as enzyme extract for mill trials. Pilot scale mill trials were carried out with 900 kg of cuttings at BJRI experimental mills. Enzymes extracts about 12 litres were mixed well with jute batching emulsion and applied to the cuttings @35% through softener machine. Enzyme treated fibres were piled up in a concrete block with coverings. After 72 hours of piling when the temperature of the bin reached 60°C the fibres were processed as per usual carding and spinning was done in slip draft with flyer speed 1700 rpm.

RESULTS

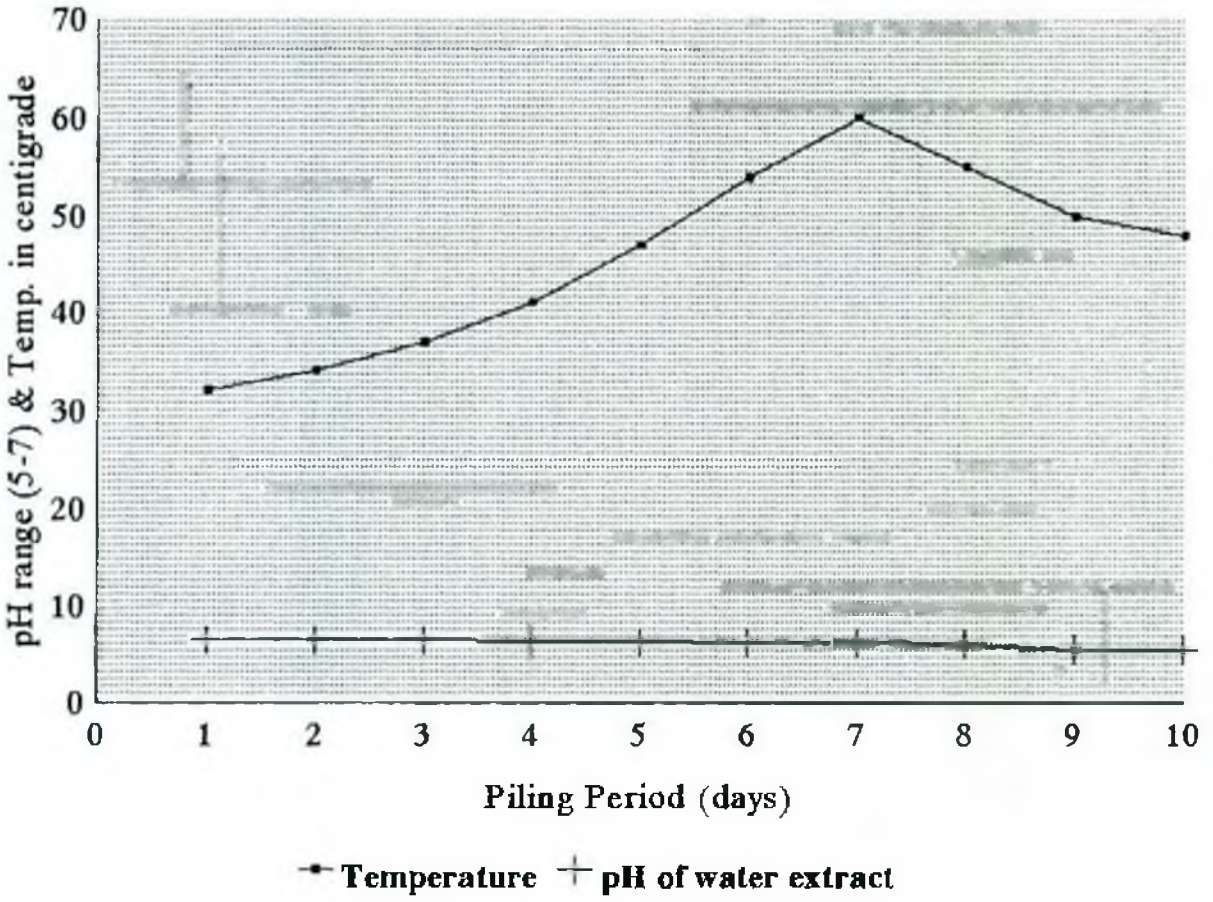
3.1 Microbial load, temp. rise and pH changes during conventional mill softening piling process :

To improve the conventional mill softening practices, a detailed study in respect of microbial load, temperature rise and pH changes were recorded during the piling process. Jute samples were collected and temperatures were recorded daily from three positions (top, middle and bottom) of jute maturation piling bin. Microbial load were counted following the procedures as described in materials and method section and a maximum number of 10.1×10^4/gm mesophilic microbes and 10.3×10^4/gm thermophilic microbes were recorded on 4th and 7th day of piling process.

A highest temperature of 60°c was recorded on 7th day of piling process and pH of water extract was changed from initial value of 6.5 to 5.47 and the results are shown in the following figure 1 and 2.



Temperature rise and pH change during conventional mill softening piling process



3.2 Screening of fungi for CMCase, PGase and Xylanase activity :

Microorganisms, specially the mesophilic fungi, were isolated from natural habitats as they are capable of secreting extracellular enzymes into the growth medium (Wood T.M. 1975). The isolated strains were randomly picked, purified and maintained on PDA slants in the refrigerator. The isolated strains (mostly fungi) were tested for softening and defibrating activities on jute cuttings. Screening of fungi for their enzyme activities were carried out by the hydrolysis of substrates incorporated in the minimal medium as the sole carbon source.

After an appropriate incubation period enzyme activities were detected by the appearance of zones either by substrate clearances or coloration and decoloration. Fungal strains were screened for their activities of CMCase, PGase and xylanase and are shown in the following photograph of petri-dishes. Out of the 20 isolated strains, A. niger showed the maximum Xylanase and pectinase activity using screening technique as mentioned in chapter -2. Results of screening tests are shown in the following petri plates.

3.3 Morphological features of isolated potential fungus :

The development of the colony of the fungus on PDA plates was observed for several days. The young vegetative mycelial growth started with whitish colour mat which turned to jet black within three days (plate-1). On the basis of the structural features the fungus was characterised as Asergillus niger.

Plate 1. Aspergillus niger grown on PDA plate showing the characteristic jet black colour in 4 days old oulture.

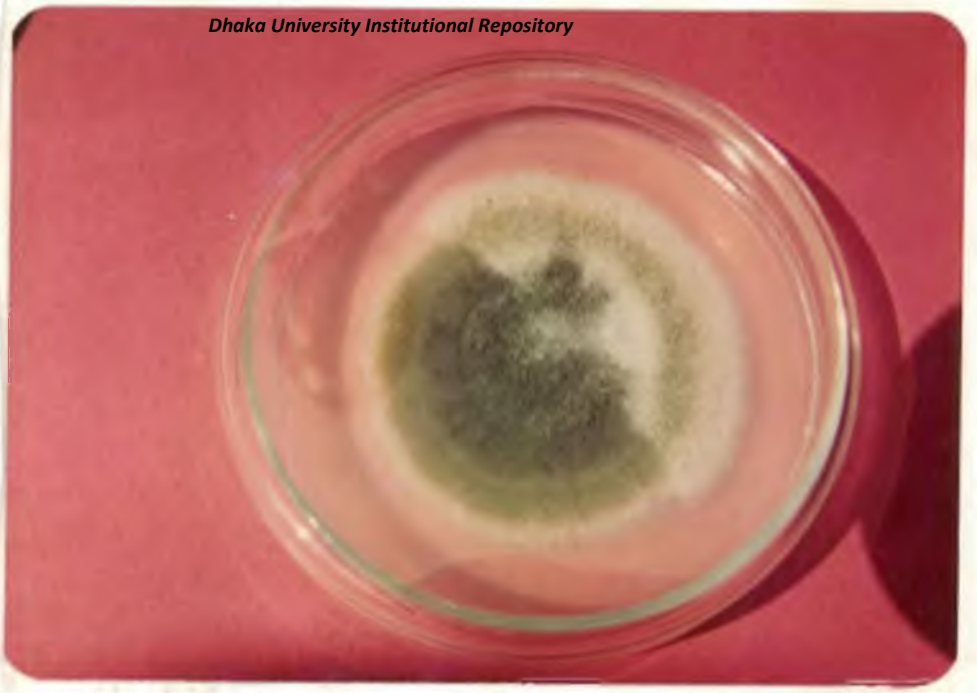


Plate 2. Aspergillus sojae (ATCC-20235) grown on PDA plate showing the light greenish colour of 4 days old culute.

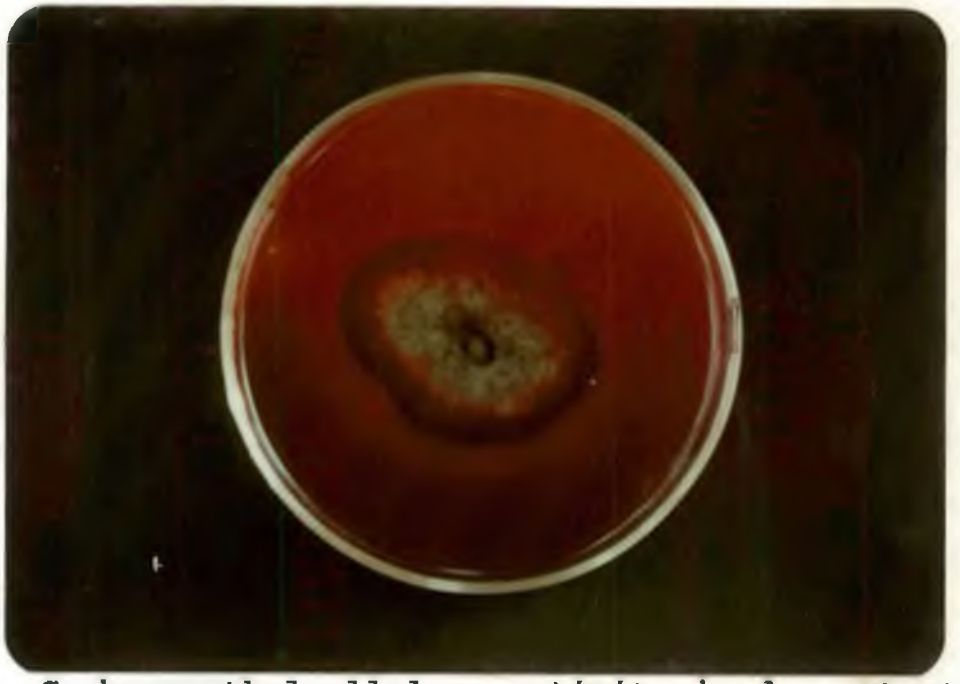


Plate 3. Carboxymethylcellulase activity is demonstrated on PDA plate by the pale orange colour zone around the fungal colony of A. niger.

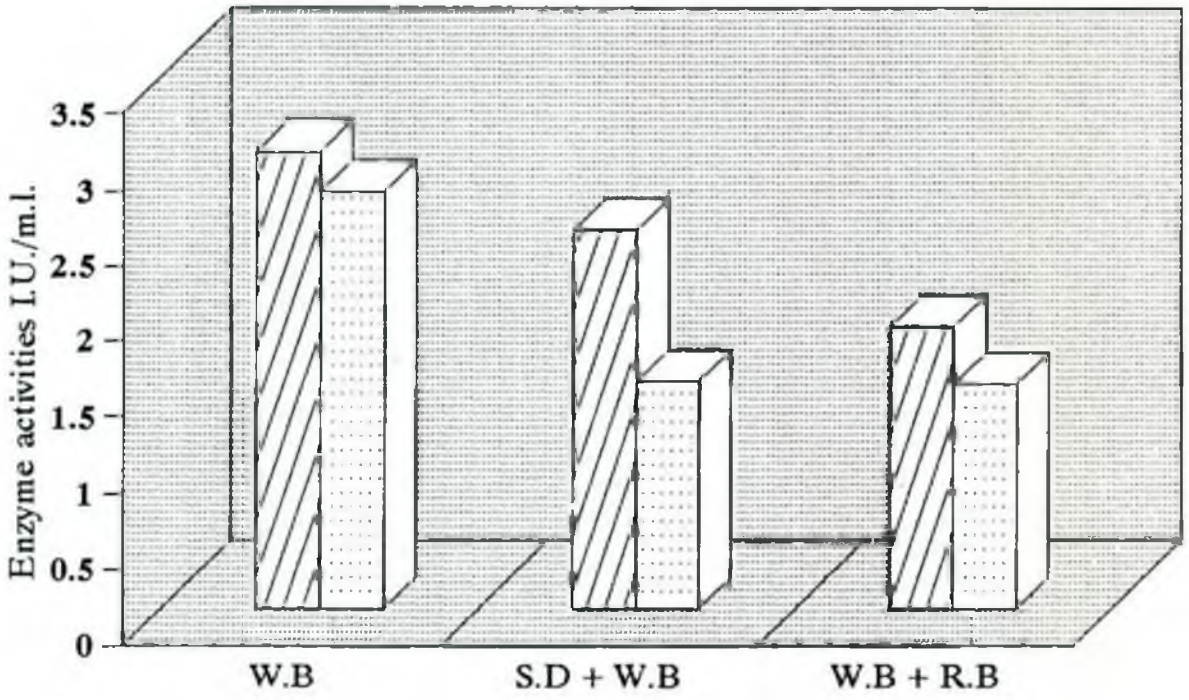
Plate 4. Xylanase activity is demonstrated on PDA plate by the clear zone around the fungal colony of A. niger.

Plate 5. Pectinase activity is demonstrated on PDA plate by the hydrolytic clear zone around the fungal colony of A. niger.

3.4 Production of enzymes on different indigenous substrates by A.niger and A.sojae :

Fungi are saprophytes and a number of indigenous lignocellulosic substrates-wheat bran, saw dust and rice bran, were used for this study as growth media. These are easily available in Bangladesh through out the year and are cheap. These substrates were sterilized and inoculated with equal volume (5 ml) of spore suspension and incubated at 32°C for fermentation. Enzymes were extracted from the fermented bran by adding five volumes (W/V) of 1% saline water. The clear culture filtrate obtained by filtration and centrifugation was used as crude enzyme preparation. Wheat bran medium with 50% added moisture produced maximum 2.56 IU/ml and 2.30 IU/ml of enzymes by A.niger and A.sojae respectably in 4 days of fermentation. Results are shown in figure No 3 (Page-123).

Production of enzymes on different substrates by A.niger and A.sojae



Substrates fermented for 4 days

A.niger A.sojae

W.B = Wheat Bran, S.D = Saw Dust, R.B = Rice Bran

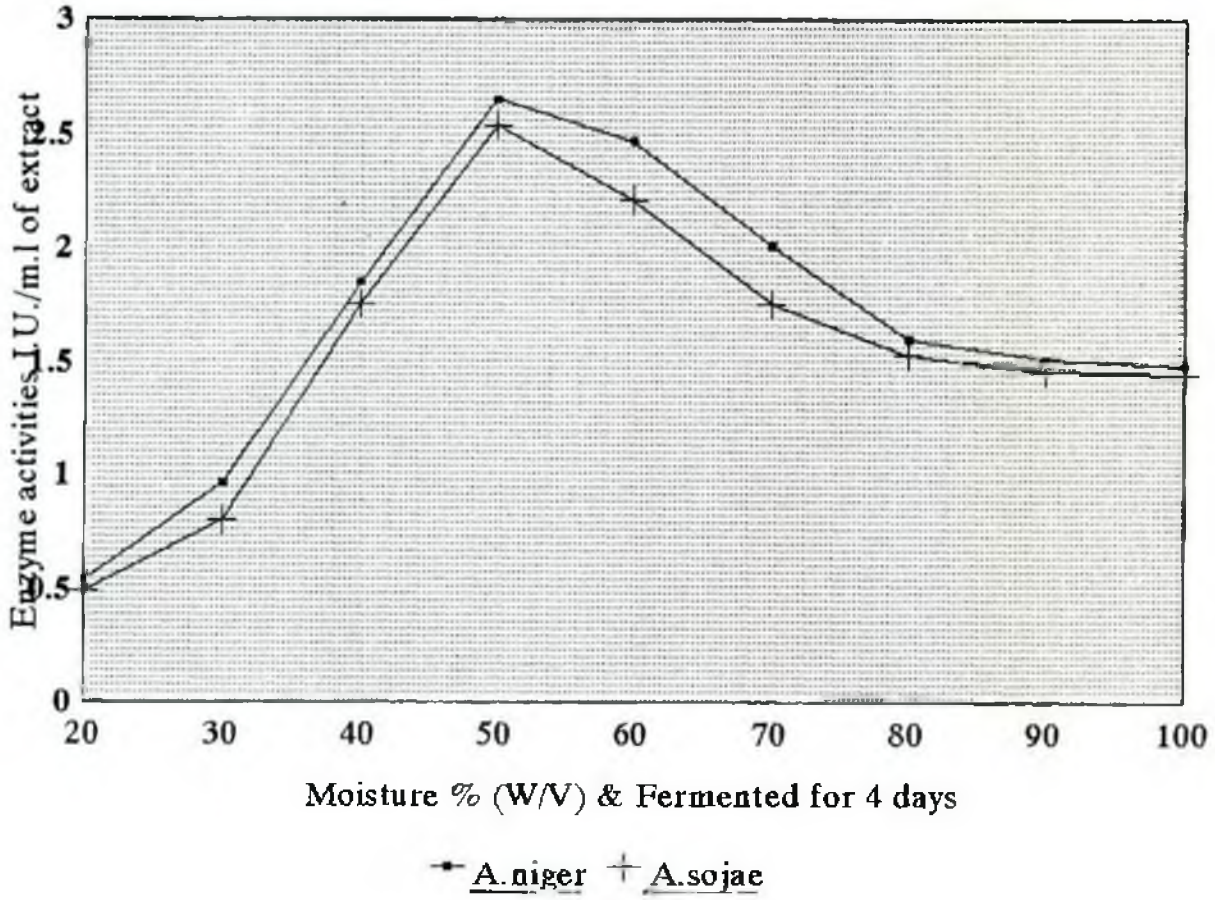
3.5 Optimization of moisture to be added to wheat bran medium for growth and enzyme production :

It is well known that presence of added moisture to solid substrate is a significant controlling factor for growth and enzyme production by the fungi in SSF (Nagai, 1980). In order to find out the optimum percent of added moisture to the bran (W/V) for growth and maximum enzyme production by the fungi were investigated with a varying degree of added moisture (20%-100% W/V). 50% added moisture was found to be optimum for growth and maximum enzyme production by both the fungi. Results are shown in figure 4.

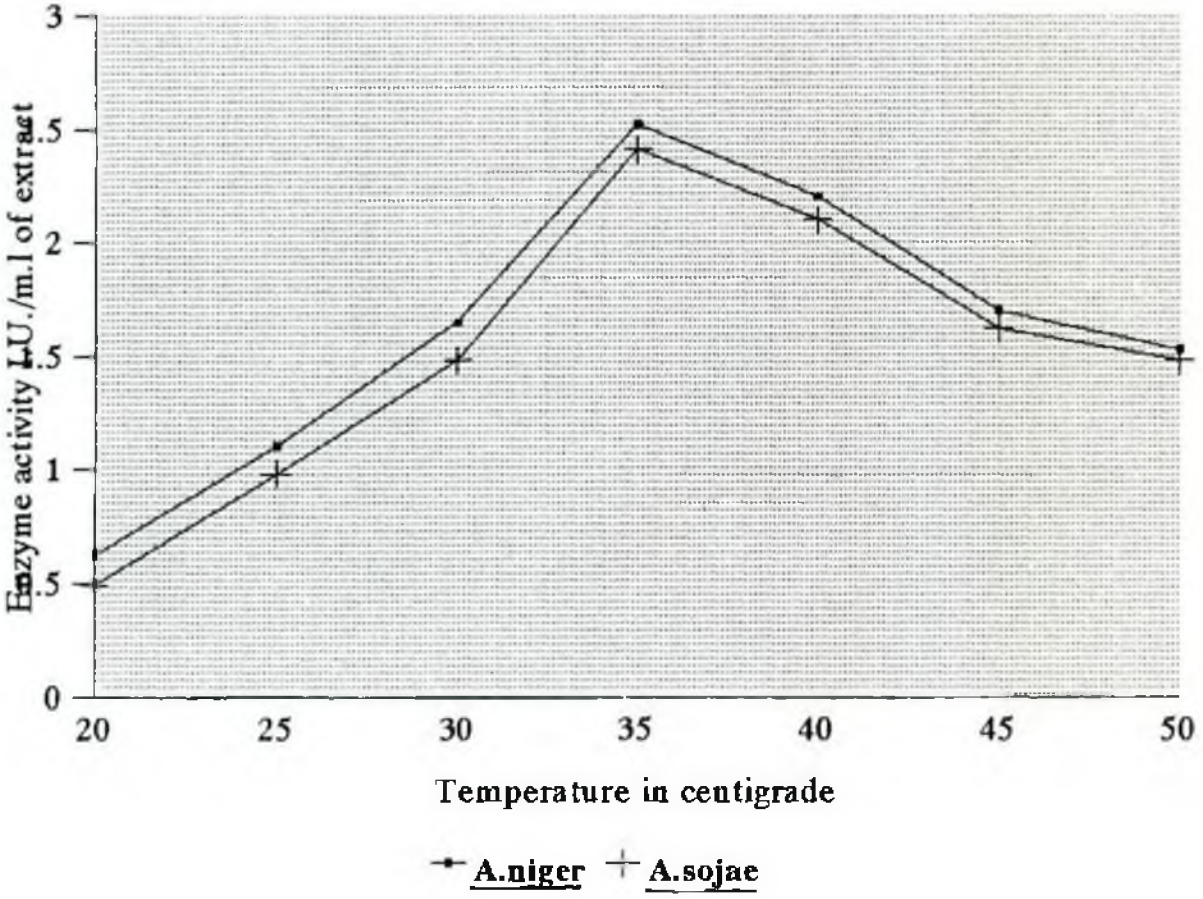
3.5.1 Optimum temperature for SSF by two fungi on wheat bran with optimum 50% moisture :

Like other microorganism fungi are very sensitive to temperature for their growth. So once the optimum level of added moisture for wheat bran medium for SSF was optimized the fungi were grown with this level of moisture at different incubation temperatures for enzyme production. The optimum temperature for growth and enzyme production by the fungi were found out from the results of enzyme production. Both the fungi were found to grow at a temperature range of 30-40°C. But at 32°C both the fungi A.niger and A.sojae. grew profusely on wheat bran medium and produced 2.56 IU/ml and 2.30 IU/ml of enzymes respectively. Results are in figure 5.

Optimization of added moisture for SSF on wheat bran by A.niger and A.sojae



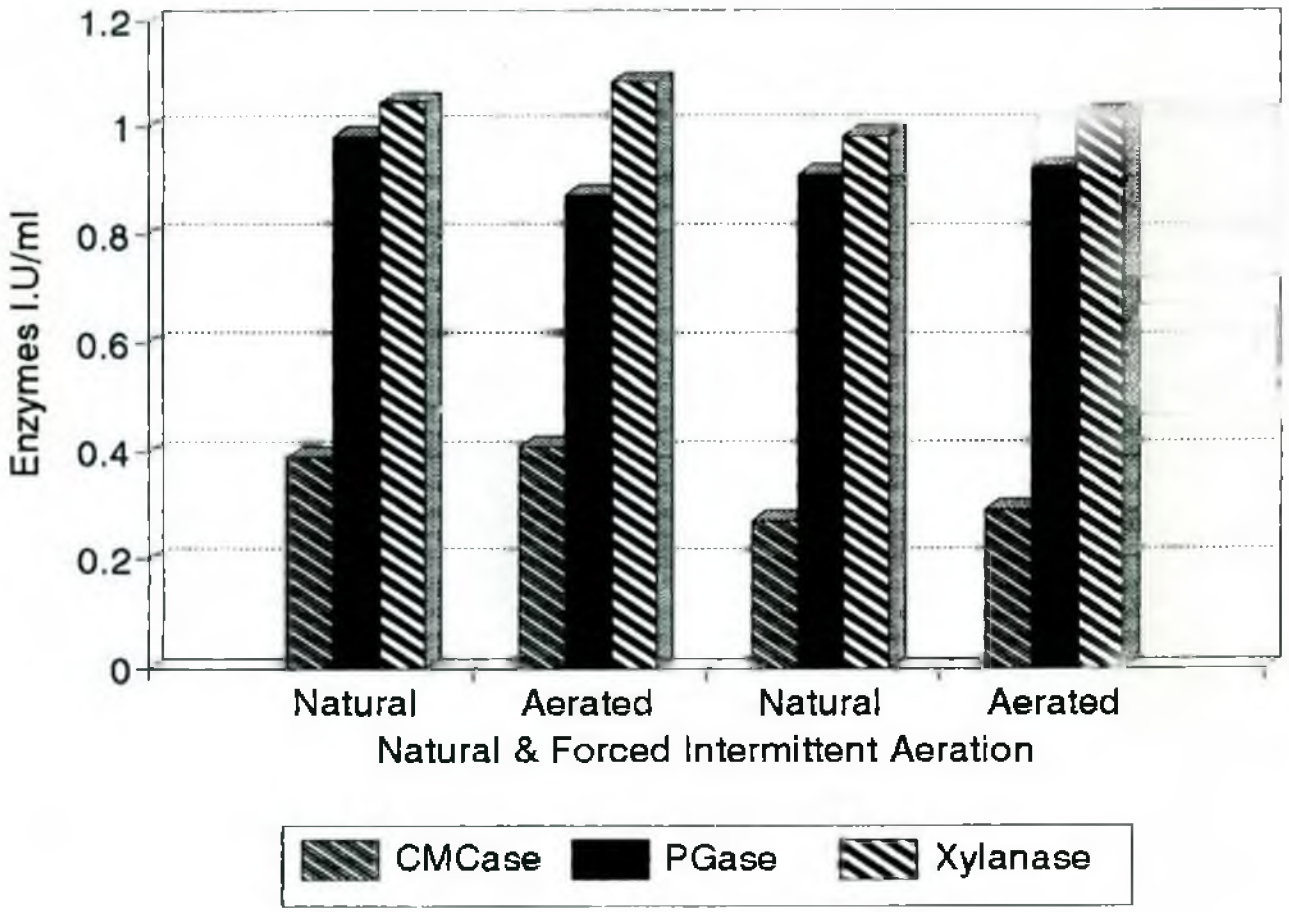
Temperature optimization for SSF by A.niger and A.sojae with 50% added moisture



3.5.2 Effect of aeration on W/B medium on growth and enzyme production by the fungi (A.niger & A.sojae):

Due to its simplicity in operation, low capital investment and easy extraction of product, the SSF technology has been and is being used for many commercial enzymes preparation (Channel and M. Moo Young, 1980). And the question was raised whether any sort of aeration is required for the optimal production of enzymes. Effect of aeration was carried out by conducting SSF in conical flask of 1 L capacity with a natural aeration and intermittent aeration at the air flow rate of 0.242 L/minute through modular fermentor. Enzyme production after 4 days of fermentation were assayed and a little variation in enzyme production was recorded between natural and intermittent ones. Results of enzyme production from 4 days fermentation under natural and intermittent aeration are shown figure 6.

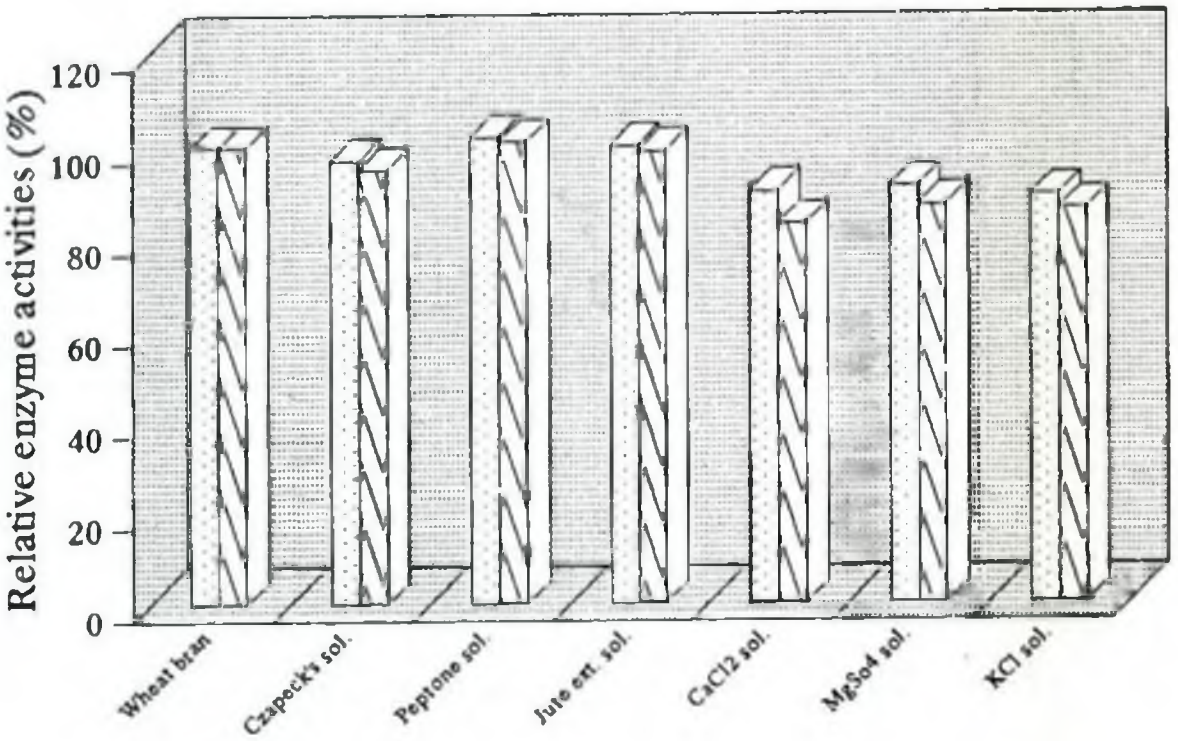
Effect of aeration on the production of enzymes by A. niger and A. soiae.



3.6 Effect of various supplements to the wheat bran medium on enzyme production by the fungi :

Among the substrates used for enzyme production, wheat bran moistened with water was found to be the best carbon source. It is known that the fungal growth is controlled by the organic materials as nutrients. In order to study the effects of various chemicals on enzyme production they were added as supplements in wheat bran medium. Among the supplements added only peptone enhanced the enzyme production though marginally and some of the rest other chemicals have a repressor effect on enzyme production. The enhancement of enzyme production might be due to increased mycelial growth with peptone. Results are shown in figure 7.

Effect of various supplements on enzyme production by A.niger and A.sojae



Chemicals (0.5 and 1%) and fermented 4 days

A.niger A.sojae

3.7 Effect of antibiotics on growth and enzyme production by the fungi :

To avoid bacterial contaminations during solid state fermentation and to enhance the enzyme productivity several antibiotics were used in the wheat bran medium. The antibiotics were added @ of 30 mg/L of added moisture at the time of inoculation. A profuse mycelial growth was observed with streptomycin compared with other antibiotics used. However, no remarkable variation of enzyme productions were recorded with the addition of antibiotics. The results are shown in the Table-4.

Table-4

Effect of antibiotics on growth and enzyme production by A.niger and A.sojae

Fungal strains	Fermentation period (days)	Enzyme components (I.U/ml)	Control	* Antibiotic used		
				Zentamycin	Crystapen-v	strepto micin
Aspergillus niger (N-5)	4	CMCase	0.37	0.37	0.31	0.48
		PGase	1.14	1.26	1.16	1.18
		Xylanase	1.01	0.97	0.88	0.98
2Aspergillus sojae(ATCC 20235)	4	CMCase	0.25	0.21	0.20	0.24
		PGase	0.72	0.74	0.64	0.76
		Xylanase	0.67	0.62	0.72	0.71

* 0.35 mg/L antibiotics solution was added to each flask of 50 gm wheat bran.

3.8 Genetic improvement of fungal strains by Gamma radiation :

Screening of fungal mutants by plate clearing zone and enzyme production in wheat bran medium :

A plate clearing assay method was used for rapid screening of CMCase, PGase and Xylanase activities of fungal mutants. The respective enzyme activities were measured as potential index (PI). The potency index was determined by dividing the clearing zone with colony diameter. Screening results of fungal mutants are shown in the Table 5 and 6.

Table - 5

Potency index of A.niger mutants

Fungal strain isolation number	Potency Index (PI)		
	CMCase	Xylanase	Pectinase
A.niger (control)	1.2	1.6	1.62
AN1	1.01	1.13	1.2
AN2	1.04	1.15	1.19
AN3	1.02	1.17	1.21

* Rest of the AN mutants did not show any significant growth and no visible clear zones were seen around their colonies and hence they are not shown in the table.

Table-6

Enzyme production by Mutants of A.niger in wheat bran medium

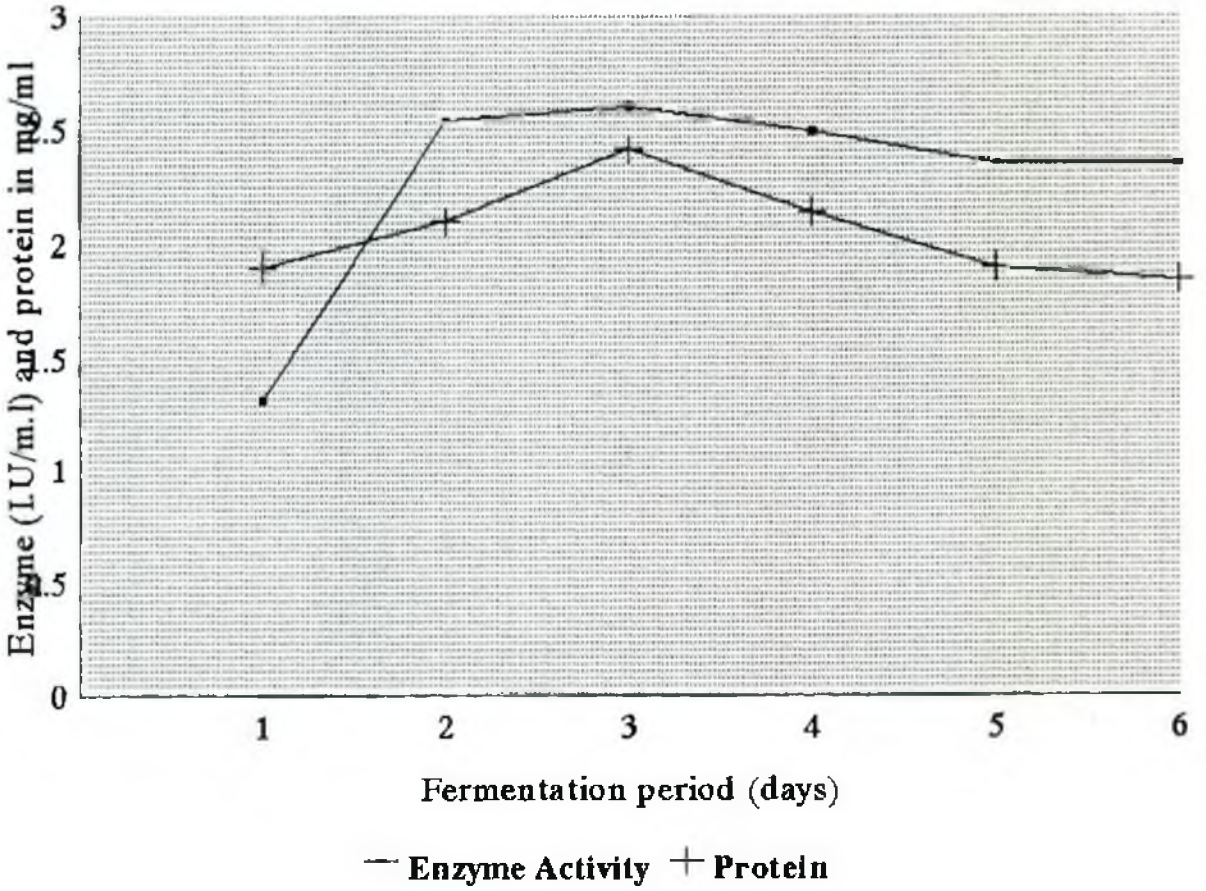
Fungal strain and mutants	Fermentation time (days)	Enzyme IU/ml		
		CMCase	Xylanase	PGase
A.niger (control)	4	0.45	1.15	1.10
AN1	4	0.15	0.39	0.45
AN2	4	0.12	0.41	0.37
AN3	4	0.18	0.54	0.51

* Rest other Mutants of A.niger did not grow well in wheat bran medium.

3.9 Optimization of extraction time and total soluble crude protein in fungal extract of A.niger :

In order to optimize the enzyme extraction time enzymes were collected after 2 days of fermentation till 7 days of fermentation. Total soluble protein of culture filtrates

Optimization of extraction time and total soluble crude protein in fungal extract of A.niger



3.10 Bio-chemical Composition of enzyme extracts of A.niger, A.sojae and Flaxzyme (Novo):

Spore free culture filtrate was used as enzyme preparation and assay of enzymes were carried out according to the procedures of A. W. Khan et al., 1981 and G. L. Miller 1959. The culture filtrate of A.niger yielded 2.56 IU/ml and A.sojae yielded 2.24 IU/ml of enzymes. The biochemical composition of enzyme extract of A.niger was 0.42 IU/ml CMCase, 1.20 IU/ml polygalacturonase and 1.00 IU/ml Xylanase enzyme activity. Similarly, the enzyme extracts of A.sojae contained 0.24 IU/ml CMCase, 0.91 IU/ml polygalacturonase and 0.97 IU/ml Xylanase enzyme activity. The biochemical composition of both enzyme extracts are shown in the Table 7.

Table - 7

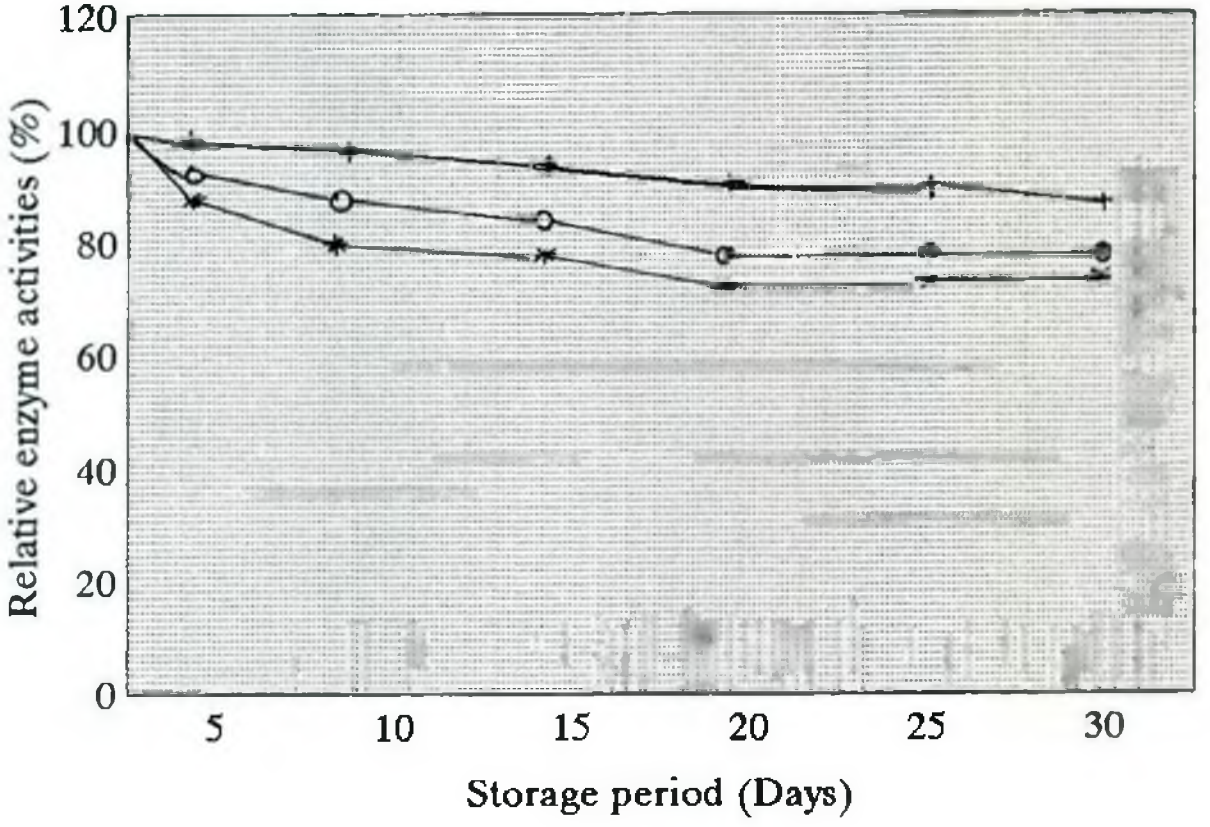
Biochemical composition of enzyme preparation of A.niger, A.Sojae and Flaxzyme (NOVO)

Enzymes of	Enzyme components (IU/ml of extracts)			Pectinlyase activity/ml	
	CMCase	PGase	Xylanase	P^H 8.5	P^H 5.5
A.niger	0.40	1.06	1.01	0.16	0.12
A.sojae (ATCC 20235)	0.37	0.95	1.02	0.21	0.81
Flaxzyme (NOVO)	4.75	35.00	34.10	-	0.88

3.11 Storage stabilities of enzyme extracts at different storage temperatures :

Enzyme extracts were kept at two different storage temperatures one at 32°C (room temperatures) and the other at 4°C in the refrigerator. Residual enzyme activities were measured after every week upto 28 days. CMCase and Xylanase activities of both the extracts did not decrease significantly at 32°C and 4°C after 28 days. But PGase activity of both the extracts decreased significantly (around 50%) at 32°C after 28 days, but at 4°C the PGase activity of both the extracts were found to be stable. So it may concluded that PGase activity is more susceptible to heat than other enzyme components of the extracts. CMCase and Xylanase activities of both the extracts were fond to be most stable and for PGase activity a cooler storage temperature is suggested. Results of storage stabilities are shown in the following figure 9.

Storage stabilities of enzyme extract of A.niger at different storage temperatures



*** 35 degree (C) o 15 degree (C) + 4 degree (C)**

3.12 Optimization of pH for the enzyme components of A.niger and A.sojae :

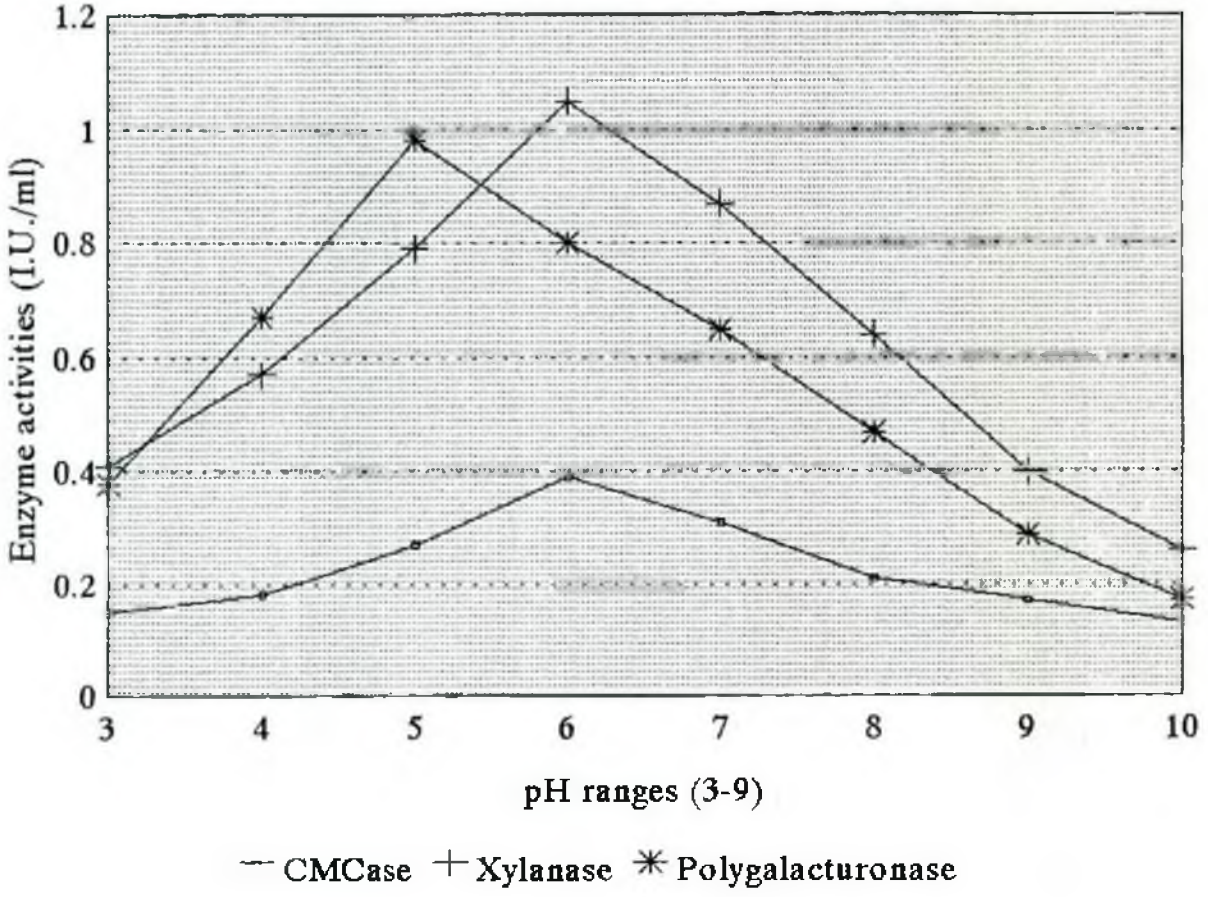
In general, enzymes are only active over a limited range of pH and in most cases a definite optimum pH is noticed. The pH effects the state of ionization and only the ionic state of enzyme is catalytically active. The pH at which the enzyme is catalytically maximum active is called the optimum pH. And optimum pH for the components of enzyme extracts of A.niger was found to be pH 6-7. All enzyme activities were measured in this study at 40°C for 20 minutes incubation at pH range of 3-9 and the results are shown in the following figure 10 and 11.

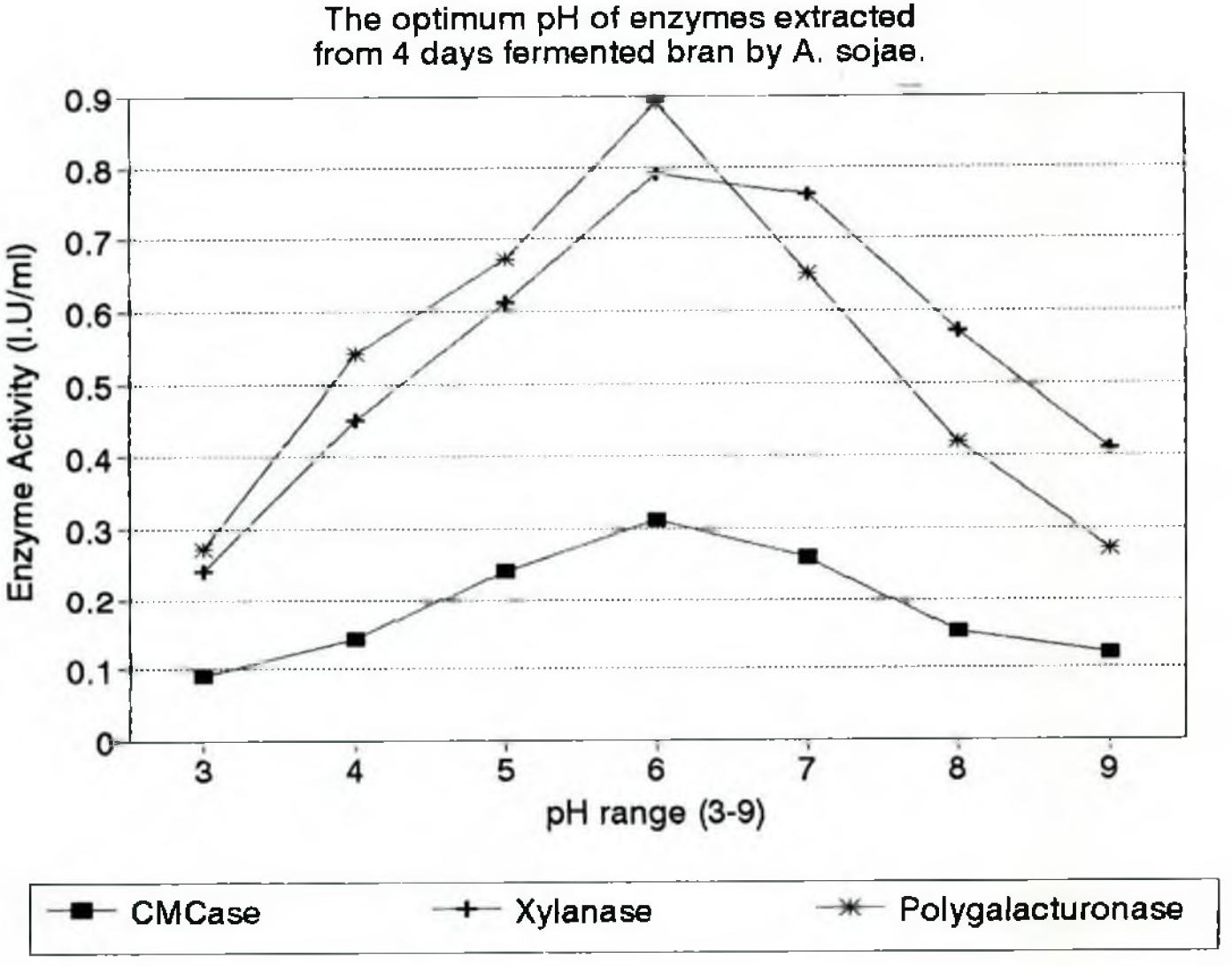
The pH was adjusted with 0.5 ml acetate buffer (for pH 2-6) and 0.5 ml phosphate buffer (for pH 6-12). The optimum pH for the A.niger CMCase was 5.5 for Xylanase 6 and for PGase 5.

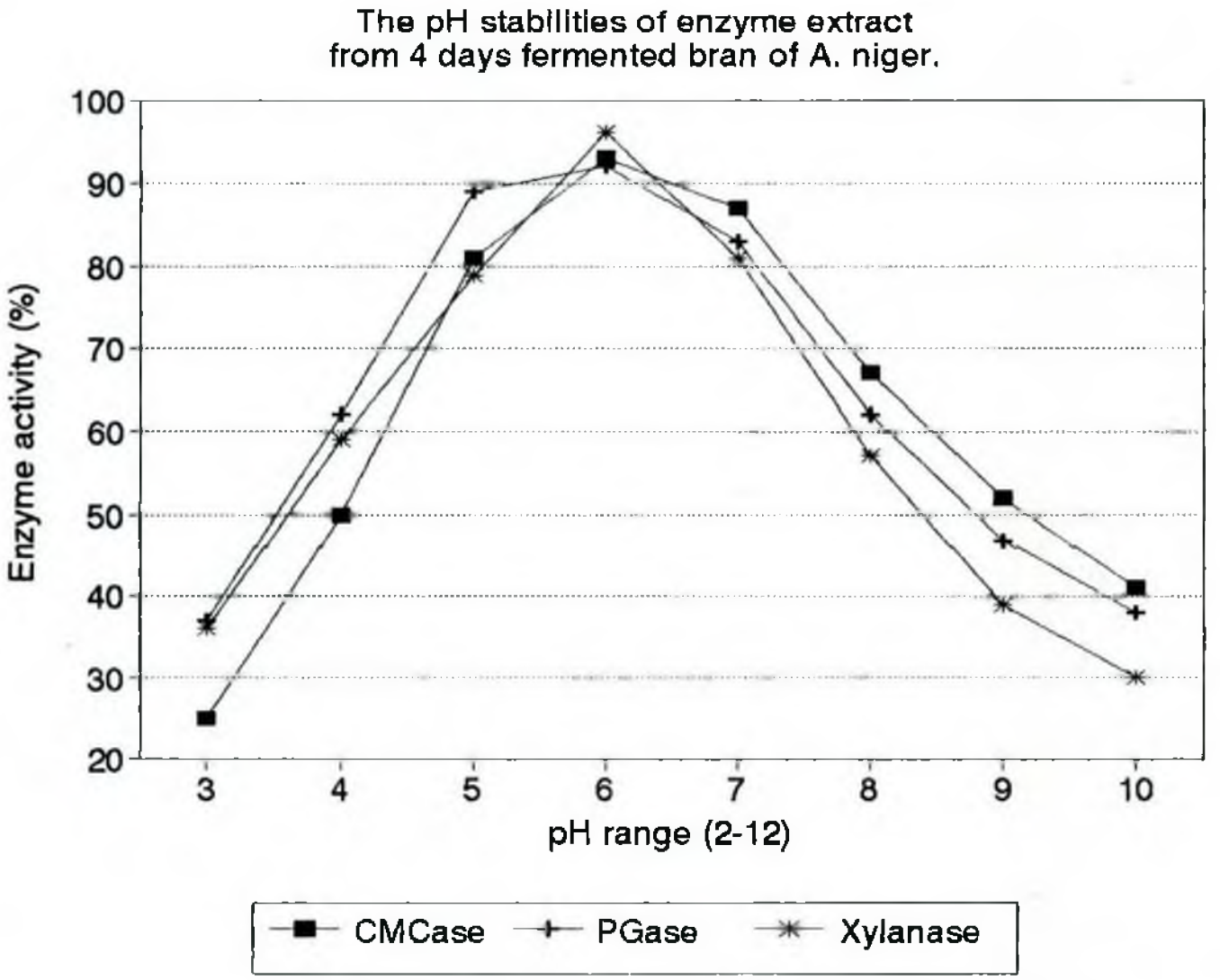
3.13 pH stabilities of enzyme extract of A.niger :

The effect of pH on the activity of enzymes is due to change in the state of ionization of the enzyme components. The pH stability of the enzyme components were carried out by pre-incubating the enzymes at 4°C for half an hour in the respective buffers. The activities were measured and expressed as relative activity and most of the enzyme components were active around the pH range of 5-7. Results are in figure 12.

The optimum pH of enzymes extracted from 4 days fermented bran by A.niger

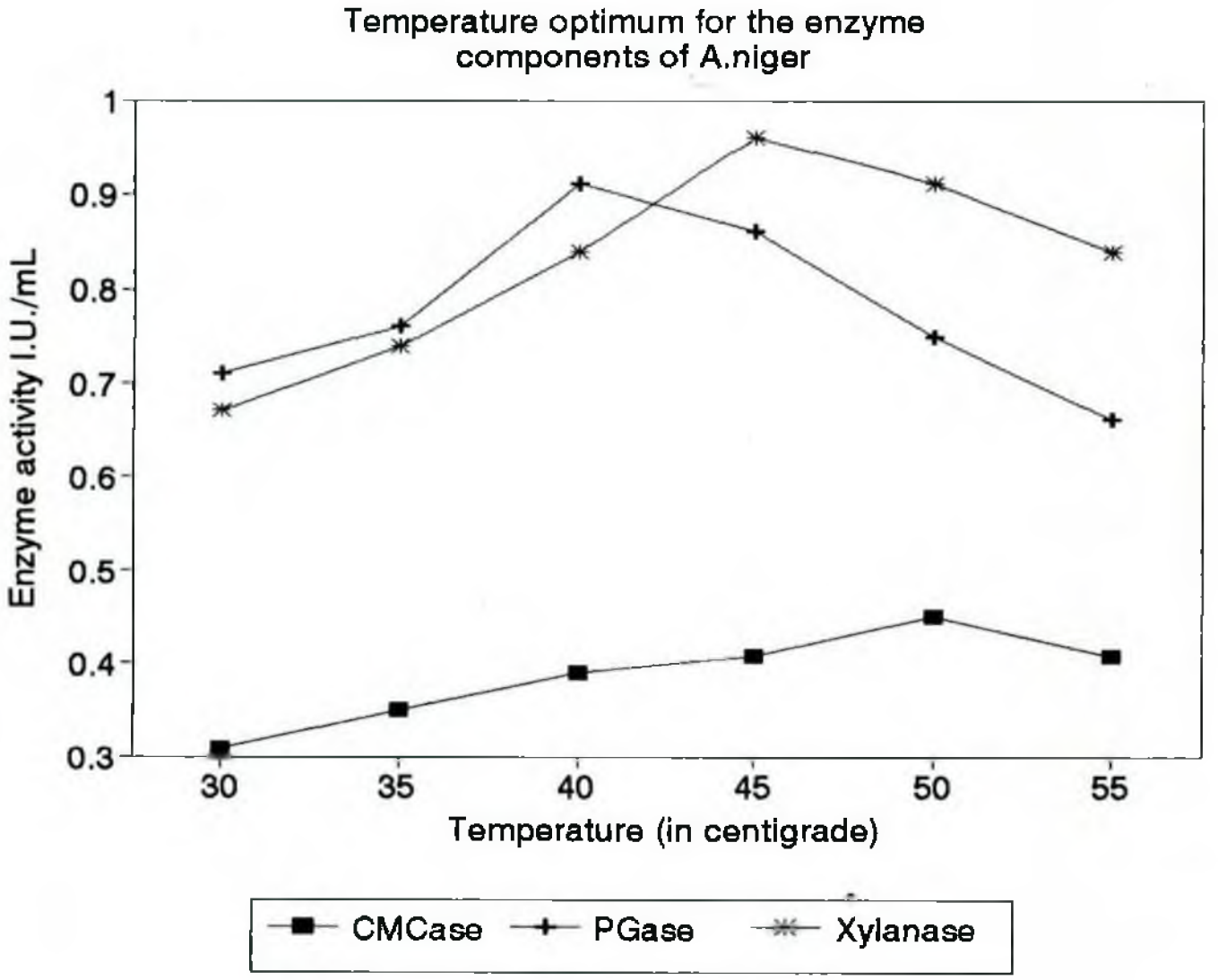






3.14 Optimization of temperature for the enzyme components of A.niger :

Temperature is an important factor and it controls the velocity of reaction mainly through effecting the stability of enzyme. It also effects the actual velocity of breakdown of enzyme substrate complex to product formation (rate limiting step) by the heat of activation of the reaction. The optimum temperature for the maximum activity of enzyme components was determined by a series of activity measurement at different temperatures (20-50°C). The optimum temperature for the maximum activities of CMCase, PGase and Xylanase were 50°C, 40°C and 48°C respective. The enzyme activities were found to decrease beyond their respective optimum temperatures. Results are shown in the following Figure 13.



3.15 Storage stabilities of enzyme extract of A.niger at different temperatures :

The pilot scale enzyme production and demonstration plant is the first venture of its kind in Bangladesh and as such the enzyme extract may be required to be transported and stored for long time before its use in the distant parts of the country. In this respect the prolonged storage stability of the enzyme extracts at room temperature and at 10°C were carried out. Enzyme extracts were stored with preservative at room temperature at 32°C and at 10°C and residual activities were monitored at every thiry days upto three months. The enzyme activity was found to decrease slowly and after 3 months the loss of activity was almost one forth of its initial activity at room temperature. Among the enzyme components the rate of loss of PGase activity was higher than other enzyme components and most stable component was Xylanase. At 10°C no significant change of activity were observed upto 3 months. Loss of activity of A. sojae and A. niger were 10.25 and 9.97 respectively. However, both the enzyme extracts were found to have prolonged storage stabilities and the results are shown in the following Table 8 and 9.

Table - 8

Prolonged storage stability of enzyme components at room temperature

Enzyme extract	Enzyme components	Activity on extraction	After 1 month	After 2 months	After 3 months	Loss of activity after 3 months (%)
A.niger	CMCase	0.42	0.39	0.37	0.40	
	PGase	1.13	0.59	0.57	0.55	24.23
	Xylanase	1.05	1.02	1.00	1.02	
A.sojae	CMCase	0.24	0.21	0.20	0.18	
	PGase	0.79	0.57	0.52	0.49	23.93
	Xylanase	0.85	0.80	0.77	0.76	

Table - 9

Storage stabilities of enzyme extracts at 10°C

Enzyme extract	Enzyme components	Activity on extraction (IU/ml)	After			Loss of activity (%)
			1 month	2 months	3 months	
A.niger	CMCase	0.41	0.42	0.39	0.41	
	PGase	0.97	0.92	0.91	0.87	9.87
	Xylanase	1.05	0.97	0.94	0.91	
A.sojae	CMCase	0.24	0.22	0.21	0.20	
	PGase	0.81	0.77	0.72	0.69	10.25
	Xylanase	0.90	0.87	0.88	0.86	

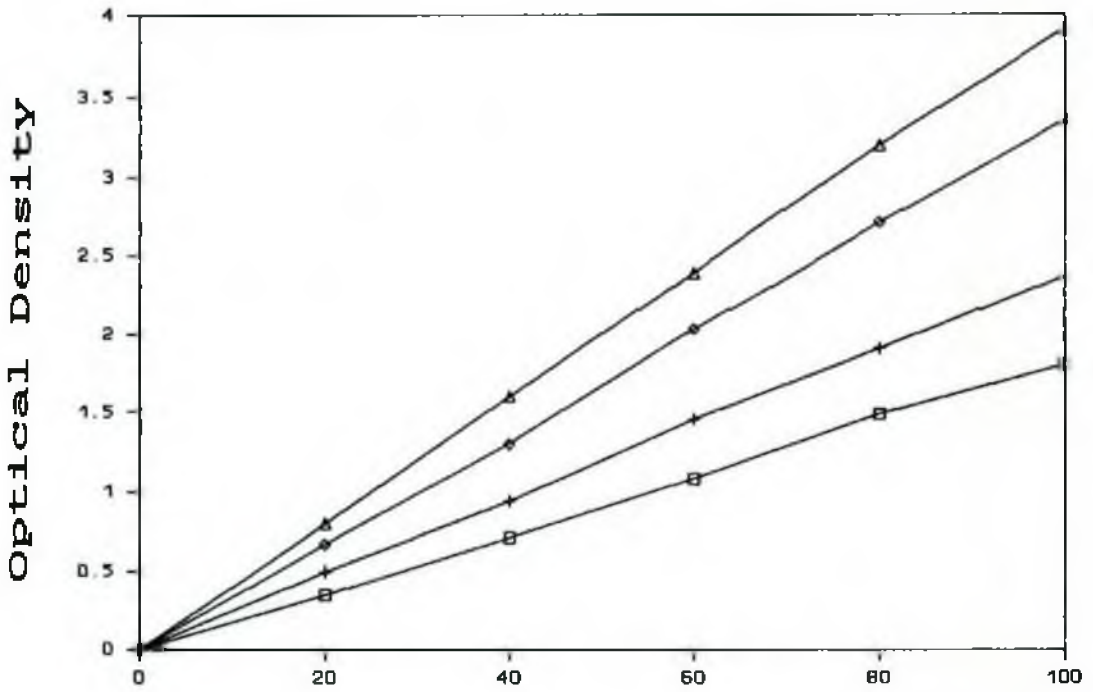
3.16 Determination of km value of Xylanase component of culture extract :

The hydrolysis of xylan (Larchwood) was conducted with xylanase (Sigma) at pH 4.8 in acetate buffer and on incubation temperature of 40°C.

Xylanase enzyme 0.5 IU/ml was prepared by dissolving 1 mg of purified enzyme in acetate buffer of pH 4.8. Time course of xylan hydrolysis was plotted at different xylan concentration (0.5%, 1.0%, 1.5 and 2%). Reducing sugar released was linear with time. Reducing sugar released increased with both time and substrate concentration. After 100 minutes of hydrolysis reducing sugar released with substrate concentration of 5, 10, 15 and 20 g/L was 1.8, 2.4, 3.4 and 4 g/L respectively.

Reducing sugar released from 5, 10, 15, 20 g/L of xylan concentration after 60 minutes of incubation was 1.10, 1.37, 2.00 and 2.4 g/L respectively. Results are shown in figure 14.

Hydrolysis of xylan with different concn.of xylan at pH-4.8, Temp.40°C

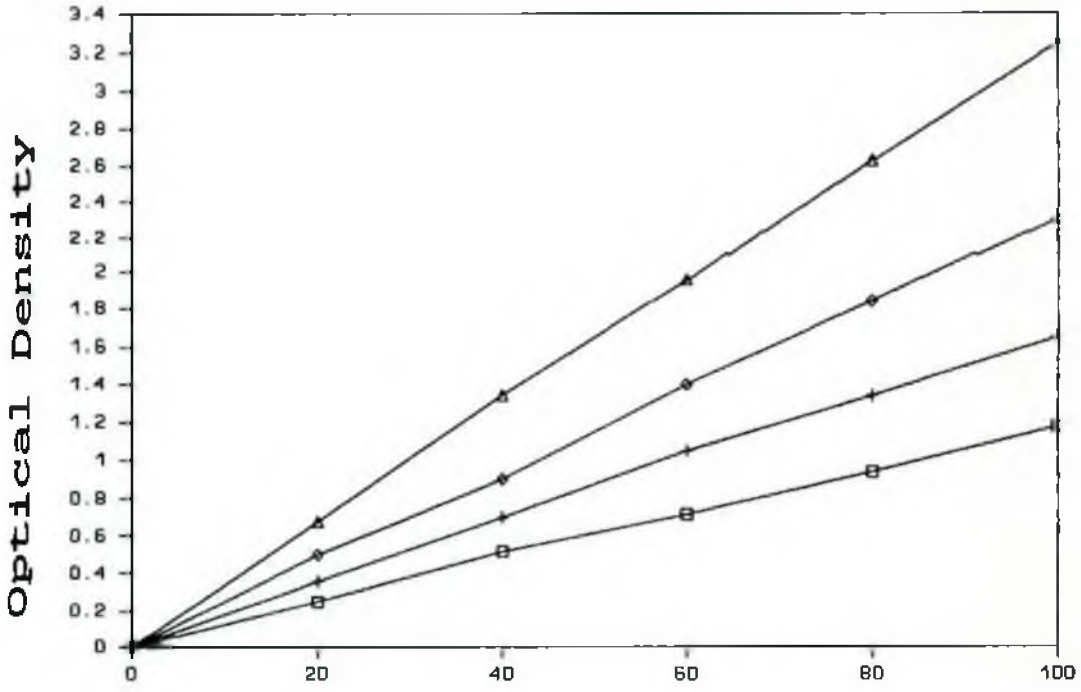


Xylan hydrolysis with xylanase in presence of product inhibitor xylose :

During hydrolysis of xylan the effect of product inhibitor D-xylose at concentrate level of 0.5 g/L was studied. Reducing sugar release was increased with both time and substrate concentration even in the presence of inhibitor xylose but the total release of sugar was less than obtained without inhibitor. After 100 minutes of incubation reducing sugar production was 3.3 g/L for substrate concentration of 20 g/L in presence of inhibitor D-xylose (0.5 g/L) where as sugar production was 4 g/L for same substrate concentration without inhibitor xylose.

The sugar product was suppressed further in presence of higher amounts of inhibitor xylose and 2.72 g/L of sugar was released in presence of 1 g/L xylose as inhibitor after 100 minutes of incubation as against 4 g/L without inhibitor. Results are shown in figure 15.

Hydrolysis of xylan with xylanase in presence of inhibitor xylose (0.5 g/L)

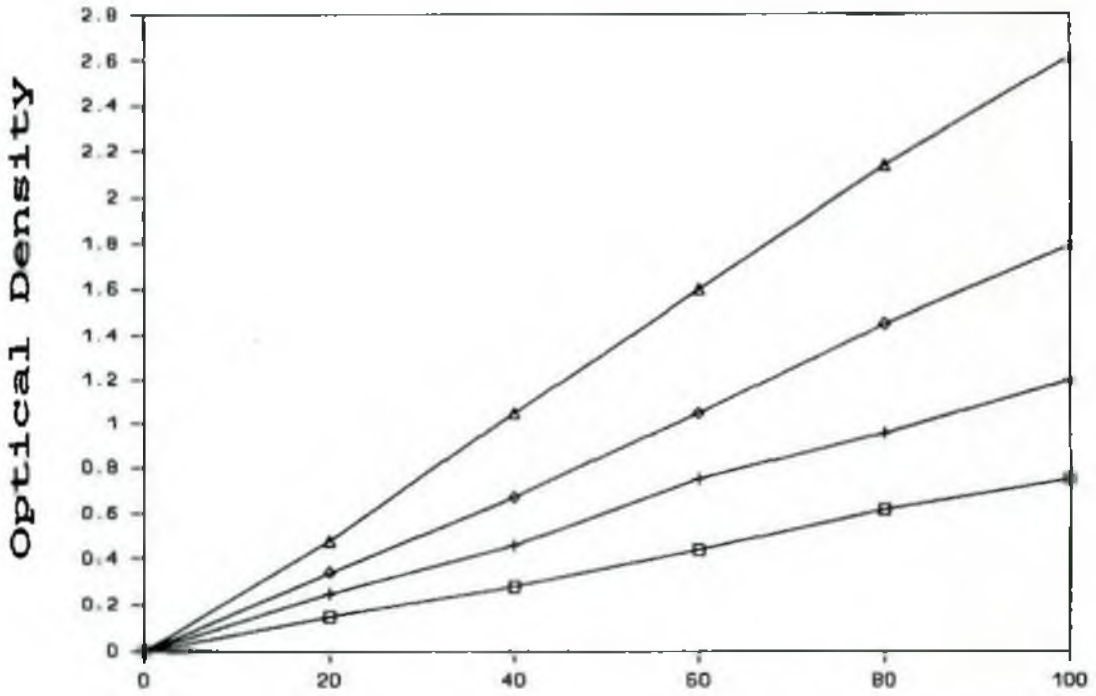


Inhibition pattern for different substrate concentration and inhibitor concentrations :

In the presence of inhibitor D-xylose at different levels of substrate concentration, the inhibitory effect is shown in Fig.-16. It was observed that percentage of inhibition decreased as the substrate concentration increased. At the substrate concentration of 20 g/L and 5 g/L percentage of inhibition was 25.00 and 51 respectively for inhibitor concentration of 0.5 g/L of xylose.

On the other hand, when the inhibitor concentration was 1 g/L, the percentage of inhibition for substrate concentration of 20 g/L and 5 g/L was 37.5 and 61.9. for constant substrate concentration. Percentage of inhibition increased with increase of inhibitor dose. At substrate concentration of 20 g/L the inhibition percent of hydrolysis was 25 to 37.5 with increasing the inhibitor dose from 0.5 g/L to 1 g/L respectively. Results are in figure 16.

Hydrolysis of xylan with xylanase in presence of inhibitor xylose (1 g/L)

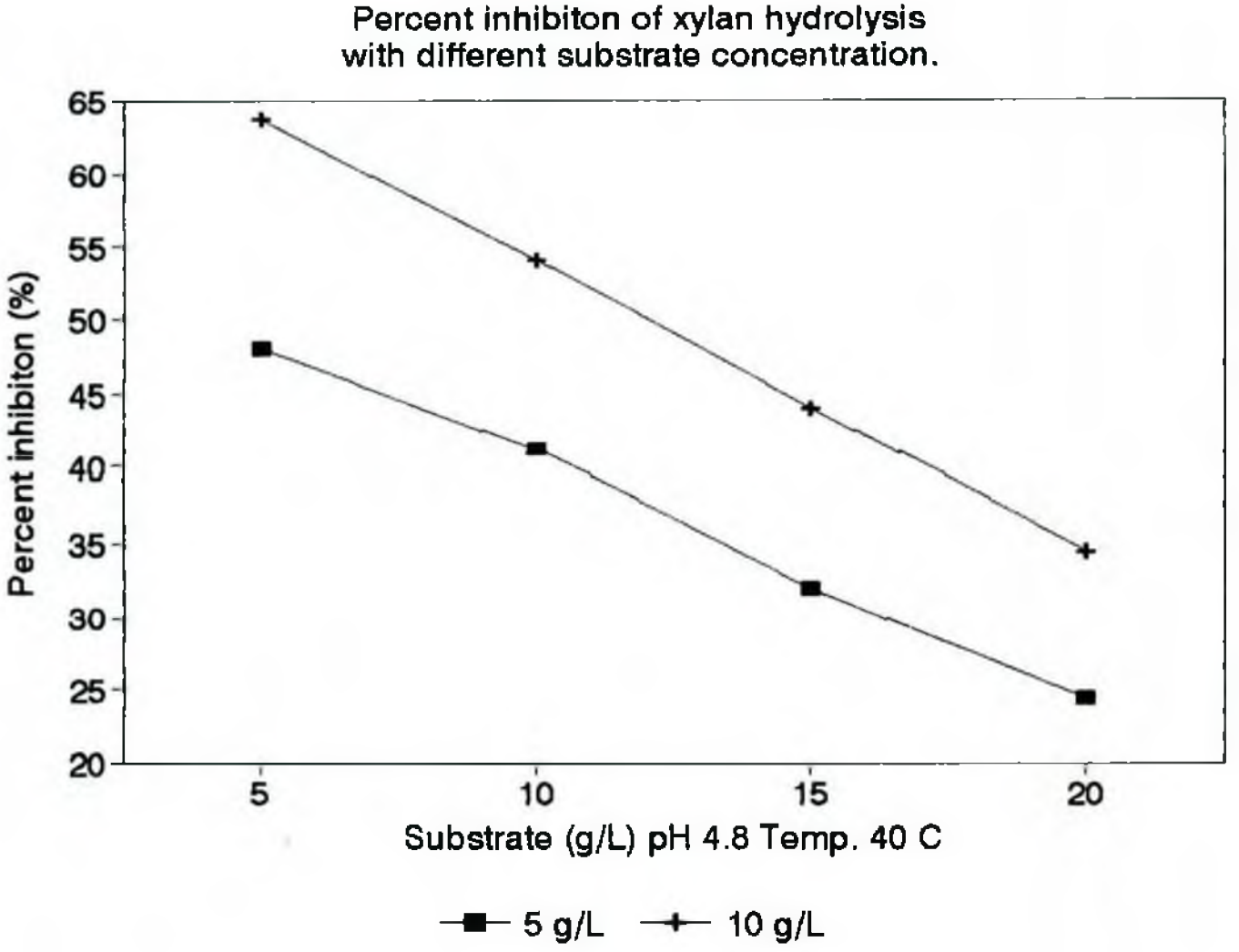


Kinetic parameters for enzymatic hydrolysis of xylan :

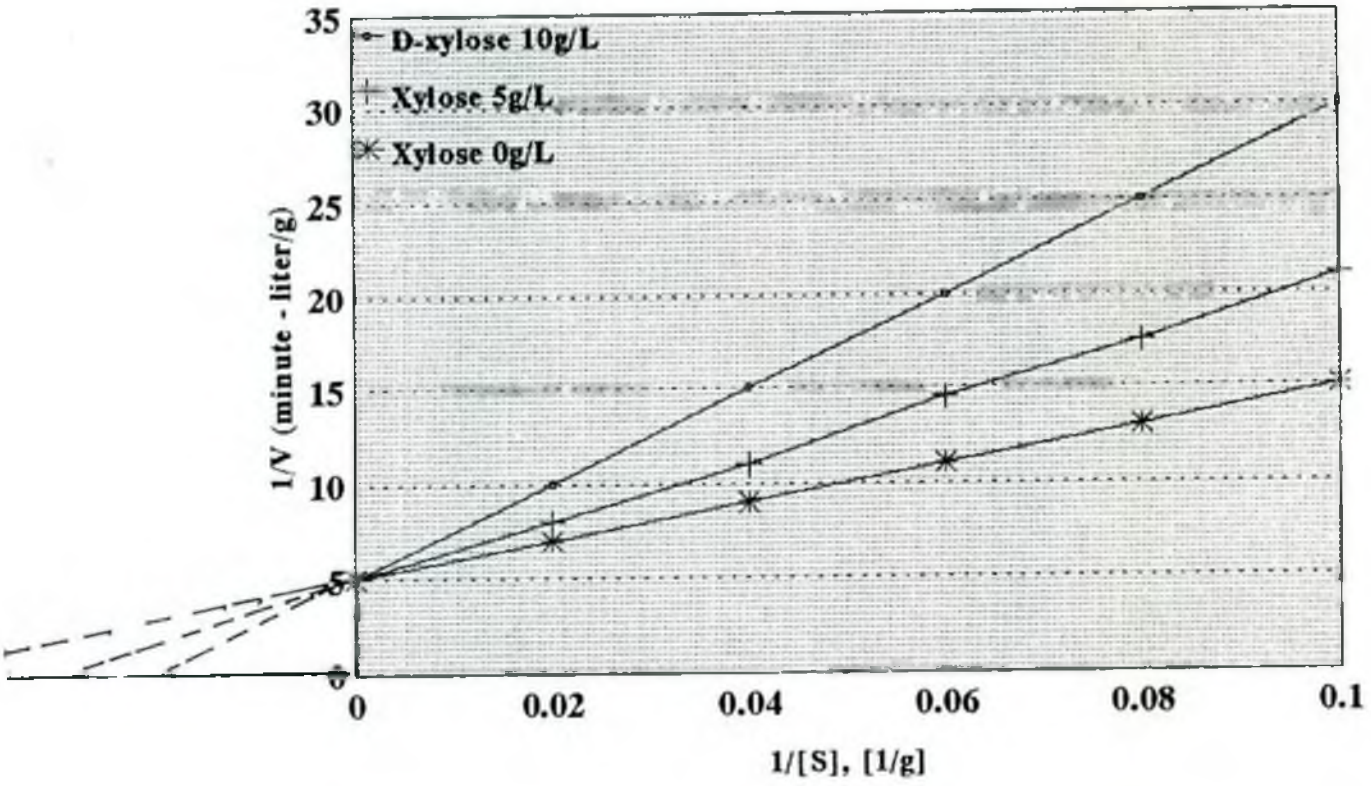
The inverse of initial rate of reducing sugar formation versus the inverse of all the substrate concentration were linear in Line Weaver - Burk coordinates as can be seen in Fig.-5. This plot was made for all inhibitor dose levels which showed similar patters.

The extrapolation of these straight lines to the abscissa gave inverse of Michaeli's constant, Km. The Km values for controlled hydrolysis (without the presence of inhibitor) and for hydrolysis in presence of inhibitors 0.5 g/L and 1 g/L of xylose were 24.28, 56.47 and 74.92 g/L respectively (figure 15 and 16). The intercept of Line Weaver-Burk plot gave the Km value which was 11.12/L per hour.

The slops of the Lineweaver-Burk plot were plotted at different level of inhibitor doses in order to obtain Ki, which was found to be 3.12 g/L for inhibitor dose of 0.5 g/L and 1 g/L of xylose. The pattern of Lineweaver-Burk plot (figure 17) represents competitor inhibitor model.



Kinetic parameters for enzymatic hydrolysis of xylan by xylanase



Line Weaver-Burk Plot for hydrolysis of xylan by xylanase

Parameters of Kinetic model :

km value obtained in presence of inhibitor 0.5 g/L of xylose

km value obtained in presence of inhibitor 1 g/L of xylose.

Sample	Vm (g/l.hour)	Km (g/l)	Ki (g/l)
xylan	11.12	24.28	
		56.47	3.12
		74.92	4.10

Km = Km (1 + [I]/Ki) [I] = 0.5 g/l xylose

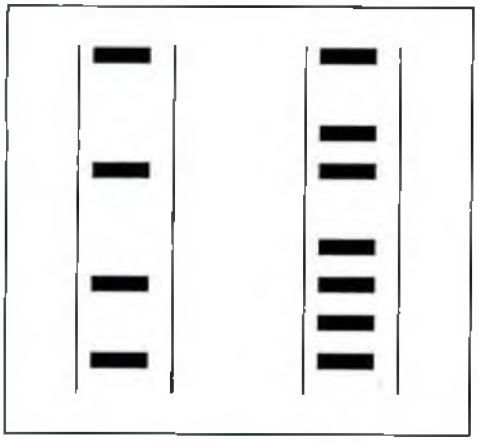
Km = Km (1 + [I]/Ki) [I] = 1 g/l xylose

3.17 Estimation of molecular weights of enzyme components by Gel Electrophoresis :

The molecular weights of enzyme components CMCase, xylanase and PGase of A.niger were calculated as Kd by SDS-PAGE using marker proteins. In the gel plate of A.niger lane the first spot is at 66k of marker protein, Bovine albumin which suggest the first spot to be of exo-polygalacturonase whose ranges are 48,000-76,000 (WA Wood et al., 1988). The next spot is at 36k which suggest it to be of endo-polygalacturonase in the range of 30,000-52,000 (Rombouts, F.M. et al., 1980).

The third spot in the plate is at 24k which suggests it to be of xylanase according to Frederick et al., 1981. The rest other minor spots of 14k which suggest it to be of cellulase fractions.

Proteins A.niger Marker

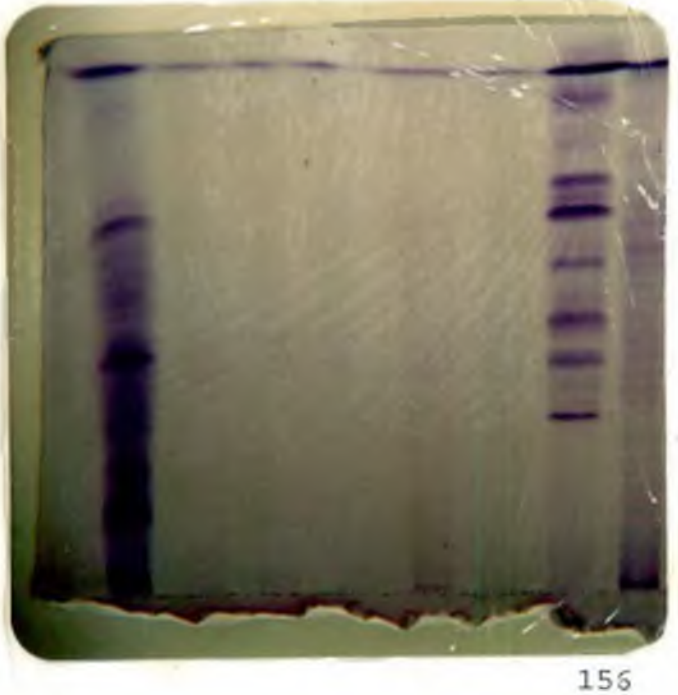


Bovine albumin 66k

Egg albumin 45k
Glyceraldehyde 3p dehydrogenase 36k

Carbonic Anhydrase 29k
Trypsinogen, Bovine 24k
Trypsin inhibitor 20.1k
α-Lacto albumin Bovine milk 14.2k

Figure 18 SDS-PAGE of crude enzymes from *Aspergillus niger* with marker proteins.



Bovine albumin 66k

Egg albumin 45k

Glyceraldehvde 3p dehydrogenase 36k

Carbonic Anhydrase 29k

Trypsinogen, Bovine 24k

Trypsin inhibitor 20.1k

α-Lacto albumin Bovine milk 14.2k

3.18 Test of different chemicals as alternate preservatives for enzyme extract :

The enzyme extracts are not fully free of fungal spore and bacteria and as such microbial growth may start during storage. Microbial growth may spoil the enzyme activity by secreting proteolytic enzymes. Sodium azide was found to be very effective as preservative but considering its health hazard effects a number of chemicals were tested as alternate preservatives. Among the chemicals tested benzoic acid, toluene and butanol were found to be effective as preservative with spore free extract in the laboratory up to two weeks. Results are in Table 10.

Table - 10

Chemical	Enzyme components	Initial activity I.U/ml	Enzyme activity I.U/ml after	
			7 days	14 days
Benzoic acid	CMCase	0.42	0.37	0.35
	PGase	1.26	0.91	0.84
	Xylanase	0.86	0.72	0.69
Toluene	CMCase	0.42	0.25	0.38
	PGase	1.26	1.11	0.88
	Xylanase	0.86	0.75	0.72
Butanol	CMCase	0.42	0.41	0.42
	PGase	1.26	1.02	1.06
	Xylanase	0.86	0.80	0.79

The above chemicals were added with enzyme extract @ of 0.25% W/V, and V/V.

3.19 Effect of enzyme extracts on chemical constituents of low grade jute and cuttings :

Low grade jute is hard and stiff and the enzyme extracts were applied to soften and defibrate them through hydrolytic degradation of non-cellulosic materials pectin and hemicellulose. This experiment was carried out by submerging the jute cuttings in enzyme extracts for 24 hours at 35°C. Chemical constituents of enzyme treated low grade fibres were estimated and a significant reduced pectin and hemicellulose content was recorded than untreated ones. A marginal reduction of cellulose content in enzyme treated fibres were also recorded. Both the enzyme extracts were found to be effective in hydrolytic removal of non-cellulosic materials from the low grade fibres. But the commercial enzyme, Flaxzyme was more effective in removing the pectin and hemicellulose from low grade jute. The results are shown in the following Table 11.

Table - 11

Chemical constituents of jute cuttings before and after enzyme treatment

Chemical constituent	Before enzyme treatment (%)	After enzyme treatment (%)			
		A.sojae	A.niger	Flaxzyme	Water
Cellulose	61.70	60.70	60.84	61.30	61.50
Hemicellulose	19.90	17.90	17.87	17.40	18.90
Lignin	14.00	14.00	14.00	13.60	13.90
Pectin	2.50	1.20	1.15	1.40	2.45
Others	5.90				

* Control experiment with distilled water.

3.20 Effect of enzymes on maturation period and physico-mechanical properties of the fibres :

Sporefree enzyme extracts were applied to low grade jute at BJRI experimental mills to assess their effectiveness of softening and difibrating activity. Enzymes were mixed with emulsion and the rest other treatments were followed according to routine practices. Application of enzyme broughtforth the radical change in maturation period to almost half of the usual time. Enzyme application has improved a number of softning parameters namely seperation index, fibre fineness and stiffness which attributed better performances during spining. Speration index of enzyme treated fibre has increased which indicats the fibre has become more seperable and easily pliable. The lower value of stiffness indicates the fibre has become softer. Decrease of fibre diameter indicates the fibre has become finer and is more suitable for fine yarns.

Table - 12

Effect of enzymes on maturation period and physico-mechanical properties of the fibres

Treatment	Time required for maturation (hours)	Separation Index	Fibre diameter μ	Stiffness index (cm)	Fibre strength (Pressely Index)
Control (without enyme)	168	48.08	70.90	16.80	2.39
Enzyme (boiled)	130	56.50	66.80	15.95	1.70
Enzyme A.niger)	68	80.67	56.40	15.10	2.20
Enzyme A.sojae)	69	79.25	57.12	15.15	2.18
Flaxzyme (Novo)	72	71.20	54.20	15.30	1.95

3.21 Thermal stabilities of enzyme extracts at a condition similar to piling bin temperature :

In the conventional mill softening practice the emulsified fibres are kept piled in a concrete bin. Temperature of the piling bin gradually rises to around 60°C after 7 days when the fibres are taken out for processing. Addition of enzyme extract with the mill softening emulsion brought forth radical improvement of piling process through quicker softening of fibres.

In this context, it is important to study the effect of piling temperature on the stability of added enzymes. In order to study the stability of added enzymes during piling process the enzyme extracts were kept in conditions similar to piling state of temperature and time. The enzyme extract was first kept at 40°C for 24 hours, then it was kept at 50°C for another 24 hours and finally the enzyme extract was kept at 60°C for 24 hours. Enzyme activity was monitored after each storage period. It was found that the enzyme activity decreased significantly at higher temperature and prolonged storage period. After 72 hours of storage period

at above conditions the enzyme extracts retained about 50% of their original activity and the results are shown in the following Table 13.

Table - 13

Thermal stability of enzyme extracts of A.niger and A.sojae at a temperature similar to temperature of piling conditions.

Fungal strain	Enzyme components	Initial activity (I.U/ml)	Activity after 24 hours at 40°C	Activity after 24 hours at 50°C	Activity after 24 hours at 60°C
Aspergillus niger	CMCase	0.42	0.39	0.36	0.28
	PGase	1.26	0.71	0.51	0.33
	Xylanase	1.06	0.84	0.72	0.51
Aspergillus sojae (ATCC-20235)	CMCase	0.24	0.21	0.18	0.18
	PGase	0.81	0.50	0.37	0.28
	Xylanase	0.93	0.79	0.67	0.47

3.22 Spinning performance of Yarn (Pilot scale trial at BJRI):

Pilot scale mill trials were carried out with 900 kgs of cuttings at BJRI experimental mills. Enzyme extracts about 12 liters were mixed well with jute batching emulsion and applied to the cuttings @ of 35%. Enzyme treated fibres were piled up in a concrete block with coverings. After 72 hours of piling when the temperature of the piling bin reached 60°C the fibres were processed as per usual carding and spinning was done in slip draft with flyer speed 1700 rpm. The results are shown in the following table - where it was noticed that though the batch compositions of experimental and control batch were same yet the spinning performances of enzyme treated experimental batch were better than that of control. As a result of enzyme treatment cuttings became softer and finer which improved their spinning performances. Results are in Table 14 and 15.

Table - 14

Spinning performances of hessian weft yarn (coarse yarn) after enzyme application at BJRI experimental mills

	Control	Flaxzyne (NOVO)	Enzyme A.niger	A.soiae
1. Average grist (lb/-spy) Liner density (tex)	31.0 (1066)	29.41 (1012)	30.26 (1046)	28.10 (968)
2. Average tensile strength (lb) (Kg)	19.21 (8.73)	21.68 (9.86)	21.14 (9.61)	19.37 (8.8)
3. C.V. %	25.54	19.06	20.57	20.60
4. Quality ratio %	61.97	70.73	69.85	68.90
5. Yarn irregularity(PMD)	35.60	39.10	34.00	34.50
6. Neps/100 yds	280	214	211	212

Spinning was done in slip draft with flyer speed 1700 RPM. Two results are shown for a parameter one in the old system and the other in the S.I. units.

Batch composition : Cuttings 72%

Low grade jute 28%

(for both control and enzyme treatment)

Table - 15

3.22.1 Spinning performance of Sacking weft (M/S Karim Jute Mills, a public sector Jute mills, Dhaka, Bangladesh):

	Control	Flaxzyme (NOVO)	Enzyme	
			A.sojae	A.niger
1. Average grist (lb/spy) Liner density (tex)	31.7 (1092)	29.63 (1019)	34.40 (1186)	31.74 (1092)
2. Average tensile strength (lb)(Kg)	20.76 (9.44)	19.53 (8.88)	19.96 (9.07)	17.42 (7.92)
3. C.V. %	19.93	20.85	20.38	27.40
4. Quality ratio %	60.96	68.11	68.05	60.38
5. Yarn irregularity (PMD)	34.67	39.87	34.25	34.24
6. Neps/100 yds	279	214	204	218

Spinning was done in slip draft with flyer speed 1700 RPM. Two results are shown for a parameter, one in the old and other in the S.I. units.

Batch composition :

Fibre	Control	Experimental (enzyme treated)
Cuttings	52.88%	72%
Low grade jute	18.18%	28%
Miscellaneous	15.71%	--
Thread	13.22%	--

DISCUSSION

In the study to improve the conventional mill softening practices a detailed study of its different aspects like temperature rise, microbial growth and pH change were recorded from beginning to the end of the piling process. Temperature rise in the jute piling bin is taken as a measure that fibre is ready for processing. A piling bin is considered to be matured (softened and ready for processing) when the temperature of the piling bin rises to 60°C. It was noticed that the temperature inside the piling bin began to rise gradually with the passing of days and reached 60°C - 65°C after seven days of piling. If the piling process was continued after seven days the temperature tended to decrease slowly. This gradual fall of temperature was due to the decrease of microbial growth and followed by dying of microbes. The microbial multiplication had stooped either by the exhaustion of available food materials or some unfavorable conditions generated in the bin that inhibited microbial growth.

Heat is produced inside the jute piling bin by the respiratory activities or other energy yielding process by the microorganisms. Most of the heat is dissipated into the area adjacent to growing microorganisms. Thus microbial growth in the piling bin causes the accumulation of enough heat to cause a marked increase in temperature. In this context, the rise of temperature inside the piling bin has a direct correlation with the microbial growth. The microbes present in fibres were characterised as molds, yeast, bacteria and fungi. At the beginning of the piling process the mesophilic microbes were predominant and continued their multiplication upto fourth day of piling when the temperature of the bin had reached around 40°C. Generation of heat was produced by the self pasteurization of the mesophilic microorganisms. On the other hand marked rise of temperature favoured the thermophilic microbes to grow. From the fifth day of piling the thermophilic microbes began to multiply and reached their maximum number at the 7th day of piling and by this time the temperature had already reached 60°C (Fig. 2). After 7th day of piling the temperature tended to decrease by the gradual fall of microbial numbers. This decrease in microbial number was mainly due to exhaustion of available

food materials and generation of some unfavorable conditions for the growth of microbes. The pH of water extract of piled fibres was also changed during the piling process. The pH value was 6.5 at the beginning of the piling process which gradually dropped to 6 at the end of the piling process. This change of pH value to acidic indicates the microbial degradations of cellulosic and non-cellulosic constituents of fibres. It is known that cellulosic degradation is associated with the increase of carboxyl number which in turn drops the pH value from 6.5 to 6 at the end of piling process.

It was revealed from the systematic study of the present mill softening piling process that a number of microbes and their enzymic action have been implicated in the softening during the piling process. However, the existing method, while making limited improvements by the uncontrolled microbial action are inadequate in softening the fibres to the desired extent of improving their spinability. The fibres thus obtained are far from satisfactory due to uneven softening, presence of large quantities of barky materials and often deterioration in the tensile strength. Generally such softened fibres are used in making coarse yarn like

sacking. Therefore, there is a scope of improvement of the present mill softening process either by enhancing the microbial action or adding a microbial crude enzyme preparation during the piling process.

The direct application of microbial strains in the piling bin is associated with several disadvantages. Growth of microorganisms depends on several factors and hetrogenocity of fibres would create varied conditions for growth of microorganism. The softening by adding one or more selected microorganisms appears to be difficult because such externally added microbes may not fully establish themselves on the barky jute owing to a changing ecosystem inside the pile. Moreover, the added microbes and their spores will have to be removed by sterilization prior to final use of the jute fibres. On the other hand added enzyme preparations are expected to be well suited to function rather synergistically, irrespective of the ecological changes in the piling bin. Thus the improvement of present mill softening process by pretreatment of jute cuttings with extracted enzyme solution is of our interest. The aim of the present project is to produce jute softening enzyme

preparation by solid state fermentation using indigenous wheat bran as substrate for softening and upgrading the jute cuttings and low grade fibres (H. S. S. Sharma - 1987).

It was decided that the process of mill softening of low grade jute and cuttings can be improved by adding a microbial enzyme preparation to the jute processing emulsion. Therefore, microorganisms specially the mesophilic fungi were isolated from natural habitats and also collected from American Type Culture Collections (ATCC) through computer search. The fungi were chosen as they appear to excrete large amount of extracellular enzymes in active form into culture media during their growth (Wood 1975).

According to the information regarding the structural decomposition of jute stem and literature survey for jute softening and upgrading, the most suitable culture should have the ability to excrete a large amount of xylanase, pectinase (PGase or pectinlyase) and little amount of cellulase activity. Therefore, fungal strain, Aspergillus sojae (ATCC 20235) was collected having very good producer of pectinlyase, polygalacturonase and hemicellulase (xylanase) and moderate amount of cellulase activity.

For isolation of mesophilic fungi dilution plate culture method was followed and a pure strain was isolated through gradual transfer from one plate to another. Thus about 20 fungal strains were isolated and maintained on PDA slants in the refrigerator for screening of their activity.

The standard procedures for the rapid screening of both isolated and collected fungi for cellulase, hemicellulase (xylanase) and pectinase (PGase) activities were established. The principle of screening for enzyme activities was based on substrate to be hydrolyzed incorporated as the main source in a solid agar medium distributed in petridishes. The medium contained all the necessary ingredients (minimal medium) to support sufficient initial growth of the inoculum. The enzyme activity was detected around the fungal colonies after an appropriate incubation period by the appearance of zones revealed either by substrate clearances, coloration or decoloration. The collected fungus A.sojae confirmed its reported characteristics through formation of clear zones during screening test (Plate - 4). A sharp clear zone around the colony of A.sojae on PDA palates having pectin as carbon

source indicated its ability as good producer of pectinase (pectinlyase and PGase) activity.

All the fungal isolates were screened for cellulase, hemicellulase and pectinase activity. Most of the isolates did not grow well in minimal medium supplemented with respective substrates as the sole carbon sources. Their poor growth on those media revealed their inability to secrete enzymes for their growth. Although some of them exhibited considerable amount of either pectinase or xylanase activity. Out of 20 screened isolates only one fungal strain exhibited very good PGase and Xylanase activity through formation of clear zones around their colonies. This particular strain also exhibited a little CMCase activity.

This was the only isolate that fulfilled our demands and was selected as potential one.

From the results of screening the best isolate was a dark blackish fungus which was isolated from a jute maturation piling bin of a jute mill near Dhaka. This potential fungus was selected for the production of jute softening enzymes under solid state fermentation on wheat bran.

In order to study the morphological structure and growth pattern the fungus was grown on PDA plates. The fungus started its mycelial growth with whitish structure which gradually turned into dark black after 3 days of culture. The fungus sporulated profusely in 4 days on PDA plates (Plates -). The characteristics of spore, hyphae and other specified structures were observed microscopically with the preparation of slide mounts with lactophenol drops. In order to avoid the interference to the arrangements of the fungal structures, microculture slide technique was also used following Banek and Rogers methods. It was observed that the mycelia of the fungus were cylindrical, branched, septate and hyaline. Fungus has the conidiophore arise directly from substrum, conidia arose from sterigmata and were borne with chain, conidial head focus carbon black (Plate -1). The overall feature of the fungus revealed it to be of Genus Aspergillus. On the basis of its morphology and growth characteristics as described by Raper and Fennel (1965) the fungus was accordingly identified and named as Aspergillus niger.

A consensus was reached by Prof. Anh LeDuy and Dr. G. Mohiuddin in 1990 that all efforts should be put into the solid state fermentation process for the production of jute softening enzymes. They produced jute softening enzymes under S.S.F. and L.S.F. with the same cultures and reached the conclusion that the tested cultures produced higher amounts of enzymes in solid state fermentation than in submerged culture fermentation. Considering the different aspects of the two processes they finally recommended that solid state fermentation process is the most suitable method for the production of jute softening enzymes by the fungi (Anh Leduy 1992).

On the basis of the above findings the two fungal isolates Aspergillus niger (Potential isolate) and Aspergillus sojae (Collected from ATCC) were used for production of jute softening enzymes under solid state fermentation process. It is known that the carbon source plays a vital role in enzyme production under SSF process.

In pursuance of this a number of indigenous materials like wheat bran, saw dust, rice bran and bagasse were used as

substrate for production of enzymes by the two fungi. Among the substrates used wheat bran alone was found to be the best medium for growth and maximum enzyme production by the two fungi. (Fig. 3). To find out the optimum extraction day for enzyme production, enzymes were extracted from 2nd day to 8th day of fermentation. The time required for maximum enzyme production by the two fungi was 3 days. The fungi A.niger and A.sojae produced highest amount 2.62 IU/ml and 2.32 IU/ml of enzymes under SSF on wheat bran medium. After 4th days of culture enzyme production by the two fungi did not increase though fermentation was continued further. This may be due to the fact that enzyme secretion normally occurs at the growing hyphae tip as reported by Brown et al., (1986). This fact was supported by the findings that reducing sugars was highest at the beginning of the fermentation process which become almost nil after 7 days of fermentation. This finding supports the fact that both the fungi stopped their mycelial growth and enzyme production after 4 days of their culture and reducing sugars were used by them for their sporulation only. This is why enzyme production did not increase any more though fermentation continued after 4 days.

The ability of both the fungi to grow on moist wheat bran without any additional supplement proved them to be the perfect degrader of the natural lignocellulosic substates. Both the fungi grew profusely on liquid on soluble sugar, glucose as carbon source. Only the isolate A. niger produced PGase enzymes which indicated that PGase secreation ability is constitutive one of the fungus. They only produced CMcase, PGase and Xylanase on lignocelluosic substrates which indicated their ability as adaptative ones.

The potential fungus A. niger was subjected to Gamma radiation for genetic modification for hyper-enzyme producing nutants at AERE, Savar. But unfortunately no hyper-enzyme producing mutent was found than that of potential fungus.

It is worthwhile to mention that wheat bran is a cheap and easily available substrate needed for enzyme production and it is a great advantage. Moreover, it is nutritionally rich enough to support growth and enzyme production by the two fungi. Thus enzyme production under solid state fermentations using wheat bran is more viable and economic one. The process of enzyme production would be attractive and profitable to millers for its simplicity in media composition and easy extraction of enzymes.

In S.S.F. added moisture level to the substrate is very important for the growth of fungi and enzyme production (Hasseltine, 1972). Optimum requirement of added moisture for growth and maximum enzyme production was determined by fermenting the bran with varied moisture level of 20-100% (W/V) (Fig. 4). It was found that at 50% (W/V) added moisture level both the fungi (A.niger and A.sojae) grew well and secreted maximum amount of enzymes. The fungal cultures though could grew below this optimum moisture level but their enzyme productions were lower than that of optimum moisture level. The decreased growth and enzyme production was due to low moisture level to initiate fungal growth. On the other hand too much moisture level (above 50%) filled up the void fraction of the substrate which forced out the trapped air and thereby the fungal growth deep into the substrate bed was depressed.

Thus the simplest method for enzyme production by this process is to inoculate the moistened solid substrate and ferment for a desired period of time. The enzymes produced are then extracted from fermented bran with saline water.

In order to study the effect of aeration on enzyme production fermentations were carried out with optimum cultural conditions under two types of aeration - natural aeration, intermittent aeration. It was seen that extra aeration had no significant impact on enzyme production by the fungus (Fig. 11). From this finding it may be concluded that no extra aeration was needed, if the substrate bed height was around 3-4 cms. The enzyme production can efficiently be carried out under natural aeration using head space air and air taped in flappy substrate.

Once the optimum level of added moisture for SSF was optimized the fungi were grown with this level at different temperatures to find out the optimum temperature for growth and enzyme production. The fungi were allowed to ferment at a temperature ranging from 20°C to 50°C for 4 days. Both the fungi were found to grow at a temperature range of 30°C-40°C. But at 32°C both the fungi grew well and produced highest amount of enzymes (Fig. 5).

A number of supplements were added with wheat bran to see their effect on the growth and enzyme production. It was found that enzyme production did not increase considerably

with the addition of various chemicals as supplement to the wheat bran medium. But only a slight increase (4%) was recorded with the addition of peptone by A.niger. The increased production by the addition of peptone was that possibly peptone had helped the fungi for its increased mycelial growth which favoured increased enzyme production.

It was seen that addition of calcium, magnesium and potassium salt solution in wheat bran was not necessary for increased enzyme production. On the other hand enzyme production was suppressed slightly with the addition of calcium, magnesium and potassium salt solution. This may be due to the presence of these ions in wheat bran. Therefore, no additional requirement of these ions were necessary for enzyme production by the fungi.

Enzymes were extracted from the fermented bran by adding 5 volumes (W/V) of 1% sodium chloride solution. The clear filtrate was used for jute softening tests and enzyme assays. Enzymes were extracted by fermenting bran for 2 days to 6 days to find the optimum extraction period for enzymes. It was noticed that depending on the desired relative ratios of the three enzymes the fermentation can be ended at any time

between 3rd to 5th day of the process. But it was better to extract enzymes after fermenting for four days when the fungi could complete their full growth deep into the medium bed and begin sporulation. On the other hand it became very difficult (specially by A.niger) to separate the spores from the culture filtrate if the fermentations were carried beyond four days.

Another interesting point was that for higher pectinlyase activity extraction of enzymes must be carried out just after 2 days of fermentation. Most of the pectinlyase activity of A.sojae was lost if extractions were made after 2 days of fermentation.

The culture filtrate was passed through milipore filter paper to make it spore free and was used for enzyme assays. Enzymes were assayed following the modified dinitro salicylic (DNS) method of A. W. Khan et al., (1981) and G. L. Miller (1959). Enzyme assay method was based upon the measure of increased reducing sugars following incubation of respective substrates with enzyme extract. The biochemical composition of culture filtrates extracted from 4 days fermented bran by A.niger and A.sojae along with the commercial enzyme

preparation (Flaxzyme) were shown in (Table 7). It was noticed from the table that both the fungi produced 3 enzymes in almost similar relative ratio of commercial enzymes. But the enzyme units (IU/ml) of commercial enzymes were much more higher than that of extracted enzymes from A.niger and A.sojae. This higher units of enzymes in flaxzyme was due to the fact that Flaxzyme was concentrated by lyophilization. Another feature regarding enzyme components between the two fungi A.sojae contained higher amounts of pectinlyase activity than that of A.niger.

Jute cuttings (30 cm by 2.5 cm) were treated with enzyme extracts of A.niger, A.sojae and commercial enzyme preparation (Flaxzyme) for twenty four hours at 35°C. Samples treated with distilled water served as control. Washed and air dried samples were used for analysis of physico mechanical properties. It appears from the table that in each case the separation index of enzyme treated fibre have improved to different degrees in comparison to the control. It is known that separation index is an important characteristic that denotes how much the fibre is separable. Enzyme action has improved the separation index value through

removal of cementing materials present in between the fibre filament and made their easy separation. But the enzyme extract of A.niger was found to be more effective in this regard. Though enzyme extracts of A.niger and A.sojae exhibited similar amount of CMCase and xylanase activity but PGase activity of A.niger was higher than that of A.sojae. The better separation index value of A.niger enzyme treated fibre was due to its higher PGase activity.

In spinning fibre fineness is of utmost importance and the fineness of individual fibre is important for spinning fine yarns. The fibre fineness was determined by measuring the diameter (in m) of individual fibre filaments with microscope (projectina). From the table 12 it appears that enzyme action has transformed the coarse fibres into finer ones. As a result of enzymatic degradation of cementing materials the fibre filaments were loosened and got splitted into finer filaments. Similar enzymatic improvement of jute and flax fibre was also reported by B. L. Ghosh et al., (1983) and H.S.S.Sharma (1987).

The softness of the fibres was measured by stiffness index and was measured as described by Ramamurthy et al., (1987). From the table (12) it appears that enzymatic degradation of pectin hemicellulose gum transformed the stiff fibres into softer ones. The distance (cm) between two hanging ends of a fibre of 20 cm length at constant weight of 1 gm was taken as the stiffness index. The lower the value of stiffness the softer the fibre is. Enzyme treated fibres have low stiffness index which indicated that the fibres have become softer compared to controls.

As a result of enzyme application the fibre strength was reduced marginally (Table 12). This marginal reduction of fibre strength was due to enzymatic degradation of non-cellulosic cementing materials (Pectin and hemicellulose) that embedded in between the fibre filaments. Enzymes can readily enter the fibre matrix and split the fibres into finer filaments. Consequently, the enzyme treated fibre suffered a slight degradation in fibre strength yet the spinning behaviour and cleanliness of the treated fibre have improved as shown by the results of mill trial experiments. (Table-14, 15)

Temperature is an important limiting factor for storage, preservation and transportation of enzyme solution. So it was necessary to observe the prolonged storage of enzyme extracts at 35°C in consideration of average daily temperature of Bangladesh before applying in jute mills. The enzyme extracts were kept at 4°C, 15°C and 32°C to examine their stability first for 28 days and finally for a long period of six months. Enzyme activities were measured after every seven days and the results are shown in Fig.9. Both the enzyme extracts retained most of their enzyme activities after 28 days and their retaining capacity was higher at lower temperature. The enzyme extracts of A.niger retained 86% of its initial activities at room temperature (32°C) for 28 days. But at 4°C the enzyme preparation was more stable and retained upto 93% of its initial activities after 28 days. Enzyme extracts of A.sojae was also stable but not as much as A.niger was. PG activity in the enzyme extract of A.sojae decreased markedly at 32°C but not at 4°C. Finally it was found that both the enzyme extracts retained more than 70% of their initial activity at 32°C after 3 months. The decrease of enzyme activity may be due to the presence of impurities

in the enzyme extracts or cleavage of enzyme by proteolytic action. The loss of enzyme activity was higher at 32°C than at 4°C which indicated that the proteolytic action became more active at 32°C than at 4°C. So it may be suggested that for prolonged storage of enzyme solutions it is to be stored at low temperature.

For prolonged storage any microbial growth in the enzyme extract need to be checked. With this aim we have tried a number of chemicals as preservative for storage. Among the chemicals used, butanol, toluene, sodium azide was found to be effective as preservatives (@ of 1% V/V and @ 0.1% W/V). But among the chemicals used sodium azide (0.1% (W/V)) was found to be most useful and it had no side effect on the enzyme activity.

It is known that after piling the temperature inside the piling bin rises to around 60°C. In this context the thermal stability of the enzyme components is an important criterion for their hydrolytic degradation at such high temperature in the piling bin. In order to study the thermal stabilities of the enzyme components, the enzyme solutions were kept at

temperatures similar to jute piling bin conditions and the residual enzyme activities were measured after each storage period. It is observed (Table 13) that the enzyme components of both the extracts were considerably thermostable. PGase activities of both the extracts were more susceptible to heat and lost more than 50% of their initial activity at 60°C after 72 hours. On the other hand, cellulase and xylanase enzyme components of both the extracts were more thermostable and retained more than 60% of their original activity at 60°C after 72 hours. On the average the enzyme extract of A.niger retained almost 50% and A.sojae 48% of their initial activity after final storage period of 72 hours at 60°C.

It is known that enzyme actions are susceptible to temperature and pH of the medium. In order to determine the optimum temperature for them, enzyme activity was determined by measuring the enzyme activity after incubation at temperatures ranging from 30°C to 60°C for 20 minutes at pH 4.8. Enzyme component of both extracts showed highest activity at 45°C on average. Temperature 50°C, 40°C and 50°C were found to be optimum for CMCase, Pectinase and xylanase respectively. Several workers (Gorbachev and Radionova 1977,

John and Schmidt 1988) reported that optimum temperature for xylanase activity (Mesophilic source) was between 40-50°C which was also similar with the optimum temperature of xylanase (Fig. 13). Among the enzyme components of both the fungal strains xylanase was found to be most stable at wide range of temperature (upto 60°C). On the other hand, PGase activity of both the enzyme extracts were most susceptible to temperature and their activity decreased sharply with the rise of temperature (above 40°C). CMCase activity did not decrease significantly with the rise of temperature and was stable upto 50°C.

pH is also an important factor of enzyme activity. So in order to determine the optimum pH for the activity of enzyme components, enzyme activity was measured at different pH values (pH 3-9). Since DNS reagent is reduced by reducing sugars under alkaline conditions, enzyme assays were carried out at pH less than 4.0 and the NaoH content of the DNS was found to increase. pH 6.0, 5.6, 6.2 were found to be optimum for CMCase, pectinase and xylanase activity respectively. These values are very close to the normal pH of water extract

of emulsified fibres before piling, and indicate their suitability for action inside the piling bin.

Various chemicals like mono and divalent ions have profound effect on enzyme activity. To determine the effect of various chemicals, 10 mM solution of various chemicals were pre-incubated for 60 minutes at room temperature with the enzyme extracts. The enzyme activity of each solution was measured. Among the chemicals tested EDTA, $MgSO_4$ and $BaCl_2$ had a pronounced inhibiting effect on the enzyme components of both A.niger and A.sojae. On the other hand, $CoCl_2$ $MnCl_2$ had pronounced stimulatory effect on the enzyme components of A.niger and A.sojae (Fig. 7).

Molecular weights of enzyme components of culture filtrate of A. niger was carriedout by Gel Electrophoresis. Three prominent bands of enzyme components-CMCase, Xylanase and PGase were matched with the marker proteins and conformed their molecular weights with the findings of W.A. wood et al, 1988 and Rombouts, F.M. et al 1980.

Enzyme kinetics of culture filtrate were also carriedout with pure Xylanase from Sigma. The Km value of Xylanase fraction were calculated by ploting Line weaver-Bark plot and

the value was found to corelate with the findings of other workers.

Application of enzyme has a profound effect on the chemical constituents of barky jute and cuttings as demonstrated by the significant variations in percentages of chemical constituents. It was seen that a-cellulose content has decreased least among the constituents which was due to comparatively low content of CMCase activity in both the fungal extracts. But the pectin and hemicellulose content of enzyme treated fibres decreased considerably (Table 11) by about 25-30%. This helped in softening the hard jute cuttings by removing a significant amount of pectin and hemicellulose which act as binding materials in between the fibre filaments. M. K. Basak et al., (1987) reported a microscopic examination of barky jute and enzyme treated jute about the huge aggregation of non-fibrous debris, mainly pectin on the epidermis which masked the fibre strands. They further reported that enzyme treated fibres were clearly visible with small amount of non-fibrous aggregates and their findings correlates with the decrease percentage of non-cellulosic materials of enzyme treated fibres. Similar improvement of

flax quality extracted with a mixture of enzymes - cellulase, xylanase and pectinase of fungal origin was reported by H. S. S. Sharma (1987) in N.Ireland.

Mill trial experiments with the application of enzyme extracts were carried out with same batch composition and batch with higher percentages of low grade jute and cuttings than controls. The spinning performances of these batches (treated and untreated) were compared with the findings of batches having same composition and other having higher percentages of quality fibres as per required composition of the mills. Enzyme extracts were mixed with the normal emulsion and enzyme mixed emulsion was applied to the fibres (about 100 IU/kg) through the softener machine. Enzyme extracts were mixed with the jute emulsion in such proportion that under normal emulsification rate of 30-35% the enzymes were 100 IU/kg of treated fibres. The enzyme treated fibres were then piled up in a concrete bin as per existing mill practices. A piling bin was taken to be matured (ready for processing) when the temperature of the piling bin reached around 60°C. One set of trial (which served as control) was carried out with the previously boiled enzyme extracts for

ascertaining whether the nutrients present in the enzyme extracts have any effect on maturation through enhancing microbial growth. Maturation time was slightly reduced by the application of boiled enzyme extracts. This marginal reduction in maturation time was due to the nutrients present in the boiled enzyme extracts have favoured increase microbial growth which inturn helped in the marginal reduction of maturation time from the usual time.

The remarkable feature of enzyme application was demonstrated by the reduction of maturation time to 72 hours from usual maturation time of 168 hours. Since the enzymes start functioning immediately after their application, the maturation time was reduced to half by the synergistic action of the natural microbes along with the enzymes. The enzymes, which remained unaffected through the ecological changes within the pile, had brought about the softening to the desired level in shorter time and thereby improved the fibre quality and spinning performances of the enzyme treated low grade fibres.

The fibres were processed after maturation with usual chartings. Blending of different qualities of fibres were made by inter carding the breaker card rolls and these rolls were finally blended with the finisher card rolls to manufacture respective yarns. The spinning performances of yarns for hessian weft after treatment with enzymes in pilot scale trial at BJRI are shown in (Table 14). Though the experimental batch contained more jute cuttings than control (52%) but the enzyme treated yarn showed better qualitative spinning performances than the untreated one's with lesser amount of cuttings. From the spinning point of view it may be concluded that enzyme treated low grade jute and cuttings appeared to be more pliable and spinnable than the costlier high quality fibres where those were replaced in experimental batches by cuttings. The spinning performances of experimental batch though contained higher amount of cuttings yet a number of spinning performances have showed higher improved values. Average grist is the weight in lbs/14,400 yards and it denotes the thickness of the yarn. The values of average grist of all the enzyme treated yarn have lower value than the control and the yarn treated with the enzymes of A.niger has the highest qualitative value. Decreased average

grist value of enzyme treated yarn indicated that the yarn has became less thicker i.e finer one. Similarly the enzyme treated yarns have developed higher values of average tensile strength. This increased tensile strength was due to the enzyme action that have removed the cementing materials from the fibre filament and transformed them into finer filaments. Thus the number of fibre filaments in a particular count of yarn have increased which intern increased the tensile strength of the yarn. The spinning parameter C.V. denotes the irregularities of yarn and all the enzyme treated yarns showed decreased values, which indicated the enzyme treated yarns were less irregular and more uniform. The other parameter quality ratio is expressed in percentage and is strength divided by jute count. The higher the value, the better is the yarn. Quality ratio was also higher for all the enzyme treated yarns which indicated that the enzyme treated yarns were stronger and finer than untreated ones. In case of production efficiency all the enzyme treated yarns have higher efficiency than the controls. Lastly enzyme treated fibres at subsequent spinning and yarn stages tended to have fewer spinning breakages than in the control batches.

Similar mill trial experiments with enzyme extracts were also carried out at M/S Karim jute mills and the spinning performance of hessian weft/sacking warp yarns with the batch composition of control and experimental are shown in Table 15. It appeared from the table that though the experimental batch consisted of 36.36% of cuttings yet application of enzymes brought about remarkable transformation which enabled the yarn to function better with better spinning performances than that of control with superior quality of fibres. These improved qualitative performances of experimental yarns indicated that the low grade jute and cuttings have been upgraded and thereby showed superior spinning performances. Enzyme treated low grade jute and cuttings at subsequent spinning and yarn stages showed superior qualitative performances and have fewer spinning breakages. From the spinning point of view it may be concluded that the enzyme treated low grade fibres appeared to be more pliable as they had better advantages over the control in respect of average grist, C.V. tensile strength, quality ratio and production efficiency. The enzyme treated fibres have developed higher values of co-efficient variation and quality ratio, which inturn helped to increase the production efficiency.

The over all improvement of low grade jute and cuttings by the application of enzyme preparations were due to the action of enzymes. Because of their high reactivity, the enzymes readily entered into the fibre matrix, split it up into finer filaments and loosened the specks of the fibres. Hydrolytic degradation of pectin hemicellulose gum that encapsulated the fibre filaments helped in the separation of individual filaments and removal of the barks. Thus enzyme application transformed the coarse, rough and stiff fibres into finer, smoother and more pliable ones and consequently their spinning behaviors have improved which attributed much in improving spinning performances and yarn properties. Lastly it may be concluded that enzyme application had not only improved the spinning performances and yarn properties but also improved the production efficiency. Enzyme treated fibres with better spinning quality could easily be blended with superior batch for quality goods production and thereby production cost could be reduced and make the jute goods more competitive to face the challenge from synthetics.

It is worth while to mention that on the basis of the above findings a Pilot Scale and Demonstration Enzyme Plant has been set up with the assistance of IDRC, France Govt. and IJO at Islam Jute Mills, Mirzapur. About 200 litres of jute softening enzymes are now being produced daily by using wheat bran as substrate with Aspergillus terreus. These enzymes are used successfully for the improved processing of jute cuttings and upgradation of different low grade jute.

CONCLUSION

Raw jute and jute goods together earn a lion share of our foreign exchange. Other countries have succeeded in finding synthetic products as substitute for jute. The relatively low price of synthetic substitute paved the way for their rapid growth and have shrinked the word jute market. At present jute is facing a tough competition with synthetic substitutes for its existence. But the awareness regarding the pollution created by the synthetics has generated a good opportunity for regaining its lost market. The most advantageous position for jute is that it is biodegradable and thus friendly to nature. In this context if the price of the jute goods could be reduced it would further Pave the way for regaining its lost glory and position.

Due to improper retting and over maturity a bulk of jute produced in Bangladesh is of low grade with cuttings. More than one million bales of jute cuttings are stock piled each year in Bangladesh alone. They are used only in limited products through inadequate conventional mill softening process.

From the study of conventional mill softening process we reached the consensus that the pretreatment of jute cuttings with enzyme preparation is a possible way for the qualitative improvements of low grade jute and cuttings. Accordingly enzyme extracts were prepared by fermenting the indigenous wheat bran as substrate. Mill trial softening experiments were carried out by the pretreatment of low grade jute and cuttings with enzyme solutions. The remarkable feature of enzyme action was demonstrated by the reduction of maturation time to half of usual time. Hydrolytic action of enzyme have partially removed the pectin hemicellulose from the low grade jute and cuttings and transformed them into better pliable, finer and smother ones. These enzyme treated low grade fibres showed better spinning performances during processing and could easily be blended with superior batches in higher percentages for production of quality goods. Enzyme pretreatment process showed a new era for improving and upgrading these low quality jute. Production cost of enzyme would be cost effective as only low priced indigenous wheat bran was needed for enzyme production by the fungi and no other imported chemicals were needed. Thus increased use of low quality (enzyme pretreated and upgraded) fibres for superior quality product would definitely reduce the cost at least by 10-20% and thereby make the jute goods more competitive in the would market.

Improvement of low grade jute through enzyme pretreatment process is going to be reality through the establishment of a Pilot scale enzyme production and demonstration plant at Islam jute mills, Mirzapur. But development of hyper enzyme producing mutants from these two fungi is a further area for future research in this regard.

REFERENCES

1. Albershim, P. 1966. Pectinlyase from fungi.Meth. Enzymol.8: 628-631

2. Banek, E.S. and Rogers, A.L. (1970) Medical Mycology Manual, 3rd edition. Burgess publishing Co., Minneapolis.

3. Biel, P.1985. Microbial xylanolytic systems. Trends Biotechnol. 3: 286-290

4. Bisira V.S and Ghose T.K. 1981. Biodegradation of cellulosic materials: Substrates, microorganisms and products. Enzyme microb. Technol. 3: 90-104.

5. Brown,J.A. Ogawa, K. and Wood.T.M. 1986. Studies on the preparation and regeneration of protoplast from the cellulolytic fungi penicillium Pinophilium Enzyme Micro. Technol. 9: 527-532.

6. Chang M.M., Chou T.Y.C. and Tsan G.T. 1981. Strature, Pretreatment and hydrolysis of cellulose Adr. Biochem. Ergin. 20: 15-40.

7. Collmer, A.J.L. Reid, and M.S. Mount 1988. Assay methods for pectic enzymes Meth. Enzymol. 161: 329-335.

8. Chowdhury, J.K. and Basu, N.N., 1932. J.Indian Chem. Soc. 9: 615

9. Chowdhury, J.K.and Bardhan, T.P., 1936. J.Indian Chem. Soc.13: 240

10. E. Channel and M. Moo-young 1980. Solid state fermentation system process, Biochemistry, June/July 1980.

11. Esterbauer H., Hayn M., Jungschaffer G., Taufrathshofer E and Schurz J. 1983. Enzymatic A conversion of lignocellulosic materials to sugars. J. wood chem. Technol. 3(3): 261-287.

12. Eriksson K.E. 1975. Enzyme mechanisms involved in the degradation of wood components. In: symposium on enzyme hydrolysis of cellulose (Bailey M., Enaric T.M. and Linko Eds.): 263-280.

13. Eriksson K.E and Jojnsrud S.C 1983. Mutants of the white rot fungus sporotrichum pulveruluntum with increased cellulase and b-glycosidase production Enzyme Microbial Technol. 5: 425-429.

14. Eriksson K.E. and Jojnsrud S.C. 1983. Mutants of the white rot fungus sporotrichum pulveruluntum with increased cellulase and b-glycosidase production Enzyme Microbial. Technol, 5: 425-429.

15. Erikson K.E. 1975. Enzyme mechanical invited in the degradation of wood components. In symposium on enzyme hydrolysis of cellulose (Bailey M.Enrai. T.M. and Linko Eds.) 263-280.

16. Fahnrich P., Ingang. K, Rotten B and Schimz K.I. 1981. Abbau von cellulose in cellulosehaltigen Material durch Mikro-organisem sowie dwcch microbial Exzymsysteme. Das papier 10: 1-9.

17. Fahnrich P., Ingang K, potten b and Schimz K.I. 1981. Abbau Von cellulose in cellulose haltigen Material durch Mikroo-rganisem sowie dwech microbial Enzyme system. Das papier 10: 1-9.

18. Gorbachev, I.V. and N.A Radionva. 1977. Studies on xylan degrading enzymes. Priochem, Biophys. Acta, 484: 93-102.

19. Hasseltine, C.W.1972. Biotechnol. Bioeng. 14: 517-532.

20. Henderson, M.E.K. and V.C.Farmer.1955. J.Gen. Microbiol, 12: 37-46.

21. Herzag, R.O. and Jancke, W.Z., Physik 3: 196.

22. Improvement of fungal culture by Gama radiation from book principles of fermentation Technology by P.F. stanburg and A. whitaker, Pergamon Press, U.K.

23. John. M. Schmidt B. and J. Schmidt 1979. Purification and some properties of five endo 1-4-3-D-xylanase and 3-D-xylosidase produced by strain of *Aspergillus niger*. Can.J, Biochem.57: 125-134.

24. John, M. and J.Schmidt 1988. Xylanases and 3-Xylosidase of Tricoderma lignorum. Methods Eznymol. 6: 662-672.

25. Khan, A.W.D. Wall and L. Vanden Berg. 1981. Fermentative conversion of cellulose to acetic acid and cellulolytic enzyme production by a bacterial mixed culture obtained from sewage sludge. Appl. Envion. Microbial. 41: 1214-1218.

26. Koneman, E.W, S.F. (1978) practical laboratory mycology 2nd edition; Williams and Wilknis Co.; Baltimore.

27. Kundu, A.B.S. Majumdar, B.S. Ghosh and J.P. Bhattacharya (1984). Rapid Kuamtiative measure ent of softness of bawcky root ends of jute. Indian Journal of Textiles, I. 112-114.

28. Khan, A.W. 1986. Assay of xylanase and xylosidase activities in bacterial and fungal cultures. Enzyme microb. Technol. 8: 373-378.

29. Lee S.B., Shin S.H. and Ryu D.D.Y. 1982. Adsorption of cellulase in cellulose: Effect of physicochemical properties of cellulose on adsorption and rate of hydrolysis. Biotechnol. Bioengin. 24: 2137-2153.

30. Lee Y.H. and Fan L.T. 1982. Kinetic studies of enzymatic hydrolysis of insoluble cellulose, Aerialists of the initial rates. Biotechnol, Bioengin. 24: 2382-2406.

31. Leduy Anh. 1992. Improved processing techniques for low grade jute and cuttings. Final report submitted to IDRC. Ottawa, Ontario, Canada KIG 3H9.

32. Lowry, O.H.,Rosebrough N.J.Farr.A.L.and Randall, R.J. 1951. Proteinmeasurement with the Folin-phenol regent. J.Biol. Chem.193: 265-175.

33. Lee S.B., Kim I.H., Hyu D.D.Y and Taguchi H. 1983. Structural properties of cellulose and cellulase reaction mechanism. Biotechnol. Bioengin 25: 33-51.

34. Martin Alexander-1961, Introduction to Soil Microbiology. John Wiley & Sons. Inc. Newyork.

35. M. Azizur Rahman, R. Siddiquellah and Jabbar Mia - Modification of jute fabrics by etherification B.J.Jute Fib. Res. 15 (1&2) 23-34, 1990.

36. Mandel, M. and E.T.Reese. 1956. J. Bacteriol 73: 269-278.

37. Majumdar,S., B.S.Ghosh, A.B.Kundu, (1987) Role of factorial growth and their extracellular enzymes in

softening of hard root-cuttings of jute. Indian J.Text.Res.12: 213-219.

38. Mandels and sternberg, D. 1976. Recent advances in cellulose technology. J. Ferment, technol. 54(4): 267-286.

39. Mohiuddin, G. S.H. Talukder, Latifa Binte Lutfur M.A. Sobhan and M. Khairul Kabir 1992, J. Text. Inst. Vol. 83, No.-4, 527-531.

40. Mandels M. Hontzs L. and Nytroon J. 1974. Enzymatic hydrosis of wastes Biotechnol. Bioengin. 16: 1471-1495.

41. Miller, G.L. 1959. Use of dinitrosalicylic acid regent for the determination of reducing sugar Anal. Chem. 31: 426-428.

42. Paice M.G., Jurasek L., Carpenter M.R. and Smillie L.P. 1978. Production, characterization and parti aminoacid sequence of xylanase A from schizophyllcm commune. Appl. Envion, Microbial 36(6) 802-808.

43. Paice M.G. Jurasek L., Carpenter M.R. and Smillier L.P. 1978. Production, characterization and partial aminoacid sequence of xylanase A from schizophyllcus commune, Appl. Envion, Microbial 36(6) 802-808.

44. Patterson L.S. 1975. The mechanism of enzymatic hydrolysis of cellulose by Trichoderma viride. In symposium on enzymatic hydrolysis of cellulose (Bailey. M, Enari T.M. and Linko M. Eds.): 255-261. Auklanka, Finland.

45. Paul, N.B., S.K.Bhattacharyya, A.C.Chakravarty: 1976. Upgrading of barky jute and mesta by a fungal culture of penicillium corylophilum Diereck. Indian J.Agric.Sa. 46: 418-420 .

46. Punja, K. and Jenking S.F.1984. Influence of medium composition on the mycelial growth and oxalic acid production in Sclerotium rolfsic. Mycologic 76 (5): 947-950.

47. Raper, K.B.and Fennel. D.I. 1965. The genus Aspergillus. Williams and Wilkens Co. Baltimore.

48. Reidly P.J. 1981. The conversion of agricultural by-products to sugars. Department of chemical rngineering Iowa State University, Ames. ISU-ERI-Ames-81100, Project 1514, Final Report. May 1981.

49. Reilly, P.J. (1977). Enzyme technology in the agricultural wastes. Dept. on Chemical Engineering and Nuclear Engineering, Engineering Research Institute, Twoa State University, Ames, Final Report,

50. Rombouts FM and Pilnik W. Pectic Enzymes in Microbial Enzymes and Bioconversions, Economic Microbiology Vol.5, AH Rose Ed., Academic Press, London, 1980, p. 227-282.

51. Reese E.T. Stu R.G.H. and Devidson H.S. 1970. The biological degradation of soluble relationship to the mechanism of cellulose hydrolysis. J. Bacterial 59: 485-497.

52. Reese E.T. 1977. Degradation of polymeric earhydrates by microbial enzymes. In recent advances in phytochemistry (Loweas F.A and Runeck V.C. Eds., 1977), vol.11. P-311.

53. Sarkar, PB. 1931. J. Indian chem. Soc. 8: 397.

54. Sarkar, P.B.Mazumdar, A.K. and Pal, K.B., 1948, J.Text.Inst.39: 46.

55. Sarkanen, K.V. and Ludwig C.H. 1971. In lignins Occurrence, formation, structure and reaction (Sarkanen K.V. and Ludwig C.H. Eds.) Wiley Interscince, New york 1971. P.I.

56. Sarkanen K.V. and Luding C.H. 1971. In lignin: Occurrence, formation struetres and reactions. (Sarkanen K.V. and Ludwig C.H. Eds.) Wiley interseience, New york, 1971, P-1.

57. Sadana J.C., Shewale J.G. and Despade M.V. 1980. High cellulose an xylanase production by Sclerotium rolfsii UN-8 mutant in submerged culture. Appl. Enviorn. Microbial 39(4): 935-936.

58. Sadana J.C., Shewale J.G. and Despande M.V.1980. High cellulase and xylanase production by Sclerotium rolfsii UV-8 mutant in submerged culture. Appl. Envion. Microbial 39 (4): 935-936.

59. Shaha, P.K., Paul,S.S. and Sarkar, P.B. 1952 Textile 78:96

60. Sharma, H.S.S. 1987. Screeing of polysaccharide degrading enzymes for retting of flax stem. International Biodeterioration 23 :181-186.

61. Seterauer H., Hayn M., Tuisel H., Mahnert W. and Taufratshofer E. 1983 (a) Enzymatischer Abbau Von Hemicellulose recsee. Berishtsband Zum workshop Uber verwertung Von pentosen: 127-154, Wein.

62. Sternberg D., Vijaykumar P and Rec E.T. 1977. b-glucosidase: Microbial production and effect on Enzymatic hydrolysis of cellulose. Can. J. Microbial 23: 139-147.

63. Schurz J., Billiani A., Honel A., Engner W.D, Janosi A; Hayn M. and Exterbauer H. 1985. Reactions mechanisms und

strkturan derungen bein enzymatischem Abbau von cellulase, Acta polymeric 36(2): 76-80

64. Schurz J. 1984. Die cellulose urdinhre Belgeitsubstanzen als energce und Rohstoffquelle. Cell. chem. Technol. 18: 257-265.

65. Ulla westermark and Karl Erik Eriksson 1974. Carbohydrate-dependent Enzymic auinone Reduction during Lignin Degradation Acta Chenica Scandinavia B 28: 204-208. No.2.

66. Urabanek H., Zalewska-Sobzak J., and Boriwinska A. 1978. Isolation and properties of Extracellular cellulase Hemicellulose complex of phoma herbernica. Arch. Microbial 118: 265-269.

67. Whistler, R.L. and E.L. Richards, 1970. In the carbohydrates, Pigman, W and D. Horton (eds.) Academic press, New York, chapter 37.

68. Wood T.M. 1975. Properties and mode of action of cellulases, in Biotechnol, Prieng. Sym. No. 5 (Garden E.L. Ltd.) 111-137. John Wiley & sons, New york London, Sydney, Toranto.

69. Wallerstein. L. 1939. Enzyme preparations from microorganisms; Commercial production and Industrial application Ind. Eng. Chem.: 31, 1218-24.

70. Whitaker, D.R.1956. Canad. J. Biochem. Physiol, 34: 488-494.

71. WA Wood and ST Kellogg Eds., 1988 Biomass part-B - Liginin, Pectin and Chitin in Methods in Enzymology Vol. 161, Academic Press San Diego, pp. 329-399.