Production and Characterization of endoxylanase from Bacillus pumilus using Solid State Fermentation and its application in Paper pulp processing

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CERTIFICATE

This is to certify that the work presented in the thesis entitled for '**Production and Characterization of Endoxylanase from *Bacillus pumilus* using Solid State Fermentation and its application in paper pulp processing'** is based on the original research done by **C. Asha Poorna,** under my guidance and supervision at Biotechnology Division, Regional Research Laboratory (CSIR), Trivandrum 695 019, India and no part of this thesis work has been included in any other thesis for the award of any degree.

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CONTENTS

LIST OF ABBREVIATIONS

AOX	- Absorbable Organic halogen
a_w	- water activity
AX	- Arabinoxylan
AXE	- Acetyl Xylan Esterase
BOD	- Biological Oxygen Demand
bp	- base pair
BSA	- Bovine serum albumin
CBB	- Coomassie brilliant blue
CE	- Capillary electrophoresis
CM	- Carboxy methyl
CMC	- Carboxy methylcellulose
CMCase	- 1, 4-(1, 3:1, 4) - β- D- glucan-4-glucano hydrolase
CMP	- Chemi-mechanical pulp
COD	- Chemical Oxygen Demand
CP	- Chemical pulp
CWDEs	- Cell Wall Degrading Enzymes
DBB	- Dry Bacto Bran
DEAE	- Diethyl- Aminoethyl
DNA	- Deoxyribonucleic Acid
DNS	- Dinitrosalicylic Acid method
DP	- Degree of Polymerization
DST	- Department of Science and Technology
EBA	- Expanded bed adsorption
EC	- Enzyme Commission
ECF	- Elemental chlorine-free
EDTA	- Ethylene diamine tetra acetic acid
FAEs	- Ferulic acid esterases
FPase	- 1, 4-β-D-glucan cellobiohydrolase
FPU	- Filter Paper Units
g	- gram
gds	- gram dry substrate

GF	- Gel filtration
GRAS	- Generally regarded as safe
h	- hour
HPLC	- High-pressure Liquid Chromatography
IU	- International unit
k	- Kappa no.
kbp	- kilo base pair
kDa	- Kilo Dalton
kg	- kilogram
K_m	- Michaelis constant
L	- Litre
Lb	- Pound
LCC	- Lignin-carbohydrate complexes
LDC	- Lignin derivative compounds
M	- Molar
mg	- milligram
min	- minute
mL	- milliliter
mm	- millimol
mM	- millimolar
µg	- microgram
µL	- microlitre
µmol	- micromole
Mol. wt. / (MW)	- Molecular weight
MR	- Methyl red
MSM	- Mineral salt medium
mV	- millivolt
N	- Normal
nkat	- nano katal units
nm	- nanometer
°C	- Degree Celsius
Od P	- Oven dried Pulp
OD	- Optical Density
PAGE	- Polyacrylamide gel electrophoresis

pH	- Hydrogen ion concentration
pI	- Isoelectric point
PIF	- Preparative Isoelectric Focusing
Pm	- Vapour pressure
pO_2	- Partial pressure of Oxygen
ppm	- parts per million
Ps	- Vapour pressure
rpm	- Rotation per minute
RRL (TVM)	- Regional Research Laboratory, (Trivandrum)
SDS	- Sodium dodecyl sulphate
sec	- Second
SEM	- Scanning electron micrograph
SmF	- Submerged Fermentation
SSF	- Solid- State Fermentation
TCA	- Tricholoro acetic acid
TCF	- Totally chlorine free
TAXI	- *Triticum aestivum* xylanase inhibitor
U	- Unit
UV	- Ultra Violet
v	- volume
V_{max}	- maximum reaction velocity
VP	- Voges-Proskauer
w	- weight
WEAX	- Water-Extractable Arabinoxylan
WUAX	- Water-Unextractable Arabinoxylan
XBE	- Xylan-Binding Endoxylanase
XPM	- Xylan Production Media
XSM	- Xylanase Screening Media
XYL	- Endoxylanase

LIST OF FIGURES

LIST OF TABLES

Chapter. 1.

Introduction and review on xylanolytic enzyme systems, and its application in biobleaching

1.0. INTRODUCTION

The current demand for better utilization of renewable resources and pressure on industry to function within environmentally compatible limits has been an inducement to the development of new enzyme- catalyzed industrial processes, leading to a steady growth of enzyme market in India (Chand and Mishra, 2003). Development in the field of Biotechnology has helped much in this aspect. The benefits of biotechnological applications are improved quality, production rate or diminished environmental impact. Utilization of enzymes will become more prevalent as biological products become less expensive through large-scale production (Wackett, 2000). Enzyme can be obtained from all living organisms but due to the fact that microorganisms can be easily and quickly grown on a large scale and in many cases, produce extracellular enzymes; they are placed in a favorable position for enzyme production. Microbes that are non-pathogenic, produce no toxins and have well established record of safety and are recognized as "generally regarded as safe" (GRAS) and preferred for the production of enzymes that can be utilized in food and health care processes (DST - 1992). Some of the major applications of enzymes and their world market rating are as follows: Paper and pulp industries, leather, laundry and detergent (25 %), textile (17 %), food, beverages and processing of fruits (17 %) and pharmaceuticals (41 %), Bioorganic synthesis and biosensors are emerging field of enzyme application. Fig. 1. 1 gives an overview of the industry break up based on application of enzyme in the world market (Biospectrum 2005).

How to use these technologies in the industrial processes has become a productive area of research. The pulp and paper industry is one of the capital –intensive with facilities specific to the tasks (Kenealy and Jeffries, 2003, Viikari *et. al.*, 2001, Bajpai, 1997). New technology must either reduce expenses or fit easily into the existing process design. Chlorinated phenolic compounds and chlorinated biphenyls are produced during conventional pulp bleaching that arises from residual lignin present in the pulps, which are environmental hazards (Lin, 2005, Yu, 2004, Zhong, 2000). Residual lignin is dark in colour because it contains extensively oxidized and modified compounds. Hemicellulose and cellulose fibers are covalently attached to it, which are difficult to remove.

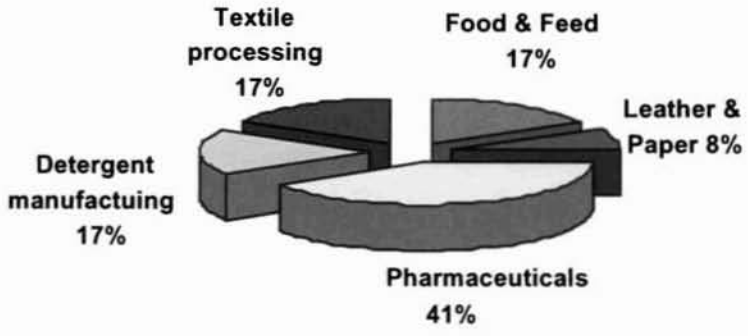


Fig. 1. 1. Industry breaks – up on application of enzymes (Biospectrum 2005)

Extensive studies were conducted to evaluate the deleterious effects of effluents from paper mills (Shoham *et. al.*, 1992). Novel enzyme technologies can reduce environmental problems and alter fiber properties (Kenealy and Jeffries, 2003, Bajpai, 1997). Viikari and co-workers were the first to demonstrate the effect of xylanase in paper and pulp industry (Viikari *et. al.*, 1986).

In cereal industry, endoxylanase hydrolyzes arabinoxylan (AX) internally (Godfrey, 2003); arabinoxylan is the non-starch polysaccharide cereal cell wall material and have a profound impact on their functionality. In industrial separation of wheat starch and gluten, AX interferes with efficient gluten coagulation (Biely 2003, Frederix *et. al.*, 2003). During bread production, flour water-extractable AX (WEAX) has a positive impact on bread quality while their water-unextractable counterparts (WUAX) have deleterious influences (Courtin *et. al.*, 1999). In swine and poultry farming, cell wall material containing high mol. wt. AX lowers feed efficiency (Bedford and Schulze, 1998). Nowadays, various microbial endoxylanase are therefore, used industrially to improve grain, production and/or product quality-related parameters (Kulkarni *et. al.*, 1999).

1.1.0. STRUCTURE OF PLANT CELL WALL

Plant biomass is the most abundant renewable energy as well as major reservoir of carbon sources and raw material for various industries particularly paper and pulp industry. It consists of three major polymeric constituents, i.e. cellulose, along with lignin and hemicellulose (Fig. 1. 2). Cellulose and hemicelluloses are macromolecules from different sugar; whereas lignin is an aromatic polymer synthesized from phenylpropanoid precursors and it varies from plant to plant and age as well as stage of growth of plant (Jeffries, 1994).

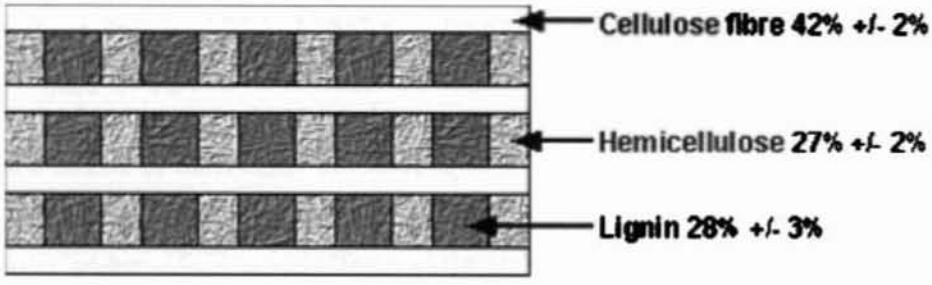


Fig. 1. 2. Diagramatic representation of plant cell wall

Cellulose (~45 % of dry weight) is a linear homopolymer composed of D-glucopyranose residues linked by 1, 4-β- glycosidic bonds forming cellobiose molecules. These linear molecules have a strong tendency to form inter and intra molecular hydrogen bonds. The strength of cellulose is due to the fact that it forms a crystalline structure called micro fibril. These form long chains (elemental fibers) linked together by hydrogen bonds and van der- Waals forces. The chain forms both crystalline (organized) and amorphous (disordered) regions (Brett and Waldron, 1996) in the micro fibril. These micro fibrils are embedded in ground substance, typically hemicellulose, lignin and pectin, which give a highly stable and crystalline structure that plays a major role in the structural characteristics of cell wall (Dickison, 2000). This leads to the strong composite structure, which is less attacked by microorganisms. The micro fibrils orientation is different in different wall levels.

Lignin is the most abundant polymer in nature. It is present in cellular cell wall, conferring structural support, impermeability, and resistance against microbial attack and oxidative stress. Structurally it is an amorphous heteropolymer, non-water soluble and optically inactive; consisting of phenyl propane units joined together by different types of linkages (Perez *et. al.,* 2002, Krik and Ferell, 1987).

'Hemicellulose' word coined by Schulze (1891) for the fraction isolated from plant material with dilute alkali. Hemicellulose or xylan is the second most abundant complex carbohydrate polymer with low mol. wt. than cellulose. It accounts to 20 to 25 % of the dry weight of hardwood and 7 to 15 % in the case of soft woods. In all terrestrial plants xylans are characterized by β- (1, 4) linked D-xylopyranosyl main chain carrying a variable number of neutral or uronic monosaccharide subunits or short oligosaccharides chains. They also vary greatly in amount and complexity in different cell types and in different plant species (Huisman *et. al.,* 2000).

Hemicellulose can be divided into three groups – xylans, mannans and galactans, which can exist as homopolymer or heteropolymer (Shallom and Shoham, 2003). The structural complexity makes xylans the most heterogeneous groups of polysaccharides (Huisman *et. al.,* 2000). Most of the hemicelluloses are heteropolymers containing two to four, and rarely five or six, different types of sugar residues. The unhydrosugar units of hemicellulose can be pentose (β- D- xylose, α L- arabinose, α - L-arabinofuranose), hexose (β-D- glucose, β- D- mannose, α –D- galactose), deoxy - hexose (α- L-rhamnose, α-L-fucose) and hexuronic acids (β- D- glucoronic acids, α - D-4- O- methylglucoronic acids) (Bastawade, 1992, Puls and Poutanen, 1989, Biely, 1985).

Hemicelluloses differs from cellulose by having much shorter molecular chains, with branching, often partially acetylated, and are easily hydrolsable polymers and they do not form aggregates when they are co-crystallized with cellulose chain. In lignocellulosic materials hemicellulose is not joined by chemical bonds with cellulose, but sufficient mutual adhesion is provided by hydrogen bonds and van der Waals forces (Perez *et. al.,* 2002). The β- (1, 4) xylan chain was reported to be more flexible than the two helix of β- (1, 4) cellulose because there is only one hydrogen

bond between adjacent xylosyl residues in contrast to the two hydrogen bonds between adjacent glycosyl residues of cellulose. (Subramaniyan and Prema, 2002).

1.1.1. Classification of wood based on the xylan side chains

Woods are classified into two types based on the side chains on xylan: hardwoods and softwoods. The xylan of hardwoods, which may account for 10-35 % of dry weight, is acetyl-4-O-methylglucuronoxylan with degree of polymerization (DP) of about 200 (Wong *et. al.,* 1988, Kulkarni *et. al.,* 1999). Xylan is found in large quantities in hardwoods from angiosperms (15 –30 % of the cell wall content) and soft woods from gymnosperms (7 –10 %), as well as in annual plants (< 30 %) (Singh *et. al.,* 2003). It is typically located in the secondary cell wall of plants, but is also found in the primary cell wall, particularly in monocots (Wong *et. al.,* 1993). Approximately $1/10^{th}$ of the β-D- xylopyranose backbone units is substituted at C-2 with a 1,2 linked 4-O-methyl-α- D- glucuronic acid residue, while 70 % acetylated at C-2 or C-3 or both (Fig. 1.3.) (Puls, 1997).

Hardwood trees lose their leaves every year, eg. birch, aspen, red gum etc. Soft wood xylans have fewer acidic side chains than hardwood xylans. It contains 10-15 % xylan as arabino-4-O- methylglucuronoxylan with DP of > 120 (Wong *et. al.,* 1993). This material, which is not acetylated, contains β-D-xylopyranose, 4 – O – methyl - α- D - glucuronic acid and L- arabinofuranose in a ratio of 100: 20: 13. The 4-O-methylglucuronose residues are attached to C - 2 and the L - arabinofuranose residues to C-3 of the relevant xylanopyranose backbone units. Softwood trees are conifers - e.g., Southern pine, Douglas fir, and spruce.

The xylan in grasses is also arabino- 4-O- methylglucuronoxylan with DP of 70. This is deacetylated during alkali treatments (Wilkie, 1983). It has less 1,2 - linked 4-O-methyl -α -D- glucuronic acid than hardwood but does not have a large content of L - arabinofuranosyl side chains (Puls, 1997) (Fig. 1.4a).

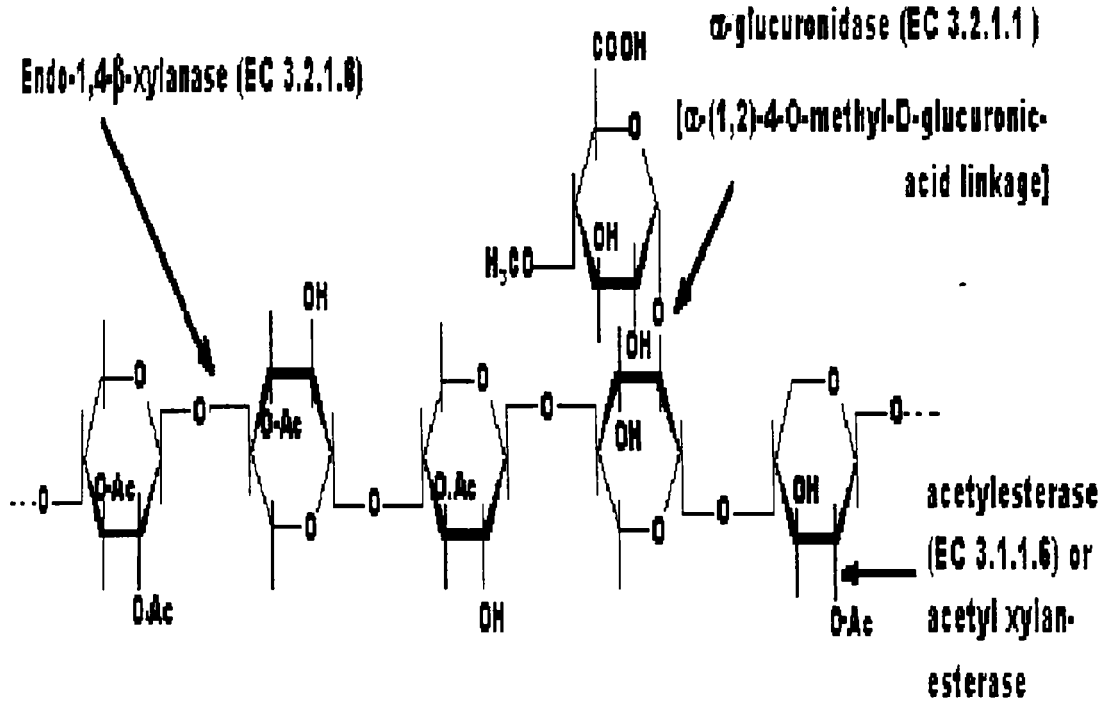


Fig. 1. 3. Structure of hardwood xylan

Polymer	Relative amount present	
	Soft woods	**Hardwoods**
4- O- methylgluronoxylan (acetate)	Small or none	Very large
4- O- methylgluronoarabinoxylan	Medium	Trace
Glucomannan	Nil	Small
Galactoglucomannan (acetate)	Very large	Nil
Arabinogalactan	Large for larch	Nil
Cellulose	42% +/- 2%	45% +/- 2%
Lignin	28% +/- 3%	20% +/- 4%
Hemicellulose	15%+/- 4%	20% +/-5%
Extractives	3% +/- 2%	5% +/- 3%
Fibre length	2-6 mm	0.6-1.5 mm
Coarseness	15-35 mg/100 mm	5-10 mg/100m

Table. 1. 1. Different component present in softwood and hardwood fibres

Table. 1. 1. gives a qualitative summary of polymer composition of hemicelluloses from hardwoods and softwoods as well as an idea about the percentage of different components. It is evident that hardwoods hemicelluloses are rich in xylan polymers with small amounts of glucomananan, whereas softwoods are rich in galactoglucomannan polymers and contain significant quantities of xylan polymers.

The glucuronoxylan consists of a linear xylan polymer backbone with methylated glucuronic acid side groups with slight branching. The D- xylose units in the backbone are pyranose form linked as in cellulose (1, 4 -β). The glucuronic acid is methylated in the 4^{th} position and is thought to attach randomly along the xylan chain by a α- linkage to the 2 (or 3) position of the xylose. This linkage is resistant to acid hydrolysis. Arabinose is attached in furanose form to the 2 or 3 positions along the xylan of soft wood and this linkage is easily hydrolyzed. There are about one or two glucuronic acid side groups and one to three arabinose units per ten xylose units. The hardwood glucuronoxylans contains a large number (7 to 8 per 10 xylose units) of acetate ester groups. These are located on both 2 and 3 position of the xylose units and it undergoes splits reaction with alkali extraction (Sunna and Antranikian, 1997) during pulping but are resistant to acid hydrolysis and make xylan partially soluble in water (Beg *et. al.,* 2001), and the average degree of polymerization is 300.

The galactomannans appears to be random mixed linear polymers having both mannose and glucose in the backbone chain, with side groups of galactose in the case of hard woods. The units in the chains are 1, 4 –β- linked glucosidically and in softwoods containabout 0.3 galactose side groups per 10 backbone units attached at the 3 position. All of the mannose present in wood appears to be tied up in glucomannan polymers. The glucose: mannose ratio ranges from 1:1 to 1:4 and degree of polymerization low, about 100.

The arabinogalactans are in significant quantity only in larch. They have not been fully characterized, but are highly branched molecules, which are basically rendered soluble during water extraction. Strong evidences exist for a 1, 3 β- linkage galactan backbone to which numerous arabinan and galactan chains are attached glucosidically through the 6-galactose position. There are about 2.0 arabinose units present per 10 units of galactose and DP is in the range of 200 to 600. Normally angiosperms contain

significantly more xylan and acetyl, where as gymnosperms contain considerably more mannan and lignin. The major hemicellulose of angiosperms is O- acetyl - 4 - O-methylglucuronoxylan and secondly glucomannan. Gymnosperms's major hemicellulose is acetyl galactogluco-mannans and arabino-4-O-methylglucurono xylan (Wilkie, 1983). Lignin bonding can occur at the C - 2 and C - 3 of the xylose monomer and at the C-5 of arabinose side chains. Arabinose, xylose and 4 - O-methylglucuronic acid are the most common sugar moities to which lignin is cross-linked. Benzyl ester, benzyl ether and phenyl glycosidic linkages are probably involved (Kirk and Cullen, 1998).

Linear unsubstituted xylan or homoxylans has also been reported, it consist of exclusively xylosyl residues e.g., in esparto grass, tobacco stalks and guar seed husk (Beg *et. al.,* 2000). Xylans with β - 1, 3- linked backbone have been reported in marine algae (Dekker and Richards, 1976). *Palmeria palmate* seaweed was repored to contain xylopyranosyl residues linked by both 1, 3 - β and 1, 4 - β linkages (Nunn *et. al.,* 1973).

The most potential sources of xylan include many agricultural crops and products such as straw, wheat, sorghum, sugar cane, corn stalks and cobs, hulls and husks from starch production as well as forest and pulping waste products (Ebringerová and Henze, 2000). Xylan from the primary walls of maize and sorghum are much more complex than from other cereals such as wheat (Huisman *et. al.,* 2000). At least three different components of arabinoxylans, each with different side branches, are known to occur in maize (Carpita, 1983). The arbinoxylans of monocots primary walls are substituted by ferulic acid and these ferulic acid components are thought to cross-link the arabinoxylans in the wall (Schooneveld-Bergmans *et. al.,* 1998, Brett and Waldron, 1996). The occurrence of these different substituents and the possible cross-linking with other polysaccharides is believed to have an influence on the enzymatic hydrolysis of xylans (Wong *et. al.,* 1988). The substituents may limit the accessibility of the residual xylosyl residues to xylanolytic enzymes thereby preventing its hydrolysis. On the other hand they may serve a positive role in substrate-enzyme binding and thus enhance enzyme action. This has been demonstrated by the preferences of some xylanase for hydrolysing branched xylans (Dekker and Richards, 1975).

1.2.0. XYLANOLYTIC ENZYMES

The complex chemical structure of xylan has been described as a linear polymer of repeating xylopyranosyl groups substituted at various carbon positions with different sugars and/or acidic compounds. Hence, complete and efficient enzymatic hydrolysis of the complex polymer requires an array of enzymes with diverse specificity and modes of action (Fig. 1.4a and 4b). Endo- 1,4-β D-xylanase (E.C. 3.2.1.8) randomly cleave the xylan backbone, β - D-xylosidases (E.C. 3.2.1.37) cleave xylose monomers from the non-reducing end of xylooligosaccharides and xylobiose while removal of the side groups is catalyzed by α- L -arabinofuranosidases (E.C. 3.2.1.55), α -D-glucuronidases (E.C. 3.2.1.139), acetylxylan esterases (E.C. 3.1.1.72), ferulic acid esterases (E.C. 3.1.1.73) and p-coumaric acid esterases (E.C. 3.1.1.-), which remove acetyl and phenolic side branches, and act synergistically on the complex polymer (Uffen, 1997, de Vries *et. al.,* 2000, Beg *et. al.,* 2001, Collins *et. al.,* 2005).

1.2.1. Endo-β-1, 4 D- xylanase (*β-1-4-D-xylan xylanohydrolase;* (E.C. 3.2.1.8) Microorganisum are reported as major producers of endoxylanase (Table. 1. 2). There are lots of reports on microbial endoxylanase from 1960's onwards (Gascoigne and Gascoigne, 1960). These reports have given prime importance to plant pathology related studies (Takahashi and Kutsumi, 1979, Dekker and Richards, 1976). 1980's have seen a great change in the application of xylanase by introducing it into the paper pulp bleaching process (Viikari *et. al.,* 2001, Vicuna *et. al.,* 1997). Endoxylanase act randomly on xylan to produce large amounts of xylooligisaccharides of various chain lengths; they release xylose and do not have activity against xylobiose.

1.2.2. Exo-β-1, 4-D-xylanase or β - Xylosidase (*β-1-4-D-xylan xylohydrolase or xylobiase*, (E.C. 3.2.1.37): These enzymes are exo acting and hydrolyze disaccharides like xylobiose and higher oligosaccharides with decreasing specific affinity. (Panbangred *et. al.,* 1984). It catalyses the hydrolysis of 1, 4 -β- D-xylooligosaccharides by removing successive D-xylose residues from the non-reducing end (IUB, 1982). Many β- xylosidase are coupled with transferase activity, which is helpful for the organism to regulate xylanolytic system (Conrad and

Noethen, 1984, Puls and Pountanen 1989). β–Xylosidases have been identified in several *Aspergilli.* These enzymes are highly specific for small-unsubstituted xylose oligosaccharides (DP up to 4), which results in the production of xylose. The activity of this enzyme is of major importance for the complete degradation of xylan, but the absence does not interfere with the induction of the xylanolytic system (van Peij *et. al.,* 1997). The ability of *A. awamori* β-xylosidase to release xylose from xylooligo-saccharides was studied to determine its substrate specificity (Kormelink *et. al.,* 1993). β-xylosidase releases xylose from the nonreducing end of branched oligosaccharides only when two contiguous unsubstituted xylose residues were present adjacent to singly or doubly substituted xylose residues. Some β-xylosidases have transxylosylation activity, allowing the production of novel xylose containing oligosaccharides using these enzymes (Coughlan and Hazlewood, 1993). Lenartovicz *et. al.,* (2003) reported the effect of temperature of growth and carbon source on the production of β -xylosidase by a thermotolerant fungi *A. fumigatus* in submerged cultures.

1.2.3. α-Arabinofuranosidases (E.C. 3.2.1.55) hydrolyse the terminal, non-reducing α-L-arabinofuranosyl groups of arabinans, arabinoxylans, and arabinogalactans. (Gomes, *et. al.,* 2000). A number of microorganisms including fungi, actinomycetes and other bacteria have been reported to produce α-arabinosidases (Saha, 2000). *Streptomyces lividans* single gene product (Vincent *et. al.,* 1997) is an examples of prokaryotic cultures characterised at the gene level for the production of α -arabinofuranosidases. The *B. pumilus* gene encoding acetyl xylan esterase (AXE) was identified and characterized as well as expressed and produced in *Escherichia coli,* which was purified and characterized. The recombinant enzyme displayed similar properties to the AXE purified from *B. pumilus* (Degrassi, *et. al.,* 2000). Ferre *et. al.,* (2000) isolated a novel type of arabinoxylan arabinofuranohydrolase from germinated barley which is able to release arabinose from both singly and doubly substituted xylose, and it hydrolyses p-nitrophenyl α-L-arabinofuranoside at a rate similar to that observed for oligosaccharide substrates.

1.2.4. α-D-glucuronidases (E.C. 3.2.1.1) are required for the hydrolysis of the α-1, 2-glycosidic linkages between xylose and D-glucuronic acid or α-D-glucuronidases

(E.C 3.2.1.1) are required for the hydrolysis of the α-1, 2-glycosidic linkages between xylose and D-glucuronic acid or its 4-O-methyl ether. The hydrolysis of the far stable α- (1, 2)-linkage is the bottleneck in the enzymatic hydrolysis of xylan and the reported α-glucuronidases are having different substrate requirements. Similar to lignin carbohydrate linkage, 4-O-methyl-glucuronic acid linkages are forming a barrier in wood degradation (Hazlewood and Gilbert, 1993). There are a number of microorganisms reported to be producing α-glucuronidases (Puls, 1997).

1.2.5. Esterases: This removes the esterified acids from xylans. Based on substrate specificities, two types are reported- acetyl xylan esterases and feruloyl esterase.

1.2.5.1. Acetyl xylan esterases (E.C. 3.1.1.6) Acetylxylan esterases (AXE) hydrolyze O-acetyl group from the C-2 and C-3 position of xylose residues in both xylan and xylooligomers (Joselau *et. al.,* 1992, Degrassi *et. al.,* 2000). Biely *et. al.,*(1986) first reported the presences of acetyl xylan esterase in fungal cellulolytic and hemicellulolytic systems of *T. reesei, A. niger, Schizophyllum commune* and *Aureobasidum pullulan.* They exhibited high specific activity towards acetylated glucuronoxylan. *Penicillium purpurogenum* secretes at least two forms of AXEs, II, and I, which demonstrate substrate specificities toward acetate esters of D - xylopyranose and belong to a new class of α / β hydrolases (Ghosh *et. al.,* 2001).

1.2.5.2. Feruloyl esterases: Feruloyl esterases (or ferulic acid esterases, FAEs) (E.C. 3.1.1.73) are a subclass of carboxylic acid esterases that catalyze the hydrolysis of the ester bond between hydroxycinnamic acids and sugars on the plant cell walls (Fig. 1. 4a). FAEs can be classified into four subclasses, types A - D, based on their activities toward a set of synthetic substrates (Humblestone, and Briggs, 2000b, Crepin, 2004). They liberate ferulic acid from arabinoxylans of monocotyledons. *Aspergillus oryaze* is also reported to be a producer of feruloyl esterase (Tenkanen *et. al.,* 1995), which is an acidic monomeric protein with pI 3.6 and mol. wt. 30 kDa and has wide substrate specificity liberating p - coumaric and acetic acids from steam extracted wheat straw arabinoxylan. Shin and Chen (2005) reported the production and characterization of a type B feruloyl esterase from *Fusarium proliferatum* NRRL 26517.

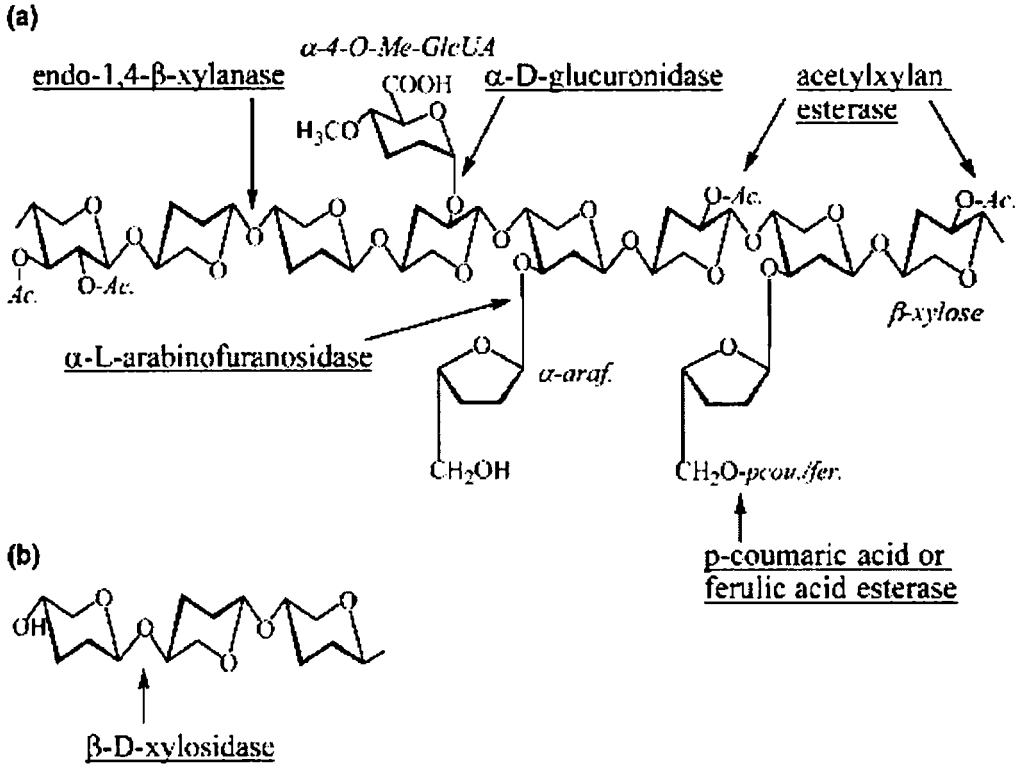


Fig. 1. 4. (a). Structure of cereal xylan and the site of attack by xylanolytic enzymes

(b). Hydrolysis of xylooligosaccharide by β-xylosidase. (Courtesy Collins *et. al.*, 2005).

1.3.0. SOURCES OF XYLANASE

Xylanase are widespread in nature, they occur both in prokaryotes and eukaryotes and have been reported from marine and terrestrial bacteria, rumen bacteria, protozoa, fungi, marine algae, snails, crustaceans, insects and seeds of terrestrial plants and germinating seeds (Taiz and Honigman, 1976, Mujer *et. al.*, 1991, Subramaniyan and Prema, 2002). Amongst the prokaryotes, bacteria and cynobacteria from marine environments produce xylanase (Dekker 1985). There are reports regarding xylanase from plants, that is endoxylanase from Japanese pear fruits during over ripening period and later Cleemput *et. al.*, (1997) purified one endoxylanase with a mol. wt. of 55 kDa from the flour of European wheat (*Triticum aestaivum*).

Microorganism	Xylanase IU/ml or U/gds	Cellulase IU/ ml or U/ gds Fp asae/ CMCase	pH of the medium	References
Bacteria				
Bacillus circulans	400	0.05/1.38		Ratto *et. al.*, (1992)
Bacillus sp.	120	0/0.05	9	Balakrishnan *et. al.*, (1992)
Bacillus sp.	11.5	0/1.2	7.2	Paul and Verma, (1993)
Bacillus -SSP-34	379	0/0.001	8.5	Subramaniyan *et. al.*, (1998)
*Bacillus*sp. Sam-3	131	-	8	Shah *et. al.*, (1999)
Bacillus pumilus	160	-	9	Duarte *et. al.*, (2003)
Bacillus sp. JB-99	3644	-	10	Virupakshi *et. al.*, (2005)
Bacillus sp.	17	-	6.8	Cordeiro *et. al.*, (2004)
Bacillus pumilus	328	-	9	Duarte *et. al.*, (2000)
Bacillus circulans	>0.871 (80°C)	-	-	Heck *et. al.*, (2006)
Fungus				
Aspergillus niger An76	357.5 ±5.52	2.04 ±0.03	4.8	Zhoa *et. al.*, (2006)
Paecilomyces themophila J18	18580	-	6.9	Yang *et. al.*, (2006)
Aspergillus foetidus MTCC 4898	210	<0.1	5	Shah and Madamwar, (2005)
Micrococcus sp (DG 10)	3.33	0/3.11		Saxena *et. al.*, (1991)
Rhizopus oryzae	32	0.06	5	Bakir *et. al.*, (2001)
T. lanuginosus ATCC 46882	5098	-	-	Christopher *et. al.*, (2005)
Cellulomanas. sp (GS2)	9.33	0/0.72		Saxena *et. al.*, (1991)
Streptomyces rosseiscleroticus	16.2	0/0.21	7	Grabski and Jeffries, (1991)
Streptomyces sp. QG-11-3	96	-	9	Beg *et. al.*, (2000)
Streptomyces T7	16.6	-		Keskar, (1992)
Streptomyces cyaneus SN32	720	-		Ninawe and Khuhad, (2005)
Streptomyces sp.strain AMT-3	70	-		Nascimento *et. al.*, (2002)
Rhodothermus marinus	1.8	0.05/0.025	7.1	Saxena *et. al.*, (1991)
Trichosporon cutaneum	14.3			Liu *et. al.*, (1998)
Thermoanaerobacterium sp. JW/SL-YS485	1290	-	6.2	Shao *et. al.*, (1995)
Aspergillus foetidus	3065	-	5.0	Shah *et. al.*,(2006)

Table. 1. 2. Microorganism that produce Xylanase and Cellulase

Higher animals like mollusc, are also able to produce xylanase (Yamura *et. al.*, 1997). There are reports related to isolation and purification of xylanase from various other sources such as anaerobic bacterium *Clostridium acetobutylicu*, immature cucumber

seeds, and germinating barley (Campenhout and Volckaert, 2005, Lee *et. al.,* 1998, Mujer *et. al.,* 1991).

1.3.1. Fungal Xylanase

Microorganisms are primarily responsible for xylan degradation. Many fungal and bacterial species can produce a mixture of xylanase, β-xylosidase and accessory side-group cleaving enzymes in order to utilize xylan. Most of the xylanase known to date are from bacterial and fungal sources (Sunna and Antranikian, 1997).

Fungi are the most potent producers of xylanase (Haltrich *et. al.,* 1996). Fungal endoxylanase are of two types, of non-branching endoxylanase that do not hydrolyse L- arabinose during their action on xylan branch points, and debranching endoxylanase that hydrolyse L- arabinose or the side chains during the hydrolysis of arabinoxylans. Fungal xylanase are extracellular (into the medium), and no cell lysis is needed. It is getting special attention because of the growing interest of their potential use in paper and pulp industries (Viikari *et. al.,* 2001) and due to their potential role in fungal pathogenicity (Carpita, 1983, Annis and Goodwin, 1997). Accordingly, xylanase have been purified and characterized from an increasing number of fungi and the genes encoding xylanase have been cloned and characterized. Xylanase are often co-induced with cellulases by pure cellulose, as in *Chaetomium thermophile* var. *coprophile* (Ganju *et. al.,* 1989), and *H. insolens* (Naren, 1992). In *M. albomyces* (Maheshewari and Kamalam, 1985) and *T. lanuginosus* (Gomes *et. al.,* 1993, Purkarthofer *et. al.,* 1993), xylanase, but little or no cellulase, was produced. Crude culture filtrates of these fungi can therefore be used for biobleaching of paper pulp (Maheshewari *et. al.,* 2000).

Trichoderma reesei (also known as *Hypocrea jecorina*) is a mesophilic fungus, which is one of the most efficient xylanase and cellulase producers. Industrial strains of *Trichoderma reesei* can achieve protein production levels of up to 100 g/L (Cherry and Fidantsef, 2003). Among the many *T. reesei* mutants, Rut C-30 is a widely studied strain (Xiong, 2004). It grows on a single carbon source, such as cellulose or xylan, and secretes both cellulases and xylanase. In addition, it produces enzymes more efficiently than the wild-type *T. reesei* (Bader, 1993). The cellulase expression

in Rut C-30 is not repressed by glucose to the same extent as in some other strains (Tangnu *et. al.,* 1981, Domingues *et. al.,* 2000). The modern production strains are genetically engineered to increase the enzyme production and often to remove the expression of unwanted enzymes, like cellulases during the production of xylanase (Paloheimo *et. al.,* 2003).

Various endoxylanase have been identified in *Aspergillus* (de- Vries and Visser, 2001). Although variation is detected in their molecular mass or pH optimum, the major difference between the enzymes is in their pI, which ranges from 3.5 (Ito *et. al.,* 1992) to 9.0 (Fujimoto *et. al.,* 1995). Endoxylanase also differ in their specificity toward xylan polymer. Some enzymes cut randomly between unsubstituted xylose residues, whereas the activity of other endoxylanase strongly depends on the substituents on the xylose residues neighbouring the attacked residues. Hydrolysis of a glucuronoxylan by an endoxylanase from *A. niger* resulted mainly in xylobiose, xylotriose, and xylose, but hydrolysis of an arabinoxylan by the same enzyme resulted mainly in oligosaccharides with a degree of polymerization of more than 3 (Gorbachev *et. al.,* 1977). This suggests that the action of this endoxylanase is reduced by the presence of arabinose residues on the xylan backbone. All xylanase that have been purified to date are produced when *Aspergillus* is grown on xylan. Most of these enzymes are also produced when xylose was used as a carbon source, but all at lower levels than on xylan. Several genes encoding endoxylanase from *Aspergilli* have been cloned. The encoded enzymes have been assigned to glycosidase families 10 and 11, which work *via* a retaining mechanism. Based on the data of the *A. kawachii* endoxylanase, it would appear that the acidic endoxylanase belong to family 11 whereas the neutral endoxylanase belong to group 10. Shah and Madamwar (2005) reported the production of xylanase from *Aspergillus foetidus* MTCC 4898, which was active at pH 5.3 and 50 °C, with negligible cellulase activity (<0.1 FPU/mL).

1.3.2. Bacterial Xylanase

Most of the bacterial xylanase reported are cellulase free or cellulase poor strains as well as active at alkaline pH and thermostable, which have high potency in various paper, pulp as well as food and feed industrial sectors. The most significant feature of the enzyme is its cellulase free nature, which is one of the necessary prerequesites for

use in the paper and pulp industries. The gene fragment coding for xylanase from *Bacillus* has been cloned and expressed in *Escherichia coli* (Yang *et. al.,* 1989, Shendye and Rao, 1993, Jeong *et. al.,* 1998). The production and characterization of xylanase from *Bucillus* sp. NCIM 59 has been reported (Dey *et. al.,* 1992, Chauthaiwale and Rao, 1993). Thetmoalkaliphilic *Bacillus* sp. strain TAR-l isolated from soil produced an extracellular xylanase R. It was purified to homogeneity with molecular mass 40 kDa and pI - 4, which was active at pH 5.0 to 10.0 at 50 °C. The optimum temperatures for activity were 75 °C at pH 7.0 and 70°C at pH 9.0, and stable up to 65°C at pH 9.0 for 30 min in the presence of xylan (Nakamura *et. al.,* 1995). Two endoxylanase were purified to homogeneity by column chromatography from *C. acetobutylicum* ATCC 824 anaerobic bacterium. Xylanase A, which has a mol. wt. of 65 kDa, hydrolyzed larchwood xylan randomly, yielding xylohexaose, xylopentaose, xylotetraose, xylotriose, and xylobiose as end products, and also active against CMC ase. Xylanase B, which has a mol. wt. of 29 kDa, hydrolyzed xylan randomly; end products are xylotriose and xylobiose and partially active against CMC ase (Lee *et. al.,* 1987). *Bacillus* sp. (V1 - 4) isolated from hardwood kraft pulp was capable of growing in diluted kraft black liquor at pH 11.5 (49 IU / mL in wheat bran at pH - 9) (Yang *et. al.,* 1995). Kulkarni and Rao, (1996) reported the application of cellulase free thermostable alklophilic xylanase from *Bacillus* sp. NCIM 59 in bagasse pulp. Thermophilic *Bacillus* isolated from soil is reported to be potent producer of xylanase which is stable for 2 hour at 30- 50 °C and 71 % of activity is retained at 100 °C and has a pH optima of 6.5 to 7.0 (Corderiro *et. al.,* 2002). Durate *et. al.,* (2000) has characterized alkaline xylanase from four different strains of *B. pumilus*. The optimal pH and temperature were pH 9.0 and 60 °C for strain 13_a, and pH 8.0 (328 U / mL) and 55 °C for strains 5_2 (131 U / mL), 5_{14} (90 U / mL), and 4_a (167 U / mL). The systematic studies on the folding and stability of cellulase-free xylanase are important, since their biotechnological applications require them to function under extremes of pH and temperature. Nath and Rao, (2000) reported pH dependent conformational and structural changes of Xyl II from an alkalophilic thermophilic *Bacillus* sp. (NCIM 59), using kinetic, circular dichroism and fluorescence spectroscopy studies. The Trp fluorescence and the kinetic constants were found to dependent on the pH. Heck *et. al.,* (2005a) reported the production of cellulase-free xylanase from *B. coagulans* BL 69 by solid-state cultivation using 2^2 factorial design using soybean residues. Heck *et. al.,* (2005b) reported water (7 °C, 40 min, 150 rpm and 1: 6 solid / liquid ratio) as the

most potent extraction medium of xylanase produced by *Bacillus circulans* BL 53 isolated from Amazon basin using SSF. Xylanase activity of *Clostridium cellulovorans*, an anaerobic, mesophilic, cellulolytic bacterium, was grown in xylan medium and has characterized the extracellular xylanolytic enzymes from both cellulosomal and noncellulosomal fractions which show that the cellulosome components are responsible for expression of xylanase (Kosugi *et. al.,* 2001). *Bacillus circulans* AB 16 isolated from a garbage dump produced extracellular thermophilic xylanase, with negligible quantities of cellulase when grown on 0.3 % xylan (19.28 IU / mL) and 20.6 U / mL with rice straw medium (Gupta *et. al.,* 2000). Ratanakhanokchai *et. al.,* (1999) reported that an alkaliphilic *Bacillus firmus* produced two xylanase, a xylan-binding endoxylanase (XBE) and an unbound xylanase, which differed in their ability to bind to insoluble xylan. The synthesis of this was investigated by incubating washed cells in alkaline mineral salt media containing various carbon sources. Five mg / mL of glucose induced production of 23 kDa XBE, which hydrolyses insoluble xylan, and glucose was found to represse the production of 45 kDa-unbound endoxylanase (Ratanakhanokchai *et. al.,* 2002). *B. thermantarcticus*, a thermophilic bacterium, isolated from Antarctic geothermal soil from Mount Melbourne reportedly produced extracellular xylanase and β-xylosidase. The optimum temperatures were 80 °C for xylanase at pH 5.6 with pI 4.8 and Mol. wt. 45 kDa and 70 °C for β -xylosidase at pH 6.0 with pI 4.2 and 150 kDa Mol. wt. (Lama *et. al.,* 2004). High production of enzyme from *Bacillus circulans* D1 in submerged fermentation was obtained during growth in media with bagasse hydrolysates (8.4 U/ mL) grass hydrolysates (7.5 U / mL) and xylan (7.0 U / mL) (Bocchini *et. al.,* 2005).

1.4.0. PROBLEMS RELATED TO FUNGAL XYLANASE

The reports show that the major producer of xylanase is fungus by different modes of fermentations (Table. 1. 2). There are about 15 leading companies, which produce xylanase of fungal origin with pH optima ranging < 5.5 (Haltrich *et. al.,* 1996). All fungal xylanase have pH optima of about 5 for the hydrolysis of xylan and are reported to be stable at a pH range of 2 - 9. Most of the fungal xylanase are active at a temperature below 50 °C and the stability is also relatively lower than 50 °C. Fungus is also reported as slow growers and requires more time for production. Application of fungi in paper pulp industries is further impaired by their growth requirements

(Christov *et. al.,* 1996, Haltrich *et. al.,* 1996). Although xylanase activity was of considerable range in fungi, major draw back is production of cellulase. *Schizophyllum commune* and *Thermomyces lanuginosus* (Purkarthofer *et. al.,* 1993) produce high level of xylanase, 1244 U/ mL and 2174 U/ mL, respectively but in *S. commune* the CMC ase activity is 65.3 U/ mL and FP ase activity 5.0 U/ mL which make it less suitable for paper pulp industry (Steiner *et. al.,* 1987). All other fungal isolates show considerable amount of cellulase activity.

Bacterial xylanase have pH optima slightly higher than the pH optima of fungal xylanase (Okazaki, 1985, Khasin *et. al.,* 1993). Most of the bacterial xylanase are active at alkaline pH and high temperature. The xylanase from *Bacillus circulans* (400 IU / mL) is active at pH-7 and and 40 % of activity is retained at pH - 9, the cellulase activity is reported to be endoglucanase of 1.38 IU / mL (CMCase) and cellulobiohydrolase of 0.05 IU / mL (FPase) (Ratto *et. al.,* 1992). *Rhodothermus marinus* was found to produce thermostable xylanase (~1.8-4.03 IU / mL) with detectable range of cellulase activity (Dahlberg *et. al.,* 1993, Hreggvidsson *et. al.,* 1996). A detailed description of microorganism producing xylanase and cellulase is given in Table. 1.2. Paper pulp industries require alkaline pH, where the application of fungal xylanase requires an additional stage in paper pulp processing for the activation of enzyme. Low pH optima and cellulase activity make fungal xylanase less suitable in paper and pulp industries. Another draw back of fungal xylanase is reduced yield when grown in fermentor. The shearing forces of fermentor originating from agitation cause the disruption of fungal mycelia and thus lower the fungal biomass (Palma *et. al.,* 1996, Krishna, 2005).

1.5.0. EXTREMOPHILIC XYLANASE

Xylanase studied are of fungal or bacterial origin which show optimum activity at, or near, mesophilic temperatures (~40 – 60 °C) (Subramaniyan and Prema, 2002) and neutral (in particular for bacterial xylanase) or slightly acidic (in particular for fungal xylanase) pHs. There are also reports related to xylanase that are active and stable at extreme pH ranging from 2 to 11 and temperature ranging from 5 to 105 °C (Kulkarni *et. al.,* 1999, Kimura *et. al.,* 2000, Collins *et. al.,* 2005) as well as at high concentration of NaCl - 30 % (Waino and Ingvorsen, 2003, Wejse *et. al.,* 2003). These are produced by microorganisms which have colonized environments that may

be said to be extreme from an anthropocentric point of view and which produce enzymes adapted to these extreme habitats.

1.5.1. Psychrophiles

Only a small number of cold-adapted, or psychrophilic xylanase are identified, which include a wide range of organisms, two-gram negative bacteria *Pseudoalteromonas haloplanktis* TAH3a (Collins *et. al.,* 2002, Collins *et. al.,* 2003) and *Flavobacterium frigidarium* sp.nov. (Wakarchuk *et. al.,* 1994), a gram positive bacterium *Clostridium strain* PXYL1 (Akila and Chandra, 2003), a yeast isolate *Cryptococcus adeliae* (Petrescu *et. al.,* 2000), two xylanase A and B identified and purified from Antartic krill *Euphasia superba* (Turkiewiz *et. al.,* 2000), fungi *Penicillium* sp., *Alternaria alternata* and *Phoma* sp.2 (Bradner *et. al.,* 1999) basidiomycetes - *Coprinus psychromorbidus* (Inglis *et. al.,* 2000), all isolated from the Antarctic environment. The bacterial family 8 xylanase from *Pseudoalteromonas haloplanktis* TAH3a (pXyl) and the family 10 xylanase from *Cryptococcus adeliae* (X_B) produced minimal xylanase.

The common features of the psychrophilic xylanase studied are a low temperature optimum, high catalytic activities at low temperatures and poor stability (Collins *et. al.,* 2002, Feller and Gerday, 2003). The comparative studies of pXyl and X_B with mesophilic xylanase showed that these enzymes have a higher catalytic activity at low and moderate temperatures. At 5 °C activity of pXyl is 60 % of the maximum while xylanase A and B from *Euphasia superba* display 30 % and 40 % of their maximum activity respectively. In comparison, a mesophilic xylanase showed less than 5 % of its maximum activity at this temperature (Turkiewiz *et. al.,* 2000). Psychrophilic xylanase have poor thermal stability indicated by short half-lives and low denaturation temperatures with low chemical stability, demonstrated by short half-lives of guanidine hydrochloride inactivation and unfolding, they display high catalytic activity at low temperatures (Collins *et. al.,* 2003). Fluorescence monitoring of acrylamide quenching indicated that it has an increased flexibility compared to a thermophilic homologous enzyme (CelA) from *C. thermocellum* (Collins *et. al.,* 2003).

1.5.2. Thermophiles

Xylanase that is thermostable are receiving considerable attention because of their application in biobleaching of pulp in the paper industry, wherein the enzymatic removal of xylan from lignin-carbohydrate complexes facilitates the leaching of lignin from the fiber cell wall, obviating the need for chlorine for pulp bleaching in the brightening process. They also have applications in the pretreatment of animal feed to improve its digestibility.

Majority of xylan degrading enzymes from thermophilic fungi are endoxylanase. *Malbranchea pulchella* var. *sulfurea* produced an extra-cellular xylosidase (Matsuo and Yasui, 1985), but in *H. grisea* var. *thermoidea* (Monti *et. al.,* 1991, Alimeida *et. al.,* 1995) and *Talaromyces emersonii* (Tuohy *et. al.,* 1993) the xylosidase was periplasmic. A number of thermophilic (optimal growth at 50 –80 °C) and hyperthermophilic (optimal growth at >80 °C) xylanase producing microorganisms have been isolated from a variety of sources (Collins *et. al.,* 2005, Maheshwari *et. al.,* 2000, Andrade *et. al.,* 1999). Majority of these xylanase belong to families 10 and 11, particularly thermophilic xylanase.

Family 10 xylanase have been isolated from various thermophilic and hyperthermophilic organisms, including *Thermotoga* sp. (Bhat and Bhat 1998) *Caldicellulosiruptor* sp. (Lamed *et. al.,* 1983), *Rhodothermus marinus* (Dahlberg and Kristijansson 1993), *B. stearothermophilus* (Li *et. al.,* 1997), *Thermoascus aurantiacus* and *C. thermocellum* (Chaudhuri *et. al.,* 1999). Family 10 xylanase, Xyn A, from *Thermotoga* sp. strain FjSS3-B.1 is the most thermostable xylanase reported with an apparent optimum temperature for activity of 105°C and a half-life of 90 minutes at 95°C (Simpson *et. al.,* 1991).

Very few reports are there related to family 11 thermophilic xylanase: *Thermomyces lanuginosus* (Singh *et. al.,* 2003), *Paecilomyces varioti* (Kumar *et. al.,* 2000), *Caldicellulosiruptor* sp Rt 69 B.1. (Morris *et. al.,* 1999), *Dictyoglomus thermophilum* (McCarthy *et. al.,* 2000), *Chaetomium thermophilum* (Hakulinen *et. al.,* 2003), *Nonomuraea flexuosa* (Hakulinen *et. al.,* 2003) and *Bacillus* strain D3 (Connerton *et. al.,* 1999). Of these *Nonomuraea flexuosa* and *Dictyoglomus thermophilum* are among

the most stable, with apparent temperature optima of 80 and 85 °C respectively. Xylanase producing hyper- thermophilic archaea have also been recently reported: *Thermococcus zilligii* (Uhl and Daniel, 1999), *Sulfolobus solfataricus* (Cannio *et. al.,* 2004), *Pyrodictium abyssi* (Andrade *et. al.,* 1999) and a number of *Thermofilum* strains (Bragger *et. al.,* 1989).

A number of comprehensive structural studies of thermal adaptation for family 10 and 11 xylanase have allowed identification of specific adaptation strategies for each family. It is evident that the structural differences between the families are the basis for this difference in adaptation strategies; family 10 enzymes have high α helix content (~ 40 %) while family 11 enzymes have a high β sheet content (greater than 50 %) (Hakulinen *et. al.,* 2003).

The reports on comparative studies of the thermophilic xylanase from *Thermoascus aurantiacus* and *C. thermocellum* with mesophilic family 10 xylanase indicated that the thermostability in this family is the outcome of improved hydrophobic packing, a favorable interaction of charged side chains with the helix dipoles as well as increased proline content in the N-termini of helices (Lo-Leggio *et. al.,* 1999). Recent comparative structural analysis of 5 thermophilic and 7 mesophilic family 11 enzymes suggested that a general thermostabilising adaptation in this family is a higher threonine to serine ratio (threonine has a high β forming propensity), an increased number of residues in the β strands and frequently an additional β strand B1 at the N-terminus (Hakulinen *et. al.,* 2003).

1.5.3. Acidophiles

Only a few reports are available related to acidiphilic xylanase. Acidophiles grow optimally between pH 1 to 5, and the most important of these are the family 10 and 11 members from *T. reesei* (Torronen and Rouveinen, 1995), *A. niger* (Krengel and Dijkstra, 1996), *A. kawachii* (Fushinobu *et. al.,* 1998), *Cryptococcus* sp. S-2 (Iefuji *et. al.,* 1996) and *Penicillium* sp.40 (Kimura *et. al.,* 2000). The latter three of these are among the most acidophilic xylanase studied with a pH optimum of 2 and stability over a broad range of pH.

1.5.4. Alkaliphiles

Ever since the demonstration that alkali-treated wood pulp could be biologically bleached by xylanase instead of the usual environmentally damaging chemical process involving chlorine, the search for thermostable alkaline xylanase has been extensive. The first report of a xylanase produced by an alkaliphilic microorganism was in 1973 from *Bacillus* sp. C-59-2 (Horikoshi and Atsukawa, 1973) and since then a number of xylanase has been isolated from various acidophilic and alkaliphilic microorganisms. Xylanase producing alkaliphilic microorganisms, which typically grow optimally at pH values above 9, have been isolated from extreme environments and also from sources like kraft pulp, pulp and paper industry wastes (Ratanakhanokchai *et. al.,* 1999, Yang, 1995), decomposing organic matter, faeces, plant sources, soils (Horikoshi, 1996, Christakopoulos, 1996, Kimura *et. al.,* 2000, Durate *et. al.,* 2000) and even from neutral environments where they are found coexisting with neutrophilic microorganisms (Horikoshi, 1999). These include family 10 and 11 xylanase from a number of *Bacillus* sp. (Nakamura *et. al.,* 1993, Khasin *et. al.,* 1993, Sabini *et. al.,* 2001), *Trichoderma* sp. (Torronen *et. al.,* 1994, Torronen and Rouveinen, 1995), *Aspergillus* sp. (Fushinobu *et. al.,* 1998, Krengel and Dijkstra, 1996), *Penicillium* sp. (Kimura *et. al.,* 2000), *Acidobacterium* sp. (Inagaki *et. al.,* 1998) and *Cryptococcus* sp. (Iefuji *et. al.,* 1996).

In addition, family 8 xylanase have been isolated from alkalophilic *B. halodurans* C-125 (Takami *et. al.,* 2000, Takami and Horikoshi, 2000) and *B. halodurans* MIR32 (Coutinho and Henrissat, 1999), however analysis of the amino-acid sequences indicates that these may be intracellular enzymes and thus may not be adapted to the environment of their hosts.

Dey *et. al.,* (1992) isolated an alkaliphilic thermophilic *Bacillus* strain (NCIM 59) that produced two types of cellulase-free xylanase at pH 10 and 50 °C. Khasin *et. al.,* (1993) reported that alkaliphilic *B. stearothermophilus* T- 6 produced an extracellular xylanase that optimally bleached pulp at pH - 9 and 65 °C. Nakamura *et. al.,* (1991, 1993, and 1994) also reported that an alkaliphilic *Bacillus* strain, 41M-1, isolated from soil, produced multiple xylanase extracellularly. One of the enzymes, xylanase J, was most active at pH 9.0. The optimum temperature for the activity at pH 9.0 was

around 50 °C. An alkaliphilic and thermophilic xylanase producing strain of *Bacillus* TAR-1, was isolated from soil, which was most active over a pH range of 5.0 to 9.5 at 50 °C. Optimum temperatures of the crude xylanase preparation were 75 °C at pH - 7.0 and 70 °C at pH - 9.0. These xylanase did not act on cellulose, indicating a possible application of the enzyme in biological debleaching processes (Nakamura *et. al.,* 1994). Blanco *et. al.,* (1995) reported that an enzyme from *Bacillus* sp. strain BP-23 facilitated the chemical bleaching of pulp, generating savings of 38 % in terms of chlorine dioxide consumption.

Many of the alkaliphilic microorganisms studied have been found to produce xylanase with pH optima in the near neutral region but with relatively high activities being retained in alkaline conditions. One of the most alkaliphilic xylanase reported is Xyl B from *Bacillus* sp. AR- 009, which has a pH optimum of 9 - 10 (Gessesse, 1998). Other highly alkaliphilic xylanase include xylanase J from *Bacillus* sp. strain 41M-1 (Nakamura *et. al.,* 1993) and a xylanase from *Bacillus pumilus* 13a (Duarte *et. al.,* 2000), both of which have a pH optimum of 9.

The pH activity profiles of enzymes are highly dependent on the pKa of the catalytic residues which are themselves dependent on the local environment and hence on the nature of the amino acids in the vicinity of the catalytic residues. A recent study of a family 11 xylanase showed that, in general, residues that contribute positive charges and hydrogen bonds serve to lower the pKa values with shorter bonds having a more pronounced effect. The chemical nature of the donor is also important, with COOH being more effective than OH and $CONH_2$ (Joshi *et. al.,* 2001). In contrast to this, neighboring carboxyl groups can either lower or raise the pKa values of the catalytic glutamic acids depending upon the electrostatic linkage of the residues involved in the interaction (Fushinobu *et. al.,* 1998). In fact it has been noted that family 11 acidophilic xylanase have aspartic acid residue hydrogen bonded to the general acid/base catalyst which is replaced by an asparagine in the xylanase active under more alkaline conditions (Fushinobu *et. al.,* 1998, Sapag *et. al.,* 2002, Joshi *et. al.,* 2000). This residue influences the pH dependence of activity, and mutation of this aspartic acid to its amide derivative in *A. kawachii* acidophilic xylanase resulted in an upward shift of the pH optimum from pH 2 to 5.

The tertiary structure analysis of this enzyme of the family 11 xylanase from *T. reesei* (Torronen and Rouveinen, 1995) and *A. niger* (Krengel and Dijkstra, 1996) indicated that adaptation to low pH is brought about by an increase in negative charge and a substitution and reorientation of residues, in particular aromatic residues, in the active sites. In contrast, a random mutagenesis study of a *Neocallimastix patriciarum* xylanase indicated that an increased negative charge and increased hydrophobicity increased the pH optimum of this enzyme (Chen *et. al.,* 2001).

Stability at the extremes of pH appears to be characterized by a spatially biased distribution of charged residues. The acidophilic and acid stable xylanase from *A.kawachii* is characterized by a concentration of acidic residues on its surface (Fushinobu *et. al.,* 1998), which is believed to reduce electrostatic repulsion of the positively charged residues at low pHs. In contrast, enzymes stable in alkaline conditions are typically characterized by a decreased number of acidic residues and an increased number of arginines. Furthermore, a recent comparative structural study of family 11 enzymes suggests a correlation between pH activity/stability and the number of salt bridges, with acidophilic xylanase having much less of these interactions than their alkaliphilic homologs (Hakulinen *et. al.,* 2003). Indeed it was even suggested that adaptation to high pH might occur *via* a similar mechanism to adaptation to high temperatures (Hakulinen *et. al.,* 2003).

1.6.0. CLASSIFICATION OF XYLANASE

Hydrolysis of β-glycosidase is carried out by acid catalytic reactions common to all glycanases. Xylanase is highly modular in structure like many cellulolytic and hemicellulolytic enzymes. Many microorganisms have multiple loci encoding overlapping xylanolytic functions, with single or number of different domains, classified as catalytic and non-catalytic domains.

Earlier microbial xylanase (E.C. 3.2.1.8) were classified into two based on the structural and physiochemical properties such as molecular mass, isoelectric point, hydrophobic cluster analysis and amino acid sequence homologies, rather than catalytic properties (Wong *et. al.,* 1998). Classifications of glycoside hydrolases, families (GH 10 - Family F and GH 11- Family G) that differ both in structure and in

catalytic properties have been distinguished (Henrissat 1991; Henrissat and Bairoch, 1993). Later, a more complete classification system was introduced (Henrissat and Coutinho, 2001) which allowed the classification of not only xylanase, but glycosidases in general (E.C 3.2.1.x), and which has now become the standard means for the classification of these enzymes. This system is based on primary structure comparisons of the catalytic domains only and groups of enzymes in families of related sequences (Henrissat and Coutinho, 2001).

Glycoside Hydrolase family	Members with a demonstrated activity on xylan	Fold	Clan	Catalytic mechanism	General acid/ base residue	Nucleophile or general base
5	8	$(\beta/\alpha)_8$	GH- A	Retaining	Glutamate	Glutamate
7	1	β– jelly roll	GH-B	Retaining	Glutamate	Glutamate
8	4	$(\alpha/\alpha)_6$	GH-M	Inverting	Glutamate	Aspartate
10	127	$(\beta/\alpha)_8$	GH-A	Retaining	Glutamate	Glutamate
11	173	β– jelly roll	GH-C	Retaining	Glutamate	Glutamate
43	1	5-blade β propeller	GH-F	Inverting	Glutamate	Aspartate

Table. 1. 3. Glycoside hydrolase families containing enzymes with a demonstrated activity on xylan. The fold, mechanism of action and catalytic residues characteristic to each family (Collins *et. al.*, 2005).

The initial classification grouped cellulases and xylanase into 6 families (A-F) (Henrissat *et. al.*, 1989), which was up dated to 77 families (Henrissat and Coutinho, 2001) and which continues to grow as new glycosidase sequences are identified. The existences of 96 glycoside hydrolase families have been confirmed and they have also been identified (Coutinho and Henrissat, 1999), with approximately one-third of these families being polyspecific, i.e., contain enzymes with diverse substrate specificities. As the structure and molecular mechanism of an enzyme are related to its primary structure, this classification system reflects both structural and mechanistic features.

Enzymes within a particular family have a similar three-dimensional structure and similar molecular mechanism and it has also been suggested that they may have a similar specificity of action on small, soluble, synthetic substrates (Henrissat *et. al.*, 1989, Claeyssens and Henrissat, 1992).

Divergent evolution has resulted in some of the families having related three-dimensional structures and thus the grouping of families into higher hierarchical levels, known as clans, have been introduced (Bourne and Henrissat, 2001). Presently, 14 different clans have been proposed (GH-A to GH-N), with most clans encompassing two to three families, apart from clan GH-A that currently consists of 17 families. Within this classification system, xylanase are normally reported as being confined to families 10 (formerly F) and 11 (formerly G) (Jeffries, 1996, Sunna and Antranikian, 1997, Berquist *et. al.*, 2001, Subramaniyan and Prema, 2002, Singh *et. al.*, 2003).

A closer look at the available literature, however, shows that only those sequences classified in families 5, 7, 8, 10, 11, 43 with truly distinct catalytic domains with a demonstrated endo-1, 4-β xylanase activity (Table. 1.3.) and bifunctional 16, 52 and 62 families containing two catalytic domains; a family 10 or 11 xylanase domain as well as a second glycosidase domain. For eg. *Ruminococcus avefaciens* enzyme contains an amino-terminal family 11 xylanase and a carboxy-terminal family 16 lichenase and is thus classified in both families 11 and 16 (Flint *et. al.*, 1993). In addition, those enzymes classified in family 26 appear not to be endo-1, 4 - β xylanase, but endo - 1, 3 - β xylanase. Thus, the current view that enzymes with xylanase activity are exclusively restricted to families 10 and 11 is not entirely correct and should be expanded to include families 5, 7, 8 and 43. The major families, which are extensively studied, are family 10 and 11.

1.6.1. Family 10

Family 10 consists of endo-1, 4-β xylanase (E.C. 3.2.1.8), endo-1, 3-β xylanase (E.C. 3.2.1.32) and cellobiohydrolases (E.C. 3.2.1.91) (Coutinho and Henrissat, 1999). The major enzymes of this family are endo-1, 4-β xylanase, though, substrate specificity studies have revealed that these may not be entirely specific for xylan and may also be

active on low molecular mass cellulose substrates (Biely, 2003, Gilkes *et. al.,* 1991) in particular on aryl-cellobiosides (van Tillbeurgh and Claeyssens, 1985, Biely *et. al.,* 1997) and certain cello-oligosaccharides (Claeyssens and Henrissat, 1992). The replacement of one or two xylose residues by glucose is normally tolerated by the xylanase of this family, generally resulting in a lower catalytic efficiency (Biely *et. al.,* 1997). In consonance with family 11 xylanase, but in contrast to the cold-adapted family 8 xylanase, members of this family are also capable of hydrolyzing aryl β glycosides of xylobiose and xylotriose at the aglyconic bond (Biely *et. al.,* 1993, Biely *et. al.,* 1997). These enzymes are highly active on short xylo-oligosaccharides, thereby indicating small substrate binding sites (Biely *et. al.,* 1997).

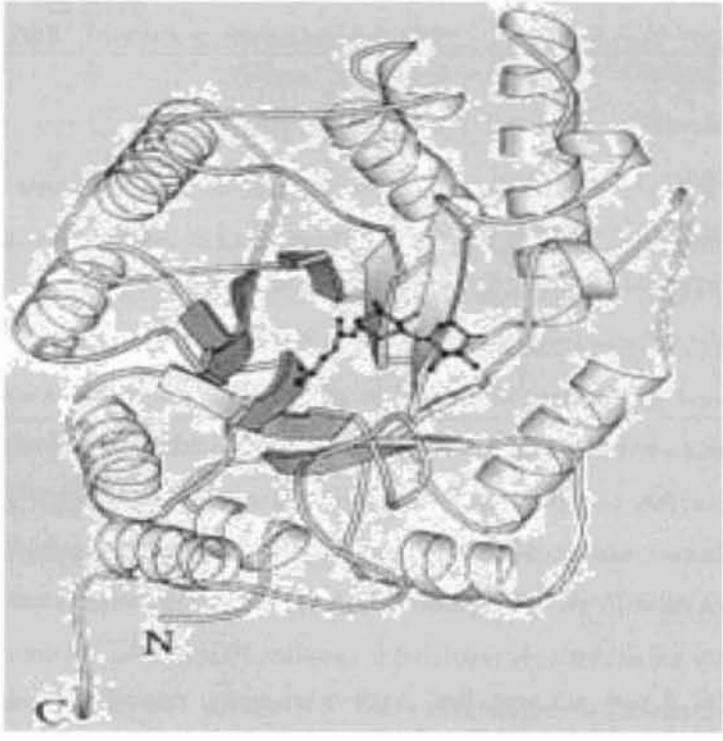


Fig. 1. 5. The three-dimensional structure of the Family 10 xylanase from *Streptomyces lividans.* The xylobioside moiety and the nucleophile Glu-236 are shown by a ball and stick representation. (Ducros *et. al.,* 2000).

The crystal structure analyses, kinetic analyses of activity on xylooligosaccharides of various sizes and end product analyses have indicated that family 10 xylanase typically have four to five substrate-binding sites as well as endo acting in nature (Biely *et. al.,* 1997). Hydrolysis studies shown that most family 10 xylanase can attack the xylosidic linkage on the non-reducing end of a substituted residue or 1, 3 - β bond, but can only cleave at the third xylosidic linkage after a substituted residue and the second after a 1, 3 - β bond (Biely *et. al.,* 1997). This indicates that the subsites on the non-reducing side (i.e., subsites $^{-}1$, $^{-}2$) are more specific than those on the reducing side (subsite $^{+}1$) of the cleavage site. The family 10 members all possess a catalytic domain, which exhibits (β/α) 8 fold architecture and displays high molecular mass of approximately 40 kDa (> 30 kDa) and low pI (Teplitsky *et. al.,* 2000, Fujimoto *et. al.,* 2000, Mechaly *et. al.,* 2000, Canals *et. al.,* 2003, Pell *et. al.,* 2004a, 2004b) as well as more complexity and more diversity (hydrolyse cellulose and xylan) (Ducros *et. al.,* 2000).

The structure (Fig. 1. 5) has been likened to a "salad bowl" with one face of the molecule having a large radius (~45 Å) due to an elaborate loop architecture, while the opposite face, which consists of simple α/β turns, has a radius of approximately 30 Å. Family 10 and 5 are quite closely related and share a common fold, they have the same type of catalytic mechanism and share several common residues and both are members of clan GH- A. (Ryttersgaard *et. al.,* 2002, Larson *et. al.,* 2003). The families 10 xylanase are more closely related with high percentage of spatially equivalent and identical residues as well as much smaller rms deviations between equivalent residues (0.95 ±0.11 Å) in its members (Larson *et. al.,* 2003).

Large amount of data reveals that family 10 enzymes display certain enzymological characteristics, which could theoretically make them better candidates for enzymatic upgrading of lignocellulosic biomass. Family 10 endoxylanase show better capability of cleaving glycosidic linkages in the xylan main chain closer to the substituents, such as methyl-glucuronic acid, acetic acid and α-L-arabinofuranoside (Biely *et. al.,* 1997). These xylanase are more permissive in terms of substrate specificity, and tolerate arabinose-decorated xylose residues in either the (3), (2), or ($^{+}1$) subsites. Fujimoto *et. al.,* (2000) suggested that some family 10 members can accommodate arabinose-

decorated xylose residues in the ($^{+}$2) subsite. These xylanase are unaffected by the presence of TAXI-like proteinaceous inhibitors (Goesaert *et. al.,* 2004), which occur in cereals. They are preferentially active against soluble substrates and can readily hydrolyse small xylooligosaccharides such as xylotriose and exhibit greater catalytic versatility.

1.6.2. Family 11

The nonspecific nature and consisting exclusively of xylanase is the oddness of family 11. These xylanase are "true xylanase" as they are exclusively active on D-xylose containing substrates. Family 11 have lower catalytic flexibility than family 10 xylanase and end products of their reaction where hydrolysed by family 10 enzymes. They can hydrolyze aryl β- glycosides of xylobiose and xylotriose at the aglyconic bond, and are inactive on aryl cellobiosides, substituents or β -1, 3 linkages and represent a more serious hindrance to their activity, resulting in the production of larger products than family 10 xylanase (Biely *et. al.,* 1997).

Xylanase from family 11 do not tolerate the presence of arabinose decorations on either the O-2 / O-3 positions of the xylose residues present in the ($^{-}$1) and ($^{+}$1) subsites or on the O-2 / O-3 of the xylose residue present in the ($^{+}$2) subsite. Hydrolysis studies of family 11 xylanase indicate that aldopentauronic acid, with an unsubstituted xylose residue at the non-reducing end (Biely *et. al.,* 1997, Christakopoulos *et. al.,* 2003), and an isomeric xylotetraose, with the 1, 3 - β bond at the non-reducing end (Ntarima, 2000), are the smallest acidic and mixed linkage fragments liberated from heteroxylans and rhodymenan, respectively. There is a suggestion that this isomeric xylotetraose may contain a 1, 4 - β bond at the non-reducing end, with the 1, 3 - β linkage occurring subsequent to this bond (Biely *et. al.,* 1997). The disturbance of xylan chain by replacing β- 1, 4 - linkages with β- 1, 3 - linkages, such as in rhodymenan, represents a more serious steric barrier for endoxylanase of family 11 than for family 10 (Biely *et. al.,* 1997). In agreement with these considerations, endoxylanase of family 10 liberate from 4-O-methyl-D-glucuronoxylan, rhodymenan (Ntarima, 2000) and with some exceptions also from

acetylxylan and arabinoxylan, smaller products than those formed with endoxylanase of family 11 (Biely *et. al.*, 1997, Christakopoulos *et. al.*, 2003).

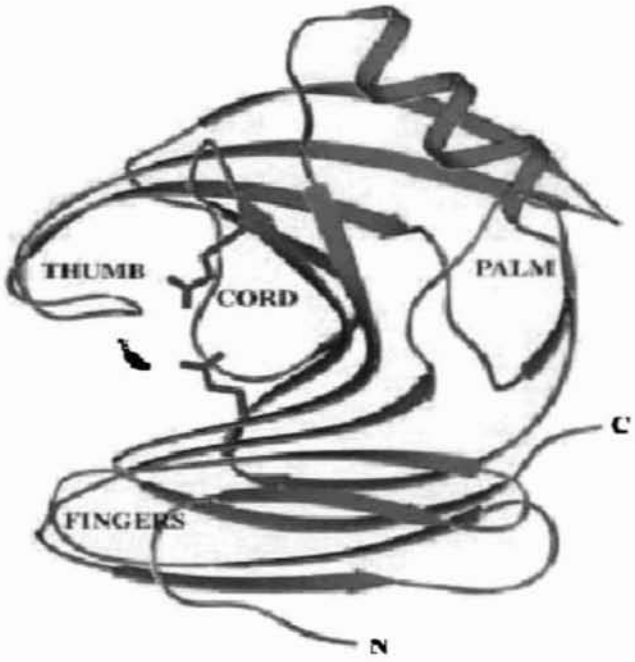


Fig. 1. 6. The protein fold of the Family 11 xylanase from *Chaetomium thermophilum*: shape of right hand- 2 β-sheets and α- helix forms fingers and a plam, a long loop between the B7 and B8 strand forms a thumb, and a loop between the B6 and B9 strands forms a cord (Haukulinen *et. al.*, 2003).

Family 11 are most active against insoluble polymeric xylans and are affected by the presence of TAXI - like proteinaceous inhibitors, which occur in cereals (Sapag *et. al.*, 2002). In common with the family 8 cold-adapted xylanase, these xylanase are most active on long chain xylo-oligosaccharides and have a larger substrate binding clefts, e.g., family 11 xylanase from *Schizophyllum commune* and *A. niger* have at least seven subsites (Bray and Clarke 1992), while, as has already been stated, family 10 enzymes are reported to have four to five subsites (Derewenda *et. al.*, 1994, Biely *et. al.*, 1997). Major differences between family 10 and 11 xylanase include their stereochemistry of protonation and the effect of ω- epoxyalkyl glycosides of xylose

and xylooligo-saccharides on their activity; family 10 xylanase are *anti* -protonators and are unaffected by the ω -epoxyalkyl glycosides while family 11 xylanase are *syn* -protonators and are inactivated by the ω- epoxyalkyl glycosides (Heightmal and Vasella, 1999, Ntarima *et. al.,* 2000, Biely, 2003).

Family 11 enzymes are generally characterized by high pI, low mol. wt. (~ 20 kDa), endo acting with double displacement catalytic mechanism, two glutamates acting as the catalytic residues and a β jelly roll fold structure (McCarthy *et. al.,* 2000, Wouters *et. al.,* 2001, Sabini *et. al.,* 2001, Oakley *et. al.,* 2003, Hakulinen *et. al.,* 2003) and are more consistent in structure and more specific for xylan (Warren, 1996). Family 11 xylanase are most frequently chosen for industrial processes.

The structure (Fig. 1. 6.) consists principally of β pleated sheets formed into a two-layered trough that surrounds the catalytic site. Two (Torronen and Rouvinen, 1997), or perhaps three (Harris *et. al.,* 1997), β sheets are present and the hydrophobic faces of these are packed against each other to form the hydrophobic core of the protein.Only one helix is present and this is typically packed against the hydrophobic face of the second β sheet. This type of structure has also been described for family 12 endoglucanases and thus both families have been grouped into the same clan, GH-C. The crystal structures of different family 11 xylanase have been resolved, including the structures of *T. reesei* (xyl I, II) and *T. lanuginosus* xylanase (Hakulinen *et. al.,* 2003).

The protein structure is composed of two- β -sheets and a single α -helix forming a right hand-like structure (Fig. 1.6). Based on the structural information, a large number of protein engineering studies have been performed with family 11 xylanase utilizing site directed mutagenesis, and also random mutagenesis techniques (Turunen *et. al.,* 2004). Recently, a method was developed to experimentally determine whether an endoxylanase belongs to family 10 or 11 (Ntarima *et. al.,* 2000). This method is based on the irreversible inhibition of family 11 endoxylanase by epoxyl glycosides of D-xylose and xylooligosaccharides, whereas family 10 endoxylanase are unaffected (Ntarima *et. al.,* 2000).

1.6.3. Family 5

Family -5 is the largest glycoside family with 7 amino acid residues, including the nucleophile and the general acid/base residue, which is strictly preserved in all members. This group formally known as family – A, consists of 467 sequences with varying activities including: endoglycosylceramidase (E.C. 3.2.1.123), cellulase (E.C. 3.2.1.4), licheninase (E.C. 3.2.1.73), β - mannosidase (E.C. 3.2.1.25), glucan 1, 3-β glucosidase (E.C. 3.2.1.58), glucan endo-1, 6 - β glucosidase (E.C. 3.2.1.75), mannan endo - 1, 4 - β mannosidase (E.C. 3.2.1.58), cellulose 1, 4 - β cellobiosidase (E.C. 3.2.1.91), endo - 1, 6 -β galactanase (E.C. 3.2.1.-), 1, 3 -β mannanase (E.C. 3.2.1.-) and endo - 1, 4 - β xylanase (E.C. 3.2.1.8) (Coutinho and Henrissat, 1999). The enzymes from *Prevotella ruminicola* 23 (Whitehead, 1993), *Clostridium cellulovorans* (Foong *et. al.,* 1991) and *F. succinogenes* S85 (Cho *et. al.,* 2000) have activity on both CMC ase and xylan, while those from *Trichoderma reesei* (*Hypocrea jecorina*) (Salohemio *et. al.,* 2003, Tenkanen *et. al.,* 2003), *Erwinia chrysanthemi* D1 (Larson *et. al.,* 2003, Hurlbert and Preston, 2001) and *E. chrysanthemi* SR120A (Braun and Rodrigues, 1993) are specific for xylan.

The structural analysis of the family 5 xylanase, XynA from *E. chrysanthemi* showed that, the catalytic domain displayed a common (β / α) 8-fold barrel (Larson *et. al.,* 2003), while the β barrels aligned well with those of another family 5 enzyme, the α helices and loops were altered, showing differences in the positioning, orientation and length, and has shown structural alignment to family 10 enzymes (Larson *et. al.,* 2003). The family 5 enzymes exhibit CMC ase (carboxymethyl cellulase) and xylanase activities, and *P. ruminicola* 23 xylanase was found to have highest activity on xylan, with 18% CMC ase activity (Whitehead, 1993). Eng B from *C.cellulovorans* was most active on lichenan, with 15 % activity on CMC ase and 14 % on xylan and no activity with other substrates (Foong *et. al.,* 1991). The endoglucanase isolated from *Fibrobacter succinogenes* S85 have the highest activity on CMC ase, as well as on oat spelt xylan (71 % as compared to CMC), p - nitrophenyl cellobioside (5.3 %), cellobiose (3.5 %), p -nitrophenyl lactoside (2.27 %) and p -nitrophenyl glucoside (0.27 %) (Cho *et. al.,* 2000).

The structure of Xyn A from *E. chrysanthemi* has been determined (1.42 Å resolution) (Larson *et. al.,* 2003) and recently the crystallization and preliminary X-ray analysis (at 2.2 Å resolution) of the *T.reesei* XYN IV was also reported (Parkkinen, 2004). Xyn A is composed of two domains, the larger domain contains the catalytic site and displays a (β/ α) 8 barrel fold while the small domain probably functions as a xylan binding domain and has a β - barrel fold. The two domains are connected by two linker peptides as well as 11 hydrogen bonds and hydrophobic interactions (Larson *et. al.,* 2003). The β barrel of the catalytic domain of Xyn A is elliptical in shape and the active site is formed by an acidic cleft situated on the carboxy-terminal side of the β strands near the larger face of the molecule. This 8-fold α/β barrel structure is indeed the most frequently encountered fold (Coutinho and Henrissat, 1999). This type of fold was originally described for triose-phosphate isomerase (TIM barrel) and a common characteristic of these structures is the position of the glutamate residues implicated in the catalysis on the C-terminal of β sheets 4 and 7, and these were originally termed the 4/7 super family, now known as clan GH-A.

1.6.4. Family 8

Family 8 (formerly family D) is mainly composed of cellulases (E.C. 3.2.1.4), but also contains chitosanases (E.C. 3.2.1.132), lichenases (E.C. 3.2.1.73) and endo1, 4-β xylanase (E.C. 3.2.1.8) (Coutinho and Henrissat, 1999). Three of the xylanase have been isolated from *Bacillus* sp. while the fourth one is cold-adapted enzyme isolated from the Antarctic bacterium *Pseudoalte romonas haloplanktis* TAH3a. Xylanase Y from the alkaliphile *Bacillus halodurans* C-125 which was identified as part of the sequencing program of the whole genome of this organism (Takami *et. al.,* 2000a Takami *et. al.,* 2000b) and the sequence of the xylanase from *B. halodurans* MIR32 is identical to this enzyme (Coutinho and Henrissat, 1999).

Initial classification as xylanase was probably based on their weak isology to the cold-adapted xylanase and xylanase Y from *Bacillus* sp. KK, which is active at pH 6.5 on birchwood than oat spelt xylan, and inactive with cellulose, CMC ase, starch, lichenan and chitosan. The cold-adapted xylanase was found to hydrolyze xylan to principally xylotriose and xylotetraose and was most active on long chain xylo-oligsaccharides.

Family 8 xylanase have a large substrate binding cleft containing at least six xylose binding residues, with the catalytic site in the middle, like family 11 xylanase (Collins *et. al.,* 2002). This enzyme was found to catalyze hydrolysis with inversion of the anomeric configuration, and was found to be inactive on aryl-β glycosides of xylose, xylobiose and xylotriose, which differ them from family 10 and 11 xylanase.

Cold-adapted xylanase folds into a distorted (α/α) 6 fold barrel formed by six inner and six outer α helices (Van Petegem *et. al.,* 2002, Van Petegem *et. al.,* 2003) and can be classified with family 48 in clan GH-M (Coutinho and Henrissat, 1999). This topology has also been observed for family 9 endoglucanases, family 15 glucoamylases, family 48 cellobiohydrolases and a family 65 - maltose phosphorylase (Egloff *et. al.,* 2001). Contrary to the common (α / α) 6 fold barrel proteins; the cold-adapted enzyme has an extra α helix near the amino terminus. The globular core has an overall distorted spherical shape with a long acidic cleft running across the molecular surface at the N-terminal end of the inner helices while the proposed catalytic residues (glutamate and aspartate) are located close to each other near the middle of the cleft. These enzymes will operate with inversion of the anomeric configuration, and with an aspartic and glutamic acid residue as proton acceptor and proton donor, respectively (Collins *et. al.,* 2002, Guerin *et. al.,* 2002, Van Petegem *et. al.,* 2003).

1.6.5. Family 7 and 43

Only one enzyme exhibiting xylanase activity has been identified and studied in family 7 and 43. The family 7 enzyme, EGI (Cel7B) from *T. reesei*, is a non-specific endo-β 1, 4-glucanase (E.C. 3.2.1.4) (Kleywegt *et. al.,* 1997, Biely *et. al.,* 1993) but is not produced during growth on xylan (Biely *et. al.,* 1993). Family 7 activity on cellulose (hydroxyethylcellulose) is only slightly higher than that on xylan (beech wood and grass), its activity on cellooligosaccharides (G 3, G 5) is 10 - fold higher than that on xylooligosaccharides (X3, X5) (Biely *et. al.,* 1991). Hydrolyses of both these substrates takes place in the same active site but a shift in the bond cleavage frequency appears to occur towards the non-reducing end linkages in xylo-oligosaccharides (Biely *et. al.,* 1993). Family 7 enzymes have characteristics in common with both family 10 and 11 xylanase. As for the former family, it has a high

mol. wt. and low pI as well as a small substrate-binding site, approximately four subsites, with the catalytic site in the middle.

Like family 11 xylanase, this enzyme displays a β jelly roll fold (Kleywegt *et. al.,* 1997) and produces aldopentauronic acid and isomeric xylotetraose as the shortest acidic and mixed linkage fragments from glucuronoxylan and rhodymenan, respectively (Biely *et. al.,* 1993). The core structures of family 7 and family 11 xylanase are similar, differences, such as: variations in the location, length and orientation of the structural elements outside of this core, the presence of four short helical segments as opposed to one in the family 11 xylanase and differences in the type and conformation of the amino acid residues lining the active site, results in family 7 enzymes being classified in clan GH-B with family 16 enzymes and not in clan GH-C with the family 11 enzymes.

Family 43 xylanase (XYND) was first identified only in *Paenibacillus polymyxa* and has both xylanase and α -L- arabinofuranosidase activities (Gosalbes *et. al.,* 1991). This enzyme (XYND) has a mol. wt. of 64 kDa and was found clustered with a lichenase gene, with only 155bp separating the two genes, in *Paenibacillus polymyxa.* Studies of the physicochemical or functional characteristics of this enzyme have not been carried out. Further, putative family 43 xylanase in *Caldicellulosiruptor* sp, *C. acetobutylicum*, *Bifidobacterium longum* (Collins *et. al.,* 2005, Gibbs *et. al.,* 2002) and *Bacillus* sp. (Kungst *et. al.,* 1997) have also been inferred on the basis of sequence homology to the above enzyme but xylanase activity has apparently not been confirmed by functional analysis.

Members of this family have not been as thoroughly studied and the structure of only one member has been determined, indicating that members of this family may display a five-blade β propeller fold. A glutamate and aspartate in the centre of a long V-shaped surface groove formed across the face of the propeller have been suggested as the catalytic residues (Nurizzo *et. al.,* 2002). The family is grouped with family 62 in clan GH-F (Coutinho and Henrissat, 1999, Nurizzo *et. al.,* 2002) and, also demonstrated for family 8 enzymes, its members are believed to catalyze hydrolysis *via* the single displacement mechanism.

1.7.0. CATALYTIC MECHANISMS OF GLYCOSIDASE FAMILY

Members of families 5, 7, 8, 10, 11 and 43 differ in their physicochemical properties, structure, mode of action and substrate specificities. Similarities exist between families; for example, families 5 and 10 are both classified in clan GH-A, thus indicating a similar three-dimensional fold. Moreover, families 5, 7, 10 and 11 contain enzymes, which catalyse hydrolysis with retention of anomeric configuration with two glutamate residues being implicated in the catalytic mechanism in all cases (Coutinho and Henrissat, 1999).

The maintenance of function may be reflected in the maintenance of sequences and therefore xylanase of individual families share common catalytic mechanism with other glycosidases. Xylanase like other cellulases and glycosidases, have been suggested to involve acid catalysis (Bray and Clarke, 1990, Chauvaux *et. al.,* 1992). It has been observed by chemical modification studies that carboxylate, cysteine, cystine, histidine, tryptophan and tyrosine residues are the most common and causing inactivation of xylanase (Kesker *et. al.,* 1992, Bray and Clarke, 1990). Thus, it is assumed that carboxylate residues are involved in catalysis, while histidine, tryptophan and tyrosine residues are involved in binding the substrate, and cystine is involved in maintenance of the confirmation of the active site (Coughlan, 1992).

Glu-93 and Glu-182 for *Bacillus pumilus* and Glu – 78 and Glu-172 for *B. subtilis* have been reported to be essential for the 20kDa enzyme (Couglan, 1992); where site directed mutagenesis research on *Bacilus circulans* has revealed the importance of Glu-78 for the catalysis of 22kDa enzyme activity (Fig.1. 7). Hydrophobic cluster analysis of enzyme from *T. saccharolyticum* has revealed the involvement of two Asp and one Glu in catalysis. Lee and co- workers have suggested the importance of three amino acids which form a catalytic triad that function in a general acid catalysis mechanism, by site directed mutagenesis by substation of Asp-537 and Asp- 602 and Glu-600 by Gln which has completely destroyed the endo-xylanase activity, (Lee *et. al.,* 1993b).

Generally, hydrolysis may result in the retention or inversion of the anomeric center of the reducing sugar monomer of the carbohydrate. This suggests the involvement of one or two chemical transition states (Sinnot, 1990). Glycosyl transfer usually results in nucleophilic substation at the saturated carbon of the anomeric center, which take place by either retention or inversion of the anomeric conjugation (Sinnot, 1990).

Most of the polysaccharide hydrolyzing enzymes such as cellulases and xylanase are known to hydrolyze their substrate with retention of the C1 anomeric conjugation. *Bacillus circulan* xylanase have been shown to catalyze like this (Gelber *et. al.*, 1992). *A. niger* xylanase proceeds with inversion of anomeric carbon (Federick *et. al.*, 1981). In all, glycanase and glycosidase inversion has been suggested to occur through single displacement reaction and its retention through double displacement reaction (Withers, 1995, Sinnott, 1990).

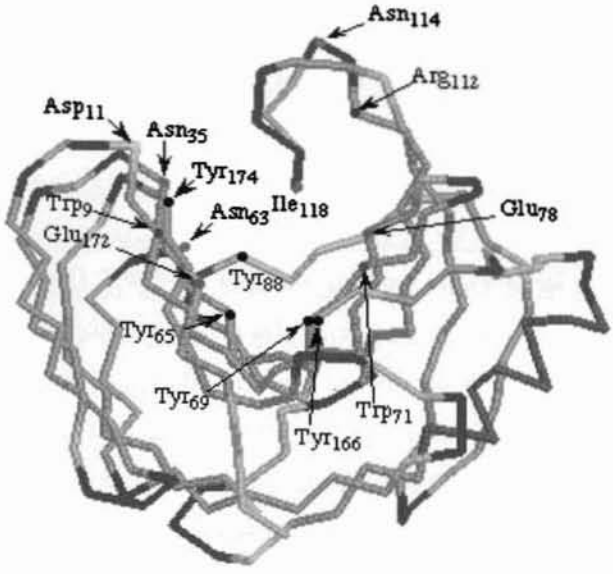


Fig. 1. 7. The three-dimensional structure of the Family 11 xylanase (1XNB) from *Bacillus circulans*. Yellow colour denotes β-pleated structure while blue and red corresponds to bends and α-helical structures. (Courtesy Jeffries, 1996).

Double displacement reaction involves nucleophilic attack by a unionized Glu or Asp residue in C1 of the incipient reducing sugar (Fig. 1. 8a), in which a covalent glycosyl-enzyme intermediate is formed and subsequently hydrolysed *via* oxocarbenium - ion like transition states (Rye and Withers, 2000, Zechel and Withers, 2000). Two carboxylic acid residues suitably located in the active site (approximately 5.5 Å apart) are involved in the formation of the intermediate; one acts as a general acid catalyst by protonating the substrate, while the second performs a nucleophilic attack which results in the departure of the leaving group and the formation of the a glycosyl enzyme intermediate (inversion β to α).

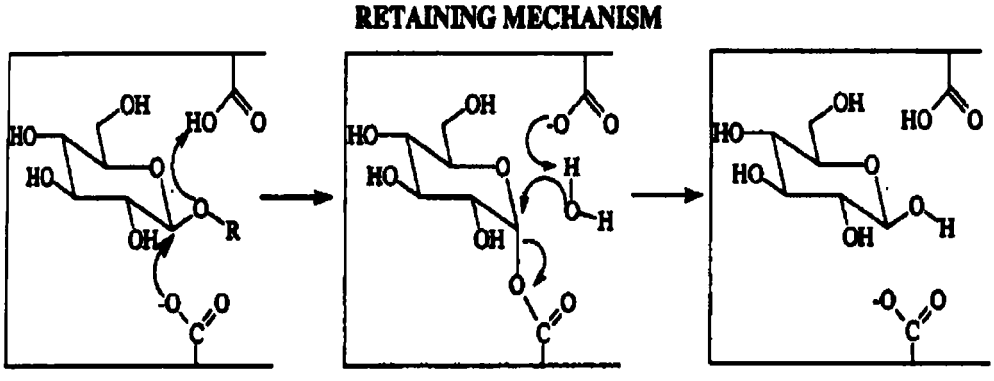


Fig. 1. 8a. **Double displacement mechanism in which a glycosyl enzyme intermediate is formed and hydrolyzed *via* oxocarbenium ion like transition states. The reaction is facilitated by acid/base catalysis, but in this case it is probable that the same group plays both roles.**

In the second step, the first carboxylate group now functions as a general base, abstracting a proton from a nucleophilic water molecule, which attacks the anomeric carbon. This leads to a second substitution in which the anomeric carbon again passes *via* an oxocarbenium-ion like transition state to give rise to a product with the β configuration (inversion α to β). Thus, the overall result is retention of the configuration at the anomeric center (Wither, 1995). In contrast, enzymes in families 8 and 43 typically operate with inversion of the anomeric centre and glutamate and

aspartate are believed to be the catalytic residues (Coutinho and Henrissat, 1999, Nurizzo *et. al.,* 2002). The resulting glycosyl fragment then diffuses away from the active center. The oxocarbonium ion intermediate is stabilized by electrostatic interaction with the ionized Glu or Asp residues. This hypothesis shows that stabilization occurs by covalent interaction with ionized Glu or Asp. Reaction is completed by the addition of hydroxyl groups to the carbonium ion and proton to the nucleophilic (Coughlan, 1992).

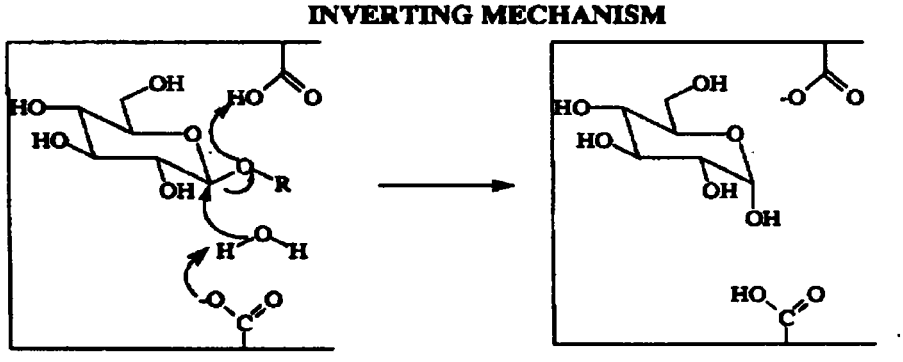


Fig. 1. 8b. Single displacement - water molecule effects a direct displacement at the anomeric centre, catalysed acid/base, with one active site amino acid acting as the general base, deprotonate the nucleophilic water molecule, the other amino acid acting as a general acid, protonating the departing oxygen atom in a concerted fashion as the bond cleaves.

Single displacement reaction in the hydrolysis of xylan involved the presences of general acid and a general base in catalysis, which attack by a nucleophilic molecule of water (Fig. 1. 8b) (Wither, 1995). Inverting enzymes function by a single displacement reaction in which one carboxylate provides for a general acid-catalyzed leaving group departure and the second functions as a general base, activating a nucleophilic water molecule to attack the anomeric carbon, thereby cleaving the glycosidic bond and leading to an inversion of the configuration at the anomeric carbon (Rye and Withers, 2000, Zechel and Withers, 2000). Typically the distance between the two residues is around 9.5 Å so as to allow for accommodation of the

water molecule between the anomeric carbon and the general base (Rye and Withers, 2000, Zechel and Withers, 2000). Alzari *et. al.*, (1996) and Guerin *et. al.*, (2002) have shown that this distance is 7.5 Å in the inverting endoglucanase CelA and have suggested that the distance between the two catalytic residues is less constrained in inverting than in retaining enzymes.

1.8.0.CHARACTERISTICS OF XYLANASE

1.8.1. Molecular weight

Xylanase from different microbial strains differ in their mol. wt. ranging from 5.5 to 350 kDa (Subramaniyan, 2000, Bastawde, 1992). The largest xylanase reported is from *Thermoanaerobacterium* sp. JW/SL- YS-485 with a mol. wt of 350 kDa. (Shao *et. al.*, 1995) and *Bacillus* CCMI 966 of 340 kDa, (Periera *et. al.*, 2000). Smallest one belongs to *Chainia* sp. with 5.5 to 6.0 kDa (Bastawde *et. al.*, 1991)

1.8.2. Amino acid sequencing

The smallest endoxylanase, 5500 to 6000 Da was isolated from *Chainia* sp. and it has 43 amino acid residues (Bastwde *et. al.*, 1991). *B. subtilis* endoxylanase is composed of 185 amino acid residues and *B. pumilus* contains 201 (Paice *et. al.*, 1986). Partial amino acid sequencing was reported for two endoxylanase from fungal sources. The first 72 amino acid residues from the N- terminal were sequenced for an endoxylanase of *Cryptococcus albidus* (Morosoli *et. al.*, 1986), while the first 27 from the N- terminal for *S. commune* endoxylanase were sequenced (Paice *et. al.*, 1978). The partial or complete amino acid analysis of xylanase from thermophiles and mesophilies of family 11 is given in Table. 1. 4. There is an increase in occurrence of Arg in thermophilic xylanase than mesophilic one (Vogt *et. al.*, 1997, Kumar *et. al.*, 2000), which was proved in *T. reesei* xylanase II (Turunen *et. al.*, 2002).

Thermophilic xylanses the frequency of Ser decreases and relatively the frequency of Thr increases (Table. 1. 4.). Ser → Thr mutation was one of the stabilizing mutations observed (Argos *et. al.*, 1979). Kumar *et. al.*, (2000) has observed decrease of Ser in thermophilic xylanase where as there is no increase in Thr. The presences of Arg and Tyr are more frequent in thermophilic protein where as Cys and Ser are less frequent

(Hakulinen *et. al.,* 2003). Increase in Thr: Ser ratio in the β- strand improves the β-formatting prospective, because half of the residues in family 11 xylanase are located in β- strands thus the thermal stability can be improved.

Aminoacids	α- Helices		β-Strands	
	Thermophiles	**Mesophilies**	**Thermophiles**	**Mesophilies**
Ala	2.0	2.0	5.4	5.3
Val	0.2	0.7	**9.8**	**14.2**
Leu	-	-	3.8	2.5
Ile	0.2	-	6.2	4.8
Pro	-	-	1.4	1.3
Met	0.2	-	0.8	1.3
Phe	1	1.3	4.4	5.0
Trp	1	1	6.4	5.0
Gly	0.6	-	11.0	9.8
Ser	0.2	0.5	12.0	13.0
Thr	0.8	0.5	**16.8**	**12.8**
Cys	0.4	-	1.0	0.3
Tyr	-	-	15.6	14.3
Asn	0.6	2.0	6.0	8.0
Gln	0.2	0.3	5.4	4.5
Asp	**0.8**	-	3.0	3.0
Glu	-	-	4.8	4.3
Lys	-	0.2	2.0	1.3
Arg	**0.8**	-	5.0	3.0
His	1	1	1.8	0.2
Total	10	9.5	122.6	114.2

Table. 1. 4. Amino acid composition in α helices and β- strands (Largest differences is given in bold) **(Hakulinen *et. al.,* 2003).**

Frequency of asparagines in thermophilic protein is low. It has low β- forming propensity, so avoided in the β- strand. Frequency of Gly is also low which improves the rigidity of loop regions. Val frequency is also low in β-strands even it has β-forming propensity, Pro do not have much influence. Crystal structure analyses revealed that thermophilic xylanase contain more amino acids then mesophilic xylanase. This high frequency of charged residues is involved in increasing the number of polar interactions (Hakulinen *et. al.,* 2003). Family 11 xylanase Aspartic acid is present at position 35 for those with "acidic" pH optima and Asparginine for those with "alkaline" pH optima (Fushinobu *et. al.,* 1998, Dijkstra, 1996, Torronen and Rouvienen, 1995, Joshi *et. al.,* 2000).

1.8.3. Crystal Structure

Inaoka and Soda first carried out studies related to crystalline structure of xylanase from *Bacillus* sp. (Inaoka and Soda, 1956). Hitherto, the crystal structures of family 11 (Mol. wt. 20 kDa) xylanase are available from several organisms: *B. pumilus.* (Ko *et. al.,* 1992, Kastusbe *et. al.,* 1990), *B. circulans* (Figure 1. 7.) (Coughlan, 1992, Wakarchuk *et. al.,* 1994, Sindhu *et. al.,* 1999), *B. stearothermophilus* T-6 (Anna *et. al.,* 1997), *B. agaradhaerens* (Sabini *et. al.,* 1999), *T. harzianum* (Campbell *et. al.,* 1993), *T. reesei* (Torronem *et. al.,* 1994, Torronem and Rouvienen, 1995), *A. niger* (Krengel and Dijkstra, 1996), *Thermomyces lanuginosus* (Gruber *et. al.,* 1998), *A. kawachii* (Fushinobu *et. al.,* 1998), *Paecilomyces varioti* (Kumar *et. al.,* 2000), *Dictyoglomu thermophilum* (McCarthy *et. al.,* 2000) and *Chaetomium thermophilum* and *Nonomuraea flexuosa* (Hakulinen *et. al.,* 2003). Three of these, *T. lanuginosus*, *P. varioti*, and *D. thermophilum* are from thermophilic organisms. A low- resolution structure has been reported for thermostable xylanase.

The crystals of *Bacillus pumilus* – IPO (Moriyama *et. al.,* 1987) were monoclinic, space group was P2, with a = 4.08 nm, b = 6.68 nm, c = 3.47 nm and β = 103.0° with mol. wt. of 22 kDa. The crystal structure of *T. harzanium* xylanase unit cell wase orthorhombic, space group P 2, 2 with a = 4.42 nm, b = 9.41 nm and c = 5.16 nm, with a mol. wt. of 20 kDa. Crystallization and X-ray analysis of xylanases from *Psedomonas fluorescens* subspecies *cellulose* expressed in *E.coli* was analyzed (Pickersgill *et. al.,* 1993), crystals are orthorhombic, space group $P2_1\ 2_12_1$ with a

=95.7 Å, b = 97.1 Å, and c = 149.8 Å (all +/- 0.2 Å). Anna and coworkers have crystallized xylanase from *Bacillus sterothermophilus* T-6 (Anna *et. al.,* 1997). These crystals belong to C- centered monoclinic crystal system, space group C2 with a = 170.6, b = 82.5, c = 80.0 Å, β = 91.43 with mol. wt. 38 kDa and pI 5.72. The needle-like crystals of TAXI -I in complex with *A. niger* xylanase belong to the trigonal space group P31 or P32, with unit-cell parameters a = b = 88.43, c = 128.99 Å, and diffract to 1.8 Å resolution. TAXI -I in complex with *B. subtilis* xylanase crystallizes in the monoclinic space group C2, with a = 107.89, b = 95.33, c = 66.31 Å, = 122.24°. Complete data sets were collected for both crystal types using synchrotron radiation (Sansen *et. al.,* 2004).

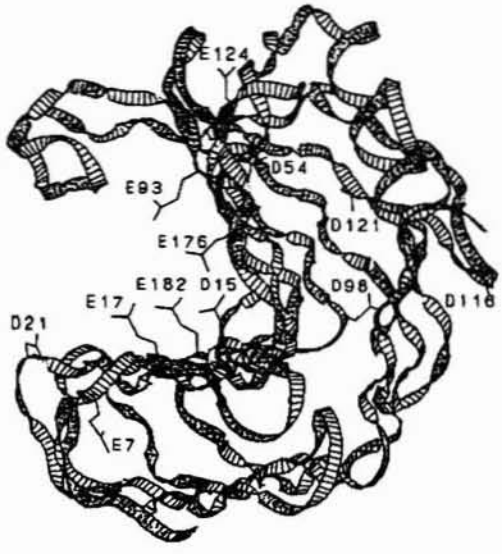


Fig. 1. 9. Computer graphics of the structure of the *B. pumilus* xylanase molecule (Essential carboxy groups in *Bacillus pumilus* xylanase) **(Courtesy: Ko *et. al.,* 1992).**

B. pumilus xylanase contains six aspartate and seven glutamate residues, which, except for Glu-165, are located on the α-carbon backbone of this enzyme (Fig. 1. 9). On the basis of the three-dimensional structure analyzed at 0.22 nm (2.2 Å) resolution, this enzyme molecule consists of two structural parts with a cleft region about 3 nm (30 Å) long and 1.5 nm (15 Å) in diameter. The size of the cleft is large enough to accommodate a xylan fibre of about 1.1 nm (11 Å) in diameter. The

candidates for the catalytic residues of this enzyme were narrowed down by presuming that the acidic amino acid residues within the vicinity of the cleft region are most likely to be the best- namely Asp-15, Asp-21, Glu-1 7, Glu-93, Glu-176 and Glu-182 (Ko *et. al.,* 1992).

Very recently, the structures of two new xylanase from *Streptomyces* sp. S38 (Wouters *et. al.,* 2001) and *B. subtilis* B230 (Dunlop *et. al.,* 1996) have also been solved. These proteins have the same general folds, yet there are major differences in their catalytic activities, specificities, pH optima and thermostabilities (Plesniak *et. al.,* 1996). Crystal structure analyses, sequence alignments and mutagenesis studies have indicated that mesophilic and thermophilic xylanase are very similar and that the enhanced stability of thermophilic xylanase is probably due to an array of minor modifications, with many xylanase using unique strategies to improve their thermostability. These modifications include: an increase in the number of salt bridges and hydrogen bonds (Hakulinen *et. al.,* 2003, McCarthy *et. al.,* 2000), an improved internal packing (Hakulinen *et. al.,* 2003), an increased number of charged surface residues (Turunen *et. al.,* 2002), present as tandem repeats, very often, in thermostabilising domains (Zverlov *et. al.,* 1996, Fontes *et. al.,* 1995), and / or the introduction of disulphide bridges, in particular, at the N or C -termini or in the a helix regions (Kumar *et. al.,* 2000, Turunen *et. al.,* 2002). Lately, the thermostabilising role of calcium on a modular family 10 xylanase was demonstrated (Abou-Hachem *et. al.,* 2003) while the *Bacillus* D3 xylanase was also shown to use a very unique adaptation strategy. Here, a series of surface aromatic residues form clusters or ''sticky patches '' between pairs of molecules and these intermolecular hydrophobic interactions are believed to contribute to the thermostability of this enzyme (Harris *et. al.,* 1997, Connerton *et. al.,* 1999). Collectively, or singly, all of the above mentioned modifications could improve the network of interactions within the protein, thereby leading to a more rigid and stable enzyme.

Crystalline structural analysis of xylanase from yeast and family 8 cold-adapted organism state that both are distinguishable by a number of discrete modifications which could give rise to a decrease in the stability, and hence an increase in the flexibility of the molecular structure. The *Pseudoalteromonas haloplanktis* TAH3a xylanase was characterized by a reduced number of salt-bridges and an increased

exposure of hydrophobic residues (Van Petegem *et. al.,* 2002, Van Petegem *et. al.,* 2003), while the family 10 yeast xylanase was characterized by a less compact hydrophobic packing, the loss of one salt bridge and destabilization of the helix macrodipoles (Petrescu *et. al.,* 2000). These modifications are extension of those observed between thermophilic and mesophilic xylanase, however, further analysis of psychrophilic xylanase, particularly related family 10 and 11, are necessary for better understand temperature adaptation in these enzymes.

1.8.4. Glycoprotein nature of xylanase

Xylanase are glycoproteins. Carbohydrate moieties are covalently linked with the protein or are present as dissociable complexes with various xylanase (Wong *et. al.,* 1988). Carbohydrate groups play an important role in stabilizing the enzyme protein structure as well as its activity and thermo stability (Woodward, 1984). The sugar moieties may be playing some role in the multiplicity of various xylanase produced by microorganisms (Wong *et. al.,* 1988) and further extensive studies are needed in enzyme catalysis reaction.

The endoxylanase X -I, X -II. A and X- II B from *Streptomyces* sp. are reported to contain less than 1% carbohydrates (Marui *et. al.,* 1985). Three thermostable endoxylanase from *T. byssochlamydoides* YH – 50 studied by Yoshioka *et. al.,* (1981), contained 36.5, 31.5 and 14.2 % carbohydrate residues respectively. These sugar residues were detected as the polymers of glucose, mannose and fructose. The glycoprotein nature of xylanase from *A. niger* was reported by Frederick *et. al.,* (1985). Four different non-debranching endoxylanase investigated by these authors, contained 5-22 % carbohydrate residues. The glycoprotein content of three-endo xylanase from *A. fumigatus* was between 46.4 and 68 % (Flannigan and Sellars, 1977), while three endoxylanase from *F. avenaceum* had 4-17 % carbohydrate (Zalewsk-Sobczak and Urbanek, 1981). *Chainia* sp. endo xylanase is also a glycoprotein (Bastawde *et. al.,* 1992). The heterogeneity between endo xylanase from prokaryotic and eukaryotic microorganism may be due to the presence of different types and amount of carbohydrate moieties. There are reports associated with broad range of multiplicity among endo xylanase from bacteria, moulds and yeast strains. There are a few known microorganisms producing a single type endoxylanase (Kusakabe *et. al.,* 1977, Keskar, 1992). Xylanase II from *A. versicolor* was reported

as a monomeric glycoprotein, exhibiting a molecular mass of 32 kDa with 14.1 % of carbohydrate content (Carmona *et. al.,* 2005).

1.8.5. Xylanase: multiplicity and multiple-domains

Hydrolysis of xylan requires multiple xylanase with overlapping or different specificities (Wong *et. al.,* 1988). Many microorganisms produce multiple xylanase such as *Stereptomyces* sp. (Godden *et. al.,* 1989), *Pencillium purpurogenum* (Belancic *et. al.,* 1995), *Cellulomonas* sp. N.C.I. M. 2353 (Chudhary and Deobagkar, 1997), and *Aeromaonas caviae* W -61 (Okai *et. al.,* 1998), which have diverse physicochemical properties, structures, specific activities and yields, as well as overlapping but dissimilar specificities, there by increasing the efficiency and extent of hydrolysis, but also diversity and complexity of the enzymes. The multiplicity of xylanase in microorganism is one of the strategies to achieve effective hydrolysis of xylan; in this each xylanase will be having a special function (Beg *et. al.,* 2001). During xylan hydrolysis, synergism is observed between enzymes acting on the β-1, 4 D- xylan backbone (β- 1, 4 – xylanase) and side chain cleaving enzymes (α- L-arabinofuranoside, acetyl xylan esterase and β- glucuronidase). The synergism between acetyl xylan esterase and endoxylanase results in the efficient degradation of acetyl xylan (Biely et. al 1986). The release of acetic acid by acetyl xylan esterase increases the accessibility of endoxylanase to xylan backbone. The endoxylanase creates shorter acetylated polymers that are preferred substrates for esterase activity (Biely *et. al.,* 1986).

How many xylanase could occur in a given species is difficult to find out because practically always the purification and characterization of xylanase are conducted from *in vitro* cultures. The purification procedures favour the "major" xylanase with high activity enough to allow purification, while "minor" xylanase remain obscured (those with too little activities for purification). Minor xylanase are so called because of the relatively low amount produced at the given culture condition but this does not necessarily mean that they have minor role in their biological functions. They have biological functions, which are not required in large quantities (Collins *et. al.,* 2005). It is also understood that some xylanase may not be detected during the assay because

they might be lost from the culture filtrate due to degradation or adsorption onto insoluble growth substrate (Wong *et. al.,* 1988).

Typical examples of microorganisms, which produce xylanase isoenzymes, include *A. niger*, which produces 15 extracellular xylanase, and *T. viride*, which secretes 13 (Biely *et. al.,* 1985). This multiplicity may be the result of genetic redundancy, but cases of differential post-translational processing have also been reported (Wong *et. al.,* 1988, Biely, 1985). The isoenzyme genes may be found as polycistronic or non-polycistronic multiple copies within the genome, and in some cases several xylanase are expressed as a distinct gene product. The xylanase, β xylosidase and acetyl esterase genes of *Caldocellulosiruptor saccharolyticus* (earlier known as *Caldocellum saccharolyticum*) are polycistronic (Luthi *et. al.,* 1990), while the XynC gene product from *Fibrobacter succinogenes* S85 encodes two different xylanase catalytic domains (Zhu *et. al.,* 1994). Further, multiple catalytic domains of many xylanase are characterized by the presence of various supplementary domains. These include xylan binding domains (Irwin *et. al.,* 1994), cellulose-binding domains (Black *et. al.,* 1997), dockerin domains (implicated in binding to multidomain complexes produced by certain microorganism, e.g., *Clostridium thermocellum*) (Hayashi *et. al.,* 1997), thermostabilising domains as well as domains for which the function has not as yet been elucidated (Winterhalter *et. al.,* 1995). These domains may fold and function in an independent manner (Black *et. al.,* 1996) and are typically separated by short junction segments enriched in hydroxyl amino acids (Kulkarni *et. al.,* 1999). The vast majority of xylanase are excreted into the extracellular environment as the large size of the substrate prevents its penetration into the cell. It is believed that small amounts of constitutively produced enzymes liberate xylooligomers which may be transported into the cell where they are further degraded by β xylosidases, or indeed by intracellular xylanase (Fontes *et. al.,* 2000, Teplitsky *et. al.,* 2000), and where they induce further xylanase synthesis.

The role of xylanase isozymes of different molecular sizes in pathogenic microorganism might be to allow their diffusion into the plant cell walls of highly variable structures. Wu *et. al.,* (1997) reported 5 xylanase from the rice blast fungus *Magnaportha grisea,* and at least four different xylanase have been identified from the maize leaf spot fungus, *Cochliobolus carbonum* (Apel-Birkhold and Walton,

1996), each differing in mol. wt. and pI values. Multiple xylanase may be allozymes, products of different allels of the same gene, or they could be distinct gene products produced by a fungus to enhance its utilization of xylan (Wong *et. al.,* 1988; Uffen, 1997). Wong *et. al.* (1986) studied the functional importance of three xylanase from the saprophytic fungus *T. harzianum* and reported a high degree of complementation of the three xylanase in the hydrolysis of aspen xylan. The conclusion from their finding was that the three xylanase are not redundant enzymes since each contributes significantly and uniquely to the hydrolysis of the xylan. In plant pathogenic fungi, it is reported that some of the xylanase are induced only during infection (Apel-Birkhold and Walton, 1996) suggesting that those different sets of endoxylanase function in saprophytic and pathogenic growth of fungi. It is also speculated that isozymes of cell wall degrading enzymes (CWDEs) are produced at different stages during infection of plant tissue (Annis and Goodwin, 1997) possibly following biochemical changes in the host environment.

In several *Aspergilli,* three different endoxylanase have been identified (de Vries and Visser 2001, Kormelink *et. al.,* 1993a). The best-studied *Aspergillus* endo xylanase, with respect to substrate specificity, are the three enzymes from *A. awamori* (Kormelink *et. al.,* 1993a). Counting from the reducing end, *A. awamori* endoxylanase I is unable to remove one unsubstituted xylose residue adjacent to singly substituted xylose residues or two unsubstituted xylose residues adjacent to doubly substituted xylose residues (Kormelink *et. al.,* 1993b). *A. awamori* endoxylanase III was not able to remove two unsubstituted xylose residues adjacent to singly or doubly substituted xylose residues toward the reducing end (Kormelink *et. al.,* 1993b).

The xylanase activity of *T. reesei* is composed of xylanase I, II, III and IV, and xylan-digesting cellulases. It also produces two β -xylosidases (E.C. 3.2.1.37), two endo-1,4- β -D-glucan cellobiohydrolases (CBH I and II, E.C. 3.2.1.91), five endo-1,4- β -D- glucan-4-glucanohydrolases (EG I, II, III, IV and V, E.C. 3.2.1.4) and two β -D-glucosidases (BGL I and II, E.C. 3.2.1.21) (Xu *et. al.,* 1998, Nogawa *et. al.,* 2001, Karlsson *et. al.,* 2001). Several other enzymes are also produced by *T. reesei*: β-mannanase (E.C. 3.2.1.78), β-mannosidase (E.C. 3.2.1.25), β-L-arabinofuranosidase (E.C. 3.2.1.55), β-galactosidase (E.C. 3.2.1.22), acetylxylan esterases (E.C. 3.1.1.72)

and laccases (benzenediol: oxygen oxidoreductases, E.C. 1.10.3.2) (Shabalin *et. al.,* 2002, Hakulinen *et. al.,* 2003). Xylanase I and II (pI 5.5 and 9) are approximately 20 kDa proteins belonging to the family 11 of glycosyl hydrolases (Törrönen and Rouvinen, 1997). Xylanase III (pI 9.1, 32 kDa) is a family 10 - glycosyl hydrolase, first characterized from *T. reesei* PC-3-7 (Xu *et. al.,* 1998). The pH optimum for xylanase I was at 4.0-4.5, for xylanase II at pH 4.0 - 6.0 and for xylanase III at pH 6.0 - 6.5. Of the total xylanase activity in *T. reesei* PC-3-7 produced on a cellulose-based growth medium, xylanase III accounted for over 25 % (Xu *et. al.,* 1998). Xylanase IV (pI 7.0, 43 kDa) was described in a recent patent (Clarkson *et. al.,* 2001). Its pH optimum is at pH 3.5 - 4.0. The activity of xylanase IV increases efficiently when it is combined with other xylanase. The xylanase of different *T. lanuginosus* strains have been characterized, and most of them have very similar mol. wt.s -25.5 kDa, and pI 4.1 (Cesar and Mrsa, 1996, Bennett *et. al.,* 1998, Lin *et. al.,* 1999). Other hemicellulases are produced in low levels (Singh *et. al.,* 2003). This is different from many other xylan-degrading organisms, which often secrete complex mixtures of xylanase and cellulases.

As in the mesophilic fungi, a multiplicity of xylanase has also been observed in some thermophilic fungi. Multiple forms of xylanase differ in stability, catalytic efficiency (Prabhu and Maheshwari, 1999), and absorption onto and activity on substrates (Tuohy *et. al.,* 1993, Maheshwari *et. al.,* 2000). Majority of xylanase have pH optima ranging from 4.5 to 6.5. *T. emersonii* xylanase unusually have acidic pH optima and the temperature optima range from 55 to 65°C. Xylanase of some strains of *T. aurantiacus* and *T. lanuginosus* are optimally active at 70 to 80 °C. The molecular masses of xylanase cover a wide range, from 21 to 78 kDa. With the exception of the dimeric Xyl I and Xyl II of *T. emersonii*, most xylanase are single polypeptides. Xylanase I of *C. thermophile* var. *coprophile* and (Ganju *et. al.,* 1989) xylanase II of *H. insolens* were remarkable in their low molecular mass (7 kDa) (Naren, 1992). Xylanase are glycoproteins, the carbohydrate content of the three xylanase of *Talaromyces byssochlamydoides* varied from 14 to 37 %. Two endoxylanase of *T. emersonii* were unusual in that they had no action on xylan unless the arabinose substituents were removed and also in their ability to hydrolyze aryl β-D-xylosides. Xylanase have not shown cooperative interaction in the hydrolysis of xylan (Gilbert *et. al.,* 1993, Prabhu and Maheshwari, 1999).

1.9.0. MOLECULAR EVOLUTION OF XYLANASE

Many microorganisms that hydrolyse xylans also degrade cellulose. In view of the similarity of the bond cleaved (1, 4- β-glucosidic linkages), and cross-specificity sometimes observed between cellulase and xylanase, the phylogenetic relationships of these enzymes have been a subject of interest. Xylanase showed no convincing sequence similarity with cellulases suggesting that the two enzyme species evolved from distinct ancestral genes.

Amino acid sequence alignment and hydrophobic cluster analysis have been utilized to assign plant cell wall hydrolases into different families (Henrissat and Bairoch, 1993). Based on these criteria, xylanase are divided into many families of which two families, family 10 and 11 are of much importance. In general the two families are not homologous in a phylogenetic sense, and it is therefore difficult to study their relationship by simple alignments. Few bifunctional xylanase that contain both family 10 and family 11 xylanase have been identified. These include the xylanase from the bacterium *Ruminococcus flavefaciens* (Accession no. P29126 and S61204) and the fungus *Neocallimastix patriciarum* (Accession no. P29127). The tight grouping of fungal and bacterial taxa, which produce bifunctional enzymes, suggests the possibility that xylanase evolution might proceed through horizontal transfer of the DNA encoding catalytic domains as suggested by Henrissat *et. al.,* (1995). Some phylogenetic studies suggest that generally less horizontal gene transfer has occurred within the family 11 than the family 10 xylanase (Georis *et. al.,* 1999).

For the comparison of the amino acid sequence a dendrogram (Fig. 1. 10) has been constructed to study the possible evolutionary relationships amoung these xylanase (Oku *et. al.,* 1993). The dendrogram studies explain that the low mol. wt. xylanase could be classified into three major groups. First group consisting of *A. tubigensis* ***A***, *A. niger* ***awa*** and *T. reesei* ***I*** xylanase. It appears that this group diverged from the other groups before other bacterial and fungal xylanase were separated to form their own groups. *S. commune* xylanase are highly similar to *T. harzanium. T. reesei* and *T. viride* xylanase. The xylanase from *B. pumilus* and *Cacetobutylicum* ***B*** are almost similar to that of *R. flavefaciens* (Oku *et. al.,* 1993).

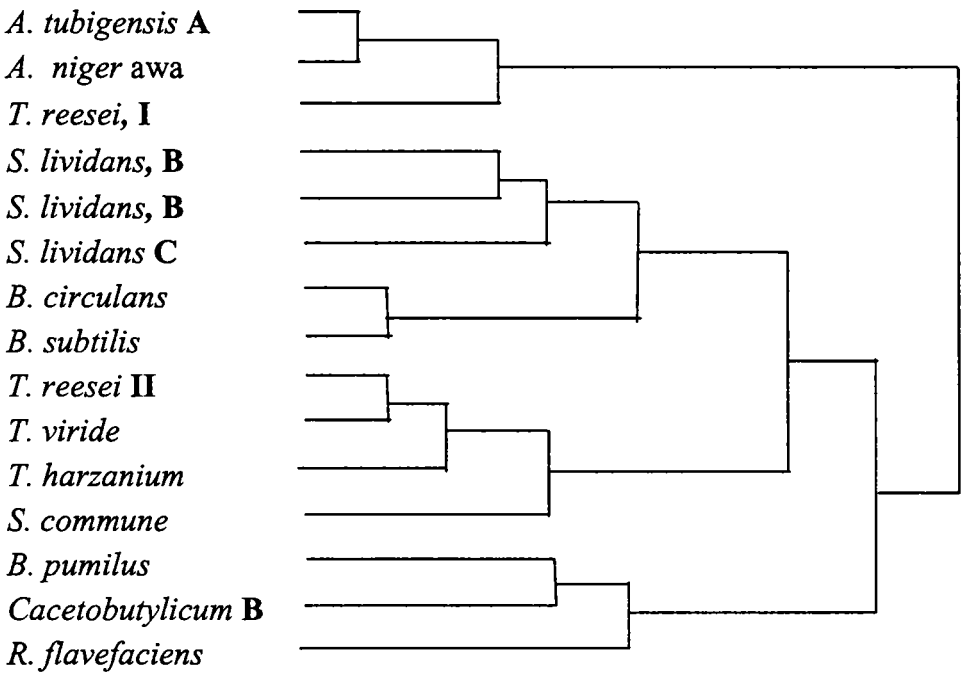


Fig. 1. 10. Evolutionary relationships amoung low mol. wt. xylanase based on the multiple alignments of amino acids sequencing (Courtesy Oku *et. al.*, 1993).

1.10.0. FACTORS INFLUENCING THE PRODUCTION OF XYLANASE

Xylanolytic enzymes appear to be inducible under natural conditions, by the products of their own action and are subject to catabolic repression by carbon sources such as glucose or xylose. Xylan, being a high molecular mass polymer, cannot enter the cell. The induction of the enzymes is stimulated by low molecular fragments of xylan namely xyloboiose, xyltriose, xylooligosaccharides, heterodisaccharides of xylose and glucose and their positional isomers, which are produced in the medium by small amount of constitutively produced enzyme (Bajpai, 1997). Xylan has been shown to be the best inducer of xylanase production in many cases (Nakamura *et. al.*, 1992, Gessesse and Mamo, 1998), pure xylan (Matsuo and Yasui, 1985, Gomes *et. al.*, 1993, Purkarthofer *et. al.*, 1993), other hemicellulose-rich materials and low mol. wt. carbohydrates such as xylose (Senior *et. al.*, 1989) and the pentosan unit of xylan in *Melanocarpus albomyces* (Maheshewari and Kamalam, 1985) and *Thermomyces*

lanuginosus (Purkarthofer *et. al.,* 1993). However, a few organisms show constitutive production of the enzyme (Debeire-Gosselin *et. al.,* 1990). Cellulose has also been observed to act as an inducer in a few cases (Morosoli *et. al.,* 1986, Stutzenberger and Bodine, 1992). Induction can also be achieved by various synthetic alkyl, aryl β-D-xylosides (Nakanishi and Yasui, 1980) and methyl β-D-xyloside (Nakanishi *et. al.,* 1992; Marui et al. 1985). These compounds enable the production of xylanolytic enzymes in the absence of xylan and xylooligosaccharides. Cheaper hemicelluloses consisting of substrates such as corncob, wheat bran, rice bran, rice straw, corn stalk, sorghum straw, wheat straw and bagasse have been found to be most suitable for the production of xylanase in certain microbes. Wheat bran was found to be the best substrate for xylanase production by alkalothermophilic *Bacillus* sp. (Dey *et. al.,* 1992), alkalophilic *Streptomyces* T-7 (Keskar *et. al.,* 1992) and *Penicillium funiculosum* (Mishra *et. al.,* 1985). Highest levels of xylanase were formed when *T. longibrachiatum* was grown on wood pulp (Royer and Nakas, 1991), corn cob (Bennett *et. al.,* 1998, Gomes *et. al.,* 1993, Purkarthofer *et. al.,* 1993), sawdust (Yu *et. al.,* 1987), sugar beet pulp (Tuohy *et. al.,* 1993), and sugarcane bagasse (Bocchini *et. al.,* 2005, Prabhu and Maheshwari, 1999). Paper of inferior quality was an excellent carbon source and inducer for xylanase in *Thermoascus aurantiacus* (Khandke *et. al.,* 1989), *Humicola lanuginosa* (Anand *et. al.,* 1990), and *Paecilomyces varioti* (Krishnamurthy, 1989).

Xylanase production has been studied under submerged (SmF) and solid-state fermentation (SSF), and Haltrich *et. al.,* (1996) reported the maximum xylanase activity (27,000 IU/ g) by SSF using fungus *S. commune*. An increase in xylanase production under SSF has also been reported from a bacterial strain *B. licheniformis* A99 (Archana and Satyanarayana, 1997) *Bacillus* sp. (Gessess and Mamo 1999), *Bacillus* sp. JB-99 (Virupakshi *et. al.,* 2005), *Bacillus pumilus* (Asha and Prema, in press) and from fungus *A. versicolor* (Jeya *et. al.,* 2005), *Thermomyces lanuginosus* (D_2W_3) (Sonia *et. al.,* 2005), and *A. fischeri* Fxn1 (Senthilkumar *et. al.,* 2005).

1.11.0. MODES OF FERMENTATION

Even with the great gains made in our understanding of microbial physiology and molecular biology, improvement of fermentation remains largely an empirical

process. In most instants the microbiologist begins with some medium and set of conditions that allow for at least modest expression of the metabolite or activity of interest. The task then is to improve that expression to a level sufficient for isolation and characterization of the desired products.

Organism	**Xylanase (U/ml)**	**References**
Bacillus pumilus	2.6	Durate *et. al.*,(1999)
Bacillus subtilis	12	Sa-Pereira *et. al.*, (2002)
Bacillus sp.	18	Corderiro *et. al.*,(2002)
Bacillus SSP-34	379	Subramaniyan *et. al.*,(2000).
Bacillus sterothermophilus T-6	2.33	Khasin *et. al.*,(1993)
Bacillus sp	11.5	Paul and Verma, (1993)
Schizophilium commune	1244	Steiner *et. al.*,(1987)
Trichodrema reesi	960	Baiely *et. al.*,(1993)
Thermomyces lanuginosis	2172	Purkathofer *et. al.*,(1993)
Cellulomonas sp.	9.33	Saxena *et. al.*,(1991)
Rhodothermus marinus	4.03	Hreggvidsson *et. al.*,(1996)
Sterptomyces roseisderoticus	16.2	Grabski and Jefferies (1991)
Aspergillus niger	~400	Yuan *et. al.*,(2005)
Rhizopus oryzae	260	Bakir *et. al.*,(2001)
Aspergillus niger GCBMX-45	195	Ikram-ul-Haq *et. al.*,(2002)

Table. 1. 5. Xylanase production by submerged fermentation by different microorganism

Preliminary experiments are done at flask level and latter transferred to pilot plants. There are extensive reports related to Xylanase production by Submerged fermentation using bacteria and fungi, and some are given in Table.1.5. Submerged fermentation is advantageous, it is well characterized as well as homogenous condition can be maintained through out the experiment and scale up is easy. But it is more energy intensive process and the production cost is high. Alternative to this is Solid-state fermentation, which is gaining interest currently (Pandey *et. al.*, 2001, Krishna, 2005). The basic difference between SmF and SSF is given in Table.1.6.

Advantage of SSF:- very high productivity, low water requirement, less wastewater, simple equipment, less expensive and no catabolic repression (Pandey *et. al.,* 2000). The general consideration for SSF is as follows: it is aerobic microbial transformation of solid materials or "Solid Substrate Fermentation" (SSF). It consists of solid porous matrix which acts as the substrate or support that can be biodegradable or not, but with a large surface area per unit volume, (range of 10^3 to 10^6 m^2/ cm^3), for a ready microbial growth on the solid/gas interface.

FACTOR	Liquid Substrate Fermentation	Solid Substrate Fermentation
Substrates	Soluble Substrates (sugars)	Polymers, Insoluble substrates: Starch, Cellulose, Pectines, Lignin
Aseptic conditions	Heat sterilization and aseptic control	Vapour treatment, non sterile conditions, heat sterilisation
Water	High volumes of water consumed and effluents discarded	Limited Consumption of Water; low *Aw* (water activity). No effluent
Metabolic Heating	Easy control of temperature	Low heat transfer capacity
Aeration (O_2)	Limitation of soluble oxygen. High level of air required	Easy aeration and high surface exchange air/substrate
pH control	Easy pH control	Buffered solid substrates
Mechanical agitation	Good homogenization	Static conditions preferred
Scale up	Industrial equipments available	Need for Engineering and New design Equipment
Inoculation	Easy inoculation, continuous process	Spore inoculation, batch
Contamination	Risks of contamination for single strain bacteria	Risk of contamination for low rate growth fungi
Energetic consideration	High energy consuming	Low energy consuming
Volume of Equipment	High volumes and high cost technology	Low volumes and low costs of equipments
Effluent and pollution	High volumes of polluting effluents	No effluents, less pollution
Concentration-Substrate/Products	30-80 g/l	100/300 g/l

Table. 1. 6. Comparison between submerged fermentation and solid substrate fermentations.

The matrix should have the capability to absorb water, one or several times its dry weight with a relatively high water activity on the solid/gas interface in order to allow

high rates of biochemical processes. Air mixture of oxygen with other gases and aerosols should flow under a relatively low pressure and mix the fermenting mash. The solid / gas interface should be a good habitat for the fast development of specific cultures of moulds, yeasts or bacteria, either in pure or mixed cultures. As to the mechanical properties of the solid matrix, it should stand compression or gentle stirring, as required for a given fermentation process.

This requires small granular or fibrous particles, which do not tend to break or stick to each other. The solid matrix should not be contaminated by inhibitors of microbial activities and should be able to absorb or contain available microbial foodstuffs such as carbohydrates (cellulose, starch, sugars) nitrogen sources (ammonia, urea, peptides) and mineral salts (Pandey *et. al.,* 2000, Krishna, 2005). The draw back is that not all organism can be grown in SSF, fungi are more suitable to SSF due to there mycelia nature and requires less amount of water, where as bacteria requires high amount of water. Some reports related to xylanase production by SSF are given in Table.1. 7. The production of xylanase by fermentation is influenced by physical and nutritional parameters. Cultural parameter optimization is an important way of enhancing production. Mostly enzyme production by microbes follows one factor - a time approach (Haltrich *et. al.,* 1993); here one factor is varied at a time keeping other factors constant. There are reports related to growth associated and non-growth associated xylanase production (Nakamura *et. al.,* 1993a, Dey *et. al.,* 1992).

Physical parameters such as pH, temperature, agitation/ aeration, inoculum sizes and incubation period in SmF and SSF together with the level of moisture, water activity and particle size of substrate are important for growth and xylanase production. The growth and production at high temperature are of great interest; there are lots of repots related to this. This is due to the importance of xylanase application in paper pulp industries. The most thermostable xylanase reported and active at 105 °C for half an hour is from *Thermotoga* sp. (Bragger *et. al.,* 1989). pH of the culture medium is important because all organism have a specific range of pH for its growth, activity and stability.

Fungal xylanase are reported to be less active at alkaline pH (Haltrich *et. al.,* 1996), where as bacterial xylanase are active at alkaline and neutral pHs (Horikoshi and Atsukawa, 1973, Tsujibo *et. al.,* 1990, Subramaniyan and Prema, 2000, Asha and

Prema, -in press). Xylanase from *Bacillus* sp 41-M was reported to be active at higher pH 10.5 than at 8 (Nakamura *et. al.,* 1993a).

Organism	Xylanase (U/gds)	References
Bacillus pumilus	~21431	Asha and Prema (in press)
Bacillus licheniformis A99	19.13	Archana and Sathynarayana, (1997)
Bacillus sp	720	Gessesse and Mamo, (1999)
Bacillus. sp. JB-99	3644	Virupakshi *et. al.,* (2005)
Fusarium oxysporum	1840	Panagioutou *et. al.,* (2003)
Thermomyces lanuginosis	20220	Purkarthofer *et. al.,* (1993)
Sterptomyces sp. QC-11-3	2360	Beg *et. al.,* (2000)
Aspergillus niger A3	5147	Cai *et. al.,* (1997)
Thermoascus aurantiacus	6193	Kalogeris *et. al.,* (1998)
Aspergillus niger USM AI 1	33.99	Kheng and Omar, (2004)
Aspergillus versicolor MKU-3	3249.9	Jeye *et. al.,* (2005)
Aspergillus sulpherureus	~1000	Lu *et. al.,* (2003)
Aspergillus fischeri	1024	Senthilkumar *et. al.,* (2005)
Thermomyces lanuginosus (D_2W_3)	48,000+/-1774	Sonia *et. al.,* (2005)
Schizophyllum commune	22700	Haltrich *et. al.,* (1992)

Table. 1. 7. Xylanase production by bacteria and fungi reported under Solid-state fermentation

Nutrients such as carbon, nitrogen, trace elements and vitamins can influence the enhancement of xylanase production. Carbon sources and their concentration were very much important for enzyme production. Xylan is widely reported to be a support for xylanase production (Esteban *et. al.,* 1982, Subramaniyan and Prema, 2000). Agro industrial residues such as wheat bran, straw etc, act as suitable natural sources for xylanase production (Asha and Prema, (in press), Yang *et. al.,* 2006, Heck *et. al.,* 2006).

Organic sources of nitrogen like tryptone; yeast extract, peptone, soymeal etc have high influence on enhancement of xylanase production. Haltrich *et. al.,*(1995) reported yeast extract as a good supporter for endoxylanase production by

Schizophillum commune , and *Bacillus* Sam-3 is reported to be highly productive in presences of soy meal (Saha *et. al.,* 1999), corn step liquor for *T. reesi* (Lappalainen *et. al.,* 2000), and tryptone for *Bacillus* sp. AB16 (Dhillon *et. al.,* 2000).

Trace elements and vitamins were important especially for thermoanerobes (Antranikian *et. al.,* 1987) and for some *Bacilli* (Ara *et. al.,* 1996). Beg *et. al.,* (2000) have reported the importance of amino acid for enhanced production by *Streptomyces* sp QC-11-3 and for Bacillus sp. AB-16 (Dhillon et. al., 2000).

1.12.0. PURIFICATION OF XYLANASE

Dekker and Richards (1975) have concentrated extracellular xylanase by evaporation under low pressure and freeze-drying. Precipitation using cold ethanol or acetone, alumina or ammonium sulphate, affinity binding to insoluble xylan, adsorption on to ion exchange like DEAE Sephadex was reported by Dekker and Richards, (1976). Column chromatography techniques are generally used which include ion exchange and size exclusion techniques; there are also reports related to the use of hydrophobic interaction column chromatography (Wong and Saddler, 1992), and Electroelution (Periera *et. al.,* 2000). Expanded bed adsorption (EBA) was used to purify a marketable xylanase often used for Kraft pulp bleaching process. It is preparative chromatography technique used in the initial steps of downstream processing. It allows that particulate-containing feedstock to be submitted directly to the column without prior clarification. It reduces the purification processing time and interaction of protease and nuclei acids with target molecules. The cost of purification is reduced and there is no need for clarification of the crude enzyme (Hamilton *et. al.,* 2000, Santos *et. al.,* 2002).

There are different methods of xylanase purification. In all cases the culture supernatant was concentrated by precipitation or ultra filtration. The fact that certain xylanase have cellulose-binding domains that interact with normal elution process restricts the use of cellulose materials as the matrix in column chromatography (Gilkes *et. al.,* 1991). Eudragit -S 100 is an enteric coating anionic polymer composed of methyl- methacrylate and methacrylic acid. This polymer is soluble at higher pH value (more than 7.0) and insoluble in acid medium (Ai *et. al.,* 2005). This was reported to be effective in the precipitation of xylanase from *B. amyloliquefaciens*

MIR-32 (Breccia *et. al.,* 1998) at pH- 4.0. Panbangred, *et. al.,* (1983) has reported the purification of endoxylanase produced by *Bacillus pumilus* from liquid medium. Endoxylanase from fungus was reported to be separated using capillary electrophoresis (CE) in a fused silica capillary at pH values close to neutral. The capillary coated with 1, 3-diaminopropane resulted in separation of the enzyme, and the reproducibility of the migration times was between 0.6 and 1.9 % (Jørgensen *et. al.,* 2003). Two xylanase from *B. subtilis* were purified by novel and convenient method, native polyacrylamide gel electrophoresis (PAGE), and homogenization extraction was used for the purification of xylanase from crude enzymes. Subsequent analysis with thin layer chromatography and high-pressure liquid chromatography (HPLC) was attempted and succeeded (Yuan *et. al.,* 2005).

1.13.0. MOLECULAR CLONING OF XYLANASE GENE

Recombinant DNA techniques are offering new area of research for construction of genetically modified microbial strains with selected characteristics for enzyme production. Pulp and paper industries require xylanase that are active at high alkaline pH, temperature as well as devoid of cellulase activity. The production cost is another criteria, which has to be considered while selecting a strain. In this respect isolation and cloning of the xylanase gene represent an essential step in the engineering of the most efficient microorganism (Kulkarni *et. al.,* 1999). There are reports related to cloning and expression of xylanase from bacteria such as *B. subtilis* (Bernier *et. al.,* 1983), *Bacillus* sp. (Jeong *et. al.,* 1998), *Bacillus* sp. (Biely, 1985) and *Acidobacterium capsulatum* (Inagaki *et. al.,* 1998) into a non-cellulase producing strain of *E. coli.* According to Biely, (1985) the main targets of cloning are - upgradation of fermentation processes of industrially important xylose fermenting microbes, by introducing genes for xylanase and xylosidase, aiming at direct fermentation of xylan, as well as the construction of xylanolytic enzyme producers devoid of cellulase activity.

Cloning helps in the introduction of novel gene as well as amplification of existing expression. The xylanase from *B. subtilis* was enhanced successfully using plasmid vector carrying *B. pumilus* gene. Three times more xylanase was produced by the transformant than the donor strain. Regulatory elements of the donor organism were

absent in the vector used for transformation making the xylanase expression constitutive (Panbangred *et. al.,* 1985). *Bacillus* strain C-125 produces two type of xylanase N with mol. wt. of 43 kDa and A with 16 kDa. The gene xyn A was located in 4.6 kbp DNA fragment and was cloned in *E.coli*, and it was observed that more than 80 % activity was detected in the culture medium (Honda *et. al.,* 1985). The thermostability of *Streptomyces lividans* xylanase B (SlxB- cat) was significantly increased by the replacement of its N- terminal region with the corresponding region from *Thermomonospora fusca* xylanase A (TfxA-cat) without observing a decrease in enzyme activity by DNA shuffling technique (Shibuya *et. al.,* 2000).

1.14.0. APPLICATION OF XYLANASE

Xylanase have high potential application in several industries, and in recent years the application of xylanase is diffusing to novel areas of research. Several commercial products have been lunched successfully in the industrial sector worldwide in the past few years, and some of the major producers, their trade name and application are given in Table. 1. 8.

In feed formulations, cooperation of xylanase, glucanases, proteinases and amylases reduces viscosity of the feed and increases the adsorption of nutrients. Enzymes liberate nutrients either by hydrolysis of non-degradable fibres or by liberating nutrients blocked by these fibres (Leisola *et. al.,* 2004). In combination with pectinases and other enzymes, xylanase have also been used in other processes such as clarification of juices, extraction of coffee, and extraction of plant oils and starch. Other potential applications include the conversion of agricultural waste and the production of fuel ethanol. Xylanase in synergism with several other enzymes like mannanase, ligninase, xylosidase, glucanase, glucosidase etc. can be used for generation of biofuels such as ethanol and xylitol from lignocellulosic biomass (Olsson and Hahn – Hagerdal, 1996, Eriksson *et. al.,* 2002, Sorensen *et. al.,* 2003). The biological process of ethanol production requires delignification of lignocellulose to liberate cellulose and hemicellulose from their complex with lignin, followed by depolymerisation of carbohydrate polymer to produce free sugars, and finally fermentation of simple sugars to produces ethanol (Beg *et. al.,* 2001).

Producer	Product name	Application
Alko Rajamaki, Finland	Ecopulp	Pulp Bleaching
Sandoz, Charlotte, N.C.	Cartzyme (HS-10, HT, SR-10)	Pulp Bleaching
Basle, Switzerland	Cartzyme (PS-10, 9407 E, NS-10)	
Clarient U. K	Cartzyme MP	
Genercor Finland	Irgazyme 40-4X/Albazyme 40-4X,	Pulp Bleaching
	Irgazyme 10 A	
Ciba Giegy, Switzerland	Albazyme – 10A,	Baking and Food
	Multifect xylanase	
Biocon India, Bangalore	Bleachzyme F	Pulp Bleaching
Enzyme development U.S.A	Enzeko xylanase	Baking, Food and Feed
Solvay Interox U.S.A	Optipulp L-8000	Pulp Bleaching
Novo Nodrisk, Denmark	Pulpzyme (HA, HB, HC)	Pulp Bleaching
	Biofeed Beta, Biofeed Plus	Feed
	Ceremix	Brewing
Voest Alpine, Austria	VAI Xylanase	Pulp Bleaching
Sankyo, Japan	Sanzyme (PX), Alpelase F,	Feed
	Sanzyme X	Food
Meito Sankyo, Nogaya, Japan	Xylanase	Research
Rohn Enzyme 0Y, Primalco,	Ecopulp (X-100, X-2000, X-200/4,	Pulp Bleaching
Finland	TX-100, TX-200, XM)	
Rohm, Germany	Rholase 7118	Pulp Bleaching
Iogen Canada	GS-35, HS70	Pulp Bleaching
Thomas Swan, U.K.	Ecozyme	Pulp Bleaching

Table. 1. 8. Major world Producers of Commercial Xylanase, their trade names and major applications

Xylanase is used in the pretreatment of forage crops to improve the digestibility of ruminant feeds and to facilitate composting (Gilbert and Hazlewood, 1993). In the food industry, xylanase are used to improve the dough properties and baking quality

of bread and other baked goods by breaking down the polysaccharides in the dough (Li *et. al.,* 2000; Bhat, 2000).

Endoxylanase are almost routinely used in bread to improve mixing, dough handling properties, oven spring and loaf volume (Courtin *et. al.,* 1999, Courtin *et. al.,* 2001). Xylanase produced from *A. foetidus* MTCC 4898 by using wheat bran was reported to be effective on dough as well as bread attributes (Shah *et. al.,* 2006). Endoxylanase (E.C. 3.2.1.8) have a strong impact on AX structure and functionality. They attack the AX backbone in a random manner, causing a decrease in the degree of polymerization of the substrate and liberating oligomers, xylobiose and xylose with retention of their configuration. The final product depends mainly on enzyme level, action time, substrate type, pH and other factors (Saha, 2003, Dekker, 1985). Arabinoxylans (AX) in cell wall are non-starch polysaccharides of cereal and an important source of dietary fiber (Saha, 2003). Wheat flour contains 1.5 - 2.5 % total AX, of which one-third to half is water-extractable (WE-AX) and the other is water-unextractable (WU-AX). WE-AX leads to a highly viscous solution, whereas WU-AX has a strong water-holding capacity. AX can be hydrolyzed by endoxylanase (XYL) (Courtin and Delcour, 2002).

Degumming of bast fiber such as flax, hemp, jute and ramie, is another import application; xylanase together with pectinase is commonly employed for this (Sharma, 1987, Puchart *et. al.,* 1999). Xylanase with pectinase was also used in the debarking of wood (Bajpai, 1999). Generally, fibers are separated by the process of retting i.e., removal of binding material present in the plant cell using enzymes particularly from the microorganisms. Pectinase is reported to be the major enzyme (Sharma, 1987). Natural retting is a slow process and produces pollutants but this can be replaced by the use of combination of enzyme system, which is environment friendly, and fast process, which could be a new fiber liberation technology in the near future.

Xylanase are used in the prebleaching of kraft pulp to reduce the use of harsh chemicals in the subsequent chemical bleaching stages. The enzymatic treatments improve the chemical liberation of lignin by hydrolysing residual xylan. This reduces

the need for chlorine-based bleaching chemicals, which is beneficial to the environment (Suurnäkki *et. al.,* 1997, Bajpai, 1997, Christov *et. al.,* 1999, Viikari *et. al.,* 2001, Kenealy and Jeffries, 2003). Xylanase are now used in mills worldwide prior to chlorine and chlorine-dioxide bleaching sequences (Tolan *et. al.,* 1995). Particularly important advantages of using xylanase as one stage in bleaching of kraft pulp are to decrease the use of ClO_2, to increase the brightness ceiling, and to decrease production of chlorinated organic materials. A xylanase from the hyperthermophilic bacterium *Thermotoga maritima,* stable at 92 °C during the time for the bleaching stage, has been cloned in *E.coli* and can now be produced in large quantities (Shah *et. al.,* 2000).

1.15.0. IMPORTANCES OF XYLANASE IN PAPER PULP PROCESS

1.15.1. Paper Making Process

Firstly, to identify the sources for cellulose fiber, it can be from wood, recovered paper or Non-wood plants that depend on the requirement of paper and its quality. Secondly, the cellulose has to be separated to a usable form, and this can be done by pulping and bleaching (if required only or for high quality paper). Thirdly, this pulp has to be converted to paper by Sheet - Formation (very dilute water solution of pulp is sprayed on a fast-moving wire) and by Pressing and drying (water is removed with pressure and heat to form paper); and finally these sheets are rolled out as paper rolls. The need for improved manufacturing efficiencies, enhanced wood utilization practices, and continuing environmental concerns has become one of the central research themes from the late 1990's.

1.15.2. Pulping and Bleaching Process

The type of pulping and the amount of bleaching used, depend on the nature of feedstock and the desired quality of the end product. There are two main types of pulp fibres - mechanical and chemical. Mechanical pulps are made by separating wood fibres using a grinding procedure resulting in "fines" (small fractions from fibres) and broken fibres. Often heat is applied during the grinding procedure and the pulp is then

referred to as thermomechanical. Chemical pulping includes sulphite process and Kraft process followed by steam cooking and grinding.

1.15.2.1. Sulphite pulp

Sulphite pulping, due to its acidity, cleaves most of the acetyl and arabinofuranose groups from xylan-type hemicelluloses, leaving methylglucuronoxylan as a residue. A marked reduction in the degree of polymerization occurs. Galactose is lost from the galactoglucomannan and it gets degraded itself, so it cannot be detected in sulphite pulp. Some part of galactomannan too is lost due to acidic condition.

1.15.2.2. Kraft pulp (sulphate processes)

The dominating process used today is sulphate pulping; a basic method. Chemical pulping disintegrates the fibres by dissolving the middle lamellae. Chemical pulp fibres are more intact in their structure as compared to mechanical pulp fibres. Kraft pulping cleaves most of the methyl-glucoronic acid groups from the xylan hemicelluloses, resulting in arabinoxylan in the case of softwoods and xylan in the case of hardwoods and prehydrolyzed softwoods. The glucomannans are partially dissolved and degraded, but large proportions remain in the pulp. The absorption of xylan polymers during Kraft pulping occurs and is attributed to removal of side groups (methylglucuronic acid and/or arabinose) and / or acetyl groups from original xylan type hemicellulose. A portion of this xylan is adsorbed to cellulose and becomes resistant to alkaline extraction.

The effect of pulping reagents on hemicellulose is important based on the yield and final paper properties of resultant pulp. These properties depend on the type, structure, and degree of polymerization and location of the various hemicellulose component polymers. Softwood fibres with their length and coarseness are generally used to provide strength to a sheet of paper. Hardwood fibres, being finer and more conformable, give a smooth printing surface and opacity to paper. Hardwood fibres are also easier to bleach to high brightness because they have less lignin. Paper generally consists of a blend of hardwood and softwood pulps to meet the strength and printing surface demands of the customer. The primary factor contributing to researcher is the well-known loss of pulping selectivity when attempting to remove

the last residue of lignin in pulps by kraft delignification. The yield can be improved by utilizing a single or double oxygen stage to delignify high lignin content pulps.

1.15.3. Delignifying agents in paper industries and their environmental effect

Wood was processed to remove lignin that causes yellowish – brown colour without damaging the cellulose and hemicellulose. The conventional commercial delignification processes produces a dark pulp because of the colour of residual modified lignin. This residual lignin is removed by various multistage bleaching procedures using chlorination and alkaline extraction steps. Some of the delignifying agents are given below.

1.15.3.1. Elemental chlorine (Cl_2) is an effective delignifying agent. As it breaks lignin bonds, it adds chlorine atoms to the lignin degradation products, thus producing significant amounts of chlorinated organic material.

Effluent from different stages of paper pulping	Major components present in the effluent	Properties and Environmental effects
Chlorination stage	Chlorinated phenols, resins, fatty acids, chlorinated hydrocarbons, hypochlorites, carbohydrates, chlorides, and their degradative products	Low molecular mass, low colour, high BOD, toxic, bacteriostatic and mutagenic, persisting and bioaccumulative.
Extraction stage	Chlorinated and oxidized kraft lignin, hemicelluloses and their degradative products	Polymeric, high colour, high BOD and COD, toxic, mutagenic, carcinogenic

Table. 1. 9. Major components in the effluent from different stages of paper pulping process and their properties and environmental effects

1.15.3.2. Chlorine dioxide (ClO_2) is a highly selective chemical that can both delignify and brighten pulp. It oxidizes lignin, but does not add chlorine atoms onto

lignin fragments; however, small amounts of elemental chlorine and other chlorine compounds formed during the chlorine dioxide bleaching process react with degraded lignin to form chlorinated organic compounds.

1.15.3.3. Sodium hypochlorite (NaOCl) is an inexpensive delignifying agent formed by mixing elemental chlorine with alkali at the mill. Mills are phasing out the use of hypochlorite because it generates large quantities of chloroform when it is used to bleach pulp.

Effluents from such bleaching processes are of great environmental concern, because it contains numerous chlorinated organic substances such as chlorophenols, which are deleterious to biological systems (Campin *et. al.,* 1991). The components of effluents, their properties and effects where given in Table. 1. 9. The paper and pulp process is the major consumer of forest and the major producer of highly polluting components to the environment. The organochlorides produced from the paper mills are responsible for high Biological oxygen demand (BOD) and Chemical oxygen demand (COD), toxicity, mutagenesis, and for carcinogenic and bioaccumulative (Viikari *et. al.,* 1994, Bajapi *et. al.,* 1994) properties. Attempts are made to develop alternative methods for effective pulp bleaching with reduction in chlorine and or chlorine dioxide or to eliminate its use by replacing with oxygen, ozone and hydrogen peroxide.

1.15.3.4. Hydrogen peroxide (H_2O_2) is mainly used to brighten pulps in the final bleaching stages. Peroxide is often used at the end of a conventional bleaching sequence to prevent the pulp from losing brightness over time. Researchers have found operating conditions under which peroxide will delignify pulp, and are working on technologies that will consume less.

1.15.3.5. Ozone (O_3) is also an effective delignifying agent. It also brightens the pulp as well. Ozone has not been used in the past because mills have not been able to improve its selectivity - ozone attacks the cellulose fibre as well as the lignin. Recent technological developments, however, have solved this problem and have allowed mills to take advantage of this cost-effective bleaching agent.

1.15.4.6. Oxygen (O_2) is an inexpensive, and highly effective delignifying agent that is usually used at the beginning of the bleaching process. It has intermediate selectivity.

Chemical consumption and environmental considerations severely limit the types of delignification technologies that can be employed with high kappa pulps. The two most promising delignification technologies for high lignin content pulps consist of using oxygen delignification or modifying the pulping process using enzyme, which is a more environment friendly way.

1.16.0. IMPORTANCE OF THE TECHNOLOGY

The paper industry has been investigating biological replacements for some of the chemicals used in the paper making process in the hope of reducing capital and operating costs and minimizing its environmental impact. One use of biological treatments, which has been of recent interest, is for reducing refining energy consumption in mechanical pulping processes. It has been shown that certain fungal treatments can achieve this end without damage to the resulting fiber and possibly with better quality fiber in the end. The use of fungus prior to pulping offers an attractive opportunity for mechanical wood pulp facilities. This technology could save an estimated 30 % of the energy consumed in refining the mechanical pulp. The technology also improves paper strength, reduces pith content, and could reduce the emissions of volatile organic compounds. The major draw back of this is that it is a time consuming process. There has also been some success in pretreating wood chips for chemical pulping processes. In this type of application more uniform delignification, improved yield, or decreased chemical usage are the goals. Research into chip treatment with cellulose and hemicellulose enzymes is just beginning. Pretreatment of hard wood chips with *Pseudomonas chrysosporium* shows an improvement in kraft pulp yield after 20 days, but is more pronounced after a period of 30 days. The resulting pulp, compared at the same kappa number, has a higher tensile strength and corresponding lower tear strength. The pulps also refine faster, thus saving refining energy to achieve the same pulp properties. Utilization of xylanase that is active at high alkalinity and temperature is an alternative that can be utilized in the plant itself without much alternation to the process, and is not time

consuming. This is an environmental friendly technology, and the by products formed are degrading type.

1.16.1. Biopulping

Biopulping is the treatment of wood chips and other lignocellulosic materials with natural wood decay fungi prior to thermo mechanical pulping. The fungal treatment process fits well into a mill's wood yard operations. Wood is debarked, chipped and screened according to normal mill operations. Then chips are briefly steamed to reduce natural chip microorganisms, cooled with forced air, and inoculated with the biopulping fungus. The inoculated chips are piled and ventilated with filtered and humidified air for 1 to 4 weeks prior to processing. The biopulping process is technologically feasible and economically beneficial. The use of biopulping as a pretreatment for the Kraft process is still an open research issue. The uses of this technology for other substrates such as l non-woody plants like straw, and corn stalks also need much attention (Sabharwal *et. al.,* 1995, Zhao *et. al.,* 2004, Zhon *et. al.,* 2006). The major advantages of this process are: Reduced electrical energy consumption (at least 30 %) during mechanical pulping; potential 30 % increase in mill through put for mechanical pulping; improved paper strength properties; reduced pitch content, and reduced environmental impact (Kenealy and Jeffries, 2003).

1.16.2. Biobleaching

The principal objective behind application of biotechnological methods is to effect selective hemicellulose removal without degrading cellulose. Degradation of cellulose is the major problem associated with conventional pulping process, which invariably affects the cellulose fibre, and thus the quality of paper (Shoham *et. al.,* 1992). Removal of xylan from the cell walls by biotechnological methods leads to decrease in energy demand during bleaching (Noe *et. al.,* 1986). Therefore, enzymatic treatments of pulp using xylanase have better prospects in terms of both lower costs and improved fibre qualities. Xylans are more accessible to hydrolytic enzymes because they do not have a tightly packed structure. As a result, the specific activity of xylanase is 2-3 times greater than the hydrolases of other polymers like crystalline cellulose (Gilbert and Hazlewood, 1993). In the pulping process, the resultant pulp gets a characteristic brown colour due to the presence of residual lignin and its

derivatives. The intensity of pulp colour is a function of the amount and chemical state of the remaining lignin. In order to obtain white and bright pulp suitable for manufacturing good quality papers, it is necessary to bleach the pulp to remove the constituents such as lignin and its degradation products, resins and metal ions (Bajpai, 1997). Biobleaching of pulp is reported to be more effective with xylanase than with lignin degrading enzymes. This is because the lignin is cross-linked mostly to the hemicellulose, which is more readily depolymerised than lignin (Viikari *et. al.,* 2001). Removal of even a small portion of the hemicellulose can be sufficient to open up the polymer and facilitate removal of the residual lignin by solvents.

New environmentally benign elemental chlorine-free (ECF) and totally chlorine free (TCF) bleaching technologies are necessary for minimizing the hemicellulose content in dissolving pulp, adjusting the brightness at a high level and improving simultaneously, the quality of the effluents in terms of toxicity and absorbable organic halogen (AOX). There are several reports related to bleaching of hardwood and softwood pulp enhanced by xylanase treatment.

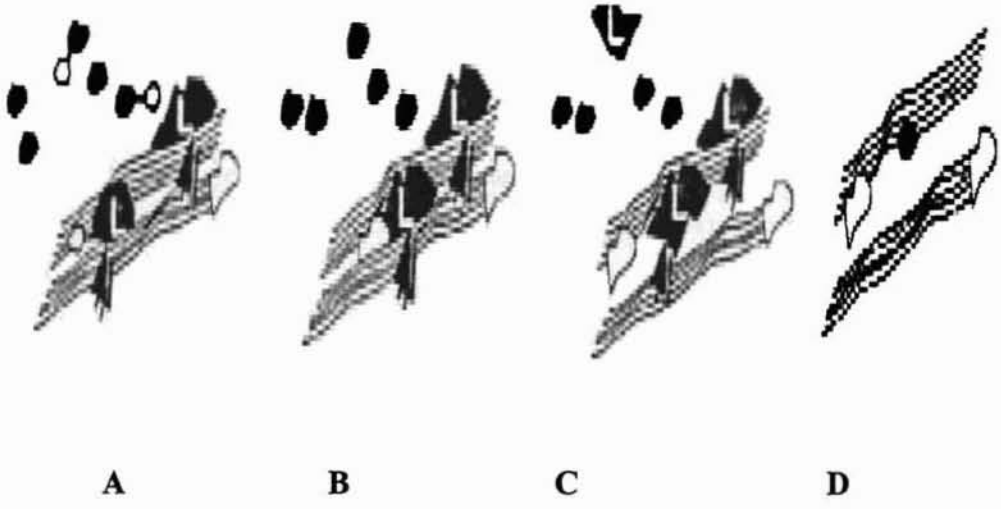


Fig. 1. 11. Digramatic representation of chemical treatment on paper pulp
L- Lignin.

Enzyme treated sheets show slight decrease in interfibre bonding strength; the mechanical strength of fiber is not affected but the interfiber bonding decreases if cellulose is present (Jeffries, 1994). Similar report is there with *Saccharomonospora viridis* (Robert *et. al.,* 1990). In the absences of cellulose, xylanase increases viscosity and hemicellulose hydrolysis enhances lignin removal (Bajpai, 1999, Kirk and Jeffries, 1996). The mechanism of pulp bleaching by chemical treatment was given in Fig. 1. 11. It explains that the use of chemical cannot fully remove the lignin from the fiber, (A, B, C) some parts of lignin get reprecipitated on to the fiber surfaces (D).

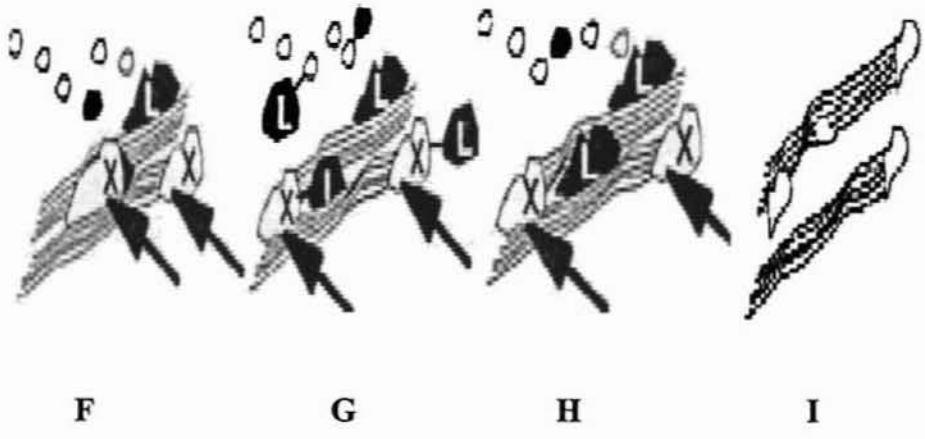


Fig. 1. 12. Effect of Xylanase treatment on paper pulp – a digramatic representation (X- Xylanase and L- Lignin).

The hypothesis of xylanase treatment is given in Fig. 1. 12. This shows that xylanase treatment helps in the removal of chromophoric groups from the pulp (F) as well as partial hydrolysis of the reprecipitated xylan or lignin carbohydrate complexes (G), thus opening up the porosity of the pulp in order to allow the free diffusion of bleaching chemicals or they split the linkage between the residual lignin and carbohydrates (H). It is proposed that the released xylan contains carbohydrate complexes and both mechanisms may allow enhanced diffusion of entrapped lignin from the fiber wall. Limited removal of pulp xylan helps to increase the pulp bleachability during subsequent bleaching stages (I) (Viikari *et. al.,* 1996).

Paice *et. al.,* (1992) and Kantelinen *et. al.,* (1993) have suggested that chromophores generated during the Kraft pulping are trapped on the surfaces of the cellulose fibers when xylan precipitates near the end of the pulping process. Biological methods of pulp prebleaching using xylanase provide the possibility of selectively removing upto 20 % of xylan from pulp and saving up to 25 % of chlorine containing bleaching chemicals. Major advantages of biobleaching are: reduced consumption of bleaching chemical; reduced absorbable organic halogen; improved pulp and paper quality; improved brightness; reduced effluent toxicity and pollution load. The discovery of new alkaline thermostable xylanase (Andrade *et. al.,* 1999, Maheshwari *et. al.,* 2000, Viikari *et. al.,* 2001, Collins *et. al.,* 2005) has also added much importance to this area.

1.17.0. CRITERIA FOR THE SELECTION OF XYLANASE

Upgradation of any technology by screening for novel enzymes with enhanced properties or developing tailor-made enzymes by site – directed mutagenisis is a continious effort and essential to be competitive. Some characteristics of xylanase, which are considered for particularly for Pulp and paper Industry, are:

1. Cellulase –free (or cellulase – poor) nature of xylanase
2. The enzyme should be of low mol. wt. to have easy access to the pulp matrix
3. Active at high alkaline pH 8 - 11, so they can be function effectively on kraft pulp or soda pulp.
4. Thermal stability – above 50 °C
5. Enzyme should be active on wide variety of pulps.
6. Affinity for acidic side chains in order to minimize hexaneuronic acid-xylooloigosaccharides and UV absorbing materials
7. The enzyme should not be inhibited by impurities from the pulp.

1.18.0. CONCLUSION

The use of environment friendly processes is becoming more popular in various Industrial sectors to avoid the deletrious effect of effluents generated. Biotechnology as well as enzyme technology has helped much to look up in this aspect, by improving the quality, production rate or diminished environmental impact. Xylanase are

hydrolytic enzymes that randomly cleave the β- 1, 4 backbone of the complex plant cell wall polysaccharide xylan. Diverse forms of these enzymes exist, displaying varying folds, mechanisms of action, substrate specificities, hydrolytic activities and physicochemical characteristics. A large variety of microorganisum with xylanase activity have been isolated as well as studied and enzymes where classiffied into different glycoside hydrolase families with each families being characterized by a particular fold and mechanism of action. Extensive studies of family 10 and 11 xylanase have been carried out, leading to the enrichment and understanding of these enzymes. New approaches, such as genome sequencing programs, functional and/or consensus PCR screening of environmental DNA libraries (metagenomics) as well as the study of extremophilic enzymes will, with-out doubt, further extend the current repertoire, understanding, and applications of xylanase and xylanase families.

The ecofriendly processes is becoming more popular in the pulp and paper industry and therefore biotechnological processes are coming to the forefront of research. An application of biotechnology in Indian pulp and paper industry is the xylanase pre-bleaching of pulp. Extensive R & D work on enzymatic prebleaching of pulp from raw materials is widely prevalent in India. Due to pressure on reducing organochlorine compounds in the effluent, more and more paper mills are getting interested in this process and have also started taking mill trials. Enzyme technology offers great potential for reducing capital and energy costs, improving properties of degraded fiber furnishes, and reducing the environmental impact of paper making processes.

1.19.0 OBJECTIVES AND SCOPE OF THE PRESENT INVESTIGATION

Scope of the present investigation was confined to the following objectives:

- Isolation of alkalophilic xylanase producing bacterial strain from RRL (TVM) culture collection.
- Identification of the strain
- Submerged fermentation using different agro industrial residues and compared with production in Oat spelt xylan medium
- Optimization of culture condition in SmF for enhanced production in wheat bran as the carbon sources
- Comparison of SmF with SSF for the selection of suitable mode of fermentation
- Selection of suitable agroindustrial residue for SSF
- Crude Enzyme profile analysis (SmF and SSF compared)
- Optimization of culture conditions for maximization of xylanase production by SSF: - particle size, initial moisture level and pH, fermentation temperature, inoculum sizes and incubation period.
- Optimization of nutritional parameters for enhances xylanase production by SSF: - inorganic and organic nitrogen sources, phosphrous sources, easily metabolisable sugars, and metal ions.
- Purification of xylanase from *Bacillus pumilus* produced by SSF
- Characterization of partially purified and purified fractions:- activity tempertature and pH, stability temperature and pH as well as effect of metal ions.

- Application of crude xylanase
    - Kraft pulp bleaching
    - Recycling of paper
    - Plant fiber separation
    - Saccharification
- Optimization of enzymatic parameters for pretreatment of kraft pulp: - Enzyme dose, temperature, pH, Chlorine dioxide dose, Peroxide dose and caustic soda dose.

Chapter. 2.

Selection of bacterial strain, comparison of different mode of fermentations for xylanase production and selection of suitable substrate for SSF

2.0. INTRODUCTION

Lignocellulosic biomass such as agricultural and forestry residues, municipal solid waste and dedicated crops provide a low cost feedstock for biological production of fuels and chemicals, which offer economic, environmental and strategic advantages (Someet *et. al.,* 2001). Effective utilization of these materials for the production of enzymes, biofuels and other metabolites needs extensive studies. (These materials generally contain up to 25 % of lignin and 40% cellulose, which cannot be easily converted to simple monomeric form due to their non-biodegradable nature. Hemicelluloses, the second most abundant polysaccharide next to cellulose, consist of β-1, 4-linked pyranosyl backbone. The break down of hemicellulose requires the action of various enzyme of which endoxylanse is the most important one. Microbial xylanase are advantageous over their counter parts from plant and animal sources because of their easier availability, structural stability and ease of genetic manipulations (Chand and Mishra, 2003, Subramaniyan and Prema, 2002). Xylanase have been isolated from a diverse range of microorganisms including fungi and bacteria of which endo β-1-4-D- xylanase (E.C. 3.2.1.8) are mainly responsible for the random hydrolysis of xylan (Puls 1997). Only few xylanase are reported to be cellulase - free as well as active and stable at alkaline pH and high temperature (Collins *et. al.,* 2005).

In view of their potential role, cost effective development of enzymes is crucial, as this will significantly benefit the overall economics of biological processes. Agricultural residues make good substrates for fermentation, provided bioreactors are designed with suitable operational control (Pandey *et. al.,* 2000, Krishna, 2005). Earlier production of enzyme was targeted using Submerged or liquid fermentation, which is easy to handle and contamination free but the cost of production is very high. One alternative for this is use of Solid State Fermentation. These fermentation systems, which are closer to natural system, may prove more efficient in producing certain enzymes and metabolites. There are many reports related to production of endoxylanase by SmF using bacteria (Archana and Sathyanarayan, 1997, Subramaniyan *et. al.,* 1997) and SSF by fungi (Souza *et. al.,*

2001, Someet *et. al.,* 2001, Holker *et. al.,* 2004, Senthilkumar *et. al.,* 2005, Yang *et. al.,* 2006). Fungi prefer to grow well on moist substrate, with feeble moisture content, where as bacteria are unable to grow in these conditions, they require high moisture contents. As a result, there are only fewer reports related to successful use of bacteria for SSF (Gessesse and Mamo, 1999, Rani and Nand, 2000, Virupakshi *et. al.,* 2005, Asha and Prema, in press). The major objective of the present study was isolation and identification of bacterial strain for the production of cellulase free endoxylanase, as well as to compare overall physiological behavior of *Bacillus pumilus* for endoxylanase production by liquid and solid fermentations Also the feasibility of agro industrial residues, in fermentation process and also to evaluate their production which can lead to reduction in the cost of enzyme production. The studies also aimed at the utilization of agro industrial residues treated with alkali and acid for SSF to enhance the production of endoxylanase.

2.1.0. MATERIALS AND METHODS

2.1.1. Selection of microorganism

Xylanase producing bacterial strains were taken from RRL (TVM) culture collection, which was isolated from soil samples collected from wood mills and forest regions of South Kerala (Subramaniyan, 2000). Selection from these cultures was based on high potency for xylanase production, which was active at high alkalinity and temperature, giving importance to bacterial strains. Nine bacterial strains were selected for qualitative screening.

2.1.2. Qualitative screening by plate method for xylanase activity

Initial screening was done on Xylanase screening media (XSM), rich in oatspelt xylan (Oatspelt xylan, Sigma Chemicals Co.). The nine bacterial cultures were spot- inoculated on plates with media composition (g/L) Oat spelt xylan 5.0, Yeast extract 5.0, Peptone 5.0, K_2HPO_4- 0.2, $MgSO_4$. $7H_2O$ 0.04 and agar 20 of pH 7 and 10 (Oat spelt xylan Agar plates), the pH was adjusted after autoclaving using 10 % Na_2CO_3 and 1N HCl. The plates were incubated at ambient temperature for 48 hour. The plates were then flooded

with Congo red solution (0.05 % w/v) for 30 min and destained with 1N NaCl solution till a clear zone of xylan hydrolysis was visible. The colonies, which have given clear zone, were preferred as xylanolytic isolates (Wood *et. al.,* 1988). Unless and other wise specified all sterilization was done at 121° C at 15 lbs for 15min.

2.1.3. Quantitative screening by shake flask method

The six xylanolytic bacterial strains were selected from the preceding step were quantitatively screened for xylanase production in Xylan production media (XPM) with xylan as the main carbon sources of composition Oatspelt xylan 5.0, Yeast extract 5.0, Peptone 5.0, K_2HPO_4- 0.2 and $MgSO_4$. $7H_2O$ 0.04 (Subramaniyan, 2000, Horikoshi, 1991a). Erlenmeyer flasks (250 mL) containing 90mL XPM was inoculated with 18 hour grown inoculum (5 % v/v) and incubated in incubator shaker for 144 hour at 120 rpm at ambient temperature. Samples were taken at regular interval of 24 hour and pH, cell count as well as dry weight was estimated. The cell free supernatant was recovered by centrifuging samples at 10,000 rpm at 4 °C for 20 min (SELECTA- Cold centrifuge, Germany) and used for measuring the xylanase activity, total soluble protein and reducing sugar.

2.1.4. Pre inoculum preparation

The inoculum was raised in the same medium (Section 2.1.3) under similar conditions in 150 mL Erlenmeyer flasks and 18 hour grown inoculum was used to initiate growth. A loop full of culture was inoculated in the medium and incubated for 18 hour in rotary shaker (120 rpm) at ambient temperature (30 +/- 2 °C).

2.1.5. Production of xylanase

Production of xylanase from selected isolates was studied in correlation with the growth profile of culture in the medium of composition Wheat bran 5.0, Yeast extract 5.0, Peptone 5.0, K_2HPO_4- 0.2 and $MgSO_4$. $7H_2O$ 0.04. Fermentation experiments were carried out using conditions already mentioned. Cells were suspended by centrifugation

at 10,000 x g for 20 min at 4°C and cells free supernatant was used for extracellular crude enzyme preparations.

2.1.6. Identification of Bacteria

Following the procedure of Bergey's manual of Systematic Bacteriology the selected bacterial strain was identified (For *Bacillus* sp. Claus and Berkeley's, 1986). Growth pattern was studied by culturing in liquid fermentation media, thus identified whether aerobic or anaerobic, spore staining done using Malachite green, motility test, gram staining for classification of the isolate to specific genera. Biochemical characterization was done this includes, M.R - V.P test, catalase test, urease test, nitrates test, indole test, starch hydrolysis test, and gelatin liquefaction test (Subramaniyan, 2000). I.M. Tech, Chandigar, (India) did identification of the selected strain.

2.1.7 Enzyme profile in SmF and SSF

Selected culture was subjected to plate assay of different carbon sources to check the production qualitatively. Cellulase production during the screening procedure was checked by incorporating plate with Carboxy methylcellulose, Protease by casein, amylase by starch and pectinase by pectin. That plate which has given clear zone was checked quantitatively for the production.

2.1.8. Analytical procedures

2.1.8.1. pH

The pH of the culture filtrate was estimated using Cyber scan 1000 pH meter.

2.1.8.2. Moisture content

The moisture content of the substrate was estimated by direct dry weight method (Pandey *et. al.*, 2001).

2.1.8.3. Reducing sugar

Reducing sugar of the extract was estimated by dinitrosalicylic acid method or DNS method (Miller, 1959). The reducing sugar was estimated using standard graph prepared with glucose. Reaction with glucose 3, 5 - Dinitrosalicylic acid (DNS) gets reduces to 3-amino 5- nitro salicylic acid while the sugar gets oxidized to gluconic acid.

2.1.8.4. Total soluble protein

Total soluble protein of the extract was estimated by Lowry's method (Lowry *et. al.,* 1951). The principle behind this method of determining protein concentrations lies in the reactivity of the peptide nitrogen[s] with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin Ciocalteau phosphormolybdic /phosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids. The protein standard was prepared using Bovine Serum Albumin (BSA).

2.1.8.5. Endoxylanase assay

Endo xylanase (1, 4, β - D - xylan xylanohydrolase- E.C. 3.2.1.8) was assayed by some modification to Bailey method (Bailey *et. al.,* 1992). Preparation of xylan suspension or substrate - 0.5 % of oat spelt xylan (Sigma Chemicals Co.) in 0.1M phosphate buffer (Na_2HPO_4/ NaH_2PO_4), at pH -7 and 0.1 M carbonate buffer at pH- 10 (Na_2CO_3/ $NaHCO_3$), by warming at 80 °C and made into paste in an homogenizer, later it was boiled and made up to required volume. The solution was cooled and stored at 4 °C. The reaction mixture contains 1.8 mL of preincubated xylan suspension and 0.2 mL of suitably diluted enzyme preparation was added and incubated at 50 °C for 10 min. The reaction was terminated by the addition of 3 mL of DNS, and boiling it in boiling water bath for 5 min followed by sudden cooling in ice bath. Enzyme blank was prepared in parallel by adding DNS reagent prior to enzyme addition so that only the reducing sugar in the enzyme will be answered. This was required only when the sample contains high reducing sugar or the dilution was small. The reagent blank too was prepared in same procedure but instead of 0.2 mL enzyme buffer was added. The concentration of reducing sugar -xylose liberated was estimated against xylose standard prepared at 540 nm. The

stock solution of xylose (Xylose, Merck) standard was prepared in 10 μ mole / mL concentration and appropriate dilution was taken for preparation of standard. One unit of endoxylanase activity was defined as 1μ mole of xylose liberated per minute per mL of enzyme preparation under the assay conditions.

2.1.8.6. Cellulase assay

The plates were incorporated with Carboxymethyl cellulose and cultures were spotted. Later during the fermentation studies selected culture was assayed for CMC ase and FP ase using cell free supernatant. Absence of cellulase activity was essential with high xylanase production, due to its importance in paper and pulp industry.

2.1.8.6.1. CMC ase -(1, 4-(1, 3:1, 4) - β- D- glucan-4-glucano hydrolase)

CMC ase (1, 4-(1, 3 : 1, 4) - β- D- glucan – 4 - glucano hydrolase (E.C. 3.2.1.4) or Carboxymethyl cellulase assay was carried out using sodium salt of Carboxymethyl cellulose in 0.1 M phosphate buffer pH - 7 as substrate (Dahlberg *et. al.,* 1993), the reducing sugar liberated was estimated by DNS method. To 1.0 mL of preincubated substrate suspension kept at 50 °C added 0.5 mL of crude enzyme suitably diluted. The reaction mixture was incubated for 15 min. The reaction was terminated by the addition of 3.0 mL of DNS reagent and heated in boiling water bath for 5 min followed by sudden cooling in ice bath. Absorbance was taken against glucose standard at 540 nm and CMC ase activity expressed as one μmol of glucose liberated per minute per mL of culture supernatant.

2.1.8.6.2. FP ase activity (1, 4-β-D-glucan cellobiohydrolase)

FP ase activity or cellobiohydrolase (E. C. 3.2.1.91) activity was estimated by modified Mandel method (Mandel *et. al.,* 1976). Whatman No.1 filter paper (0.05g) was used as substrate; Filter paper of 1x 6 cm was cut into pieces. The reaction mixture contain 0.5 mL of preincubated phosphate buffer (pH - 7, 0.1 M) with filter paper, to this added 0.5 mL of suitably diluted enzyme and incubated at 50 °C for 1 hour. The reaction was stopped by the addition of 3 mL DNS reagent and boiled for 5 min followed by sudden

cooling. The absorbance of the reaction was taken at 540nm against glucose standard; the reducing sugar liberated was expressed as one μmol of glucose produced per mL per minute under standard assay conditions.

2.1.8.7. Protease assay

The protease was assayed by TCA method (Subramaniyan *et. al.,* 2001). Two percent casein was used as substrate in carbonate buffer pH - 10. The preincubated reaction mixture contain 0.5 mL of suspension of casein to this added 0.5 mL of suitably diluted enzyme and incubated at 40 °C for 10 min. The reaction was terminated by the addition of 10 % TCA (tricholoro acetic acid). The mixture was centrifuged at 10,000 rpm at 4 °C for 10 min and supernatant was collected. From this supernatant 0.5 mL of reaction mixture was mixed with 5 mL of 0.5 M Na_2CO_3, and kept for 10min; to this add 1mL of 1N Folin Ciocalteau's reagent and the tubes were incubated for 30 min in dark for colour development. Enzyme blank and reagent blank were prepared in parallel with test solution. The readings were taken at 600 nm against tyrosine standard and one unit of enzyme was expressed as one μmol of tyrosine liberated per mL per minute.

2.1.8.8. β- Xylosidase assay

Incubating 1 mL of 1mg/mL of O-nitro phenyl- β-D- xylopyranoside with 0.1 mL suitably diluted enzyme in 50 mM phosphate buffer pH - 7 at 50 °C for 30 min reaction was terminated by addition of 2 mL of 1 M Na_2CO_3. The liberated O-nitro phenol was measured at 410 nm (Flanigan and Sellars, 1977).

2.2.0. Fermentation experiments

Submerged or liquid fermentation using different agro industrial residues was studied and the results were compared with that of Oat spelt xylan as carbon sources. Optimization of culture conditions for Submerged fermentation using wheat bran was tried. Solid-state fermentation was done with different agro industrial residues and production was compared with that of acid and alkali treated residues. Comparison of SmF and SSF were done to select suitable mode of fermentation.

2.2.1 Pre inoculum preparation for SmF and SSF

The inoculum was raised in the same medium (Section 2.1.3) in 150 mL Erlenmeyer flasks and 18 hour grown inoculum was used to initiate growth. Properly inoculated media was incubated for 18 hour in water bath shaker (LAB-LINE Instruments Incorp. Orbit water bath shaker) set at 150 rpm at ambient temperature (32 +/- 2 °C). This was used as preinoculum for both SmF and SSF studies. The cell count was estimated using haemocytometer and it was estimated as ~3.6 x 10^6 cell count /mL.

2.2.2. SUBMERGED FERMENTATION

2.2.3. Effect of different carbon sources on xylanase production

Liquid mode of fermentation was carried out using media composition as follows (g/L): Yeast extract 5.0, Peptone 5.0, K_2HPO_4- 0.2 and $MgSO_4$. $7H_2O$ 0.04 and Oat spelt xylan (5.0) was replaced with other simple agro industrial residues like wheat bran, rice straw, saw dust, coconut pith, sugarcane bagasse, rice bran and compared with the production related to oat spelt xylan. The flasks were incubated in a water bath shaker set at 150 rpm. Enzyme was extracted at regular interval of 24 hour and assayed for reducing sugar, endoxylanase, biomass and total soluble protein.

2.2.4. Effect of pH on xylanase production

In order to study the effect of initial media pH on enzyme production by *Bacillus pumilus* by SmF, initial media pH was adjusted. The experiment was carried out in modified Horikoshi -II basal media of composition as follows wheat bran 5.0, Yeast extract 5.0, Peptone 5.0, K_2HPO_4- 0.2 and $MgSO_4$. $7H_2O$ 0.04. The initial media pH was adjusted within the range of 5 to 10 with an increment of 1.0 unit (after autoclaving). In all cases the initial pH was uncontrolled through out the experimentation. The variation in pH was also recorded through out the studies. Enzyme was extracted at regular interval of 24 hour and estimated for pH, reducing sugar, endoxylanase, biomass and total soluble protein. Growth was correlated with the enzyme production and variation in pH.

2.2.5. Effect of temperature on endoxylanase production

Effect of temperature on enzyme production by *Bacillus pumilus* was analyzed by varying the incubation temperature ranging from 20 to 65 °C (20, 30, 35, 40, 45, 50, 55 and 65 °C). The temperature was set in the environmental incubator shaker (New Bruenswik, Scientific Edison, N.J., USA). The initial pH in all case was set at 8.5 with wheat bran as substrate. The fermentation was carried out for 120 hour and samples were taken at regular interval of 24 hour for estimation.

2.2.6. Effect of inoculum sizes on xylanase production

Inoculum was raised with media composition similar to the fermentation media; this was done to curtail the lag phase in fermentation process (Lincoln, 1960). Inoculum was transferred at logarithmic phase of growth. Different concentration of inoculum was studied (%) 1, 2.5, 5, 7.5 and 10. Cell growth was monitored by measuring the optical density (OD) of suitably diluted culture broth at 600 nm. The cell count was taken by serial dilution in sterile saline and 0.1 mL was plated on nutrient agar plate. Number of cell was counted in plate counter and mean of the data was taken. Growth can also estimate by dry weight method (Pandey *et. al.,* 2000). Concentration above 10 % was not feasible to industrial process (Lincoln, 1960). Samples were taken at regular interval and estimated for 120 hour.

2.2.7. Effect of inducers on xylanase production

Different simple sugars were added to the liquid fermentation medium as carbon sources (1 %) replacing xylan from the Horikoshi basal media II. The worked aimed at the effect of different carbon sources on the enhancement of xylanase production by *Bacillus pumilus.* Different carbon sources supplemented are mono-sugars like- xylose, glucose, fructose, galactose, disaccharides like- sucrose, lactose, maltose and polysaccharides like- xylan, starch, wheat bran and rice bran.

2.3.0 SOLID-STATE FERMENTATION

Erlenmeyer flask (250 mL) containing 10 g of substrate, was mixed with basal medium of composition (g/L) Yeast extract 5.0, Peptone 5.0, K_2HPO_4 2.0, and $MgSO_4$ 0.4, were autoclaved at 15-lbs / inch pressure for 45 min. The cooled sterilized medium was inoculated with 18 hour grown inoculum and incubated for 120 hour. The samples were taken and extracted at regular interval of 24 hour, the content was extracted with 10 fold (v/w) distilled waster. The content was filtered using cheesecloth and filtrate was centrifuged at 10,000-x g for 20 min at 4 °C (SELECTA cold centrifuge, Germany), the clarified supernatant was used as crude enzyme. All experiments were done in triplicate, individually and no optimized condition of one is carried over to another.

2.3.1. Enzyme production using agro-industrial residues

The fermentation media was prepared as described earlier in Section 2.3.0. The different substrates selected for SSF studies were: - rice bran, rice husk, rice straw, sawdust, coconut pith, sugarcane bagasse, thur-dal husk, Soy meal flakes and wheat bran. The autoclaved media was cooled and inoculated with 18 hour grown culture, preinoculum prepared as mentioned in section 2.1.4. The flasks were incubated at ambient temperature, samples were taken at regular intervals and extracted as mentioned earlier. The supernatant obtained after centrifugation was used as the crude enzyme source.

2.3.2. Effect of pretreatment of agro industrial residues on xylanase production

In order to study the effect of acid and alkali treatment on xylanase production, different agro industrial residues like straw, coir pith, sawdust, bagasse, wheat bran, rice husk and rice bran were pretreated. 1 N NaOH for alkali treatment and 1 N HCl for acid treatment followed by autoclaving at 15 lbs for 30 min. It was then washed to neutral pH and dried at 60 °C before use in fermentation studies. Fermentation studies were carried out as mentioned earlier and results compared with untreated.

2.4.0. RESULTS AND DISCUSSION

2.4.1. Importance of alkaline thermostable microorganism

The significance of xylanase and its application in wide area of industries has made it a key enzyme. The interest in xylanase has been raised markedly in the recent years, mainly because of their use in various industries like bread and baking, food and feed and particularly pulp and paper industry, mainly for the bleaching process (Viikari *et. al.,* 1994. Beg *et. al.,* 2001, Subramaniyan and Prema, 2002). For such biotechnological applications xylanase should be devoid of cellulase activity as well as stable at elevated temperatures and active at alkaline pH. Manufacturers continuously search for better performing enzymes by screening different microorganisms or by developing tailor-made enzymes using molecular engineering. Only few bacterial and actinomycete have been reported earlier, which are active at high alkalinity or neutral pH (Horikoshi and Atsukawa, 1973, Nakamura *et. al.,* 1994, Durate *et. al.,* 2000, Kamal *et. al.,* 2004, Asha and Prema, in press). Various methods have been adopted for the screening of microorganism that was capable of degrading lignocellulosic matter. The screening was complicated by the fact that each main components of lignocellulosic material; requires various enzymes which were controlled by elaborate pathways of biochemical and genetic regulation (Kluepfel *et. al.,* 1986).

2.4.2 Screening for xylanolytic potential

The major aim of the study was to isolate a potent producer of xylanase form the various sources, mainly soil isolates. Of the nine bacterial isolates taken for xyloanlytic activity by spot inoculation on xylan agar plates, the plates that have shown clear zone by xylan hydrolysis were selected by measuring the diameter of clearance. Oat spelt xylan used as the sole carbon sources, this help to eliminate those strains that are not specific for xylanase. Observing the area of clear zone produced differentiated the potent xylanase producers; six of those, which have shown clear zone, were selected for further studies. Since, xylan polymer is of large molecular weight, it could not enter the microbial cell,

the endoxylanase produced by most of the microorganism were extracellular in nature (Biely, 1985). All cultures that has produced clear zone might not be good producers of endoxylanase.

Isolate No.	pH	Soluble protein mg/mL	Reducing sugar mg/mL	Biomass mg/mL	Maximum hour of production	Xylanase activity	
						pH-7	pH-10
1	9.1	2.9	0.314	2.1	96	0.15	1.01
13	9.1	2.5	0.346	2.2	96	1.34	1.16
20	9.2	2.4	0.0994	1.2	144	0.18	0.1
21	8.4	2.5	0.452	4.7	144	11.75	0.05
34	**8.5**	**3.3**	**0.058**	**2.5**	**96**	**92.3**	**4.41**
53	8.1	2.8	0.36	4.4	120	2.95	0.58

Table. 2. 1. Growth profile and xylanase production of selected bacterial strains in quantitative screening

When the isolates grown on solid agar medium the presences of membrane bound hydrolyses may produces the clearing by xylan hydrolysis. This can also be due to the regional presences of enzyme secreted near the growing colony in the agar plates. This will not occur in liquid medium, so all above selected strains were subjected to liquid fermentation using oat spelt xylan, followed by xylanase assay to get a clear picture of xylanolytic enzyme produces as well as a quantitative estimation was also possible (Table. 2. 1.). Of all the isolates selected only one has highest production, indicating that all others have produced clearing due to the occurrence of regional presences of xylanase and/or membrane bound xylanase that has lead to good clearing zone on solid agar plate (Aunstrup, 1974).

2.4.3. Detection of production by plate assay

The selected bacterial strain (Fig. 2. 1.) produced clear halo of xylan hydrolysis on xylan agar plates when stained with Congo red and destained with NaCl (Fig. 2. 2.). A clear

zone of hydrolysis was noticed when the culture filtrate was dropped in wells on xylan plates stained with Congo red. I. M. Tech, Chandigar, identified the selected bacterial stain as *Bacillus pumilus,* based on the morphological and biochemical characters as given in Table. 2. 2.

Bacillus species are dominant heterologous enzyme producing microorganism (Priest, 1992) which are very much attracted by industries for a varying reasons, including their high growth rates leading to short fermentation cycle times, their capacity to secrete proteins into extracellular medium and the GRAS status (Boren *et. al.,* 1998), with food and drug administration for species such as *B. subtilis* and *B. lichineniformis*. The biochemistry, physiology and genetics are well studied, facilitating further development and greater exploitation for industrial purposes (Schallmey *et. al.,* 2004). *Bacilli* are gram positive they lack lipo-polysaccharide in the cell wall, so will not produce endo-toxins, which is encountered with gram negative strains. There are number of reports related to endo-xylanase production by *Bacilli* (Uchino and Nakane, 1981, Khasin *et. al.,* 1993, Subramaniyan *et. al.,* 1997, Gessess and Mamo, 1999, Dhillon *et. al.,* 2000, Virupaskhi *et. al.,* 2005, Asha and Prema, in press).

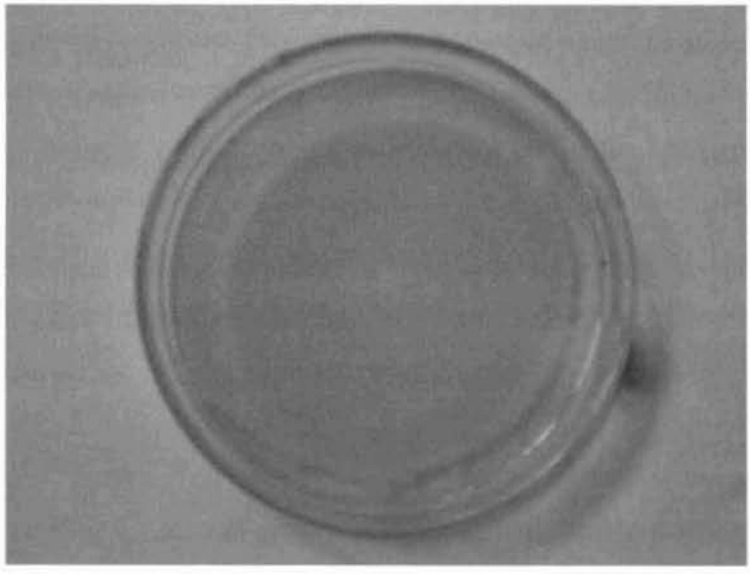


Fig -2.1. Xylan Agar plate with 24 hour grown culture

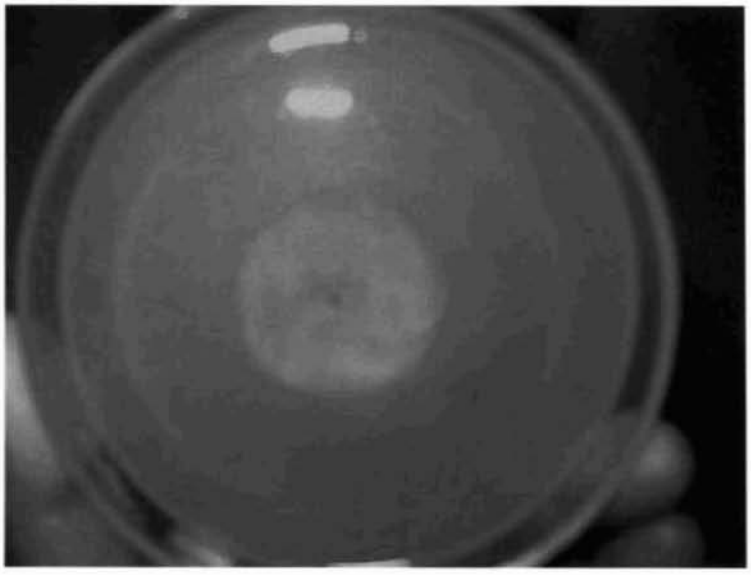


Fig. 2. 2. Plate assay for xylan hydrolysis

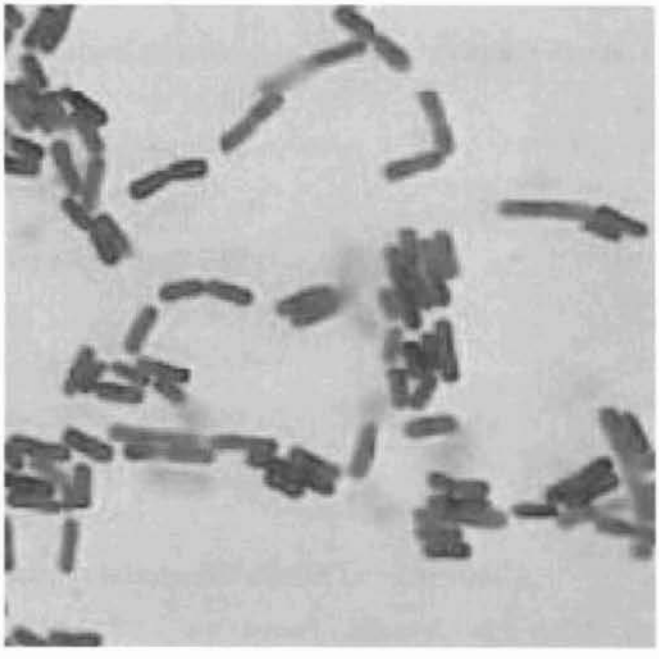


Fig. 2. 3. Micrograph of *B. pumilus* culture

Name of test	Result
Gram staining	+ve
Spore staining	+ve, Shape oval
Sporangia bulging	+ve
Position of the spore	Lateral
Anaerobic growth	-ve
Voges -Proskaur	+ve
Casein hydrolysis	+ve
Starch hydrolysis	-ve
Citrate utilization	+ve
Acid production	
Glucose	+ve
Arabinose	+ve, (weak)
Xylose	-ve
Manitol	+ve
Growth temperature (^{o}C)	
4	Nill
15	+ve
25	+ve
30	+ve
37	+ve
42	+ve
50	+ve, weak

Table. 2. 2. Identification character of selected bacterial strain provided by I. M. Tech, Chandigar, India. Based on Fatty Acid profile and few diagnostic characters of *B. pumilus* the strain has been identified as *Bacillus pumilus* – GC Sub Group- B.

2.4.4. Culture characteristics of *Bacillus pumilus*

Colour - Creamish
Shape - circular
Margin - irregular
Elevation - flat
Opacity - opaque
Texture - smooth, with wrinkles on aging
Consistency - mucoid

2.4.5. Growth in liquid medium

In liquid medium, uniform growth was observed which settle to form sediment in static conditions.

2.4.6. Pigment production

No pigment productions observed on prolong incubation.

2.4.7. Morphological characteristics

Cell shape: - long, slender rods (Fig. 2. 3).

2.4.8. Taxonomy of *Bacillus pumilus*

Kingdom: *Bacteria*
Phylum: *Firmicutes*
Class: *Bacilli*
Order: *Bacillales*
Family: *Bacillaceae*
Genus: *Bacillus*
Species: *Bacillus pumilus*

2.4.9. Growth profile of *Bacillus pumilus*

Growth profile of the *Bacillus pumilus* shows (Fig. 2. 4.) that xylanase production seldom occur at the early hour of incubation, the activity was observed at the post exponential period of incubation or during the stationary phase (Saiman *et. al.*, 1992, Nakamura *et. al.*, 1993a). There was a shift in pH toward 8.5 during maximization of production (Table. 2. 3).

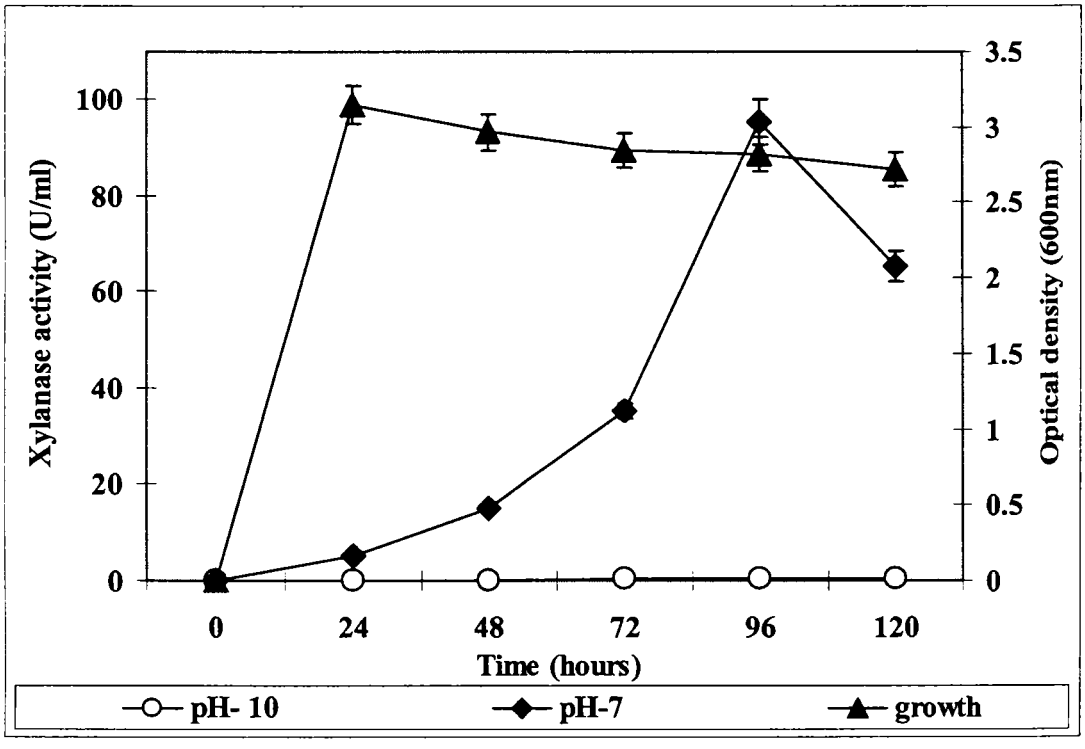


Fig. 2. 4. Fermentation profile of *B. pumilus* in liquid medium, growth and enzyme production (pH- 10 and 7).

The culture has attained stationary phase of growth by 24 hour of incubation, but during this period low amount of xylanase was detected, where as during the end of stationary phase there was a drastic increase in enzyme production. Similar reports were with

Aspergillus (Espinar *et. al.,* 1992), *Aspergillus niger* in acidic pH range (Ikram-ul- Haq *et. al.,* 2002) and with *Bacillus* (Nakamura *et. al.,* 1993, Subramaniyan *et. al.,* 1997, Shabeb, 2000).

Low xylanase activity might be due to the adsorption of xylanase on the surface of insoluble xylan particles present in the culture medium (Espinar *et. al.,* 1992, Irwin *et. al.,* 1994). The higher xylanase activity during the lag phase of growth can be counted as the reflection of small amount of xylanase liberated by the cell undergoing autolysis (Espinar *et. al.,* 1992, Subramaniyan *et. al.,* 1997) as well as non availability of insoluble particle in the medium which if present might have bounded to the xylanase (Connerton *et. al.,* 1999). Decline in production after 120 hour of incubation can be considered as the result of intracellular protease from autolysed cells that inactivate xylanase.

Time (hour)	Total soluble protein (mg/mL)	Reducing Sugar (mg/mL)	Variation in pH of the medium
0	0.348	4.1	6.5
24	0.395	3.7	7.5
48	0.120	3.3	8.3
72	**0.118**	**2.7**	**8.5**
96	0.059	2.2	8.6
120	0.075	2.1	9.1

Table. 2. 3. Fermentation profile of *B. pumilus*, the variation in pH of the fermentation medium at regular interval, reducing sugar and total soluble protein was monitored.

Reducing sugar was high during the early hour of fermentation due to the presences of xylanase in significant amount in the inoculum, which causes the hydrolysis of xylan in the medium. During maximization of production the level of reducing sugar was considerably low. This can be explained as depletion of reducing sugar related to growth

of bacteria which causes the reversion of catabolic repression, as well as the insufficient amount of xylan at later stage of fermentation which leads to adsorption of xylanase molecules and autolysis of cell (Espinar *et. al.,* 1992).

Bocchini *et. al.,* (2005) reported that during the cultivation of *Bacillus circulans*, for xylanase production, there was a decrease of sugar concentration, concurrently with an increase of cellular growth, indicating that the sugar was used as substrate by the microorganism. The highest enzyme production coincided with the smallest sugar concentration level. Total soluble protein and reducing sugar together with variation in pH during the time course of fermentation was explained in Table. 2. 3. There was no considerable variation in total soluble protein and as production starts there was a slight decrease. pH of the medium has shown considerable variation during the latter fermentation hour, it has changed more towards alkaline side. Maximum xylanase production (Fig. 2. 4.) was observed at 96 hour of incubation (95.3 IU/ mL). Earlier similar reports were there related to xylanase production by *Bacillus* of 6.23 IU/ mL (Liu *et. al.,* 1998), 2.6 - 4 U/ mL (Duarte *et. al.*, 1999), 4.8 U/ mL (Ratanakhanokchai *et. al.,* 1999) and 118.5 IU/ mL (Shabeb, 2000). The production of *Bacillus pumilus* was comparable to earlier reports, which can be maximized by optimization of culture conditions.

2.5.0. SUBMERGED FERMENTATION

2.5.1. Effect of different carbon sources on xylanase production

To minimize the production cost various agro industrial residues were tried and the result on time course of xylanase production was given in Fig. 2. 5. The results explained that wheat bran (65.5 IU / mL) as suitable sources for xylanase production but not relative to oat spelt xylan (94.9 IU / mL). Rice straw and rice bran can also be used as good sources for xylanase production where as coconut pith and sugar cane bagasse has considerable less production, the biomass as well as reducing sugar and soluble protein were also very less.

The industrial enzymes production is often limited by the costs of substrates for the cultivation of the producer microorganisms. The use of low cost substrates, such as agricultural wastes has been suggested as an alternative to reduce the production costs (Dhillon *et. al.*, 2000, Bocchini *et. al.*, 2005). With wheat bran high level of xylanase was reported by *Bacillus* sp. NCIM 59 (Dey *et. al.*, 1992, Archana and Sathyanarayan, 1997).

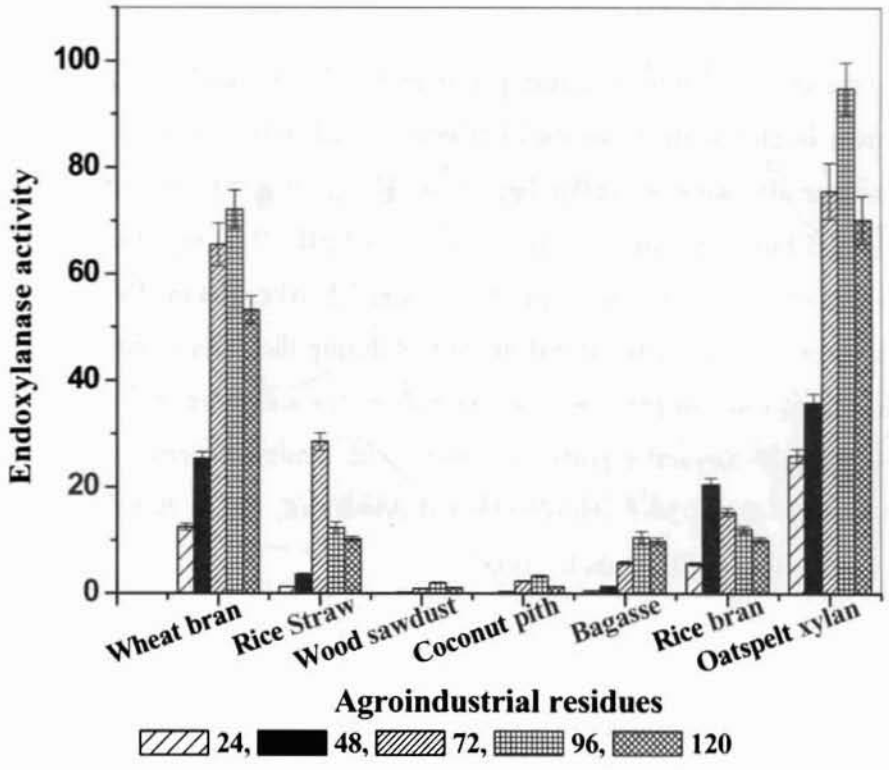


Fig. 2. 5. Time course of xylanase production by *B. pumilus* on various agro industrial residues at 30°C under SmF.

Subramanıyan has reported oats pelt xylan as suitable carbon sources for the enhancement of xylanase production by *Bacillus* SSP-34 (Subramaniyan and Prema, 1998). Wheat bran has been reported as a suitable source of xylanase production in SmF by fungus *A. niger* GCBMX-45 (Ikram-ul- Haq *et. al.*, 2002) and *A. nidulans* KK-99 of

40 IU/ mL (Taneja *et. al.,* 2002). There were reports of efficient utilization of sugar cane bagasse (8.4 U / mL) and grass hydrolysates (7.5 U / mL) as carbon sources for xylanase production by *B. circulans* by SmF (Bocchini *et. al.,* 2005). To minimize the cost of production wheat bran was taken for further optimization studies. *Thermomyces lanuginosus* IOC-4145 was able to produce a very high level of cellulase-free xylanase (266 U / mL) in shake flasks using corncob as substrate (Damaso *et. al.,* 2000).

2.5.2. Effect of pH on xylanase production

In order to study the effect of initial medium pH on endoxylanase production by *Bacillus pumilus* was grown in Horikoshi basal medium with varied initial medium pH ranging from 5 to 10 and results were given in Fig. 2. 6. The culture has shown maximum production at pH- 8.5 but maximum growth was observed pH - 9. A significant increase in pH towards 8 was observed with initial pH 5, 6, 7 and 7.5, where as medium with pH - 8.5, 9, 9.5 and 10 there was a decline in pH towards 8 during the early period of growth. Later as growth progress and enzyme production maximizes and there was considerable increase in pH towards 9. Several reports were there with similar pattern of growth and change in pH, *Bacillus* no. C-59-2 (Horikoshi and Atsukawa, 1973) *Bacillus*- SSP-34 (Subramaniyan and Prema, 1998, Shabeb, 2000).

Important factor in any fermentation process is pH, and it may change in response to metabolic activities. Each microorganism possesses a pH range for its growth and activity with an optimum value within the range. The initial pH of the medium has strong influence on enzyme production; it influences many enzymatic system and transport of several species of enzymes across the cell membrane (Moon and Parulkar, 1991). The H^+ concentration of the external environment of the organism, to which it gets adapted, is an important factor that influences growth, production and stability of metabolite produced by it (Horikoshi, 1999). The most obvious reason is the secretion of organic acids that will cause the pH to drop. On the other hand, the assimilation of organic acids, which may be present in some media, will lead to an increase in pH, and urea hydrolysis will result in alkalinization (Krishna, 2005). The shift in media pH towards alkalinity during

maximization of production indicates that the organism was alkalophilic. Similarly *A. niger* has also reported to show a shift in pH towards alkalinity during maximization of production in SmF (Yuan *et. al.*, 2005).

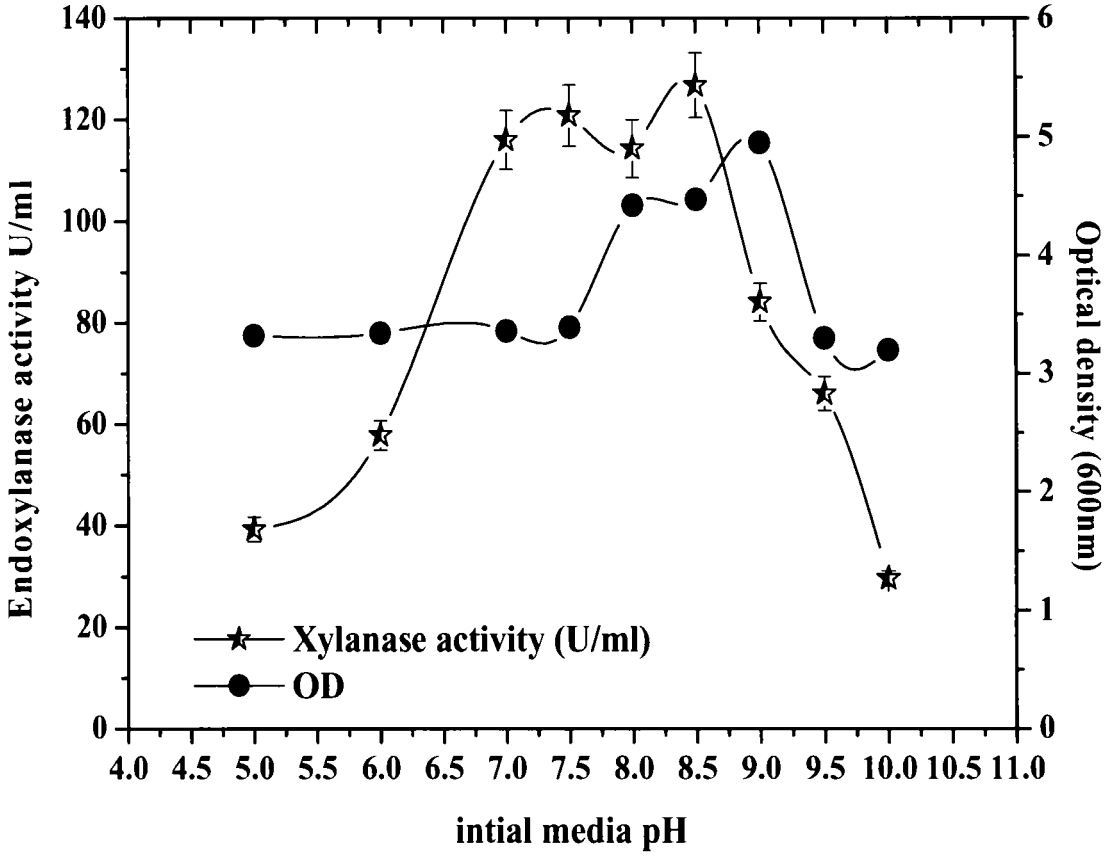


Fig. 2. 6. Influences of initial medium pH on xylanase production by *B. pumilus* with wheat bran as carbon sources at 30°C under SmF.

Xylanase production near neutral was reported in case of *B. circulans* WL-12 (Esteban *et. al.*, 1982). Many bacterial species reported to be productive at alkaline range. Similar reports related to alkalophilic organism were there (Subramaniyan, 2000, Horikoshi, 1999, Kubata *et. al.*, 1994). Most of the alkalophilic xylanolytic enzyme producers have similar optimum pH range from 5.5- 9 (Kulkarni *et. al.*, 1999, Shabeb, 2000).

2.5.3. Effect of temperature on endoxylanase production

Generally microbes produce enzymes optimally during their optimum growth temperature. The enzyme activity of crude extract from *Bacillus pumilus* grown at different temperature ranging from 20 to 65 °C at a constant pH of 8.5 and substrate wheat bran 0.5 % was assayed and results were given in Fig. 2. 7.

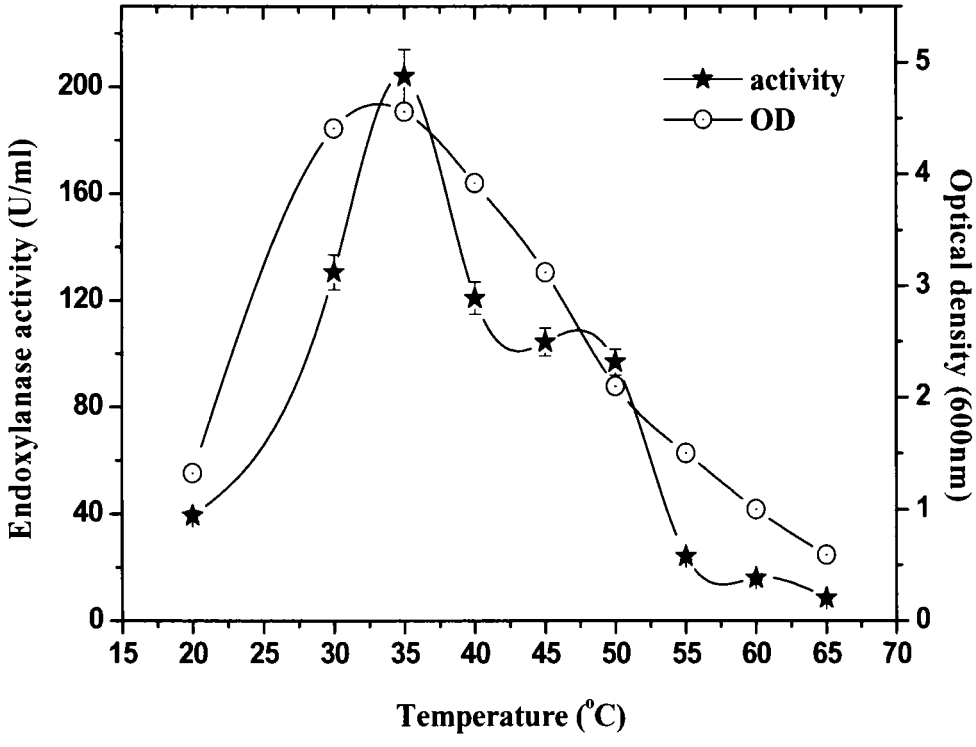


Fig. 2. 7. Influences of temperature on xylanase production by *Bacillus pumilus* with wheat bran as carbon sources at pH 8.5 under SmF

Optimum activity was recorded at 35 °C, it was relative to the production reported by *Bacillus* sp. BP - 7 (Lopez *et. al.*, 1998). At temperature 30 and 40 °C almost 59 % activity was retained at 96 hour of incubation and at 45 and 50 °C 49 % of activity was retained. Increase in growth temperature above 50 °C has lowered the xylanase production, but even at 60 °C there was ~7 % of activity. Maximum growth was recorded

at 35 °C related to enzyme production. The growth and enzyme production by the microorganism indicates that this is a mesophilic culture but its production even at 50 °C could classify this organism under thermo tolerant bacteria (Dubeau *et. al.,* 1987, Subramaniyan and Prema, 1998). There were reports of xylanase production at high temperature by *B. stearothermophilus* T-6 at 65-70 °C (Khasin *et. al.,* 1993), where as Esteban has reported growth and maximum enzyme production at 65 and 37 °C (Esteban *et. al.,* 1982). There was a report related to production of alkaline xylanase from fungus *A. nidulans* KK-99 at 37 °C at pH-10 by SmF (Taneja *et. al.,* 2002).

2.5.4. Effect of inoculum sizes on enzyme production

Several advantages have been cited in the use of spores rather than vegetative cells for inoculum. They can serve as a biocatalyst in bioconversion reactions because they are often able to carry out the same reactions as the corresponding mycelium (Larroche, 1996). The fermentation profile of an organism is affected by the inoculum concentration and physiological conditions. It also helps to minimize the time lag in fermentation process, by growing preinoulum in same fermentation medium (Krishna, 2001, Lincoln, 1960). Some organisms require vegetative inocula in which the amount of spores will be very high and young, those are highly metabolically active and can initiate the fermentation in much faster rate. The results were given in Fig. 2. 8., which show that incoulum concentration of 2.5 to 5 %, were almost equal in the production rate, where as high concentration above 7.5 to 10 % has reduced the production by 20 %. Low percentage of inoculum less than 1 % observed to be less productive and it takes more time to start the production and the biomass was also very low.

There are reports related to high production with low inocula concentration by *Bacillus* sp. (Sen and Satyanarayana, 1993, Subramaniyan and Prema, 1998). The transfer of small quantities of xylanase synthesized in the xylan preinoculum medium increases the initial reducing sugar level in the fermentation medium. Higher percentage of spore in the inoculum has reported to be a leading factor for long lag phase and thus slow down the fermentation rate (Meyrath and Suchanek, 1972). This was the reason why industries

prefer low percentage of preinoculum in fermentation process (Lincoln, 1960). It was noticed that low concentration of inocula (1 %) was efficient in enzyme production, may be due to low cell density. Higher concentration of more than 7.5 % might transfer compounds with preinocula that may resist higher enzyme production (Lincoln, 1960).

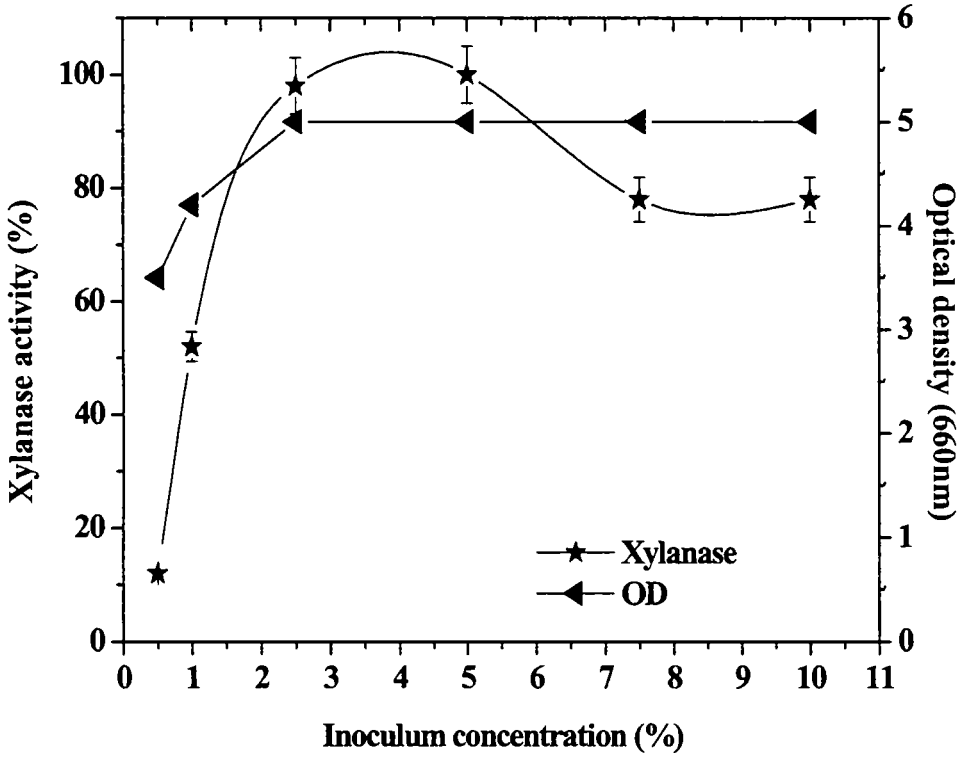


Fig. 2. 8. Influences of inoculum concentration on xylanase production by *B. pumilus* with wheat bran as carbon sources at pH - 8.5 and 35°C under SmF.

2.5.5. Effect of inducers in xylanase production

Carbon sources supplemented in the medium have a profound effect on the production and growth behavior of the organism. Some carbon sources promote good growth and production, but some promotes growth and inhibits production. The rate of incorporation

of a carbon sources could often influences the formation of biomass and production of primary or secondary metabolites.

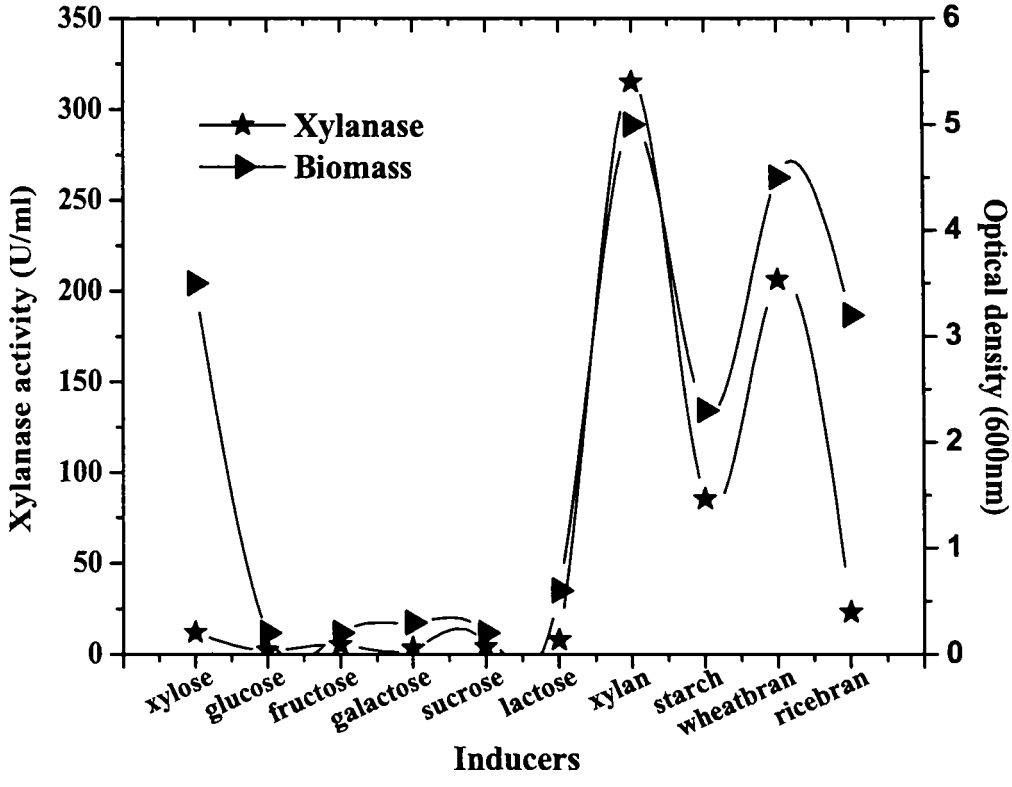


Fig. 2. 9. Effect of inducers on xylanase production by *Bacillus pumilus* with wheat bran as carbon sources at pH - 8.5 and 35°C under SmF.

Rapid growth from high concentration of easily available metabolites always affects the production (Hoq *et. al.,* 1994). Single carbo-hydrate material can act both as an energy sources and carbon sources and the main final product of fermentation help to decide the choice of carbon sources. The results of utilization of easily metabolisable sugars as additives (1 %), replacing wheat bran in the medium and combined with the production related to Oat spelt xylan as well as wheat bran in optimized condition (Fig. 2. 9).

The graph shows that xylose in low concentration have an inducing effect on xylanase production. Where as all other sugars have an inhibitory effect. There was report related to the inducing effect of xylose and xylan and inhibitory effect of glucose and other sugars xylanase production by *Bacillus subtilus* CD- 4 (Gessesse and Mamo, 1998). When xylanase fermentation was carried out in complex heterogeneous substrates, various factors have to be noticed for effective xylanase expression. This includes substrate accessibility and the rate of amount of xylooligosaccharides released, that act as a carbon sources and represses xylanase synthesis. Generally a slow release of inducers and the possibility of converting the inducers to its non-metabolisable forms are known to improve the level of xylanase production (Kadowaki *et. al.,* 1997). *Thermomyces lanuginosus* IOC-4145, xylan or xylan containing substrates, such as corncob (266 U/mL) and their precursor, xylose, induced maximum level of xylanase free of cellulase, while the easily metabolisable sugars (fructose, glucose and maltose) appeared to repress xylanase synthesis (Damaso *et. al.,* 2000). *A. foetidus* MTCC 4898 maximum xylanase yield (210 U/mL) was obtained in SmF with negligible cellulase activity at 30 °C with 1 % birch wood xylan as substrate in 3 days (Shah and Madamwar, 2005). Bocchini *et. al.,* (2005) reported that grass and sugarcane hydrolysates, which contain sugars, mainly xylose, mannose, arabinose and galactose, have an inducing effect on xylanase production in SmF by *B. circulans* D1.

2.6.0. SOLID-STATE FERMENTATION

In Western countries, important problems have originated recently, such as pollution of soils, the potential use of bioremediation, as well as the necessity to find alternatives for animal feeding (Durand, 2003) which has directed the search for new technologies to produces by product utilizing these sources. SmF is advantageous as it is well characterized, and homogeneous conditions are maintained throughout and it is easier to scale up. However, SmF being an energy intensive process, SSF is gaining more importance. Economic analysis has indicated that SSF technology can considerably reduce the capital investment and total production cost as well as increases profitability, thereby making it an ideal technology in several industrial sectors (Castilho, 2000). It is

gaining more and more attention in recent years, due to the possibility of using cheap and abundant agro industrial waste as substrates, higher productivity, simplicity, low energy requirement, better recovery of product, lesser waste water output, and there is no catabolic repression (Pandey *et. al.,* 2000, Krishna, 2005). Nevertheless, its use is limited by the fact that not all organisms are able to grow in SSF, and the process cannot be well characterized. Fungi produce higher levels of xylanase than bacteria or yeasts. However, fungal xylanases are generally associated with cellulase (Steiner *et. al.,* 1987) and more active in acidic range (Haltrich *et. al.,* 1996). Utilization of oat spelt xylan is very costly in the production of xylanase enzyme (Bocchini *et. al.,* 2005), as well as SmF has limitations, so SSF was carried out.

2.6.1. Xylanase production using agro industrial residues by SSF

The selection of a suitable substrate for SSF process depends on several factors mainly related with cost and availability, and the heterogeneous nature of the substrates makes the problem difficult. Fig. 2. 10. illustrate xylanase production from *B. pumilus* on various agro-industrial residues by solid-state fermentation. Wheat bran (5582 U/gds) was found to be the best substrate followed by soya meal untoasted (4215 U/gds) and rice straw (1876 U/gds). The results also indicated that these substrates promoted high biomass, which would have been the reason for better production; however, in rice bran biomass was high but production was comparatively low. Production was very low with sawdust, rice husk and coir pith; the presence of polyphenol in high quantity in these substrates would have inhibited the growth and enzyme production. Xylanase was produced by *T. lanuginosus* ATCC 46882 (5098 U/g) with bagasse pulp by SSF (Christopher *et. al.,* 2005). Substrates for SSF are heterogeneous products from agriculture or by-products of agro-industries. This basic macro-molecular structure confers the properties of a solid to the substrate. The structural macromolecules may provide an inert matrix within which the carbon and energy sources are adsorbed. Preparation and pretreatment are the necessary steps to convert the raw substrate into a form suitable for use (Krishna, 2005). Wheat bran is produced worldwide in enormous quantities as an important by-product of the cereal industry. The outer tissues of the

wheat kernel imply that the wheat bran consists mainly of cell wall polysaccharides. Arabinoxylan is the main polysaccharide in wheat bran, but only a minor fraction of the flour. It is an integral part of the cell walls of endosperm material in the flour and of aleuronic layer cells present in bran.

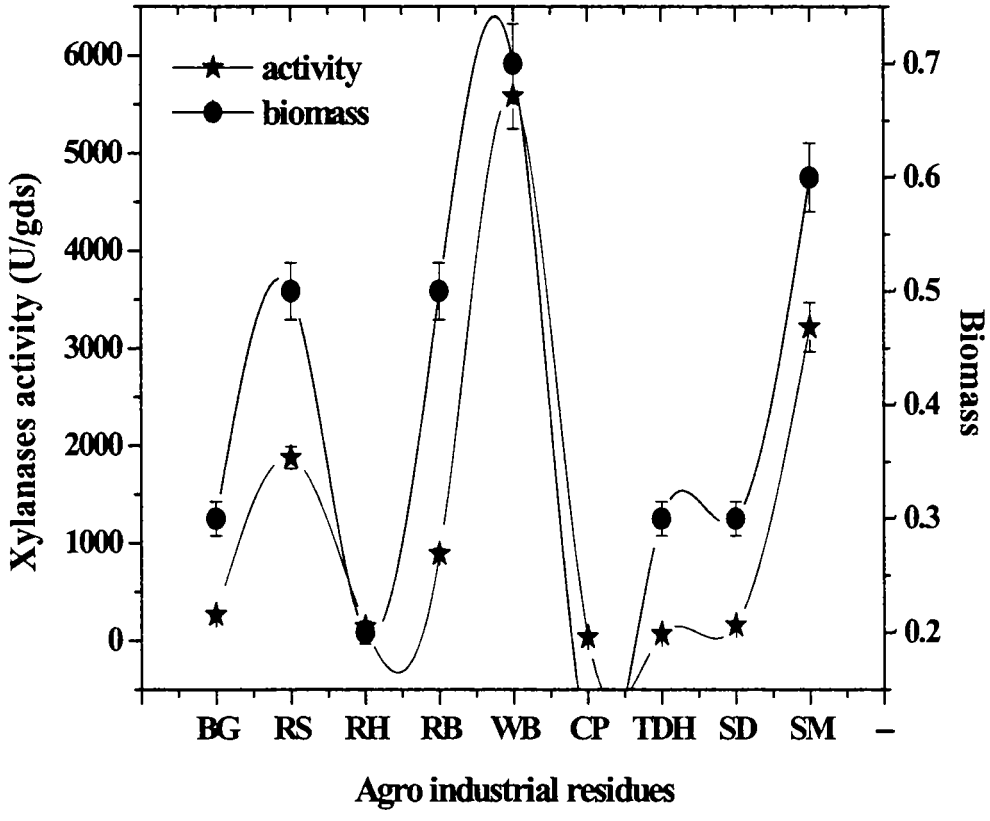


Fig. 2. 10. Xylanase production by *Bacillus pumilus* on various agro industrial residues (BG- sugarcane bagasse, RS-rice straw, RH- rice husk, RB- rice bran, WB- wheat bran, CP- coconut pith, TDH- thur dal husk, SD- sawdust, and SM- Soy meal flakes at 30^{0}C under SSF.

It has been shown that wheat aleurone cell walls contain about 65 % arabinoxylan, with an arabinose to xylose ratio of 0: 35 (Beldman, 1996). Xylans consist of a linear backbone of β -(1, 4) linked D-xylopyranosyl residues containing individual α -L-arabinofuranosyl residues attached through O-2 and/or O-3 (Izydorczyk *et. al.*, 1993). A

feature of some xylans is the existence of feruloyl residues that are esterified with L-arabinofuranose. Feruloyl groups can form cross-linkages between polysaccharide chains by peroxidase-catalysed dimerization or between lignin and polysaccharides via ether-ester bonds (Perez *et. al.,* 2002). Due to their structural complexity several specific enzymes are required for the complete depolymerization of wheat bran xylans.

2.6.2. Effect of pretreatment on enzyme production

Different agro industrial residues, all pretreated with acid and alkali and untreated were used for endoxylanase production by *Bacillus pumilus* by SSF. The results were given in Fig. 2. 11. Of different agro industrial residues used, untreated wheat bran was more effective for enhanced production of endoxylanase; next to this was rice straw and then rice bran, while alkali and acid treatment has inhibited enzyme production in wheat bran by ~16 to 20 fold. Rice straw alkali treatment has not effected very much whereas in rice bran acid and alkali treatment has decreased enzyme production. The biomass was also very low when the production has decreased. On the other hand, alkali treated saw dust, as well as acid and alkali treated sugar cane bagasse were found to be effective to enhance enzyme titer, but the overall production was very less compared to rice straw and wheat bran. In order to increase the accessibility of the cellulose and hemicellulose present in the substrates, mild alkali (1N NaOH) and/or acid treatment (1N HCl) is a widely accepted method for solubilizing lignin from lignocellulose. In this study, xylanase activity did not increased even after mild alkali or acid treatment relatively it was reduced. Such negative effects of alkali treatment on xylanase production were also reported by studies on *A. foetidus*. A possible explanation for this observation has been suggested that rapid consumption of carbon sources and concurrent release of monomeric sugars would lead to a repression in enzyme synthesis (Shah and Madamwar, 2005). There were similar reports on endoxylanase production by *Bacillus* sp. on SSF using wheat bran (Archana and Sathyanarayana, 1997, Gessesse and Mamo, 1999, Virupakshi *et. al.,* 2005) wheat straw by *Thermoascus aurantiacus* (Kalogeris *et. al.,* 1998).

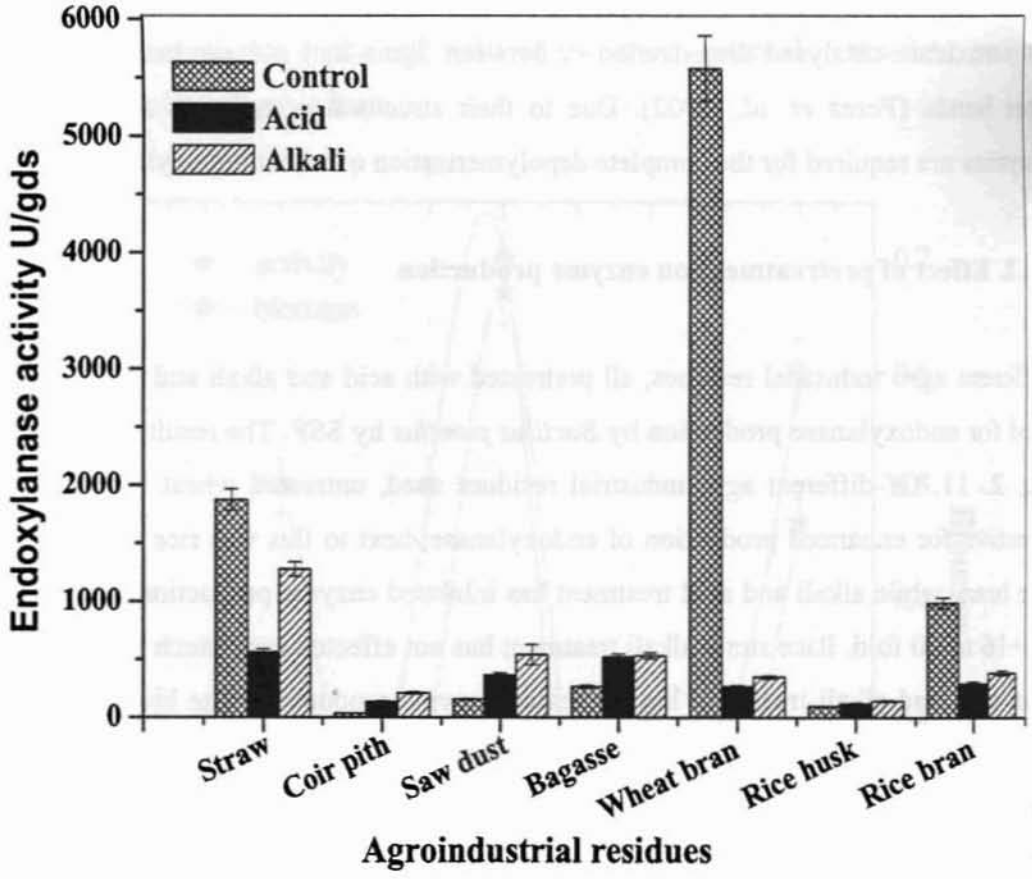


Fig. 2. 11. Xylanase production by *Bacillus pumilus* on various agro industrial residues (production with acid and alkali treated substrate compared with untreated substrates) at 30 °C under SSF.

Pretreatment with acid and alkali did not enhance xylanase production in wheat bran. The removal of major part of hemicelluloses from wheat bran during pretreatment (Koullas *et. al.,* 1993, Kalogeris *et. al.,* 1998) was the reason for this reduction in enzyme production. The α- (1-6) linkages of galactose are very sensitive to acid and alkali and may be cleaved during alkaline extraction (Timell, 1965, Jeffries, 1994). It was reported that agro industrial residues from graminaceous plants contain arabinoxylan, which supports high xylanase activity (Singh *et. al.,* 2000). The biochemical composition of wheat bran

(Lequart *et. al.,* 1999) indicated that wheat bran contained considerable amount of soluble sugar like glucose, xylose, arabinose and galactose required for the initiation of growth and replication of the microorganism.

Arabinoxylan is the main polysaccharide in wheat bran; aleurone cell walls of it contain about 65 % arabinoxylan, the degree of substitution of the main chains by arabinose is higher it is approximately 1 in 5 xylose residues (Lequart *et. al.,* 1999). This might be the reason for enhanced production of endoxylanase on wheat bran, where the substrate itself is highly nutritive sources for *Bacillus pumilus*. This study highlighted that choosing an appropriate source of carbon could improve enzyme production markedly. Developing a process for the production of xylanase using wheat bran as a potential substrate is very attractive as it is readily available and inexpensive source of carbon. Ninawe and Kuhad (2005) reported wheat bran and corncob as an enhancer for xylanase production by *Streptomyces cyaneus* SN32. *Thermomyces lanuginosus* (D_2W_3) was reported to give high xylanase activity on sorghum straw by SSF (Sonia *et. al.,* 2005), *A. fischeri* Fxn by wheat bran (Senthilkumar *et. al.,* 2005) and *Bacillus* JB-99- (3644 U/g) (Virupakashi *et. al.,* 2005) on rice bran. But these reports on xylanase activities were lower compared to that obtained by *B. pumilus* on wheat bran. The universal suitability of wheat bran as substrate is that it contains sufficient nutrients and able to remain free even in high moist condition providing large surface area (Archana and Sathynarayana, 1997).

2.7.0. Enzyme profile in SSF and SmF

Related to plate assay the crude enzyme extract was subjected to enzyme profile studies. Related to xylanase activity, protease, β- xylosidase, CMC ase and FP ase activity were estimated as explained in the procedure. Enzyme profiles of the culture in agro industrial residue like wheat bran, rice straw and soybean flakes are recorded and related with that of submerged fermentation using Oat spelt xylan and well explained in Table. 2.4. The results indicate that wheat bran was the best source for endoxylanase production by SSF. The production of proteases and cellulase was low in the case of wheat bran. In agro

industrial residues, generally protease synthesis is inhibited by C-sources indicating the presence of catabolic repression of protease biosynthesis (Pandey *et. al.,* 2000). Heck *et. al.,* (2002) reported soybean industrial residue as suitable sources for cellulase and xylanase production by *Bacillus* strains.

Enzymes	SSF (IU gds^{-1})			SmF (U/mL)
	RS	WB	SBF	
CMCase	3.2	1.8	ND	ND
Filter paper	0	0.0117	0	0
Xylanases	1873	5875	4215	352
β- xylosidases	0.11	2.845	ND	ND
Protease	124.1	121.1	293.3	303.3

Table. 2.4. Enzyme profile of *Bacillus pumilus*

Ferreira and coworkers has reported high proteolytic activity with wheat bran as substrate in SSF by *Aspergillus tamari* (Ferreira, 1999). Soya meal has the highest level of protease. This can be explained based on the chemical composition of these agro industrial materials, compared to wheat bran (11.5-18 %) and rice straw (4.5 %), soya meal (44 - 47 %) is having high protein content. The production of protease affects the storage of enzyme, which can be rectified by using proteolytic inhibitors or by protein purification methods (Walsh and Headon, 1994). In this study it was found that the production of protease was comparatively lower in SSF when compared to SmF. Protease production was very low in all agro-industrial residues. The β-xylosidase production was found to be very low and about 2 IU/ gds in wheat bran. There was a report of high secretion of β- xylosidase (45 U/mL) by *Aspergillus fumigatus* with 3 % corncob as carbon sources in SmF (Lenartovicz *et. al.,* 2003). Minor cellulosic activity was found in the crude filtrate. The results point out that, the culture was a poor producer of cellulase

related to xylanase, indicting that this enzyme was a persuasive source for biobleaching process.

2.8.0. Conclusion

From these data we can conclude that the isolate *Bacillus pumilus* was highly alkalophilic and thermo tolerant. Xylanase production was investigated using different carbon sources in different mode of fermentation, where SSF has shown 58- fold increase in production related to SmF. It was possible to verify that a lignocellulosic waste, wheat bran, was an important substrate for the production of xylanase. Use of wheat bran as substitute, was an efficient alternative to reduce the costs of xylanase production by *B. pumilus* in SmF as well as in SSF, since these materials are often available in tropical countries like India. It is as an inexpensive source of components that propitiate the bacterial growth and the enzyme production. The thermo tolerances of the microbe and enzyme production as well as its high stability at alkaline pH values are superiors to the mesophilic counterparts used for prebleaching of pulp. These properties together with the production of extremely high levels of xylanase, with poor cellulolytic activity making this strain a promising entrant for industrial applications.

Chapter. 3.

Culture and nutritional parameter optimization for enhanced production of endoxylanase by Bacillus pumilus *using Solid -State Fermentation*

3.0. INTRODUCTION

Xylanase production with bacteria was mostly reported under SmF (Cordeiro *et. al.,* 2002, Subramaniyan *et. al.,* 1998, Khasin *et. al.,* 1993). For SmF generally Oat spelt xylan was preferred, which is costly and thus the production cost will be high. In order to make the enzyme applications more cost effective at industrial level, its production using low cost substrates such as agro-wastes has been recommended by many (Asha and Prema, in press, Gupta *et. al.,* 2001). The cost of production can be lowered by SSF, which is closer to natural system and has proved to be more efficient in producing certain enzymes and metabolites (Pandey *et. al.,* 2000). There is two type of SSF, firstly Solid-substrate fermentation where the substrate itself act as the carbon sources, secondly Solid support fermentation where the substrate act as the support (like polyurethane beads) that help the organism to anchor and grow, and this is generally supplemented in liquid medium, major advantage is that the support can be reused (Ooijkaas *et. al.,* 2000).

There are various factors that affect the SSF process, which vary from process to process depending upon the type of substrates and microorganisms used, as well as the scale of the process. The factors can be broadly divided into biological, physico-chemical, and environmental factors. A factor can be independently related to a biological process but dependent when it is related to a physico-chemical process and *vice versa* (Pandey *et. al.,* 2001).

The most important factor that controls the process of SSF is - lignocellulosic substrate used. All of them have the basic macromolecular structure consisting of starch, cellulose, hemicelluloses (xylan, mannan, and galactan), pectin, lignin and other polysaccharides that act as carbon or energy sources. Substrates for SSF are products from agriculture or by-products of agro- industries, with heterogeneous nature and the macromolecular structure decide the property of solid substrate (Mitchell *et. al.,* 1992). Particle size of the substrate is also of considerable effect during the initiation of fermentation, later the size of particle changes as fermentation progresses. The size of the substrate determines the void space, which is occupied by air. Since the rate of oxygen transfer into the void space

affects growth, the substrate should contain particles of suitable size to enhance mass transfer. (Nanadakumar *et. al.,* 1995, Pandey *et. al.,* 2001, Krishna, 2005).

One of the important factors of SSF is level of moisture. Fungus prefers low moisture and there are many reports (Senthilkumar *et. al.,* 2005, Jeye *et. al.,* 2005, Kheng and Omar, 2004, Lu *et. al.,* 2003,). However use of bacteria (Gessess and Mamo, 1999, Virupakshi *et. al.,* 2005, Asha and Prema, in press) in SSF system is less exploited due to their high moisture requirement. Moisture content of the substrate greatly depends on the water activity; any small change in the latter could lead to great effect on the former. Water requirements of microorganisms are defined in terms of the water activity (a_w) rather than the water content of the solid substrate. Water activity represents the availability of water for reaction in the solid substrate. It is a thermodynamic parameter defined in relation to the chemical potential of water, and is related to the condensed phase of absorbed water but is well correlated to the relative humidity (RH) (Lu *et. al.,* 2003, Raimbault, 1998). The water activity ($a_w = P_m/P_s$) of the medium depended upon its moisture content and composition. It was obtained by measuring the vapour pressure (P_m) in equilibrium with SSF medium of different moisture content and saturated vapour pressure (P_s) in equilibrium with pure water at 30 °C. The measurements were carried out in a closed chamber equipped with a hygrometer and a wet bulb thermometer (Lu *et. al.,* 2003).

Each organism has a range of pH for optimum growth and activity. Another important factor in any fermentation process is pH, and it may change in response to metabolic activities, due to secretion of organic acids, which lead to decrease or increase in pH, and urea hydrolysis will result in alkalinization (Raimbault, 1998).

Growth and production of enzymes or metabolites are usually sensitive to temperature so it is important parameter for the growth of microorganisms. Optimum growth temperature will be different from product formation (Yuan *et. al.,* 2005, Yadav, 1988). The rate of heat generation is directly proportional to the level of metabolic activity in the system (Yuan *et. al.,* 2005, Zhang *et. al.,* 2004, Raimbault, 1998) and there is generally a thermal gradient in the system related to the local moisture and void spaces available.

Lenartovicz *et. al.,* (2005) has reported that temperature and carbon sources have great influences on enzyme production by *Aspergillus fumigatus*. Temperature control is quite tedious factor in SSF (Pandey *et. al.,* 2000), because the conventional convection or conductive cooling devices are inadequate for dissipating metabolic heat due to poor thermal conductivity of most solid substrates, as well as the predominant static nature of SSF. Heat generated can be removed by forced aeration and Evaporative cooling (Gowthaman *et. al.,* 2001, Raimbault, 1998, Trilli, 1986).

An inoculum size is the next important factor, it provides vegetative cell than spores that initiate the fermentation and reduce time of fermentation. Major advantages include convenience, greater flexibility in the coordination of inoculum preparation, prolonged storability for subsequent use, and higher resistance to mishandling during transfers (Krishna and Nokes, 2001).

The composition of the fermentation medium is another important factor for enhancement of production. Cellular biomass presents an average of 40–50 % carbon, 30–50 % oxygen, 6–8 % hydrogen and 3–12 % nitrogen (Pandey *et. al.,* 2001). Other elements such as phosphorus, nitrogen, sulfur, and metals are also important, although in small quantities. The nutrients, which can promote the sporulation and production, are important in fermentation processes. This include carbon and nitrogen sources, minerals, and vitamins or co-factors, carbon can be pure monosaccharide compound such as glucose or complex molecules such as cellulose or starch, that act as a energy source that will be available for the growth of the microorganism. Presences of nitrogen sources stimulate the microorganism; where as mineral salts enhance the production.

The use of purified xylan in the production media increases the cost of production and hence different lignocellulosic residues have been used as growth substrate. The purpose of the study was to investigate the effect of culture and nutritional factors on the production of xylanase by *Bacillus pumilus* on SSF. In culture condition optimization one optimized condition was carried over to the next experiment where as during nutritional parameter optimization no optimized condition was carried over to next experiment. All

experiments were done individually, latter various combination were tried. Special attention was given to develop a simple culture medium using low cost ingredients in order to reduce the cost of enzyme production. The simple media composition was used for scale up studies by tray and tank fermentation.

3.1.0. MATERIALS AND METHODS

3.1.1. Xylanase production by SSF and enzyme extraction

Ten gram of the solid substrate (wheat bran) was well mixed with mineral salts solution containing (g/L) KH_2PO_4, 2.0; $MgSO_4 .7H_2O$, 0.4 in Erlenmeyer flasks (250 mL) to get an initial moisture of 1: 1 ratio, pH – 7 (upto optimization). It was autoclaved at 121 °C for 20 min; cooled, inoculated with 18 h old seed culture (~ 3.6 x 10^6 counts / mL) and incubated at 30 °C. Flasks were removed at regular interval of 24 h for 5 days and content was extracted in 0.05 M phosphate buffer pH - 7 (1: 30 w/v) by mixing the contents on a shaker for 30 min and filtered through cheese cloth. The filtrate was centrifuged for 20 min at 10, 000 x *g* at 4 °C and the supernatant was used as crude extract. All experiments were carried out in triplicate and the results were present as the mean of three. The crude enzyme extracted was subjected to xylanase assay, total soluble protein and reducing sugar estimation, as mentioned in the analytical procedures explained in Chapter. 2.

3.2.0. CULTURE CONDITION OPTIMIZATION

3.2.1. Effect of particle size of wheat bran

The effect of particle size of wheat bran on enzyme production was evaluated by culturing the organism on wheat bran of different particle sizes: 2 mm, 1 mm, 0.5 mm and 0.3 mm.

3.2.2. Effect of initial moisture level on xylanase production

The influence of initial moisture level on the enzyme production was evaluated by varying the ratio of moisture (w/v) of wheat bran to mineral salt (1: 0.5, 1: 1, 1: 1.5, 1:2, 1: 2.5 and 1: 3). The level of mineral salts was maintained constant in all experiments where as the water level was varied.

3.2.3. Effect of media pH, temperature and inoculum size on xylanase production

Effect of medium pH on enzyme production was estimated by culturing the strain in wheat bran of different initial pH -7, 8, 9 and 10. Effect of temperature on enzyme production by *B. pumilus* was studied by incubating at different temperatures - 25, 30, 35, 40, 45 and 50 °C for 120 h. Effect of inoculum size on enzyme production was estimated by inoculating flasks with 2.5, 5.0, 7.5 and 10 % (v/w) of 18-h inocula and incubated at 35 °C.

3.3.0. NUTRITIONAL PARAMETER OPTIMIZATION

3.3.1. Effect of glycerol and tween on xylanase production

Glycerol was used as a depressant of water activity in culture flasks (Grejek and Gervais, 1987) and tween - 80 a detergent was added as a supplement to the substrate wheat bran and their effect on enzyme production was evaluated. The substrate was treated with varying concentration of glycerol and tween -80 (0.1 and 2 % v/v) and mineral salt medium before autoclaving. The enzyme extracted as mentioned earlier and estimated.

3.3.2. Effect of easily metabolizable sugars on xylanase production

To study the effect of additives with wheat bran on enhanced production of xylanase, wheat bran was supplemented with different carbon sources like glucose, sucrose, xylose, galactose, xylan and starch (SRL chemicals) at different concentrations (0.01, 1.0, 5.0 and 10 %).

3.3.3. Effect of phosphorus salts on xylanase production

Different phosphorus salts were supplemented with wheat bran in SSF for the production of endoxylanases by *Bacillus pumilus*. Phosphorus salts supplemented are K_2HPO_4, KH_2PO_4. NaH_2PO_4, Na_2HPO_4 and $(NH_4)_2$ H_2PO_4 at different concentration (0.05, 0.1, 0.15 and 0.2 M). The concentration of phosphorous in the salt was calculated and added accordingly.

3.3.4. Effect of nitrogen sources on xylanase production

Different mineral nitrogen sources like ammonium sulphate, ammonium chloride, ammonium tartrate, potassium nitrate and sodium nitrate (SRL chemicals), in varying concentrations (0.1 and 1 g) were added to mineral salt solution and mixed with wheat bran. The effects of different organic nitrogen sources like peptone, yeast extract, soy meal, tryptone, urea, and malt extract (SRL chemicals) on xylanase production by *Bacillus pumilus* was studied by mixing them to the solid substrate in varying concentrations (0.2. 1.0, 5.0 and 10 g).

3.3.5. Effect of metal ions on xylanase production

The solid medium was supplemented with different concentrations (0.01 to 0.5 mM) of various metal ions to study their effects on enzyme production. The various metal salts tried were KCl, NaCl, $CuSO_4$, $MnSO_4$, $CoCl_2$, $FeSO_4$, $MgSO_4$, $ZnSO_4$, $CaCl_2$ and $K_2Cr_2O_4$.

3.3.6. Effect of Sodium carbonate on enzyme production

Different concentration of Na_2CO_3 (ranging from 0.5, 1, 2, 2.5, 3.0, 3.5 4.0, 5.0 and 10 % w/w) was added to wheat bran with basal media and autoclaved. The pH of the medium was adjusted to high alkaline range by adding Na_2CO_3. These flasks were inoculated and

incubated as explained earlier. Samples were withdrawn at regular interval and estimated for pH, xylanase activity, protein and reducing sugar.

3.4.0. Moisturizing agent

All the nutritional parameter optimization was done individually, so a combination of different nitrogen and phosphorus sources as well as metal ions were tried. Wheat bran was moistened with different mineral salt solutions (MSM), in all experiments pH where adjusted to ~ 9; with contents (g/L) as given below:-

MSM_1 – tap water

MSM_2 – Distilled water

MSM_3 - K_2HPO_4 1.0, $MgSO_4.7H_2O$ 0.50

MSM_4 -Yeast extract 4.0, Peptone 4.0, K_2HPO_4 3.0, $MgSO_4$.$7H_2O$ 1.0.

MSM_5 -K_2HPO_4 2.0, $MgSO_4.7H_2O$ 1.0, Na_2HPO_4 3.0, $CaCl_2.2H_2O$- 0.2, $FeSO_4$-0.2, $MnSO_4$-0.2

MSM_6 -K_2HPO_4 2.0, $MgSO_4.7H_2O$ 0.4

MSM_7 -K_2HPO_4 2.0, $MgSO_4.7H_2O$ 0.40, $MnSO_4$-0.2 NaCl- 10, $(NH_4)_2$ SO_4 -2.0, Yeast extract – 2.0, Peptone – 4.0

MSM_8 -K_2HPO_4 2.0, $MgSO_4.7H_2O$ 0.40, $MnSO_4$-0.2 NaCl- 2, $(NH_4)_2$ SO_4 -6.0, Yeast extract – 6.0

MSM_9 -K_2HPO_4 2.0, $MgSO_4.7H_2O$ 0.4, $MnSO_4$-0.2, NaCl- 2, $(NH_4)_2$ SO_4 -6.0, Yeast extract – 2.0, $NaNO_3$- 2, xylan –0.01

MSM_{10}- K_2HPO_4 2.0, $MgSO_4.7H_2O$ 1.0, $MnSO_4$-0.04, KCl- 1.0, Yeast extract – 1.0

MSM_{11}- K_2HPO_4 2.0, $MgSO_4.7H_2O$ 0.4, Na_2CO_3-35

All were prepared in distilled water.

3.5.0. SCALE UP STUDIES IN SSF

3.5.1. Cultivation in enamel trays

The fermentation was carried out in enamel trays of sizes 18 x 16 x 2 inch. Wheat bran (900g) of particle size 0.5 mm was moistened in trays with simple mineral salt medium of composition K_2HPO_4 2.0, $MgSO_4.7H_2O$ 0.4 (g / L) in the ratio 1: 1.5, autoclaved at 15 lb for 45 min, cooled and inoculated with 24 hour grown bactobran (100g), which was prepared as mentioned earlier for flask fermentation. The inoculated trays were inserted into steel frames in humidity chamber humidified with sterile distilled water set at 35 °C. The time needed for SSF by *Bacillus pumilus* in the culture room was 48–72 h. The medium was then transferred to the drying room. After removing the trays, the chamber was disinfected by spraying 5 % formaldehyde solution and then sealed for 10 h. Airflow was used to clear the formaldehyde for 10 h. The whole process of disinfections and preparation for the next culture lasted 20–22 h. The fermented matter was withdrawn at every 24 h and extracted in phosphate buffer of pH-7 and activity determined.

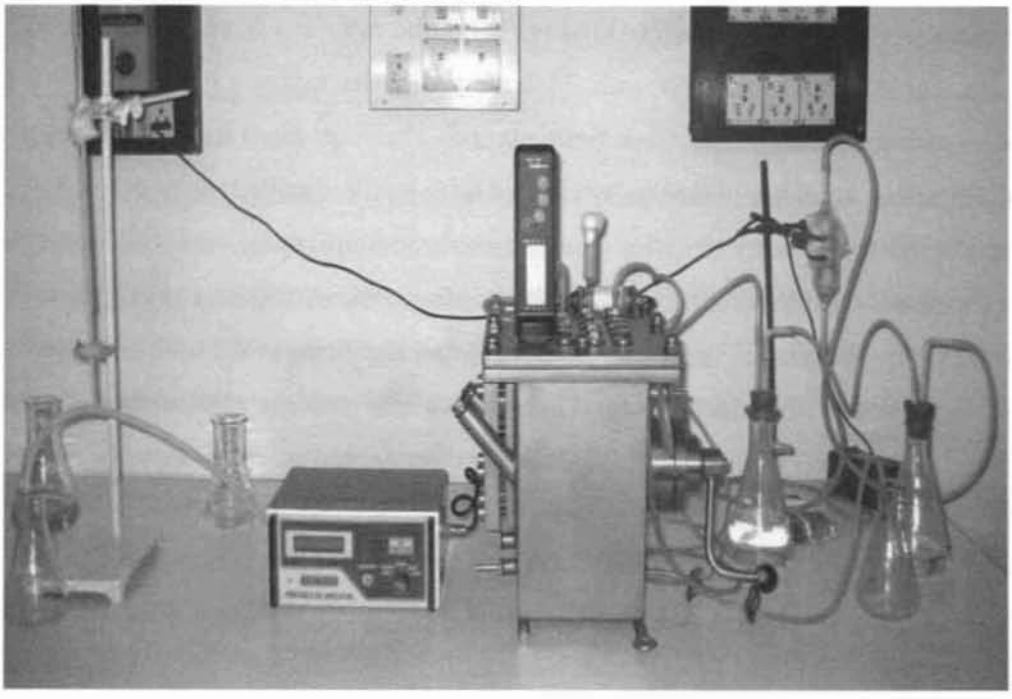
3.5.2. Fermentation in Tank fermentor

Keeping the moisturizing agent as mineral salt solution of composition K_2HPO_4 2.0, $MgSO_4.7H_2O$ 0.4, scale up studies was performed in tank fermentor of 1 Kg capacity (Fig. 3. 1). Autoclaved moisturized wheat bran was transferred to the tank under aseptic condition, which was sterilized by spraying 5 % formaldehyde solution and then sealed for 10 h, there after airflow was used to clear the formaldehyde. The pre-inoculum was transferred through sterilized syringe and mixed with the moisturized wheat bran. The temperature of the tank was maintained at 35 °C by passing lukewarm water through the jacket layer of fermentor. Filtered air was passed through the fermentor to control the oxygen and carbon dioxide in balance. The fermentation was carried out for 120 h and samples were with drawn at regular interval.

3.6.0. CRUDE ENZYME EXTRACTION BUFFER ESTIMATION

The extraction buffer for crude xylanase was studied by extracting the enzyme in different buffer solution ranging from 6 to 12 as well as in distilled water and sterilized

membrane sterilization. The buffer 6-8 was from phosphate buffer and 9-12 Glycine – OH buffer both of 100mM concentration.



ş. 3. 1. Tank fermentor showing the airflow and CO_2 estimation probe as well as humidity and temperature measuring instruments attached

.1. Storage stability of crude extract

e storage stability of crude xylanase was studied by extracting the enzyme in different fer solution ranging from 6 to 12 and sterilized by membrane sterilization. This nple was stored for 2 weeks in deep freezer (-10 to -20 °C), room temperature (25 - 30 and refrigerator (5 - 7 °C), estimation was done and from the result phosphate buffer s selected. The enzyme was stored at 0.1 M and 0.6 M phosphate buffer (pH - 7) in p freeze, refrigeration and room temperature. The enzyme solution was stored in ile Eppendorf vials. Xylanase activity was assayed at regular intervals for 1 month. rage stability of dried fermented matter was also estimated. The fermented bacterial n was dried in a hot air oven set at 30 °C for 48 hours in sterilized trays. The moisture s removed and dried matter was estimated and results compared with that of wet

sample. The dried matter was transferred to polythene bags and stored at 4 °C and activity was checked weekly for one month.

3.7.0. RESULTS AND DISCUSSION

3.7.1. Effect of particle size of wheat bran

Particle size of the substrate is important, as it is related to substrate characterization and system capacity to interchange with microbial growth, heat and mass transfer during SSF process. It also affects the surface area to volume ratio of the particle, which determines the fraction of the substrate, which is initially accessible to the microorganism and the packing density within the surface mass (Krishna, 1999). The size of the substrate determines the void space, which is occupied by air. Since the rate of oxygen transfer into the void space affects growth, the substrate should contain particles of suitable size to enhance mass transfer (Pandey *et. al.,* 2000). Influence of particle size of wheat bran on enzyme production was shown in Fig. 3. 2. Maximum enzyme production was recorded when the particle size was at 0.5 mm (6532 U/gds), followed by 1mm (5081 U/gds). Enzyme production and biomass was too low and the protein content was too high at 0.3 mm, due to particle agglomeration, which would have inhibited oxygen transfer. Generally smaller substrate particles would provide larger surface area for microbial action but too small particles may result in substrate agglomeration, which may interfere with microbial respiration/aeration and thus result in poor growth. The results clearly indicated that particle size of the substrate influenced xylanase production. Particle size of substrates is one of the most critical among several factors that influence fermentation process.

The microorganism derives its energy by oxidation of organic carbon. The adherence and penetration of microorganism as well as enzyme action on the substrate depends upon the physical properties of the substrate, such as the crystalline and amorphous nature, the accessible area, surface area, porosity, particle size, etc, (Krishna, 2005). In SSF, particle

size of the substrate determines the efficiency of the process and it is necessary to choose appropriate particle size for a particular process (Pandey *et. al.*, 2000).

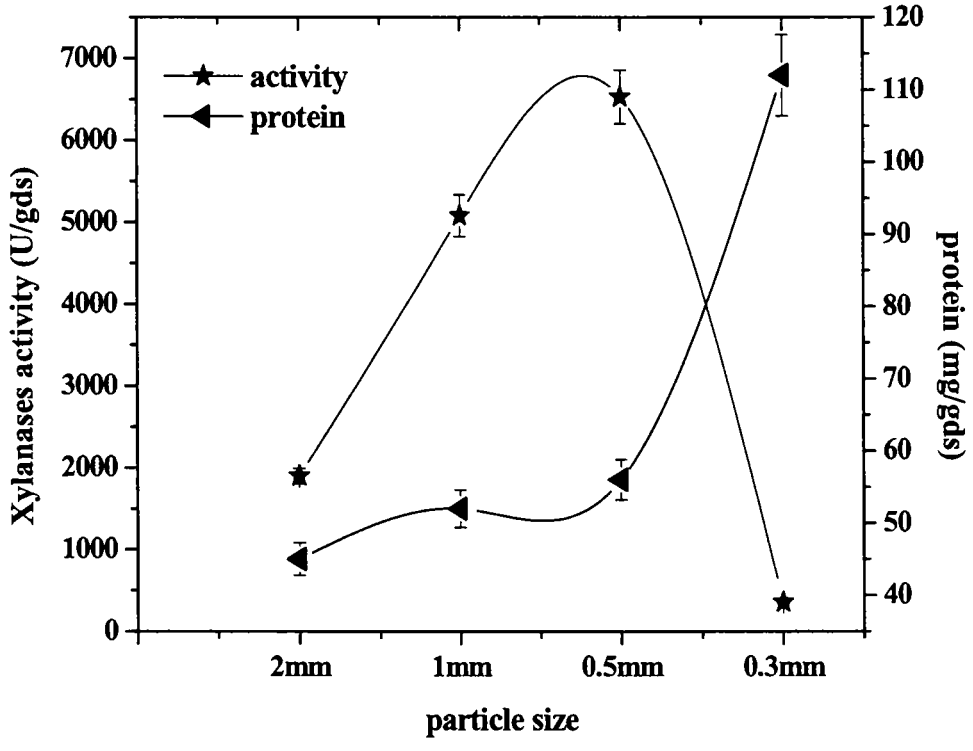


Fig. 3. 2. Effect of wheat bran particle size (2.0, 1.0, 0.5 and 0.3 mm size) on endoxylanase and protein production by *Bacillus pumilus* in SSF at 30 °C at 96 hour of incubation.

The primary organic degradable carbohydrate components of wheat bran can be represented in the order of preference of the microorganisms as free sugars, starch, hemicellulose, and cellulose. This may be responsible for the deviation of production in different particle sizes. Smaller particles contain starch that results in agglomeration of particles, which restrict the air, flow leading to reduction in production as well as growth, where as large particle contain more hemicellulose and cellulose which helps to increase the production. As the fermentation progresses, depletion of these carbohydrate

components take place in sequential order and compounds like cellulose and lignin, which are not easily degradable by the microorganism employed remains which results in reduction of xylanase production (Nadakumar *et. al.,* 1996).

3.7.2. Effect of initial moisture level

Effect of initial moisture content on enzyme production was investigated by varying the initial moisture content of the substrate ranging from 1: 0.5 to 1: 3 (w/v). Mineral salt solution used as the moisturizing agent to support xylanase production to a varied extent. The enzyme titer was relatively high when wheat bran was moistened with mineral salt in a 1: 2.5 ratio ie: 70 % moisture (Table. 3. 1.). The importance of water in any SSF system is attributed to the fact that majority of the viable cells require the moisture content of 70 - 80 % for the synthesis of new cells. The significance of moisture and water activity (a_w) implies that, while preparing a substrate, it is necessary to consider the exact quantity of water addition to the substrate to satisfy the requirement of the system (Pandey *et. al.,* 2000). Babu and Satynarayana, (1996) did pioneering work in this area. There could be some correlation between microbial growth and product synthesis with the level of moisture content selected. Low moisture may reduce the solubility of lignin and swelling capacity of substrate, and so higher water tension minimizes microbial growth. Higher moisture content results in swelling of substrate, thereby facilitating better utilization of the substrate by the microorganism (Pandey *et. al.,* 2000). A reduction in enzyme yield at very high moisture level more than 75 %, may be due to the steric hindrance of growth of the organism through reduction in inter particle space, decreased porosity, gummy texture, alteration in wheat bran particle structure and impaired oxygen transfer (Feniksova *et. al.,* 1960).

3.7.3. Effect of medium pH, temperature and inoculum size

The result of influence of initial medium pH on enzyme production is shown in Fig. 3. 3. The optimum pH for the production of xylanase by *B. pumilus* was in the range of pH 8 (8853 U/gds) to 9 (10125 U/gds). At pH 7 (6931 U/gds) and 10 (6521 U/gds) the

production of xylanase were slightly decreased. There were reports related production of xylanase at alkaline pH- 9 by *Aspergillus fischeri* Fxn 1 (1024 U/g) in wheat bran (Senthilkumar *et. al.,* 2005) and *Streptomyces cyaneus* SN32 using wheat bran (632 IU / mL) and corn cob (616 IU / mL) at 42°C (Ninawe and Kuhad, 2005). There was considerable increase in the pH value after the growth was established. Each microorganism holds a range pH for its growth and activity with optimum value between these ranges. The initial pH influences many enzymatic systems and the transport of several species of enzymes across the cell membrane. Most bacterial species are unable to grow at reduced moisture level and alkaline pH. The optimum pH for the production of endoxylanase by *B. pumilus* ranged from 8.0 - 9.0.

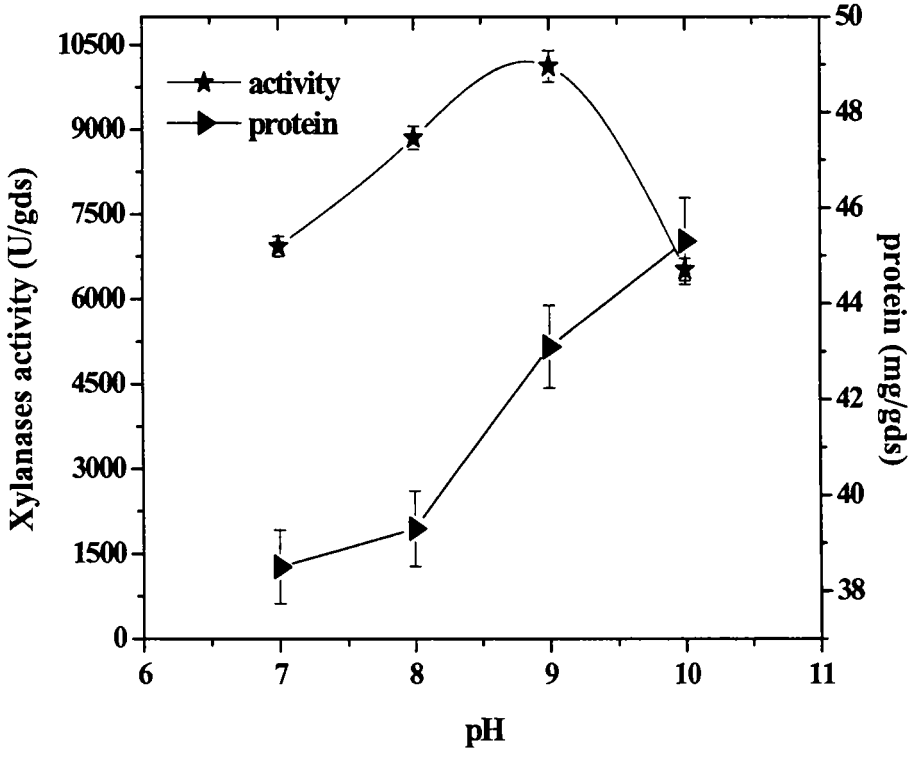


Fig. 3. 3. Effect of initial medium pH on endoxylanase and level of protein production by *B. pumilus* on wheat bran as substrate in SSF at a particle size of 0.5 mm.

From the analysis of result, it was evident that the optimum temperature (Table. 3. 1.) for the production of extracellular xylanase was 35 °C (13032 U/gds) at 72 h of incubation,

followed by 40 °C (8545 U/gds), and was productive even at 50 °C (7214 U/gds). At 25 °C (158 U/gds), a very low titer of enzyme was recorded. Results indicated that temperature was influencing factor for enhanced production. The maximum cell growth and enzyme yield of *B. pumilus* coincide at 35 °C.

Highest levels of xylanase production in fungal system have already been reported to occur generally at temperatures optimum for growth of culture in SSF (Sudgen and Bhat, 1994). Archana and Sathyanarayana (1997) have reported xylanase activity (19.8 U / DBB) at 50 °C. There were reports related to synthesis of endoxylanase, pectinase and cellulase when supplemented with other carbon sources (Olsson *et. al.,* 2003). Similar reports were there for xylanase production by *A. sulphureus* (Lu *et. al.,* 2003) and *S. cyaneus* SN32 (Ninawe and Kuhad, 2005). The fungal strain *Paecilomyces themophila* J18 reported to grow well at 50 °C and produced high-level of xylanase (18580 U/ g) using wheat straw (Yang *et. al.,* 2006).

Activity *	**Activity ****	**Activity ****
(Sub: initial moisture)	**(Temp - °C)**	**(Incoclum size -v/w)**
780.8 (1:0.5)	158 (25)	543 (2.5)
1568 (1:1)	9387 (30)	8531 (5.0)
3081 (1:1.5)	13032 (35)	11531 (7.5)
7070.9 (1:2)	8545 (40)	16252 (10)
9290.7 (1:2.5)	7901 (45)	
7473 (1:3)	7214 (50)	

Table. 3. 1. Effect of initial moisture level, ie: substrate to mineral salt plus inoculum ratio, temperature (°C) and inoculum size (v/w) on endoxylanase production by *B. pumilus* grown on wheat bran of particle size 0.5 mm and initial pH - 9. (Fermentation period * 96 hour, **72 hour).

The inoculum level at 10 % (v / w) with a cell count of ~3.6 x 10^6 counts/mL (16252 U / gds) was found to be optimum for xylanase production at 72 hour of incubation by SSF using wheat bran as substrate by *Bacillus pumilus*. An inoculum size below 5 % was inadequate for good growth and enzyme production in SSF (Table. 3. 1.). Inoculum size more than 7.5 % found to be suitable for high titer of enzyme in SSF. Similar reports on α- amylase by *B. coagulans* B49 have been described with maximum production at 10 % (Babu and Satyanarayana, 1996) and *B. licheniformis* A99 (Archana and Sathyanarayana, 1997) at 15 % inoculum size.

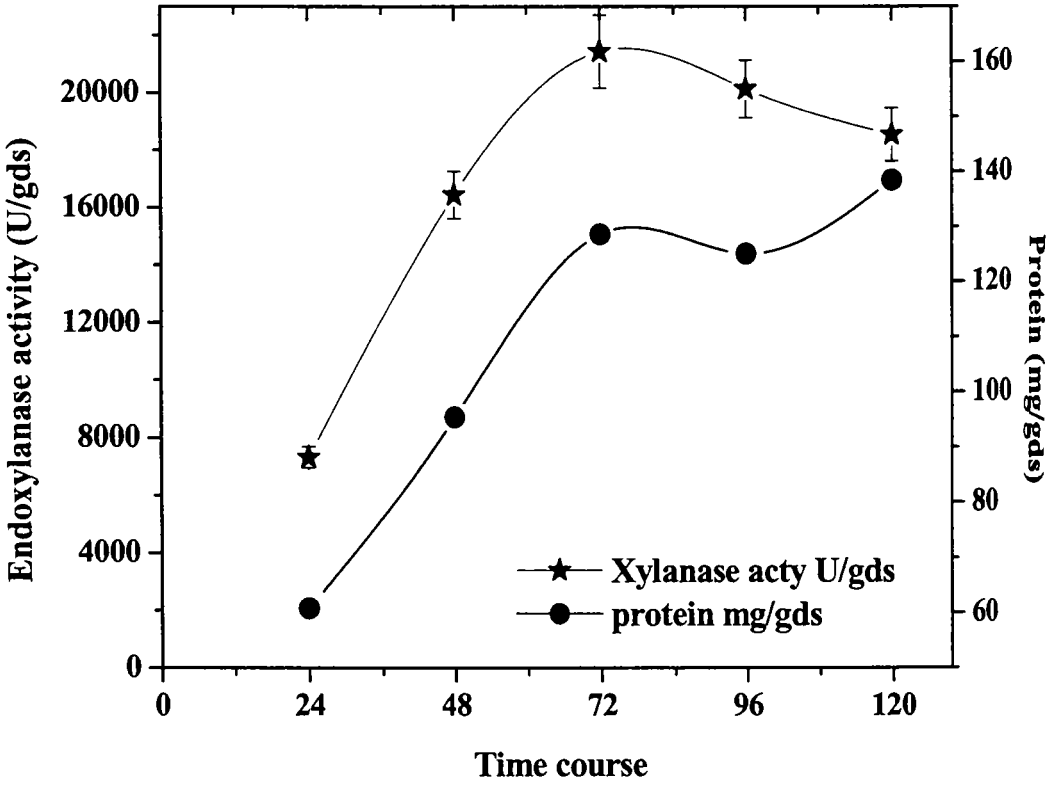


Fig. 3. 4. Time course of endoxylanase production and level of protein produced by *Bacillus pumilus* using fresh wheat bran of particle size 0.5 mm, initial pH and moisture level of mineral salt added as 9 and 70 % respectively, at 35 °C and 18 hour grown inoculum of 10 % (v/w) by SSF.

All optimized cultural conditions were incorporated and batch culturing was performed to compare the activity of optimized as well as non-optimized conditions. An incubation period of 72 h (21431 U / gds) has given maximum production and has retained 93 % activity at 96 h (20128 U / gds), the total soluble protein was very less (Fig. 3. 4). There was an increase of 3.8-fold by optimization from initial value of 5582 U/gds of cultivation conditions. The bactobran retained 86 % of activity at 120 h. The cost of production of endoxylanase using Oat spelt xylan by SmF is very high. Hence in order to develop a cheaper process for the production of this enzyme, different agro industrial wastes were used as carbon sources.

3.7.4. Effect of glycerol and tween -80 on xylanase production

The effect of glycerol and tween-80 on xylanase production by SSF using wheat bran by *Bacillus pumilus* was evaluated and explained in Fig. 3. 5. Glycerol (0.1 and 2 %) and Tween –80 (2 %) has shown considerable effect on xylanase production by *B. pumilus*. Tween- 80 (2 mg / mL) increased the yield of xylanase in *Thermoascus aurantiacus* by about 43 % (Gomes *et. al.,* 1994), while *Trichosporon cutaneum* SL-409 reported to be highly productive at about 0.6 % tween-80 (Liu *et. al.,* 1997). Xylanase production by *Thermomyces lanuginosus* (D_2W_3) by SSF of sorghum straw (39726 U / gds) was enhanced by the addition of glycerol (0.25 % v/v) (Sonia *et. al.,* 2005). The inducing effect of glycerol on xylanase production has also been observed with *T. lanuginosus* RT9 in liquid cultivation (Hoq *et. al.,* 1994). Glycerol can act as water depressant which help to retain the moisture where moisture level and water activity is relative. With the increase of moisture content of the medium, the porosity of the medium was decreased proportionally. This lead to the difficulties of oxygen transfer and heat release in the medium (Lu *et. al.,* 2003). By lowering the amount of glycerol, the water activity was increased, which resulted in an enhancement of xylanase production. Water activity in the range of 0.982 – 0.986 was found to be suitable for the production of hydrolytic enzymes (Archana and Satyanarayana, 1997). In the present study, glycerol content of 0.1 % was found to be most suitable for enhanced xylanase production that will result on 97 % water activity. Sodium chloride and sorbitol can also be used as water depressant. *B.*

licheniformis A99 produced maximum xylanase at an a_w value of 0.95 using wheat bran as substrate (Archana and Sathyanarayana, 1997).

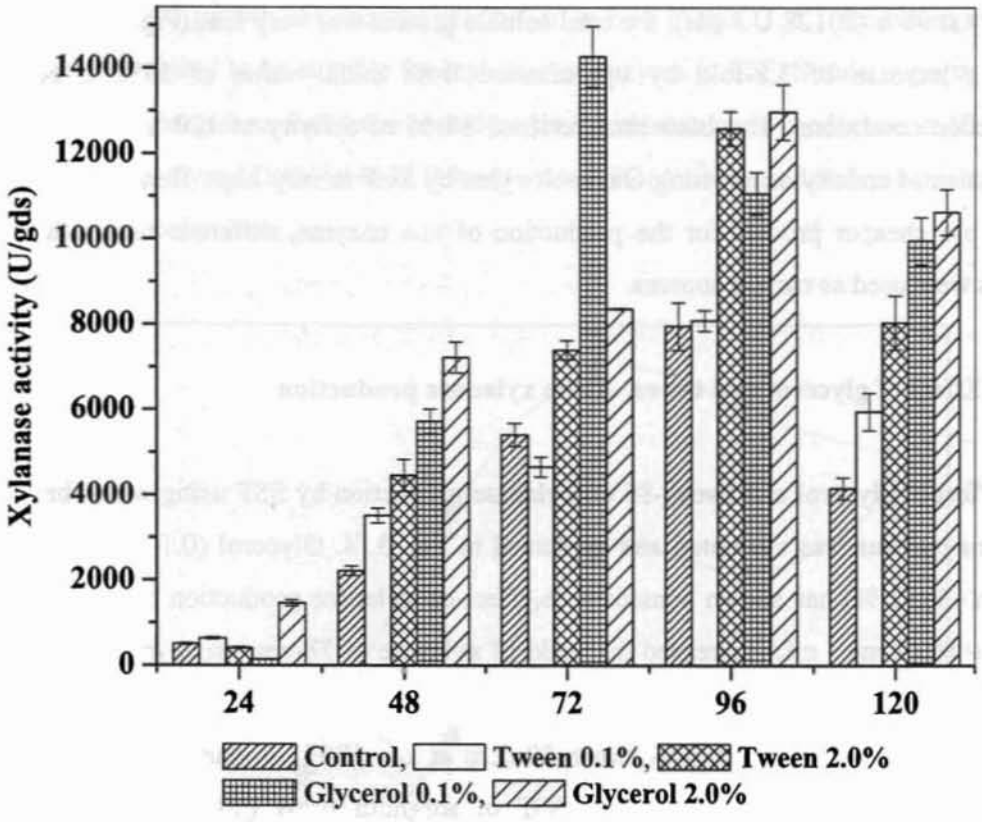


Fig. 3. 5. Time course of endoxylanase production by *Bacillus pumilus* using fresh wheat bran of particle size 0.5 mm, pH -9 treated separately with different concentration of glycerol and tween- 80 (0.1 and 2.0 %), incubated at 35 °C.

Shah *et. al.,* (1999) reported that tween -80 (0.1-0.3 %) have no considerable effect on xylanase production by *Bacillus* sp. Sam-3, where as Duarte *et. al.,* (1999) reported a medium with tween - 80 (1.0 g) in the growth medium of *Bacillus* sp. for enhanced production of xylanase. *Trichoderma viridie* TS, water activity of 0.995 was reported to be optimum using sugar beet pulp (Grejek and Gervais, 1987). For *A. sulphureus* the most suitable moisture content of the medium for enzyme production was in the range of

40-50 % with a water activity in the range of 93–96 % for xylanase biosynthesis. Increasing air humidity was reported as very good way to maintain the water activity of the medium during tray fermentation (Lu *et. al.*, 2003).

3.7.5. Effect of easily metabolisable sugars on xylanase production

The effect of additional carbon sources on enzyme production was evaluated and the results were given in Fig. 3. 6. The graph explains the production of endoxylanase by *Bacillus pumilus* at 72 hour. Xylan (~21034 +/- 1060 U/gds) and starch (~ 16582 +/- 1060 U/gds) 0.01 % found to be the best additional carbon sources for maximum xylanase production.

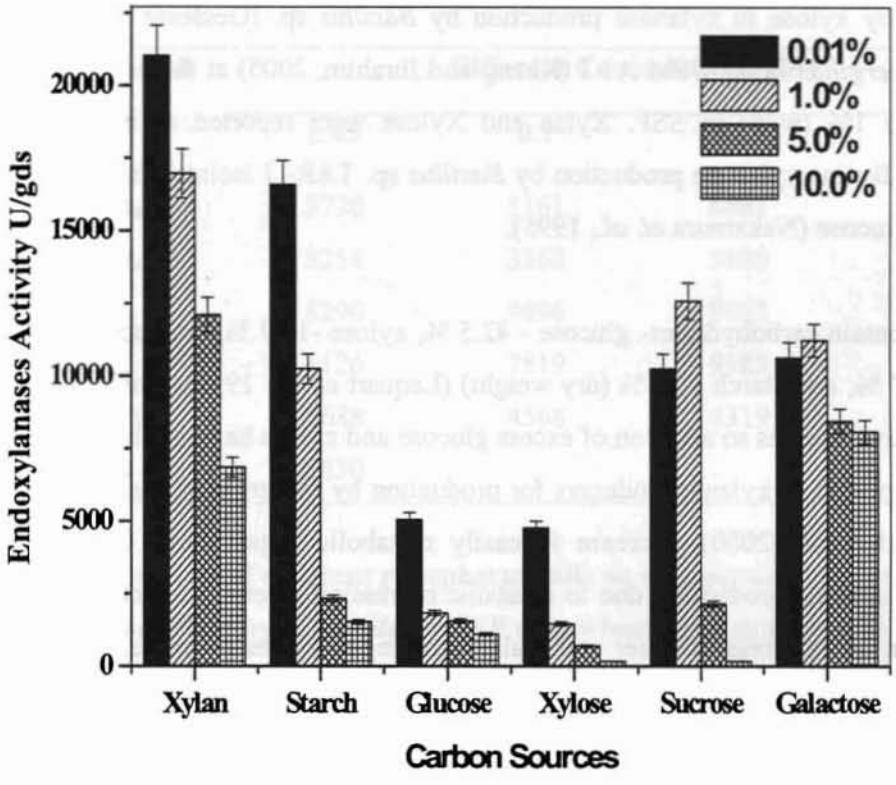


Fig. 3. 6. Influence of different concentration of additives supplement to wheat bran as inducers to enhance of xylanase production by *B. pumilus*.

The production was not much effected by increasing the concentration of galactose (0.01 % ~11237 U/gds) upto 10 % (~10635 U/gds), but in case of Oat spelt xylan (arabinose: glucose 10: 15 %) only 57 % activity retained upto 5 % (~12106 U/gds) and upto 1 % (~10256 U/gds) in case of starch; beyond which production decreased. The optimum concentration of xylan and starch for the production of xylanase was 0.01 %. However, when starch concentration was increased upto 5 % (~2335 U/gds), the production of xylanase was decreased 4.3 fold, while that of xylan (10 %) has shown 1.5 fold decreases. Xylose (~ 4761 U/gds) and glucose (~ 5049 U/gds) in low concentration (0.01 %) have repressed the enzyme production by 4.5 fold and in high concentration it has shown inhibitory effect. Lemos and Pereira (2002) explained the effect of some sugars on xylanase production by *A. awamori* in SSF. There are reports related to the phenomenon of repression by xylose in xylanase production by *Bacillus* sp. (Gessesse and Mamo, 1999) and *Aspergillus niger* USM A1 I (Kheng and Ibrahim, 2005) at the concentration of higher than 1% (w/w) in SSF. Xylan and Xylose were reported as inducers for thermophilic alkaline xylanase production by *Bacillus* sp. TAR- 1 isolated from soil and repressed by glucose (Nakamura *et. al.,* 1995).

Wheat bran contain carbohydrates- glucose - 42.5 %, xylose -16.7 %, arabinose - 3.3 %, galactose - 2.7 %, and starch - 35 % (dry weight) (Lequart *et. al.,* 1999), which by itself acts as a nutritive sources so addition of excess glucose and xylose have inhibitory effect. Xylose was reported as xylanase inducers for production by *Clostridium absonum* CFR-702 (Rani and Nand, 2000). Increase in easily metabolic sugars have inhibited or decreased the enzyme production due to catabolic repression or end product inhibition. The repression of xylanase or other cell wall degrading enzymes by preferred carbon sources such as glucose or xylose (Asymeric *et. al.,* 1988) is an efficient energy conserving mechanism because the activity of enzymes that degrade xylan and other polysaccharides may not be required when simple sugars like glucose is abundant in growth medium. Xylanase excretion is activated in presences of oligosaccharides with high degree of polymerization and substitution such as xylan. Xylan's backbone structured with mixed 1- 3 and 1- 4 linkages, like in oat spelt and birch wood xylan, was preferentially attacked, inducing the excretion of xylanolytic enzymes (Kohli *et. al.,*

2001, Sa- Perira *et. al.,* 2002, Saiman *et. al.,* 1997). Enzyme production was related to the type and concentration of carbon source used (Gawande and Kamat, 2000).

3.7.6. Effect of phosphorus salts on enzyme production

The effect of different concentration of various phosphorus salts on endoxylanase production was explained in Table. 3. 2. Where Na_2HPO_4, K_2HPO_4, and KH_2PO_4 in low concentration has shown enhancing effect on xylanase production by *B. pumilus*. Inorganic phosphates are important constituent of cellular biomolecules like cAMP, nucleic acid and co -enzymes and are known to play a regulatory role in the synthesis of both primary and secondary metabolites (Demain *et. al.,* 1972).

Phosphorous- salt	Different Concentration (M)			
	0.05	**0.1**	**0.15**	**0.2**
Na_2HPO_4	3720	5161	**8881**	6303
NaH_2PO_4	3258	3360	5880	6768
K_2HPO_4	6290	**9896**	9623	8925
KH_2PO_4	4126	7819	**9983**	9841
$(NH_4)_2H_2PO_4$	4688	4568	4319	3055
Control	5830			

Table. 3. 2. Influences of different phosphorus salts on enhancement of xylanase production by *B. pumilus* in SSF with wheat bran as substrate

An increase in enzyme production was observed with increasing phosphate concentration upto 0.15 M followed by a decline in higher concentration of more than 0.15M. It was reported that high concentration of phosphorus lead to protein accumulation in the system (Shaku *et. al.,* 1980). Ninawe and Kuhad, (2005) have reported that the presences of $(NH_4)_2H_2PO_4$ have completely ceased the production of xylanase by *Streptomyces cyaneus* SN32. Orthophosphate anions (HPO_4^{2-}) reported to have a significant effect on

the enzyme structure and thermal stability; it improves the stability as well as the activity of xylanase (Park *et. al.,* 2000).

3.7.7. Effect of nitrogen sources on enzyme production

The productivity of xylanase is greatly influenced by both the source and concentration of nitrogen (Kulkarni *et. al.,* 1999). In the present work different inorganic nitrogen like ammonium sulphate, ammonium chlorides, ammonium tartrate, potassium nitrate and sodium nitrate were evaluated (Fig. 3. 7). The results showed maximum xylanase production (~14752 U / gds) with Sodium nitrate (0.01 g) as nitrogen source. Sodium nitrate is a basic salt containing nitrate nitrogen, so it was easy to get nitrogen, as well as Na^+ ions present that help in the growth of bacteria. Jeye and coworkers has reported similar effect of low concentration of sodium nitrate on endoxylanase production by *A. versicolor* MKU3 (3249. 9 U/g) in wheat bran (Jeya *et. al.,* 2005). Similarly *Streptomyces cyaneus* SN32 was found to exhibit maximum xylanase production in liquid medium in presences of sodium nitrate (Ninawe and Kuhad, 2005). The results show that enzyme production was very low in presences of $(NH_4)_2SO_4$. Ammonium sulphate, an acidic salt containing ammonium nitrogen has inhibitor effect on endoxylanase production. It was reported that increased concentration of $(NH_4)_2SO_4$ leads to the toxicity so that microorganisms cannot survive therefore, xylanase production decreased (Kansoh and Gammal, 2001). NH_4NO_3 was found to be effective for enhanced xylanase production with *Schizophyllum* and $(NH_4)_2HPO_4$ with *Thermomyces lanuginosus* RT9 (Haltrich *et. al.,* 1993, Hoq *et. al.,* 1994). Bakir *et. al.,* (2001) reported the enhancement of xylanase production by *Rhizopus oryzae* with the addition of 1 % inorganic nitrogen sources like $(NH_4)_2SO_4$, KNO_3 and $NaNO_3$ with 1 % soybean bagasse, resulting in an increase almost six fold for the first two salts and fivefold for the third. Among the nitrogen sources tested for xylanase production by *Aspergillus niger* USM A1 I in SSF using palm kernel cake, $NaNO_3$ -0.075% (w/w) was found to enhance the production by 46 % compared to the cultivation without any other nitrogen sources (Kheng and Ibrahim, 2005). In *T. harzianum* 1073 D3 presences of $NaNO_3$ has enhanced xylanase production (Seyis, and

Aksoz, 2005), where as in *T. harzianum* presences of $NaNO_3$ and peptone as nitrogen sources enhanced production (Abdel-Sater and El-Said, 2001).

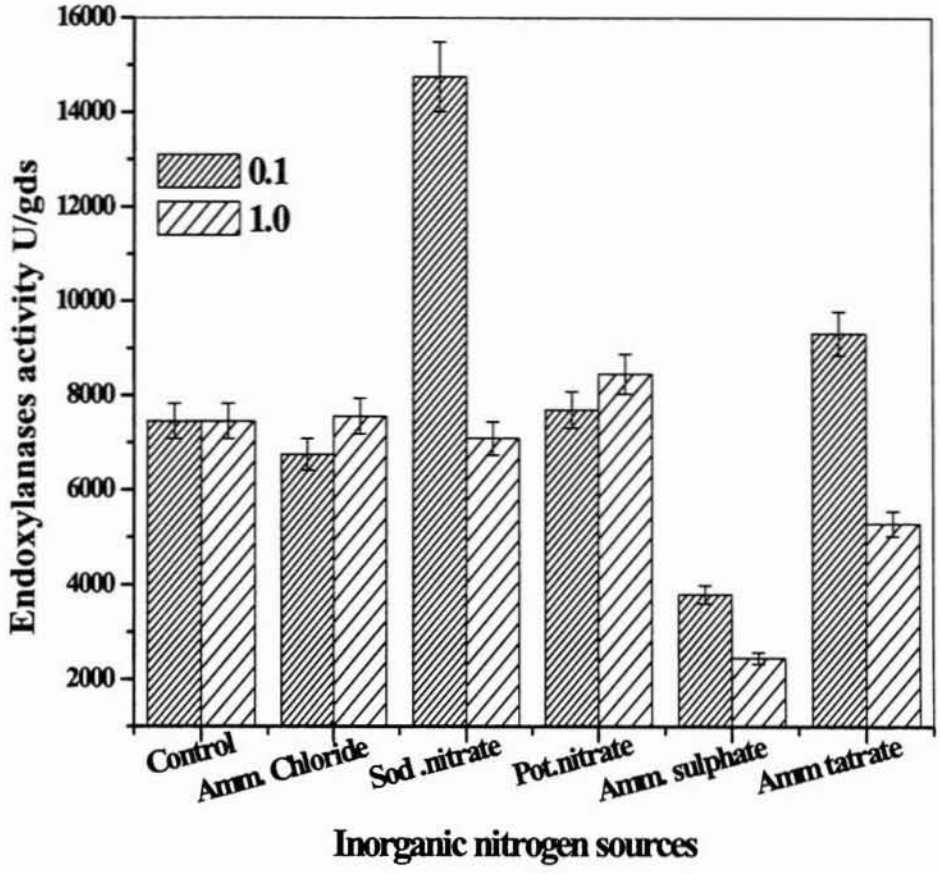


Fig. 3. 7. Effect of different inorganic nitrogen sources (0.1 and 1 g nitrogen) on xylanase production by *B. pumilus* growing in SSF at 35 °C with wheat bran.

The result of effect of organic nitrogen sources with wheat bran was well explained in Table. 3. 3. This shows that supplementary nitrogen sources do not have a considerable effect on enzyme production except malt extract, which has shown inhibitory effect. Yeast extract (5.0 %) has given highest production (~19546 U / gds) at 96 hour, Soy meal

also found to be very productive; it is a balance source of protein (42 %) in terms of composition and accessibility. Yeast extract has high influence to enhance endoxylanase production at 72 hour of incubation.

Nitrogen sources	Different concentration (g %)			
	0.2	1.0	5.0	10
YE	14185	**16912**	19546*	10453*
Pep	10221	10006	9320.8	9267.0
ME	7815	5680	Nd.	Nd.
SM	**15266**	14404	13720	13639.2
Try	13690	13128	11821.8	10538.26
CSL	10338	10520	Nd.	Nd.
Urea	10192	10124	Nd.	Nd.
Control	7544	-	-	-

Table. 3. 3. Influence of different organic nitrogen sources (0.2, 1.0, 5.0 and 10 enhancement of xylanase production by *B. pumilus* growing in SSF at 35 °C wheat bran as carbon sources (* Fermentation period 96 hour, Nd- Not detected).

Urea was reported as a strong recalcitrant source of protein, which represses the endoxylanase production (Sa- Perira *et. al.,* 2002, Kalogeris *et. al.,* 1998), but here it has not repressed endoxylanase production, in low concentration it has an inducing effect. The result shows that even low concentration of all nitrogen sources have significant effect on enhanced enzyme production except malt extract, which might be due to presence of high sugar content. This result was in good agreement with xylanase production for *Streptomyces* sp. using peptone, whereas yeast extract has negative effect on xylanase production by *Streptomyces* sp. (Techapun *et. al.,* 2002). Shah *et. al.,* (1999) reported that addition of soybean meal has resulted in 25 % increase in xylanase activity

related to yeast extract by *Bacillus* sp. Sam-3. Addition of yeast extract was reported to be effective for the enhancement of xylanase activity by *Bacillus* sp. AR-009, but bacteriological peptone and tryptone have no sizeable effect on xylanase production (Gessess and Mamo, 1999).

3.7.8. Effect of metal ions on enzyme production

Metal ions have been classified as hard and soft acids and bases (Pearson, 1973) and Class -A (e.g. Mg^{2+}, Ca^{2+}, Na^{2+} etc) Class -B (e.g Hg^{2+}, Pb^{2+} etc) and borderline ions (e.g. Cu^{2+}, Fe^{2+}, Ni^{2+}, Zn^{2+} etc) (Nieboer and Richardson, 1980). Some Class -A ions are essential for growth of microorganism while borderline ions are required as micronutrients, where as class B ions are toxic and not necessary for biological functions. The effect of potential activity enhancers on xylanase production was given in Fig. 3. 8. The result indicates that additives like NaCl, KCl, $MgSO_4$ and $MnSO_4$ have significant effect on enzyme production where as $CuSO_4$, $CoCl_2$, $FeSO_4$, $ZnSO_4$, and $CaCl_2$ have decreased enzyme production and in high concentration has shown inhibitory effect. The presence of potassium dichromate in low concentration has not inhibited enzyme production. The xylanase excretion and its extracellular performances depend directly on the type of ions present in solution, since the effect is overall kinetics (Buchert *et. al.,* 1997). NaCl can be used as a water activity depressant that can enhances xylanase production (Archana and Sathynarayana, 1997).

There was report related to enhanced xylanase production by *Streptomyces cyaneus* SN32 in presences of NaCl and KCl in the mineral medium supplemented with wheat bran by liquid fermentation (Ninawe and Kuhad, 2005). The presences of KCl showed a positive effect where as low concentration of $FeSO_4$ negative effect and the addition of four components - $NaNO_3$, $MgSO_4$, peptone and K_2HPO_4 has enhanced xylanase production by *Aspergillus versicola* MKU3 in SSF (Jeye *et. al.,* 2005). It was reported that $FeSO_4$ did not have any significant effect for xylanase production by *Aspergillus terrus*, whereas $MgSO_4$ showed a contrary effect (Ghanem *et. al.,* 2000). Xylanase production by *Rhizopus oryzae* reported to be enhanced by a medium composition containing 3 % corn

cobs, 0.5 % NaCl, 1 % soybean bagasse, and 1 % ammonium sulphate (Bakir *et. al.*, 2001).

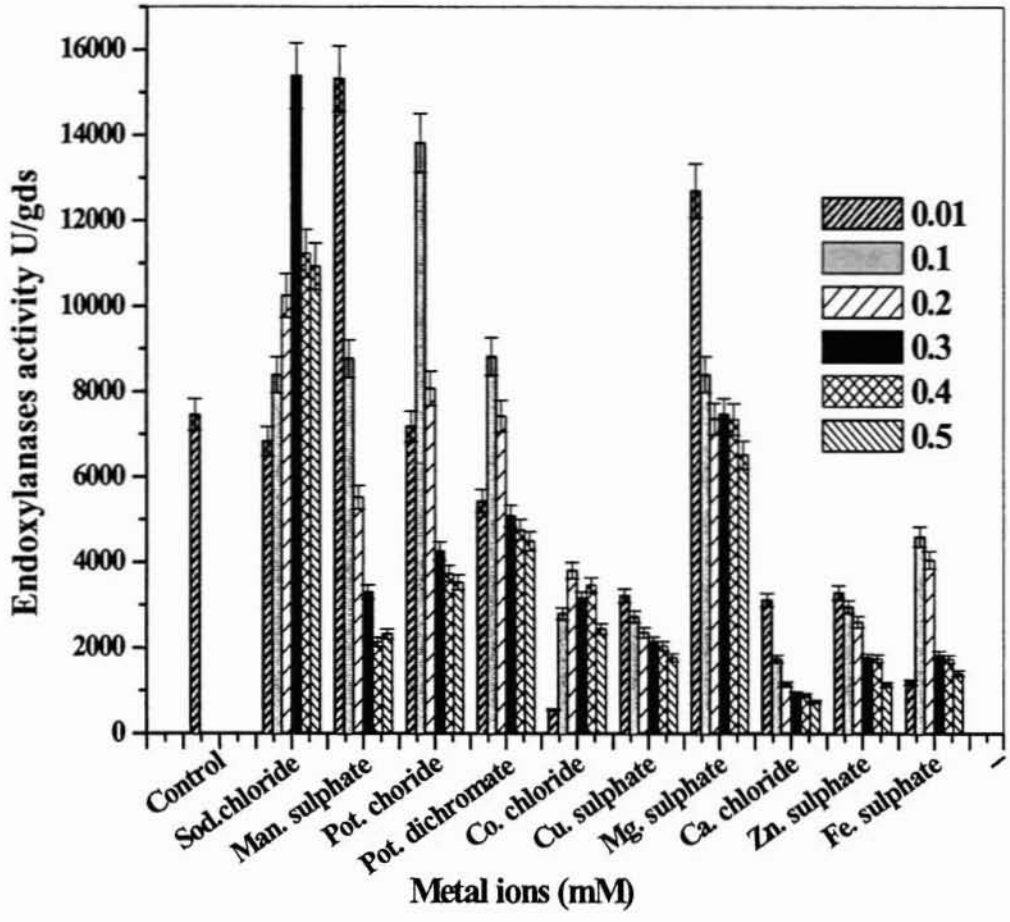


Fig. 3. 8. Effect of different metal ions in different concentration (0.01, 0.1, 0.2, 0.3, 0.4 and 0.5 mM) on enhancement of xylanase production by *B. pumilus* grown in wheat bran at 35 °C.

3.7.9. Effect of Sodium carbonate on xylanase production

Xylanase production with varying concentration of Na_2CO_3 was studied and the results were explained in Fig. 3. 9. As the concentration of Na_2CO_3 increases there was enhanced production of xylanase up to 3.5 % (pH - 9.8) and as concentration increased there was an observable drop in production. With 10 % total inhibition of xylanase production was observed.

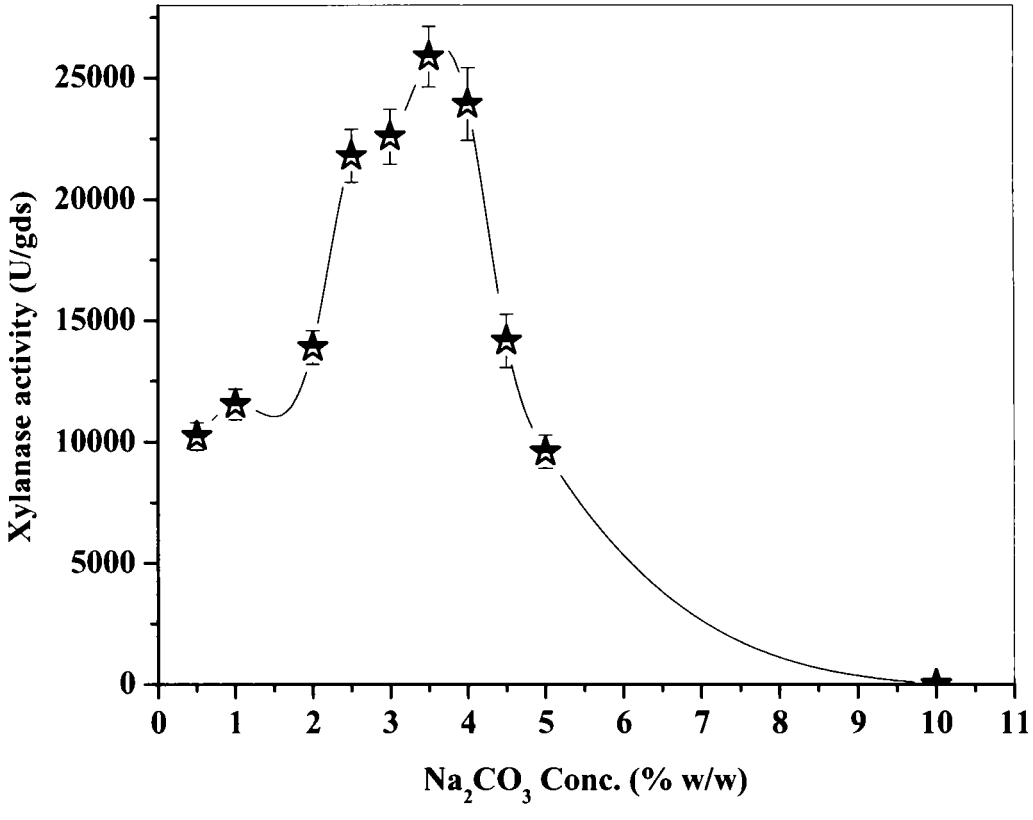


Fig. 3. 9. Influence of different concentration of Na_2CO_3 (ranging from 0.5, 1, 2, 2.5, 3.0, 3.5 4.0, 5.0 and 10 % w/w) on enhancement of xylanase production by *B. pumilus* growing in wheat bran at 35 °C.

This might be due to high alkaline condition (pH - 12) of the medium, which has inhibited the growth as well as enzyme production. Na_2CO_3 act as a nitrogen sources as well as presences of this help to increases the pH toward high alkaline range and it was

maintained through out the growth of the organism. There was no much decrease in pH as the growth and enzyme production proceeds. Also the mild alkaline treatment of the substrate help in the break down of cell wall as well as release of simple sugars, which can enhances xylanase production. Sodium carbonate is generally used to adjust the pH to around 10, because alkalophiles usually require at least some sodium ions (Horikoshi, 1999). Various alkaliphilic bacterial strains are reported to grow in sodium carbonate medium and do not grow at pH 7- obligatory sodium-dependent alkalophiles (Horikoshi, 1999). There are various reports related to the use of Na_2CO_3 for adjusting the pH of the medium after autoclaving towards alkalinity for SmF and SSF by *Bacillus* sp. (Dimitrov *et. al.*, 1997, Subramaniyan *et. al.*, 1998, Shah *et. al.*, 1999, Sa-Pereira *et. al.*, 2002) and for *Streptomyces* sp. QG-11-3 (Beg *et. al.*, 2000). There was report related to maximum xylanase production in SSF by *Bacillus* sp. JB-99 in rice bran moistened with mineral salt and Na_2CO_3-5.0 % with a pH of 10.0 (Virupakshi *et. al.*, 2005). Gessesse and Mamo, (1999) reported the effect of Na_2CO_3 different concentration (2.5, 5 and 10 %) of which 10 % reported as enhancer for xylanase production in wheat bran as substrate in SSF by *Bacillus* sp.

3.7.10. Different Moisturizing agent

The mineral salt solutions, distilled water, and tap water were used as moistening agents to support xylanase production with wheat bran to a varied extent (Fig. 3. 10). Tap water supported xylanase production where as enzyme production using distilled water was very low than any other MSM, which shows the importance of essential elements and phosphorus salts in low concentration to enhances production. Xylanase titers were relatively high when wheat bran was moistened with MSM_{11} which contains 3.5 % Na_2CO_3 with K_2HPO_4 and $MgSO_4.7H_2O$ followed by MSM_5 with composition as follows (g / L) K_2HPO_4 2.0, $MgSO_4.7H_2O$ 1.0, Na_2HPO_4 3.0, $CaCl_2.2H_2O$- 0.2, $FeSO_4$-0.2, $MnSO_4$-0.2 in a 1 : 2.5 ratio. The presence of NaH_2PO_4 together with other mineral salts would have effected in the enhanced production of xylanase by *B. pumilus*. MSM_9 the medium contains $NaNO_3$ and xylan that has an enhancing effect on xylanase production,

where as in MSM_8 the amount of YE was in very high concentration and that of MSM_7 the NaCl content was very high that have affected the xylanase production.

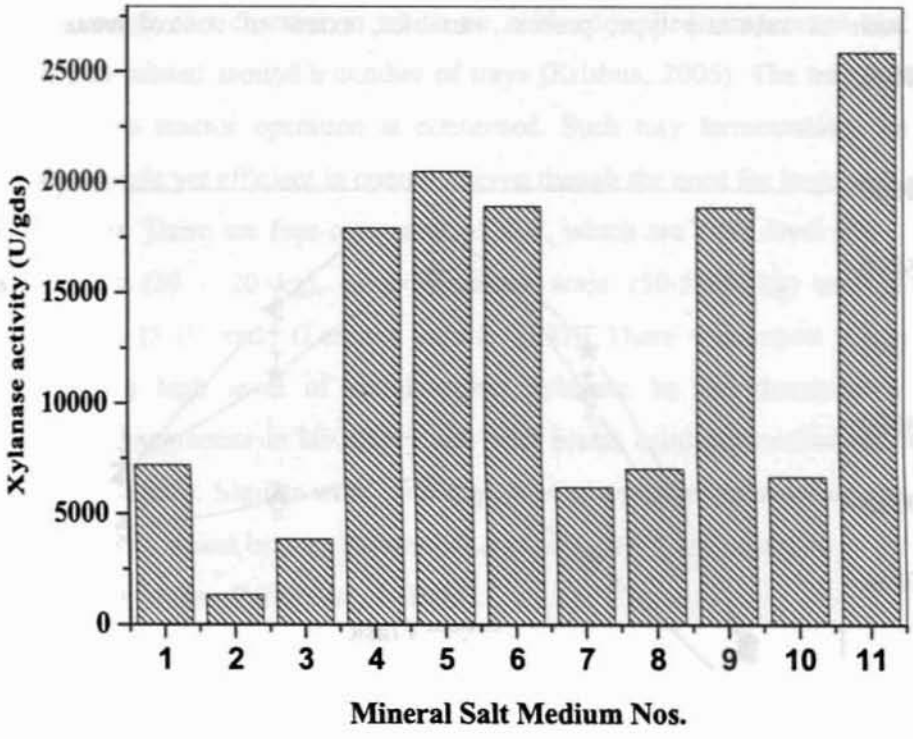


Fig. 3. 10. Xylanase production by *B. pumilus* with different moisturizing agent used in the ratio 1:2.5 with wheat bran as substrate and incubated at 35 °C for 72 hour.

Addition of YE and Peptone in MSM_4 has increased the production but relatively equal to the production as medium MSM_6 with out these nitrogen sources. It was reported that the use tap water and distilled water as moisturizing agents was effective in the enhancement xylanase production (Archana and Satayanarayan, 1997). Maximum xylanase production was reported in SSF using *Bacillus* sp. JB-99 in rice bran moistened with mineral salt of composition K_2HPO_4 2.0, NaCl- 1.0, $MgSO_4.7H_2O$ 0.1, $CaCl_2.2H_2O$- 0.05 and Na_2CO_3-5.0 with a pH 0f 10.0 (Virupakshi *et. al.*, 2005).

3.7.11. Xylanase production in enamel trays

Unlike submerged fermentation, in SSF the choice of a suitable reactor system is difficult, given the heterogeneity of the substrate matrix. Therefore, selection will depend on factors such as substrate type, process variables, extent of control required etc (Krishna, 2005).

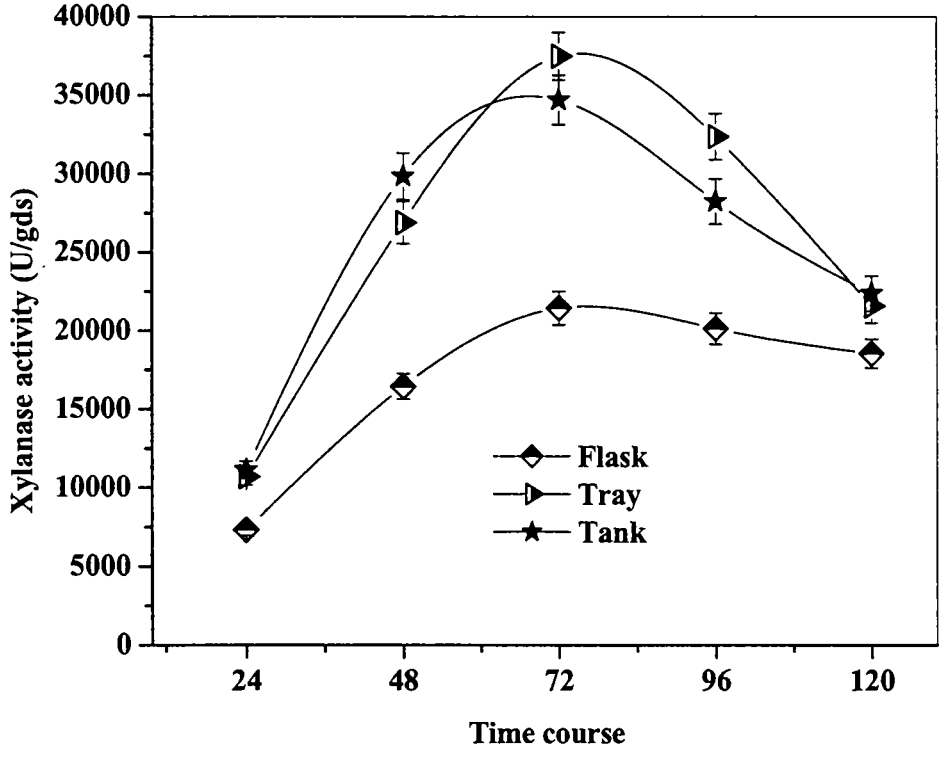


Fig. 3. 11. Comparison of xylanase production by *B. pumilus* in flask fermentor, tray fermentor and tank fermentor in a time course of 120 h. Wheat bran was used as the substrate and incubated at 35 °C.

Xylanase production in enamel tray (~37482 +/- 1000 U/gds) as well as in tank fermentor (~34698+/- 1000 U/gds) was 1.78 fold higher than that of culture flasks (~21034 +/- 1000 U/gds), there by indicating that scaling up does not results in a reduction of xylanase titer (Fig. 3. 11). The humidity inside the tank was almost 100 %, which has directly effected

the moisture level inside the tank and adversely effected the enzyme production, as the fermentation proceeds. Suitable measure to control the humidity in tank fermentor will be advantageous to improve enzyme production. Tray fermentors are the simplest of all types and the trays are usually constructed using wood, plastic, or metals. A tray bioreactor consists of a chamber in which air, with controlled temperature and relative humidity, is circulated around a number of trays (Krishna, 2005). The tray systems are simple as far as reactor operation is concerned. Such tray fermentations have been described as simple yet efficient in operation even though the need for large area is one of their drawbacks. There are four categories of SSF, which are flask level (50 - 1000 g), laboratory-scale (50 - 20 kg), pilot fermentor scale (50-5000 kg) and production fermentor scale (5-10 tons) (Lonsane *et. al.,* 1992). There was report related to the production of a high level of cellulase-free xylanase by the thermophilic fungus *Thermomyces lanuginosus* in laboratory and pilot scales using lignocellulosic materials (Gomes *et. al.,* 1993). Similar work with high titer of xylanase production was there in wooden trays with wheat bran as substrate (Lu *et. al.,* 2003) and pectinase production by *Aspegillus niger* (Solis - Pereira *et. al.,* 1993).

3.7.12. Extraction buffer estimation

The extraction ability of buffers for crude xylanase extraction from fermented bran was tried using different buffers (pH 6-12) and explained in Fig. 3. 12. The results show that phosphate buffer with pH-7 (0.1 M) was effective in the recovery of enzyme. Buffer solutions are observed to be highly effective for maximum recovery of crude enzyme as well as they help to maintain the stability of enzyme, than simple water extraction. Enzyme from *Bacillus* sp. JB-99 was extracted from rice bran with 10 mM glycine - NaOH buffer, pH 10.0 (Virupakshi *et. al.,* 2005). Cold distilled water was used effectively in extraction of xylanase in SSF (Christopher *et. al.,* 2005, Kheng and Ibrahim, 2005). Beg *et. al.,* (2000) reported glycine NaOH buffer (pH 8.6), for the extraction of xylanase from *Streptomyces* sp. QG-11-3. K_2HPO_4 increases the binding affinity of xylanase to xylan resulting in enhanced activity and stability.

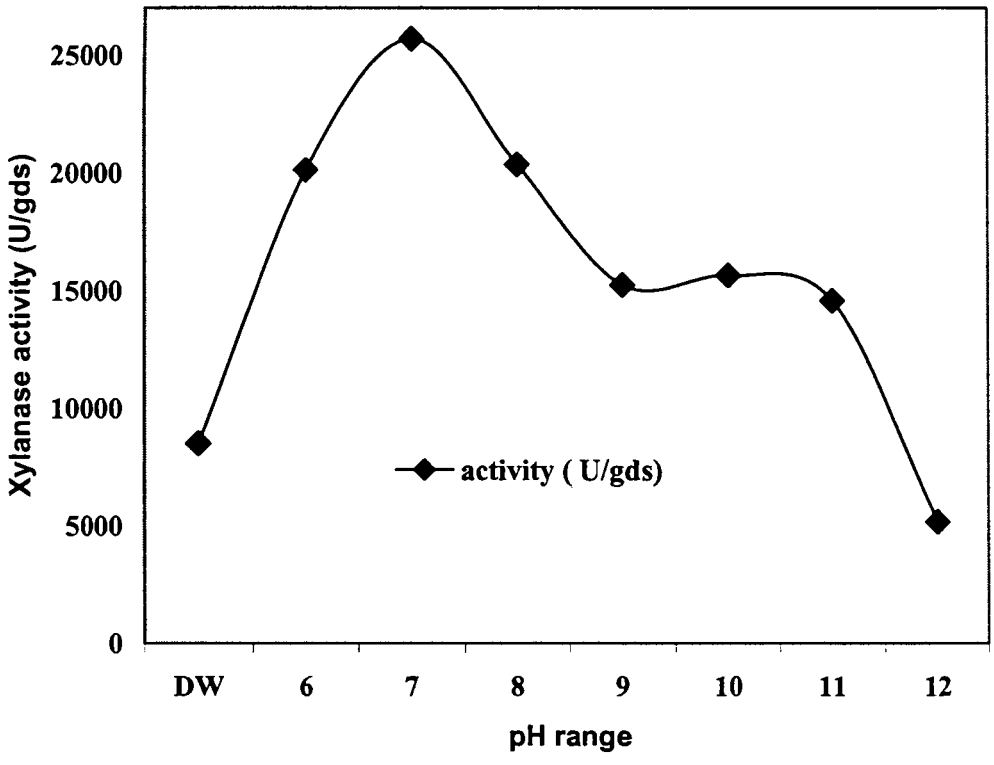


Fig. 3. 12. The effect of different buffers and distilled water on effective extraction of xylanase from *B. pumilus*

3.7.13. Storage stability

For industrial applications, storage of the enzymes at room and/or refrigerated temperature, without appreciable loss of activity is one of the most important and desirable characteristics. Fig. 3. 13 give the storage ability of crude enzyme at different pH under different conditions after 2 weeks. The results shows that 93 % activity was retained after 2 weeks in deep freezer, where as in refrigerator only 60 % activity retained and at room temperature almost all activity was lost after 2 weeks. The activity observed

o be decreasing gradually in deep freezer and refrigerator with in one week and there fter no considerable reduction observed, where as at room temperature reduction ccurred drastically within 3 days. The crude enzyme was extracted in phosphate buffer pH-7) of 0.1 M and 0.6 M concentration. The enzyme retained 98 % activity (0.6 M) and bout 95 % activity (0.1M) when stored in deep freeze for more than 4 months with out ddition of any preservatives. At refrigeration temperature no loss of activity was found p to 1 week but after 3 weeks a marginal decrease (10–15%) was found.

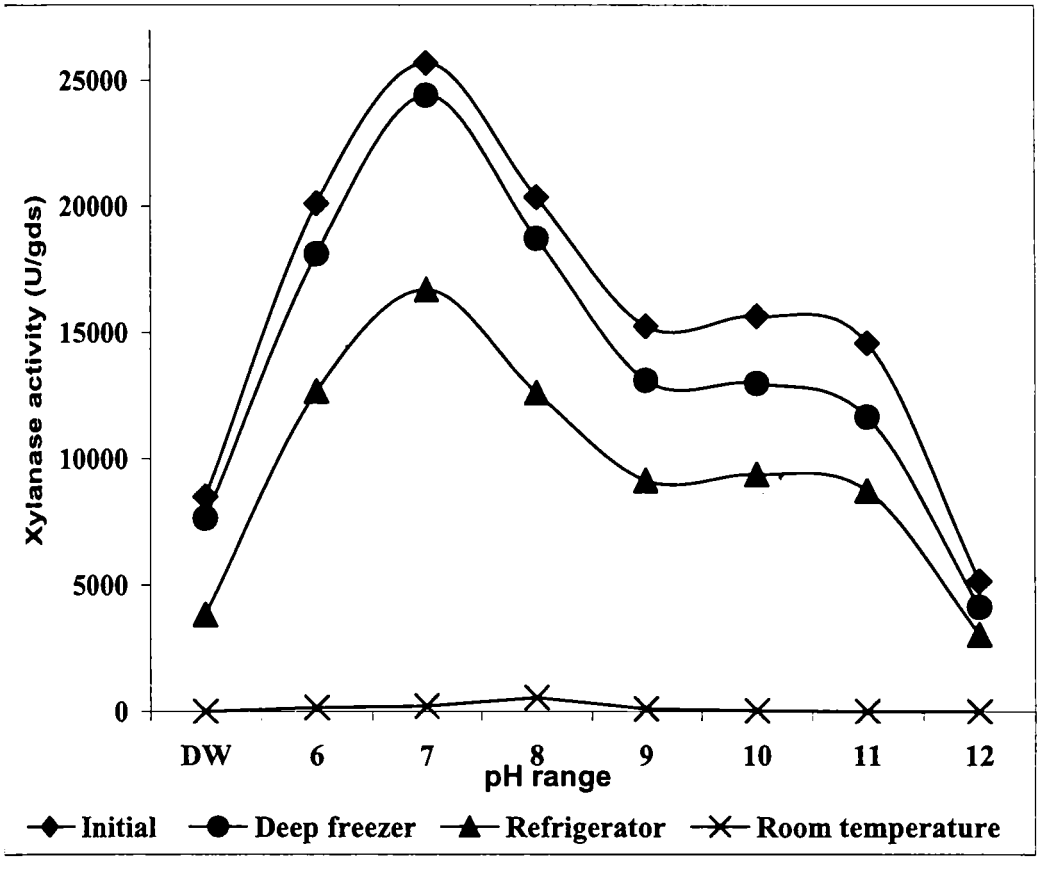


Fig. 3. 13. **Effect of different buffers (pH ranging from 6 to 12 and distilled water) on the storage of xylanase from *B. pumilus*. (Initial and final activity after 2 weeks) in different storage conditions.**

At room temperature decrease in activity was not observed upto 1 day but at the end of the week there was 20 % loss in activity. The activity of xylanase from *Bacillus* sp. has been reported to be enhanced 2.3 fold with 0.6 M K_2HPO_4 than 50 mM or 1 M concentration, suggesting that biding residues as well as catalytic residues were affected by K_2HPO_4 (Park *et. al.*, 2001). The concentration of K_2HPO_4 was reported to be effective on enhancing the thermal stability of endoxylanase from *Bacillus* sp. probably because of the conformational change in xylanase cause by phosphate anions. Phosphate anions are reported to improve the stability of xylanase significantly and the stabilizing effect was reported to be due to ionic strength effect. The studies made by Park *et. al.*, (2001) give an information that relatively small phosphate anions may intrude in to the active site (10Å) of xylanase and effect the glutamic acid residues. The fermented matter was dried and activity was estimated, it was observed that 92 % of activity was retained in the dried fermented matter for more than 1 months when stored in deep freezer.

3.8.0. Conclusion

In conclusion, the results obtained from this work strongly indicate that the SSF system using wheat bran as substrate was an economical method for the production of xylanase at extremely low operational cost based on the fact that wheat bran was one of the cheap and abundant agro waste by-products of agricultural industry. The use of low- cost raw materials leads to reduction in the culture medium cost that generally range from 25 to 50 % of total production cost. The cultivation system was easily been modified to enhance the production of the enzyme by the bacteria, which has facilitated the scale up processes for mass production. The xylanase from *B. pumilus* has been produced in tray and tank fermentor and characterized. This increase in yield and decrease in production cost was promising methodology for hyper production of alkaline thermostable xylanase. The presences of additives in low concentration does not effected the growth and enzyme production by this strain indicating that this can also be used as biopulping agent.

Chapter. 4.

Purification and characterization of xylanase from B. pumilus produced by SSF

4.0. INTRODUCTION

The characteristics of biotechnological systems make purification the most expensive part of biomaterials process production. Spalding (1991) has commented that values as high as 50 % of overall costs in the biotechnology industries are related to downstream processing. Thus, the development of new and economically advantageous purification methods is a challenging area. Purification of target proteins requires their separation from medium or from the raw extract used for the maintenance of the biomolecules. Various methods are used in sequence for the purification of enzymes, which includes Ultrafiltration or membrane filtration, ammonium sulphate or acetone precipitation, ion exchange chromatography, and gel filtration chromatography. Extracellular xylanase can be purified with one or combination of one or more methods. The usual steps in xylanase purification include concentration, ion exchange and gel filtration chromatography. Ammonium sulphate precipitation is the most commonly used concentration technique followed by combination of conventional chromatography methods like ion exchange or gel permeation chromatography for xylanase purification. Ion exchange can be done by cationic resins like- amberlite, CM - Sephadex, hydroxyapatite, SE- Sephadex or anionic resins like- DEAE - Cellulose, DEAE - Sephadex, Duolite A-2. Gel permeation chromatography was done using Sephadex beads and Biogel. Ultrafiltration reported to be effective for the concentration of xylanase from *Chainia* sp. in combination with anionic exchanger (Bastawde *et. al.,* 1991) and cationic exchanger for *S. lividans* (Morosoli *et. al.,* 1986) ionic exchanger and gel permeation chromatography for *Sporotrichum dimorphosporum* (Comtat, 1983). The purification of xylanase from thermophilic and alkalophilic *Bacillus* sp. was done in single step using DEAE – Toyopearl 650M (Okazaki *et. al.,* 1984). *Bacillus* sp. strain TAR- 1 xylanase was purified to homogeneity by ammonium sulfate fractionation and anion-exchange chromatography using DEAE- Toyopearl 650M (Nakamura *et. al.,* 1995). Xylanase from *Bacillus* sp- K-1 was purified by single step by affinity chromatography (Ratanakhanokchai *et. al.,* 1999). Electroelution has been reported as simple, efficient and time saving method for xylanase purification from *Bacillus* sp. CCMI- 1996 (Periera *et. al.,* 2000). Purification of xylanase I from *Bacillus circulans* AB-16 was done using anion exchanger, Sepharose - Q and

Sepharose - 6B leading to the purification of two form of xylanase A and B (Dhillon *et. al.*, 2000). Santos *et. al.*, (2002) reported the purification of xylanase from *Bacillus pumilus* by expanded bed adsorption, a cationic adsorbent (Streamline SP) in the presence of cells. *B. thermantarcticus*, a thermophilic bacterium isolated from Antarctic geothermal soil produced extracellular xylanase and β- xylosidase, which was separated by gel filtration with Sephacryl S-200 (Lama *et. al.*, 2004). Rizzatti *et. al.*, (2004) reported the use of DEAE – cellulose and Biogel –P- 60 column for the purification of xylanase from *A. phoenicis*. Eudragit - S100 an anionic polymer was reported to be effective in the precipitation of xylanase from *B. amyloliquefaciens* MIR-32 and *Streptomyces olivaceoviridis* E-86 (Breccia *et. al.*, 1998, Ai *et. al.*, 2005). Endoxylanase from *Rhizopus oryzae* fermentation was partially purified from the culture medium by ammonium sulfate precipitation and cation exchange filtration (Bakir *et. al.*, 2001). Carmona *et. al.*, (2005) reported the purification of xylanase II (28 - fold) from *A. versicolor* by DEAE-Sephadex and HPLC GF-510 gel filtration.

4.1.0. MATERIALS AND METHODS

4.1.1. Crude enzyme preparation

The microorganism *Bacillus pumilus* was grown on wheat bran by SSF as mentioned in Chapter. 3. The enzyme was extracted after 72 hour of incubation, the cell free supernatant was obtained by centrifugation at 10, 000 x g for 20min at 4°C (SELECTA Cold centrifuge, Germany). The initial activity was estimated together with total soluble protein (Lowry's method) as mentioned in Chapter. 2, or by direct method where the absorbance was taken at 280 nm, here free Tyrosine (Tyr) and Tryptophan (Trp), uric acid, and bilirubin interfere at 280 nm.

4.1.2. Fractional ammonium sulphate precipitation

The cell free supernatant was kept at 4 °C and required amount of ammonium sulphate was added slowly with mild stirring. The mixture after addition was stored for 1 hour and

centrifuged at 10,000 x g for 20 min at 4 °C. The precipitate was dissolved in minimal volume of 50mM phosphate buffer ($Na_2 HPO_4$ / NaH_2PO_4, pH-7) and the supernatant was again subjected to precipitation, thus the separation was followed for each fraction of precipitate upto 100 % fraction with gradual 10 % increase between each fraction. The fractions collected were dialyzed against the same buffer at 4 °C for 24 hour with buffer change at every 4-hour. The dialyzed samples were subjected for the estimation of total soluble protein and endoxylanase activity. The samples were loaded on SDS - PAGE incorporated with 0.5 % Oats spelt xylan. For further experiments the 30- 70% was precipitated and taken. The partially purified samples (70 % ammonium sulphate ppt.) from SmF and SSF medium where subjected to Native – PAGE followed by activity analysis by zymogram analysis.

4.1.3. Concentration of enzyme by Ultrafiltration

The cell free supernatant (200 mL) was concentrated by Ultrafiltration membrane system (Amicon, Millipore) with a cut off 10,000 kDa (Mol. wt). The volume was reduced to 110 mL.

4.1.4. Purification by Ion exchange chromatography

Concentrated enzymes were loaded onto Q-Sepharose Fast Flow column (2.5 × 12 cm) pre-equilibrated with 50 mM Tris HCl buffer (pH 8.0) for xylanase. The column was washed with 200 mL of the respective buffers at a flow rate of 3.0 mL / min followed by the sample elution with 5-bed-volume of NaCl salt in gradient (0, 0.01, 0.5 and 1.0 M) in the equilibrating buffers. The active fractions were pooled and concentrated by Ultrafiltration membrane (Amicon, Millipore).

4.1.5. Purification of xylanase by isoelectric focusing

All purification steps were carried out at 4 °C.

Step 1. The organism was inoculated into the wheat bran medium (5 g) in 250 mL Erlenmeyer flasks and incubated at 35 °C in a humidity chamber for 72 h. The bactobran was extracted in 10 mM Tris HCl buffer pH – 8.0, the culture supernatant was obtained by centrifugation at 10,000 x g for 20 min at 4 °C.

Step 2. Solid $(NH_4)_2SO_4$ was added to the culture supernatant (150 mL) until 20 % saturation was obtained. After standing overnight, the precipitate was discarded by centrifugation (10000 x g, 20 min). The resultant supernatant was again precipitated with $(NH_4)_2SO_4$ to 70 % saturation. The precipitate was collected by centrifugation, suspended in 80 mL of 10 mM Tris HCl buffer (pH 8.0), and 10 mL from this was dialyzed five times against 10 L of the same buffer for 20 h with 5 changes.

Step 3. The sample was dialyzed (final volume, 18 mL) and 0.1 mL of this was subjected to preparative isoelectric focusing (PIF). The isoelectric focusing was performed according to the instructions provided by the manufacturer Rotofor system (BIORAD) equipped with water recirculating- chiller and vacuum pump (Biolyte Ampholyte from Biorad - 1 % was added with pH ranging from 3 to 10). The anion exchanger membrane consist of 0.1 N NaOH and the cation exchanger membrane consist 0.1 N H_3PO_4, the mini focusing camber can hold 18 mL which consist of sample, ampholyte and glycerol solution. Xylanase sample (1mg / mL protein) was used for the experiment. Twenty fractions (1mL each) were automatically collected, scanned for their absorbance at 280nm, and assayed for xylanase activity as well as the pH of each fraction was monitored. The fractions were subjected to Native – PAGE and those, which has shown activity in zymogram was subjected to SDS – PAGE.

4.1.6. Gel electrophoresis (SDS - PAGE)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970) using 12 % gels of thickness 1.5 mm. The sample loaded in denatured condition - partially purified fraction (30 - 70 %), PIF fraction (10 - 20 active fractions only) and the 3 fraction obtained from DEAE-

Sepharose column. Samples were dissolved in a solution containing 2 % (w/v) SDS, 5 % (v/v) 2-mercaptoethanol, 10 % (v/v) glycerol, and 15 mM Tris- HCl (pH 6.8) and heated in a boiling water bath for 3 min with 0.9 mL of water. The electrophoresis was run at 200Volts for 4 hour until the tracking dye reached 1 mm above the bottom line. After electrophoresis, the gels were stained with sliver staining using Sliver nitrate. The molecular mass markers used were the SDS-PAGE standards (medium range). The molecular weight of protein marker phosphorylase b (97.0 kDa), albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa), α - lactalbumin (14.4 kDa).

4.1.8. Native – PAGE electrophoresis and zymogram preparation

The samples were loaded under non-denaturing condition; Polyacrylamide gel was prepared according to the method of Laemmli (1970). The separating gel of 12 % and stacking gel of 5 % with a slab gel thickness of 1.5 mm was found to be optimum. The crude sample, $(NH_4)_2SO_4$ fractions, DEAE – Sepharose fraction and PIF sample fractions were loaded. After solidification of the gel the well were loaded with 50 µL samples and subjected to electrophoresis at 200 Volts until the protein bands reached 1 mm above the bottom of separating gel. The gel before staining was overlaid on substrate gel prepared with Oat spelt xylan (0.5 %) and agarose (2 %) (SIGMA). The overlaid gel was incubated for 30 min at 50 °C. The substrate gel was soaked in 0.1 % Congo red solution for 15 min at room temperature and washed with 1 M NaCl until excess dye was removed from the active band. A zymogram was prepared after an introduction of the gel into 0.2 % acetic acid. The background turned dark blue and clear zones were observed in areas exposed to xylanase activity. The polyacrylamide gel was stained overnight with Coomassie brilliant blue (CBB) R-250 and. The excess stain was washed by de-staining solution containing methanol and acetic acid in deionized water. The partially purified samples – (30- 70 % $(NH_4)_2SO_4$ precipitate) from SSF and SmF medium were analyzed primarily.

4.1.7. Zymogram analysis

Zymogram analysis was performed by the method of Hager and Burgess (1980). Samples were run on an SDS gel of 12 % polyacrylamide, incorporated with 0.1 % oat spelt xylan. The sample was run and then, the gel was washed four times for 30 min at 4 °C in a 100 mM phosphate buffer (pH 7.0) (the first two washes contained 25 % (v/v) isopropyl alcohol) to remove SDS and renature proteins in the gel and further incubated in the buffer for 10 min at 50 °C. The gel was soaked in 0.1 % Congo red solution for 15 min at room temperature and washed with 1 M NaCl until excess dye was removed from the active band, and zymogram was prepared by soaking the gel in 0.2 % acetic acid. The background turned dark blue and clear zones were observed in areas exposed to xylanase activity.

4.2.0. CHARACTERIZATION STUDIES

The enzyme was characterized related to its optimum activity pH, pH stability range, activity temperature, temperature stability range as well as the effect of metal ions on partially purified and purified fractions.

4.2.1. Effect of temperature on partially purified and purified xylanase

The effect of temperature on enzyme activity was determined by performing the standard assay procedure for 10 minute at pH- 7 within a temperature range of 30-65 °C. Thermostability was determined by incubation of the purified enzyme at temperatures ranging from 25-65 °C. The residual enzyme activities were measured at every 10 minute interval for a period of two hour. The purified fractions obtained from DEAE- Sepharose column was subjected to temperature optimization and stability studies at a range of 30-60 °C.

4.2.2. Effect of pH on partially purified and purified xylanase

The optimum pH of the xylanase was determined by incubating the xylanase from *Bacillus pumilus* with Oat spelt xylan in appropriate buffers. Effect of pH on the activity was measured by essentially adopting Bailey's method (Bailey *et. al.,* 1992) using the following buffer systems. The following buffer (100 mM): citrate phosphate pH- 5 and 6, phosphate buffer pH- 6.5, 7, 7.5 and 8, Glycine - NaOH buffer pH 8.6, 9, 9.6, 10, 10.5 and 11. pH stability was determined after incubating the enzyme in the buffers described above for 2 hour at 30 °C and measuring the residual activity. The purified fraction obtained from DEAE – Sepharose column were also subjected to pH optimization and stabilization studies at a range of pH-5 to 11 (pH 5, 6 and 7 from citrate phosphate buffer pH-6, 7 and 8 from phosphate buffer and pH 9, 10 and 11 from Glycine - NaOH buffer).

4.2.3. Effect of metal ions on partially purified and purified xylanase

The effect of various additives on endoxylanase activity was determined by incubating enzyme partially purified as well as purified enzyme fraction at room temperature (30 +/- 2 °C) with different metal ions. The different monovalent and divalent metals like (10 mM) Na^{+}, Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Zn^{2+}, Cu^{2+} Co^{2+}, Hg^{2+}, EDTA (metal chelator), Potassium dichromate (oxidizing agent) and SDS (protein disulphide reducing agent) was taken as additives. Activity was monitored at every 15 min for 30 min and activities were determined using Oat spelt xylan prepared at pH - 7. Residual activity was expressed as the percentage of the activity observed in the absence of any compound.

4.2.4. Effect of substrate concentration on the purified xylanase

The Michaelis - Menten constant (K_m) and maximum velocity (V_{max}) were determined by using Oat spelts xylan as the substrate. Initial reaction rates of Oat spelt xylan hydrolysis were determined at different substrate concentrations ranging from (2 - 24 mg / ml) 1.0 - 30.0 mg/mL in phosphate buffer (0.05 M, pH - 6.5). Higher xylan concentrations could not be used due to low xylan solubility. Reaction rate vs. substrate concentration curve

was plotted to determine whether the enzyme obeys Michaelis-Menten kinetics, and constants were determined from a Lineweaver-Burk plot (Lineweaver and Burk, 1934).

4.2.5. Effect of organic solvents on partially purified enzyme

The partially purified enzyme was stored in different organic solvents like acetone, chloroform, ethanol, proponol, butanol and methanol (1 and 10 %) in order to find their stability and effect on enzyme action and the activity was estimated at regular interval of 15 min for 2 hour.

4.3.0. RESULTS AND DISCUSSION

Purification of enzymes to homogeneity is crucial to study the characteristics of enzyme in the absences of any interfering factors present in the cell or culture broth. The characteristic of enzyme reflects the properties, which may be important from the industrial point of view. Purification to homogeneity often proves very difficult owing to the complexity of the systems and usually multiple steps required before the enzyme is sufficiently pure to be considered homogenous (Warren, 1996). The purification of protein from *Bacillus* sp. requires special consideration and combination of various approaches. Cell free culture supernatant 200mL obtained after centrifugation of culture broth, which serves as crude xylanase preparation, with a total activity of 31992 Units. The specific activity of crude xylanase preparation was 216.16 U/mg of protein (Table. 4. 1.).

4.4.0. CONCENTRATION OF CRUDE ENZYME

4.4.1. Concentration by $(NH_4)_2SO_4$ precipitation

An initial experiment with $(NH_4)_2SO_4$ fraction precipitation was performed on separate sample from 20 to 100 % precipitation with 10 % increase and was explained in Fig. 4. 1., and fraction samples loaded on SDS – PAGE incorporated with xylan was given in

Fig. 4. 2. The graph shows that the activity was distributed between 30- 70 % fraction with maximum at 50 % and 72 % of activity was present in 60 % fraction where as 40% fraction has retained 48% of activity and 30 % with 25 % activity where as 70 % has shown mild activity. The SDS – PAGE incorporated with 0. 5 % oats spelt xylan shows that fraction 30 to 70 % have activity. Ultrafiltration sample too were subjected to $(NH_4)_2SO_4$ precipitation from 30 - 70 %.

4.4.2. Concentration by Ultrafiltration

The crude xylanase obtained from the culture broth after centrifugation was concentrated by Ultrafiltration and $(NH_4)_2SO_4$ precipitation prior to chromatographic separation (Table.4. 1.). The crude preparation was concentrated by Ultrafiltration using Ultrafiltration membrane (Amicon, Millipore) filtration unit having a molecular exclusion limit of 10,000 kDa. The xylanase activity in the ultrafilterated solution (110mL) was 22394.4 Units with 70 % recovery and 1.3 purification fold, with specific activity of 297.79 U / mg of protein. The activity recovered from the ammonium sulphate fraction ranging from 30 – 70 % with maximum activity at 40 - 50 %. There were reports related to the use of ammonium sulphate for the partial purification of xylanase from various microorganisms (Kubata *et. al.,* 1992, Honda *et. al.,* 1993, Battallion *et. al.,* 2000). The crude enzyme samples extracted from SmF and SSF medium were administered to 70 % ammonium sulphate precipitated and loaded on Native - PAGE, also zymogram analyzed done and result given in Fig. 4. 3. The zymogram analysis shows that there was more than one type of fraction present in SSF extract where as in SmF there was only one type. Protein from *Rhizopus oryzae* strain ATCC 9363 precipitated between 40 and 80 % ammonium sulfate saturation and dissolved in 50 mM citrate buffer at pH 4.8 (Bakir *et. al.,* 2001). There are various reports related to purification of xylanase from bacteria. Honda *et. al.,* (1985a) *Bacillus* sp. no. C-125, Okada and Shinmyo, (1988), reported poor recovery of xylanase from *Bacillus pumilus* with ammonium sulphate and used ethanol for first step concentration. Yang *et. al.,* (2006) reported ammonium sulphate fraction (20 – 60 % saturation) of crude xylanase from fungus *Paecilomyces themophila* J18, yielded 74 % of the enzyme with 3.6-fold purification. Crude xylanase (80%) precipitated by

cetone from wild strain of *Aspergillus nidulans* was subjected to SDS-PAGE, which revealed the occurrence of four isoxylanases with molecular weights of 50, 43, 20 and 18 kDa (Reis *et. al.*, 2003).

Procedure	Fraction (mL)	Total protein (mg)	Total activity (Units)	Specific activity (U/mg protein)	Purification (fold)	Yield (%)
Crude	200	148	31992	216.16	1	100
Ultrafiltration	110	75.2	22394.4	297.79	1.3	70
$(NH_4)_2SO_4$ precipitated	80	22.8	13436.64	589.32	2.7	42
DEAE - Sepharose (0.01N NaCl)	20	2.01	1357.1	675.14	3.12	4.2
DEAE - Sepharose (0.5N NaCl)	10	4.28	980.8	229.15	1.06	3.1
DEAE - Sepharose (1.0N NaCl)	6	0.81	470.28	580.59	2.68	1.46

Table. 4. 1. Purification of xylanase from *Bacillus pumilus* produced by SSF on wheat bran as the substrate

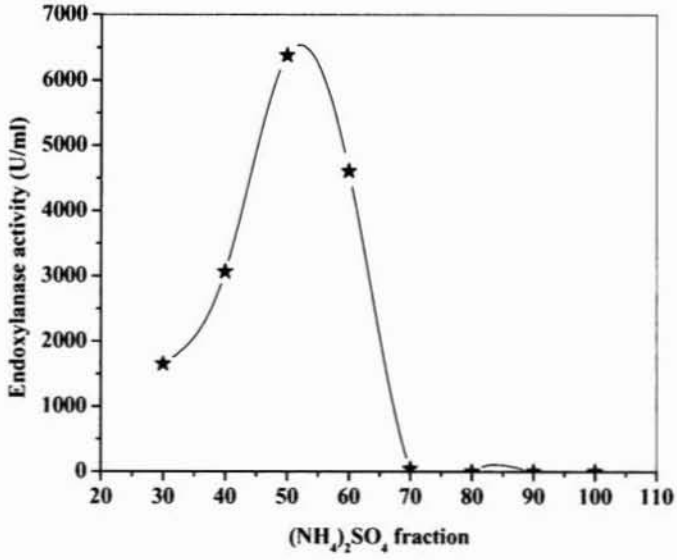


Fig. 4. 1. Xylanase activity graph of $(NH_4)_2SO_4$ precipitated fractions from SSF medium (30, 40, 50, 60, 70 %)

Fig. 4. 2. SDS - PAGE incorporated with 0.1 % xylan, different $(NH_4)_2SO_4$ precipitated fractions (30, 40, 50, 60, 70 %)

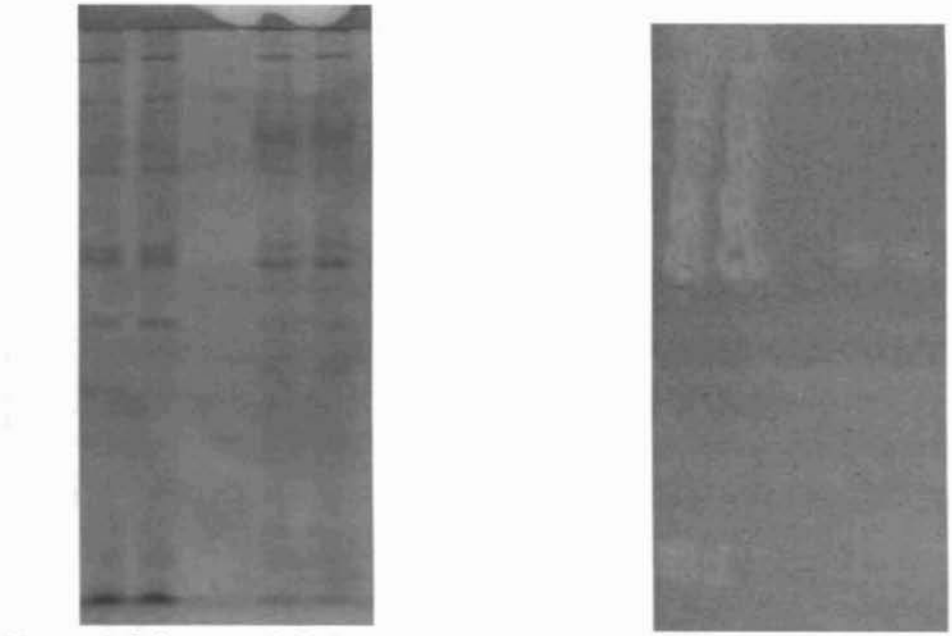


Fig. 4. 3. Native - PAGE of partially purified samples from SSF and SmF

(a) Native – PAGE- line 1 & 2 SSF sample, 3 & 4 SmF sample.

(b) Zymogram - 5 & 6 SSF zymogram, 7 & 8 SmF zymogram.

4.5.0. PURIFICATION BY ION EXCHANGE CHROMATOGRAPHY

The dialyzed enzyme was applied to DEAE Sepharose column. Elution was followed using NaCl (0.0, 0.01, 0.5 and 1.0M). The elution profile of xylanase activity from the column at pH - 8 was shown in Fig. 4. 4. All the active fractions of each salt concentration were pooled and concentrated by ammonium sulphate precipitation for 100 % concentration and dialysis in sodium phosphate buffer (0.05 M pH - 7 for 24 hour at 4 ^{o}C with regular change at every 4 hour. The specific activity of DEAE - Sepharose purified fraction of 0.01 M NaCl was 675.14 U / mg protein and purification was of 3.12 fold, 0.5 M NaCl fraction was 229.15 U / mg protein and purification was of 1.06 fold and that of 1.0 M NaCl fraction was observed to be having a specific activity of 580.59 U / mg protein and purification of 2.68 fold (Table.4.1.). Sepharose and Sephadex column were universally used for protein purification. Sepharose column often with stand the change in salt concentration and pH without much change in column bed volume were as Sephadex column concentration in bed volume at high concentration as well as high and low pH values which leads to very low flow rate. There was no report related to the purification of xylanase from bacteria produced by solid-state fermentation and this is the first of that kind. There were reports related to the use of DEAE – cellulose for the purification of xylanase from *Bacillus* sp. (Okada and Shinmyo 1998). Carmona *et. al.,* (2005) reported the purification of xylanase II from *Aspergillus versicolor*, with DEAE-Sephadex and HPLC GF-510 gel filtration which help to obtain a purification of 28-fold.

Purification of xylanase from *T. longibrachiatum* was reported to follow four-step purification scheme involving Ultrafiltration, ammonium sulphate precipitation and cation exchange (CM - Sepharose CL - 6B) and gel filtration chromatography (Chen *et. al.,* 1997). Alkaline xylanase was purified from alkalophilic *Micrococcus* sp AR-135 ammonium sulfate precipitation of 70 % saturation followed by DEAE - Sepharose column chromatography, equilibrated with 10 mM Tris - HCl buffer, pH - 8 and elution was performed as linear gradient of 0 - 0.5 M NaCl at a flow rate of 90 mL / h (Gessesse and Mamo, 1998).

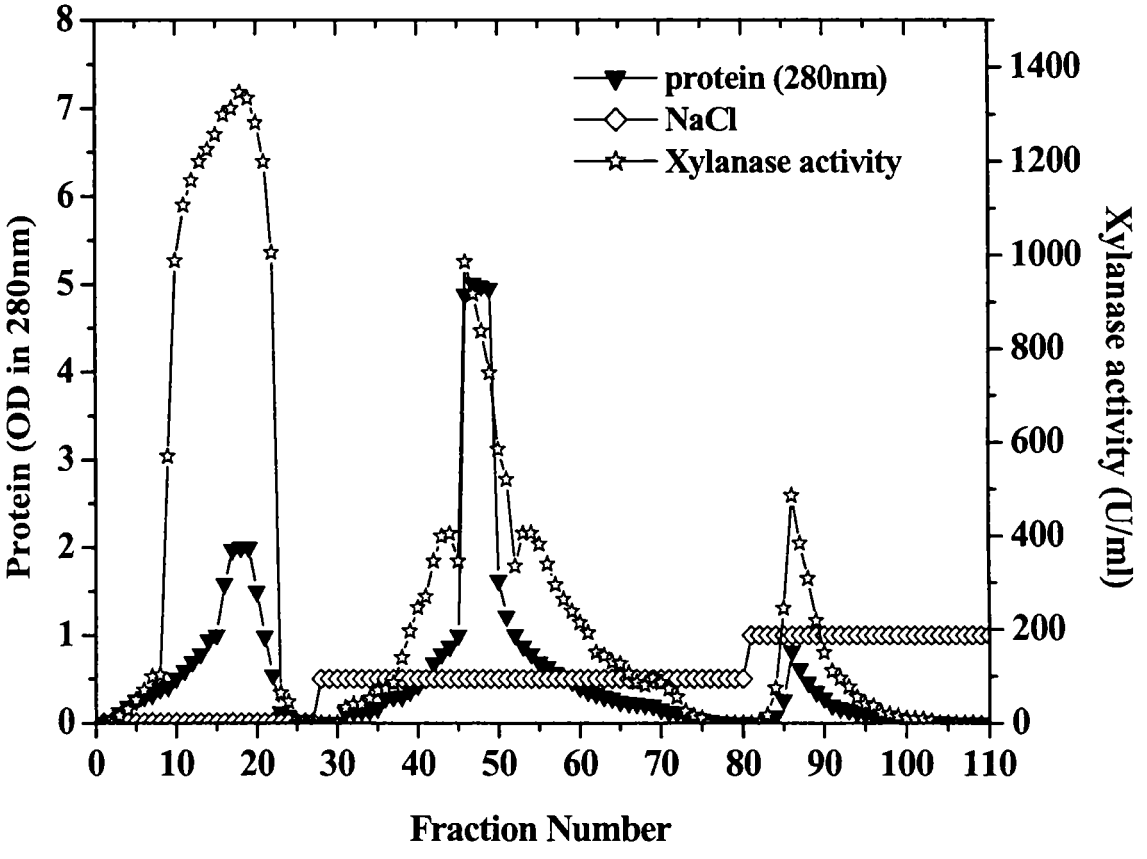


Fig. 4. 4. Elution profile of Xylanase on DEAE - Sepharose, Protein profile at 280 nm, and NaCl elution gradient (0.01, 0.5 and 1.0M)

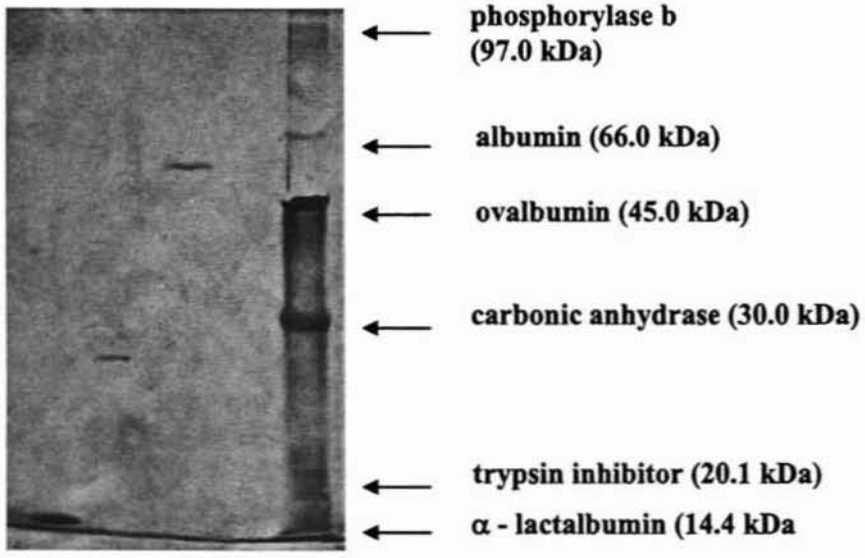


Fig. 4. 5. SDS- PAGE Lane - 1 0.01 M NaCl (~14kDa),
Lane - 2 0.5M NaCl (~35kDa),
Lane - 3 1.0 M NaCl (~60kDa),
Lane - 4 Molecular markers

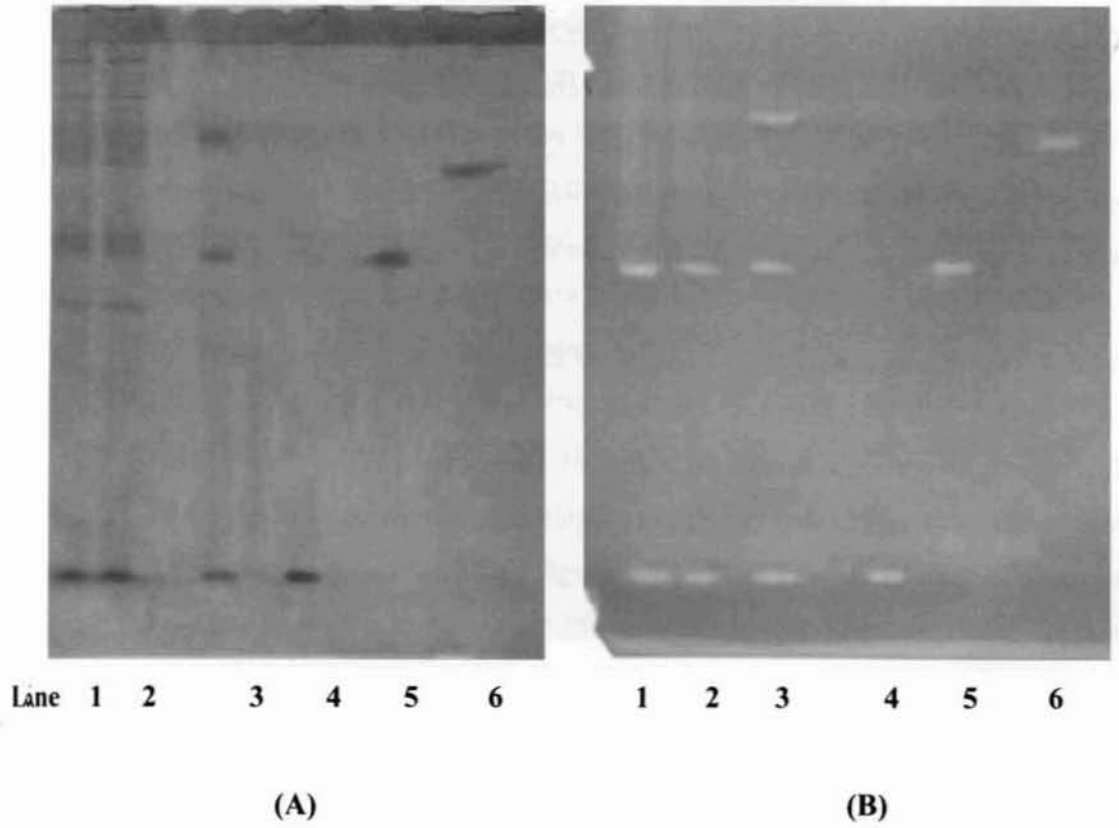


Fig. 4. 6. Native – PAGE (A) and Zymogram analysis (B):

Lane –1 Crude,

Lane –2 $(NH_4)_2 SO_4$,

Lane –3 combined fraction from DEAE- Sepharose column,

Lane –4 0.01M NaCl,

Lane – 5 0.5 M NaCl,

Lane –6 1.0 M NaCl.

Xylanase from *T. lanuginosus* ATCC 46882 was purified to homogeneity by DEAE Sepharose and Bio-Gel P-30 column chromatography (Bennett *et. al.,* 1998). Combination of DEAE –Sepharose and Q-Sepharose column were used for the purification of xylanase from *Acrophialophora nainiana* at pH 7 and column was washed with liner gradient of NaCl (0-1 M) (Cardoso and Filho, 2003).

4.6.0. PAGE ELECTROPHORESIS AND ZYMOGRAM ANALYSIS

The activity of xylanase coincided well with the protein. SDS –PAGE (Fig. 4. 5.) will help to estimate the molecular weight of purified enzyme as well as the extension of purification obtained. The molecular weight of xylanase was calculated from the electrophoretic mobility. The purity and nature of enzyme was analyzed by running the samples obtained from each step of purification as, crude sample, ammonium sulphate fraction and pooled active fraction from each concentration of NaCl fraction. The band appearing in the crude and ammonium sulphate fraction was all the most of same pattern, where as the band obtained from purified fraction are single bands. The zymogram analysis of Native – PAGE (Fig. 4. 6.) as well as the xylan incorporated SDS-PAGE.

(Fig. 4. 1.), showed trailing which shows that there was more than one form of xylanase present. The protein gets concentrated in Ultrafiltration and ammonium sulphate precipitation and all other protein expect for a single band was purified in column chromatography (Fig. 4. 6.). DEAE- column helped to eliminate the pigment as well as the unwanted protein from the broth. There were various reports related to similar observation (Gessesse and Mamo, 1998, Cardoso and Filho, 2003). From the SDS-PAGE analysis it was found that the molecular weight of fraction obtained from 0.01 M NaCl as ~ 14 to 20 kDa, that of 0.5 M NaCl as ~ 30 to 35 kDa and that from 1 M NaCl as ~ 60 kDa. There were only few reports related to low molecular weight xylanase which has application in paper pulp industry, due to its high penetration in the Kraft cooked pulp and help in the easy removal of re-precipitated xylan. Xylanase from *Acrophialophora nainiana* was reported to be of 54 kDa (Cardoso and Filho, 2003). Two xylanases from *Bacillus subtilis* were purified by native polyacrylamide gel

electrophoresis (PAGE) and homogenization extraction was used for the purification and analyzed using HPLC which showed that both xylanase were endoacting, designated xyl I and xyl II (activity range pH 5.0 to 9.0 at 50 °C with optimum activities at pH - 7 and 50 °C) (Yuan *et. al.,* 2005). *A. versicolor* produces a xylanolytic complex with two components, the minor component designated xylanase II (5 days in 1 % wheat bran) a monomeric glycoprotein with molecular mass of 32 kDa with 14.1 % of carbohydrate content (Carmona *et. al.,* 2005). Endoxylanase from *T. lanuginosus* ATCC 46882 was purified by DEAE- Sepharose and the molecular mass of native (non-denatured) and denatured endoxylanase were 26.3 and 25.7 kDa, respectively (Bennett *et. al.,* 1998). A xylanase from *S. roseiscleroticus* (*Chainia rosea*) was purified and the had a very low apparent molecular weight of 5,500 by native gel filtration, but its denatured molecular weight was 22,600 by SDS –PAGE (Grabsk and Jeffries, 1991). The molecular mass of xylanase and β- xylosidase from *Bacillus thermantarcticus* was reported as 45 kDa and 150 kDa, respectively (Lama *et. al.,* 2004). Ratanakhanokchai *et. al.,* (2002) reported xylanase from alkaliphilic *Bacillus firmus* which has a molecular weight of 23 kDa. Xylanase from thermoalkaliphilic *Bacillus* sp. strain TAR-1 was purified to homogeneity by ammonium sulphate fractionation and anion-exchange chromatography and the molecular mass was 40 kDa (Nakamura *et. al.,* 1995).

4.7.0. PURIFICATION OF XYLANASE BY ISOELECTRIC FOCUSING

The xylanase activity as well as the protein concentration and pH of 20 fractions were given in Fig. 4. 7. The fractions were subjected to Native – PAGE as well as zymogram analysis (Fig. 4. 8.) and those, which have shown activity in zymogram was subjected to SDS – PAGE (Fig. 4. 9.). The pI of the xylanase was determined by using an Ampholyte (Pharmacia Biotech), with pH values ranging from 3 to 10, and the pH of the fraction collected were tested. The pI value was estimated to be between 8 - 9 (Fig. 4. 7.) from the plot of migration distance versus the pI values of the standards. The results showed that the fraction obtained from preparative isoelectric focusing was of high alkaline range and have very low molecular weight of ~ 14 to 20 kDa other fraction which has shown activity have not produced any band in the electrophoretic analysis. This might be due

low concentration of protein in the fraction (minor xylanase). Xylanase with pI 4- 10 have been reported in literature. *Bacillus* sp. W1 was reported to have a pI of 8.5 (Okazaki *et. al.,* 1984), while pI of 9.1 was reported for xylanase from *Bacillus circulans* (Esteban *et. al.,* 1982). Xylanase and β- xylosidase from *Bacillus thermantarcticus* was reported to have a pI of 4.8 and 4.2 respectively (Lama *et. al.,* 2004). Another xylanase from thetmoalkaliphilic *Bacillus* sp. strain TAR-l reported to have a pI 4.1 (Nakamura *et. al.,* 1995). Xylanase from *S. roseiscleroticus* (*Chainia rosea*) was reported to have high pI of 9.5 (Grabski and Jeffries, 1991). Park *et. al.,* (2000) reported xylanase from *Bacillus* sp. with a pI of 10.6 and the net charge of this enzyme at pH - 7 was reported to be positive. Gerber *et. al.,* (1997) reported two types of xylanase from *T. harzianum* one with a molecular weight of 20 kDa and pl - 9.4, and a second fraction of molecular weight of 29 kDa and pl - 9.5. *Bacillus* V1-4 grown at pH - 9 in alkaline medium was reported to have a pI of 9.1 (Yang *et. al.,* 1995). Two extra-cellular endoxylanases were purified to homogeneity from thermophilic fungus, *Myceliophthora* sp. IMI 387099. Xyl Ia and Ib, having a molecular mass of approximately 53 kDa and pI of 5.2 and 4.8, respectively (Chadha *et. al.,* 2004).

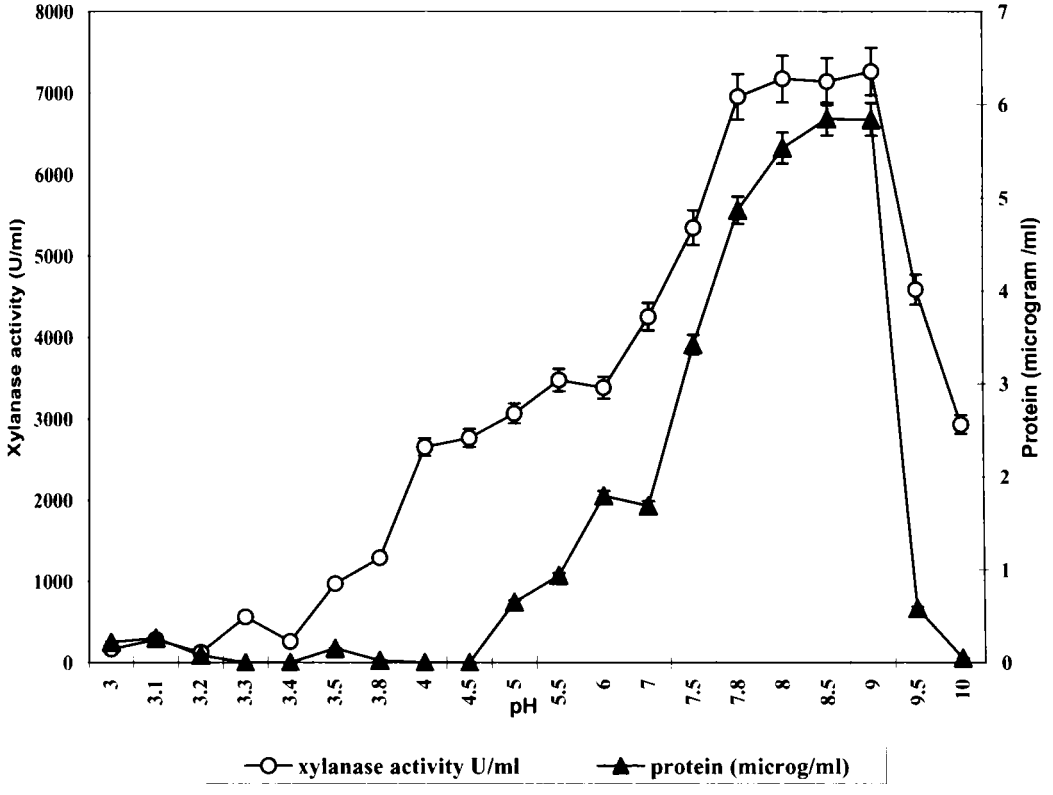
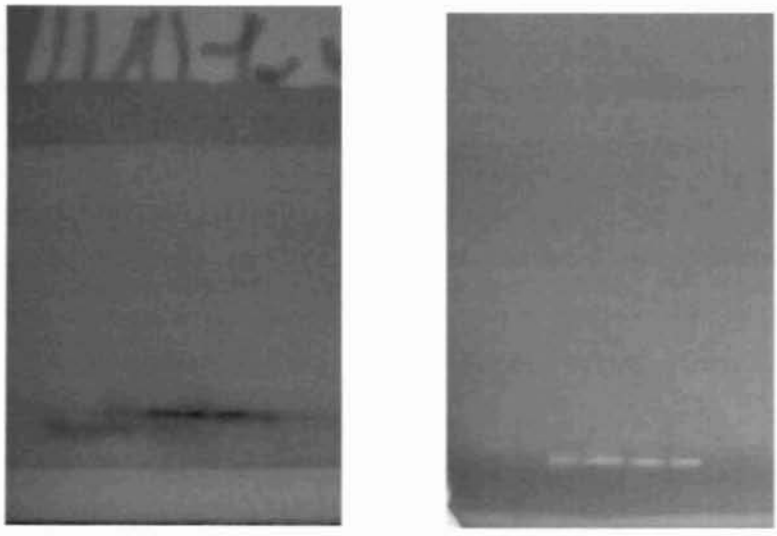


Fig. 4. 7. The 30- 80% $(NH_4)_2SO_4$ precipitate applied to Isoelectric focusing chamber, 20 fraction were collected (1 mL each) the protein (µg/ mL) and Xylanase activity (U/ mL) was estimated together with pH.



Native – PAGE Zymogram

Fig. 4. 8. Native - PAGE and zymogram analysis of fraction obtained after PIF (Active fraction 15, 16, 17 and 18).

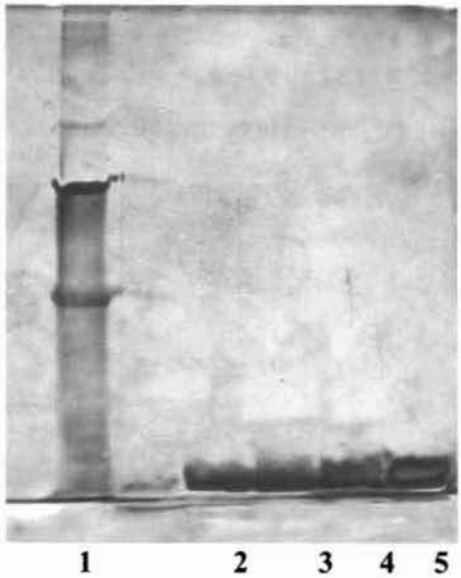


Fig. 4. 9. SDS - PAGE (15, 16, 17 and 18 Fraction from PIF (~14kDa) Lane 1 – molecular marker, 2, 3, 4, and 5 active fractions

4.8.0. CHARACTERISATION OF XYLANASE

4.8.1. Effect of temperature on partially purified and purified xylanase

Temperature profile of partially purified endoxylanase from *Bacillus pumilus* was given in Fig. 4. 10, and that of purified enzymes fractions were given in Fig. 4. 11. Partially purified xylanase exhibited and optimum activity at 50 °C and it has retained 20 % of activity 60 °C and 10 % activity at 65 °C. Similar result was obtained for xylanase producted by actinomycetes (Georis *et. al.*, 2000). The purified fractions also has shown similar trend with maximum activity at 50 °C and 93 % of activity was retained at 55 °C for 0.01M and0.5 M NaCl fractions where as 1M NaCl fractions has shown maximum activity at 55 °C.

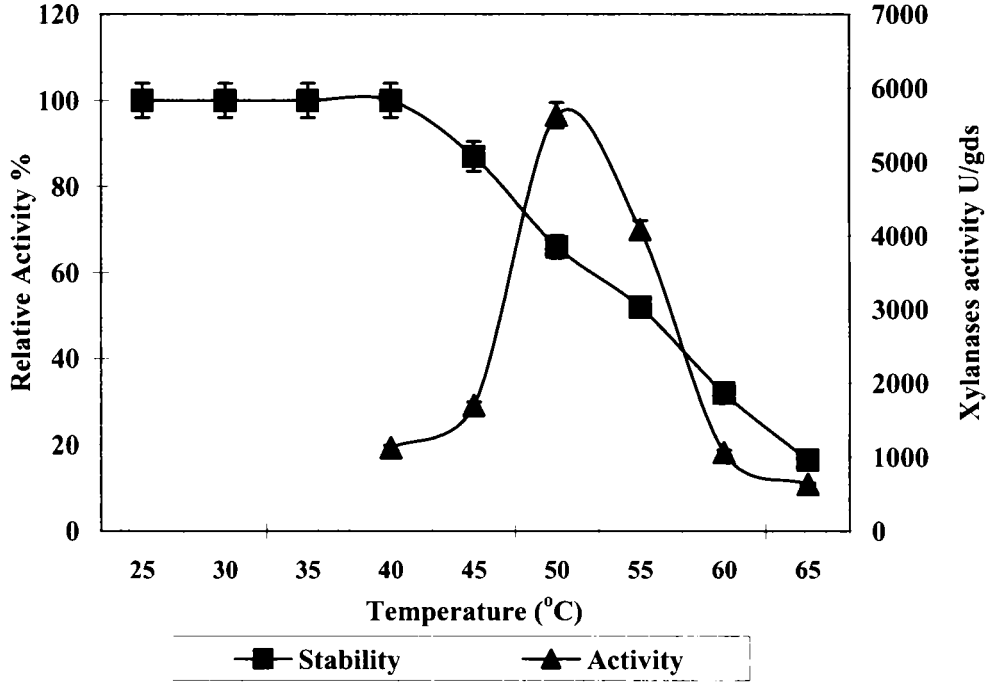


Fig. 4. 10. Effect of temperature on activity and stability of partially purified xylanase enzyme from *B. pumilus*.

Effect of temperature stability of partially purified (Fig. 4. 10.) and purified enzyme was explained in Fig. 4. 12, 4. 13, and 4. 14. Partially purified enzyme has shown stability over a wide range of temperature 25 to 40 °C. 32 % of activity was retained at temperature 60 °C and 60-50 % of activity at 50 °C even after 1 hour of incubation. It was reported that xylanase from *Streptomyces*. sp AMT-3, was active over a range of temperature 55 - 60 °C and pH - 6 (Nascimento *et. al.*, 2002). Similar reports were there related to fungi that are active at acidic pH 4.5 and 55 °C (Beg *et. al.*, 2000) and thermophilic fungi *T. lanuginosus* that was stable at wide range of pH from 4.4 to 7.6 (Bennet *et. al.*, 1998).

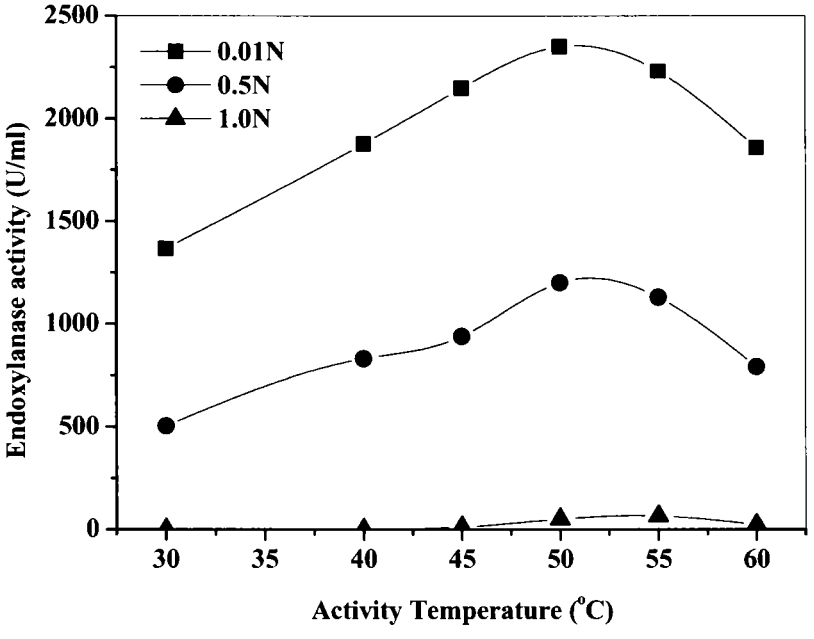


Fig. 4. 11. Effect temperature on the activity of purified fractions collected from DEAE- Sepharose column.

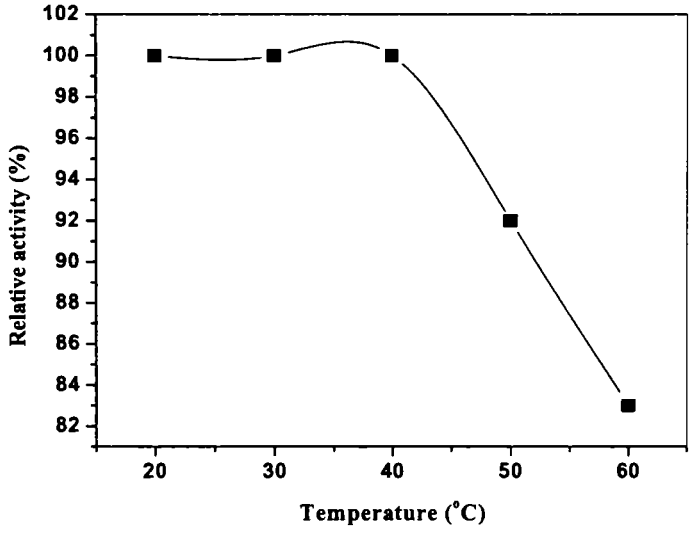


Fig. 4. 12. Temperature stability of fraction (0.01 M NaCl) collected from DEAE – Sepharose column.

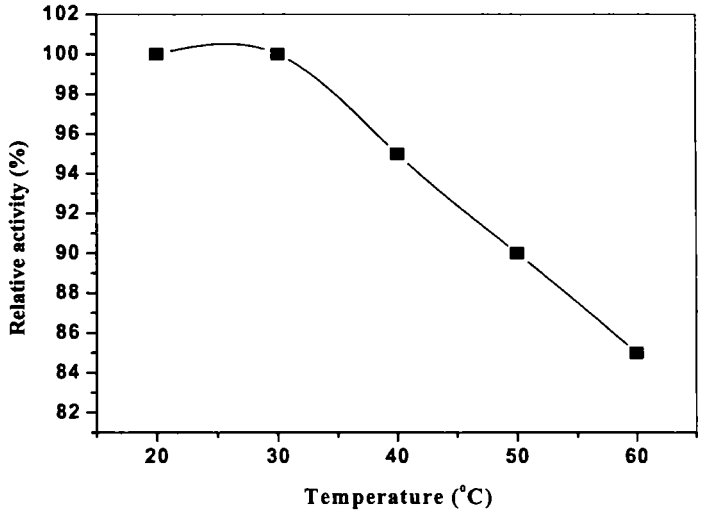


Fig. 4. 13. Temperature stability of fraction (0.5 M NaCl) collected from DEAE – Sepharose column.

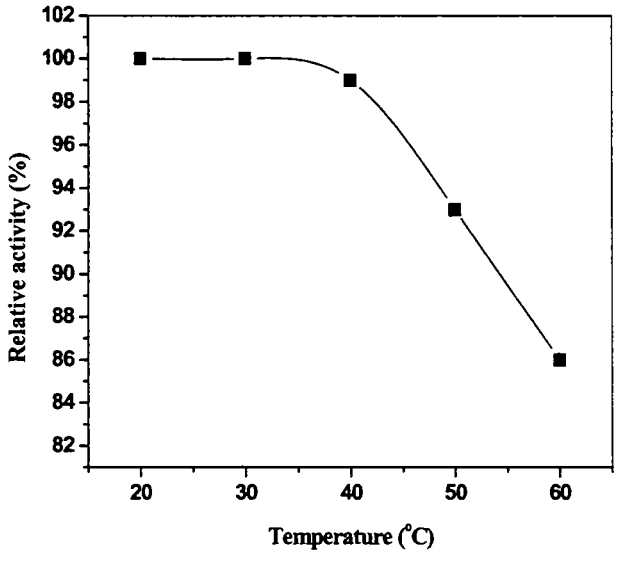


Fig. 4. 14. Temperature stability of fraction (1.0 M NaCl) collected from DEAE - Sepharose column.

Pereira and coworkers explained the difference in pH and temperature tolerance for xylanase excreted might be due to the effect of different enzymes mixtures excreted, and/ or the post - translational modifications in xylanase excretion process, such as glycosylation, that improve stability in more extreme pH and temperature conditions (Pereira *et. al.,* 2002).

4.8.2. Effect of pH on partially purified and purified xylanase

A pH range from 5 to 11 was used to study the effect of pH on partially purified xylanase activity and the result given in Fig. 4. 15. The optimum activity was at 6.5, but a significant level of activity was observed at a range of pH from 5 to 7.5 (above 80 % of activity) and there was another peak at pH 8.5 (about 60 % of activity) as well as at pH - 11 (about 10 % of activity). This behavior was similar to the xylanase activity of *Streptomyces* at varying pH (Bakir *et. al.,* 2001). The effects of pH on purified fractions

were explained in Fig. 4. 16, 4. 17 and 4. 18. The results show that the fraction 0.01 M NaCl has highest activity at pH - 7 in phosphate buffer (0.05 M) as well as 0.5 M NaCl fraction with highest activity at pH - 6 in citrate - phosphate buffer (0.05 M) and 1 M. NaCl fraction with activity at pH- 6 in phosphate buffer (0.05 M).

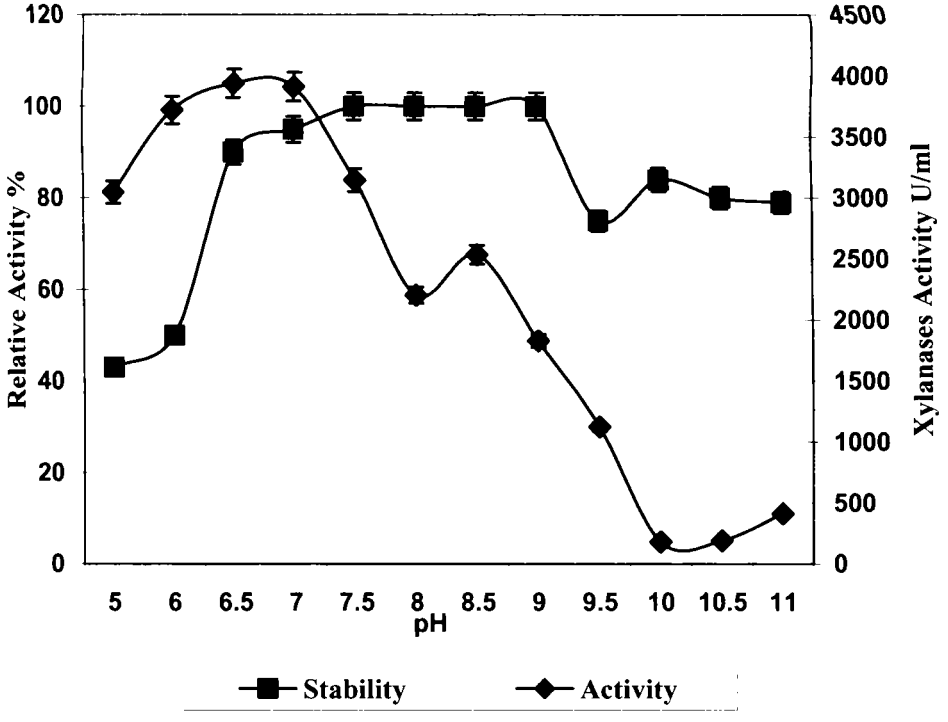


Fig. 4. 15. Effect of pH on activity and stability of partially purified xylanase enzyme from *B. pumilus*.

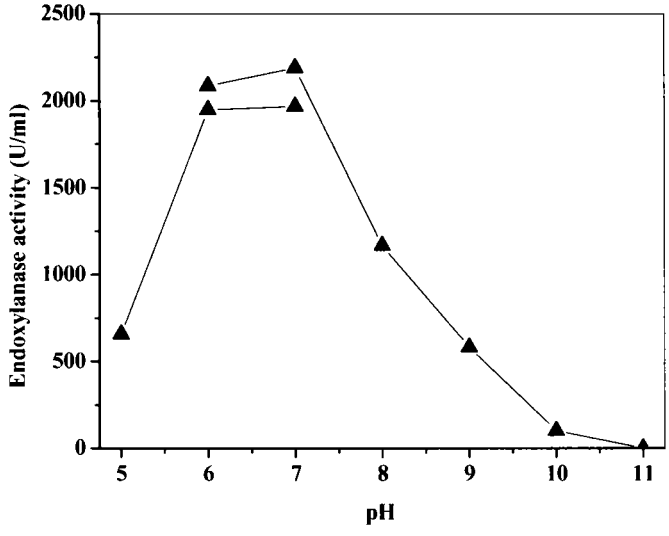


Fig. 4. 16. Effect of pH on activity purified xylanase enzyme from *B. pumilus* (DEAE-fraction – 0.01 M)

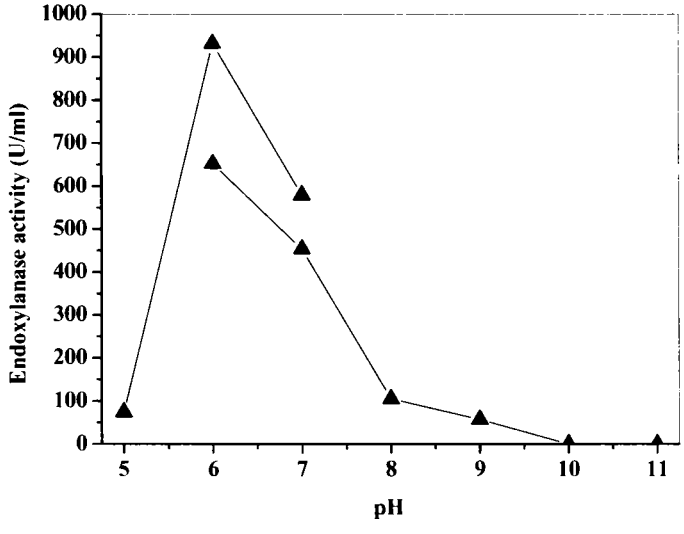


Fig. 4. 17. Effect of pH on activity purified xylanase enzyme from *B. pumilus* (DEAE-fraction – 0.5 M)

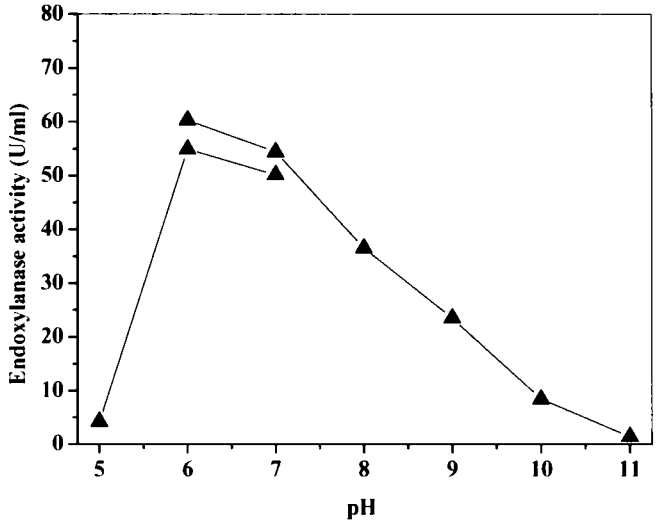


Fig. 4. 18. Effect of pH on activity purified xylanase enzyme from *B. pumilus* (DEAE-fraction – 1.0 M)

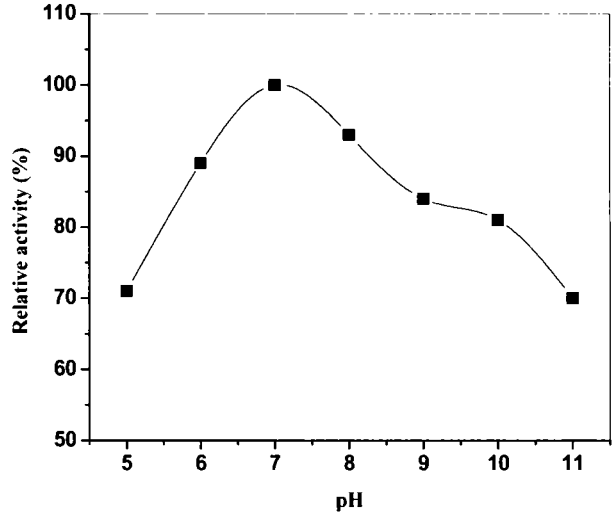


Fig. 4. 19. Effect of pH on stability of purified xylanase enzyme from *B. pumilus* (DEAE-fraction – 0.01 M)

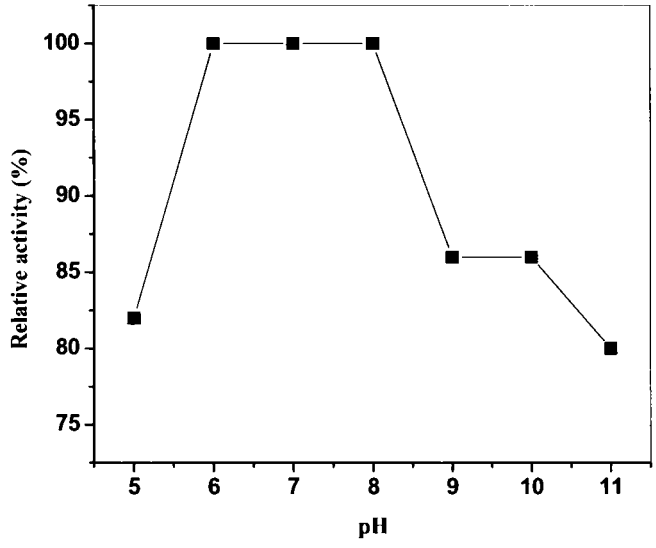


Fig. 4. 20. Effect of pH on stability of purified xylanase enzyme from *B. pumilus* (DEAE-fraction – 0.5 M)

Fractions from 0.01 M and 1M NaCl have shown activity at pH-10 and 11 where as fraction from 0.5 M NaCl has no activity at this pHs. pH and temperature stability of the enzyme are very important factors when we intend to study the industrial importance of the enzyme. The stability of enzyme at different pH ranging from 5 - 11 was shown in Fig. 4. 15. and that of purified fraction in Fig. 4. 19, 4. 20 and 4. 21. The DEAE fraction 0.01 M and 1.0 M fraction was stable at pH - 7 for 1 hour, where as 0.5 M was stable at a range of 6 to 8. Xylanase was almost completely stable at different pH 6.5 to 9 after 1 hour of incubation after 24 hour pH - 5 has retained 30 % of activity, where as from pH 7 to 9 has retained 90 % of activity even after one week. Two xylanase where isolated from *Aspergillus versicolor* both reported to have optimal pH and temperature values for the enzyme activity were about 6.0–7.0 and 55 °C, respectively (Carmona *et. al.,* 2005). Endoxylanase from *Rhizopus oryzae* reported to have an optimal pH and temperature values at about 4.5 and 55 °C, respectively (Bakira *et. al.,* 2001). Xylanases (Xyl Ia and Ib) from *Myceliophthora* sp. IMI 387099, were optimally active at 75 °C and at pH 6.0

and stable at pH 9.2 at 60 °C for 2 h, but less stable at pH 6.0 and above 50 °C (Chadha *et al.*, 2004).

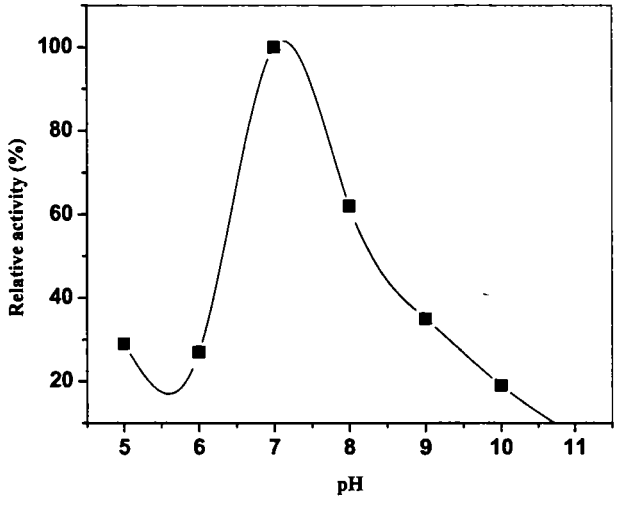


Fig. 4. 21. Effect of pH on stability of purified xylanase enzyme from *B. pumilus* (DEAE-fraction – 1.0 M)

Xylanase from *Paecilomyces themophila* J18 exhibited remarkable stability and was completely stable at temperature up to 65 °C for 60 min at pH 6.0 - 9.0, while at 70 °C it showed complete stability at pH 7.0 - 8.0 for 30 min. But, xylanase retained over 70 % of its original activity for 2 h at pH 7.0 - 9.0 at 70 °C while retained over 50 % of its original activity for 4 h at pH 7.0 –8.0 (Yang *et. al.*, 2006).

4.8.3. Effect of metal ions on partially purified and purified xylanase

Xylanase activity of partially purified enzyme was assayed with and without additives was given in Fig. 4. 22. Enzyme activity was enhanced 1fold with Mn^{2+} salt and there was no decrease in activity even after 30 min of incubation. Presence of Mg^{2+}, Ca^{2+} and Co^{2+} has no inhibited enzyme activity, where as presence of potassium dichromate, Fe^{2+} and SDS in high concentration in the system may inhibit enzyme activity on long run. Presence of other metal ion like Zn^{2+}, Na^{2+}, Cu^{2+} and EDTA also positively influenced

enzyme activity. This results relates to the use of this preparation in industrial processes, that how the presences of these ions in relevant concentration affect the enzyme activity.

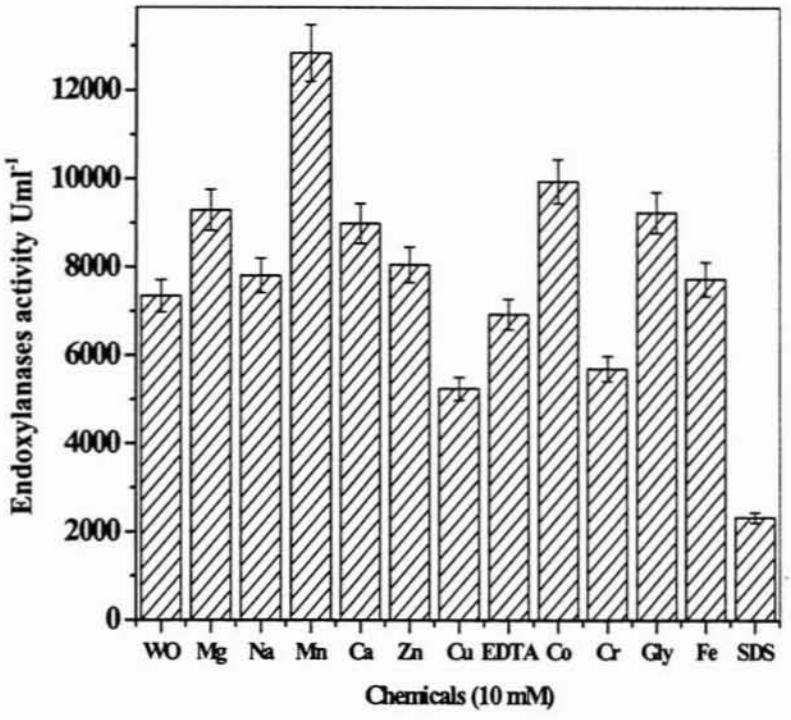


Fig. 4. 22. Effect of different metal ions on the activity of partially purified xylanase from *B. pumilus*

The effect of metal ions on purified fraction was given in Fig. 4. 23. In this we can observe that Fe^{2+}, SDS and Hg^{2+} has completely inhibited the enzyme activity in all fractions, where as Mg^{2+} enhanced enzyme activity in 0.01 M NaCl fraction and Mn^{2+} in other two fraction. Similar effect was reported by (Park et. al. 2002, Chandha *et. al.*, 2004). Xylanase from *Bacillus* sp. SSP-0 was completely inhibited by Hg^{2+} (Battallion *et. al.*, 2000). Yuan *et. al.*, (2005) reported Mn^{2+} ions enhanced xylanolytic activities to 2.7-fold whereas Fe^{3+} completely inhibited.

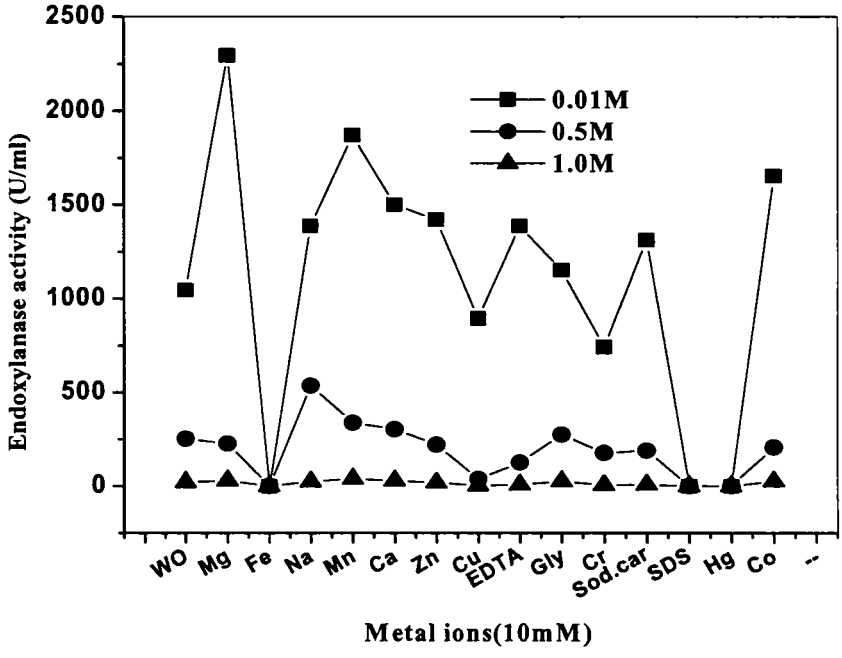


Fig. 4. 23. Effect of metal ions on purified fractions of xylanase obtained from DEAE- Sepharose column

Xylanase II from *Aspergillus versicolor* reported to show thermo inactivation at 50 °C with a biphasic curve, the ions Hg^{2+}, Cu^{2+} and the detergent SDS were strong inhibitors, while Mn^{2+} ions and dithiothreitol were stimulators of the enzyme activity (Carmona *et. al.*, 2005). *B. circulans* BL53 has shown considerable decreases on activities in the presence of Cu^{2+}, Zn^{2+}, Ba^{2+}, and EDTA, while Ca^{3+}, Mn^{2+} and Mg^{2+} produced discrete inhibitions. These effects might preclude the use of this enzyme in industrial processes were these chemicals are present in relevant concentrations (Heck *et. al.*, 2006). Stimulatory effects were, however, obtained with Fe^{2+}, and Co^{2+}. The increase on xylanase activities by Co^{2+} was reported by Panagioutou *et. al.*, (2003). These results

suggest that the xylanases from *Bacillus coagulans* are metalloproteins, probably with Co^{2+}, or Fe^{2+}, at the active site. b- Mercaptoethanol enhanced activity, presumably by counteracting the oxidation effects of S –S linkage of cysteine residues, thus stabilizing xylanase. This effect has been previously reported for other microbial xylanases (Sa'-Pereira *et. al.,* 2002). Xylanases (Xyl Ia and Ib) from thermophilic, *Myceliophthora* sp. IMI 387099, Mg^{2+}, Zn^{2+}, Ca^{2+}, Co^{2+} and DTT increased their activity by 1.5–3.0-folds, while SDS and NBS completely inhibited their activity (Chadha *et. al.,* 2004).

4.8.4. Effect of solvents on partially purified enzyme

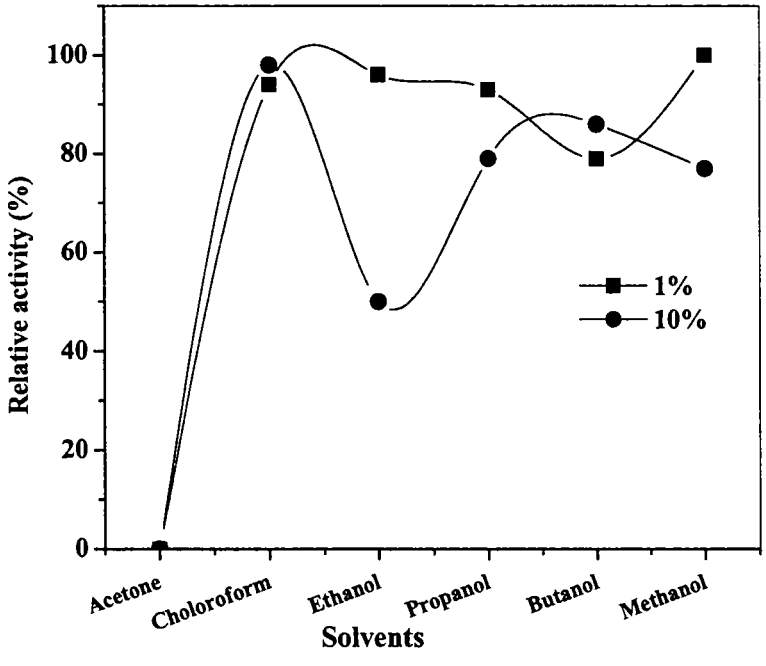


Fig. 4. 24. Effect of solvents on the activity of partially purified xylanase from *B. pumilus*

The effect of various solvents on partially purified xylanase was explained in Fig. 4. 24. The enzyme was observed to be very tolerant to organic solvents. The results show that acetone has completely inhibited enzyme activity where as 1 % of chloroform, ethanol

and methanol have retained 100 %, while 10 % not well suited. Enzymes from thermophiles in general are expected to be more tolerant to organic solvents (Sonnleitner and Fiechter, 1983).

4.8.5. Effect of substrate concentration on the purified xylanase

Substrate concentration is one of the most important factors, which determines the velocity of enzyme reaction, which can be determined by the Michaelis –Menten constant (K_m). The velocity against substrate concentration of two fractions (0.01 M and 0.5 M NaCl fraction) where tested for K_m and the activity graph was given in Fig. 4. 25 and 4. 26. The Lineweaver-Burk plot of two above-mentioned fractions were given in Fig. 4. 27, and Fig. 4. 28.

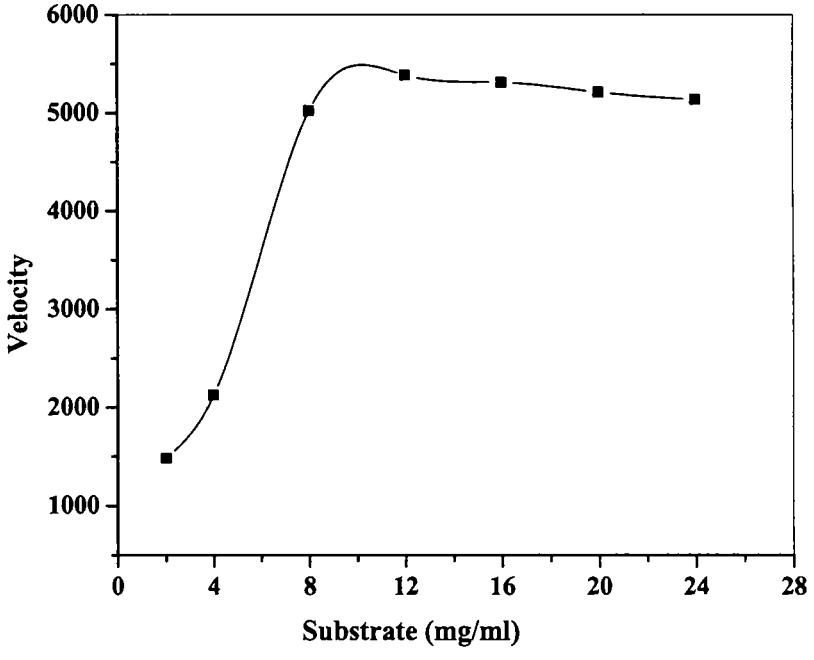


Fig. 4. 25. Effect of xylan concentration on activity / velocity of reaction on purified fraction (0.01 M NaCl) obtained from DEAE- Sepharose column.

The Michaelis –Menten constant (K_m) for oatspelt xylan for fraction obtained by 0.01 M NaCl was observed as 4.0 mg / mL and V_{max} as 5000 µmol / min / mg protein and that of fraction obtained by 0.5M NaCl as K_m - 3.5 mg / mL and V_{max} 3448 µmol / min / mg of protein. Xylanase from *Trichoderma* sp. was reported to have similar high specific activity. It has wide range of K_m values (0.5 - 12.5 mg / ml), V_{max} value (4,025 U mg / protein). These differences could be, to a certain extent, attributed to different xylans or temperatures used in the xylanase assay and to methods used in determination of sugar concentration (Chen *et. al.,* 1997). Similar K_m for birchwood xylan was reported as 2.3 mg / mL while maximal velocity (V_{max}) was 233.1 µmol / mg / min of protein from *A. versicolor* (Carmona *et. al.,* 2005).

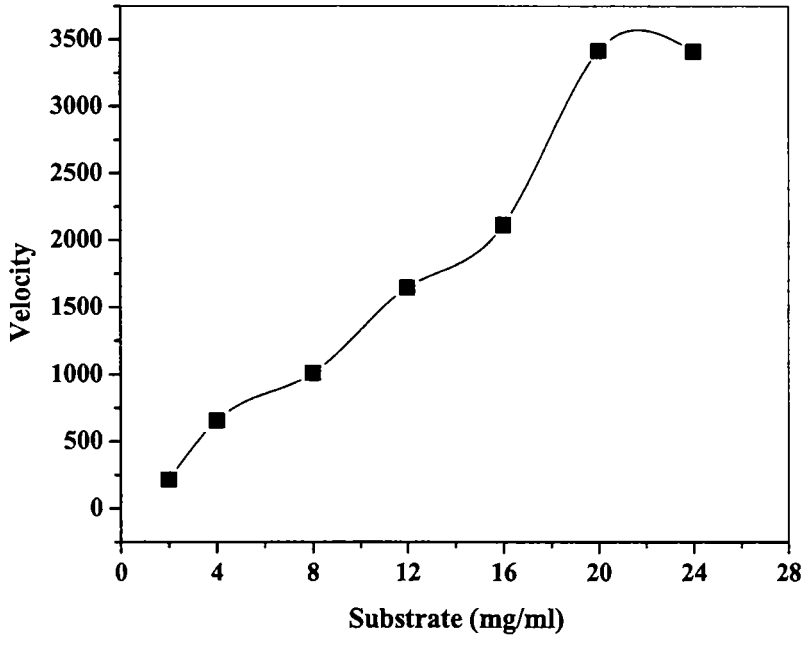


Fig. 4. 26. Effect of xylan concentration on activity / velocity of reaction on purified fraction (0.5 M NaCl) obtained from DEAE- Sepharose column.

The enzyme obtained from *Rhizopus oryzae* reported to obeys Michaelis-Menten kinetics with K_m and V_{max} values being 18.5 mg xylan / mL and 90 IU / mg proteins, respectively (Bakira *et. al.,* 2001). There were reports related to xylanase from *Bacillus* .sp, which has K_m value ranging from 3-8 mg of Oat spelt xylan. Only few reports were there related to

Bacillus sp. with low K_m values like *Bacillus* sp. 11-1S (1.68mg / mL) (Uchino and Nakane, 1981), *Bacillus* strain XE (0.6mg / mL) (Debeire –Gosselin *et. al.,* 1992b), *Bacillus sterothermophilus* (1.63 mg / mL) (Khasin *et. al.,* 1993). Reports related to high K_m were of *Trichoderma longibracchiatum* (10.14 mg / mL) (Chen *et. al.,* 1997), *Aeromonas caviae* ME1- 9.4 (Kubata *et. al.,* 1992). The *Streptomyces* sp. No 317, reported to produce highest activity with 40 mg / mL with substrate xylotriose, which was far smaller than the xylan molecule with which the same xylanase (Xyn-2b) showed a K_m of 11.2 mg / ml (Nakamura *et. al.,* 1992). Thermophilic xylanase from *Thermotoga thermarum* was reported to have a larger molecule with mol. wt. of 266 kDa (Dimer of 105 and 150kDa), which has low K_m of 0.36, and a low molecular weight (33 kDa) fraction with K_m of 0.24 (Sunna *et. al.,* 1997). Xylanase I and II, with mol. wt. 20.8 and 23.5 kDa from *Fusarrium oxysporum* F3 were reported to have a K_m value 0.41 and 0.37 mg / mL respectively (Christakopoulos *et. al.,* 1996).

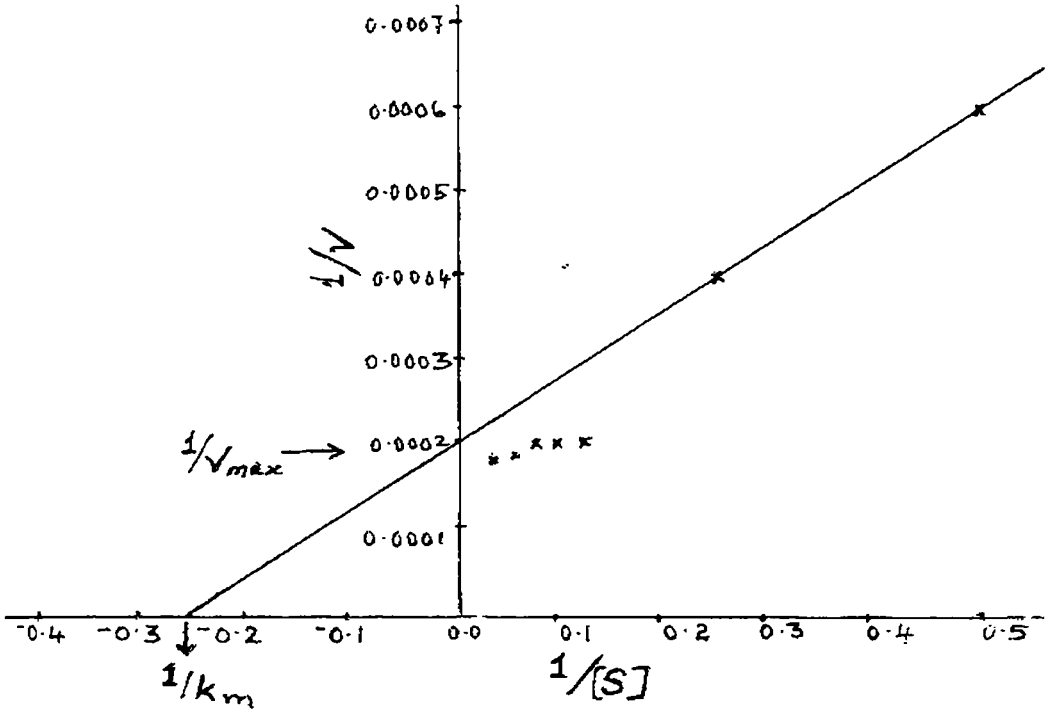


Fig. 4. 27. Lineweaver-Burk plot for xylanase fraction (0.01 M NaCl) obtained from DEAE- Sepharose column.

Bataillon *et. al.,* (2000) reported low K_m of 0.7mg / mL and V_{max} of 2420 nkat / mg with brich wood xylan as substrate for *Bacillus* sp. Strain SPS- 0. Various strains of *Bacillus pumilus* was reported to have quit different K_m and V_{max} values for the same substrate, strains 5_2 and 5_{14} (8.9 and 1.1 mg / mL) requires low substrate concentration to reach the V_{max} (178.57 and 112.36 µmol / mL / min) for catalysis where as 13_a and 4_a require high substrate (K_m 33.3 and 71.4 mg / mL) concentration to reach V_{max} (1666.67 and 1428.57 µmol / mL / min) which shows that these strains have high catalytic power and could use for higher technology efficiencies (Durate *et. al.,* 2000).

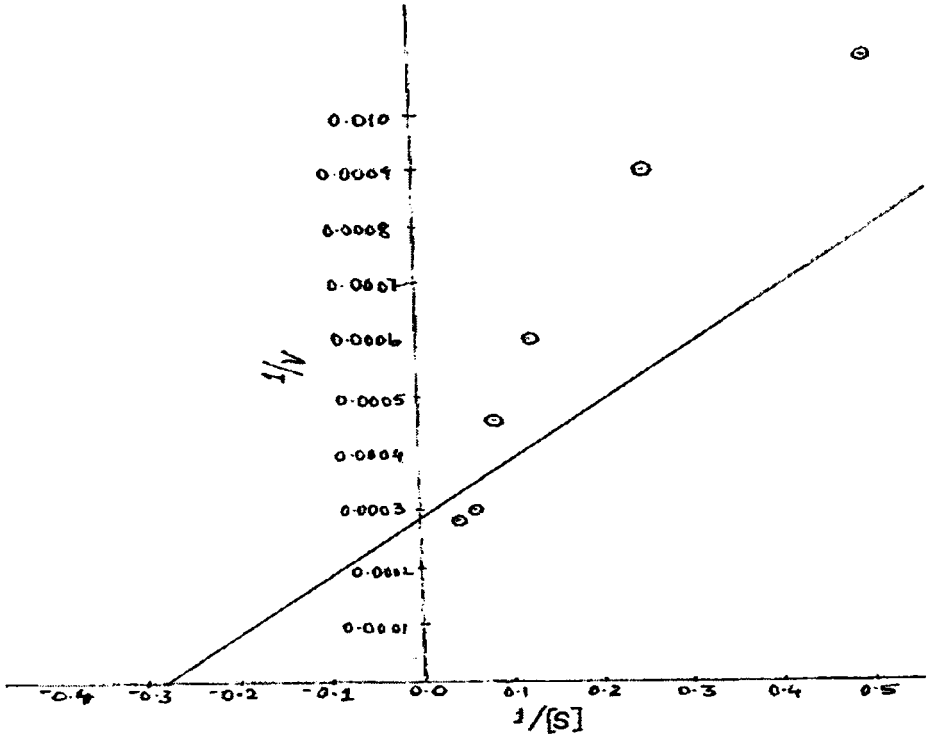


Fig. 4. 28. Lineweaver-Burk plot for xylanase fraction (0.5 M NaCl) obtained from DEAE- Sepharose column.

4.9.0. CONCLUSION

In conclusion, *Bacillus pumilus* produces minor and major xylanases with different molecular weight of which a low molecular weight endoxylanase forms the major

action. This fraction was observed to be having a pI in the alkaline range. Low olecular weight and alkaline pI are the important property of industrial xylanase, which ake this enzyme industrially important. Partially purified or crude enzyme can be irectly used for industrial purpose because of its stability at wide range of pH and mperature. The production was from low-cost lignocellulosic byproducts thus making e cost of production in a reasonable rate. Since *Bacillus pumilus* is a GRAS icroorganism, the xylanase produced can be used in the food industry as well as in other dustrial areas.

Chapter. 5.

Xylanase from alkalophilic B. pumilus, its effects on kraft pulp as a biobleaching agent, in recycling of paper, in plant fiber separation and Saccharification.

5.0. INTRODUCTION

Pulp and paper industry in India is the sixth largest energy consumer in the industrial sector and its energy costs account for about 24.5% of the total manufacturing cost. Paper can be made from wood, agricultural residues or from waste paper, having a share of 43 %, 28 % and 29 %, respectively. The use of wood based technology is gradually on the decline because of capital and raw material availability constraints. The share of waste paper (secondary fiber) based technology, which is less energy intensive, is expected to increase in future. The production of pulp and paper involves three major processing steps - pulping, bleaching, and paper production. The conventional cooking and bleaching processes have caused serious water pollution problems (Yu, 2004, Lin, 2005). There are two types of pulping sulphite and kraft pulping. Less degree of polymerization occurs in Kraft pulp than sulphite pulp. Sulphite pulps from softwoods and hardwoods contain cellulose, glucomannan and methyl- glucuronoxylan, where as Kraft pulps contain arabinoxylan and galactomannan. Kraft pulping of hardwood results in cellulose, xylan and glucomannan. The presence of hemicelluose in less quantity has positive effect on fiber preparation and bonding of paper making fiber. It helps in water retention, swelling and plasticization of fibers by water, rendering them easier to prepare mechanically and promoting improved fiber – fiber contact, bonding and paper strength. The presence of hemicelluose in high quantity has negative effect, it reduces the time and power required to soften and fibrillate fibers during mechanical action in water. It absorbs and swells in water, due to its lack of crystallinity, their low molecular weight, and branched molecular configuration. Pulp with high uronic acid and highly soluble in dilute alkali solubility is most effective in this regard. Excellent papers can be made out of cotton and flax fiber, which is free of hemicelluose, but more mechanical energy is need here due to the fibrillar structure of the fibers. Biopulping and biobleaching processes have been investigated frequently over the past 15 years and it has been proven from numerous published studies that enzyme (mainly xylanase) pre-bleaching is a clear environmentally friendly, economically attractive technology, and can decrease the amount of bleaching chemicals required to attain a given brightness in subsequent

chemical bleaching stage (Patel *et. al.*, 1994, Jacobs-Young *et. al.*, 2000, Zhao *et. al.*, 2004, Zhao *et. al.*, 2006).

The potential application of xylanase and the need for enzymes that might be more appropriate for use in pulp bleaching has caused many researchers to look for xylanase in different organisms and environment. Interest in xylanase has markedly increased during the last few years, mainly due to their use in aiding the bleaching of kraft pulp (Viikari *et. al.*, 1993, Bajpai, 1997). Limited hydrolysis of xylans has been found to facilitate the extractability of lignin by conventional bleaching chemicals, thus enzymatic pretreatment results either in higher final brightness values or in a reduction of the consumption of bleaching chemicals. The Kraft process results in pulps that are alkaline and high temperature, consequently enzymes that do not require adjustment of pH or temperature are better suited for this process (Kenealy and Jeffries, 2003). Xylans are the most abundant non-cellulosic polysaccharides, which is easily degradable, the basic structure of it consist of (1- 4) - β - D - xylopyranosyl residues. Typically, these linear chains carry short side chains to a varying extent, whereas pure, unsubstituted xylans are extremely rare. Due to heterogeneous nature of xylan, complete degradation of it requires several hydrolytic enzymes, of these the best known are endo-β-1, 4-xylanases and β-xylosidases, the former attack the main chain of xylans and latter hydrolyze xylo-oligosaccharides to D-xylose.

Alkaline stable lignin-carbohydrate complexes (LCC) present in the wood seem to be the major obstacles for solubilisation of the residual lignin. During conventional bleaching, these linkages are cleaved by acidic bleaching stages, e.g. chlorine or chlorine dioxide. Delignification is mainly done using chlorine compounds that generate toxic and highly persistent chlorinated organic by products, which are mutagenic, bioaccumulating and harmful to biological system (Bajpai, 1999). Oxygen delignification and application of new agents like xylanase enzyme selectively hydrolyze polysaccharide chains attached to lignin, thereby decreasing the amount of chemicals required for pulp bleaching. The use of xylanase for prebleaching kraft pulp has been one of the greatest success stories of enzyme in the pulp and paper industry, which helps to solve some of the environmental

concerns associated with the use of chlorine in bleaching (Viikari *et. al.,* 1994). In corporation of xylanase into pulp mill bleaching sequence is simple and economically feasible. Xylanase is primarily used for the removal of LCC that are generated in the kraft process and act as physical barriers to the entry of bleaching agent (Subramaniyan and Prema, 2002). The enzyme treated pulp is more difficult to refine and requires more beating to achieve an equivalent tensile strength and freeness (Vidal *et. al.,* 1997, Torres *et. al.,* 2000). Earlier industries have been using mildly acidic pH range by lowering the concentration of xylanase. Now there is a new generation of enzymes that operate at conditions close to mill conditions, like alkaline pH, thermostale and short retention time (Bajpai, 1999, Kenealy and Jeffries, 2003). Use of xylanase improves the fiber swelling which results in good refining that upshot in better physical properties of paper. The break down of lignin carbohydrate bonds improves delignification and also supposed to hydrolyze the xylan in the fiber thus enhancing the free penetration of bleaching chemicals (Viikari, 1994, Shobhit *et. al.,* 2001, Zhao *et. al.,* 2006, Khandeparkar and Bhosle, 2006).

5.1.0. Materials and Methods

5.1.1.0. Specificity of the culture

As the crude enzyme obtained from the culture filtrate of *Bacillus pumilus* grown in wheat bran by Solid-state fermentation was poor in cellulase activity (xylanase to cellulase ratio 1: 0.0117), the enzyme could be evaluated for prebleaching properties.

5.1.2.0. Kraft pulp preparation

The xylanase applications on Chemi-mechanical pulp (CMP) -*Acacia* and *Eucalyptus* and Chemical pulp (CP) -*Bamboo* and *Reed*, pulps were carried out in a commercial paper mill (Hindustan News Print Ltd, India). The investigation aimed at the effectiveness of xylanase treatments on conventional sequence used in the mill. The pulp with kappa number CMP- 15.3 and CP-18.2 with pH - 9 at 106 ^{o}C was taken shred and packed in polythene bags separately; the moisture content of the pulp was estimated. Known weight

of pulp was taken filtered through Buchner funnel, pressed to sheet, dried in hot air oven, and dry weight was taken, from this the weight of oven dried pulp (Od pulp) was calculated which help to fix on the consistency of the pulp for further experiments. For CMP enzyme treatment and peroxide treatment were given and blank was run parallel. For CP enzyme treatment, chlorine dioxide treatment and peroxide treatment were given. The enzyme treatment was given before chlorination as well as control was run parallel without treatment and results were compared. For this purpose, crude enzyme extracts obtained from fermentation of *B. pumilus* on wheat bran by SSF, prepared as described previously, was used.

Conditions	D_O	Eop
Time (min)	45 min	3 hour
Temperature (^oC)	Ambient temperature	78 ^oC
Consistency (%)	3 %	12 %
Initial pH	4	10.8
Final pH	2	9.5
NaOH Dosage (%)	6	6
Cl_2 dosage (%)	3.5- 4.5	-
H_2O_2 (%)	-	1.5 - 4
$MgSO_4$ (%)	0.05	0.05

Table. 5. 1. Conventional treatment stages

The conventional treatment sequence for pulp as follows -Firstly the wood was made to chips and cooked in active alkali mixture (Na_2S and NaOH) at 160 ^oC for 4 hour, which partially remove the hemicelluloses and lignin. This was followed by thickening process where the water content was lowered and unbleached pulp was stored in storage tower and then chemical bleaching stage using Chlorine dioxide followed by alkali extraction.

The pulp was exposed to Chlorination, and alkali extraction bleaching sequences to obtain pulp with different properties.

D_0 → Eop (A)

where D_0 is the chlorine dioxide treatment; Eop is the hydrogen peroxide reinforced extraction. The conditions employed in the industrial bleaching sequence are given in Table. 5. 1. During CM pulp bleaching there is no chlorination stage, directly the pulp is subjected to alkali extraction after cooking (here chemical treatment with alkali mixture and mechanical – grinding is given to separate fibre).

5.1.3.0. Xylanase application on kraft pulp

The enzymatic treatment (X) was made as follows in the conventional process

X → D_0 → Eop (B)

In all sequences studies 20g oven-dried pulp were used as samples, separate experiments were carried out for CMP and CP pulp. The xylanase treatment was given to the pulp separately; by treating the pulp with different dose of xylanase the enzyme dose optimization was done (raw pulp, 2, 4, 8, 12 and 14 U/g Od pulp). The enzymes were diluted in distilled water before application, and applied in the final pulp (consistency 3 % w/v). After application, the mixture was incubated in a thermostatic bath at 50 ^{o}C for 60 min. At 15 min intervals the pulp suspension was mixed well for proper distribution of enzyme. The pulp was filtered on a Buchner funnel and washed with distilled water (3 L / 20 g). The pulp samples were subsequently submitted to the other bleaching stages. At the D_o stage in Chemical pulp was subjected to chlorine dosage (5 % consistency), where it was tested with different concentrations of Chlorine dioxide: 1, 2, 3, and 4 % (w/w) as well as without chlorine. The pulp was then subjected to Hydrogen peroxide treatment (12 % consistency), at different concentration ranging from 1.5, 2, 3, 4, 5 and 6 %. The treated pulp was washed and hand sheets were prepared by standard Test Method T 236 cm-85 (TAAPI methods). The enzyme treated CM pulp was subjected to different peroxide dose (12 % consistency). The ISO brightness of pulp was measured on a brightness analyzer (GLRPHO- brightness color tester according to the TAPPI standards)

and pulp properties were analyzed by Scanning electron micrographs (SEM). All experiments were run in duplicate and results were the average of the two.

5.1.4.0. Optimization of condition for xylanase treatment

5.1.4.1. Chemi – Mechanical pulp

The optimization of enzyme dose and reaction time for biobleaching was carried out by treating pulp with varying dose of xylanase- 0, 2, 4, 6, 8, 12 and 14.0 U/g in (all enzyme treatment was done at 3 % consistency, ie. 20 g of Od pulp, w/v) for variable time intervals upto 2 hours and pulp properties were studied at regular intervals. Optimum enzyme active was at 50 °C, so optimization of temperature was done only at 40 and 60 °C. The effect of pH of the pulp on enzyme action was studied by treating the pulp suspension at different pH - 6, 7, 8, 9 and 10 (pH of the pulp was adjusted using 1N - H_2SO_4 / NaOH) with (4 U/g) xylanase enzyme in a total volume of 200 mL at 50 °C. The enzyme treated and untreated pulp where washed and subjected to alkali extraction alone and the brightness attained was estimated. The pulp samples were subjected to scanning electron micrograph studies too.

5.1.4.2. Chemical pulp

The chemical pulp requires more bleaching to attain required brightness so it was subjected to chlorination before alkali extraction. The optimization of enzyme dose for biobleaching was carried out by treating pulp with different dose of xylanase- 0, 2, 4, 6, 8, 12 and 14.0 U/g in (enzyme treatment was done at 3 % consistency, ie. 20 g of Od pulp, w/v) for variable time intervals upto 2 hours and pulp properties were studied at regular intervals. Optimization of temperature was done only at 40, 50 and 60 °C. The effect of pH of the pulp on enzyme action was done at different pH - 8, 9 and 10 (pH of the pulp was adjusted using 1N -H_2SO_4 / NaOH) with xylanase in a total volume of 200 mL at 50 °C. Untreated as well as enzyme treated pulp where washed to neutral pH and subjected to chlorination with chlorine dioxide (4 %) at 3 % consistency and treated for

45 min at room temperature. Kneading at regular interval help to attain proper action of chemical on the pulp. The pulp was washed to neutral pH, which was followed by alkali extraction (1.5 % peroxide) at 12 % consistency for 3 hour at 75 ^{o}C. The pulp washed to neutral pH, hand sheets were made and the brightness attained was estimated. The pulp samples were subjected to scanning electron micrograph.

5.1.4.3. Optimization of Chlorine dioxide Peroxide and Caustic soda dose in Chemical pulp bleaching

For the Chemi- mechanical pulp brightness obtained was not of much variation where as Chemical pulp there was a significant variation in the pulp brightness so it was subjected to other optimization studies. The chemical pulp after enzyme treatment was subjected to peroxide treatment of different dose devoid of chlorination (3, 4, 5 and 6 %). Enzyme dose (4 and 7 U/g OdP) was then optimized for 4 and 5 % peroxide treatment. In the next experiment low dose of Chlorine treatment (1, and 2 %) was given to enzyme (4 U/g OdP) treated pulp and it was subjected to peroxide treatment (4 and 5 %). Next experiment the enzyme treated (4 U/g OdP) pulp was subjected to Chlorine treatment (2 %) and hydrogen peroxide 4 % followed by different concentration of sodium hydroxide (1N) 11, 12, 13, 14 and 15 mL (v/v).

5.1.5. Release of chromophoric material and reducing sugar from pulps

For estimation of chromophoric material released the pulp samples treated with different enzyme dose as mentioned above were sealed in plastic bags (3 % consistency) in 100 mM phosphate buffer, pH -8 at 50 ^{o}C for 1hour, intermittent kneading was given. Control sample was treated under the same conditions with inactivated (boiled) enzyme. The enzyme-mediated release of lignin-derived compounds and chromophoric material from pulp was monitored in filtrates by measuring the absorbance at 280 nm and 465 nm, respectively (Wong *et. al.*, 1997). Amount of reducing sugar released from pulp was determined spectrophotometrically at 540 nm according to the DNS method (Miller,

59). The degree of solubilisation of pulp was determined from the reducing sugar leased.

egree of solubilisation = Reducing sugar released / weight of pulp x 100

1.6. Effect of Kappa Number

he brown and bleached pulps were treated with xylanase at optimum pH – 9 at 50 °C of olubilisation. The kappa numbers of unbleached and the bleached pulp were estimated y potassium permagnate method. The sample of pulp (3-4 g) was exposed to the action f 0.1 N $KmnO_4$, (acidified) in a volume of 1000 mL at room temperature for 10 min. he reaction was stopped by adding excess KI solution and $KMnO_4$, consumed was etermined from the results of back-titrating the liberated iodine with standard sodium hiosulphate. The *k* number so obtained was the mL of 0.1 N $KmnO_4$, consumed per gram of pulp (Tappi method T -236). It is a measure of residual lignin in the pulp. The amount of reducing sugar and degree of solubilisation were calculated.

5.1.7. Scanning electron micrograph (SEM)

Change of the surface morphology of the enzyme and non-enzyme bleached sequence were examined by SEM to obtain a better understanding of the effect of xylanase treatment on enhanced bleachabilty. Samples of control and enzyme treated pulp were processed for normal scanning procedure. The fibers were suspended in distilled water and placed on a cover slip and air-dried. The preparation was coated with gold particles (24 carat, 12 nm- layer 20 nm- thick) and examined at 15kV under SEM (JOEL JSM-6400) at various magnifications and micrographs were prepared.

5.2.0. ENZYMATIC TREATMENT OF CARTON AND OFFICE PAPER

For the preparation of paper pulp for this experiment, firstly carton boxes and office papers were cut in to small pieces and soaked in distilled water for 10 min, then grinded in wet grinder for 15 min. The fiber freeing process included enzymatic pulping ie.

treating the pulp with enzyme and separation stage by beating ie. grinding the pulp for separation. By agitation at 900 rpm the pulp suspension of 400 g (consistency of 3.76 %) was disintegrated in water at pH 6.0, 32 °C for 10 min. To this, enzyme was added at 2 U/g oven dry pulps and stirred for 1 h at 900 rpm at 50 °C. The enzyme was diluted in distilled water before addition to get better dispersion in the pulp suspension (10 % of the total reaction volume). The enzyme was inactivated by boiling the pulp for 5 min. Physical and mechanical properties of the pulp and paper were determined by preparing hand sheet, by passing the pulp through Buchner funnel which was layered with filter paper, the water was removed by filtering and sheets were pressed and dried in hot air oven at 50 °C until the sheets has shown constant weight (Tappi Test Method). The fiber surface samples were examined and recorded using a JOEL JSM-6400 scanning electron microscope.

5.3.0. CRUDE ENZYME IN THE PLANT FIBER SEPARATION

The crude enzyme extract was used for fiber separation of plantain and coconut husk. Five gram of each day sample was made into small pieces and soaked with suitably diluted enzyme (4 U/g in distilled water) in a conical flask; control without enzyme was done parallel. The flasks were placed in a thermostat at 50 °C for 1 hour for plantain and 3 hour for coconut husk. The deactivated of enzyme was done by boiling the mixture for 5 min. Scanning electron micrograph was done to observe the surface of fibers. Samples were dried and mounted on a small metal stub using fixing gum. Thereafter, samples were coated with gold and observed in a JEOL JSM-6400 -SEM operated at 15 kV using the secondary electron mode with images collected digitally.

5.4.0. HPLC ANALYSIS OF THE HYDROLYSIS PRODUCTS OF POLYSACCHARIDES

Hydrolysis of Oat spelt xylan, brich wood xylan, wheat bran and rice bran (1 %) was studied by treating these substrates with crude xylanase by incubating 50°C for 10 min, the reaction was terminated by heating at 100°C for 5 min. To remove any residual solids

the samples were centrifuged for 2 min at maximum speed (10000 g) in an Eppendorf Microfuge. The supernatants were filtered through 0.45 μm pore-size disc filters (Gelman Science, MI, U.S.A.). These filtrates were analyzed by injecting 20 µL on to a C- 18 column fitted in the HPLC system with Milli - Q water as the mobile phase at a flow rate of 0.5 ml/min.

5.5.0. SACCHARIFICATION OF LIGNOCELLULOSIC SUBSTRATES

Saccharification of natural lignocellulosic materials such as wheat bran and rice straw was carried out by the method of Okele and Obi, (1995). The substrates were taken as suspension (1 g) in phosphate buffer (0.1 M, pH 7) and 4U of enzyme was added to make the final volume of 100mL of reaction mixture. Sodium azide (0.2 %) was added to each to avoid growth of other microorganisms. Saccharification was performed at 50 °C for 2 hour, the resultant supernatant was collected at regular interval, centrifuged (7000 x g, 4 °C 15 min) and assayed for total reducing sugar using DNS reagent (Miller, 1959).

5.6.0. RESULTS AND DISCUSSION

The crude xylanase from *Bacillus pumilus* was cellulase poor. The zymogram studies revealed that multiple form of xylanase present. The characterization studies of the enzyme revealed that it was stable and active at wide range of pH and temperature 50°C. The rationale for the production of multiple xylanase was the heterogeneous nature of the wheat bran substrate used for the production of the enzyme (Gunasekaran, 2001, Biely, 1985). The simultaneous action of these multiple enzymes improves the solubilisation of pulp, by action of each of these enzymes on different location of the xylan polymer. Due to these factors the crude enzyme was as such used for the application studies. In the process of biobleaching the xylanase enzyme added to the pulp act upon the left over xylan, and degrade it, which enhance the penetration of chemical bleaching agents with ease (Bajpai, 1997, Coughlan and Hazelwood, 1993). So prior to the treatment of xylanase in the pulp bleaching sequence, the solubilisation of residual xylan in the pulp

be at its maximum, so maximization of solubilisation can be achieved by optimization of enzyme dose, pH and temperature.

5.6.1. Optimization of Xylanase bleaching for Chemi- Mechanical pulp

The optimization of enzyme dose and reaction time for biobleaching was carried out by treating pulp with varying dose of xylanase, for variable time intervals up to 2 hour and the results were given in Table. 5. 2. The results show that there was considerable effect of enzyme dose on CM pulp at 1 hour of incubation. The xylanase treatment of CM pulp using different dose of xylanase from *B. pumilus* when incubated at 50 °C for 1 h resulted in the release of chromophore with an OD of ~1.25 (280 nm) and brightness 60.1 with enzyme dose of 4 U/g Od pulp. As enzyme dose increase there was obvious increase in reducing sugar and absorbance at 280 nm, where as the final brightness obtained was only of marginal differences, so to make the bleaching process cost effective low enzyme dosing was selected for further studies.

Enzyme Dose	Reducing Sugar (mg/g Od Pulp)	Absorbance at 280nm	Absorbance at 465nm	Brightness (%)
Raw pulp	0.58	0.017	0.15	58.0
2	2.06	0.195	0.18	59.8
4	**2.05**	**1.25**	**0.45**	**60.1**
8	2.09	1.65	0.49	59.8
12	2.11	1.89	0.48	59.7
14	2.13	1.85	0.48	59.7

Table. 5. 2. Optimization of enzyme dosage in Chemi- mechanical pulp, reducing sugar, UV spectrum of coloured compounds released (280 and 465nm) during enzyme treatment at different enzyme dose and Brightness checked after alkali extraction.

Temperature (°C)	Blank		Enzyme treated	
	Residual peroxide (%)	Brightness (%)	Residual peroxide (%)	Brightness (%)
40	28	59.1	31	60.1
50	36	59.2	33	**61.4**
60	35	59.9	33	60.7

Table. 5. 3. Effect of temperature on enzyme treatment, residual peroxide and brightness of CM-Pulp treated and untreated with enzyme after peroxide treatment.

pH	Blank		Enzyme treated	
	Residual peroxide (%)	Brightness (%)	Residual peroxide (%)	Brightness (%)
6	27	58.1	30	58.9
7	36	58.2	33	59.9
8	36	58.9	34	**61.7**
9	42	58.8	34	61.5
10	46	58.0	34	59.2

Table. 5. 4. Effect of pH on enzyme activity, residual peroxide and brightness of CM-Pulp treated and untreated with enzyme after peroxide treatment.

The effect of temperature from 40 to 60 °C was studied by treating pulp suspension with 4 U/g OdP of xylanase at alkaline pH and given in Table. 5. 3. The enzyme activity on Chemi – mechanical pulp was observed to be maximum at 50 °C for the treatment of the

pulp. The effect of pH on CM- pulp was studied by treating the pulp suspension with (4 U/g OdP) xylanase in a total volume of 200 mL from pH 6, 7, 8, 9 and 10. The results were given in Table. 5. 4. The enzymatic treatment with CM- pulp has shown a slight variation in brightness. There was not much effect for the use of enzyme in the bleach sequencing of CM- pulp where an ISO brightness of 60 Units was sufficient for paper for Newspaper manufacturing. If high quality final paper is required the enzymatic treatment will be more efficient, so further optimization studies with CM- pulp was not pursued. Christov *et. al.,* (1998) reported the effect of xylanase from white - rot fungus as means of improving the selectivity of both pulping and bleaching processes, thereby increasing either the final pulp yield or brightness of *Eucalyptus* pulp. Due to lignin modification by the white-rot fungi at the biopulping stage, its degradation and removal from pulp apparently becomes easier at the bleaching stage. This could possibly be translated into savings of pulping and/or bleaching chemicals at a given pulp yield and therefore advantageous toward improving the effluent quality, reduction of pulping time and there by saving energy. Duarte *et. al.,* (2003) reported the effective utilization of D- xylanase and other enzymes from *B. pumilus* CBMAI 0008 on *E. grandis* kraft pulp which resulted in a reduction of 0.3 % of chlorine usage as well as an increased brightness, compared to conventional bleaching. Since xylanase appear to be more efficient, it should not be necessary to modify existing processes, which is favorable for its industrial employment.

5.6.2. Optimization of xylanase treatment conditions for Chemical pulp

The effect of different dose of xylanase on Chemical pulp was explained in Table. 5. 5. The samples were incubated for 1 h resulted in the release of chromophore with an OD ~1.45 at 280 nm and ISO brightness of 63.0 % with 4U/g. After 1 hour there was no considerable change in the result so 1 hour of incubation time for enzyme treatment was selected. There was no observable change with higher dose of enzyme treatment, 4U/g Od pulp observed to be more effective than higher dose. The results show that higher dose of enzyme has increased the release of reducing sugar and chromophoric material with absorbance at 280 nm, but the ISO brightness of resulting sheet was only of marginal difference and higher dose has given very low brightness. So to make the

process more economical, low enzyme dose was used for optimization studies. The effect of temperature at 40, 50 and 60 °C was studied by treating pulp suspension with 4 U/g of xylanase and given in Table. 5. 6.

Enzyme Dose	Reducing Sugar (mg/g Od Pulp)	Absorbance at 280nm	Absorbance at 465nm	Brightness (%)
Raw pulp	0.37	0.012	0.11	58.1
2	0.535	0.145	0.19	61.2
4	**1.187**	**1.45**	**0.293**	**63.0**
8	1.590	1.564	0.392	62.5
12	2.170	1.982	0.318	62.1
14	2.750	1.859	0.310	59.9

Table. 5. 5. Optimization of enzyme dosage in Chemical pulp, reducing sugar released, UV spectrum of coloured compounds released (280 and 465nm) during enzyme treatment at different enzyme dose and Brightness observed after chlorination and alkali extraction.

Parameter (°C)	Blank			Enzyme treated		
	Residual Chlorine (%)	Residual peroxide (%)	Brightness (%)	Residual Chlorine (%)	Residual peroxide (%)	Brightness (%)
40	1	18.5	57.0	0.05	20.3	57.9
50	**1**	**14.7**	**54.2**	**0.05**	**16.8**	**63.4**
60	1	15.9	52.8	0.05	22.6	59.0

Table. 5. 6. Effect of temperature on enzyme treatment, residual chlorine, peroxide and brightness of Chemical Pulp treated and untreated with enzyme after chlorination and alkali extraction.

The effect of pH on was studied by treating the pulp suspension (3 % consistency) with (4 U/g OdP) xylanase in a total volume of 200 mL from pH 8, 9 and 10 and the results were given in Table. 5. 7. From the results of enzyme activity on chemical pulp has shown that pH – 9 and temperature 50 °C was optimum for the enzyme treatment of the pulp.

Parameter	Blank			Enzyme treated		
	Residual Chlorine (%)	Residual peroxide (%)	Brightness (%)	Residual Chlorine (%)	Residual peroxide (%)	Brightness (%)
8	1	14.5	54.7	0.05	21.3	60.2
9	**1**	**14.9**	**54.8**	**0.05**	**22.1**	**63.6**
10	1	15.1	53.9	0.05	21.2	58.0

Table. 5. 7. Effect of pH of pulp on enzyme treatment, residual chlorine, peroxide and brightness of Chemical Pulp treated and untreated with enzyme after chlorination and alkali extraction.

The effectiveness of xylanase treatments has been evaluated by at least two aspects: First, by determining the amount of sugars present after enzyme incubations, where 0.5 % to 1.0 % of the pulp carbohydrate content is liberated; and second, by observing increased bleachability with conventional methods after xylanase treatments (Viikari *et. al.,* 1993). The increases in enzyme dose and treatment time enhances the amount of reducing sugar released from the brown pulp, similar observation was made by the application of xylanase from *Auerobasedium pullulans* (Christov *et. al.,* 1993, 1998) and in *Aspergillus fischeri* Fxn 1 (Gunansekaran, 2001). Christov *et. al.,* (1993) has reported maximum amount of reducing sugar released with 1500 Unit enzyme dose and further increase has not resulted in any improvement of solubilisation, This was due to the restricted accessibility of substrate for xylanase action or saturation of enzyme substrate complex. Kulkarni and Rao (1996) reported an OD of about 0.1900 at 465 nm when 10 IU of the

xylanase from *Bacillus* NCIM 59 was used per gram of washed and Od bagasse pulp at 50 °C for 4 h. Similarly 5 g of eucalyptus pulp using 7 IU/g of xylanase from *B. circulans* AB 16 when incubated at 55 °C for 3 h resulted in the release of chromophore with an OD of 0.2414 at 465 nm (Dhillon *et. al.,* 2000). The UV absorption spectrum of the compounds released by enzyme treatment showed a characteristic peak at 280 nm, indicating the presence of lignin in the released colouring matter. Chromophore release by xylanase treatment is one of the significant factors of enzyme attack on the pulp. The enzyme when act upon the pulp help in the release of material that have absorbance at UV and visible region, which originates from the colour of the pulp. Reducing sugar, chromophore release and kappa number have been widely monitored to check the solubilisation of pulp following xylanase treatment (Shah *et. al.,* 1999, Jeffries, 1996, Ratto *et. al.,* 1994) Release of chrompohores is a better indication of kinetics of enzyme attack on the pulp as reducing sugar will continue to be generated by xylanase hydrolysis of soluble oligosaccharides released by the initial depolymerisation of the xylan coating the fiber surface (Garg *et. al.,* 1998). Xylanase from *Bacillus* NCIM 59 was reported effective in biobleaching of bagasse pulp, maximum reduction was obtained at 10 U/ g Od pulp. Increase in the enzyme dosage has no effect on reduction of the *k* number but there was an increase in the absorbance at 280 nm, which shows that the carbohydrate degradation products also attribute to the coluoring matter (Kulkarni and Rao, 1996).

5.6.3. Degree of solubilisation and Kappa No. reduction of pulp

The reducing sugar released was measured using DNS method, the degree of solubilisation of pulp was determined from the reducing sugar released and the kappa number as well as the brightness of optimized condition was well explained in Table. 5. 8. The pulp was treated with enzyme 4 U/g Od pulp and the kappa no. before and after treatment was measured. The final Kappa number was measured after the chlorination and alkali extraction. The results shows that there was observable change in the enzyme treated pulp than untreated pulp samples. The major factors affecting enzyme treatment efficiency includes pH, temperature, reaction time, enzyme dosage and dispersion consistency.

Pulp	Reducing sugar (mg/g pulp)	Kappa No. Initial and final	Degree of solubilisation	Brightness (%)
CM Pulp untreated	0.68	15.2 and 2.8	68	59.4
CM- Pulp treated	**2.18**	**12.2 and 2.1**	**218**	**61.2**
CP-Untreated	0.40	18.2 and 3.5	40	54.7
CP- treated	**1.57**	**14.1 and 2.3**	**157**	**63.2**

Table. 5. 8. Reducing sugar, Kappa No. (Initial and final-after treatment), Degree of solubilisation and brightness of untreated and xylanase treated (4U/g Od pulp) CM and CP- pulp.

The optimum pH for xylanase treatment varies amoung enzymes, generally enzyme from bacterial origin were effective at pH range 6 to 9 and fungal 4 to 6 and temperature ranges from 35 to 60°C as well as optimum enzyme dosage ranges from 2 to 5 U/g of dry pulp. The pulp consistency should be such as effective distribution of enzyme through out the system (Bajpai, 1997, Suurnäkki *et. al.,* 1997). During alkaline digestion, modified carbohydrates were deposited on the cellulose fibers imparting colour (Wong *et. al.,* 1992). When xylans break down xylooligomers and LCC is formed and some persist in the pulp, which later effect in the reprecipitation and thus the brightness of the paper was effected. All breakdown products are susceptible potassium permagnate oxidation and are easily removed by alkali extraction after enzyme treatment of pulp (Paice *et. al.,* 1992). Garg *et. al.,* (1998) has described the effect of xylanase from *S. thermoviolaceus* which reduced the kappa number and increased the brightness of kraft pulp in conventional CEDED bleach sequence at 4 % chlorine dose. Earlier reports pertained to xylanase treatment at neutral or acidic pH (Senior et. al 1988, Patel *et. al.,* 1993), which was made possible by adjusting the pH after alkaline extraction.

Alkali stable xylanase have clear advantage in that there was no need of washing the pulp to reduce its pH. In this study it was observed that pH- 8 and 9 was more effective in the

inal brightness. So adjustment pH of the pulp was not required. In case of *Bacillus* sp. am -3, a pH of 8.0 was suitable for solubilisation of pulp, it was also reported that ncrease in enzyme dosage 1.2 U/g pulp has effected in increased production of reducing ugar and lignin derivative compounds (LDC) (Shah *et. al.,* 1999). Beg *et. al.,* (2000) has eported xylanase from *Streptomyces* sp. QG-11 –3 (3.5 U/g of Od pulp) which support naximum bleach boosting effect at 50°C and pH- 8.5 for a 2 hour treatment using. ndoxylanase from *Thermotoga maritime* cloned into *E. coli* was capable of releasing educing sugar in the pH range between 5.0 to 10.0 with maximum at 5.0 (Shah *et. al.,* 000). Pulp processing is generally carried out at high temperature and so thermostable ylanase are more acceptable for bleaching process. Xylanase from *Bacillus* sp. Sam-3 olubilized pulp maximally at 60°C (Shah *et. al.,* 1999) and xylanase from *Dictyoglomus* p. reported to be capable of solubilising pulp at 80 °C and pH- 5.0. (Ratto *et. al.,* 1994). iarg *et. al.,* (1998) has reported xylanase from *Streptomyces thermoviolaceus* active at 0°C. Xylanase from *Thermomyces lanuginosus* produced by SSF was applied on agasse pulp at pH 6.0; 60 °C; 10 % pulp consistency for 3 h (Christopher *et. al.,* 2005).

.6.4. Optimization of Chlorine dioxide, Peroxide and Caustic soda dose in Chemical ulp bleaching

he optimization studies with Chemical pulp, which has shown significant variation in ie final brightness. Experiment was tried to make the process total chlorine free. For this ie chemical pulp was subjected to peroxide treatment, of higher dose devoid of hlorination and results was explained in Table. 5. 9.

he results show that 4 and 5 % of hydrogen peroxide has significant effect on the pulp rightness. The use of low concentration of H_2O_2 is more economical so for further tudies 4 and 5 % was used. Enzyme dose (4 and 7 U/g OdP) was then optimized for 4 nd 5 % peroxide treatment and result given in Table. 5. 10. The pulp treated with ylanase 4 U/g Odp has given a final brightness of 55 units with 4% peroxide treatment /hich was of 5 unit rise related to the control.

Peroxide (%)	Blank	
	Residual Peroxide (%)	Brightness (%)
3.0	35.0	47.7
4.0	**37.0**	**49.8**
5.0	**42.0**	**50.3**
6.0	57.0	50.7

Table. 5. 9. Effect of higher dose of peroxide on CP pulp sample without enzyme treatment and chlorination.

Peroxide Dose (%)	Enzyme dosage (4U/g OdP)		Enzyme dose (7U/g OdP)	
	Residual peroxide (%)	Brightness (%)	Residual peroxide (%)	Brightness (%)
4	**33**	**55**	**34**	**54.5**
5	30	54.8	36	54.2

Table. 5. 10. Effect of higher dose of peroxide (4 and 5 %) on CP pulp sample treated with xylanase (4 and 7 U/g OdP) without chlorination.

The pulp after enzyme (4 U/g OdP) was subjected to low dosage of Chlorine treatment (1, and 2 %) and peroxide treatment (4 and 5 %) and results were given in Table. 5. 11. The results shows that there was no residual chlorine left over in the effluent which shows that the enzyme treatment has helped in the complete action of chlorine on the pulp. The residual peroxide was also low than the blank, which was run parallel with a ISO brightness of 52.8 with 2 % chlorination and 4 % hydrogen peroxide treatment. The final ISO brightness of the pulp was observed to be 60.1 and 60.5 for 1 % chlorine treated followed by 4 and 5 % H_2O_2 treatment where as the pulp, which was given 2 %, chlorination has shown 62.7 and 62.5 % ISO brightness. Residual chlorine was not present in the effluent, which show that complete utilization of chlorine occurred in pulp

treated with enzyme. The left over peroxide was also of low amount that shows the effect of xylanase treatment in pulp bleaching.

Chlorine Dose (%)	Peroxide dose (4%)			Peroxide dose (5%)		
	Residual Chlorine (%)	Residual peroxide (%)	Brightness (%)	Residual Chlorine (%)	Residual peroxide (%)	Brightness (%)
1	0	30.8	60.1	0	26.3	60.5
2	0	11.5	62.7	0	8.0	62.5
Control	1	42.0	52.2	1	42.0	53.2

Table. 5. 11. Effect of higher dose of peroxide (4 and 5 %) and Chlorine dioxide (1 and 2 %) on CP pulp sample treated with xylanase (4 U/g OdP).

Table. 5. 12. gives the result of higher dose of chlorination (3 and 4 %), which shows a marginal increase in final brightness, but residual chlorine was present. Use of high chlorination lead to the presence of chlorine in the effluent as well as chlorination and enzyme treatment together was not economical for paper processing.

To make the process more economical low dose of enzyme, chlorine dioxide and peroxide was selected and amount of alkali was varied. The enzyme treated (4 U/g OdP) pulp was subjected to Chlorine treatment (2 %) followed by hydrogen peroxide (4 %) and different concentration of sodium hydroxide (1N) 11, 12, 13, 14 and 15 mL (v/v) and result given in Table. 5. 13. The results show that the pulp treated with 15 mL of 1 N NaOH has shown higher brightness of 65.9 and followed by 14 mL 65.1 and 64.4 and 63.2 % by 13 and 12 mL respectively. The control has shown only 57.4 % brightness with low amount of chlorination and peroxide treatment. There were about 8.5 units difference related to control. There was no residual chlorine and residual peroxide in the effluent was observed to be very low. This shows that the treatment of enzyme prior to chlorination and peroxide treatment has made the fibers to swell and have effected in easy penetration of chemical to the interior.

Xylanase treatment has been tested in several bleaching sequences containing peroxide stage (Garg *et. al.,* 1996). Peroxide has been used as a primary reagent of bleaching stage and to reinforce alkaline extraction (Vicuna *et. al.,* 1995). The improvement of alkaline peroxidase bleaching was demonstrated using *Dictyoglomus* sp. xylanase (Ratto *et. al.,* 1994, Wong *et. al.,* 1997). The direct use of SSF enzymes in bleaching was a relatively new biobleaching approach and xylanase from *T. lanuginosus* strain ATCC 36350 and ATCC 46882 were most efficiently used in biobleaching, resulted in improving brightness by 2.0 and 1.8 points respectively (Christopher *et. al.,* 2005). There were reports related to the utilization of xylanase enzyme for the bleach boosting of baggase soda pulp (Bissoon *et. al.,* 2002) and wheat straw enriched soda pulp (pH 9.5 - 10) at 65 ^{o}C (Techapun, *et. al.,* 2003, Ninawe and Kuhad, 2005, Li *et. al.,* 2005).

Chlorine Dose (%)	**Peroxide dose (4%)**		
	Residual Chlorine (%)	**Residual peroxide (%)**	**Brightness (%)**
3	0.05	25	63.4
4	0.05	23.8	63.8
Control	1.0	40.1	52.8

Table. 5. 12. Effect of higher dose of Chlorine dioxide (3 and 4 %) with 4 % peroxide treatment on CP pulp sample treated with xylanase (4 U/g OdP).

Crude enzymes produced from *Penicillium* A10 and *Aspergillus* L22 were used in the production of bleached wheat straw with xylanase dose of 4 IU/g prior to pulping, decreased pulp kappa number by 6.29 % and 12.07 % respectively as compared to the control (Zhao *et. al.,* 2006). Cellulase-free xylanase produced from *Arthrobacter* sp. MTCC 5214 by SSF using wheat bran, which was thermoalkalophilic was evaluated for prebleaching of kraft pulp that resulted 20 % reduction in kappa number of the pulp

ithout much change in viscosity. And also reduced the amount of chlorine by 29 % ithout any decrease in brightness (Khandeparkar and Bhosle, 2006).

Caustic Soda (v/v)	Enzyme treated (4 U/g ODP)	
	Residual Peroxide (%)	Brightness (%)
15	7.9	65.9
14	11.9	65.1
13	13.4	64.4
12	19.3	63.2
11	23.9	61.4

able. 5. 13. Effect of higher dose of Caustic soda with 4 % peroxide treatment on CP pulp sample treated with xylanase (4 U/g OdP) and chlorination (2 %).

6.5. Scanning electron micrograph (SEM)

. order to obtain a better under standing of the effect of xylanase treatment on enhanced eachability, the surface morphology of the pulps was studied by SEM. The ıotomicrographs showed significant changes on the surface of xylanase treated pulps as result of xylan hydrolysis. Scanning electron micrograph of Chemi – mechanical (Fig. 1 (a). untreated (b). enzyme treated and chemical pulp (Fig. 5. 2. (a). untreated + ClO_2,). enzyme treated + ClO_2, (c). untreated + ClO_2 + H_2O_2, (d). enzyme treated + ClO_2 + $_2O_2$). Fiber of treated pulp underwent a peeling processes giving rise to flakes and laments of material detached from the fiber surface. The surface of untreated fiber looks nooth than that of xylanase treated ones. No significant change was observed in the ıtreated pulps. This suggests that the enzyme not only assisted in surface modification ıt also penetrated pulp fibres allowing for a much improved xylan hydrolysis. This was

related to the findings of Pham *et. al.,* (1995) for TCF bleaching with commercial xylanase. Torres *et. al.,* (2000) has also reported similar results of the effect of xylanase on ECF bleaching of oxygen delignified *Eucalytus* kraft pulps. Xylanase treatment observed to be effective in the opening the closed cell – wall – pores of kraft pulps. Changes in the fiber morphology such as cracks, flakes filaments and peeling were observed during the bleaching due to enzyme treatment. These cracks facilitate the diffusion out the fiber cell wall of the larger lignin macromolecules (Kantelinen *et. al.,* 1993, Li *et. al.,* 1996, Torres *et. al.,* 2000). Thus the xylanase enzyme treatment improves the accessibility of pulps for the bleaching chemicals; it decreases the diffusion resistances to the outward movement of the degraded lignin fragments and allowed the removal of the less degraded lignin fragments from the fiber wall. This was the reason for the decreased kappa number and higher brightness of the enzyme treated pulps at the same bleaching reagents consumption (Torres *et. al.,* 2000). Unbleached bagasse soda pulp was pretreated with a purified xylanase (150 U/g) from *Thermomyces lanuginosus* SSBP, exhibited direct brightening abilities on bagasse pulp improving brightness by 1.5 points, however, kappa number decreased by 0.5 points Scanning electron microscopy revealed significant surface modification of bagasse pulp fibres without marked fibre disruptions (Bissoon *et. al.,* 2002).

5.7.0. ENZYME APPLICATION IN RECYCLING OF WASTE PAPER

The effect of crude enzyme on waste paper utilization was tried. The SEM (Scanning electron micrograph) micrographs of pulp were given in Fig. 5. 3. (a). Untreated (b). treated. The SEM micrograph of enzyme treated pulp showed a significant change on the surfaces; the surface of untreated pulp appeared smoother than that of xylanase treated fibers. Fibers of treated pulp underwent a peeling process giving rise to flakes and filaments of materials detached from fiber surfaces, due to xylan hydrolysis. Enzyme treatment increases the fiber swelling, which facilitates refining, which in turn results in better physical properties (Kenealy and Jeffries, 2003). Enzyme addition prior to refining can improve strength properties at a fixed refining level.

5.8.0. EFFECT OF XYLANASE ENZYME ON PLANT FIBER

The plant fiber was treated with crude xylanase at 50 °C and fibers were separated and dried in air at ambient temperature. The scanning electron micrograph was taken Fig. 5. 4. (a). untreated (b). treated plantain fiber and 5. 5 (a). untreated (b). treated coconut fiber. SEM is a microscopic method, which can be used for analysis of surfaces of material. The micrograph clearly shows that the enzyme has effect on fiber freeing process. The untreated fiber surface observed to be smooth where as the treated fiber appears to be rough with small micro fibrils.

5.9.0. HYDROLYSIS PRODUCTS OF POLYSACCHARIDES

Hydrolysis of Oat spelt xylan, brich wood xylan, wheat bran and rice bran and results given in Fig. 5. 6 (a). and (b). The products from hydrolysis, corresponding to different peaks, the main products of hydrolysis of xylan was xylose and xylobiose in trace. The hydrolysis pattern of the enzyme treated substrate resulted mainly in xylose and xylobiose and minor fraction of the other two sugar (xylotriose and xylopentose) moieties indicating that the enzyme was mainly endoxylanase. Similar pattern of hydrolysis of xylan using xylanase from yeast strain was estimated by paper chromatography, which has resulted mainly in xylose with traces of xylobiose (Bastawde *et. al.,* 1993). Similar reports was there related to xylanase extracted from hyperthermophilic archaeon *Pyrodictium abyssi*, the analysis of hydrolysis products performed by HPLC showed as main product xylotriose and xylotetraose, indicating the presence of an endoxylanase (Andrade *et. al.,* 1999). Similar result of HPLC analysis of the hydrolysis production of birchwood xylan, wheat and rye arabinoxylans acted up by xylanase (Xyl Ia and Ib) from thermophilic fungus *Myceliophthora* sp. IMI 387099, shows xylobiose as major product followed by xylotriose (Chadha *et. al.,* 2004). *Bacillus flavothermus* strain LB3A reported to produce mainly xylotriose, xylotetrose and xylopentose as hydrolysis product (Sunna *et. al.,* 1997). *Bacillus* - SSP-34 has also shown similar patter of hydrolysis with oat spelt xylan (Subramaniyan, 2000). According to Biely *et. al.,* (1993) there was a relation between enzyme action and mol. wt. of the enzyme. High mol. wt. endoxylanase

(Family 10) exhibit high catalytic versatility than family 11. Exception to this was the report xylanase from *Aeromonas caviae* ME- 1 liberating only xylobiose and xylotetrose (Usui et. al., 1999) and three xylanase from *Streptomyces lividans* resulted in the release of xylobiose, xylotriose, xylotetrose and xylopentose (Marui *et. al.,* 1985). Beily *et. al.,* (1997) suggested the relationship between the molecular size and hydrolytic properties. Family 10 liberates more efficiently the terminal xylopyranose residues than family 11, because they have smaller substrate binding sites than family 11. This factor alone allowed a possible conformational flexibility of the larger enzyme than smaller ones. Substrate binding site of family 10 xylanase are not located in deep clefts as of family 11 xylanases.

5.10.0. SACCHARIFICATION OF LIGNOCELLULOSIC SUBSTRATES

Saccharification of natural lignocellulosic materials such as wheat bran rice bran and rice straw was tried. The maximum solubilisation of wheat bran, rice bran and rice husk in 2 hour of incubation at 50 °C was given in Fig. 5. 7. The observation shows that wheat bran saccharified rapidly while rice straw and bran was hydrolyzed at a very slow pace. *Bacillus pumilus* (cellulase – poor), so the released reducing sugar will be from lignocellulosic materials by the hydrolysis of hemicellulose. Report related to saccharification of lignocellulosic materials using cellulase was there but that related to xylanase were few. Ball and McCarthy, (1988) reported significant differences in enzymatic profiles of over 200 strains, and their release reducing sugars from ball milled straw. Hemicelluloses act as a cementing material that binds cellulose to lignin and the separation of cellulose fiber from lignin can be by the degradation or chemical removal of lignin or by physically tearing fibers apart (Erikksson *et. al.,* 1990). Chemical process is harmful, mechanical process is energy intensive where as biological process is environment friendly. Biopulping by saccharification using cellulase – free xylanase of *Bacillus* 518 was reported to be a beneficial process, since it reduced energy requirements, improve tensile strength and brightness yield (Tremblay and Archibald, 1993).

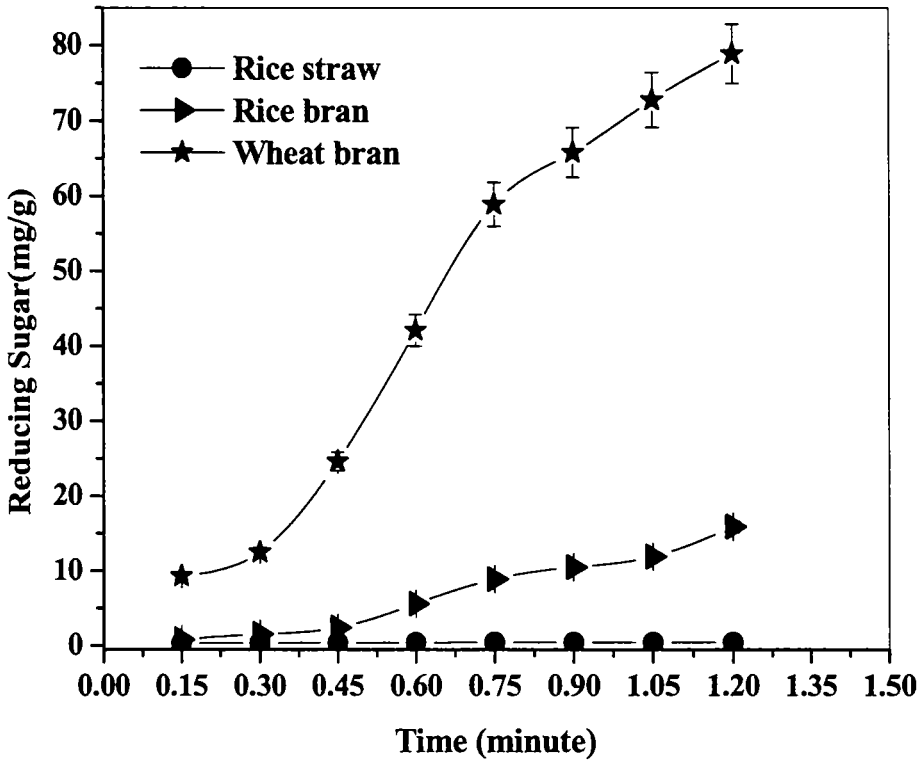


Fig. 5. 7. Effect of xylanase from *B. pumilus* in the saccharification of lignocellulosic material like rice straw, rice bran and wheat bran upto 2 hour of incubation at 50 °C.

5.1.0. CONCLUSION

To satisfy the specific conditions of the bleaching process at the pulp and paper mills, xylanase that are active and stable at elevated temperatures and alkaline pH are needed. A considerable amount of research worldwide has focused on the search for novel microbial isolates having the ability to produce thermotolerant and alkalophilic xylanase. Use of this crude xylanase from *B. pumilus* observed to be highly valuable for the pulp and paper industry as well as in the saccharification of lignocellulosic material. The significance of this enzyme was that it help in the reduction of use of Chlorine making the effluent

elemental chlorine free (ECF) as well as there was an increase of brightness by 8.5 unit related to control. This suggested that xylanase could be useful in the paper and pulp industry as it produced better quality kraft pulp than that obtained with xylanase produced by other sources. The SEM analysis shows that it was highly beneficial in fiber freeing process as well as in recycling of waste paper, which opens a new area of research.

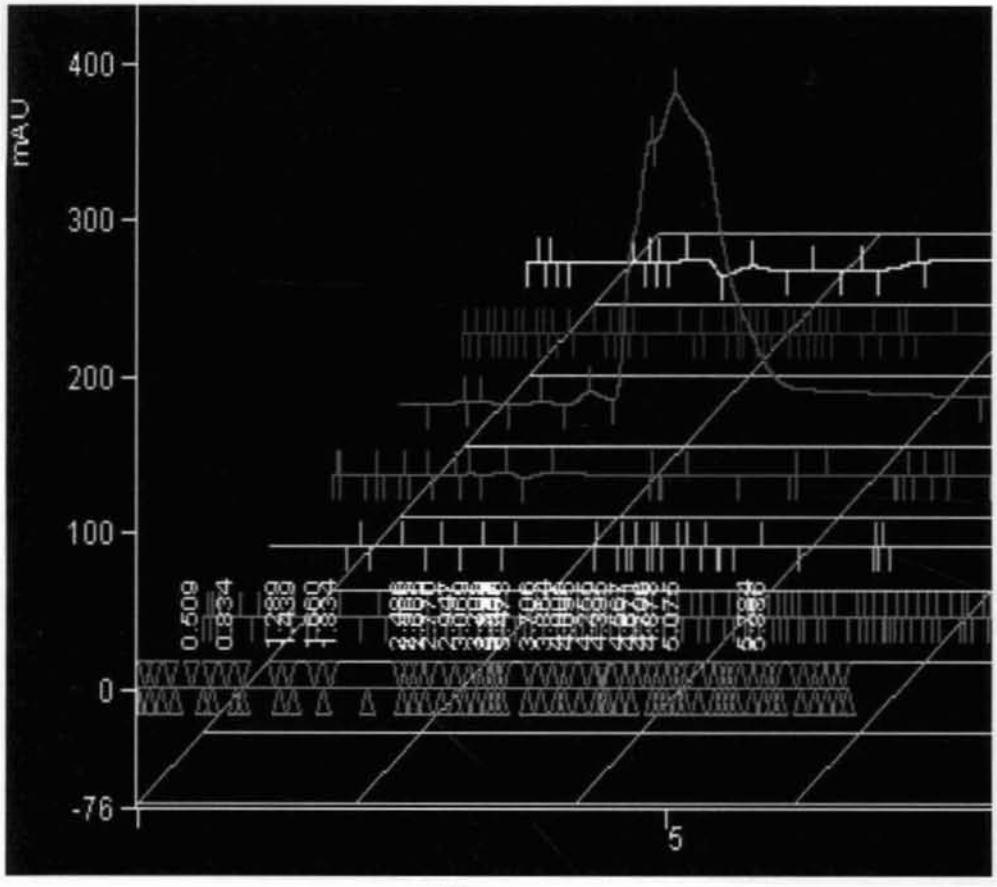
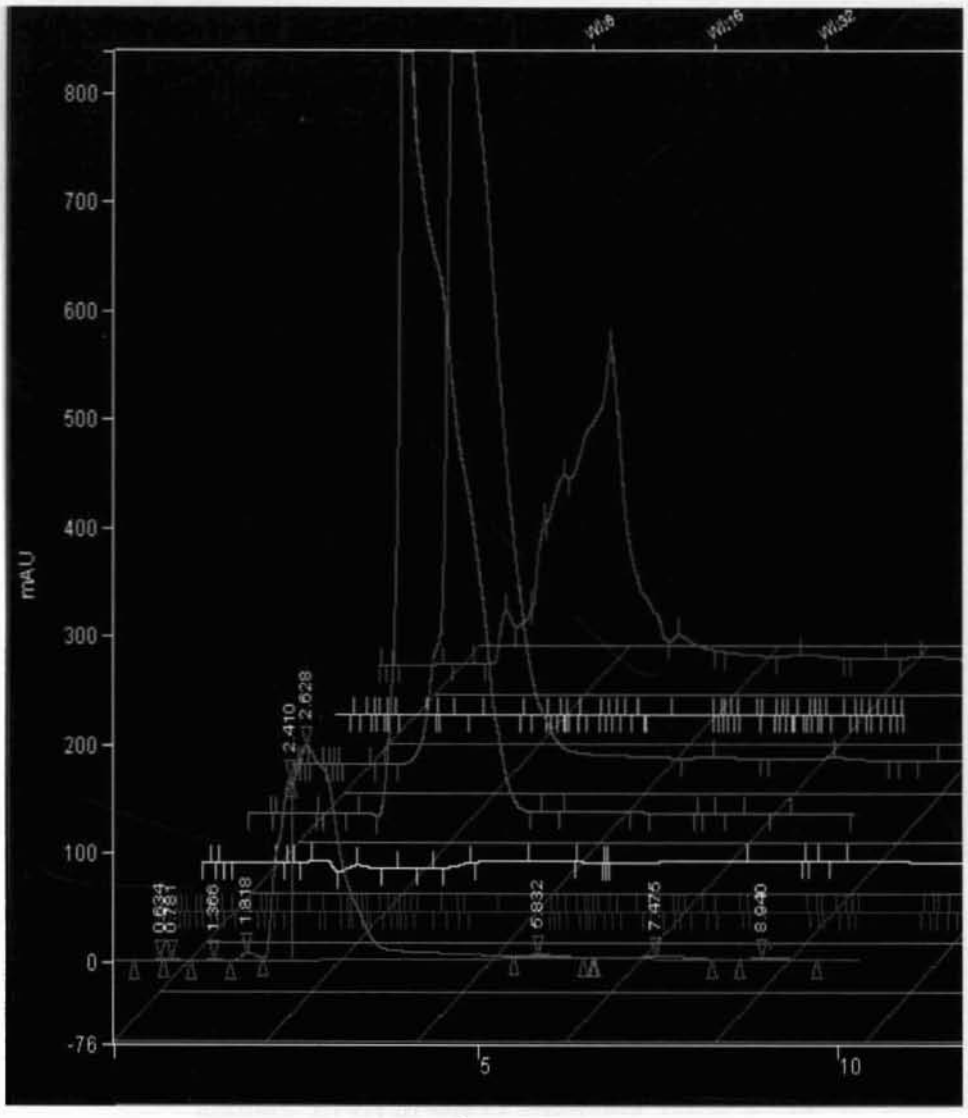
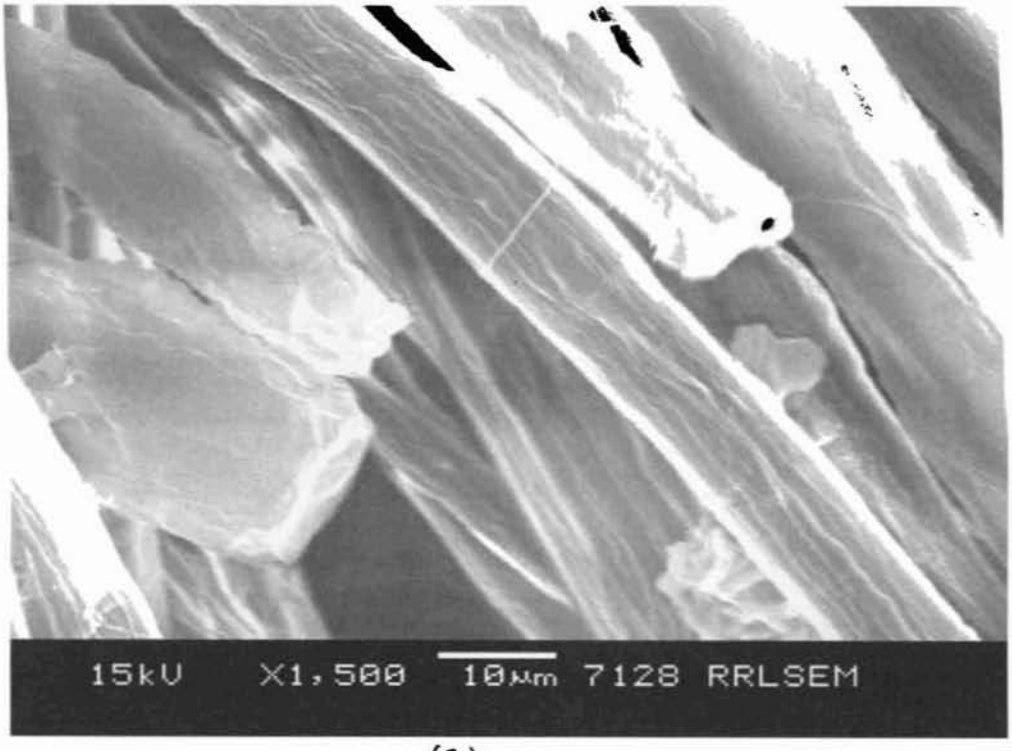


Fig. 5. 6. (a). Standard Xylose in HPLC column

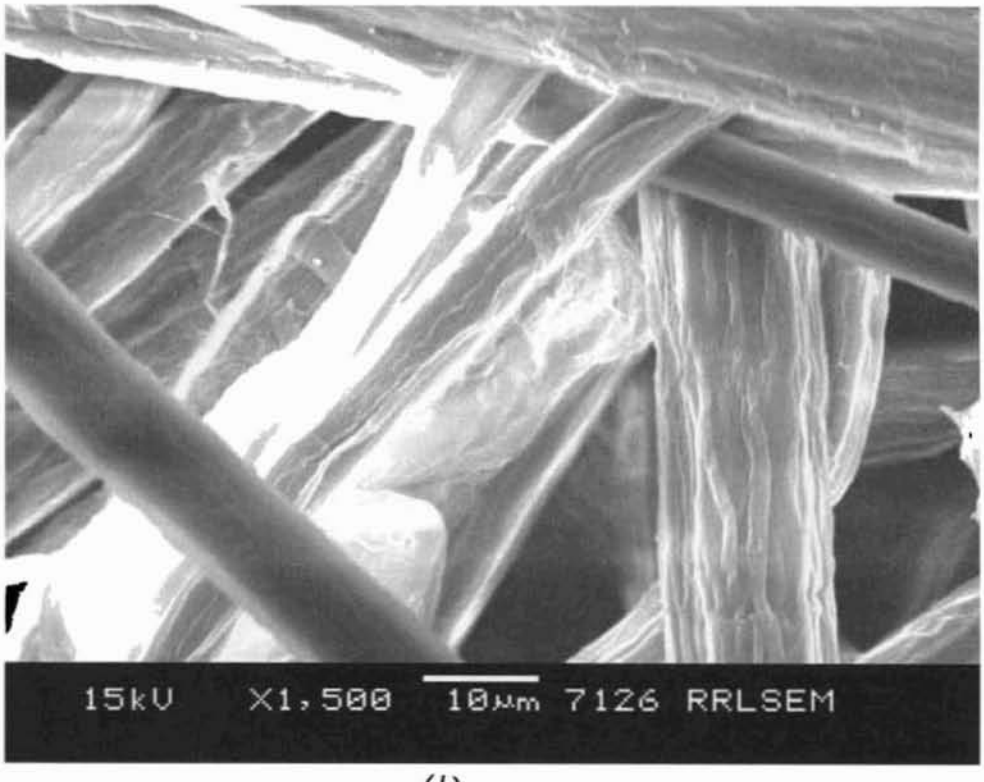


(b)

Fig. 5. 6. (b). Hydrolysis pattern of Oat spelt xylan (Green), Wheat bran (rose), Brich wood xylan (light blue) and rice bran (red), obtained from HPLC column the major peak corresponds to xylose.

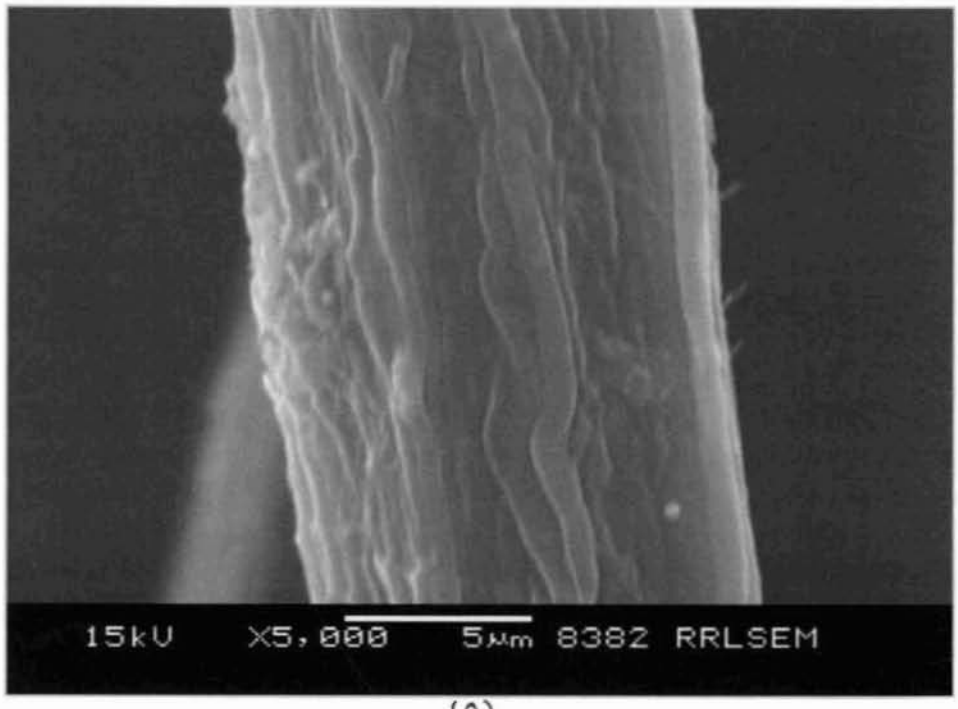


(a)

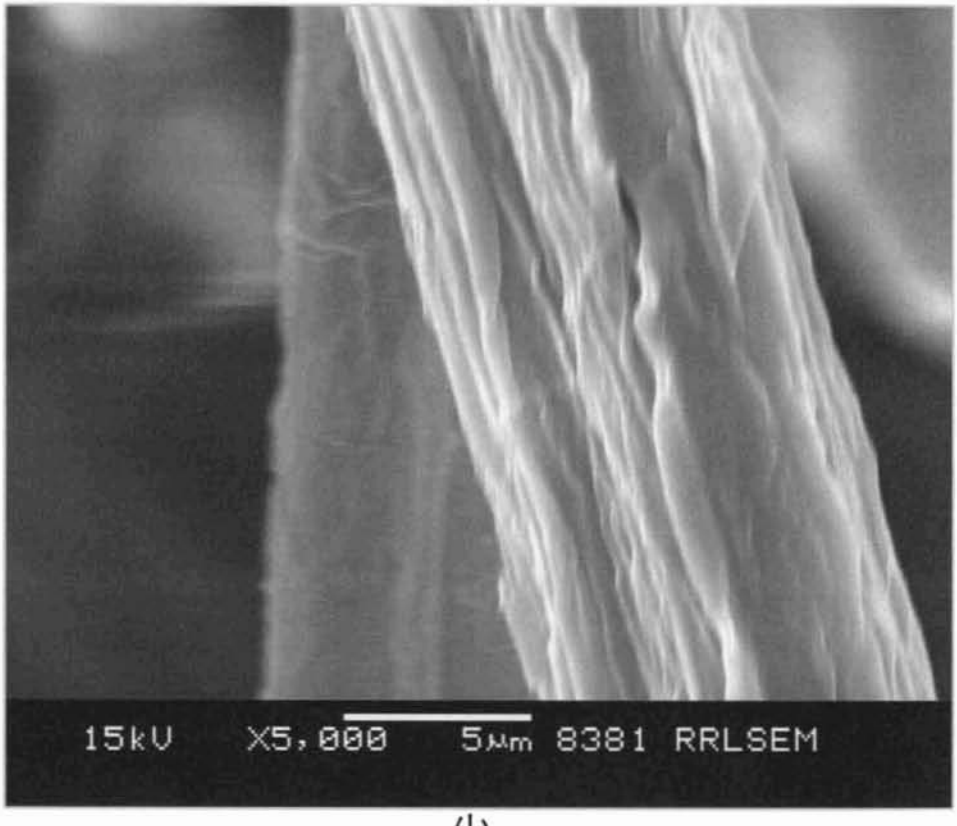


(b)

Fig. 5. 1. (a). Scanning electron micrograph of CM pulp untreated (b). Xylanase treated (after peroxide treatment).

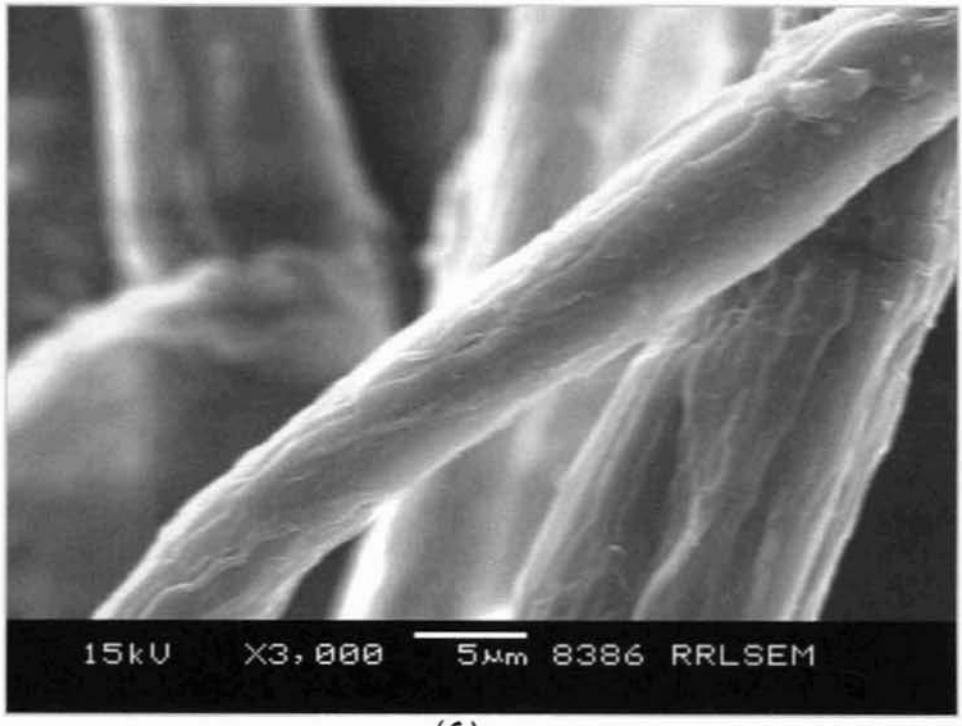


(a)

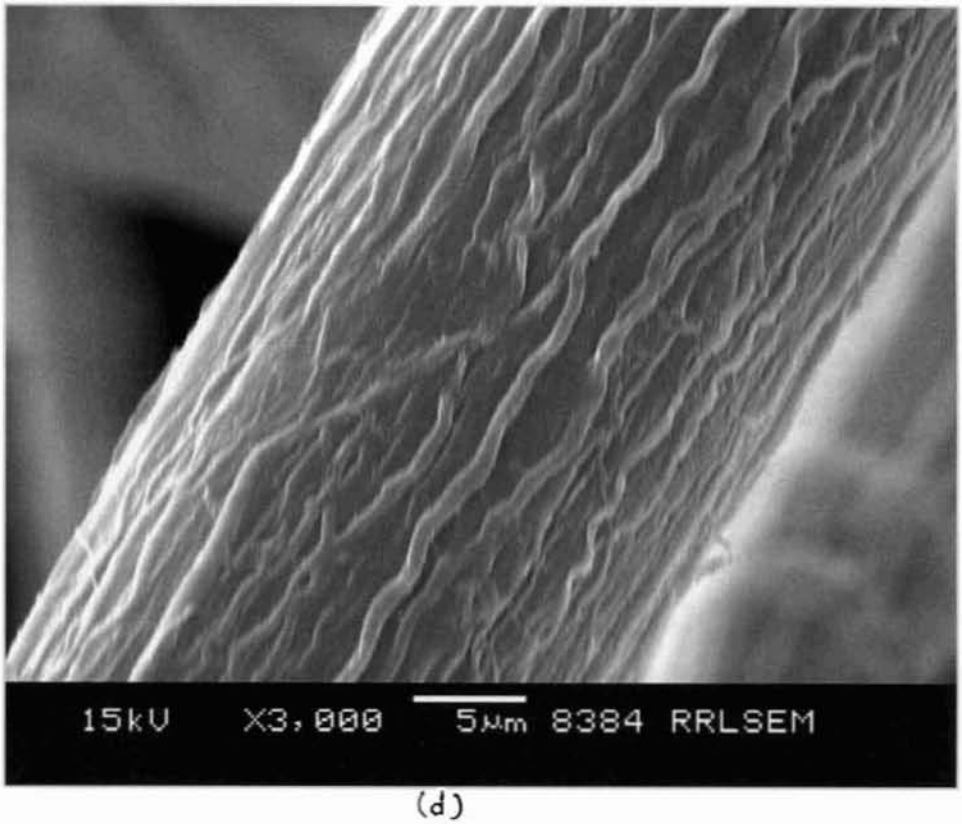


(b)

Fig. 5. 2. (a). Micrograph of Chemical pulp untreated (b). treated with xylanase after chlorination.

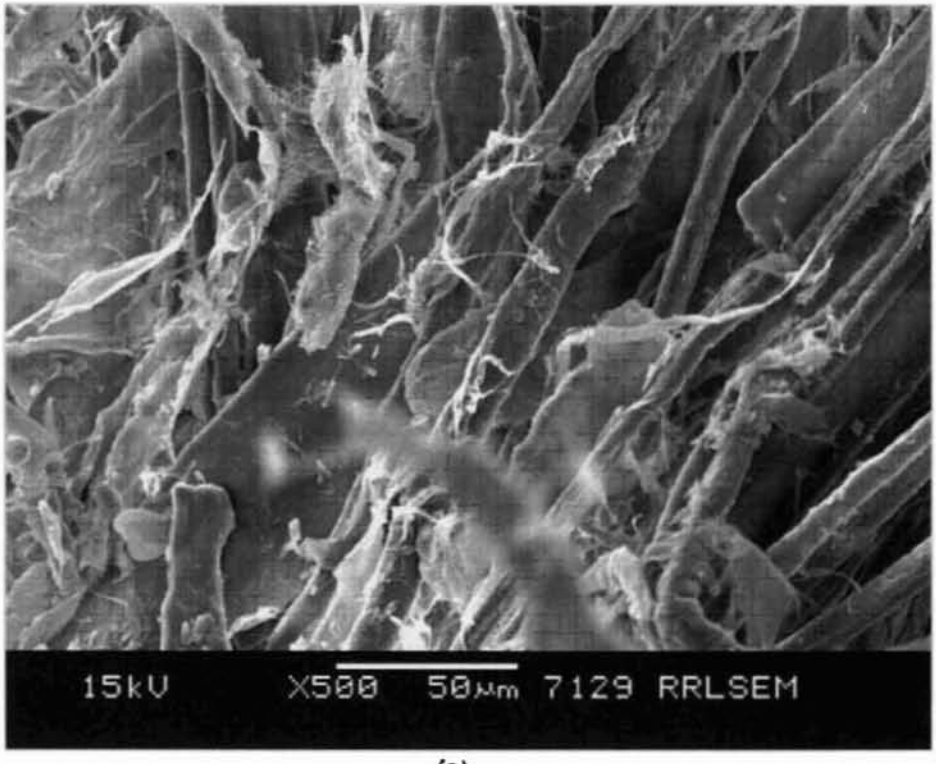


(c)

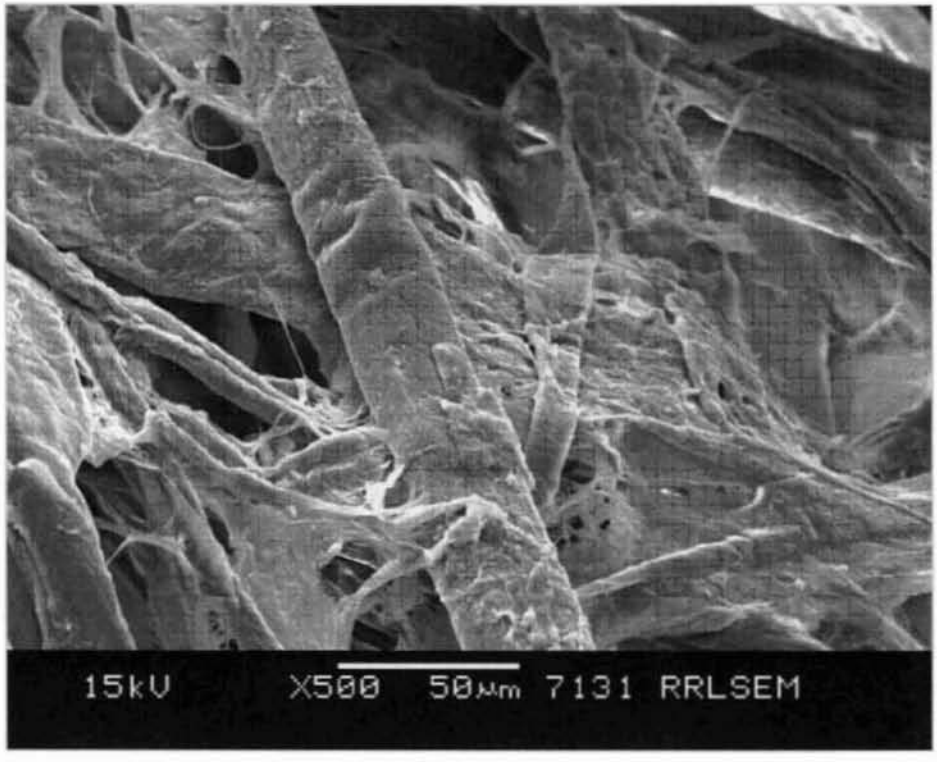


(d)

Fig. 5. 2. (c). Micrograph of Chemical pulp untreated. (d). treated with xylanase after chlorination alkali extraction.

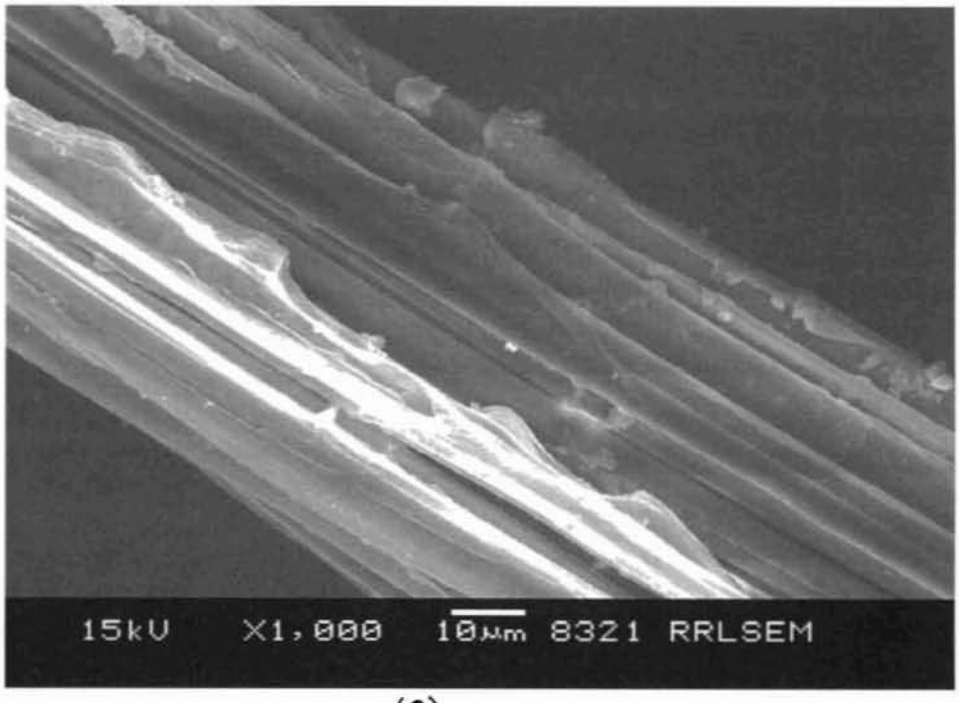


(a)

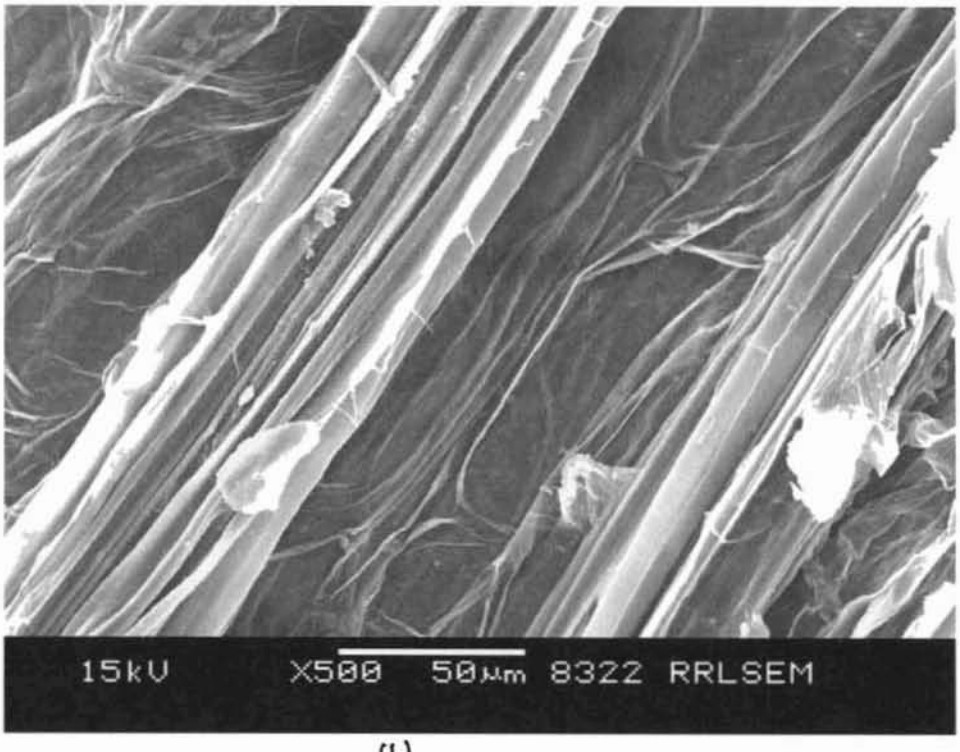


(b)

Fig. 5. 3. (a). SEM of waste paper untreated. (b). treated with xylanase



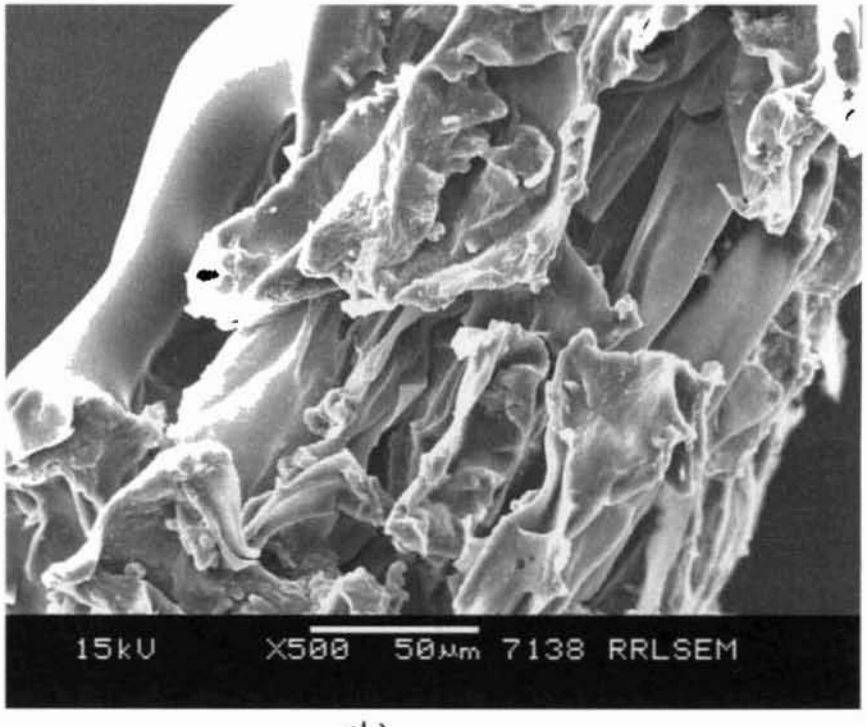
(a)



(b)

Fig. 5. 4. (a). SEM of plantain fiber untreated (control) (b). treated with crude xylanase

(a)



(b)

Fig. 5. 5. (a). SEM of coconut fiber untreated (control). (b). treated with crude xylanase for three hour.

Chapter. 6.

Summary and Conclusion

The thesis illustrates the experiments and observation related to xylanase production by *Bacillus pumilus* using Solid-State Fermentation and its application particularly in paper pulp processing.

6.1.0. SUMMARY

- The nine bacterial cultures from RRL (TVM) culture collection were spot-inoculated on xylan agar plates (pH - 7 and 10) and based on the clear zone produced at 24 hour of incubation six cultures were selected.
- The six xylanolytic bacterial strains were subjected to quantitatively screened for xylanase production in liquid medium and based on the xylanase titer *Bacillus* – 34 was selected for further studies
- Based on the morphological as well as biochemical characteristic and fatty acid profile the strain was identified as *Bacillus pumilus* -GC Sub Group- B (I. M. Tech, Chandigar).
- Xylanase production was investigated using different carbon sources in different mode of fermentation- SmF and SSF.
- SSF has shown 58- fold increase in production related to SmF.
- Wheat bran was observed as an important substrate for the production of xylanase by SSF.
- *Bacillus pumilus* was highly alkalophilic and thermo tolerant.
- High levels of xylanase, with poor cellulolytic activity.
- Highest production has been resulted with a particle size of 0.5 mm, with initial pH - 9 and moisture level in the ratio 1: 2.5 (70 %) with inoculum size of 10 v/w at 35°C with in 72 hour of incubation.
- Glycerol in low concentration has enhanced xylanase production related to control
- Xylan has an inducing effect on xylanase production with wheat bran where as xylose and glucose totally inhibits xylanase production in high concentration.
- Na_2HPO_4, K_2HPO_4, and KH_2PO_4 in low concentration have enhancing effect on xylanase production by *B. pumilus*.

- Sodium nitrate (0.01 g) as nitrogen source enhanced xylanase production where as Ammonium sulphate has inhibited xylanase production by *B. pumilus* in wheat bran medium.
- Presences of organic nitrogen like malt extract has shown inhibitory effect in high concentration where as all other nitrogen sources were individually effective in enhancing xylanase activity in SSF.
- Presence of NaCl, $MnSO_4$, $MgSO_4$ and KCl individually, has relatively enhanced xylanase activity in wheat bran medium.
- Na_2CO_3 at a concentration of 3.5 % (pH- 9.8) has shown high influence on xylanase production than any other nutritional sources and as concentration increased there was an observable drop in production. Where as with 10 % total inhibition of xylanase production was observed.
- Moisturizing agent analyzed, simple medium incorporated with Na_2CO_3 has resulted in maximum xylanase activity, which was ~4.6 fold increase than the unoptimized condition.
- Xylanase production with simple moisturizing agent with out addition of Na_2CO_3 has resulted in ~37482 +/- 1000 U/gds in enamel tray, ~34698+/- 1000 U/gds in tank fermentor, which was ~1.78 fold higher than that of culture flasks (~21034 +/- 1000 U/gds).
- Extraction was effective using phosphate buffer (0.1 M)
- Extraction in buffer improves stability as well as effective storage in deep freezer or drying the bactobran also help in the storage of xylanase with out the addition of any preservatives for more than one month.
- Distinctive purification procedure was followed for purification of xylanase from *B. pumilus* extracted from wheat bran SSF medium.
- Ultra filtration followed by ammonium sulphate precipitation and Ion exchange chromatography was done. Only DEAE – Sepharose elution was subjected which has resulted in three purified fraction with different salt gradient.
- Isoelectric focusing of the partially purified fraction was done.
- SDS and Native –PAGE analysis of the crude, partially purified, and xylanase was carried out, which shows that there are three isoforms of xylanase with

different molecular weight (major and minor fraction). The major xylanase with ~ 14 kDa has been purified by PIF, which has shown a pI value ranging from 8 - 9.0.

- The partially purified xylanase was active at 50°C and stable upto 40°C for more than 2 hour. The enzyme was optimally active at a range of 6 to 7.5 and stable at a range of 6 to 9 pH. The presence of acetone has inhibitory effect on xylanase activity. Manganese have enhancing effect on xylanase where as SDS has inhibitory effect.
- The purified fraction also has shown optimum activity at 50°C and stability upto 40°C, activity pH ranging from 6 to 7 and stability at for 0.01 M fraction and 1.0 M fraction and 6 - 8 for 0.5 M fraction. SDS, Hg^{2+} and Fe^{2+} have total inhibitory effect in purified fraction, where as Mg^{2+}, Mn^{2+} and Co^{2+} have enhanced xylanase activity.
- The Michaelis –Menten constant (K_m) for oatspelt xylan for fraction obtained by 0.01 M NaCl was observed as 4.0 mg / mL and V_{max} as 5000 μmol / min / mg protein and that of fraction obtained by 0.5 M NaCl as K_m -3.5 mg / mL and V_{max} 3448 μmol / min / mg of protein.
- As a prebleaching agent in Chemi - Mechanical pulp has resulted marginal increase of 2 units in final brightness.
- In Chemical pulp (kraft pulp) the significance of this crude xylanase was that it help in the reduction in Chlorine usage, making the effluent elemental chlorine free (ECF) as well as there was an increase in ISO % brightness by 8.5 unit related to control.
- The SEM analysis clearly indicated that the use of this xylanase was highly effective in paper pulp bleaching, recycling of waste paper as well as in plant fiber separation.
- Industrial applications involving this xylanase, especially after modification of its properties by protein engineering, could bring interesting results, which requires further research.

6.2.0. CONCLUSION

From the present studies it is clear that *Bacillus pumilus* xylanase is having the characteristic suited for an industrial enzyme (xylanases that are active and stable at elevated temperatures and alkaline pH are needed). SSF production of xylanases and its application appears to be an innovative technology where the fermented substrate is the enzyme source that is used directly in the bleaching process without a prior downstream processing. The direct use of SSF enzymes in bleaching is a relatively new biobleaching approach. This can certainly benefit the bleaching process to lower the xylanase production costs and improve the economics and viability of the biobleaching technology. The application of enzymes to the bleaching process has been considered as an environmentally friendly approach that can reduce the negative impact on the environment exerted by the use of chlorine-based bleaching agents. It has been demonstrated that pretreatment of kraft pulp with xylanase prior to bleaching (biobleaching) can facilitate subsequent removal of lignin by bleaching chemicals, thereby, reducing the demand for elemental chlorine or improving final paper brightness. Using this xylanase pre-treatment, has resulted in an increased of brightness (8.5 Unit) when compared to non-enzymatic treated bleached pulp prepared using identical conditions. Reduction of the consumption of active chlorine can be achieved which results in a decrease in the toxicity, colour, chloride and absorbable organic halogen (AOX) levels of bleaching effluents. The xylanase treatment improves drainage, strength properties and the fragility of pulps, and also increases the brightness of pulps. This positive result shows that enzyme pre-treatment facilitates the removal of chromophore fragments of pulp there by making the process more environment friendly.

BIBLIOGRAPHY

Abdel - Sater, M. A. El-Said, A. H. (2001). Xylan-decomposing fungi and xylanolytic activity in agricultural and industrial wastes. Int. Biodeter. Biodeg. 47: 15 - 21.

Ai, Z. Jiang Z. Li, L. Deng, W. Kusakabe, I. and Li, H. (2005). Immobilization of *Streptomyces olivaceoviridis* E - 86 xylanase on Eudragit S-100 for xylo-oligosaccharide production. Proc. Biochem. 40: 2707 - 2714.

Akila, G. and Chandra, T. S. (2003). A novel cold - tolerant *Clostridium* strain PXYL1 isolated from a psychrophilic cattle manure digester that secretes thermolabile xylanase and cellulase. FEMS Microbiol. Lett. 219: 63 - 67.

Almeida, E. M. de, M. de L. T. M. Polizeli, H. F. Terenzi, and J. A. Jorge. (1995). Purification and biochemical characterization of β-xylosidase from *Humicola grisea* var. *thermoidea*. FEMS Microbiol. Lett. 130: 171 - 176.

Alzari, P. M. Souchon, H. and Dominguez, R. (1996). The crystal structure of endoglucanase CelA, a family 8 - glycosyl hydrolase from *Clostridium thermocellum* Structure, 4: 265 - 275.

Anand, L. S. Krishnamurthy, and P. J. Vithayathil. (1990). Purification and properties of xylanase from the thermophilic fungus, *Humicola lanuginose* (Griffon and Maublanc) Bunce. Arch. Biochem. Biophys. 276: 546 - 553.

Andrade, C. M. M. C. Pereira, N. and Antranikian, G. (1999). Extremely thermophilic microorganisms and their polymer - hydrolytic enzymes. Rev. Microbiol. 30: 287 - 298.

Anna, T. Hadar, F. Rotem, G. Aviva, L. Adra, M. Vivian, S. Capel, M. Shoham, Y. and Shoham, G. (1997). Crystallization and preliminary X - ray analysis of the thermostable alkaline xylanase from *Bacillus stearothermophilus*. Acta. Crystallogr, D. 53: 608 - 611.

Annis, S. L. and Goodwin, P. H. (1997). Recent advances in the molecular genetics of plant cell wall - degrading enzymes produced by plant pathogenic fungi. Eur. J. Plant Pathology, 103: 1 - 14.

Antranikian, G. Zablowski, P. and Gottschalk, G. (1987). Conditions for the overproduction and extraction of thermostable a - amylase and pullalanase from *Clostridium thermohydrosulfuricum.* DSM - 567. Appl. Microbiol and Biotechnol. 27: 75 - 81.

Apel-Birkhold, P. C. and Walton, J. D. (1996). Cloning, disruption and expression of endo - β, 1, 4 - xylanase genes, *XYL2* and *XYL3* from the maize pathogen *Cochliobolus carbonum.* Appl. Envt. Microbiol. 62: 4129- 4135.

Ara, K. Igarashi, K. Hagihara, H. Sawada, K. Kobayashi, T. and Ito. S. (1996). Separation of functional domains for the α - 1, 4 and α - 1, 6 hydrolytic activities of a *Bacillus* amylopullulanase by limited proteolysis with papain. Biosci. Biotechnol. Biochem. 60: 634 - 639.

Archana, S. Sathyanarayana, T. (1997). Cellulase - free xylanase production by thermophilic *Bacillus liformis* A99. Ind. J. Microbiol. 38: 135 - 139.

Archana, S. Sathyanarayana, T. (1997). Xylanase production by thermophilic *Bacillus licheniformis* A99 in Solid - state fermentation. Enz. Microbial. Tech. 21: 12 - 17.

Argos, P. Rossman, M. G. Gra, U. M. Zuber, H. Frank, G. and Tratschin, J. D. (1979). Thermal stability and protein structure. Biochem. 18: 5698 - 5703.

Asha, P. C. and Prema, P. Production of cellulase - free endoxylanase from novel alkalophilic thermotolerent *Bacillus pumilus* by Solid - state fermentation and its application in wastepaper recycling, Biores. Technol. (in Press).

Asymeric, J. L. Guiseppi, A. Pascal, M. C, Chippaux, M. (1988). Mapping and regulation of the *cel* genes in *Erwinia chrysanthemi.* Mol. Gen. Genet. 211 : 95 - 101.

Aunstrup, K. (1974). In Industrial aspects of biocehemistry (B. Spencer, Ed.) Federation of European Biochemical Socities Symposium. Pp. 23 Elsevier, Amsterdam.

Babu, K. R. Satyanarayana, T. (1996). Production of bacterial enzymes by Solid - state fermentation. J. Sci. and Ind Res. 55: 464 - 467.

Bader J. (1993). Modelling and simulation of the growth and enzyme production of *Trichoderma reesei* Rut C30. J. Biotechnol. 29: 121 - 135.

Bailey, M. J. Buchert, J. and Vikari, L. (1993). Effect of pH on production of xylanase by Trichoderma reesi on xylan - and cellulose - based. Appl. Microbiol. Biotechnol. 40: (2 - 3), 224 - 229.

Bailey, M. J. Biely, P. Poutanen, K. (1992). Interlaboratory testing of methods for assay of xylanase activity. J. Biotechnol. 23: 257 - 270.

Bajapi, P. Bhardwaj, N. K. Maheshwari, S. and Bajpai, P. K. (1993). Use of xylanase in bleaching of eucalyptus kraft pulp. Appita. 46: (4), 274.

Bajpai, P. (1997). Microbial Xylanolytic enzyme system: properties and applications. Adv in Appl. Microbial. 43: 141 - 194.

Bajpai, P. (1999). Application of enzymes in the pulp and paper industry. Biotechnol. Progr. 15: 147 - 157.

Bajpai, P. Bhardwaj, N. K. Bajpai, P. K. Jauhari, M. B. (1994). The impact of xylanases on bleaching of eucalyptus kraft pulp. J. Biotechnol. 38: 1 - 6.

Bakir, U. Yavascaoglu, S. Guvenc, F. and Ersayin, A. (2001). An endo - β - 1, 4 - xylanase from *Rhizopus oryzae*: production, partial purification and biochemical characterization. Enz and Microbial Technolo. 29: 328 - 334.

Balakrishnan, H. Dutta - Choudhary, M. Srinivasan, M. C. and Rele, M. V. (1992). Cellulase - free xylanase production from an alkalophilic *Bacillus* sp. World. J. Microbiol. Biotechnol. Letts. 19: (7). 599 - 601.

Ball, A. S. and McCarthy, A. J. (1988). Saccharification of straw by actinomycete enzymes. J. Gen. Microbiol. 134: 2139 - 2147.

Bastawade, K. B. (1992). Xylan structure, microbial xylanases, and their mode of action. World J. Microbiol. Biotechnol. 8: 353 - 368.

Bastawde, K. B. Puntambekar, U. S. and Gokhale. D. V. (1993). Optimisation of cellulsae – free production by a novel yeast strain. J. Ind. Microbiol. 13: 220- 224.

Bastawde, K. B. Tabatabia, L. B. Meagher, M. M. Srinivasan, M. C. Vartak, H. G. Rele, M. V. and Reilly, P. J. (1991). Catalytic properties and partial

amino acid sequencing of an actinomycete endoxylanase from *Chainia* sp. American Chemical Society Symposium Series. 460: 417 - 425.

Bataillon, M. Nunes, A. P. Cardinali, A. P. Castillon, N. and Duchiron, F. (2000). Purification and characterization of a moderately thermostable xylanase from *Bacillus* sp. strain SPS - 0, Enz. Microbiol. Technol. 26: 187 - 192.

Bedford M. R, Schulze H. (1998). Exogenous enzymes for pigs and poultry. Nutr Res Rev. 11: 91 - 114.

Beg, Q. K, Kapoor, M. Mahajan, L. Hoondal, G. S. (2001). Microbial xylanase and their industrial application: a review. Appl. Microbiol. Biotechnol. 56: 326 - 338.

Beg, Q. K. Bhushan, B. Kapoor, M. Hoondal, G. S. (2000). Enhanced production of a thermostable xylanase from *Streptomyces* sp. QG - 11 - 3 and its application in biobleaching of eucalyptus kraft pulp. Enzyme Microb. Technol. 27: 459 - 466.

Belancic, A. Scarpa, J. Peirano, A. Diaz, R. Steiner, J. and Eyzaguirre, J. (1995). *Penicillium purpurogenum* produces several xylanases: purification and properties of two of the enzymes. J. Biotechnol. 41: 71 - 79.

Beldman, G. Osuga, D. and Whitaker, J. R. (1996). Some Characteristics of β - D - xylopyranosidases, α - L - arabinofuranosidases and an arabinoxylan α - L - arabino furanohydrolase from wheat bran and germinated wheat. J. Cereal Sci. 23: 169 - 180.

Belien, T. Campenhout, S. V. Acker M. V. and Volckaert, G. (2005). Cloning and characterization of two endoxylanases from the cereal phytopathogen *Fusarium graminearum* and their inhibition profile against endoxylanase inhibitors from wheat. Biochem. Biophy. Res. Comm. 327: 407 - 414.

Bennett, N.A. Ryan, J. Biely, P. Vrsanska, M. Kremnicky, L. Macris, B.J. Kekos, D. Christakopoulos, P. Katapodis, P. Claeyssens, M. Nerinckx, W. Ntauma, P. Bhat, M.K. (1998). Biochemical and catalytic properties of an endoxylanase purified from the culture filtrate of *Thermomyces lanuginosus* ATCC 46882. Carbohydr. Res. 306: 445 - 455.

Bergquist, P. L. Gibbs, M. D. Morris, D. D. Thompson, D. R. Uhl, A. M. and Daniel, R. M. (2001). Hyperthermophilic xylanases. Methods Enzymol. 330: 301 - 319.

Bernier, R. Jr. Driguez, H. and Desrochers, M. (1983). Molecular cloning of a *Bacillus subtilis* xylanase gene in *Escherichia coli*. Gene 26: 59 - 65.

Bhat, M. K. (2000) Cellulases and related enzymes in biotechnology. Biotech. Adv. 18: 355 - 383.

Bhat, M. K. and S. Bhat. (1998). *Clostridium thermocellum* cellulosome: dissociation, isolation and characterisation of subunits and the potential biotechnological implications. Recent Res. Dev. Biotechnol. Bioeng. 1: 59 - 84.

Biely, P. (2003). Diversity of microbial endo - β - 1, 4 -xylanases In: Applications of Enzymes to Lignocellulosics (Mans. eld, S. D. and Saddler, J. N. Eds.), American chemical Society, Washington. pp. 361 - 380.

Biely, P. Kluepfel, D. Morosoli, R. and Shareck, F. (1993) Mode of action of three endo - beta - 1, 4 - xylanases of *Streptomyces lividans*. Biochim. Biophys. Acta. 1162: 246 - 254.

Biely, P. M. Vrsanska, and M. Claeyssens. (1991). The endo- 1, 4-beta -glucanase I from *Trichoderma reesei* - action on beta - 1, 4 - oligomers and polymers derived from D - glucose and D - xylose. Eur. J. Biochem. 200: 157 - 163.

Biely, P. Markovic, O. and Mislovicova, D. (1985). Sensitive detection of endo - 1, 4 -beta -glucanases and endo- 1, 4 -beta -xylanases in gels. Anal. Biochem. 144: 147 - 511.

Biely, P. (1985). Microbial xylanolytic systems. Trends Biotechnol. 3: 286 - 290.

Biely, P. McKenzie, C. R. Puls, J. Schneider, H. (1986). Co-operativity of esterases and xylanases in the enzymatic degradation of acetyl xylan. Biotechnology. 4: 731 - 733.

Biely, P. Vrsanskh, M. Tenkanen, M. and Kluepfel, D. (1997). Endo - β - 1, 4 - xylanase families: differences in catalytic properties. J. Biotechnol. 57: 151 - 166.

Biospectrum (2005). BioIndustrial makes silent progress - Segment analysis, biospectrumindia. com. A CyberMedia Publication. 136 - 137.

Bissoon, S. Christov, L. and Singh, S. (2002). Bleach boosting effects of purified xylanase from *Thermomyces lanuginosus* SSBP on bagasse pulp. Proc. Biochem. 37: 567 - 572.

Black, G. W. Rixon, J. E. Clarke, J. H. Hazlewood, G. P. Ferreira, L. M. Bolam, D. N. and Gilbert, H. J. (1997). Cellulose binding domains and linker sequences potentiate the activity of hemicellulases against complex substrates. J. Biotechnol. 57: 59 - 69.

Black, G. W. Rixon, J. E. Clarke, J. H. Hazlewood, G. P. Theodorou, M. K. Morris, P. and Gilbert, H. J. (1996). Evidence that linker sequences and cellulose - binding domains enhance the activity of hemicellulases against complex substrates. Biochem. J. 319: (Pt 2), 515 - 520.

Blanco, A. T. Vidal, J. F. Colom, and F. I. J. Pastor. (1995). Purification and properties of xylanase A from alkali - tolerant *Bacillus* sp. strain BP- 23. Appl. Environ. Microbiol. 61: 4468 - 4470.

Blum, L. (1996). The Production of Bleached Kraft Pulp and Kraft Pulp Production, Envt. Defence Fund (available online).

Bocchini, D. A. Oliveira, O. M. M. F. Gomes, E. and da Silva, R. (2005). Use of sugarcane bagasse and grass hydrolysates as carbon sources for xylanase production by *Bacillus circulans* D1 in submerged fermentation. Proc. Biochem.

Boren, S. Bolhuis, A. Tjalsma, H. Holsappel, S. Venema, G. and van Dijil, J. M. (1998). Protein secretion and possible roles for multiple signal peptidases for precursor processing in *Bacilli*. J. Biotechnol. 64: 3 - 13.

Borght, A. V. D. Goesaert, H. Veraverbeke, W. S. and Delcour, J. A. (2005). Fractionation of wheat and wheat flour into starch and gluten: overview of the main processes and the factors involved. J. Cereal Sci. 41: 221 - 237.

Bourne, Y. and Henrissat, B. (2001). Glycoside hydrolases and glycosyl transferases: families and functional modules. Curr. Opin. Struct. Biol. 11: 593 - 600.

Bradner, J. R. Sidhu, R. K. Gillings, M. and Nevalainen, K. M. (1999). Hemicellulase activity of antarctic microfungi. J. Appl. Microbiol. 87: 366 - 370.

Bragger, J. M. Daniel, R. M. Coolbear, T. and Morgan, H. W. (1989). Very stable enzymes from extremely thermophilic archae bacteria and eubacteria. Appl. Environ. Microbiol. 31: 556 - 561.

Braun, E. J. and Rodrigues, C. A. (1993). Purification and properties of an endoxylanase from a corn stalk rot strain of *Erwinia chrysanthemi.* Phytopathology 83: 332 - 338.

Bray, M. R. and A. J. Clarke. (1990). Essential carboxy groups in xylanase A. Biochem. J. 270: 91 - 96.

Bray, M. R. and Clarke, A. J. (1992). Action pattern of xylooligosaccharide hydrolysis by *Schizophyllum commune* xylanase A. Eur. J. Biochem. 204: 191 - 196.

Breccia, J. D. Sifieriz, F. Baigori, M. D. Castro, G. R. and Hatti - Kaul, R. (1998). Purification and characterization of a thermostable xylanase from *Bacillus amyloliquefaciens.* Enz. Microbial Technol. 22: 42 - 49.

Brett, C. and Waldron, K. (1996). Physiology and biochemistry of plant cell walls, 2nd Ed. Champman and Hall, New York.

Buchert, J. Tamminen, T. and Viikari, L. (1997). Impact of the Donnan effect on the action of xylanases on fiber substrates. J. Biotechnol. 57: 3217 - 22.

Cai, J. M. Z. Jie, H. Xiagang and R. Ruipen, (1997). Production of xylanase by *Aspergillus niger* A3 using Solid - state fermentation. Quonguo Gongye Weishengwu Keji Qingbaozhan, 27: 1 - 4.

Campbell, R. L. Rose, D. R. Wakarchuk, W. W. To, R. J. Sung, Z. and Yagushi, M. (1993). High - resolution structures of xylanases from *Bacillus circulans* and *Trichoderma harzianum* identify a new folding pattern and implications for the atomic basis of the catalysis. Foundation for biotechnical and industrial fermentation research In: *Trichoderma reesei* Cellulases and Other Hydrolases (Souminen, P. and Reikainen, T. Eds.), pp. 63 - 72. Espoo, Finland.

Campin, D. N. Buckland, S. J. Hannah, D. J. and Taucher, J. A. (1991). Water. Sci. Technol. 24: 65 - 74.

Canals, A. Vega, M. C. Gomis - Ruth, F. X. Diaz, M. Santamaria, R. R. and Coll, M. (2003). Structure of xylanase Xys1 delta from *Streptomyces halstedii*. Acta Crystallogr. D: Biol. Crystallogr. 59: 1447 - 1453.

Cannio, R. Di Prizito, N. Rossi, M. and Morana, A. (2004). A xylan - degrading strain of *Sulfolobus solfataricus*: isolation and characterization of the xylanase activity. Extremophiles. 8: 117 - 124.

Cardoso, O. A. V. and Filho, E. X. F. (2003). Purification and characterization of a novel *Acrophialophora nainiara*. FEMS Microbiol. Lett. 223, 309 - 314.

Carmona, E. C. Fialho, M. B. Buchgnani, E. B. Coelho, C. D. Brocheto - Braga, M. R. and Jorge, J. A. (2005). Production, purification and characterization of a minor form of xylanase from *Aspergillus versicolor* Proc. Biochem. 40: 359 - 364.

Carpita, N. C. (1983). Hemicellulose polymers of the cell walls of *Zea coleoptiles*. Plant Physiology. 75: 515 - 521.

Castilho L. R. Medronho R. A. Alves T. L. M. (2000). Production and extraction of pectinase obtained by Solid - state fermentation of agro - industrial residues with *Aspergillus niger*. Biores. Technol. 71 : 45 - 50.

Cesar T, and Mrsa V. (1996). Purification and properties of the xylanase produced by *Thermomyces lanuginosus*. Enz. Microb. Technol. 19: 289 - 296.

Chadha, B, S. Ajay, B. K. Mellon, F. Bhat, M. K. (2004). Two endoxylanases active and stable at alkaline pH from the newly isolated thermophilic fungus, *Myceliophthora* sp. IMI 387099. J. Biotechnol. 109: 227 - 237.

Chand, S. and Mishra, P. (2003). Research and application of microbial enzymes - India's contribution. Adv Biochem Engin/ Biotechnol 85: 95 - 124.

Chaudhuri, A. Bharadwaj, G. and Maheshwari, R. (1999). An unusual pattern of invertase activity development in the thermophilic fungus *Thermomyces lanuginosus*. FEMS Microbiol. Lett. 177: 39 - 45.

Chauthaiwale, J. and Rao, M. (1993). Specific interaction of guanidine hydrochoride with essential carboxyl group of xylanase from alkalo

thermophilic *Bacillus* sp. Biochem. Biophys. Res. Commun. 191: 922-927.

Chauvaux, S. Beguin, P. and Aubert, J. P. (1992). Site directed mutagenisis of essential carboxyl residues in *Clostridium thermocellum* endoglucanase cel D. J. Biol. Chem. 26: 4472 - 4478.

Chen, J. C. Yang, G. H. Li, Z. C. Wang, B. M. Qu, Y. B. Gao, P. J. (1997). Improvement of bleached wheat straw pulp with xylanases. China Pulp and Paper 16 (6): 31 - 35 (in Chinese).

Chen, C. Chen, J. L. and Lin, T. Y. (1997). Purification and characterization of a xylanase from *Trichoderma longibrachiatum* for xylooligosaccharide production. Enz. Microbiol. Technol. 21: 91- 96.

Chen, Y. L. Tang, T. Y. and Cheng, K. J. (2001). Directed evolution to produce an alkalophilic variant from a *Neocallimastix patriciarum* xylanase. Can. J. Microbiol. 47: 1088 - 1094.

Cherry J. R. and Fidantsef, A. L. (2003). Directed evolution of industrial enzymes: an update. Curr. Opin. Biotech. 14: 438 - 443.

Chistov, L. P. and Prior, B. A. (1994). Enzymatic prebleaching of sulphite pulps. Appl. Microbial. Biotechnol. 42: 492 - 498.

Chivero, E. T. Mutukumira, A. N, and Zvauya, R. (2001). Partial purification and characterization of a xylanase enzyme produced by a microorganism isolated from selected indigenous fruits of Zimbabwe. Food Chemistry. 72: 179 - 185.

Cho, K. K. Kim, S. C. Woo, J. H. Bok, J. D. and Choi, Y. J. (2000). Molecular cloning and expression of a novel family A endoglucanase gene from *Fibrobacter succinogenes* S85 in *E. coli*. Enz. Microb. Technol. 27: 475 - 481.

Christakopoulos, P. Katapodis, P. Kalogeris, E. Kekos, D. Macris, B. J. Stamatis, H. and Skaltsa, H. (2003). Antimicrobial activity of acidic xylo-oligosaccharides produced by family 10 and 11 endoxylanases. Int. J. Biol. Macromol. 31: 171 - 175.

Christakopoulos, P. Nerinckx, W. Kekos, D. Macris, B. and Claeyssens, M. (1996). Purification and characterization of two low molecular mass alkaline xylanases from *Fusarium oxysporum* F3. J. Biotechnol. 51: 181 - 189.

Christopher, L. Bissoon, S. Singh, S. Szendefy, J. and Szakacs, G. (2005). Bleach - enhancing abilities of *Thermomyces lanuginosus* xylanases produced by Solid - state fermentation. Process Biochemistry 40: 3230 - 3235.

Christov, L. P. and Prior, B. A. (1993). Xylan removal from dissolving pulp - using enzymes of *Aureobasidium pullulans.* Biotechnol. Lett. 15: 1269 - 1274.

Christov, L. P. Akhtar, M. and Prior, B. A. (1996). Impact of Xylanase and Fungal Pretreatment on alkali Solubility state and Brightness of Dissolving Pulp. Holzforschung, 50: 579 - 582.

Christov, L. P. Akhtar, M. and Prior, B. A. (1998). The potential of biosulfite pulping in dissolving pulp production. Enz. Microbial. Technol. 23: 70 - 74.

Christov, L. P. Szakacs, G. Balakrishnan, H. (1999). Production, partial characterization and use of fungal cellulase - free xylanases in pulp bleaching. Proc. Biochem. 34: (5), 511 - 517.

Chudhary, P and Deobagkar, D (1997). Purification and Characterisation of xylanse from *Cellulomonas* sp. N. C. I. M 2353. Biotechnol. Appl. Biochem. 25: 127 - 133.

Claeyssens, M. and Henrissat, B. (1992). Specificity mapping of cellulolytic enzymes: classification into families of structurally related proteins confirmed by biochemical analysis. Protein Sci. 1: 1293 - 1297.

Clarkson, K, Siika - Aho, M, Tenkanen, M, Bower, B. S, Penttilä, M. E, Saloheimo M. L. A. (2001). *Trichoderma reesei* xylanase. Patent WO 0149859.

Claus, D. and Berkeley, R. C. W. (1986). *Bacillus.* In: Bergey's Manual of Systematic Bacteriology Vol. 2 (Sneath, P. H. A. Ed.). Williams and Wilkins, Baltimore. 1105 - I 138

Cleemput, G. Hessing, M. van Oort, M. Deconynck, M. and Delcour, J. A. (1997). Purification and characterization of a β - D - xylosidase and an endo - xylanase from wheat *(Triticum aestivum)* flour. Plant Physiology 113: 77 - 386.

Collins, T. Gerday, C. Feller, G. (2005). Xylanase, xylanase families and extremophilic xylanase. FEMS Microbial. Rev. 29: 3 - 23.

Collins, T. Meuwis, M. A. Gerday, C. and Feller, G. (2003). Activity, stability and flexibility in glycosidases adapted to extreme thermal environments. J. Mol. Biol. 328: 419 - 428.

Collins, T. Meuwis, M. A. Stals, I. Claeyssens, M. Feller, G. and Gerday, C. (2002). A novel family 8 xylanase: functional and physico - chemical characterization. J. Biol. Chem. 277: 35133 - 35139.

Comtat, J. (1983). Isolation, Properties and postulated role of some of the xylanase from the Basidiomycete *Sporotrichum dimorphosporum.* Carbohy. Res. 8: 215 - 231.

Connerton, I. Cummings, N. Harris, G. W. Debeire, P. and Breton, C. (1999). A single domain thermophilic xylanase can bind insoluble xylan: evidence for surface aromatic clusters. Biochim. Biophys. Acta. 1433: 110 - 121.

Conrad, D. and Noethen, W. (1984). Hydrolysis of cyclodextrins (DP 2 - 6) by xylanolytic enzymes from *Aspergillus niger.* Proc. 3rd Eur. Cong. Biotechnol. 2, Munchen, Verlag Chemie, Germany, pp. 169 - 177.

Cordeiro, C. A. M. Martins, M. L. L. Luciano, A. B. and da - Silva, R. F. (2002). Production and properties of xylanase from *Bacillus* sp. Brazilian Archives of Biology and Technology. 45: 413 - 418.

Coughlan, M. P. (1992). Towards an understanding of the mechanism of action of main chain - hydrolyzing xylanases. In: Visser, J. Beldman, G. Kustersvan Someren, M. A. Voragen, A. G. J. (eds). Xylans and Xylanases. Elsevier Sci. Publ. Amsterdam, p. 111 - 139.

Coughlan, M. P. Hazlewood, G. P. (1997). β - 1, 4 - D - xylan degrading enzyme systems: biochemistry molecular biology and applications. Biotechnol. Appl. Biochem. 17: 259 - 289.

Courtin C. M, Roelants A, Delcour J. A. (1999). Fractionation reconstitution experiments provide insight into the role of endoxylanases in bread - making. J. Agric. Food. Chem. 47: 1870 - 7.

Courtin, C. W. and Delcour, J. A. (2002). Arabinoxylans and endoxylanases in wheat flour bread - making. J. Cereal Sci. 35: 225 - 243.

Courtin, C. W. Gelders, G. G. Delcour, J. A. (2001). Use of two endoxylanases with different substrate selectivity for understanding arabinoxylan functionality in wheat flour breadmaking. J. Cereal Chem. 78: 564 - 571.

Coutinho, P. M. and Henrissat, B. (1999). Carbohydrate - active enzyme server (CAZY) at URL:http://afmb. cnrs - mrs. fr/~cazy/ CAZY/.

Crepin VF, Faulds CB, Connerton IF. (2004). Functional classification of the microbial feruloyl esterases. Appl Microbiol Biotechnol. 63: 647 - 52.

Dahlberg, L. H. and Kristijansson, J. K. (1993). Thermostable xylanolytic enzymes from *Rhodothermus marinus* grown on xylan. Appl. Microbiol. Biotechnol. 40: 63 - 38.

Damaso, M. C. T. Rasse, T. M. Sant'Anna Jr, G. L. Andrade, C. M. M. C. and Pereira Jr, N. (2000). Properties of the cellulase - free xylanase produced by the thermophilic strain *Thermomyces lanuginosus* IOC - 4145 in laboratory scale. XIX - International Congress of Chemical Engineering. 1 Brazillian Congress of Phase equilibrium and fluid properties for Chemical Process Design.

de - Vries, R. P. and Visser, J. (2001). *Aspergillus* enzymes involved in degradation of plant Cell wall polysaccharides. Microbiol. Mole. Biol. Reviews, vol. 65: (4), 497 - 522.

De Vries, R. P. Kester, H. C. M. Poulsen, C. H. Benen, G. A. E. and Visser, J. (2000). Synergy between enzymes from *Aspergillus* involved in the degradation of plant cell wall polysaccharides. Carbohy. Res. 327: 401 - 410.

Debeire, P. Priem, B. Strecker, G. and Vignon, M. (1990). Purification and properties of an endo - 1, 4 - xylanase excreted by hydrolytic thermophilic anaerobe *Clostridium thermolacticum*. Eur. J. Biochem. 187: 573 - 580.

Degrassi, G. Kojic, M. Ljubijankic, G. and Venturi, V. (2000). The acetyl xylan esterase of *Bacillus pumilus* belongs to a family of esterases with broad substrate specificity. Microbiol. 146: 1585 - 1591.

Dekker R. F. H. (1985). Bidegradation of hemicellulose. In: Biosynthesis and Biodegradation of wood componenets. Ed. T. Higuchi pp. 505-533 Academic Press Orland, USA.

Dekker, R. F. H. and Richards, G. N. (1975). Hemicellulases IV. Structure of the oligosaccharides from the enzymic hydrolysis of hemicellulose by a hemicellulase of *Ceratocystis paradoxa*. Carbohydr. Res. 43: 335 - 344.

Dekker. R. F. H. Richards, G. N. (1976). Hemicellulases: their occurrence, purification, properties and mode of action. Adv. Carbohydr. Chem. Biochem. 32: 277 - 352.

Demain, A. C. R. (1972). Influnce of environment on the control of enzyme synthesis. J. Appl. Chem. Biotechnol. 22: 245 - 259.

Derewenda, U. Swenson, L. Green, R. Wei, Y. Morosoli, R. Shareck, F. Kluepfel, D. and Derewenda, Z. S. (1994). Crystal structure at 2. 6 - A ¢^{a} resolution, of the *Streptomyces lividans* xylanase A, a member of the F family of β - 1,4 - D - glycanases. J. Biol. Chem. 269: 20811 - 20814.

Dey, D. J. Hinge, A. Shendye, and M. Rao. (1992). Purification and properties of extracellular endoxylanases from alkalophilic thermophilic *Bacillus* sp. Can. J. Microbiol. 38: 436 - 442.

Dhillon, A. Gupta, J. K. Jauhari, B. M. and Khanna, S. (2000). A cellulase - poor, thermostable, alkalitolerant xylanase produced by *Bacillus circulans* AB 16 grown on rice straw and its application in biobleaching of eucalyptus pulp. Biores. Technol. 73: 273 - 277.

Dickison, W. C. (2000). Integrative plant anatomy. Academic press. San Diego, San Francisco, New York, Boston, London, Toronto, Sydney Tokyo.

Domingues, F. C. Queiroz, J. A. Cabral, J. M. S. Fonseca, L. P. (2000). The influence of culture conditions on mycelial structure and cellulase production by *Trichoderma reesei* Rut C - 30. Enz. Microb Technol. 26: 394 - 401.

Duarte, M. C. T. da Silva, E. C. Gomes, I. M. B. Ponezi, A. N. Portugal, E. P. Vicente, J. R. Davanzo, E. (2003). Xylan - hydrolyzing enzyme system from *Bacillus pumilus* CBMAI 0008 and its effects on *Eucalyptus grandis* kraft pulp for pulp bleaching improvement. Biores. Technol. 88: 9 - 15.

Duarte, M. C. T, Pellegrino, A. C. Portugal, E. P. Ponezi, A. N. and Franco, T. T. (2000). Characterization of alkaline xylanases from *Bacillus pumilus*. Braz. J. Microbiol. 31: 90 - 94.

Duarte, M. C. T. Portugal, E. P. Ponezi, A. N. Bim, M. A. Tagliari, C. V. and Franco T. T. (1999). Production and purification of alkaline xylanases Biores. Technol. 68: 49 - 53.

Dubeau, H. Chahal, D. S. and Ishaque, M. (1986). Production of xylanases by *Chaetomium cellulolyticum* during growth on lignocellulosis. Biotechnol. Lett. 8: 445 - 448.

Dubeau. H. Chabal, D. S. and Bruschi, C. V. (1987). Xylanase of *Chaetmium cellulolyticum*: its nature of production and hydrolytic potential. Biotechnol Lett. 9: (4), 275 - 280.

Ducros, V. Charnock, S. J. Derewenda, U. Derevwenda, Z. S. Dauter, Z. Dupont, C. Shareck, F. Morosoli, R. Kluepfel, D. and Davies, G. J. (2000). Substrate specificity in glycoside hydrolase family 10: Structure and kinetic analysis of the *Streptomyces lividans* xylanase 10A. J. Biolo. Chem. 275: 23020 - 23026.

Dunlop, R. W. Wang, B. Ball, D. Ruollo, A. B. and Falk, C. J. (1996). Bacterial protein with xylanase activity. Patent US - 6200797. Biotech International Limited, Australia.

Durand, A. (2003). Bioreactor designs for Solid - state fermentation. Biochem. Eng. J. 13: 113 - 125.

Ebringerová, A. and Heinze, T. (2000). Xylan and xylan derivatives- biopolymers with desirable properties, 1 Naturally occurring xylans structures, isolation procedures and properties. Macromolecular Rapid Communications 21: 542 - 556.

Egloff, M. P. Uppenberg, J. Haalck, L. and van Tilbeurgh, H. (2001). Crystal structure of maltose phosphorylase from *Lactobacillus brevis*: unexpected evolutionary relationship with glucoamylases. Structure (Camb) 9: 689 - 697.

Eriksson K. E, Blanchette A, and Ander M. (1990). Microbial and enzymatic degradation of wood and wood components. Berlin: Springer Verlag, p. 181 - 213.

Eriksson T, Börjesson J, Tjerneld F. (2002). Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose, Enz. Microb. Technol. 31: 353 - 364.

Eriksson, K. E. (1991). Biotechnology: Three Approaches to Reduce Environmental Impact Caused by the Pulp and Paper Industry", Sci. Prog. 75: 175 - 189.

Esteban, R. Villanueva, J. R. and Villa, T. G. (1992). β - D - Xylanases of *Bacillus circulans* WL - 12. Can. J. Microbiol. 28: 733 - 793.

Feniksova, R. V. Tikhomrova, A. S. Rakhleeva, B. E. (1960). Conditions for forming amylases and proetinase in surface culture of *Bacillus subtilis*. Mikrobiologica. 29: 745 - 748.

Fernandez - Espinar, M. T. Romon, D. Pinaga, F. and Valles, S. (1992). Xylanase production by *Aspergillus nidulans*. FEMS Microbiol. Lett. 91: 91 - 96.

Ferre, H. Broberg, A. Duus, J. and Thomsen, K. K. (2000). A novel type of arabinoxylan arabinofuranohydrolase isolated from germinated barley (Analysis of substrate preference and specificity by nano - probe NMR). Eur. J. Biochem. 267: 6633 - 6641.

Ferreira, G. Boer, C. G. and Peralta, R. M. (1999). Production of xylanolytic enzymes by *Aspergillus tamarii* in Solid - state fermentation. FEMS Microbiol. Lett. 173: 335 - 339.

Flanigan, B and Sellars, P. N. (1977). Amyalase, β - glucosidase and β - xylosidase activity of thermotolerant and thermophilic fungi isolated from barley. Transaction of the British Mycological Society. 69: 316 - 317.

Flint, H. J. Martin, J. McPherson, C. A. Daniel, A. S. and Zhang, J. X. (1993). A bifunctional enzyme, with separate xylanase and beta (1, 3 - 1, 4) - glucanase domains, encoded by the *xyn*D gene of *Ruminococcus avefaciens*. J. Bacteriol. 175: 2943 - 2951.

Fontes, C. M. Gilbert, H. J. Hazlewood, G. P. Clarke, J. H. Prates, J. A. McKie, V. A. Nagy, T. Fernandes, T. H. and Ferreira, L. M. (2000). A novel *Cellvibrio mixtus* family 10 xylanase that is both intracellular and expressed under non - inducing conditions. Microbiol. 146: (Pt 8), 1959 - 1967.

Fontes, C. M. Hall, J. Hirst, B. H. Hazlewood, G. P. and Gilbert, H. J. (1995). The resistance of cellulases and xylanases to proteolytic inactivation. Appl. Microbiol. Biotechnol. 43: 52 - 57.

Foong, F. Hamamoto, T. Shoseyov, O. and Doi, R. H. (1991). Nucleotide sequence and characteristics of endoglucanase gene *eng*B from *Clostridium cellulovorans*. J. Gen. Microbiol. 137: (Pt 7), 1729 - 1736.

Frederick, M. M. Chin - Hen, K. Fredericks, J. R. and Reilly, P. J. (1985). Purification and characterization of endoxylanases from *Aspergillus niger* I two isoenzyme active on xylan backborn near branch points. Biotechnol. Bioeng. 27: 527 - 532.

Frederick, M. M. Frederick, J. R. Fratzke, A. R. and Reilly, P. J. (1981). Purification and characterization of a xylobiose and xylose - producing endo - xylanase from *Aspergillus niger*. Carbohydr. Res. 97: 87 - 103.

Frederix, S. A. Courtin, C. M. and Delcour, J. A. (2003). Impact of endoxylanases with different substrate selectivity on gluten starch separation In: Recent Advances in Enzymes in Grain Processing (Courtin, C. M. Veraverbeke, W. S. and Delcour, J. A. Eds.), pp. 247 - 254. Kat. Univ. Leuven, Leuven.

Fujimoto, H. T. Ooi, S. L. Wang, T. Takiwaza, H. Hidaka, S. Murao, and M. Arai. 1995. Purification and properties of three xylanases from *Aspergillus aculeatus*. Biosci. Biotechnol. Biochem. 59: 538 - 540.

Fujimoto, Z. Kuno, A. Kaneko, S. Yoshida, S. Kobayashi, H. Kusakabe, I. and Mizuno, H. (2000). Crystal structure of *Streptomyces olivaceoviridis* E - 86-β - xylanase containing xylan - binding domain. J. Mol. Biol. 300, 575 - 585.

Fushinobu, S. Ito, K. Konno, M. Wakagi, T. and Matsuzawa, H. (1998). Crystallographic and mutational analyses of an extremely acidophilic and acid stable xylanase: biased distribution of acidic residues and importance of Asp-37 for catalysis at low pH. Protein Eng. 11: 1121 - 1128.

Ganju, R. K. Vithayathil, P. J. and Murthy. S. K. (1989). Purification and characterization of two xylanases from *Chaetomium thermophile* var. *coprophile*. Can. J. Microbiol. 35: 836 - 842.

Garg, A. P. Roberts, J. C. and McCarthy, A. J. (1998). Bleach boosting effect of cellulase - free xylanase of *Streptomyces thermoviolaceus* and its comparison with two commercial enzyme preparations on birchwood kraft pulp. Enz. Microbial. Technol. 22: 594 - 8.

Garg, A. P. McCarthy, A. J. and Roberts, J. C. (1996). Biobleaching effect of *Streptomyces thermoviolaceus* xylanase preparations on birchwood kraft pulp, Enz. Microbial. Technol. 18: 261 - 267.

Gascoigne, J. A. and Gascoigne, M. M. (1960). The xylanase of *Fusarium roseum*, J. Gen. Microbiol, 22: 242 - 247.

Gawande, P. V. and Kamat, M. Y. (2000). Production of *Aspergillus* xylanase by lignocellulosic waste fermentation and its application. J. Appl. Microbiol. 87: 511 - 519.

Georis, J. de Lemos - Esteves, F. Lamotte - Brasseur, J. Bougnet, V. Devreese, B. Giannotta, F. Granier, B. Frere, J. M. (2000). An additional aromatic interaction improves the thermostability and thermophilicity of a mesophilic family 11 xylanase: structural basis and molecular study. Prot. Sci. 9: 466 - 475.

Georis, J. Giannotta, F. Lamotte - Brasseur, J. Devreese, B. Van Beeumen, J. Granier, B. and Frere, J. M. (1999). Sequence, overproduction and purification of the family 11 endo - β - 1, 4 - xylanase encoded by the xyl1 gene of *Streptomyces* sp. S38. Gene 237: 123 - 133.

Gerber, P. J. Heitmann, J. A. and Joyce, T. W. (1997). Purification and characterization of xylanase from *Trichoderma.* Biores. Technol. 61: 127 - 140.

Gessesse, A. (1998). Purification and properties of two thermostable alkaline xylanases from an alkaliphilic *Bacillus* sp. Appl. Environ. Microbiol. 64: 3533 - 3535.

Gessesse, A. and Mamo, G. (1998). Purification and characterization of an alkaline xylanase from alkaliphilic *Micrococcus* sp AR - 135. J. Ind. Microbiol. Biotech. 20: 210 - 214.

Gessesse, A. and Mamo, G. (1999). High -level xylanase production by alkaliphilic *Bacillus* sp. by using Solid-state fermentation. Enz. Microbiol. Technol. 25: 68 - 72.

Ghanem, N. B, Yusef, H. H. Mahrouse, H. K. (2000). Production of *Aspergillus terreus* xylanase in Solid-state cultures: application of the Plackett-Burman experimental design to evaluate nutritional requirements. Bioresour. Technol. 73: 113 - 121.

Ghosh, D. Sawicki, M. Lala, P. Erman, M. Pangborn, W. Eyzaguirre, J. Gutierrez, R. Jornvall, H. and Thiel, D. J. (2001). Multiple Conformations of Catalytic Serine and Histidine in Acetylxylan Esterase at 0. 90 Å. J. Biolo. Chem. 276: (14), 11159 - 11166.

Gibbs, M. D. Reeves, R. A. Farrington, G. K. Anderson, P. Williams, D. P. and Bergquist, P. L. (2000). Multidomain and multifunctional glycosyl hydrolases from the extreme thermophile *Caldicellulosiruptor* isolate Tok7B. 1. Curr. Microbiol. 40: 333 - 340.

Gilbert, H. J. and Hazlewood, G. P. (1993). Bacterial cellulases and xylanases. J. Gen. Microbiol. 139: 187 - 194.

Gilbert, M. M. Yaguchi, D. C. Watson, K. K. Y. Wong, C. Breuil, and J. N. Saddler. (1993). A comparison of two xylanases from the thermophilic fungi *Thielavia terrestris* and *Thermoascus crustaceus*. Appl. Microbiol. Biotechnol. 40: 508 - 514.

Gilkes, N. R. Henrissat, B. Kilburn, D. G. Miller Jr. R. C. and Warren, R. A. (1991). Domains in microbial 4 - glycanases: sequence conservation, function, and enzyme families. Microbiol. Rev. 55: 303 - 315.

Godden, B. Legon, T. Helvenstein, P. and Penninckx, M. (1989). Regulation of the production of hemicellulolytic and cellulolytic enzyme by a Streptomyces sp. growing on lignocellulose. J. Gen. Microbiol. 135: 285 - 292.

Godfrey, T. (2003). The enzymes market for grain processing In: Recent Advances in Enzymes in Grain Processing (Courtin, C. M. Veraverbeke, W. S. and Delcour, J. A. Eds.), Kat. Univ. Leuven, Leuven. 401 - 406.

Goesaert, H. Elliott, G. Kroon, P. A. Gebruers, K. Courtin, C. M. Robben, J. Delcour, J. A. and Juge, N. (2004). Occurrence of proteinaceous endoxylanase inhibitors in cereals. Biochimica Biophysica Acta, 193 - 202.

Gomes, J. Gomes, I. Kreiner, W. Esterbauer, H. Sinner, M. and Steiner, W. (1993). Production of high level of cellulase - free and thermostable xylanase by a wild strain of *Thermomyces lanuginosus* using beechwood xylan. J. Biotechnol. 30: 283 - 297.

Gomes, J., H. Purkarthofer, M. Hayn, J. Kapplmufller, M. Sinner, and W. Steiner. (1993). Production of a high level of cellulase-free xylanase by the thermophilic fungus *Thermomyces lanuginosus* in laboratory and pilot scales using lignocellulosic materials. Appl. Microbiol. Biotechnol. 39: 700–707.

Gomes, J. Gomes, I. Terler, K. Gubala, N. Ditzelmuller, G. and Steiner, W. (2000). Optimisation of culture medium and conditions for α-L-Arabinofuranosidase production by the extreme thermophilic eubacterium *Rhodothermus marinus*. Enz. Microbial Technol. 27: 414 - 422.

Gorbacheva, I. V. and Rodionova, N. A. (1977). Studies on xylan degrading enzymes. I. Purification and characterization of endo - l, 4 - β - xylanase from *Aspergillus niger* str. 14. Biochim. Biophys. Acta. 484: 79 - 93.

Gosalbes, M. J. Perez - Gonzalez, J. A. Gonzalez, R. and Navarro, A. (1991) Two beta - glycanase genes are clustered in *Bacillus polymyxa*: molecular cloning, expression, and sequence analysis of genes encoding a xylanase and an endo - β - (1, 3) - (1,4) - glucanase. J. Bacteriol. 173: 7705 - 7710.

Gowthaman, M. K. Krishna, C. and Moo - Young, M. (2001). Fungal Solid-state fermentation - An overview. In: Appl. Mycolo. Biotechnol. Agriculture and Food Productions. 1: 305 - 352.

Grabski, A. C. and Jeffries, T. W. (1991). Production, purification, and characterization of β-(1-4)-endoxylanase of *Streptomyces roseiscleroticus*. Appl. Environ. Microbiol, 57; 987 - 992.

Grejek, W. Gervais, P. (1987). Influence of water activity on the enzyme biosynthesis and enzyme activities produced by *Trichoderma viridies* TS in Solid -state fermentation. Enzyme Microb. Technol. 9: 658 - 662.

Gruber, K. Klintschar, G. Hayn, M. Schlacher, A. Steiner, W. and Kratky, C. (1998). Thermophilic xylanase from *Thermomyces lanuginosus*: high - resolution X - ray structure and modeling studies. Biochemistry. 37: 13475 - 13485.

Guerin, D. M. Lascombe, M. B. Costabel, M. Souchon, H. Lamzin, V. Beguin, P. and Alzari, P. M. (2002). Atomic (0. 94 A ¢[a]) resolution structure of an inverting glycosidase in complex with substrate. J. Mol. Biol. 316: 1061 - 1069.

Gunansekaran, P. (2001). Alkaline xylanase in pulp bleaching. Interaction Meet on Biotechnology in Paper Industry - A fresh look. 99 - 107.

Gupta, S. Bhushan, B. Hoondal, G. S. (2000). Isolation, purification and characterization of xylanase from *Staphylococcus* sp. SG-13 and its application in biobleaching of kraft pulp. J. Appl. Microbiol. 88: 325- 334.

Gupta, S. Kuhad, R. C. Bhushan, B. and Hoondal, G. S. (2001). Improved xylanase production from a haloalkalophilic *Staphylococcus* sp. SG-13

using inexpensive agricultural residues. W. J. Microbiol. Biotechnol. 17: 5 - 8.

Hager D. A, and Burgess R. R. (1980). Elution of proteins from sodium dodecyl sulfate - polyacrylamide gels, removal of sodium dodecyl sulfate, and renaturation of enzymatic activity: Results with sigma subunit of *Escherichia coli* RNA polymerase, wheat germ DNA topoisomerase, and other enzymes. Anal Biochem. 109: 76 - 85.

Hakulinen N, Turunen O, Jänis J, Leisola M, Rouvinenl J. (2003). Three - dimensional structures of thermophilic β- 1, 4- xylanases from *Chaetomium thermophilum* and *Nonomuraea flexuosa* comparison of twelve xylanases in relation to their thermal stability. Eur. J. Biochem. 270: 1399 - 1412.

Haltrich D, Nidetzky B, Kulbe KD, Steiner W, Zupancic S. (1996). Production of fungal xylanases. Biores. Technol. 58: 137 - 161.

Haltrich, D. Prei B, M. and Steiner, W. (1993). Optimization of a culture medium for increased xylanase production by a wild strain of *Schizophyllum commune.* Enz. Microb. Technol. 15: 854 - 860.

Haltrich, D. Prenner, E. Prieb, M. and Steiner, W. (1992). Production of extremely high values of xylanase activity by *Schizophyllum commune.* In: Biotechnology in the Pulp andPaper Industry. Proc. 5th Int. Conf. Biotechnol. Pulp Paper Ind. (Kuwahara, M. and Shimada, M. Eds.), 123 - 128.

Haltrich, D. Sebesta, B. and Steiner, W. (1995). Induction of xylanase and cellulase in *Schizophyllum commune.* In: Enzymatic Degradation of Insoluble Carbohydrates. ACS Symp. Series, Ed. J. N. Saddler and M. H. Penner. American Chemical Society, Washington, 618: 305 - 318.

Hamilton, G. E. Luechau, F. Burton, S. C. and Lyddiatt, A. (2000). J. Biotechnol. 79 - 103.

Harris, G. W. Pickersgill, R. W. Connerton, I. Debeire, P. Touzel, J. P. Breton, C. and Perez, S. (1997). Structural basis of the properties of an industrially relevant thermophilic xylanase. Proteins 29: 77 - 86.

Hayashi, H. Takagi, K. I. Fukumura, M. Kimura, T. Karita, S. Sakka, K. and Ohmiya, K. (1997) Sequence of *xyn*C and properties of *Xyn*C, a major component of the *Clostridium thermocellum* cellulosome. J. Bacteriol. 179: 4246 - 4253.

Hazlewood, G. P. and Gilbert, H. J. (1993). Molecular biology of hemicellulases, In: Hemicelluloses and Hemicellulases (Coughlan, M. P. and Hazlewood, G. P. Eds.), Portland Press, London. 103.

Heck, J. X. Flores, S. H. Hertz, P. F. Ayub, M. A. Z. (2005a). Optimization of cellulase-free xylanase activity produced by *Bacillus coagulans* BL69 in Solid-state cultivation. Proc. Biochem. 40: 107 - 112.

Heck, J. X. Flores, S. H. Hertz, P. F. Ayub, M. A. Z. (2005b). Extraction optimization of xylanases obtained by Solid - state cultivation of *Bacillus circulans* BL53. Proc. Biochem. 40: 2891 - 2895.

Heck, J. X. Flores, S. H. Hertz, P. F. Ayub, M. A. Z. (2006). Statistical optimization of thermotolerant xylanase activity from Amazon isolated *Bacillus circulans* on Solid-state cultivation. Biores. Technol. 97: 1902 - 1906

Heck, J. X. Hertz, P. F. and Ayub, M. A. Z. (2002). Cellulase and xylanase production by isolated amazon *Bacillus* strains using soybean industrial residue based Solid - state cultivation. Braz. J. Microbiol. 33: 213 - 218.

Heightman, T. D. and Vasella, A. (1999). Recent insights into inhibition, structure, and mechanism of configuration - retaining glycosidase. Angew. Chem. Int. Ed. 38: 750 - 770.

Henrissat, B. (1991). A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem. J. 280: 309 - 316.

Henrissat, B. and Coutinho, P. M. (2001). Classification of glycoside hydrolases and glycosyltransferases from hyperthermophiles. Meths. Enzymol. 330: 183 - 201.

Henrissat, B. (1992). Analysis of hemicellulases sequences. Relationships to other glycanases. In: Xylans and Xylanases. J. Visser. G. Beldman, M. A.

Kusters - van Someren and A. G. J. Voragen (Eds.), Elsevier Science Publishers, Amstardam. 463 - 466.

Henrissat, B. and Bairoch, A. (1993). New families in the classification of glycosylhydrolases based on amino acid sequence similarities. Biochem. J. 293: 781 - 788.

Henrissat, B. Callebaut, I. Fabrega, S. Lehn, P. Mornon, J. - P. and Davies, G. (1995). Conserved catalytic machinery and the prediction of a common fold for several families of glycosyl hydrolases. Proceedings of the National Academy of Sciences USA 92: 7090 – 7094.

Henrissat, B. Claeyssens, M. Tomme, P. Lemesle, L. and Mornon, J. P. (1989). Cellulase families revealed by hydrophobic cluster analysis. Gene 81: 83 - 95.

Holker, U. Hofer, M. and Lenz, J. (2004). Biotechnological advantages of laboratory - scale Solid- state fermentation with fungi. Appl. Microbiol. Biotech. 64: 175 - 186.

Honda, H. T. Kudo, and K. Horikoshi. (1985). Molecular cloning and expression of the xylanase gene of alkalophilic *Bacillus* sp. strain C-125 in *Escherichia coli*. J. Bacteriol. 161: 784 - 785.

Honda, H. T. Kudo, and Horikoshi, K. (1985). Purification and partial characterization of alkaline xylanse from *Escherichia coli* carrying pCX 311. Agric. Biol. Chem. 49: 3165 - 3169.

Honda, H. T. Kudo, Ikura,Y. and K. Horikoshi (1993). Two types of xylanases of alkalophilic *Bacillus* sp. No C - 125. Can. J. Microbiol 31: 538 - 542.

Hoq, M. M. Hempel, C. and Deckwer, W. (1994) Cellulase - free xylanase by *Thermomyces lanuginosus* RT9: Effect of agitation, aeration and medium components on production, J. Biotechnol. 37: 49.

Horikoshi, K. (1991a). Microorganisms in alkaline environments. Kodansha-VCH, Tokyo, Japan.

Horikoshi, K. (1996). Alkaliphiles - from an industrial point of view. FEMS Microbiol. Rev. 18: 259 - 270.

Horikoshi, K. (1999). Alkaliphiles: some applications of their products for biotechnology. Microbiol. Mol. Biol. Rev. 63: 735 - 750.

Horikoshi, K. and Atsukawa, Y. (1973). Xylanase produced by alkalophilic *Bacillus* no C - 59 - 2. Agric. Biol. Chem. 37: 2097 - 2103.

Hreggvidsson, G. O. Kaiste, E. Holst, O. Eggertsson, G. Palsdottir, A. and Kristjansson, J. K. (1996). An extremely therostable cellulase from the thermophilic eubacterioum *Rhodothermus marinus*. Appl. Environ. Micro biool. 62, (8): 3047 - 3049.

Huisman, M. M. H. Schols, H. A. and Voragen, A G. J. (2000). Glucurono-arabinoxylan from maize kernel cell walls are more complex than those from Sorghum kernel cell walls. Carbohyd. Polym. 43: 269 - 279.

Humblestone, F. J. Briggs, D. E. (2000b). Extraction and assay of ferulic acid esterase from malting barley. J.Inst. Brewing. 106: 21 - 29.

Hurlbert, J. C. and Preston 3rd, J. F. (2001). Functional characterization of a novel xylanase from a corn strain of *Erwinia chrysanthemi*. J. Bacteriol. 183: 2093 - 2100.

Iefuji, H. Chino, M. Kato, M. and Iimura, Y. (1996). Acid xylanase from yeast *Cryptococcus* sp. S-2: purification, characterization, cloning, and sequencing. Biosci. Biotechnol. Biochem. 60: 1331 - 1338.

Ikram - ul - Haq, Ehsan, A. Butt, W. A. and Ali, S. (2002). Studies on the Biosynthesis of Enzyme Xylanase by Submerged Fermentation from *Aspergillus niger* GCBMX - 45. Pak. J. Biolo. Sci. 5, (12): 1309 - 1310.

Inagaki, K. Nakahira, K. Mukai, K. Tamura, T. and Tanaka, H. (1998). Gene cloning and characterization of an acidic xylanase from *Acidobacterium capsulatum*. Biosci. Biotechnol. Biochem. 62: 1061 - 1067.

Inaoka, M and Soda, H (1956). Crystalline Xylanase. Nature, London 178: 202 - 203.

Inglis, G. D. Popp, A. P. Selinger, L. B. Kawchuk, L. M. Gaudet, D. A. and McAllister, T. A. (2000). Production of cellulases and xylanases by low - temperature basidiomycetes. Can. J. Microbiol. 46: 860 - 865.

Irwin, D. Jung, E. D. and Wilson, D. B. (1994). Characterization and sequence of a *Thermomonospora fusca* xylanase. Appl. Environ. Microbiol. 60: 763 - 770.

Ito, K. Iwashita, K. and Iwano, K. (1992). Cloning and sequencing of the xynC gene encoding acid xylanase of *Aspergillus kawachii*. Biosci. Biotechnol. Biochem. 56: 1338 - 1340.

IUB (International Union of Biochemisty) (1982). Enzyme. Nomenclature Academic Press. London. UK.

Izydorczyk MS, Biliaderis CG. (1993). Structural heterogeneity of wheat endosperm arabinoxylan. Cereal Chem. 70: 641 - 6.

Jacobs - Young, C. J. Gustafson, R. R. Heitmann, J. A. (2000). Conventional kraft pulping using enzyme pretreatment technology: role of diffusivity in enhancing pulp uniformity. Paperi ja Puu - Paper and Timber 82: 114 - 118.

Jeffries, T. W. (2001). Enzymatic Treatments of Pulps: Opportunities for the Enzyme Industry in Pulp and Paper Manufacture (available online).

Jeffries, T. W. (1996). Biochemistry and genetics of microbial xylanases. Curr. Opin. Biotechnol. 7: 337 - 342.

Jeffries, T. W. Klungness, J. H. Sykes, M. S. Rutledge, C. K. R. (1994). Comparison of enzyme enhanced with conventional deinking of xerographic and laser - printed paper. Tappi. J. 77, (4): 173 - 179.

Jeong, K. J. Lee, P. C. Park, I. Y. Kim, M. S, and Kim, S. C. (1998). Molecular cloning and characterization of an Endoxylanse gene of *Bacillus* sp. in *Escherichia coli*. Enz. Microb. Technol. 4: 297 - 304.

Jeya, M. Thiagarajan, S. and Gunasekaran, P. (2005). Improvement of xylanase production in Solid - state fermentation by alkali tolerant Aspergillus versicolor MKU3 Lett. Appl. Microbiol. 41: 175 - 178.

Jorgensen, H. Kutter, J. P. and Olsson, L. (2003). Separation and quantification of cellulases and hemicellulases by capillary electrophoresis. Analytical Biochemistry. 317: 85 - 93.

Joseleau, J. P. Comptat, J. and Ruel, K. (1992). Chemical structure of xylans and their interaction in the plant cell walls. In: Visser, J. Beldman, G. Kusters - van Someren, M. A. and Voragen, A. G. J. (Eds.), Xylan and Xylanases. Elsevier, Amsterdam. 1 - 15.

Joshi, M. D. Sidhu, G. Nielsen, J. E. Brayer, G. D. Withers, S. G. and McIntosh, L. P. (2001). Dissecting the electrostatic interactions and pH - dependent activity of a family 11 glycosidase. Biochem. 40: 10115 - 10139.

Joshi, M. D. Sidhu, G. Pot, I. Brayer, G. D. Withers, S. G. and McIntosh, L. P. (2000). Hydrogen bonding and catalysis: a novel explanation for how a single amino acid substitution can change the pH optimum of a glycosidase. J. Mol. Biol. 299: 255 - 279.

Kadowaki, M. K. Souza, C. G. M. Simão, R. C. G. and Peralta, R. M. (1997). Xylanase production by *Aspergillus tamarii*, Appl. Biochem. Biotechnol. 66, (3): 97 - 106.

Kalogeris, K. Christakopoulos, P. Kekos, D. Macris, B. J. (1998). Studies on the Solid-state production of thermostable endoxylanases from *Thermoascus aurantiacus*: Characterization of two isozymes. J. Biotech. 60: 155 – 163.

Kamal Kumar, B. Balakrishnan, H. and Rele, M. V. (2004). Compatibility of alkaline xylanases from an alkaliphilic *Bacillus* NCL (87 - 6 - 10) with commercial detergents and proteases. J. Ind. Microbiol. Biotechnol. (Epub ahead of print).

Kansoh, A. L. and Gammal, A. (2001). Xylanolytic activities of *Stretomyces* sp. 1, taxonomy production, partial purification and utilization of agricultural wastes. Acta Microbiol. Immunol. Hung. 48: 39 - 52.

Kantelinen, A. Hortling, B. Sundquist, J. Linko, M. and Viikari, L. *(1993)* Proposed mechanism of the enzymatic bleaching of kraft pulp with xylanases. Holzforschung 47: 318 - 324.

Karlsson O. Pettersson, B. and Westermark, U. (2001). Linkages between residual lignin and carbohydrates in bisulphite (manefite) pulps, J. Pulp and Paper Sci. 27: 310.

Katusbe, Y. Hata, Y. Yamaguchi, H. Moriyama, H. Shinmyo, A. and Okada, H. (1990). Estimation of the xylanase active site from crystalline structure. Proc. Protein Eng. 3: 289 - 290.

Kenealy, R, W. and Jeffries, T, W. (2003). Enzyme Processes for pulp and paper: A review of recent development in Wood deterioration and preservation Ed. by Goodell, B. Nicholas, D, D. and Schultz, T, P. 1953 - IV. American Chemical Society Meeting (221st: 2001: San Diego, Calif) V. Series. Chap 12: 210 - 238.

Keskar, S. S. (1992). High activity xylanase from thermotolerant *Streptomyces* T: cultural conditions and enzyme properties. Biotech. Letts, 14, (6): 481 - 486.

Khandeparkar, R. and Bhosle, N. B. (2006). Application of thermoalkalophilic xylanase from *Arthrobacter* sp. MTCC 5214 in biobleaching of kraft pulp. Biores. Technol. (in press).

Khandke, K. M, Vithayathil, P. J and Murthy, S. K (1989). Purification of xylanase, β -glucosidase, endocellulase and exocellulase from a thermophilic fungus *Thermoascus aurantiacus*; Arch. Biochem. Biophys. 274: 491 - 500.

Khasin, A. Alchanati, I. and Shoham, Y. (1993) Purification and characterization of a thermostable xylanase from *Bacillus stearothermophilus* T - 6. Appl. Environ. Microbiol. 59: 1725 - 1730.

Kheng, P. P. and Omar, I. C. (2005). Xylanase production by a local fungal isolate, *Aspergillus niger* USM AI 1 via Solid-state fermentation using palm kernel cake (PKC) as substrate. Songklanakarin J. Sci. Technol. 27, (2): 326 - 336.

Kimura, T. Ito, J. Kawano, A. Makino, T. Kondo, H. Karita, S. Sakka, K. and Ohmiya, K (2000). Purification, characterization, and molecular cloning of

acidophilic xylanase from *Penicillium* sp. 40. Biosci. Biotechnol. Biochem. 64: 1230 - 1237.

Kirk T. K. and Jeffries, T. W. (1996). Role of microbial enzymes in Pulp and paper processing. Published by Amerian Chemical Society. 1 - 14.

Kirk, T. K. and Farell, R. L. (1987). Enzymatic "combustion": the microbial degradation of lignin. Annual Review of Microbiology. 41: 465 - 505.

Kirk, T. K. and Cullen, D. (1998). Enzymology and molecular genetics of wood degradation by white - rot fungi. In: Young RA, Akthar M, Eds. Environment friendly technologies for the pulp and paper industry. New York: Wiley. 273 - 307.

Kleywegt, G. J. Zou, J. Y. Divne, C. Davies, G. J. Sinning, I. Stahlberg, J. Reinikainen, T. Srisodsuk, M. Teeri, T. T. and Jones, T. A. (1997). The crystal structure of the catalytic core domain of endoglucanase I from *Trichoderma reesei* at 3. 6 A ¢^{a} resolution, and a comparison with related enzymes. J. Mol. Biol. 272: 383 - 397.

Kluepfel, D. Shareck, F. Mandau, F. and Morosoli, R. (1986). Characterization of cellulase and xylanase activities of *Streptomyces lividans*. Appl. Microbiol. Biotechnol. 24: 230 - 234.

Ko, E. P. Akatsuka, H. Moriyama, H. Shinmyo, A. Hata, Y. Katsube, Y. Urabe, I. and Okada, H. (1992). Site - directed mutagenesis at aspartate and glutamate residues of xylanase from *Bacillus pumilus*. Biochem. J. 288: 117 - 121.

Kohli, U. Nigam, P. Singh, D. and Chaudhary, K. (2001). Thermostable, alkalophilic and cellulase free xylanase production by *Thermoactinomyces thalophilus* subgroup C. Enz. Microbial. Technol. 28: 606 - 10.

Kormelink, F. J. M. H. Gruppen, R. J. Vietor, and A. G. J. Voragen. (1993a). Mode of action of the xylan - degrading enzymes from *Aspergillus awamori* on alkali - extractable cereal arabinoxylans. Carbohydr. Res. 249: 355 - 367.

Kormelink, F. J. M. H. Gruppen, and A. G. J. Voragen. (1993b). Mode of action of (1,4) - β - D - arabinoxylan arabinofuranohydrolase (AXH) and α - L - arabinofuranosidases on alkali - extractable wheat-flour arabinoxylan. Carbohydr. Res. 249: 345 - 353.

Kormelink, F. J. M. M. J. F. Searle - van Leeuwen, T. M. Wood, and A. G. J. Voragen. (1993). Purification and characterization of three endo - (1, 4) - β - xylanases and one α - xylosidase from *Aspergillus awamori*. J. Biotechnol. 27: 249 - 265.

Kosugi, A. K. Murashima, Y. Tamaru, and R. H. Doi. (2002). Xylanase and acetyl xylan esterase activities of XynA, a key subunit of the *Clostridium cellulovorans* cellulosome for xylan degradation. Appl. Environ. Microbiol. 68: 6399 – 6402.

Koullas, D. P. Christakopoulos, P. F. Kekos, D. Koukios, E. G. and Macris, B. J. (1993). Effect of alkali - delignification on wheat straw saccharification by *Fusarium oxysporium* cellulose. *Biomass. Bioener. 4: 9 - 13.*

Krengel, U. and Dijkstra, B. W. (1996) Three - dimensional structure of Endo - 1,4 - beta - xylanase I from *Aspergillus niger*, molecular basis for its low pH optimum. J. Mol. Biol. 263: 70 - 78.

Krishna, C. (1999). Production of bacterial cellulases by Solid state bioprocessing of banana wastes. *Bioresource Tech*. 69: 231 - 239.

Krishna, C. (2005). Solid-State Fermentation Systems - An Overview, Crit. Rev. Biotech. 25: 1 - 30.

Krishna, C. and Nokes, S. E. (2001). Influence of inoculum size on phytase production and growth in Solid - state fermentation by *Aspergillus niger*. Trans. ASAE. 44: 1031 - 1036.

Krishnamurthy, S. (1989). Purification and properties of xylanases and β - glucosidases elaborated by the thermophilic fungus *Paecilomyces varioti* Bainier. Ph. D. thesis. Indian Institute of Science, Bangalore.

Kubata, B. K. Suzuki, T. Horitsu, H. Hawai, K. Takamizawa, K. (1994). Purification and characterization of *Aeromonas caviae* ME - 1 xylanase V,

which produces exclusively xylobiose from xylan. Appl. Environ. Microbiol. 60: 531 - 535.

Kubata, K. B. Horitsu, H. Kawai, K. Takamizawa, K. and Suzuki. T. (1992). Xylanse I of *Aeromonas caviae* ME - I isolated from the industine of a herbivorous insect (*Samia cynthia pryeri*) Biosci. Biotech. Biochem. 56: 1463.

Kulkarni, N. and M. Rao (1996). Application of Xylanase from Alkaliphilic Thermophilic *Bacillus* SP NCIM 59 in biobleaching of bagasse pulp. J. Biotech. 51: 167 - 173.

Kulkarni, N. Shendye, A. and Rao, M. (1999). Molecular and biotechnological aspects of xylanases. FEMS Microbiol. Rev. 23: 411 - 456.

Kumar, P. R. Eswaramoorthy, S. Vithayathil, P. J. and Viswamitra, M. A. (2000). The tertiary structure at 1. 59 A ¢^{a} resolution and the proposed amino acid sequence of a family-11 xylanase from the thermophilic fungus *Paecilomyces varioti bainier*. J. Mol. Biol. 295: 581 - 593.

Kungst, F. Ogasawara, N. Moszer, I. Albertini, A. M. Alloni, G. Azevedo, V. Bertero, M. G. Bessieres, P. Bolotin, A. Borchert, S. Borriss, R. Boursier, L. Brans, A. Braun, M. Brignell, S. C. Bron, S. Brouillet, S. Bruschi, C. V. Caldwell, B. Capuano, V. Carter, N. M. Choi, S. K. Codani, J. J. Connerton, I. F. and Danchin, A. (1997). The complete genome sequence of the gram - positive bacterium *Bacillus subtilis*. Nature 390: 249 - 256.

Kusakabe, I. Yasui, T. and Kobayashi, T. (1977). The action of the *Streptomyces* xylanase on various xylans and xylooligosaccharides (in Japanese). Nippon Nogeikagaku Kaishi 51: 439 - 448.

Laemmli U. K. (1970). Cleavage of structural proteins during the assembly of the head of bateriophage T4. *Nature* 227: 680 - 685.

Lama, L. Calandrelli, V. Gambacorta, A. and Nicolaus, B. (2004). Purification and characterization of thermostable xylanase and β - xylosidase by the thermophilic bacterium *Bacillus thermantarcticus*. Res. Microbiol. 155: 283 - 289.

Lamed, R. E. Stetter, R. Kenig, and E. A. Beyer. (1983). The cellulosome - a discrete cell surface organelle of *Clostridium thermocellum,* which exhibits separate antigenic, cellulose binding and various cellulolytic activities. Biotechnol. Bioeng. Symp. 13: 163 - 181.

Lappalainen, A, Siika - Aho, M, Kallkkinen, N. Fagerström, R. Tenkanen, M. (2000). Endoxylanase II from *Trichoderma reesei* has several isoforms with different isoelectric points. Biotechnol. Appl. Biochem. 31: 61 - 68.

Larroche, C. (1996). Microbial growth and sporulation behaviour in Solid- state fermentation. J. Sci. Ind. Res. 55: 408 - 423.

Larson, S. B. Day, J. Barba de la Rosa, A. P. Keen, N. T. and McPherson, A. (2003). First crystallographic structure of a xylanase from glycoside hydrolase family 5:implications for catalysis. Biochem. 42: 8411 - 8422.

Leathers, T. D. and Gupta, S. C. (1996). Saccharification of corn fiber using enzymes from *Aureobasidium* sp. Strain NRRl Y-2311- 1. Appl. Biochem. Biotech. 59: 337 - 347.

Lee, S. F. Forsberg, C. W. and Rattray, J. B. (1987). Purification and characterization of two Endoxylanases from *Clostridium acetobutylicum* ATCC -824. Appl. Envt. Microbiol. 53, (4): 644 - 650.

Lee, Y. E. Lowe, S. E. Henrissat, B. and Gregory Zeikusl. J. (1993), Characterization of the active site and thermostability regions of Endoxylanase from *Thermoanaerobacterium saccharolyticum* B6A - RI. J. Bact. 175, (81): 5890 - 5898.

Lee, Y. Lowe, S. Zeikus, J. (1998). Molecular biology and biochemistry of xylan degradation by thermoanaerobes. In: Kalegoris, E. Christakopoulos, D. Kekos, D. Macris, B. (Eds.), Studies on the Solid-state production of thermostable endoxylanases from *Thermoascus aurantiacus*: characterization of two isoenzymes. J. Biotechnol. 60: 155 - 163.

Leisola, M. Jokela, J. Pastinen, O. Turunen, O. Schoemaker, H. (2004). Industrial use of enzymes, in Encyclopedia of Life Support Systems (EOLSS), UNESCO, Eolss Publishers, Oxford, UK.

Lemos. J. L. S. and Pereira Junior (2002). Influences of some sugars on xylanase production by *Aspergillus awamori* in SSF Braz. Arch. Biol. Tech. 45: 431 - 437.

Lenartovicz, V. de Souza, C. G. M. Moreira, F. G. and Peralta, R. M. (2003). Temperature and carbon source affect the production and secretion of a thermostable β- xylosidase by *Aspergillus fumigatus* Proc. Biochem. 38: 1775 - 1780.

Lequart, C. Nuzillard, J, M. Kurek, B. Debeire, P. (1999). Hydrolysis of wheat bran and straw by an endoxylanase: production and structural characterization of cinnamoyl - oligosaccharides. Carbo. Res. 319: 102 - 111.

Li, B. Nagalla, S. R. and Renganathan, V. (1997). Cellobiose dehydrogenase from *Phanerochaete chrysosporium* is encoded by two allelic variants. Appl. Environ. Microbiol. 63: 796 - 799.

Li, K. Azadi, P. Collins, R. Tolan, J. Kim, J. and Eriksson, K. (2000). Relationships between activities of xylanases and xylan structures. Enzyme Microb. Technol. 27: 89 - 94.

Li, X. T. Jiang, Z. Q. Li, L. T. Yang, S. Q. Feng, W. Y. Fan, J. F. and. Kusakabe, I. (2005). Characterization of a cellulase-free, neutral xylanase from *Thermomyces lanuginosus* CBS 288. 54 and its biobleaching effect on wheat straw pulp. Biores. Technol. 96: 1370 - 1379

Lin, J. Ndlovu, L.M. Singh, S. and Pillay, B. (1999). Purification and biochemical characteristics of β-D-xylanase from a thermophilic fungus, *Thermomyces lanuginosus* - SSBP. Biotechnol. Appl. Biochem. 30: 73 - 79.

Lin, W. Y. (2005). The present situation and developmental prospect of China's non - wood fiber pulping and alkali recovery. China Pulp and Paper Indust. 26, (4): 22 - 26 (in Chinese).

Lincoln, R. E. (1960). Control of stock culture preservation and inoculum build in bacterial fermentation. J. Biochem. Microbiol. Tech. Eng. 2: 481 - 500.

Lineweaver, H, and Burk, D. (1934). The determination of the enzyme dissociation. J. Am. Chem. Soc. 56: 658 - 66.

Liu, R. T. Qu, Y. B. Wang, B. M. Chen, J. C. Gao, P. J. Jiang, Y. H. Yang, G. H. Li, Z. C. (1998). Enzymatic modification of straw pulp and characterization of alkaline xylanases from *Pseudomonas* sp. In: Enzyme Engineering XIV, Annals of the New York Academy of Sciences, 864: 474 - 478.

Liu, X. M. Qi, M. Lin, J. Q. Wu, Z. H. Qu, Y. B. (2001). Asparagine residue at position 71 is responsible for alkali - tolerance of the xylanase from *Bacillus pumilus* A - 30. J. Microbiol. Biotechnol. 11, (3): 534 - 538.

Lo Leggio, L. Kalogiannis, S. Bhat, M. K. and Pickersgill, R. W. (1999) High resolution structure and sequence of *T. aurantiacus* xylanase I: implications for the evolution of thermostability in family 10 xylanases and enzymes with β / α - barrel architecture. Proteins, 36: 295 - 306.

Lonsane, B. K. Saucedo-Castaneda, G. Raimbault, M. Roussos, S. Vinigera - Gonzalez, G. Ghildyal, N. P. Ramakrishna, M. and Krishnaiah, M. M. (1992). Scale up strategies for Solid-state fermentation systems. Proc. Biochem. 27: 259 - 273.

Lowry, O. H. Rosebrough, N. J. Farr, A. and Randall, R. L. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 93: 267 - 75.

Lu, W. Li, D. Wu, Y. (2003). Influence of water activity and temperature on xylanase biosynthesis in pilot - scale Solid-state fermentation by *Aspergillus sulphureus*. Enz. Microbiol. Technol. 32: 305 - 311.

Luthi, E. Jasmat, N. B. and Bergquist, P. L. (1990). Xylanase from the extremely thermophilic bacterium *Caldocellum saccharolyticum*: overexpression of the gene in *Escherichia coli* and characterization of the gene product. Appl. Environ. Microbiol. 56: 2677 - 2683.

Maheshwari, R. and P. T. Kamalam. (1985). Isolation and culture of a thermophilic fungus, *Melanocarpus albomyces*, and factors influencing the production and activity of xylanase. J. Gen. Microbiol. 131: 3017 - 3027.

Maheshwari, R. Bharadwaj, G. Bhat, M. K. (2000). Thermophilic fungi: their physiology and enzymes. Microbiol. Mol. Biol. Rev. 64: 461 - 488.

Mandels, M. and Stenberg, D. (1976). Recent advances in cellulase technology. J. Ferment. Technol. 54: 267 - 286.

Marui, M. Nakanishi, K. and Yasui, T. (1985). Purification and properties of three types of xylanases induced by methyl β - xyloside from *Streptomyces* sp. Agric. Biol. Chem. 49: 3399 - 3407.

Matsuo, M. and Yasui, T. (1985). Properties of xylanase of *Malbranchea pulchella* var. *sulfurea* no. 48. Agric. Biol. Chem. 49: 839 - 841.

McCarthy, A. A. Morris, D. D. Bergquist, P. L. and Baker, E. N. (2000). Structure of *Xyn*B, a highly thermostable β- 1, 4 - xylanase from *Dictyoglomus thermophilum* Rt 46 B. 1, at 1. 8 A ¢[a] resolution. Acta Crystallogr. D: Biol. Crystallogr. 56 (Pt 11): 1367 - 1375.

Mechaly, A. Teplitsky, A. Belakhov, V. Baasov, T. Shoham, G. and Shoham, Y. (2000). Overproduction and characterization of seleno-methionine xylanase T - 6. J. Biotechnol. 78: 83 - 86.

Meyrath, J. and Suchanek, G. (1972). Inoculation techniques- effects due to quality and quantity of inoculum. In: Methods in Microbiology, (Norris, Jr. and Ribbons, D. W. Eds.) Acadmic Press London 7B: 159 - 209.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31: 426 - 428.

Mishra, C. Seeta, R. and Rao, M. (1985). Production of xylanolytic enzymes in association with the cellulolytic activities of *Penicillium funiculosum.* Enz. Microb. Technol. 7: 295 - 299.

Mitchell, D. A. Targonski, Z. Rogalski, J. Leonowicz, A. (1992). Substrates for processes. In: Solid Substrate Cultivation. Doelle, H. W. Mitchell, D. A. Rolz, C. E. Eds. Elsevier Applied Biotechnology Series, Elsevier, London. 29 - 52.

Monti, R. Terenzi, H. F. and Jorge, J. A. (1991). Purification and properties of an extracellular xylanase from the thermophilic fungus *Humicola grisea* var. *thermoidea*. Can. J. Microbiol. 37 : 675 - 681.

Moon, A. G. and Parulekar, S. J. (1991). A parametric study of protease production in batch and fed batch cultures of *Bacillus firmus*. Biotechnol Bioeng 37: 467 - 483.

Moriyama, H. Y. Hata, H. Yamaguchi, M. Sato, A. Shinmyo, N. Tanaka, H. Okada, and Y. Katsube. (1987). Crystallization and preliminary X - ray studies of *Bacillus pumilus* IPO xylanase. J. Mol. Biol. 193: 237 - 238.

Morosoli, R. J. Bertrand, J. L. Mondou, F. Shareck, F. and Klupfel, D. (1986) Purification and properties of a xylanases from *Streptomyces lividans*. Biochem. J. 239: 587 - 592.

Morris, D. D. Gibbs, M. D. Ford, M. Thomas, J. and Bergquist, P. L. (1999). Family 10 and 11 xylanase genes from *Caldicellulosiruptor* sp. strain Rt 69B. 1. Extremophiles, 3: 103 - 111.

Mozammel Hoq, M. Hempel, C. and Deckwer, W. D. (1994). Cellulase-free xylanase by *Thermomyces lanuginosus* RT9 effect of agitation, aeration, and medium components on production. J. Biotech. 37: 49 - 58.

Mujer, C. V. Kretchmen, D. W. and Miller, R. (1991). Partial purification and properties of endo xylanase from cucumber seeds. Physiologia Plantarum, Copenhagen, 81: 327 - 338.

Nadakumar, M. P. Thakur, M. S. Raghavarao, K. S. M. S. and Ghildyal, N. P. (1996). Substrate particle sizes reduction by *Bacillus coagulans* in Solid - state feremtation. Enz. Microbiol. Technol. 18: 121 - 125.

Nakamura, A. F. Fukumori, S. Horinouchi, H. Masaki, T. Kudo, T. Uozumi, K. Horikoshi, and T. Beppu. (1991). Construction and characterization of the chimeric enzymes between the *Bacillus subtilis* cellulase and an alkalophilic *Bacillus* cellulase. J. Biol. Chem. 266: 1579 - 1583.

Nakamura, S. Aono, R. Wakabayashi, K. Horikoshi, K. (1992). Alkaline xylanase produced by newly isolated alkaliphilic *Bacillus* sp. In: Visser, J. Beldman, G. Kusters - Van - Someren, M. A. Voragen, A. G. J. (Eds.), Xylans and Xylanases. Elsevier, Amsterdam. 443 - 446.

Nakamura, S. Ishiguro, Y. Nakai, R. Wakabayashi, K. Aono, R. and Horikoshi, K. (1995). Purification and characterization of a thermophilic alkaline

xylanase from thermoalkaliphilic *Bacillus* sp. strain TAR - 1 J. Mole. Catalysis B: Enzymatic 1: 7 - 15.

Nakamura, S. K. Wakabayashi, R. Nakai, R. Aono, and K. Horikoshi. (1993a). Production of alkaline xylanase by a newly isolated alkaliphilic *Bacillus* sp. strain 41M - 1. World J. Microbiol. Biotechnol. 9: 221 - 224.

Nakamura, S. K. Wakabayashi, R. Nakai, R. Aono, and K. Horikoshi. (1993b). Purification and some properties of an alkaline xylanase from alkaliphilic *Bacillus* sp. strain 41M - 1. Appl. Environ. Microbiol. 59: 2311 - 2316.

Nakamura, S. R. Nakai, K. Wakabayashi, Y. Ishiguro, R. Aono, and K. Horikoshi. (1994). Thermophilic alkaline xylanase from newly isolated alkaliphilic and thermophilic *Bacillus* sp. strain TAR- 1. Biosci. Biotechnol. Biochem. 58: 78 - 81.

Nakanishi, K. and Yasui, T. (1980). Production of xylanase by *Streptomyces* sp. using non - metabolizable inducer. Agric. Biol. Chem. 44: 2729 - 2730.

Nakanishi, K. Marui, M. and Yasui, T (1992). Comparison of xylan and methyl - β - xyloside induced xylanase *Streptomyces* sp. J. Ferm. Bioeng. 74, (6): 392 - 394.

Naren, A. P. (1992). Structure and function of xylanases from four thermophilic fungi. Ph. D. thesis. Indian Institute of Science, Bangalore.

Nascimento, R. P. Colho, R. R. R. Marques, S. Alves, L. Girio, F. M. Bon, E. P. S. Amaral - Collaco, M. T. (2002). Production and partial characterization of xylanase from *Streptomyces* sp. Strain AMT - 3 isolated from Brazilian cerrado soil. Enz. Microbiol. Technol. 549 - 555.

Nath, D. and M. Rao (2001). pH dependent conformational and structural changes of xylanase from an alkalophilic thermophilic *Bacillus* sp (NCIM 59). Enz. Microb. Technolo. 28: 397 – 403.

Nieboer, E. and Richardson, D. H. S. (1980). The replacement of non-descript terms "Heavy metals", by a biologically and chemically significant classification of metal ion. Environ. Pollut. Ser. B. 1: 3 - 26.

Ninawe, S and Kuhad, R. C. (2005). Use of xylan - rich cost effective agro - residues in the production of xylanase by *Streptomyces cyaneus* SN32. J. Appl. Micrbiol. 99: 1141 - 1148.

Noe, P. J. Chevalier, F. Mora, and J. Comtat. (1986). Action of xylanases on chemical pulp fibers. Part II. Enzymatic beating. J. Wood Chem. Technol. 6: 167 - 184.

Nogawa M, Goto M, Okada H, Morikawa Y. (2001). L - Sorbose induces cellulase gene transcription in the cellulolytic fungus *Trichoderma reesei.* Curr. Genet. 38: 329 - 334.

Ntarima, P. (2000). Les xylanases des familles 10 et 11: differentiation et caracterisation, Laboratory of Biochemistry, University of Gent, Gent. 96.

Ntarima, P. Nerinckx, W. Klarskov, K. Devreese, B. Bhat, M. K. Van Beeumen, J. and Claeyssens, M. (2000). Epoxyalkyl glycosides of D - xylose and xylooligosaccharides are active - site markers of xylanases from glycoside hydrolase family 11, not from family 10. Biochem. J. 347, (Pt 3): 865 - 873.

Nunn, J. R. Parolis, H. and Russel, I. (1973). Polysaccharides of the red algae *Chaetangium erinaceum.* Part I: Isolation and characterization of the water - soluble xylan. Carbohydr. Res. 26: 169 - 180.

Nurizzo, D. Turkenburg, J. P. Charnock, S. J. Roberts, S. M. Dodson, E. J. McKie, V. A. Taylor, E. J. Gilbert, H. J. and Davies, G. J. (2002). *Cellvibrio japonicus* α - L - arabinanase 43A has a novel five blade β-propeller fold. Nat. Struct. Biol. 9: 665 - 668.

Oakley, A. J. Heinrich, T. Thompson, C. A. and Wilce, M. C. (2003). Characterization of a family 11 xylanase from *Bacillus subtillis* B230 used for paper bleaching. Acta Crystallogr. D: Biol. Crystallogr. 59: 627 - 636.

Okada, H. and Shinmyo, A (1988). Xylanase of *Bacillus pumilus.* In: Meth. Enz. 160 (A): 632 – 637.

Okai, N. Fukasaku, M. Kaneko, J. Tomita, T. Muramotu, K. and Kamio, Y. (1998). Molecular properties and activity of a carboxyl- terminal truncated

form of xylanase 3 from *Aeromonas caviae* W - 61. Boiosci. Biotechnol. Biochem. 62: 1560 - 1567.

Okazaki, W. T. Akiba, K. Horikoshi, K. and Akahoshi, R. (1985). Purification and characterization of xylanases from alkalophilic thermophilic *Bacillus* spp. Agric. Biol. Chem. 49: 2033 - 39.

Okeke, B. C. and Obi, S. K. C. (1995). Production of cellulolytic and sylanolytic enzymes by an *Arthrographis species*. World J. Microbiol. Biotechnol. 9: 345 - 349.

Oku, T. Roy, C. Watson, D. C. Wakarchuk, W. Campbell, R. Yaguchi, M. Jurasek, L. and Paice, M. G. (1993). Amino acid sequence and thermostability of xylanase A from *Schizophyllum commune*. FEBS Lett. 334: 296 - 300.

Olsson, L. and Hahn-Hagerdal, B. (1996). Fermentation of lignocellulosic hydrolysates for ethanol production. Enz. Microb. Technol. 18: 312 - 331.

Olsson, L. Christensen, T. M. I. E. Hansen, K. P. Palmqvist, E. A. (2003). Influence of the carbon sources on the production of cellulase, hemicelluases and pectinases by *Tricoderma reesi* Rut C-30. Enz. Microb. Technol. 33: 612 - 619.

Ooijkaas, L. P. Weber, F. J. Buitelaar, R. Tramper, J. and Rinzema, A. (2000). Defined media and inert supports: Their potential as Solid - state fermentation production systems. TIBTECH. 18: 356 - 360.

Paice, M. G. Gurnagul, N. Page, D. H. Jurasek, L. (1992). Mechanism of hemicellulose-directed prebleaching of kraft pulps. Enz. Microb. Technol. 14: 272 - 276.

Paice, M. G. Jurasek, L. Carpenter, M. R. and Smillie, L. B. (1978). Production, characterization, and partial amino acid sequence of xylanase A from *Schizophyllum commune*. Appl. Environ. Microbiol. 26: 802 - 808.

Paice, M. G. Bourbonnais, R. Desrochers, M. Jurasek, L. Yaguchi, M. (1986). A xylanase gene from *Bacillus subtilis*: nucleotide sequence and comparison with *Bacillus pumilus* gene. Arch. Microbiol. 144: 201 - 206.

Palma, M. B. Milagres, A. M. F. Prata, A. M. R. and de Mancilha, I. M. (1996). Influence of aeration and agitation rate on the xylanase activity from *Penicillium janthinellum.* Proc. Biochem. 31: 141- 145.

Paloheimo, M. Mäntylä, A. Kallio, J. and Suominen, P. (2003). High-yield production of a bacterial xylanase in the filamentous fungus *Trichoderma reesei* requires a carrier polypeptide with an intact domain structure. Appl Environ. Microbiol. 69: 7073 - 7082.

Panagioutou, G. Kekos, D. Macris, B. J. and Christakopoulos, P. (2003). Production of cellulolytic and xylanolytic enzymes by *Fusarium oxysporum* grown on corn stover in Solid state fermentation. Ind. Crops Prodts. 18: 37 - 45.

Panbangred W, Kawaguchi O, Tomita T, Shinmyo A, and Okada H. (1984). Isolation of two β - xylosidase genes of *Bacillus pumilus* and comparison of their gene products. Eur. J. Biochem. 138: 267 - 73.

Panbangred, W. A. Shinmyo, S. Kinoshita, and H. Okada. (1983). Purification and properties of endoxylanase produced by *Bacillus pumilus*. Agric. Biol. Chem. 47: 957 - 963.

Panbangred, W. Fukusaki, E. Epifanio, E. C. Shinmyo, A. and Okada, H. (1985). Expression of a xylanases gene of *Bacillus subtilis*. Appl. Microbiol. Biotechnol. 22: 259 - 264.

Pandey, A (2003). Solid-state fermentation. Biochem. Eng. J. 13: 81 - 84.

Pandey, A. Soccol C. R. Rodriguez - Leon J. A. and Nigam. P. (2001). Solid-State Fermentation in Biotechnology Published by Asiatech Publishers, INC. New Delhi, First edition.

Pandey, A. Soccol, C. R. Mitchell, D. (2000). New development in Solid -State Fermentation: I - bioprocesses and products. Proc. Biochem. 35: 1153 - 1169.

Park, T. M. Lee, M. S. Choi, J. Y. Kim, S. C. and Lee, G. M. (2000). Orthophosphate anion enhances the stability and activity of endoxylanase from *Bacillus* sp. Biotechnology and Bioengineering, 72, (4): 434 - 440.

Park, Y. S. Kang, S. W. Lee, J. S. Hong, S. I. and Kim, S. W. (2002). Xylanase production in Solid - state fermentation by *Aspergillus niger* mutant using statistical experimental designs. Appl. Microbiol. Biotechnol. 58: 761-766.

Parkkinen, T. Hakulinen, N. Tenkanen, M. Siikaaho, M. and Rouvinen, J. (2004). Crystallization and preliminary X - ray analysis of a novel *Trichoderma reesei* xylanase IV belonging to glycoside hydrolase family 5. Acta Crystallogr. D: Biol. Crystallogr. 60: 542 - 544.

Patel R. N, Grabski A. C. Jefrries T. W. (1993). Chromophore release from kraft pulp by purified *Streptomyces roseiscleroticus* xylanases. Appl. Microbiol. Biotechnol. 39: 405 - 12.

Patel, R. N. Thakker, G. D. Rao, K. K. (1994). Potential use of a white - rot fungus *Antrodiella* sp. RK1 for biopulping. J. Biotechnol. 36: 19 - 23.

Paul, J. and Varma, A. K. (1990). Influence of sugars on endo -glucanase and β - xylanase activities of a *Bacillus* strain. Biotechnol. Lett. 12: 61 - 64.

Pearson, R. G. (1973). Hard and Soft acid and bases. John Wiley and Sons New York.

Pell, G. Szabo, L. Charnock, S. J. Xie, H. Gloster, T. M. Davies, G. J. and Gilbert, H. J. (2004a). Structural and biochemical analysis of *Cellvibrio japonicus* xylanase 10C: how variation in substrate binding cleft influences the catalytic profile of family GH - 10 xylanases. J. Biol. Chem. 279: 11777 - 11788.

Pell, G. Taylor, E. J. Gloster, T. M. Turkenburg, J. P. Fontes, C. M. Ferreira, L. M. Nagy, T. Clark, S. J. Davies, G. J. and Gilbert, H. J. (2004b). The mechanisms by which family 10 glycoside hydrolases bind decorated substrates. J. Biol. Chem. 279: 9597 - 9605.

Perez, J. Munoz-Dorado, J. de-la-Rubia, T. and Martinez, J. (2002). Biodegradation and biological treatment of cellulose, hemicellulose and lignin: an overview. Int Microbiol. 5: 53 - 63.

Petrescu, I. Lamotte - Brasseur, J. Chessa, J. P. Ntarima, P. Claeyssens, M. Devreese, B. Marino, G. and Gerday, C. (2000). Xylanase from the psychrophilic yeast *Cryptococcus adeliae*. Extremophiles, 4: 137 - 144.

Pham, P. L. Alric, I. and Delmas, M. (1995). Incorporation of xylanase in total chlorine free sequences using ozone and hydrogen peroxide. Appita, 48, (3): 213 - 217.

Pickersgill, R. W. Jenkins, J. A. Connerton, M. S. I. Hazelwood, G. P. and Gilbert, H. J. (1993). Crystallization and preliminary X - ray analysis of the catalytic domain of xylanase A. from *Pseudomonas fluorescens* subspecies cellulose. J. Mol. Biol. 229: 246 - 248.

Plesniak, L. A. Wakarchuk, W. W. and Mcintosh, L. P. (1996). Seconday structure and NMR assignment of *Bacillus ciruculans* xylanase. Protein Sci. 5, 1118 - 1135.

Prabhu, K. A. Maheshwari, R. (1999). Biochemical properties of xylanases from a thermophilic fungus, *Melanocarpus albomyces*, and their action on plant cell walls. J. Biosci. 24: 461 - 470.

Priest, F. G. (1992). Biology of *Bacilli.* In *Bacilli*: applications to industry. Ed. R. H. Doi and M. McGloughlin. Butterworth - Heinemann, Boston, Mass. 293 - 320.

Puchart, V. Katapodis, P. Biely, P. Kremnicky, L. Christakopoulos, P. Anska, M.V. Kekos, D. Macris, B. J. Bhat, M. K. (1999). Production of xylanases, mannanases, and pectinases by the thermophilic fungus *Thermomyces lanuginosus*. Enz. Microb. Technol. 24: 355 - 361.

Puls, J. and Poutanen, K. (1989). Mechanism of enzymatic hydrolysis of hemicelluloses (xylans) and procedures for determination of the enzyme activities involved. In Enzyme Systems for Lignocellulose Degradation ed. Coughlan, M. P. London: Elsevier Applied Science. 151 - 165.

Puls, J. (1997). Chemistry and biochemistry of hemicelluloses: Relationship between hemicellulose structure and enzymes required for hydrolysis. Macromol Sym. 120: 183 - 196.

Purkarthofer, H. Sinner, M. and Steiner, W. (1993a). Cellulase - free xylanase from *Thermomyces lanuginosus*: Optimization of production in submerged and Solid - state culture. Enz. Microb. Technol. 15: 677 - 682.

Purkarthofer, H. Sinner, M. and Steiner, W. (1993b) Effect of shear rate and culture pH on the production of xylanase by *Thermomyces lanuginosus*. Biotechnol. Lett. 15: 405 – 410.

Raimbault, M. (1998). General and microbiological aspects of Solid substrate fermentation. Elect. J. Biotech. 1: 1 - 15.

Rani, D. S. and Nand, K. (2000). Production of thermostable cellulase-free xylanase by *Clostridium absonum* CFR -702. Proc. Biochem. 36: 355 -62.

Ratanakhanokchai, K. Kyu, K. L. and Tanticharoen, M. (1999). Purification and properties of a xylan - binding endoxylanase from alkaliphilic *Bacillus* sp. strain K - 1. Appl. Environ. Microbiol. 65: 694 - 697.

Ratanakhanokchai, K. Noiduang, P. and Kyu, K. L. (2002). Two extracellular endoxylanases from alkaliphilic *Bacillus firmus* differ in their synthesis. Biotech. Lett. 24: 1487 - 1490.

Ratto, M, Poutanen, K. and Viikari, L. (1992). Production of xylanolytic enzymes by an alkalitolerant *Bacillus circulans* strain. Appl Microbiol. Biotechnol. 37: 470 - 473.

Ratto, M. Mathrani, I. M. Ahring, B. and Viikari, L. (1994). Application of thermostable xylanase of *Dictyoglomus* sp. In enzymatic treatment of kraft pulps. Appl. Microbiol. Biotechnol. 41: 130 - 133.

Reis, S. Costa, M. A. F. and Rosane Marina Peralta, R. M. (2003). Xylanase production by a wild strain of *Aspergillus nidulans* Acta Scientiarum: Biological Sciences Maringá, 25, (1): 221 - 225.

Rizzatti, A. C. S. Sandrim, V. C. Jorge, J. A. Terenzi, H. F. Polizeli, M. L. T. M. (2004). Influence of temperature on the properties of the xylanolytic enzymes of the thermotolerant fungus *Aspergillus phoenicis*. J. Ind. Microbiol Biotechnol. 31: 88 - 93.

Roberts, J. C. McCarthy, A. J. Flynn, N. J. and Broda, P. (1990). Modification of paper properties by the pretreatment of pulp with *Scrccharomonospora viridis* xylanase. Enz. Microb. Technol. 12: 210 - 213.

Royer, J. C. Nakas, J. N. (1991). Purification and characterization of two xylanases from *Trichoderma longibrachiatum*. J. Biochem. 202: 521- 529.

Rye, C. S. and Withers, S. G. (2000). Glycosidase mechanisms. Curr. Opin. Chem. Biol. 4: 573 - 580.

Ryttersgaard, C. Lo Leggio, L. Coutinho, P. M. Henrissat, B. and Larsen, S. (2002) *Aspergillus aculeatus* beta - 1,4 - galactanase: substrate recognition and relations to other glycoside hydrolasesin clan GH - A. Biochemistry, 41: 15135 - 15143.

Sa - Pereira, P. Mesquita, A. Duarte, J. C. Barros, M. R. A. and Costa - Ferreira, M. (2002). Rapid production of thermostable cellulase - free xylanase by a strain of *Bacillus subtilis* and its properties. Enz. Microbiol. Technol. 30, 924 - 933.

Sabharwal, H. S. Akhtar, M. Blanchette, R. A. Young, R. A. (1995). Refiner mechanical and biomechanical pulping of jute. Holzforschung. 49: 537-544.

Sabini, E. Sulzenbacher, G. Dauter, M. Dauter, Z. Jorgensen, P. L. Schulein, M. Dupont, C. Davies, G. J. and Wilson, K. S. (1999). Catalysis and specificity in enzymatic glycoside hydrolysis: a 2,5B conformation for the glycosyl - enzyme intermediate revealed by the structure of the *Bacillus agaradhaerens* family 11 xylanase. Chem. Biol. 6: 448 - 455.

Sabini, E. Wilson, K. S. Danielsen, S. Schulein, M. and Davies, G. J. (2001). Oligosaccharide binding to family 11 xylanases: both covalent intermediate and mutant product complexes display (2,5) B conformations at the active centre. Acta Crystallogr. D: Biol. Crystallogr. 57: 1344 - 1347.

Saha B. C. (2000). α - L - Arabinofuranosidases: biochemistry, molecular biology and application in biotechnology. Biotechnol. Adv. 18: 403 - 23.

Saha, B. C. (2003). Hemicellulose bioconversion. J. Ind. Microbiol. Biotechnol. 30: 279 - 291.

Saiman, E. Debire, P. H. and Touzel, J. P. (1997) High - level production of a cellulase free xylanase in glucose limited fed batch cultures of a thermophilic *Bacillus* strain. J. Biotechnol. 58: 71 - 8.

Saiman, E. Touzel, J. P. Brodel, B. and Debeire, P. (1992). Isolation of a thermophilic bacterium producing high levels of xylanase. In: Xylans and Xylanases. Progress in Biotechnology, (Visser, J. Beldman, G. Kurster - van Someran, M. A, and Voragen, A. G. J. Eds), Elsevier Science Publishers, Amsterdam. 7: 467 - 470.

Saloheimo, M. Siika - Aho, M. Tenkanen, M. and Penttila, M. E. (2003). Novel xylanase from *Trichoderma reesei*, method for production there of, and methods employing this enzyme. In: US-Patent Application 20030054518.

Sansen, S. De - Ranter, C. J. Gebruers, K. Brijs, K. Courtin, C. M. Delcour, J. A. and Rabijns, A. (2004). Crystallization and preliminary X - ray diffraction study of two complexes of a TAXI- type xylanase inhibitor with glycoside hydrolase family 11 xylanases from *Aspergillus niger* and *Bacillus subtilis*. Acta Cryst. D 60: 555 - 557.

Santos, E. S. D. Guirardello, R. and Franco, T. T. (2002). Preparative chromatography of xylanase using expanded bed adsorption, J. Chromato. A, 944: 217 - 224.

Sapag, A. Wouters, J. Lambert, C. de Ioannes, P. Eyzaguirre, J. and Depiereux, E. (2002). The endoxylanases from family 11: computer analysis of protein sequences reveals important structural and phylogenetic relationships. J. Biotechnol. 95: 109 - 131.

Saxena, S. Bahadur, J. and Varma, A. (1991). Production and localization of Carboxymethylcellulase, Xylanase and β - glucosidase from *Cellulomonas* and *Micrococcus* sp. Appl. Microbiol. Biotechnol. 34: 668 - 670.

Schallmey, M. Singh, A. and Ward, O. P. (2004). Developments in the use of *Bacillus* sp. for industrial production. Can. J. Microbiol. 50: 1 - 17.

Schooveneveld - Bergmans, M. E. F. Hopman, A. M. C. P. Beldman, G. and Voragen, A. G. J. (1998). Extraction and partial characterisation of feruloylated glucurono-arabinoxylans from wheat bran. Carbohyd. Polym. 35: 39 - 47.

Schulze, E. (1891). In formation regarding chemical compostion of plant cell membrane. Beriche der Deutschen Chemischem Gesellschaft. 23: 2277 - 2287.

Sen, S. and Satyanarayana, T. (1993). Optimization of alkaline protease production by thermophilic *Bacillus licheniformis* S- 40. Ind. J. Microbiol. 33, (1): 43 - 47.

Senior, D. J. (1988) Selective solubilisation of xylan in pulp using a purified xylanase of *Trichoderma harzianum*, Biotech. Lett. 11: 907 - 912.

Senior, D. J. Mayers, P. R. and Saddler, J. N. (1989). Production and purification of xylanases. In: Plant cell wall polymers. (Eds. Lewis, N. G. and Paice, M. G.) Washington D. C. American Chemical Society. 641 - 654.

Senthilkumar, S. R. Ashokkumar, B. Chandra Raj, K. Gunasekaran, P. (2005). Optimization of medium composition for alkali-stable xylanase production by *Aspergillus fischeri* Fxn 1 in Solid- state fermentation using central composite rotary design. Biores. Technol. 96: 1380 - 1386.

Seyis, I. and Aksoz, N. (2005). Effect of carbon and nitrogen sources on xylanase production by *Trichoderma harzianum* 1073 D3. Int. Biodete. Biodeg. 55: 115 - 119.

Shabalin, K. A, Kulminskaya, A. A, Savel, A. N, Shishlyannikov, S. M, Neustroev, K. N. (2002). Enzymatic properties of α-galactosidase from *Trichoderma reeei* in the hydrolysis of galacto oligosaccharides. Enz. Microb. Technol. 30: 231 - 239.

Shabeb, M. S. A. (2000). A new record of bacterial alkaline thermostable xylanase from an Egyptian soil. Egypt. J. Biotechnol. 7: 193 - 205.

Shah, A. K. Sidid, S. S. Ahmed, A. and Rele, M. V. (1999). Treatment of bagasse pulp with cellulase-free xylanases from an alkalophilic *Bacillus* sp. Sam-3. Biores. Technol. 68: 133 - 40.

Shah, A. K. Cooper, D. Adolphson, R. Eriksson, K. E. L. (2000). Xylanase treatment of oxygen - bleached hardwood kraft pulp at high temperature and alkaline pH levels gives substantial savings in bleaching chemical. J. Pulp Paper Sci. 26: 8 - 11.

Shah, A. R. and Madamwar, D. (2005). Xylanase production by a newly isolated *Aspergillus foetidus* strain and its characterization. Proc. Biochem. 40: 1763 - 1771.

Shah, A. R. Shah, R. K. and Madamwar, D. (2006). Improvement of the quality of whole wheat bread by supplementation of xylanase from *Aspergillus foetidus*. Biores. Techn. 97: 2047 - 2053.

Shaku, M. Koike, S. and Udaka, S. (1980). Cultural conditions for protein production by *Bacillus brevis* No. 47. Agri. Biol. Chem. 44: 99 - 103.

Shallom, D. and Shoham, Y. (2003). Microbial hemicellulases. Curr. Opin. Microbiol. 6: 219 - 228.

Shao, W. S. K. C. Obi, J. Puls, and J. Wiegel. (1995). Purification and characterization of the α- glucuronidase from *Thermoanaerobacterium* sp. strain JW/SL-YS485, an important enzyme for the utilization of substituted xylans. Appl. Environ. Microbiol. 61: 1077 - 1081.

Sharma, H. S. S. (1987). Enzymatic degradation of residual non-cellulosic polysaccharides present on dew-retted flax fibers. Appl. Microbiol. Biotechnol. 26: 2714 - 2723.

Shendye, A. and Rao, M. (1993). Molecular cloning and expression of xylanases from an alkalophilic thermophilic *Bacillus* (NCIM 59) in *Bacillus subtilis* A8. Enz. Microb. Technol. 15: 343 - 347.

Shibuya, H. Kaneko, S. Hayashi, K. (2000). Enhancement of the thermostability and hydrolytic activity of xylanase by random gene shuffling. Biochem. J. 349: 651 - 656

Shin, H, D. and Chen, R. R. (2005). Production and characterization of a type B feruloyl esterase from *Fusarium proliferatum* NRRL- 26517. Enz. Microb. Technol.

Shobhit, M. Kumar, S. Rao, N. J. (2001). Application of commercial xylanases in bleaching - a review. IPPTA. 13: 13 - 23.

Shoham, Y. Schwartz, Z. Khasin, A. Gat, O. Zosim, Z. and Rosenberg, E. (1992). Biodegradation. 3: 207 - 218.

Sidhu, G. Withers, S. G. Nguyen, N. T. McIntosh, L. P. Ziser, L. and Brayeer, G. D. (1999). Biochemistry 38: 5346 - 5354.

Simpson, H. D. Haufler, U. R. Daniel, R. M. (1991). An extremely thermostable xylanase from the thermophilic eubacterium *Thermotoga.* Biochem. J. 277: 413 - 417.

Sinnot, M. L. (1990). Catalytic mechanisms of enzymic glycosyl transfer. Chem. Rev. 90: 1171 - 1202.

Solis -Pereira, S. Favela -Torres, E. Viniegra -Gonzalez, G. and Gutierrez- Rojas, M. (1993). Effects of different carbon sources on the synthesis of pectinases by *Aspergillus niger* in submerged and Solid-state fermentation. Appl. Microbiol. Biotechnol. 39: 36 - 41.

Someet, N. S. Vikram and S. B. Virendra, (2001). Optimization of xylanase production by *Melanocarpus albomyces* IIS 68 in Solid-state fermentation using response surface methodology. J. Bioscie Bioeng. 91: 425 - 427.

Sonia, K. G. Chandha, B. S. and Saini, H. S. (2005). Sorghum straw for xylanase hyper - production by *Thermomyces lanuginosus* (D_2W_3) under Solid - state fermentation. Biores. Techn. 96: 1561 - 1569.

Sonnleitner, B. and Fiechter, A. (1983). Advantages of using thermophiles in biotechnological processes: expectations and reality. Trend. Biotech. 1, (3): 74 - 80.

Sorensen, H. R. Meyer, A. S. Pedersen, S. (2003). Enzymatic hydrolysis of water -soluble wheat arabinoxylan: Synergy between α-L-arabinofuranosidases, Endo - 1, 4 - β - xylanases, and β - xylosidase activities. Biotechnol. Bioeng. 81: 726 – 731.

Souza, D. F. Souza, C. G. M. Peralta, R. M. (2001). Effect of easily metabolizable sugars in the production of xylanase by *Aspergillus tamarii* in Solid - state fermentation. Proc. Biochem. 36: 835 - 838.

Spalding, B. J. (1991). Bio/ Technology 9: 229.

Steiner, W. Lajerty, R. M. Gomes, I. and Esterbauer, H. (1987). Studies on a wild type strain of *Schizophyllum commune*: Cellulase and xylanase production and formation of the extracellular polysaccharide Schizophyllan. Biotechnol. Bioeng. 30: 169 - 178.

Stutzenberger, F. J. and Bodine, A. B. (1992). Xylanase production by *Thermomonospora curvata*. J. Appl. Bacteriol. 72: 504 - 511.

Subramanian, S. and Prema, P. (1998). Optimization of cultural parameters for the synthesis of endo - xylanases from *Bacillus* sp. SSP - 34. J. Sci. Ind. Res. 57: 611 - 616.

Subramaniyan S, (2000). Studies on production of bacterial xylanases. Ph. D Thesis, Cochin University of Science and Technology.

Subramaniyan S. Prema P. and Ramakrishna S. V. (1997). Isolation and screening for alkaline thermostable xylanase. J. Basic Microbiol. 37: 431 - 437.

Subramaniyan, S. and Prema, P. (2002). Biotechnology of microbial xylanases: enzymology, molecular biology, and application. Crit. Rev. Biotechnol. 22: 33 - 64.

Subramaniyan, S. Sandhia, G. S. and Prema, P. (2001). Control of xylanase production without protease activity in *Bacillus* sp. by selection of nitrogen source. Biotechnol. Lett. 23: 369 - 371.

Sudgen, C. Bhat, M. K. (1994). Cereal straw and pure cellulose as carbon sources for the growth and production of plant cellwall degrading enzyme by *Sporotrichum thermophilie*. World J. Microbial. Biotechnol. 10: 444- 451.

Sunna A, Puls J, Antranikian G. (1997). Characterisation of the xylanolytic enzyme system of the extreme thermophilic anaerobic bacteria *Thermotoga maritim*a, *T. neapolitana* and *T. thermarum*. Comp. Biochem. Physiol. 118 (A): 453 - 61.

Sunna, A. and Antranikian, G. (1997). Xylanolytic enzymes from fungi and bacteria. Crit. Rev. Biotechnol. 17: 39 - 67.

Suurnäkki A, Tenkanen M, Buchert J, Viikari L. (1997). Hemicellulases in bleaching of chemical pulps. In: Adv Biochem. Eng. Biotechnol. 57: 261-287.

Taiz, L. and Honigman, W. A. (1976). Production of cell wall hydrolyzing enzymes by barley aleurone layers in response to gibberellic acid. Plant Physiol. 58: 380 - 386.

Takami, H. and Horikoshi, K. (2000). Analysis of the genome of an alkaliphilic *Bacillus* strain from an industrial point of view. Extremophiles. 4: 99- 108.

Takami, H. Nakasone, K. Takaki, Y. Maeno, G. Sasaki, R. Masui, N. Fuji, F. Hirama, C. Nakamura, Y. Ogasawara, N. Kuhara, S. and Horikoshi, K. (2000). Complete genome sequence of the alkaliphilic bacterium *Bacillus halodurans* and genomic sequence comparison with *Bacillus subtilis*. Nucleic Acids Res. 28: 4317 - 4331.

Takanashi M, and Kutsumi S. (1979). Purification and properties of xylanase from *Gliocladium virens*. J. Ferment. Technol. 57: 434 -39.

Taneja, K. Gupta, S. and Kuhad, R. C. (2002). Properties and application of a partially purified alkaline xylanase from an alkaophilic fungus *Aspergillus nidulans* KK - 99. Biores. Technol. 85: 39 - 42.

Tangnu, S. K. Blanch, H. W. Wilke, C. R. (1981). Enhanced production of cellulase, hemicellulase, and β - glucosidase by *Trichiderma reesei* Rut C-30. Biotechnol. Bioeng. 23: 1837 - 1849.

TAPPI Test Methods, (1996, 1997). Technical association of the Pulp and Paper Industry, TAPPI Press, Atlanta, GA, USA

Techapun, C. Poosaran, N. Watanabe, M. Sasaki, K. (2003). Thermostable and alkaline - tolerant microbial cellulase - free xylanases produced from agricultural wastes and the properties required for use in pulp bleaching bioprocess: a review. Proc. Biochem. 38: 1327 - 1340.

Techapun, C. Sinsuwongwat, S. Watanabe, M. Sasaki, K. and Poosaran, N. (2002). Production of cellulase-free xylanase by a thermotolerant *Streptomyces* sp. grown on agricultural waste and media optimization

using mixture design and Plackett - Burman experimental design methods. Biotech. Lett. 24: 1437 - 1442.

Technomarket survey reports on enzymes (1992). Department of Science and Technology, New Delhi.

Tenkanen, M. Burgermeister, M. Vrsanska, M. Biely, P. Saloheimo, M. and Siika - Aho, M. (2003). A novel xylanase XYN IV from *Trichoderma reesei* and its action on different xylans In: Recent Advances in Enzymes in Grain Processing (Courtin, C. M. Veraverbeke, W. S. and Delcour, J. A. Eds.), Kat. Univ. Leuven, Leuven. 41 - 46.

Tenkanen, M. Thornton, J. and Viikari. L. (1995). An acetylglucomannan esterase of *Aspergillus oryzae*; purification, characterization and role in the hydrolysis of O-acetyl -galactoglucomannan. J. Biotechnol. 42: 197 - 206.

Teplitsky, A. Shulami, S. Moryles, S. Shoham, Y. and Shoham, G. (2000). Crystallization and preliminary X- ray analysis of an intracellular xylanase from *Bacillus stearothermophilus* T-6. Acta. Crystallogr. D: Biol. Crystallogr. 56, (Pt 2): 181 - 184.

Timell, T. E. (1965). Wood hemicelluloses: part II. Adv. Carbohydr. Chem. Biochem. 19: 409 - 483.

Tolan, J. S. Olson, D. and Dines, R. E. (1995). Survey of Xylanase Enzyme Usage in Bleaching in Canada, 81st Annual Meeting Technical Section, 31 Jan. - 3 Feb. Montreal, Canada. B91 - B96.

Torres, A. L. Roncero, M. B. Colom, J. F. Pastor, F. I. J. Blanco, A. and Vidal, T. (2000). Effect of novel enzyme on fiber morphology during ECF bleaching of oxygen delignified Eucalyptus kraft pulps. Biores. Techn. 74: 135 - 140.

Torronen, A. and Rouvinen, J. (1995). Structural comparison of two major endo - 1,4 - xylanases from *Trichoderma reesei*. Biochem. 34: 847 - 856.

Torronen, A. and Rouvinen, J. (1997). Structural and functional properties of low molecular weight endo -1,4 -β - xylanases. J. Biotechnol. 57: 137 - 149.

Torronen, A. Harkki, A. and Rouvinen, J. (1994). Three - dimensional structure of endo - 1,4 - β - xylanase II from *Trichoderma reesei*: two conformational states in the active site. EMBO J. 13: 2493 - 2501.

Torronen, A. Mach, R. L. Messner, R. Gonzalez, R. Kalkkinen, N. Harkki, A. and Kubicek, C. P. (1992). The two major xylanases from *Trichoderma reesei*: characterization of both enzymes and genes. Biotechnology (NY) 10: 1461 - 1465.

Tremblay, L. and Archibald, F. (1993). Production of a cloned xylanase in *Bacillus cereus* and its performance in kraft pulp prebleaching. Can. J. Microbiol. 39: 853 - 860.

Trilli, A. (1986). Scale up of fermentation. In: Industrial Microbiology and Biotechnology. Demain, A. L. and Solomon, N. A. Eds. American Society of Microbiology, Washington, U. S. A. 227 - 307.

Tsujibo, H. T. Sakamoto, N. Nishino, T. Hasegawa, and Y. Inamori. (1990). Purification and properties of three types of xylanases produced by an alkalophilic actinomycete. J. Appl. Bacteriol. 69: 398 - 405.

Tuohy, M. G. Puls, J. Claeyssens, M. Vrsanska, M. and Coughlan, M. P, (1993). The xylan - degrading enzyme system of *Talaromyces emersonii*: novel enzymes with activity against aryl -β-D-xylosides and unsubstituted xylans. Biochem. J. 290: 515 - 523.

Turkiewiz, M. Kalinowska, H. Zielinska, M. and Bielecki, S. (2000). Purification and characterization of two endo - 1, 4 - xylanases from Antarctic krill, *Euphasia superba* Dana. Comp. Biol. Physiol. Part. B 127: 325- 335.

Turunen, O. Jänis, J. Fenel, F. and Leisola, M. (2004). Engineering the thermotolerance and pH optimum of family 11 xylanases by site - directed mutagenesis. Meths. Enz. 388: 156 - 167.

Turunen, O. Vuorio, M. Fenel, F. and Leisola, M. (2002). Engineering of multiple arginines into the Ser/Thr surface of *Trichoderma reesei* endo - 1, 4 - β - xylanase II increases the thermotolerance and shifts the pH optimum towards alkaline pH. Protein Eng. 15: 141 - 145.

Uchino, F. and T. Nakane. (1981). A thermostable xylanase from a thermophilic acidophilic *Bacillus* sp. Agric. Biol. Chem. 45: 1121 - 1127.

Uffen, R. L. (1997) Xylan degradation: a glimpse at microbial diversity. J. of Ind. Microbiolo. Biotech. 19: 1 - 6.

Uhl, A. M. and Daniel, R. M. (1999). The first description of an archaeal hemicellulase: the xylanase from *Thermococcus zilligii* strain AN1. Extremophiles. 3: 263 - 267.

Usui, K. Ibata, K. Suzuki, T. and Kawai, K. (1999). Xyn X, a possible exo-xylanase of *Aeromonas caviae* ME-1 that produces exclusively xylobiose and xylotetrose from xylan. Biosci. Biotechnol. Biochem. 63, 8: 1346-1352.

van Peij, N. N. M. E. J. Brinkmann, M. Vrs¡anska, J. Visser, and L. H. de- Graaff. (1997). β - Xylosidase activity, encoded by *xln*D, is essential for complete hydrolysis of xylan by *Aspergillus niger* but not for induction of the xylanolytic spectrum. Eur. J. Biochem. 245: 164 - 173.

Van Petegem, F. Collins, T. Meuwis, M. A. Gerday, C. Feller, G. and Van Beeumen, J. (2002). Crystallization and preliminary X - ray analysis of a xylanase from the psychrophile *Pseudoalteromonas haloplanktis*. Acta. Crystallogr. D: Biol. Crystallogr. 58: 1494 - 1496.

Van Petegem, F. Collins, T. Meuwis, M. A. Gerday, C. Feller, G. and Van Beeumen, J. (2003). The structure of a cold - adapted family 8 xylanase at 1. 3 A ¢ªresolution. Structural adaptations to cold and investigation of the active site. J. Biol. Chem. 278: 7531 - 7539.

van Tilbeurgh, H. and Claeyssens, M. (1985). Detection and differentiation of cellulose components using low molecular mass fluorogenic substrates. FEBS Lett. 187: 283 - 288.

Vicuna, R. Escobar, F. Osses, M. and Jara, A. (1997). Bleaching of eucalyptus kraft pulp with commercial xylanases. Biotechnol. Lett. 19: 75 - 78.

Vicuna, R. Oyarzun, E. and Osses, M. (1995). Assessment of various commercial enzymes in the bleaching of radiata pine kraft pulps. J. Biotech. 40: 163 - 168.

Vidal, T. Torres, A. L. Colom, J. F. and Siles, F. J. (1997). Xylanase bleaching of Eucalyptus kraft pulp - an economical ECF process, Appita. J. 50, (2): 144 - 148.

Viikari, L. Tenkanen, M. Suurnäkki, A. (2001). Biotechnology in the pulp and paper industry. In: Biotechnology, 2nd, Weinheim, Wiley - VCH, 523 - 546.

Viikari, L. Suurnakki, A. Buchert, J. (1996). Enzymatic bleaching of kraft pulps: fundamental mechanisms and practical applications. Abstracts of Paper of the American Chemical Society, Meet, Pt-1, CELL -211.

Viikari. L. Tenkanen, M. Buchert. J. Ratto, M. Bailey, M. Siika-Aho, M. and Linko, M. (1993). Hemicellulases for industrial applications. In: Saddler, J. N. (Ed.), Bioconversion of Forest and Agricultural Plant Residues, C. A. B. International, U. K. I31 - 181.

Viikari, L. Kantelinen, A. Sundquist, J. and Linko, M. (1994). Xylanase in bleaching: from an idea to the industry. FEMS. Microbiol. Rev. 13: 335-350.

Viikari, L. Sundquist, J. and Kettunen, J. (1991). Xylanase enzymes promote pulp bleaching. Paperi ja Puu (Pap Timber) 73: 384 - 389.

Viikari, L. Ranua, M. Kantelinen, A. Sudquist, J. Linko, M. (1986). In: Proceedings of Third International Conference Biotechnology Pulp and Paper Industry, Stochkolm. 67.

Vincent, P. F. Shareck, C. Dupont, R. Morosoli, and D. Kluepfel. (1997). New α - L - arabinofuranosidase produced by *Streptomyces lividans*: cloning and DNA sequence of the *abf*B gene and characterization of the enzyme. Biochem. J. 322: 845 - 852.

Virupakshi, S. Gireesh Babu, K. Satish R. G, Naik, G. R. (2005). Production of a xylanolytic enzyme by a thermoalkaliphilic *Bacillus* sp. JB - 99 in Solid - state fermentation. Proc. Biochem. 40: 431- 435.

Vogt, G. Woell, S. and Argos, P. (1997). Protein thermal stability, hydrogen bonds, and ion pairs. J. Mol. Biol. 269: 631- 643.

Wackett, L. P. (2000). Environmentalo Biotechnology. TIBTECH. (Vol - 8)

Waino, M. and Ingvorsen, K. (2003). Production of β- xylanase and β- xylosidase by the extremely halophilic archaeon *Halorhabdus utahensis*. Extremophiles. 7: 87 - 93.

Wakarchuk, W. W. Sung, W. L. Campbell, R. L. Cunningham, A. Watson, D. C. and Yaguchi, M. (1994) Thermostabilization of the *Bacillus circulans* xylanase by the introduction of disulfide bonds. Protein. Eng. 7: 1379 - 1386.

Walsh, G. and Headon, D. (1994). Downstream processing. In: Wiley John and Sons (Eds). Protein Biotechnology. 107 - 109.

Warren, R. A. J. (1996). Microbial hydrolysis of polysaccharides. Annu. Rev. Microbiol. 50: 183 - 212.

Wejse, P. L. Ingvorsen, K. and Mortensen, K. K. (2003). Purification and characterization of two extremely halotolerant xylanases from a novel halophilic bacterium. Extremophiles. 7: 423 - 431.

Whitehead, T. R. (1993). Analyses of the gene and amino acid sequence of the *Prevotella (Bacteroides) ruminicola* 23 xylanase reveals unexpected homology with endoglucanases from other genera of bacteria. Curr. Microbiol. 27: 27 - 33.

Wilkie, K. C. B. (1983). Hemicellulose. Chemtech 13: 306 - 319.

Winterhalter, C. Heinrich, P. Candussio, A. Wich, G. and Liebl, W. (1995). Identification of a novel cellulose- binding domain within the multidomain 120 kDa xylanase *Xyn*A of the hyperthermophilic bacterium *Thermotoga maritima*. Mol. Microbiol. 15: 431- 444.

Wither, S. G. (1995). Enzymatic cleavage of glycosides: How does it happen? Pure and Appl. Chem. 67 (10): 1673-1682.

Wong, K. K. Y, and Saddler, J. N. (1992). *Trichoderma* xylanases, their properties and application, Crit. Rev. Biotechnol. 12: 413 - 435.

Wong, K. K. Y. de Jong, E. Saddler, J. N. Allison, R. W. (1997). Mechanisms of xylanase - aided bleaching of kraft pulp. Appita J. 50: 415 - 422.

Wong, K. K. Y. Tan, L. U. L. and Saddler, J. N. (1988) Multiplicity of beta - 1,4 - xylanases in microorganisms: functions and applications. Microbiol. Rev. 52: 305 - 317.

Wong, K. K. Y. and Saddler, J. N. (1993). Applications of hemicellulases in the food, feed and pulp and paper industries In: Hemicelluloses and Hemicellulases (Coughlan, M. P. and Hazlewood, G. P. Eds.), Portland Press, London. 127 - 143.

Wong, K. K. Y. Tan, L. U. L. Saddler, J. N. and Yaguchi, M. (1986). Purification of a third distinct xylanase from the xylanolytic system of *Trichoderma harzianum*. Can. J. Microbiol. 32: 570 - 574.

Wood, P. J. Erfle, J. D. Teather, R. M. (1988). Use of complex formation between Congo red and polysaccharides in detection and assay of polysaccharide hydrolases. Meths. Enzymol. 160: 59 - 74.

Woodward, J. (1984). Xylanases functions. Top Enz. Ferment. Biotech. 8: 9- 30.

Wouters, J. Georis, J. Engher, D. Vandenhaute, J. Dusart, J. Frere, J. M. Depiereux, E. and Charlier, P. (2001). Crystallographic analysis of family 11 endo - β - 1, 4 -xylanase *Xyl*1 from *Streptomyces* sp. S38. Acta. Crystallogr. D: Biol. Crystallogr. 57: 1813 - 1819.

Wu, S. C. Ham, K. S. Darvill, A. G. and Albersheim, P. (1997). Deletion of two endo - β - 1, 4 - xylanase genes reveals additional isozymes secreted by rice blast fungus. Molecular Plant - Microbe Interactions 10: 700 - 708.

Xiong, H. (2004). Production and characterization of *Trichoderma reesei* and *Thermomyces lanuginosus* xylanases. Ph. D Thesis. Helsinki University of Technology, Department of Chemical Technology, Laboratory of Bioprocess Engineering.

Xu J, Takakuwa N, Nogawa M, Okada H, Morikawa Y. (1998) A third xylanase from *Trichoderma reesei* PC- 3-7. Appl. Microbiol. Biotech. 49: 718 -724.

Yadav, J. S. (1988). SSF of wheat straw with alcaliphilic *Coprinus*. Biotechnol. Bioeng. 31: 414 - 417.

Yamaura, I. T. Koga, T. Matsumoto, and T. Kato. (1997). Purification and some properties of endo - 1, 4 - β - D - xylanase from a fresh - water mollusc,

Pomacea insularus (de Ordigny). Biosci. Biotechnol. Biochem. 61: 615 – 620.

Yang, V. W. Zhuang, Z. Elegir, G. and Jeffries, T. W. (1995). Alkaline-active xylanase produced by an alkaliphilic *Bacillus* sp isolated from kraft pulp. J. Ind. Microbiol. 15: 434 - 441

Yang, R. C. MacKenzie, C. R. Bilous, D. and Narang, S. A. (1989). Identification of two distinct *Bacillus circulans* xylanases by molecular cloning of the genes and expression in *Escherichia coli*. Appl. Environ. Microbiol. 55: 568 - 572.

Yang, S. Q. Yan, Q. J. Jiang, Z. Q. Li, L. T. Tian, H. M. Wang, Y. Z. (2006). High-level of xylanase production by the thermophilic *Paecilomyces themophila* J18 on wheat straw in Solid-state fermentation. Biores. Technol. 97: 1794-1800.

Yoon, K. H. Yun, H. N. and Jung, K. H. (1998). Molecular cloning of a *Bacillus* sp. KK-1 xylanase gene and characterization of the gene product. Biochem. Mol. Biol. Int. 45: 337 - 347.

Yoshioka, H. Nagato, N. Chavanich, S. Nilubol, N. and Hayashida (1981). Purification and properties of thermostable xylanases from *Talaromyces byssochlamydoides* YH - 50. Agric. Biol. Chem. 45: 2425 - 2432.

Yu, E. K. C. Tan, L. U. L. Chan, M. H. K. Deschatelets, L. and Saddler, J. N. (1987). Production of thermostable xylanase by a thermophilic fungus, *Thermoascus aurantiacus*. Enz. Microb. Technol. 9: 16 - 25.

Yu, Y. J. (2004). Present status and prospect of China's paper making raw material. Paper and Paper Making 23, (3): 17 - 20 (in Chinese).

Yuan, X. Wang, J. Yao, H. and Venant, N. (2005). Separation and identification of endoxylanases from *Bacillus subtilis* and their actions on wheat bran insoluble dietary fibre. Proc. Biochem. 40: 2339 - 2343.

Zalewska - Sobczak, J. and Urbanek, H. (1981). Cellulose and Xylan degrading enzyme of *Fusarium avenaceum* Archiev. Microbiol. 129: 247- 250.

Zechel, D. L. and Withers, S. G. (2000). Glycosidase mechanisms: anatomy of a finely tuned catalyst. Acc. Chem. Res. 33: 11 - 18.

Zhao, J. Li, X. Z. Qu, Y. B. Gao, P. J. (2004). Alkaline peroxide mechanical pulping of wheat straw with enzyme treatment. Appl. Biochem. Biotechnol. 112, (1): 13 - 24.

Zhao, J. Li, X. Qu, Y. (2006). Application of enzymes in producing bleached pulp from wheat straw. Biores. Technol. 197: 1470 - 1476.

Zhong, X. J. (2000). Marching onwards into the 21st century - Aprospective view of the Chinese pulp & paper industry. Paper presented at Fourth International Nonwood Fiber Pulping and Papermaking Conference. Jinan, Shandong Province, China. 1: 43 - 51.

Zhu, H. Paradis, F. W. Krell, P. J. Phillips, J. P. and Forsberg, C. W. (1994). Enzymatic pecificities and modes of action of the two catalytic domains of the XynC xylanase from *Fibrobacter succinogenes* S 85. J. Bacteriol. 176: 3885 - 3894.

Zverlov, V. Piotukh, K. Dakhova, O. Velikodvorskaya, G. and Borriss, R. (1996). The multidomain xylanase A of the hyper - thermophilic bacterium *Thermotoga neapolitana* is extremely thermoresistant. Appl. Microbiol. Biotechnol. 45: 245 - 247.