BIOBLEACHING : ENZYMATIC COLOUR REMOVAL FROM PULP

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CHAPTER I: INTRODUCTION

The worldwide charm of environmental friendliness has resulted in the development of innovative biotechnological processes in various processing industries. Biotechnology seems to be a major player in solving the major problems of pollution created by these industries. A vast pulp and paper industry exists around the world to supply an ever increasing demand of multitude of paper products. Paper manufacturing industries are considered to be the worst offenders as far as deteriorating health of the environment is concerned. Organochlorine compounds, mainly produced by reactions between residual lignin present in pulp and chlorine used for bleaching, have found to be toxic, mutagenic and nondegradable. This causes significant harm to the environments and release of spent liquors in water bodies represents the most important environmental problem posed by the pulp and paper industry. Till date, about 300 different chlorinated organic compounds in bleaching pulp mill effluents have been identified (Sharma *et al. 1997)*. About 200 of these include chlorinated resin acids, chlorinated phenolics and dioxin. These compounds have been classified as acidic, phenolic and neutral and are partly responsible for oxygen demand (BOD and COD), effluent colour, toxicity, mutagenicity and carcinogenicity. Untreated pulp and paper mill effluents can be extremely toxic to fish at a low concentration. Ministry of environment and Forest, Government of India has categorized the paper and pulp industry as one of the most polluting industries. Confronted with the market, environmental and legislative pressures, the pulp

and paper industry is modifying its pulp bleaching and effluent treatment technologies to reduce the environmental impact of mill effluents with the help of ECF (elemental chlorine free), TCF (total chlorine free) and TEF (totally effluent free) bleaching strategies. Chlorine gas and chlorinedioxide are very effective and selective for bleaching purposes and their substitution with other chemicals has been very difficult. Oxygen, chlorine dioxide, ozone, hydrogen peroxide and enzyme based technologies are being examined for replacing elemental chlorine based bleaching of pulp. Much work has been devoted to find alternatives bleaching agents such as oxygen, which is now widely used but requires considerable investments, or hydrogen peroxide, which is an expensive chemical to be used industrially. Despite good bleaching performance by ozone this technology is not very viable due to degradation of cellulose causing a loss of strength in final paper product. Advantage of using biological process is several folds. Products of biological origin are often biodegradable. The enzymes can be produced by fermentation process.

In response to growing concerns for environment, restrictions on release of waste bleach waters are becoming stringent, thus necessitating an urgent need to lower the impact of pulp bleaching on the environment. A suggested alternative for chlorine in bleaching is to use biological agents to degrade and remove lignin. Many fungi in nature are capable of degrading wood. The white rot fungi have been used for pulp bleaching but the process is too slow. Lignin peroxidase, first produced from *Phanerochaete chrysosporium* was considered a suitable candidate for the same.

Another promising area for improving bleaching technologies relies on an understanding of the relation between lignin and other cell wall components. Lignin is covalently linked to at least some of cell wall polysaccharides to from lignin-carbohydrate complexes, which are mainly responsible, for difficulties encountered in separating lignin from wood. Since hemicellulose results in the mutual dissociation of the latter two. Hence, hemicellulose removal leads to enhanced removal of lignin, thus reducing chlorine charge. Viikari *et al.* first reported in 1986 that endoxylanases decrease chemicals needed for bleaching kraft pulp. Many researchers (Paice *et al.* 1988, Clark *et al.* 1990) have confirmed and extended this observation and the technology is being commercialized. Hemicellulase treatments are effective on both hardwoods and softwoods, but they affect hardwood kraft pulps more (Vikari *et al.* 1990). Although promising results were obtained initially with mannanases (Clark *et al.* 1990, Buchert *et al.* 1992), they are not as effective as xylanases, even with softwood pulps (Saake *et al.* 1995).

There are at least two explanations for how xylanases and mannanases enhance pulp bleaching. The first model suggests that they increase access of bleaching chemicals to pulp fibers by removing precipitated xylan (Kantelinen *et al.* 1993). The uncoated fibers are then more susceptible to bleaching chemicals and lignin extraction. Essentially, this model proposes that xylan physically entraps lignin and chromophores in the pulp matrix. The second model suggests that hemicellulases release chromophores and lignin from the cellulosic pulp matrix by breaking covalent linkages between the hemicellulose and lignin moieties.

Rather than postulating a physical entrapment, it holds that residual lignin and chromophores are chemically bound in the pulp. Recent evidence supports the role of xylanase in breaking lignin-carbohydrate bonds (Wong *et al.* 1996), and Suurnäkki *et al.*1996 recently found that no extensive relocation of xylan to the outer surface occurs during pulping, so the occlusion model might not have a sound premise.

There is increasing evidence that chromophores arise at least in part from the degradation of hemicellulose rather than lignin (Zibiro 1990a 1990b). During kraft pulping, methylglucuronic acid and other hemicellulosic components are degraded to acidic chromophoric entities that remain bound to the xylan backbone. There are many different degradation and condensation products, and they are not fully characterized or documented. Degradation products of lignin and hemicellulose can cross-react with xylan and become bound into the hemicellulosic matrix. Hemicellulose hydrolysis can release the bound chromophores and lignin, but xylan removal, per se, is not good because it decreases the pulp yield, and if carried to an extreme, xylan removal can decrease pulp strength. So the objective is not to take out the xylan, but rather to remove the chromophores and lignin. The reason for using enzymes is to do that in a more specific, economical and environmentally benign manner.

1.1 Chemical structure of xylan: Plant cell walls have three major polymeric constituents: cellulose (insoluble fibers of β-1-4-glucan), hemicellulose (non-cellulosic polysaccharides including glucans, mannans, and xylans) and lignin (a complex poly phenolic structure). Xylan is the major hemicellulose in wood from

angiosperms but is less abundant in wood from gymnosperms. Xylan is also found in the primary walls of growing cells, seeds. Xylan is composed of a backbone of glycosidically β-1,4- linked xylopyranose units in terrestrial plants but in marine algae β-1,3- linked backbone are found (Dekker and Richards 1976). The mixed links β-1,3- and β-1,4- are found in certain seaweeds such as *Palmeria palmata* (Barry and Dillon 1940). Isolated β-1,4- xylans are generally polydispersed and highly branched (with more than one type of substituents) heteropolymers, though homoxylans that consists of exclusively of xylosyl residues have been isolated from esparto grass (Chanda *et al.* 1950). Xylan of hardwood where it contains 10-35% of dry weight is acetyl-4-O-methyl glucuronoxylan with a degree of polymerization of about 200. Approximately 10% β-D-Xylopyranose backbone units are substituted at C-2 with a 1,2 linked 4-O-methyl-α-D glucoronic acid residue while 70% are acetylated at C-2 or C-3 or both. The structures of oligosaccharides, isolated after the xylanase treatment of hardwood suggest that xylan has two kinds of xylose and β-1-4 xylobiose attached to main chain of 1,4 linked β-D-xylopyranosyl residues and both of them are branched through the O-3position of xylose residues of the main chain. Moreover the xylan also had β-1-4 xylobiose stubs that attached through the O-2 position of xylose residues of the main chain. Softwoods contain 10-15% xylan as arabino–4-O methyl glucuronoxylan with Dp of >120 (Plus and Schuseil, 1993). This material which is not acetylated contains β-D- xylopyranose, 4-O-methyl -α-D-glucuronic acid and L-arabinose in a ratio of 100:20:13. The 4-O-methyl glucuronosyl residues attached to C2 and L-arabinofuranosyl residues to

C-3 of the relevant xylopyranose backbone units. Xylan is categorized as linear homoxylan, arabionxylan, glucuronoxylan and glucuronoarabinoxylan. The O-acetyl groups present at C2 and C3 positions of xylosyl residues inhibit xylanases from completely degrading acetylxylan, probably by steric hindrance. So the synergistic action of acetylxylan esterases and xylanases necessary for complete hydrolysis of acetylxylan. The presence of small amounts feruloyl and p-coumaroyl acids linked via L-arabinose residue has been shown in xylan structure. The presence of covalent bond between lignin and hemicellulose perhaps through xylan substituents in many cases has been documented. Evidence for the existence of an ether linkage between arabinose and lignin and ester linkage between glucuronic acid and lignin has also been shown. Feruloyl groups may also crosslink xylan and lignin. The side chains determine the solubility, physical conformation and reactivity of the xylan molecule with other hemicellulosic components and hence greatly influence the mode and extent of enzymatic cleavage.

1.2 Xylan degrading enzymes:

The main enzymes responsible for the hydrolysis of xylan backbone are endo-β-xylanases, β-xylosidases and exo-β-xylanases. Besides the main chain cleaving enzymes some side chain cleaving enzymes also play an important role in xylan hydrolysis. Side chain cleaving enzymes include acetyl esterase, α-L-arabino furanosidase and α-D-glucuronidase and it has been reported that many of these enzymes act synergistically for xylan hydrolysis (Lee and Forsberg 1987).

1.3 Xylanase Classification

Xylanases can be classified at least three ways. The first, as suggested by Wong *et al.*.1988, is based on molecular weight and pI. They are either high or low molecular weight and have either a high (basic) or low (acidic) pI. Information for this sort of classification is readily obtained during purification and initial characterization. The second is based on crystal structure. This can be derived indirectly by a determination of DNA sequence. Xylanases can be structurally classified into family F or (now known as glycosidase family 10), and family G (now known as family 11). The third classification is based on kinetic properties, substrate specificity, or product profiles. Virtually all xylanases are "endo" acting, as readily determined by chromatography, but the more detailed determination of kinetic properties -- measuring the relative reaction rates on various substrates and determining the kinetics of intermediate product formation -- is much less common.

Classifications based on molecular weight and pI are necessarily related to those based on sequence, and sequence analysis can reliably predict crystal structure, but few studies have been performed that relate sequence or structural family to action patterns and substrate specificity. Even fewer studies have explored the specificity of hemicellulases with respect to substrate branching patterns or substitution.

Family 10 xylanases occasionally exhibit endocellulase activity; they generally have a higher molecular weight, and they occasionally will possess a cellulose-binding domain. Members of family 10 will act on both PNP-xylobiose and PNP-

cellobiose, however, the overall catalytic efficiency on PNP-xylobioside is about 50 times higher (White *et al.* 1994). This suggests that family 10 enzymes act mainly on xylan.

Even though all xylanases are endo acting, they can show variations in their product profiles. Some enzymes form predominantly xylose and xylobiose and others predominantly (or exclusively) form xylotriose and other higher oligosaccharide products. This difference appears to result from the number of substrate-binding subsites on the enzyme surface. The number of pyranose rings that the enzyme will bind effectively determines the nature of the oligoproducts.

The family 10 catalytic domain is a cylindrical [[alpha]]/b barrel resembling a salad bowl with the catalytic site at the narrower end, near the C-terminus of the [[beta]]-barrel (Derewenda *et al.* 1994, Harris *et al.* 1994). There are five xylopyranose-binding sites. Catalytic domains of these enzymes belong to a "super family" that includes Family A cellulases, [[beta]]-glucosidase, [[beta]]-galactosidase, [[beta]]-(1-3)-glucanases, and [[beta]]-(1-3, 1-4)-glucanases (Jenkins *et al.* 1995). Family 10 xylanases have relatively high molecular weights, and they tend to form oligosaccharides with a low degree of polymerization (DP).

Family 11 xylanases are true xylanases. They don't have cellulase activity; they consistently exhibit a low molecular weight, and they can have either a high or low pI. They are formed by both bacteria and fungi. Family 11 catalytic domains consist principally of [[beta]]-pleated sheets formed into a two layered trough that surrounds the catalytic site (Miao *et al.* 1994, Whithers *et al.* 1995). Protruding

down into the trough, and located toward one side of the protein is a long loop terminating in an isoleucine. Törrönen and Rouvinden 1995 have likened the trough to the palm and fingers and the loop to the thumb of a right hand. The positions of many amino acids are essentially identical in the family 11 xylanases from bacterial (*Bacillus circulans*) and fungal (*Trichoderma harzianum*) origins. Thus, there has been a tremendous conservation of the basic structure of the catalytic site of family 11 xylanases during evolution.

Two family 11 xylanases are produced by *Trichoderma*. Xyn1 has an acidic pI (5.5), possesses a smaller, tighter groove than Xyn2, and a lower pH optimum (Törrönen *et al.* 1992). It also exhibits a fifteen-fold higher turnover number (Tenkanen *et al.* 1992) and a three-fold lower Km than Xyn2. The latter has a basic pI (9.0), a more open structure, and a wider pH range. Xyn2 tends to produce larger oligo-saccharides. Both Xyn1 and Xyn2 release xylobiose in the retained [[beta]]-configuration, indicating that the product is transiently attached to the enzyme surface (Biely *et al.* 1994). The difference in pI between Xyn1 and Xyn2 is attributable to the presence of more lysine an arginine residues on the sides of the isoleucine "thumb" of the enzyme. The function of these charged groups is not well established, but they could assist in binding to acidic side chain substituents on the xylan backbone. Binding of *Trichoderma* xylanases to polysaccharides is affected by the pH and the ionic strength (Tenkanen *et al.* 1995). Enzymes are totally bound to xylan when the pH is below their pI, but are mainly unbound at pH values above the pI.

The pH optimum depends on properties of the acid/base catalyst (Törrönen *et al.* 1994). In Xyn1, this is Glu164; in Xyn2, it is Glu177. In Xyn1, Asp33 makes a strong hydrogen bond (2.9 angstroms) to Glu164, thereby lowering the pKa. In Xyn2, an asparagine residue (Asn44) takes the place of Asp33; the hydrogen bond is much longer (3.7 angstroms), and the interaction is weaker. All acidic pI xylanases of family 11 have an aspartic residue in this position; all basic xylanases have an asparagine residue.

The enzyme from *Bacillus* sp. V1-4 has a rather broad pH optimum extending well into the alkaline region. Other enzymes have even more alkaline optima. Pulpzyme HC, has a distinct optimal activity around pH 9 to 9.5.

1.4 Microbial source of xylanase enzyme:

Considerable progress has been made in the isolation of extremophilic microorganims and their successful cultivation in the laboratory. Commercial applications of the xylanase demand identification of highly stable enzymes active under routine handling conditions. Many advantages such as reduced contamination risk and faster reaction rates have been proposed for the use of thermophiles in biotechnology processes. In, general parameters such as temperature, pH and chemical and enzymatic stability for any enzyme. One of the ways to identify the industrially suitable xylanase preparations is to look for the enzymes from extreomophilic microorganims. For obtaining industrially relevant xylanase enzyme, two types of organism have been preferred and they are alkaliphilic organism and thermophilic organism. Alkaline xylanases gained importance due to their applications for the development of biobleaching of pulp.

The enzymes are able to hydrolyze xylan, which is soluble in alkaline solutions. The first report on xylanase from alkaliphilic bacteria was published in 1973 by Horikoshi and Atsukawa. The purified enzyme of Bacillus sp. C-59-2 exhibited a broad pH optimum ranging from 6 to 8. Many of the xylanases produced by alkaliphilic organisms such as *Bacillus* sp. (Okazaki *et al.* 1984) and *Aeromonas* Sp. 212 (Okoshi *et al.* 1985) with optimum growth at pH 10 showed remarkable stability at pH 9-10.The enzymes from Bacillus sp. TAR-1, C-125 (Nakamura *et al.* 1994, Honda *et al.* 1985) and alkaliphilic *Bacillus* sp.(NCL-86-6-10) (Balakrishna *et al.* 1992) were optimally active at pH 9-10. The xylanase from *Cephalosporium* was the only one reported from an alkaliphilic fungus having activity at broad pH range of 6.5-9 (Bansod *et al.* 1993).

The xylanases from thermophilic bacteria such as *Thermonospora fusca* (McCarthy *et al.* 1985) , *Bacillus stearothermophilus* (Khasin *et al.* 1993) show an optimum in the range of 65-80^{0}C. Even, thermostable xylanase produced by *Aspergillus* strain (Gilbert *et al.* 1993) grows at 37^{0}c has an optimum at 80^{0}C (Mendicuti *et al.* 1997). Thermophilic anaerobe *Clostridium stercorarium* has temperature optimum of 70^{0}C and half life of 90min at 80^{0}C whereas *Thermotoga* sp. xylanase has temperature optimum at 105^{0}C with half-life of 90 min at 95^{0}C (Simpson *et al.* 1991). However, fungal source of enzyme also shows higher thermal stability and it was reported that *Thermoascus auranticus* has been reported to be stable at 70^{0}C for 24 hr and half life of 54 min at 80^{0}C (Yu *et al.* 1987). Other source of thermophilic fungal xylanase are from *Paecilomyces variota* (Krishnamurthy and Vithayathil 1989) and *T. byssochlamydoides*

(Yoshika *et al.* 1981) and having enzyme optimum temperature of 65-75^{0}C at pH 5-6.5. Recently endoxylanases from thermophilic actinomycete *Microtetraspora flexuosa* SIIX found to have optimum temperature of 80^{0}C at pH 6 (Berens *et al.* 1996). Despite the prevalence of xylan degrading enzymes in actinomycetes , comparatively little information is available on xylanases from thermophilic actinomycetes.

Table 1.1 : Xylanases from extremophilic organisms:

	Optimum growth pH	Optimum temperature °C	Optimum activity pH	Optimum temperature °C
Thermophilic bacteria				
1. *Bacillus acidocaldarius*	3.5-4	65	4	80
2. *Bacillus licheniformis* A 99	7	60	7	50
3. *Bacillus stearothermophilus* T-6	7-7.3	60	9	65
4. *Bacillus stearothermophilus* no 21				
5. *Bacillus thermoalkalophilus*	7	55	7	60
6. *Clostridium acetobutylicum* ATCC 824				
7. *Clostridium stercorarium* HX-1	9	60	6-7	60,70
8. *Clostridium stercorarium* F-9	6	37		
9. *Clostridium thermolacticum* TC 21				
10. *Dictyoglomus thermophilum* strain B1	6-7	60	5	50
11. *Microtetraspora flexuosa*	6-7	65	6.5	75
12. Thermophilic *Bacillus* strain XE	6-7	65	6.5	75
13. Thermophilic *Bacillus* sp.			6.5	80
14. Thermophilic bacteria ITI 283, ITI 236	7	68		
15. *Thermobacterium* sp. JW/SL-YS485			7	80
16. *Thermotoga* sp. (Fjss3-B.1)	6	80		
17. *Thermotoga maritima* (MSB 8)	7	55	9	52
18. *Thermotoga thermarum*	7	65	6	75
19. *Thermomonospora curvata*	7.5	65	6.5-7	78
20. *Thermomonospora chromogena* MT814			8	80
21. *Thermomonospora fusca* BD 21	6	60		
22. *Thermomonospora fusca* YX	6.8-7	80	6.2	80
23. *Streptomyces thermoviolaceus* OPC-520	7	80	5.3	105-110
Thermophilic fungi	6-7	77	6.2	90
1. *Gloephyllum trabeum*	6-7	55	6	80
2. *Talaraomyces byssochlamydoides* YH 50	8	50	7.8	75
3. *Thermoascus aurantiacus*	8	50	5-8	75
4. *Thermomyces lanuginosus* DSM 5826			6-8	
5. *Talaromyces emersonii* CBS 814.7	8	50		65
Alkaiphilic bacteria			6-8	
1. Alkalophilic *Bacillus* 41M-1	7	50		70
2. *Bacillus sp.* TAR-1	6.2	50	7	70
3. *Bacillus* C-59-2	6	45	4	80
4. *Bacillus* C-125	6.5	50	5	70
5. *Bacillus* NCIM 59	4.5	45	5	75
6. *Aeromonas* Sp. 212			6.5	50
7. *Bacillus sp.* Ng-27	10.3	37	4.2	78
8. *Bacillus*	10.5	50	9	50
W1	8	37	9	70
W2	10.5	-	5.5-9	60
W3	10	50	6-10	70
W4	10	37	6	50-60
9. Bacillus NCL-87-6-10	9-10	27	7-8	50
	9-10	45-50	7	70
			6	
			7-9	65
			6	70
			6-7	65
	9.5	28	8	70
				60

1.5 Production of xylanase:

The production of xylanase enzyme depends on the medium composition as well as on the inducing substrate. Filamentous fungi are particularly interesting producers of xylanases since they excrete the enzymes into the medium and their enzyme levels are much higher than those of yeast and bacteria. However, fungal xylanases are generally associated with cellulases (Steiner *et al.* 1987). Selective production of xylanases by *Trichoderma* and *Aspergillus* species is possible by using pure xylan as substrate. On cellulose these strains produce both cellulase and xylanase which may due to traces of hemicellulose present in the cellulosic substrates. The mechanisms that govern the formation of extracellular enzymes with reference to carbon sources present in the medium are influenced by the availability of precursors for protein synthesis. Therefore, in some fungi, growing the cells on xylan not contaminated with cellulose under a low carbon/nitrogen ratio in the medium is one of the strategies adopted for xylanase enzyme production. However, cellulosic substrates are also found to be essential for maximum xylanase production by *Clostridium stercorarium* (Berenger *et al.* 1985), *Thermomonospora curvata* (Stutzenberger and Bodine 1992) and *Neurospora crassa* (Deshpande *et al.* 1986). Cheaper hemicellulose substrates like corncob, wheat bran, rice bran; rice straw; corn stalk and bagasse have also been found to be most suitable for the production of xylanases by certain organisms (Haltrich *et al.* 1996, Dey *et al.* 1992). The xylanase activity is found to be higher with fungi and maximum activity of 3350lu/ml was reported in

Trichoderma reesi (Haapala *et al.* 1994). Maximum activity in solid state fermentation was achieved with *Schizophyllum commune* (22 700 Iu/g) (Haltrich *et al.* 1992) . In case of *Trichoderma hamatum* is reported to produce 7000 IU /g using wheat straw as substrate (Grajek 1987). Number of cellulase free xylanase producer has been reported in *Bacillus* sp. and fungi (Dey *et al.* 1992, Gilbert *et al.* 1992, and Biswas *et al.* 1990). Actinomycetes and bacteria exhibit near neutral pH optima for growth and enzyme production in contrast to the generally acidic pH requirements of fungi.

The yield of xylanases in fermentation process is function of various key factors besides standard parameters. During growth of organism in complex media various factors have combined effect on expression of xylanase enzyme. Some of the key factors are substrate accessibility, rate and amount of release of the xylooligosaccharides and their chemical nature and quantity of xylose released- which acts as the carbon source as well as inhibitor of xylanase synthesis. Generally slow release of the inducer molecules and the possibility of the culture filtrate converting the inducer to its non-metabolizable derivatives are believed to boost the enzyme production.

Xylanases generally bind tightly to the substrate as result of which some of the enzyme produced is lost during fermentation as bound enzyme. The metabolic enzymes of xylanase producer such as proteases (Penbroke *et al.* 1992) and transglycosidases (Hrmova *et al.* 1991) also effect the actual yield and these enzymes are generally expressed in the exponential phase. Due to these phenomena, time of harvesting is a very important parameter in xylanase

production. *Bacillus licheniformis* A99 has been used for xylanase production in solid state fermentation (Archana and Satanarayana 1997) .

1.6 Regulation of xylanase synthesis:

Despite vast knowledge of microbial xylanolytic systems, proper understanding on induction and secretion of xylanases by xylanase producer is necessary. Generally it was reported that xylanase production by Bacteria and fungi are inducible. However, rare examples of constitutive xylanase expression have also been reported (Srivastava and Srivastava 1993).

1.7 Xylan hydrolysis: The enzymes responsible for xylan hydrolysis are endoxylanase, β-xylosidase, and xylan debranching enzymes such as acetyl esterase, α-L-arabinofuranosidase, and α-D-glucuronidase. The cooperative effect of different xylanases on polymeric xylan substrates has been reported (Deshpande *et al.* 1986, Wong *et al.* 1986, Rana *et al.* 1996)). The cooperative effects can either be synergistic or additive. An example of the additive cooperativity is exhibited by xylanases $X_{34}E$ and X_{22} from *Bacillus polymyxa* (Morales *et al.* 1993*)*. The cooperative effect of xylanase I and II from *Neocallimastix frontalis* was also reported where combined action of both from releases 15% more reducing sugars than the individual enzyme. Besides, oligosaccharides of smaller size were produced which is due to synergistic action. Coughan and Hazelwood 1993 have classifieds the synergistic interactions into four categories namely homo synergistic, hetero synergistic, uniproduct synergy and multi product synergy. The extent of xylan hydrolysis by the combined action of the main chain and the side chain-cleaving enzymes

exceed, in most cases, the sum of that achieved by each enzyme acting alone. The product of xylan hydrolysis have been analyzed and it was reported that main hydrolysis products are xylobiose in case of *Cephalosporium* sp. RYM 202 (Kang *et al.* 1996), xylobiose and xylotriose in case of *Cellulomonas fimi* (Clarke *et al.* 1996) and *Aureobasidium pullulans (Li et al. 1993), Bacillus circulans, Bacillus polymyxa, Bacillus pumilis, Microtetraspora flexuosa.* Some of the xylanases responsible for releasing oligosaccharides larger than X_3 belonged to *Aeromonas caviae* W-61, *Aureobasidium pulluans, Chaetomium cellulolyticum, Neocallimastix.* In some cases, xylose was also released due to xylanase action and it was observed that small sugars were detected only on incubating the polymeric carbohydrate with endosplitting hydrolytic enzyme for long duration.

1.8 Characterization of xylanases:

Endoxylanases are the best-characterized hemicellulases. An examination of xylanase multiplicity in *Bacillus sp.* suggested that these bacteria produce two types of xylanases: one is basic (pI 8.3-10.0) with low molecular weight (16,000-22,000) and the other is acidic (pI 3.6-4.5) with high molecular weight (43,000-50,000). Microbial xylanases are single subunit proteins with molecular masses in the range of 8-145kDa (Suna and Antranikian1997). The optimum temperature for endoxylanases from bacterial and fungal sources varies between 40 and 60^{0}C. Fungal xylanases are generally less thermostable than bacterial xylanases. Fungi, which are mesophilic in origin but produce thermostable xylanases, include *Ceratocystis paradoxa,* the xylanase of which is stable at 80^{0}C for 1 hour (Dekker and Richards 1975). A low temperature active enzyme

having both carboxymethyl cellulase and xylanases activities from *Acremonium alcalophilum* JCM 7366 has been reported recently. The xylanases and cellulases activities at 0^0C were 25 and 48.8% respectively, of their activities at the optimum temperature of 40^0C(Kazuya *et al.* 1997). D-Xylanases from different organisms is usually stable over a wide pH range (3-10) and show optimum pH in the range of 4-7. The xylanases from fungi such as *Aspergillus kawachii* (Ito *et al.* 1992) and *Penicillum herque* (Funaguma *et al.* 1991) exhibit optimum pH towards acidic side (pH 2-6). The isoelectric points for endoxylanases from various sources ranged from 3 to 10. Generally, bacteria are known to produce two xylanases but this phenomenon is not observed with fungi.

1.9 Measurement of xylanase activity:

Xylanase activity has been assayed by various methods, which includes colourimetric, turbidometric and viscometric methods. Colourimetric method is the most commonly used and range of substrate has been utilized. Some of substrates are beech wood xylan, birchwood xylan, carboxymethyl xylan, larchwood xylan, oatspelt xylan, aspenwood xylan, ricestraw arabino xylan and wheat straw glucuroxylan (Hrmova *et al.* 1991, Lie *et al.* 1993, Irwin *et al.* 1994, Biely *et al.* 1980, Maheswari and Kamalan 1985, Deshpande *et al.* 1990, Adhi *et al.* 1989, Tan *et al.* 1985, Kimura *et al.* 1995, Agosin *et al.* 1987). In addition to the variation in substrate used, different groups has assayed xylanase activity on the basis of different reaction time and detection procedure of reducing sugars. Reaction time of 5 min to 30 min has been reported and as high as 2 hr has also been used (Lee at al. 1993). In order to detect the reducing sugars, generated by

xylanase action, mainly three methods have been used mainly Nelson method, Somogyi method and Dinitro salicylic acid. On the basis of interlaboratory testing Bailey *et al.*. (1992) have suggested that birchwood 4-O-methyl glucuroxylan should be used in all the xylanase assays owing to low turbidity of 1% substrate solution, extended range of linearity of the reaction on using the above substrate and its commercial availability. Bailey *et al.* 1992 have suggested that enzyme : substrate ratio should be 1:9 and incubation time should be 5 min. Reducing sugar detection should be done involving dinitrosalicylic acid method and after addition of DNS the mixture should be boiled for 5 min. The use of turbidometric and viscometric methods though are not common but has also been reported (Nummi *et al.* 1985).

1.10 Molecular weight:

The molecular weight of majority of purified xylanases fall between 11-85 kDa (Wong et al 1988) although xylanases with molecular weight as high as 537 kDa (Matte and Forsberg 1992) and as low as 5.5 kDa (Bastawade et al 1992) has also been reported. It has been observed that in case of multiple xylanase-secreting organism mostly the low molecular weight xylanase is the dominant one . Comparative analysis of both high and low molecular weight xylanase reveals that high molecular weight xylanase shows greater catalytic versatility due to greater conformational flexibility (Biely *et al.* 1993). Low molecular weight xylanase are generally well packed and form βsheets and due to structural rigidity they don't posses conformational flexibility. Some of the xylanases especially those having large molecular weights are found to possess more than

one subunits which are either are of same molecular weight or of different molecular weights. The main methods for molecular weight determination are gel filtration and SDS-PAGE. On comparing the molecular weights in SDS-PAGE and gel filtration one can have information on the number of subunits.

1.11 Substrate specificity:

Xylanases have been found to have different specificities on different kind of xylans. The substrate specificities of the xylanases, produced by a microorganism may be same or different on various substrates. *Streptomyces* sp. B-122 showed the maximum specificity on birchwood xylan and minimum on acetyl glucuronoxylan. Moreover, it was observed that although no arabinose releasing property was present in xylanase 2 of *Streptomyces* sp. yet it showed maximum activity on arabinoxylan. Since the degree of substitution is directly proportional to solubility, the preferences of more substituted xylans by some xylanases could be due to higher solubility. But the overall hydrolysis of unsubstituted xylans is surprising in view of the fact that substitutents on the backbone known to hinder the efficient hydrolysis of xylans.

1.12 Thermal and pH stability:

The thermal and pH stability of a xylanase is highly important effective applications in biobleaching of pulp. Thermostability of proteins can be attributed to subtle changes in hydrophobic interactions, hydrogen bonds, extra disulphide bonds and salt bridges (Klibanov *et al.* 1983, Utpal *et al.* 1990). Generally, xylanase pH stability lies in the range of 3.9-8 (Marui *et al.* 1985). However, *Bacillus* sp. Bp-23 (Blanco and Paster 1993) has been reported to be stable at

pH 10 and that of *Bacillus* sp. W1 and W2 in the broad pH range of 4.5-10 (Okazaki *et al.* 1983). Recently, xylanases CXI and CxII from *Cephalosporium* sp. have been reported to be stable over a wide pH range 5.5-12 (Kang *et al.* 1996). Stability at one of the highest pH value (11-12) has been reported for *Bacillus* sp.(Honda *et al.* 1985) whereas stability at one of the lowest pH values (75% activity at pH 1) has been reported for the xylanase of *Cryptococcus* sp (Fefugi *et al.* 1996).Thermostability of enzyme has been defined interms of half-life of enzyme and T*hermotaga* sp. is active at a very high temperature of 105^0C and its half life at 105^0C was 15-20 min and 90 min at 95^0C(Simpson *et al.* 1991). Half life of 1 hr was reported for xylanase of *Thermoanaerobacterium* sp at 70^0C and 14-16 h at 50^0C for xyl-I and Xyl-II of *Streptomyces* sp. A451 (Shao *et al.* 1995). The xylanase of *Thermomyces lanuginosa* has been reported to have half life of 96 h at 60^0C (Cesar and Mrsa 1996).

Table 1.2 :Thermostability of xylanases

Organism	Incubation Temp	Half life in min
Bacillus thermoalkalophilus	65	60
Dicrtyglomus strain B1	90	78
Thermoanaerobacterium sp. JW/SL-YS 485	70	60
Thermoascus Aurianticus	70	480
Thermoascus aurianticus	80	54
Thermophilic fungus	85	45
Thermotaga sp. Fjsss- B1	95	90
Thermotoga sp.	105	20

In general, extracellular enzymes are more stable than intracellular enzymes (Bragger *et al.* 1989). Some of the methods used to stabilize proteins include attachment on solid supports as multiple points, cross linking by covalent disulphide bridges, cross linking by noncovalent slat bridges, pressure stabilization, and substitution of specific aminoacid residues in the protein and chemical modifications. Besides addition of some divalent cations and anions, addition of salts such as ammonium sulphate and addition of some substances like BSA, gelatin, glycerol, polyethylene glycol etc, can also confer thermostability

to enzyme. Thermostability of xylanase from *Thermotoga* sp. FjSS3-B1 was improved by its immobilization onto porous glass beads and half-life was improved by five times (Simpson *et al.* 1991).

1.13 Kinetic parameters:

The most important kinetic parameters K_m and maximum velocity are measured for most of the purified xylanases. This value can be of various use like improving assay conditions and evaluation of various enzymes with respect to affinity towards a substrate. The values of K_m which depend on the substrate used for xylanase action generally are in the range of 0.1-14mg/ml (Dekker and Richards 1976, Woodwards *et al.* 1984, Anand *et al.* 1990, Khasin *et al.* 1993, Kromelink *et al.* 1993, Vakamura *et al.* 1993). One of the lowest K_m value (0.14 mg/ml) has been reported for the xylanase Xln2 of *Trochederma reesei* (Torronen *et al.* 1992). The $K_m$value of 22.3 mg/ml (on glucurono xylan) for 19 kDa xylanase from *T.reesei* was the highest reported value. In addition to a high K_m the xylanase of *Bacillus polymyxa* showed substrate inhibitions at concentrations exceeding 32 mg/ml (Morales *et al.* 1995).

Comparison of V_{max} values of various xylanases is difficult due to nonuniformity in units. Many researchers have also calculated the values of K_{cat} the enzyme turnover number which is directly proportional to V_{max}.

1.14 Purification of xylanases:

Purified xylanase is a prerequisite for various biochemical studies. Various methods have been adopted to purify xylanase. Mostly, xylanases are extracellular but intracellular enzyme has been reported for *Bacillus firmus*,

Bacteroid amylogens, *Bacteroids ruminicola*, *Ruminicoccus flavefaciens* (Hespell *et al.* 1988). Cell associated xylanases has also been reported for *Thermoanaerbacterium* sp. (Shao *et al.* 1995) Although extracellular enzyme purification is comparatively easier than intracellular enzymes but it also involves multistage purification.

In the initial stage, removal of polyphenols that is generated during growth of organisms is necessary as it interferes in the ionexchange process. This step has been achieved by various means and some of the examples are use of bioprocessing acid, precipitation with polyethylenimine etc.

After clarification of crude extract xylanase purification is initiated by concentration of crude extract and this has been achieved by precipitation with ethanol, acetone, iospropanol and ammonium sulphate (Flaningan and Sellars 1978, Paice *et al.* 1978, Anand *et al.* 1990, Tsujibo *et al.* 1990, Tuohy *et al.* 1993, Honda *et al.* 1985, Okazaki *et al.* 1985, Biswas *et al.* 1990). In addition to concentration, ammonium sulphate has been used to bring about purification by adjusting its concentration in the crude extract. Besides these concentration has also been accomplished using polyethyleneglycol and ultrafiltraion (Honda *et al.* 1985, Dahlberg *et al.* 1993).

Purification involving various chromatography matrices is an integral part of standard xylanase purification protocol. These matrices based purification has been carried out in specialized chromatography columns. Both cation and anion exchangers have been used for xylanase purification but anion exchange based purification is more common with xylanase purification. Some of the frequently

used anion exchange based matrices are DEAE-Sepharose (Tenkanen *et al.* 1992, Shao *et al.* 1995), Q-Sepharose(Shao *et al.* 1995), QAE-Sephadex (Anand *et al.* 1990) and DEAE-Toyopearl (Okazaki *et al.* 1985, Nakamura *et al.*1993) Cation exchange based matrices used in xylanase purification are CM-Sepharose (Tan *et al.* 1985, Tenkanen *et al.* 1992) SP-Sephadex (Anand *et al.* 1990, Okazaki *et al.* 1985, Khasin *et al.* 1993).

Gel filtration materials have also been used for xylanase purification and they are Biogel, Sephacryl, Sephadex and ultrogel (Khasin *et al.* 1993). However in all the cases gel filtration has been used in combination with other column chromatographic procedures.

Adsorption chromatography has also been used for xylanase purification. Biogel HPHT hydroxyaptite column chromatography has also been used to bring about 66 fold xylanase purification (Simpsol *et al.* 1991).

In some cases hydrophobic chromatography has also been used with most commonly used groups are phenyl and n-octyl. One-step affinity based purification of xylanase has been reported for *Thermoanaerobacterium saccharolyticum* B6A-R1 xylanase using xylan coupled Sepharose Cl-4B (Lee *et al.* 1993). Similarly, affinity based adsorbent has been prepared by crosslinking oatspelt xylan with epichlorohydrin in aqueous NaOH (Rozie *et al.* 1992). Using the above adsorbent 80 fold purification has been achieved which is due to electrostatic interactions. An affinity based matrix Cellobiose-Sepharose –6B (Teunissan *et al.* 1993) has been reported to indirectly improve xylanase purification by removing β-glucosidase.

Generally various combinations of column chromatography procedures have been adopted for purification of xylanases from diversified sources. Tan *et al.* 1985 have reported the purification of two xylanases from *Trichoderma harzianum* in which 20kDa xylanase was purified 4.45 times after CM-Sepharose and Phenyl-Sepharose chromatography whereas 29Kda xylanase was purified 0.9 fold using CM-sepharose and Sephadex G-75 column chromatography.

Using gel filtration-ionexchange-hydrophobic chromatography chromatofocusing sequence 13 xylanases have been purified from the crude extract of *Talaromyces emersonii* whereas in ion exchange-ion exchange- hydrophobic chromatography- gel filtration- chromatofoccusing sequence of protein separation purified 350 kDa xylanase from *Thermoanaerobacterium* sp. by 340 fold (Shao *et al.* 1995). Ammonium sulphate precipitation- ion-exchange- ion exchange – gel filtration purification strategy has purified four xylanases, namely W1-I (100 fold), W1-II (96 fold), W2-I (333 fold) and W2-II(135 fold) from Bacillus sp. W1 and W2 (Okazaki *et al.* 1985) respectively. Gel filtration- hydrophobic chromatography – ion exchange scheme purified xyl –III xylanase of *Talaromyces emersonii* 1590 fold (Tuohy *et al.* 1993). Using a series of chromatographic procedures Gorbacheva and Rodionova 1977 achieved 5000 fold purification of xylanase from the culture filtrate of *Aspergillus niger.*

1.15 Protein engineering:

Biotechnological applications of the xylanases require thermostability with wide temperature and pH range. However, availability of ideal enzymes with all desirable characteristics is limited so application of protein engineering is imminent. Protein engineering studies is also a means of identifying active site residues and its role in catalysis. Site directed mutagensis provide means to redesign the protein with desirable characteristics. Identification of active side residues by chemical modifications, X-ray crystallographic data and site directed mutagensis has provided basic information regarding the structure-function correlation of the xylanases. These studies have formed the basis for the protein engineering of xylanases for specific manipulation of the gene for desired enzymatic properties. Identification of active site residue can be achieved my amino acid modification, active site peptide and X-ray crystallographic studies. Amino acid modification is usually carried out chemically or by site directed mutagensis. Chemical modifications, using group specific reagents, may suggest the type of residue involved in catalysis or in substrate binding. Substrates and competitive inhibitors, which can bind to the active site frequently, protect the enzyme against inactivation. The participation of trytophan in the active site of xylanases from *Chainia* (Deshpande *et al.* 1990) and *Streptomyces* (Keskar *et al.* 1989) has been reported. Chemical modifications of xylanases from the fungus *Schizophyllum commune* (Bray and Clarke 1990) and an alkaliphilic thermophilic *Bacillus* sp. (Chauthaiwale and Rao 1994) indicated the involvement of carboxyl groups in the catalysis. The presence of cysteine in the active site of a few

bacterial xylanases has been reported. However, the role of such residues has not been addressed. In case of xylanases, inhibitors can be used to identify the active site residues. The characterization and sequencing of the cystein containing active site peptide of the xylanase from *Streptomyces* T-7 (Keskar *et al.* 1992) and *Chainia* (Rao *et al.* 1996) have been reported. The peptides showed the presence of a conserved aspartic acid residue consistent with the catalytic regions of other glucanases.

The three dimensional structures of low molecular mass xylanases (family 11, M_r 20) from *B. pumilus* (Katusbe *et al.* 1990) *B. circulans*, (Campbell *et al.* 1993) *T. harzianum*, thermophilic *Bacillus* sp. (Pickersgill *et al.* 1993) and *B. stearothermophilus* T-6 (Anna *et al.* 1997) have been reported. These studies have helped to determine the overall structure of xylanases, in possible identification of specific residues involved in substrate binding and catalysis.

F10 catalytic domain is a cylindrical [(α)/ (β)] barrel resembling a salad bowl with catalytic site at the narrower end, near the c terminus of [(beta)] barrel. There are five xylopyranose binding sites. Catalytic site domains of these enzymes belong to a "superfamily" that includes Family A cellulases, [(β)]-glucosidases, [(β)]-galactosidase, [(β)]-1,3- glucanases. F10 xylanases have relatively high molecular weights, and they tend to form low DP oligosaccharides. Based on Cex from C. fimi, the overall structure resembles a tadpole with a catalytic (N-terminus) "head" and a cellulose binding domain (C-terminus) "tail".

F11 catalytic domains consist principally of β- pleated sheets formed into a two layered trough that surrounds the catalytic site. Protruding down into the trough,

and located toward one side of the protein is a long loop terminating in an isoleucine. The positions of many amino acids are essentially identical in the F11 xylanases from bacterial and fungal origins. The *Trichoderma* enzyme, however is more complex. It has at least one extra course of β-pleated sheets.

1.16 Site directed mutagensis for altered desirable properties:

Commercial applications of xylanases in biobleaching need highly active and thermostable enzymes. Site directed mutagensis of the cloned gene offers interesting research opportunities to change the properties of protein suitable for its application. In the case of xylanase from *S. lividans* 1326 (Moreau *et al.* 1994) the thermostability is increased by replacing Arg^{156} by glutamic acid. The modified enzyme has shown temperature optimum 5^0C higher than that of the wild type. The half life of Arg^{156} Glu was 6 min higher than that of wild type of enzyme, suggesting that although the engineered xylanase had a higher optimal temperature, the stability was not significantly affected. Previous report suggested that the same substitution occurs naturally in the xylanases produced by *Bacillus* sp. C-125 (Luthi *et al.* 1992) and *C. saccharolyticum.* Both xylanases have an optimum temperature of 70^0C, which is the same for the modified enzyme. The introduction of disulphide crosslinks into proteins, to protect them from unfolding, requires the creation of cystein residues that form disulphide bonds spontaneously in solution and do not obstruct functional domains. In *B. circulans* (Wakarchuk *et al.* 1994) mutant xylanase proteins, disulphide bridges conferred thermoprotection as observed by 15^0C increase in thermostability.

Even though protein engineering is the most powerful tool to redesign the protein, the reports on xylanases to date suggest that it have not been fully exploited to improve the properties of xylanases. The future scope includes alternation of the residues to improve the pH stability and shift in pH optimum of the enzyme for its commercial applications at alkaline pH.

1.17 Applications of xylanases in pulp and paper technology:

Xylanases from different organisms have been evaluated for their interaction with various kinds of pulps. On the laboratory scale xylanases from *Streptomyces roseiscleroticus* (Patel *et al.* 1993), actinomycetes (Davis *et al.* 1992), *T. harzianum* (Senoir *et al.*.1988) and *Humicola* sp (Silva *et al.* 1994) have been used for enzymatic pulp treatment to test their bleach boosting abilities. The biotreatment of bagasse pulp using xylanase from an alkanophilic *Bacillus* sp. and subsequent peroxide bleaching resulted in a Kappa number by 10 points and an increase in brightness by 2.5% (Kulkarni and Rao 1996).Recently xylanase enzyme from *Thermotoga mari* tima was compared with commercial pulpzyme Hc and was found to be efficient in releasing lignin from kraft pulp (Chen *et al.* 1997). The cloned xylanase expressed in *Bacillus cereus* (Tremblay and Archibald 1993) and *E. coli* (Paice *et al.*. 1988) have also been reported to improve the delignifications of unbleached kraft pulps. Many alkali tolerant strains of *Bacillus* produce xylanase with pH optima around 9 and have been used for biobleaching. Thermostable xylanase produced by *Dictyoglomus* sp. has been evaluated for its suitability in pulp bleaching (Ratto *et al.* 1994). Thermostable xylanase from *Bacillus stererothermophilus* T-6 bleached the pulp effectively at

65^0C and pH 9 and has been industrially used in successful mill trial (Lapidot 1996) . The first commercially available xylanase enzyme was marketed by Novo Nordisk A/S under the barnd name of "pulpzyme HA" has been produced by a strain of *T. reesei*. Later on new enzyme from bacterial source has been marketed under the same brand name. Another enzyme manufacturer Sandoz chemicals also marketed "Cartazyme HS" for same purpose. Similarly Indian manufacturers also produced indigenous enzyme for pulp bleaching. Recently three commercial enzymes namely Ecopulp (from Alko-ICI), Cartazyme NS-10 (from Clariant) and Pulpzyme Hc (From Novo Nordisk) were tested on Eucalyptus kraft pulps and results indicated significant decrease in consumption of ClO_2 and H_2O_2 (Vicuna *et al.* 1997).

Mill scale trial has first been used in Finish forest companies in 1988 and since 1991 this methods has been continuously used on industrial scale in Finland. The chlorine requirements in prebleaching has been shown to be reduced by 20-30% and as a result of which AOX load of the effluents has reduced by 15-20%. The greatest number of mill trials has been carried out in Europe mainly in Scandinavia where most of the kraft pulp is produced.

Recently total chlorine free bleaching methods has been adopted along with enzyme. In TCF bleaching sequences the addition of enzymes increases the final brightness value which is key parameter in marketing chlorine free pulps (Farell *et al.* 1996).

1.18 Desirable Enzyme Characteristics for pulp bleaching

Several features determine which xylanases are effective for bleaching. First, it is desirable to have a low molecular weight xylanase that is able to penetrate the fiber. An enzyme must have access to the substrate if it is going to release chromophoric groups and lignin. Second, it must have an appropriate pH optimum. Kraft pulps are alkaline and the enzyme must be able to function at the ambient pH. Third, the enzyme should have an alkaline pI in order to bind to negatively-charged fiber surfaces. Fourth, the enzyme must be thermally stable at the temperature prevailing in the pulp stock. Fifth, it should possess appropriate substrate specificity. The objective is to release chromophores and extract residual lignin, not remove the bulk xylan.

Let us consider the molecular weight requirement in the light of the first mechanistic model for xylanase bleaching. Xylanases are proposed to remove precipitated xylan thereby enhancing the access of chemicals to pulp fibers and increasing bleachability. That is to say, xylanases with approximate diameters ranging from 10 to 30 angstroms are proposed to increase the accessibility of chlorine, which has an approximate diameter of 1 angstrom. Given this size comparison, it seems likely that chlorine would penetrate more readily into pulp than any enzyme, and that xylanases could not significantly enhance its access. The micropore structure of kraft pulp is on the order of 30 to 50 angstroms and is sufficient to allow enzyme diffusion.

Enzyme penetration into pulp, however is not simply determined by molecular weight or porosity. Some xylanases possess cellulose binding domains that

promote substrate adhesion and limit the rate of enzyme diffusion. Other enzymes, once absorbed, do not readily dissociate from the substrate, and their actions become localized. Both of these factors favor maximal enzyme action on the surface of the fibers, and various experiments suggest that enzyme action is greatest on the outer layers.

Perhaps the better question is whether precipitated hemicellulose forms a physical barrier that prevents the subsequent extraction of residual lignin. What we know from chemical analysis of enzymatically digested kraft pulps is that covalently-bound lignin or chromophores generated during the kraft cook (Gierer *et al.* 1984) cross-react with the hemicellulose (and in some cases between the lignin and cellulose), and that those linkages must be broken in order to extract the residual lignin and to remove the chromophores. Hemicellulases liberate residual lignin and kappa by breaking covalent lignin-carbohydrate linkages, and thereby increasing extraction. The more residual lignin and chromophores one can remove by extraction, the less chlorine (or other oxidant) is required.

Enzyme penetration throughout the cellulose fiber is essential in order to fully access and extract residual lignin. Complete penetration might not be achieved in a single enzyme application. Enzyme penetration is determined in part by molecular weight, but other factors such as substrate binding may be more critical. High substrate affinity may be a positive factor when enzymes are applied to pulps in a dilute suspension, (because they would keep the enzyme in the pulp rather than the free solution) but at the relatively high consistencies used

in bleaching plants (>10% total solids), they could work against enzyme diffusion to the interior of the fiber.

The second important characteristic, at least with respect to kraft pulp, is that the enzymes should have an alkaline pH optimum. If one uses a xylanase with an acidic pH optimum, it is possible to wash most of the alkali out of the pulp then neutralize the residual with sulfuric acid. Not much acid is required, but alkali keeps leaching out of these pulps even after extensive washing and pH adjustment. In fact, enzyme activity actually enhances the leaching of the alkali, and so one can observe a constantly increasing pH in the bulk solution. Moreover, neutralization may be uneven, and local pH may be critical. In order to maximize effectiveness, it is essential for the enzyme to act in the interior of the fiber where the residual alkali concentration is highest, so an alkaline pH optimum is very important.

A third feature that' s useful in a xylanase is an alkaline isoelectric point. With an alkaline pI, the enzyme binds more readily to the fibers under the pH prevailing in the pulp, and this is important for determining its ability to attack the substrate. Pulp fibers are negatively charged due to the presence of sugar acids, and if an enzyme has an alkaline pI, and hence a positive charges at the operational pH, it will bind more effectively.

The fourth factor, thermal stability is not generally a problem for xylanases. Most microbial xylanases are stable at 50-60^{0}C, and this is within the range of prevailing temperatures in pulp mills. The combination of alkaline and thermal stability, however, is more difficult to come by.

The final factor, substrate specificity, is not well understood with respect to pulp bleaching and is the principal subject of ongoing studies. We know that the best prebleaching enzymes are xylanases, but these enzymes differ significantly in their effectiveness.

1.19 Chromophore Release

Some xylanases release chromophores from pulps in rather significant amounts. It was first observed with xylanases derived from *Streptomyces* (Patel *et al. 1993).* The crude enzyme mixture releases material that absorbs very strongly in the UV and also into the visible region. The latter absorptivity shows up as a color that one sees in the pulp.

Chromophore release correlates with enzyme dose over the effective range of enzyme addition. In fact we can also see that the kappa number following enzyme treatment and alkali extraction decreases with the enzyme dose. Absorptivity is much greater in the UV region than in the viable region, so monitoring UV absorptivity is a sensitive means to determine the extent of enzyme action on pulp. Effective xylanases show a high ability to release chromophores.

CHAPTER II : Materials and Methods

In this project, xylanase enzyme production for biobleaching of pulp has been attempted with *various* isolated cultures, most of which are bacterial cultures. *Melanocarpus albomyces* IIS 68 which is a fungal culture and known producer of xylanase has been obtained from IIT Delhi. All the other cultures used in this work have been isolated from various sources and method for isolation will be described in the next section. As it is well known that bacterial cultures are more suitable for biobleaching of pulp due to their optimum pH which is mostly in the alkaline range. Method of isolation is usually enrichment culture technique and source of carbon is Birchwood xylan, which is also known as an inducer for xylanase production. The media used for isolation are ML-30 and M-9 which are usually used for cultivation of bacterial cultures. As we are interested in isolating alkalophilic culture so culture medium used in the present medium has pH above 8 and temperature of incubation was 50^{0}C. The composition of ML-30 and M-9 are as follows.

Modified ML-30	**M-9**
KH_2PO_4 1.37 g/l $(NH_4)_2SO_4$ 2g/l Peptone 5g/l Yeast extract 5g/l $MgSO_4, 7H_2O$ 0.2 g/l $FeSO_4, 7H_2O$ 0.005 g/l Birchwood xylan 10g/l Temperature 50^{0}C pH 8-8.5	Na_2HPO_4 6g/l KH_2PO_4 3g/l NH_4Cl 1g/l NaCl 0.5 g/l Birchwood xylan 10g/l $MgSO_4, 7H_2O$ 1ml/l Thiamine, HCl 1ml/l $CaCl_2, 2H_2O$ 1ml/l *Stock solution*: $MgSO_4, 7H_2O$ 246.5g/l Thiamine, HCl 10mg/l $CaCl_2, 2H_2O$ 14.7g/l pH 9.5-10 Temperature 50^{0}C

Standard screening and isolation techniques have been followed in all the cases. Initially, soil or sludge samples of measured quantity has been added in sterilized liquid media and incubated in shaker for appropriate period of time at suitable temperature (50^0C). After incubation for suitable period of time (Generally 48-72 hrs), liquid broth suitably diluted and plated on agar plates (Having the same medium composition as the liquid culture) and incubated at the same temperature till growth appears in the plate. This process of plating from one stage to other has been carried out for several times till pure cultures were not obtained. Purity of the culture was checked by microscopic and morphological observations. Once pure cultures was obtained it was further characterized by microscopic observation. As these cultures were grown in xylan agar plates so probability of obtaining suitable xylanase producer is much higher. Confirmation of xylanase production has been done by culturing pure isolates in liquid media (having the same composition as the agar plates) at suitable conditions. Liquid broth has been analyzed for xylanase activity and filter paper activity for deciding on the suitability of the isolate for biobleaching applications.

Xylanase activity determination:

Xylanase assay: The substrate solution was prepared by adding the finely powdered birchwood xylan (SIGMA) (1% w/v) to appropriate buffer (50 mM) (preheated at 60°C for 10 min) and stirring the mixture at 25-35°C for 10 min. Birchwood xylan formed a suspension which was stirred uniformly to distribute the xylan in the buffer.

Xylanase activity was measured by adding 0.2 ml of appropriately diluted enzyme solution to 1.8 ml preheated birchwood xylan suspension in buffer. The reaction mixture, having a final substrate concentration of 9.0 mg/ml, was incubated for 15 min at 55 °C or at the optimum temperature. The incubation time used in the present study was generally either 15 min at 55°C or 5 min at 70°C. The reaction was terminated by adding 3 ml of DNS reagent and boiling the reaction mixture for 10 min in a boiling water bath and immediately cooling it in melting ice. The undegraded xylan was removed by centrifugation at 2000 rpm at room temperature for 5 min. The reducing sugars generated by xylanase action were estimated in the supernatant, by deducting the A_{540} value of enzyme blank (heat inactivated) from the absorbance value of enzyme assay sample. The xylanase activity was measured in terms of international unit. One international unit (IU) of xylanase is defined as one µmole of xylose produced by 1 ml undiluted enzyme in 1 min. The µmoles of xylose produced by xylanase, were deduced from xylose standard plot.

Filter paper activity:

Filter paper activity was measured by using the method recommended by IUPAC using filter paper as a substrate. A suitably diluted enzyme solution (0.5ml) was mixed with 1 ml buffer at 50 ° C. One filter paper strip (6X3 cm) was added to the enzyme solution and the reaction mixture was incubated at 50°C for 60 min. in a thermostat water bath with constant shaking. The reaction was stopped by adding 3 ml of DNS reagent and boiling for 5 min in a boiling water bath. The reaction mixture was immediately cooled and mixed with 20 ml distilled water and

OD was taken at 540 nm. The activity was calculated in accordance with the following definition given by IUPAC – one unit of enzyme activity was equivalent to one mmole of glucose formed per min per ml of undiluted enzyme solution.

β-Xylosidase determination:

The substrate for measuring β-Xylosidase activity was para-nitrophenyl -β -D xylopyranoside. A suitably diluted enzyme solution or crude extract of 0.1 ml was incubated with 0.9 ml of 4mM para-nitrophenyl--β -D xylopyranoside (pNPX) solution in sodium phosphate buffer (0.05M, pH 7) at 50^0C for 10 min in a thermostat water bath with constant shaking. The final concentration of the substrate was 3.6 mM. The enzyme reaction was terminated by adding 1 ml of 1M sodium carbonate solution. The released *para*-nitrophenol was analyzed by measuring absorbance at 410 nm against the substrate blank. The absorbance value of enzyme blank, if any, was deducted from the absorbance values of enzyme assay. One unit of β-xylosidase activity was defined as one μmole of para-nitrophenol released by the enzyme per min by 1 ml of undiluted enzyme. The μmoles of para-nitrophenol released by the enzyme were calculated from the para-nitrophenol standard plot.

Alpha L-arabinofuranosidase determination:

The substrate used for measuring Alpha L-arabinofuranosidase activity was para-nitrophenyl-α-L-arabinofuranoside. Other conditions of the assay were exactly the same as described for β-Xylosidase assay.

Alpha –D-Glucuronidase:

The substrate used for measuring α-D-glucuronidase activity was para-nitrophenyl-β-D-glucuronide. Other conditions for the assay were exactly the same as described for β-Xylosidase assay

Acetyl esterases: The substrate used for measuring acetyl esterase activity was para-nitrophenylacetate. A suitably diluted enzyme solution of 0.1 ml was incubated with 0.9 ml of a saturated solution of the substrate in phosphate buffer(0.05M, pH7) at 50°C for 10 min in thermostat water bath with shaking. Immersing the tubes in icewater bath terminates the reaction. The released paranitrophenol was analyzed by measuring the absorbance at 410nM against substrate blank. The absorbance value of enzyme blanks, if any, were deducted from the absorbance values obtained with any specific enzyme samples. One unit of acetyl esterase activity was defined as one μ mole of para-nitrophenol released per min by 1ml of undiluted enzyme.

Culture maintenance:

M. albomyces IIS 68 has been preserved and maintained in PDA slants and plates. It was observed that PDA plates and slants resulted in better sporulation in comparison to YPSS agar plates.

Medium composition for Inoculum (*M. albomyces* IIS 68)

Glucose	10 g/l
Yeast extract	0.1 g/l
Urea	0.5 g/l
$MgSO_4$, 7 H_2O	0.5 g/l
KH_2PO_4	0.6 g/l
K_2HPO_4	0.4 g/l
Final pH	6.0
Incubation temperature	45°C
Incubation time	48 hr

10% (v/v) of inoculum was transferred aseptically in the production medium.

After 96 hr, cells and unutilized wheat straw were separated by centrifugation and the supernatant was used for enzyme assay and enzymatic treatment of pulp.

***Bacillus* and Culture 2 maintenance and production media:** Culture 2 has been maintained in the media with composition same as mentioned earlier. Culture has been grown in agar media and kept at cold room for storage. Every one month, subculturing has been carried out. For *Bacillus* culture, medium with following composition has been used for subculturing and storage. The production of enzyme with *Bacillus culture* has been carried out with media having same composition. For culture 2, an enriched media has also been used with the following composition.

Media composition for *Bacillus* culture	
Birchwood xylan	10-30 g/l
Yeast extract	5g/l
Peptone	5 g/l
K_2HPO_4	1 g/l
$MgSO_4$	0.2 g/l
pH	7

Enriched Media composition for culture no 2	
Wheat straw powder	10g/l
Yeast extract powder	5g/l
Peptone	5g/l
Na_2HPO_4	3g/l
KH_2PO_4	1.5g/l
NaCl	0.5g/l
$MgSO_4$ Soln	1ml/l
Thiamine HCl	1ml/l
$CaCl_2$ soln	1ml/l
Stock solution:	
$MgSO_4, 7H_2O$	246.5 g/l
$CaCl_2$	14.7 g/l
Thiamine .HCl	10mg/l
pH	8

Wheat straw xylan isolation: The dried powder of wheatstraw powder was used for isolation of wheat straw xylan. The method followed was similar to the method described by Biely *et al.* 1988. Dried wheat straw powder of 100 gm was stirred with preheated distilled water (1000ml) at 50^0C. Delignification of wheat straw

was carried out by acetic acid(99%, 14 ml) and sodium chlorite (70gm) with equal proportions and stirred at 70^0C for one hour. The mixture was maintained at 70^0C and kept in well-ventilated fume hood. The mixture was filtered through a cloth on a Buchner funnel and washed with distilled water (1000ml). Wet wheat straw powder was transferred to a beaker and stirred with 1% aqueous ammonia solution (700 ml) at room temperature for 2hr. The extract was filtered off and the wet wheat straw powder was stirred again with 5% aqueous Sodium hydroxide (700ml) at room temperature for 2hour. The Sodium hydroxide extract was collected by filtration through a Buchner funnel and the latter extraction was repeated. The combined extracts were added to 4l of 96% ethanol, the resultant precipitate was allowed to settle and supernatant was collected by filtration. The precipitate was first washed with 75-80% ethanol containing 2% acetic acid to neutralize the residual alkali and then with 5l of 80% ethanol. The wet polysaccharide was suspended in 100ml distilled water and lyophilized for further use. The xylan yield was around 20.2 gm per 100 gm of wheat straw powder.

Fermentation experiment:

All the fermentation was carried out in INFORS 7.5 liter fermentor with working volume of 4 lit. Autoclavable fermentor was sterilized at 121^0C for 20 mins and 10% (v/v) inoculum was used for inoculation. Temperature and pH was maintained automatically. pH was maintained at desired value by the addition of 2(N) HCl and 2(N) NaOH. Samples were collected at certain interval of time and analyzed for enzyme activity. After the fermentation is over, cell broth was centrifuged at 10,000 RPM for 20 min and cell free broth was used for further

processing. After harvesting the broth, all operations have been carried out at low temperature (<10^{0}C).

Protein concentration by ultrafiltration: Enzyme broth has been concentrated in tangential filtration system using 10kD cutoff polysulphone membrane. Broth was kept in water bath maintained at 4^{0}C and a peristaltic pump was used for feeding broth inside the membrane. To achieve sufficient permeate flux in ultrafiltration system, a peristaltic pump has been used to feed cell free broth (after centrifugation of fermentation broth at 10,000 rpm, 4^{0}C for 20 min) to the membrane system at a flow rate of 40-50 lit/hr. Inlet pressure of the membrane was initially 2.5 Bar and retentate outlet pressure was adjusted at 1 Bar by regulating a valve in the retentate line. Permeate was collected in a glass beaker and retentate was collected in the original vessel. After the desired the reduction in volume of broth ultrafiltration process has been stopped. Retentate, permeate has been analyzed for enzyme activity. Membrane has been washed with 2(N) NaOH for 1hour by feeding base solution to the membrane using a peristaltic pump. After the cleaning operation, it has been rinsed with distilled water and then kept in cold room for storage.

Pulp making process: The pulps were produced from the typical method of "open hot digestion" where raw materials were cooked with 8% NaOH for a period of three hours at the boiling temperature. This pulping procedure is commonly used in the Handmade paper industry.

Enzymatic prebleaching: The pulps were subjected to enzyme –treatment in polythene bags. For this, two lots of each pulps were taken .One lot was added

with enzyme and the other lot as a control was subjected to the similar conditions without adding enzyme. The pulp consistency used was 6% .The initial pH of the pulp was maintained at 7.0 and 8.5. For maintaining initial pH of the pulp to the desired value 4N NaOH and 4 N H_2SO_4 were used. Enzyme dose of 20IU/gm OD pulp was used for all the studies. The pulps were then kept in water bath at a temperature of 55^0C for a period of 3 hours. After the retention time, final pH of the pulps were recorded .The pulps were then squeezed to collect the water extracts or enzyme filtrates and then washed with hot water and normal tap water .The enzyme filtrates collected were then analyzed for lignin at 280nm, colour at 465 nm and reducing sugars as xylose to find out the efficacy of the enzyme.

Bleaching methods: Both the treated and control pulps were subjected to the Peroxide bleaching after enzyme treatment. All the pulps were given an additional EDTA-treatment (0.2%EDTA, pH:4-5, temperature: ambient, time: 45 min. Consistency:5%) before Peroxide bleaching so as to increase the effect of Peroxide, because EDTA is a chelating agent which traps the hindering metal ions in the pulps. Peroxide bleaching was carried out at a consistency of 8%, NaOH:1%, hydrogen peroxide 2-3 %, temperature:70^0C, time:2 hrs., final pH:>9.0.

Evaluation of optical and strength properties: The bleached pulps were used to make handsheets (T-205om-88) so as to evaluate their optical (brightness:ISO-2471) and strength properties (tensile strength: ISO-1924, tear strength:ISO-1974) using the standard procedures.

CHAPTER III: RESULTS

The applications of xylanase enzyme as a pre-bleaching agent has been established in various laboratories and also been commercially exploited in Western counties. There are number of commercially available enzymes but due to the differences in paper making process in the developed counties and India, it has been felt to develop enzymatic pre-bleaching process indigenously from locally isolated cultures. One of the major differences is the use of different kind of raw materials for pulp making. In this context present project aims to isolate and screen xylanase producing culture from local sources. Some of the desirable characteristics of xylanase, which has been identified for effective bleaching has already been mentioned in Chapter 1. Some of the desirable characteristics are as follows:

- Thermo-stable enzyme with high stability at process temperature
- Optimum enzyme activity at higher pH (>7) and stability at higher pH

Besides these usual characteristics of enzyme some critical parameters has recently been identified and these are as follows.

- Enzyme with higher affinity for acidic functional groups in pulp.
- Low molecular weight of enzyme for easy diffusion into the pulp matrix.

With these theoretical backgrounds, suitable plan has been made to effectively implement the project with the ultimate goal to develop enzyme as a pre-bleaching agent.

The work carried out in this project has been presented in the following section:

1. Isolation of alkanophilic organism from various local sources and characterization of isolate
2. Selection of suitable isolate based on enzyme activity at higher pH.
3. Characterization of enzyme
4. Optimization of enzyme production in shake flask and fermentor level.
5. Enzyme concentration in ultrafiltration membrane
6. Storage stability of enzyme
7. Development of preliminary purification protocol.
8. Application of enzyme for different pulp treatment.

3.1 Isolation of alkalophilic organism from various local sources and characterization of isolate

First set of isolation and screening of thermo-tolerant xylanase producing organism has been carried out in first year (July 1998- June 1999) of the project duration. Sources selected for this purpose are garden soil samples, rotting wood samples and Cowdung. Enrichment culture method was followed and modified ML-30 (composition shown in the materials and methods section) has been used. The possibility of isolating xylanase-producing culture has been ensured by using 1% Birch wood xylan (SIGMA, USA) as the only carbon source. Major objective of the present study was to isolate xylanase-producing culture having higher stability in the alkaline range. So, all the liquid cultures for isolation of xylanase producing organism has been carried out at a pH of 9.5-10. Incubation at 30-35^{O} C for 48 hrs has resulted growth in all the liquid media and suitably diluted broth has been plated on ML30 agar plates and incubated at 30-35^{O} C for 48 hrs. After

two to three cycle of repeated streaking, pure isolate was obtained. Morphological and microscopic characterization of each isolate was studied and the results can be seen in Table 3.1.

Table 3.1: Morphological characteristics and microscopic observations of isolates

Source of microbes	Isolate codes	Colony morphology	Microscopic observations
Garden soil sample # 1	S1	Bacterial colony , Creamish in color	Long and short chain, thin rods in chains
	S2	Bacterial colony, white in color	Short highly motile rod
	S3	Bacterial spreader, with undefined periphery, light brown in color	Medium to short motile rods
Garden soil sample # 2	S4	Bacterial colony similar to S3	Short motile rods in chain
	S5	Bacterial colony light brown with glossy shine	Medium to short thin rods some in pairs
	S6	Bacterial colony light in color	Long and short thin nonmotile rods
Rotting wood sample # 1	W1	Bacterial colony with white colony with a glossy shine	Short thick and thin rods, thin rods nonmotile , some thick rods in pairs
	W2	Bacterial colony, creamish in color	Short thick and thin rods, mostly nonmotile .
Rotting wood sample # 2	W3	Bacterial colony translucent with glossy shine creamish in color	Short and thin highly motile rods
	W4	Bacterial spreader with undefined periphery, light brown in color	Medium to short motile rods
Air	B1	Bacterial colony, light brown in color, translucent with glossy shine	Short and thin highly motile rods
	B2	Bacterial colony, creamish brown and opaque	Short thick and thin rods, nonmotile
	B3	Bacterial colony, yellow and opaque	Medium to Short thin rods
	B4	Bacterial tiny colony light orange in color	Short and thin highly motile rods
	B5	Bacterial colony, white in color and opaque	Short thick and thin rods, nonmotile
	B6	Bacterial colony, dark yellow in color	Long and short, thin rods in chains
	B7	Fungal colony, central black in color with mycelia radiating outwards	Septate hylaine mycelium
	B8	Fungal colony, no definite periphery with white fluffy mycelia radiating in all direction	Septate hylaine mycelium

Enzyme production capabilities of these isolates have been checked by culturing in liquid media containing xylan as the carbon source. Cultured broth free of cells has been used for xylanase activity determination. The results of these studies have been presented in the next section.

Search for suitable xylanase producing culture from various sources has been a continuous process and after the first screening, number of other sources has been used for this purpose. Independent isolation has also been carried out using soil sample from alkaline source and xylan has been used as a carbon source. Medium composition is similar to the earlier case but pH of the isolation media was 8-8.5 and temperature was 50^0C. From this isolation rod shaped isolate was obtained, which has been identified as *Bacillus*.

Another attempt to isolate xylanase producing culture from an unusual source has resulted in successful isolation. The source of isolation was unusual, as there are no report on isolation of xylanase producing culture from saline lake. We have used sludge sample from Gudha Kyar lake, a saline lake in Rajasthan. Although, we are not interested in isolation of halophilic organism but our interest on thermophilic and alkanophilic culture has compelled us to use this unusual source (pH >8). This time we have used M9 medium for isolation containing 1% Birchwood Xylan as the carbon source. One of the advantages of using M9 medium in comparison to complex media ML30 is higher probability of isolating desired organism. Methods followed are almost similar with the earlier one except the temperature of incubation and pH were 50^{O}C and 10 respectively. Liquid medium incubated with 1gm of sludge sample for 72 hrs and thereafter

suitably diluted sample were spread on M9 xylan agar plates. The source of sample is tabulated as follows:

Table 3. 2: Source of sample for isolation of xylanase producing culture

Code name	Source
1	Slush from condenser no 7, Gudha kyar lake
2	White algae from crystallizer no 3, Gudha kyar lake
3	Normal sample from crystallizer no 1, Gudha kyar lake
4	Slush sample 9 from crystallizer no 1, Gudha kyar lake
5	Lake slush , Gudha kyar lake
6	Slush sample from discharged drain 1B, Gudha kyar lake
7	Slush sample from crystallizer no 2 , Gudha kyar lake
8	Slush sample from pan no 5, Gudha kyar lake
9	Slush from common drain , Gudha kyar lake
10	Slush from 2C reservoir, Gudha kyar lake

After 24-48 hrs of incubation, growth was observed in all the plates and further purification of isolates were carried out by repeated streaking on M9 agar plates. The purity of the culture was checked by microscopic observations. After ensuring purity of the isolates, characterization of the cultures was carried out and an effort has been made to identify the isolates using Biologe plates. The results of microscopic observation along with photograph are presented in Table 3.3 and Fig 1-10. Some of the slide photographs shows similarity in morphology. During characterization of isolates it was observed that all the cultures can grow at pH 10 in liquid media and also M9 xylan agar plate containing 2(M) NaCl thus indicating halo-tolerant nature of the isolate. This is a unique characteristic of isolates, which was rarely reported for any xylanase producing organism. In case

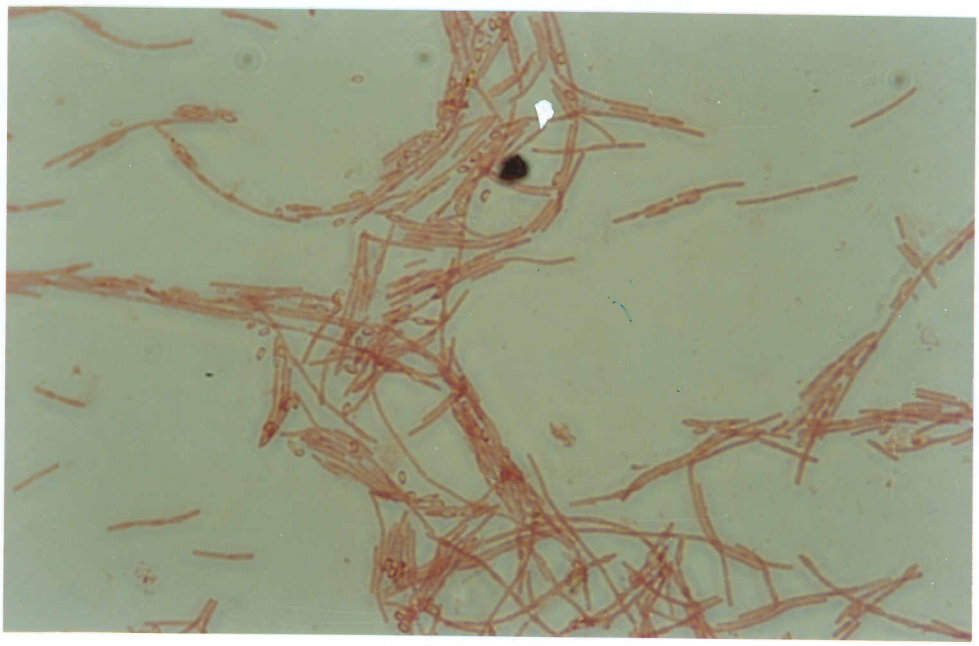


Fig 1: Photograph of isolate number 1

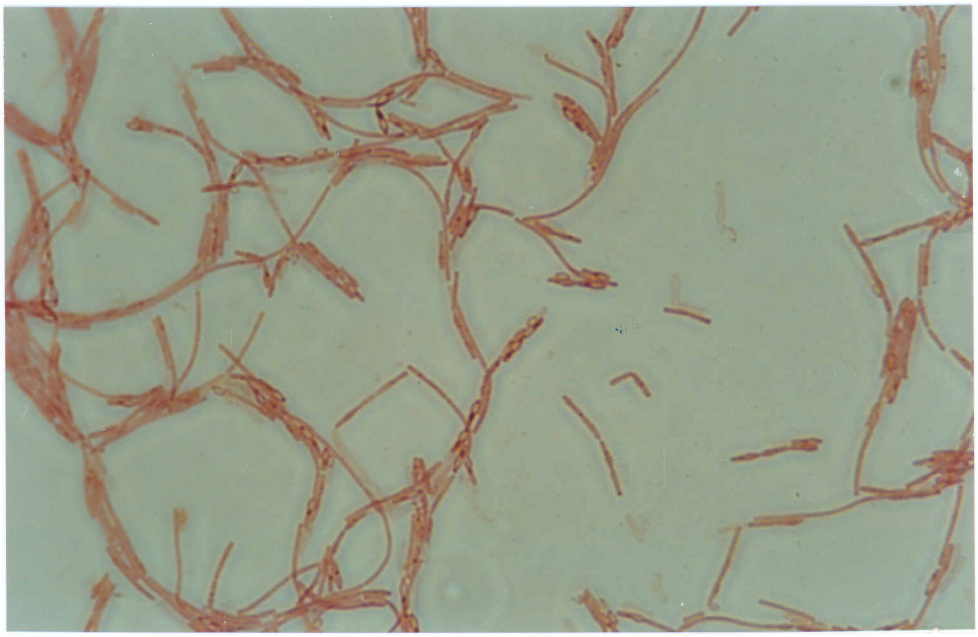


Fig 2: Photograph of isolate number 2

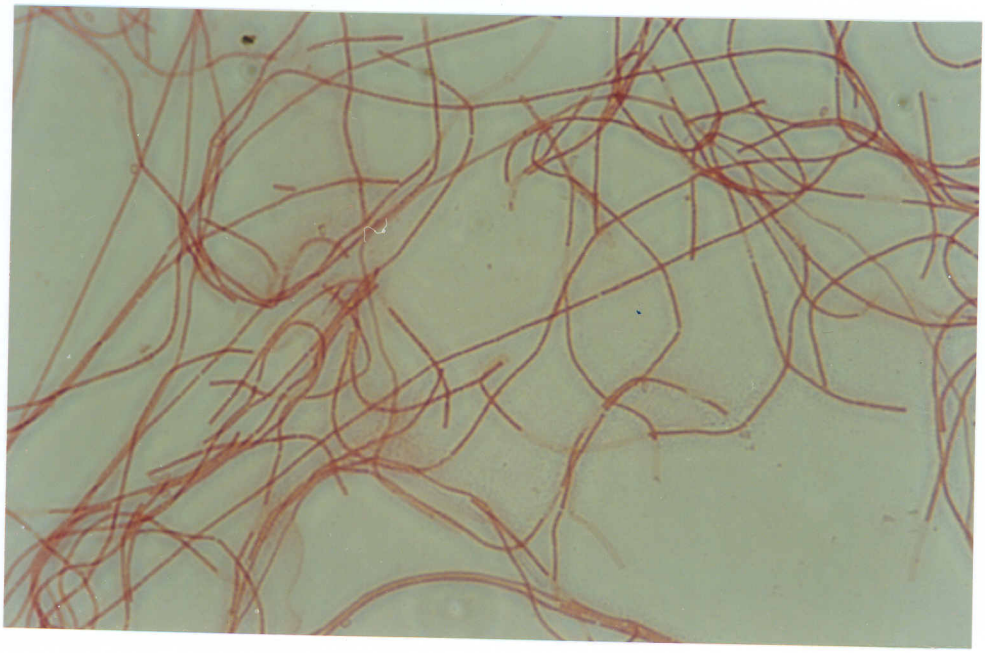


Fig 3: Photograph of isolate number 3

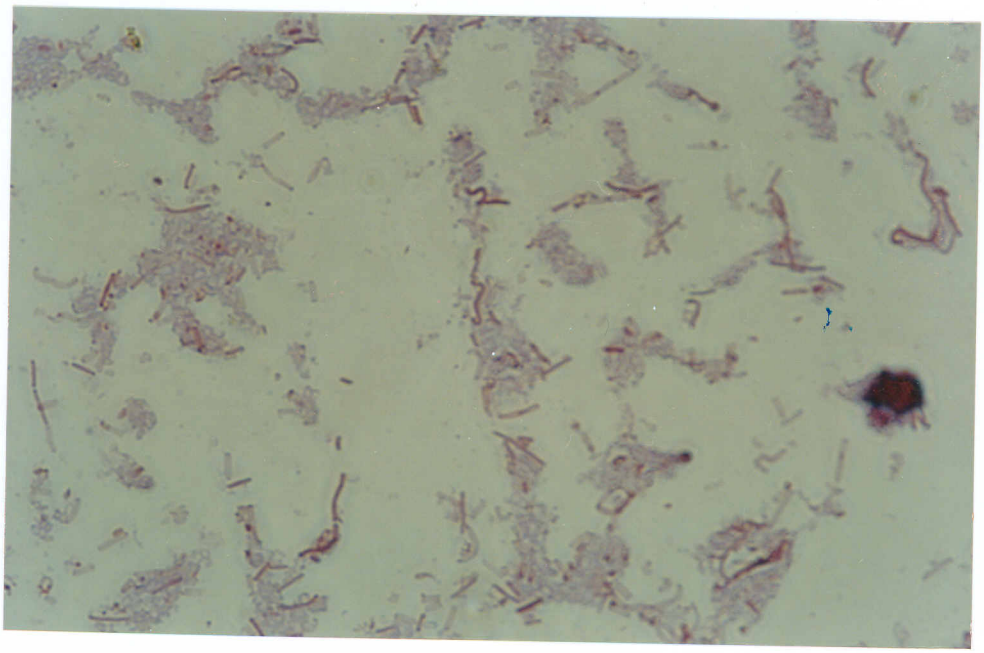


Fig 4: Photograph of isolate number 4

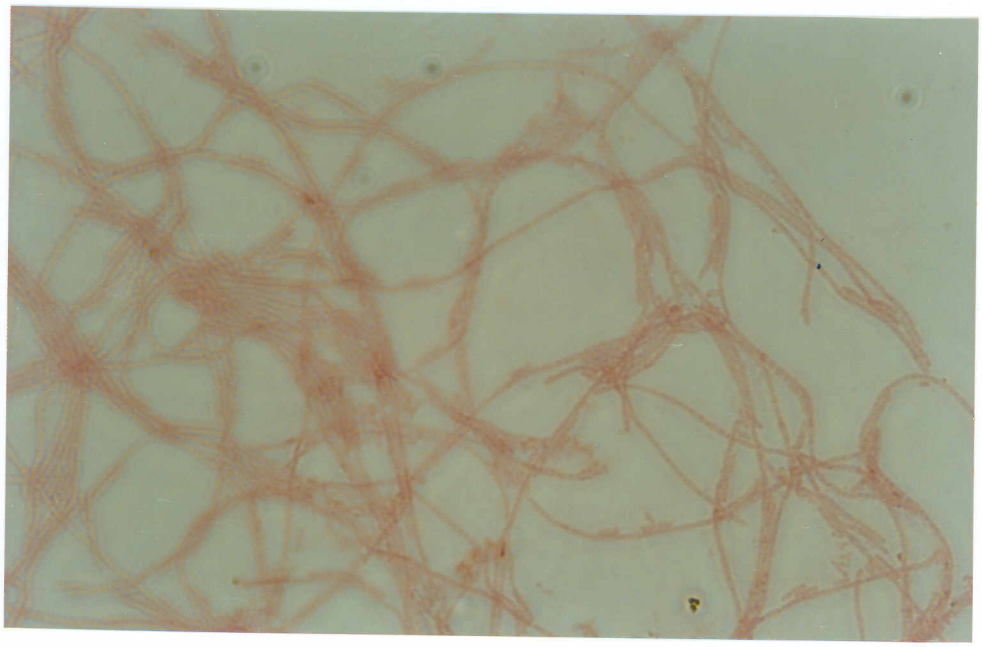


Fig 5: Photograph of isolate number 5

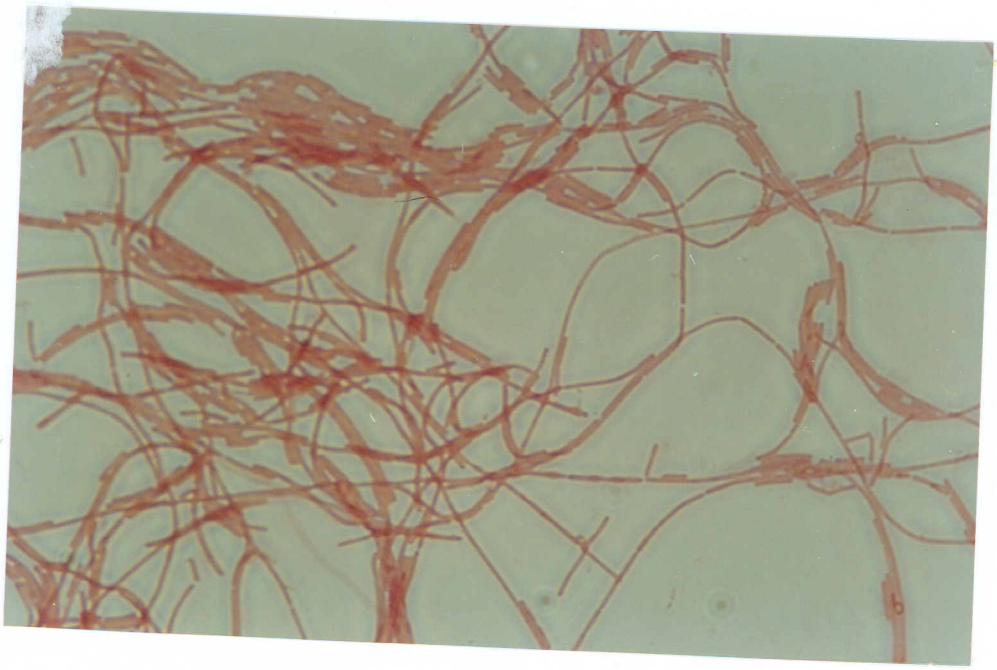


Fig 6: Photograph of isolate number 6

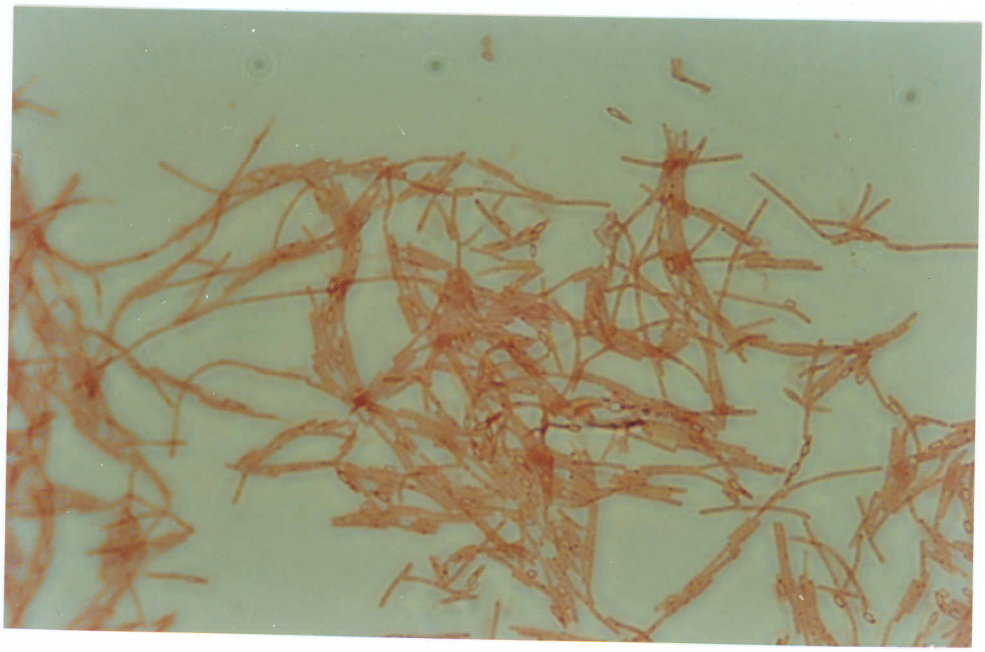


Fig 7: Photograph of isolate number 7

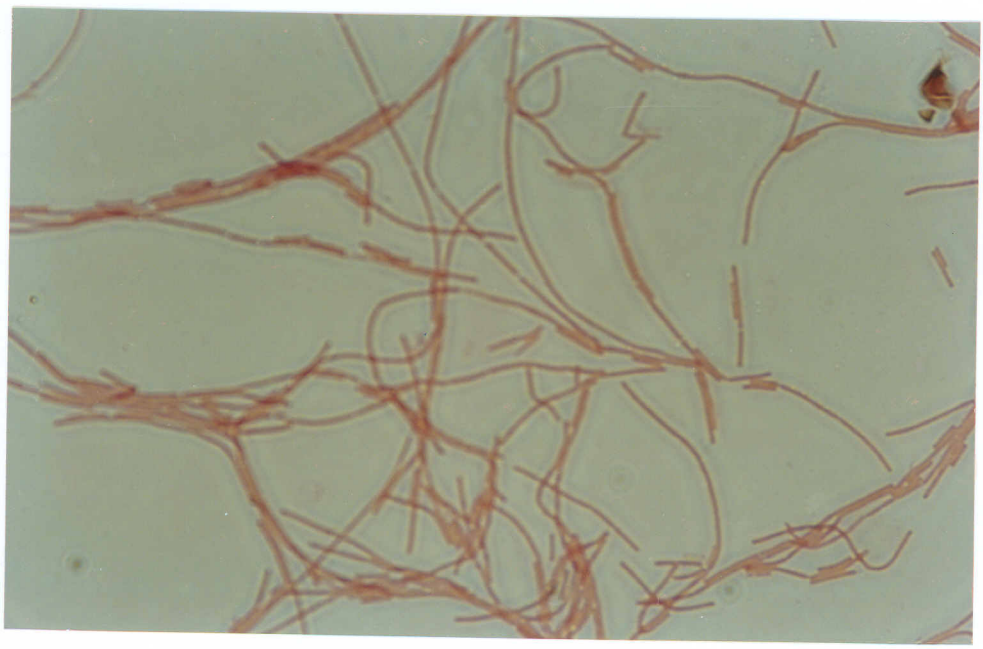


Fig 8: Photograph of isolate number 8

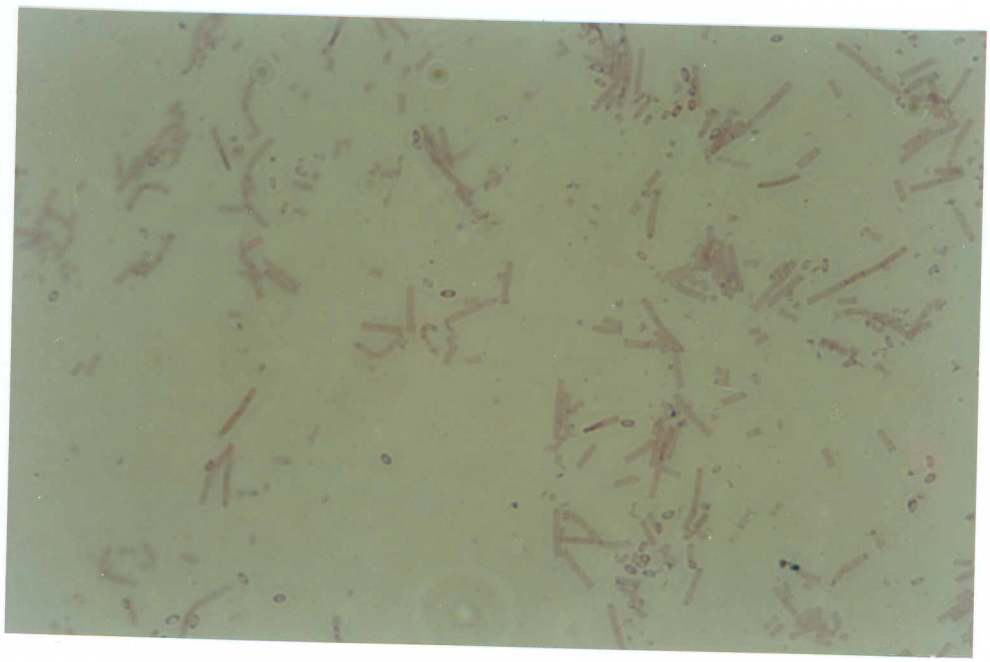


Fig 9: Photograph of isolate number 9

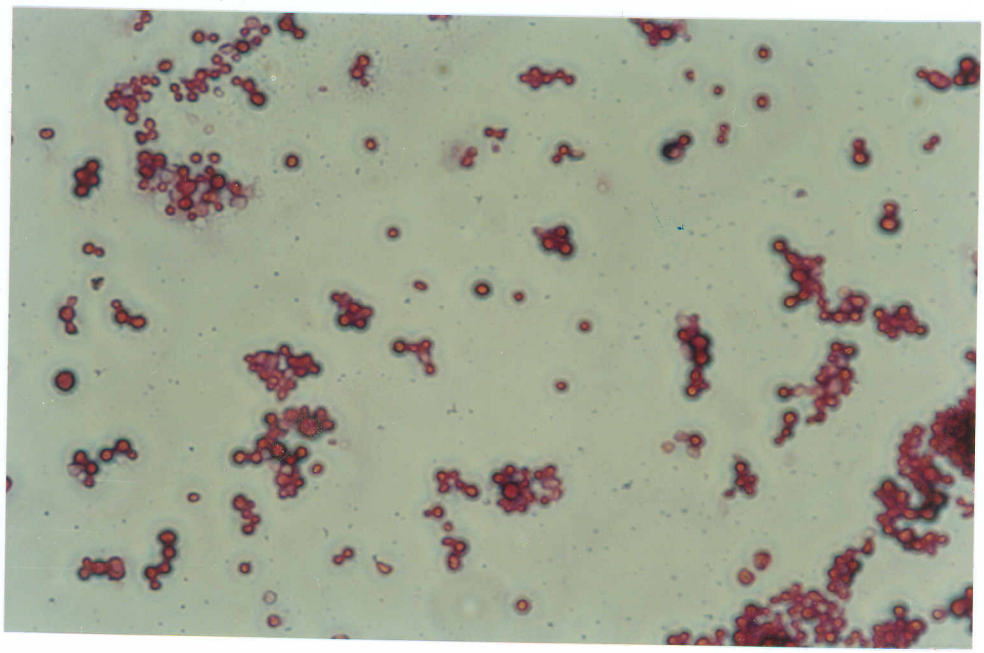


Fig 10: Photograph of isolate number 10

of Biolog plate study, some of the culture shows unusual response, which needs to be characterized and optimized. Identification of isolate with code 2 has been carried out and it has been observed that no match was found in database. Identification of other cultures is in progress. Once the cultures have been characterized, liquid culturing has been carried out and the results of these studies will be presented in next section.

Table 3 .3: Microscopic observations of isolates obtained from Gudha Kyar lake samples

Code Number of isolate	Microscopic observations
1	Very long filamentous string like broken fragments
2	Filamentous, interspersed dark clots, spores
3	Very long filamentous with interspersed dark clots
4	-
5	Numerous spores filamentous
6	Very long filamentous, string like, broken fragments
7	-
8	Very long filamentous, string like, broken fragments
9	Flat transparent filamentous
10	Disc shaped structure

Identification of isolates:

Two-prong stragedy has been adopted for identification of ten isolates. One method based on Biolog^{R} plates and other one depends on 16s RNA sequence matching with known databank. Although both methods has been frequently used for identification of organism but 16S RNA method is more elaborate. 16S

RNA sequence has been determined in TERI, New Delhi and based on the sequence results, search has been carried in Ribosomal Database.

In Biolog plate study, number of isolates showed improper results (false positive) which indicates that there is need for some modifications. However, isolates that showed proper result are not able to identify the isolates based on the Biolog databank. So, there was a need for more elaborate method to be adopted and 16S RNA seems to be fittest for this purpose. The sequence match for isolate number 2 is as follows:

Sequences producing significant alignments:

	Score (bits)	E Value
gi|18765901|gb|AF411223.1| Oryza sativa	40	1.9
gi|18449987|gb|AC094104.2| Homo sapiens chromosome 5 clone ...	40	1.9
gi|7689098|gb|AF223296.1| Uncultured gamma proteobacterium ...	40	1.9
gi|4585773|emb|AL035251.1|HS705O1 Human DNA sequence from ...	40	1.9
gi|19851286|gb|AF365734.1| Uncultured bacterium clone BT60D...	38	7.5
gi|18042436|gb|AC098677.2| Homo sapiens BAC clone RP11-416E...	38	7.5
gi|14585888|gb|AY038902.1| Marine bacterium SE105 16S ribos...	38	7.5
gi|11992253|gb|AF317783.1|AF317783 Unidentified bacterium w...	38	7.5
gi|11992220|gb|AF317750.1|AF317750 Unidentified bacterium w...	38	7.5
gi|12740602|gb|AC005079.6|AC005079 Homo sapiens BAC clone C...	38	7.5
gi|12407642|gb|AF277994.1|AF277994 Mus musculus chromosome ...	38	7.5
gi|4507260|ref|NM_003154.1| Homo sapiens statherin (STATH),...	38	7.5
gi|9910030|gb|AC063956.7|AC063956 Homo sapiens 4 BAC RP11-5...	38	7.5
gi|7689100|gb|AF223298.1| Uncultured gamma proteobacterium ...	38	7.5
gi|16519591|emb|AL603842.9|AL603842 Mouse DNA sequence from...	38	7.5
gi|9368570|emb|AL390092.1|NCB2A19 Neurospora crassa DNA lin...	38	7.5
gi|16501199|emb|AL592490.8|AL592490 Human DNA sequence from...	38	7.5
gi|5733807|gb|AF171697.1|AF171697 Neurospora crassa chromos...	38	7.5
gi|4510438|gb|AC007052.4|AC007052 Homo sapiens chromosome 1...	38	7.5
gi|13234794|emb|AL136095.25|AL136095 Human DNA sequence fro...	38	7.5
gi|4995933|emb|AJ240988.1|UGA240988 uncultured gamma proteo...	38	7.5
gi|4995931|emb|AJ240986.1|UGA240986 uncultured gamma proteo...	38	7.5
gi|17736867|dbj|AP004500.1|AP004500 Lotus japonicus genomic...	38	7.5
gi|14349208|dbj|AB062830.1|AB062830 uncultured bacterium ge...	38	7.5
gi|338610|gb|M18371.1|HUMSTTRNA Human statherin mRNA, compl...	38	7.5

From the matching no conclusive identification can be made. However, sequence matching with a marine bacterium SE105 but E value is relatively higher.

3.2 Selection of suitable isolate based on enzyme activity at higher pH

In the first phase of screening, total eighteen isolates have been purified and the isolates have been further cultured in liquid medium containing ML30 media and 1% Birchwood Xylan as the carbon source. After 48 hrs of culturing at a temperature of 45^{O} C, liquid broth free of cell and insoluble substrates has been utilized for xylanase enzyme assay. The enzyme assay protocol has been described in the previous chapter.

Enzyme activities at a pH 9 and temperature of 70^{O}C have been determined for all the isolates and the results (Fig 11) shows that isolates with code number W3, B4, B5 and S5 gave maximum xylanase activity under the assay conditions.

So, from the point of view of isolating alkanophilic isolates, these four isolates seem to be suitable. Further characterization needs to be carried out for selection of suitable isolates. Another important parameters studied for judging the suitability of enzyme in pulp bleaching applications is filter paper activity. The filter paper activity of xylanase enzyme is an important parameter as cellulase contamination adversely affects the pulp bleaching process. So, a study has been carried out to determine the filter paper activity for these four enzymes. The result (Table 3.4) indicates that filter paper activity of all the enzymes were significantly higher than the desired value. So, all these four isolates have not been further studied for biobleaching applications.

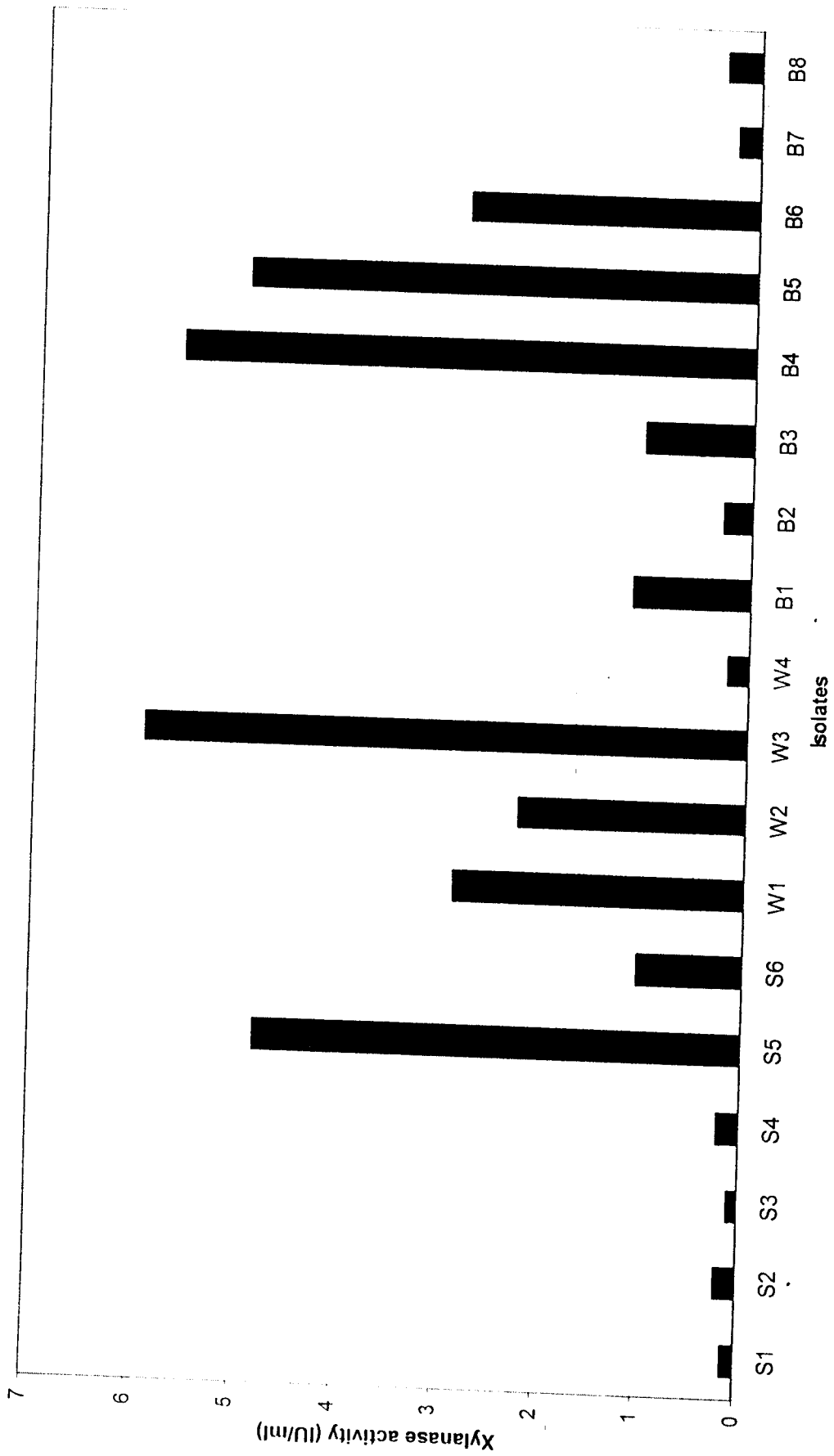


Fig. 11 Xylanase activity (IU/ml) of the various isolates

Table 3.4 : Filter paper activity of xylanase enzyme

Culture	Filter activity IU/ml
M. albomyces IIS 68	0.08
B4	0.69
B5	0.89
W3	0.46
S5	0.47

Other than these isolates, known xylanase producing cultures has also been employed in this project. *Melanocarpus albomyces* IIS 68 obtained from IIT Delhi has also been tried for xylanase enzyme production. The medium for production of xylanase enzyme contains Birchwood xylan and wheat straw as the carbon source and also as inducers. The method for cultivation of the organism has been adopted from the literature and it was observed that it took almost 72-96 hrs to produce significant amount of xylanase. Xylanase activity of 30-40 IU/ml was obtained with 1% wheat straw as a substrate. In a different experiment, it was observed that increase in wheat straw concentration upto 1% resulted in increase in enzyme yield but increase beyond 1% decreases the yield. Besides, agitation also plays an important role and optimum was found to be 120rpm. In case of *Melanocarpus albomyces* IIS68 enzyme filter paper activity has also been determined and it was found that cellulase activity is five to ten times lower than the isolates (Table 3.4). So, this enzyme can be used further for optimization and also as a pre-bleaching agent. However, it is well known that

most of the fungal enzyme are more active in the acidic range and so there is a need for developing alkanophilic enzyme.

Isolates obtained from Gudha Kyar Lake were further cultured in liquid medium containing 1% wheat straw as carbon source and medium composition is similar to M9. pH of the initial culture medium was 9-9.5 and incubated at 50^0C for 48 hrs. After growth was observed in all cases, cell free broth collected and used for xylanase enzyme assay. Enzyme assay was carried out with 1% Birchwood xylan and at a pH of 11 and temperature of 70^0C. The results (Table 3.5) shows that enzyme is active at a pH of 11 for all the isolates except for isolate with code number 4. Besides, xylanase activity was also checked on Xylan Agarose plates using Congo red as dye. It can be seen that isolates with code number 2 and 9 shows highest activity at pH 11 and temperature of 70^0C. Thus indicating that culture 2 can be used for further optimization.

An another important criterion, for xylanase enzyme application as pre bleaching agent is filter paper activity. Minimum filter paper activity of enzyme solution is desirable. So, filter paper activity of enzyme solutions has been determined it was observed that filter paper activity is minimal for all the cases.

So, isolate with code number 2 has been chosen for further optimization.

Table 3. 5 : Xylanase and Filter activity of isolates obtained from Gudha Kyar Lake

Isolate code number	Xylanase activity IU/ml at pH11, temperature 70^{0}C	Filter paper activity IU/ml at pH 7, Temperature 50^{0}C
1	3.6	0.013
2	4.64	0.019
3	3.19	0.017
4	-	-
5	3.68	0.0148
6	2.75	0.025
7	2.57	0.017
8	2.76	0.013
9	4.94	0.018
10	2.76	0.018

Similarly *Bacillus* culture has also been cultured in 1% Wheat straw medium containing yeast extract and peptone as nitrogen source and cultivated for 48 hrs at 50^{0}C. Sample has been taken at 24 and 48 hrs and it was observed that maximum activity achieved at 24 hrs and thereafter activity decreased. Maximum activity of 14.2 IU/ml (at pH 7) was obtained and with a corresponding filter paper activity of 0.025 IU/ml.

So altogether, two cultures namely, *Bacillus*, Isolates with code number 2 have chosen for further characterization and optimization.

3.3 Characterization of enzyme

Characterization of enzyme is necessary as it provides useful data for biobleaching applications. Important parameters like activity-temperature, activity-pH profiles are deciding factors in enzymatic treatment of pulp. So two enzymes from the chosen isolates have been used for determining activity-temperature and pH profiles. Cell free broth used for this purpose and results are presented in the following sections.

Temperature-activity profiles of enzyme samples:

Methods followed for determining temperature-activity has been described in the previous chapter. From the results (Fig 12) it can be seen that *Bacillus* has an optimum temperature of 55^0C whereas culture no 2 showing optimum activity at temperature of 70^0C. Even at a temperature of 80^0C it (culture no 2) retain 69% of its maximum activity whereas *Bacillus* enzyme retain 28% of its maximum activity at same temperature. Thus it indicates that probably culture no 2 has higher thermal stability in comparison to *Bacillus* enzyme.

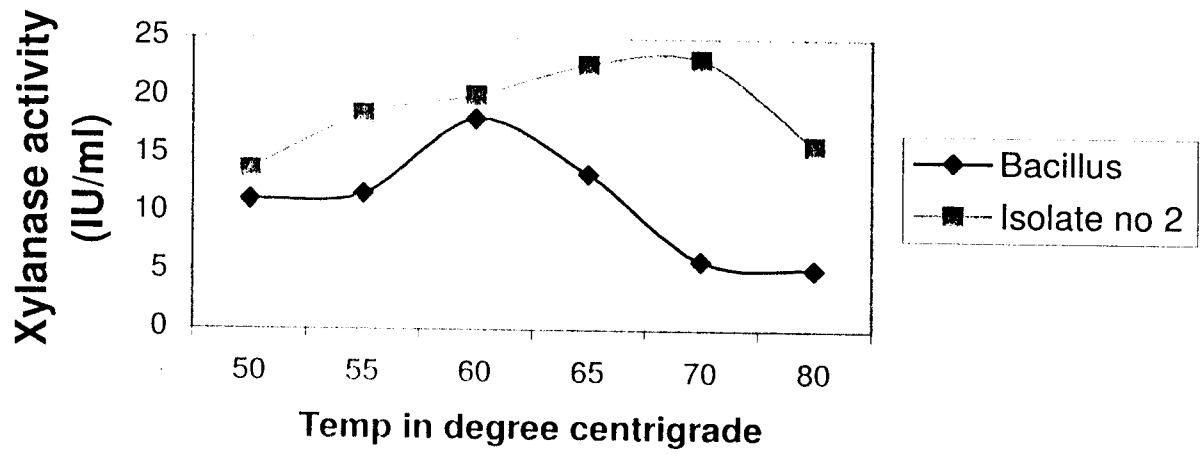
pH-enzyme activity profile:

In biobleaching applications using xylanase enzyme one of the major important parameter is pH optima and enzyme stability at higher pH (>7). Although large number of fungal source of xylanase enzyme has been used in biobleaching applications but due to low pH optima these class of enzyme finds limited

applications. Search for new alkaophilic enzyme has resulted in isolating number of bacterial enzymes with higher pH optima. In the present study, We are able to isolate two distinct cultures with enzyme activity in the alkaline range. The enzyme activity at various pH was determined and the results (Fig 13) shows that enzyme form culture no 2 is active in broader pH range. Enzyme from culture 2 is active even at p H above 10 whereas *Bacillus* enzyme is active upto a pH of 9. Although both the enzymes have pH optima around 7 but broader pH activity profile of culture 2 enzyme thus makes it more suitable for biobleaching applications. However, a recent report shows that higher pH optimum is not the sole criteria for successful biobleaching applications. For successful biobleaching applications enzyme stability at pulp treatment conditions is a prerequisite.

Thus xylanase enzyme from isolate number 2 is not only suitable as pre-bleaching agent from the point of higher optimum temperature but also due to activity in the alkaline range. However it should be noted that higher pH optima is desirable for biobleaching applications but enzyme stability at higher pH is also necessary.

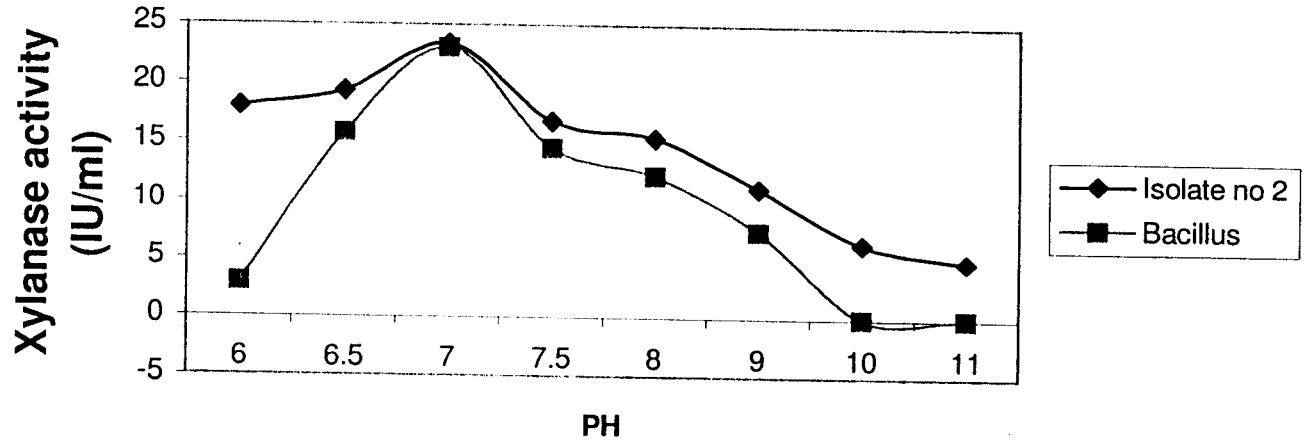
Fig 12: Optimum temperature of enzmes



Enzyme stability:

In the previous section it has already been mentioned that stability of enzyme at operating conditions is important. So the stability of enzyme has been studied at optimum temperature and pH. Besides, stability of enzyme also determined at other pH to find out its suitability. In the present study, enzyme from isolate number 2 has been concentrated in ultrafiltration and this concentrated enzyme has been used for stability studies. Results (Fig 14) indicates that enzyme from culture number 2 has higher stability at 55^0C than at optimum temperature of 70^0C. Enzyme stability of *Bacillus* culture is comparatively less at temperature of 55^0C and pH of 7 (Fig 15). Enzyme from culture number 2 has lost 50% of its activity within 2hrs at 55^0C whereas *Bacillus* enzyme loose 85% of its activity within the same time duration. Thus it indicates that enzyme from culture number 2 has higher stability at operating temperature (around 50^0C). Similar studies have also been carried out to check the stability of both the enzymes at different pH. *Bacillus* enzyme found to be more stable at the optimum pH and with decrease in pH, stability of enzyme also decreased. At higher pH of 8 enzyme also loose its activity at higher rate than at pH 7. In case of enzyme from isolate number 2, stability has been carried out at two different pH values. It was observed that enzyme is more stable at optimum pH (=7) but at higher pH of 8 enzyme is relatively less stable (Fig 16). Although enzyme of isolate number 2 is relatively less stable at pH 8 but half life at this pH is almost similar to pH 7. Thus it indicates that enzyme of isolate number 2 has higher thermostability and also stable in the alkaline range.

Fig 13: Optimum pH of enzymes



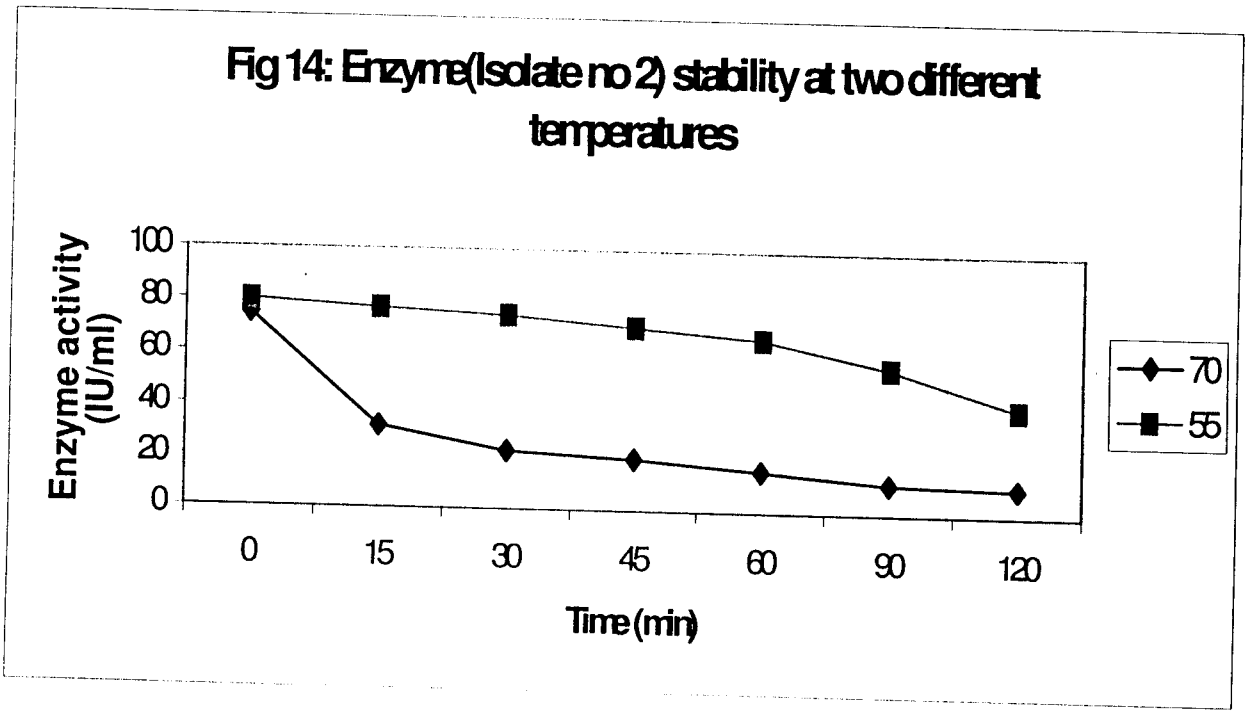


Fig 14: Enzyme(Isolate no 2) stability at two different temperatures
Enzyme activity (IU/ml)
100
80
60
40
20
0
0
15
30
45
60
90
120
Time (min)
70
55

Fig 16: Enzyme(Isolate no 2) stability at two different pH values at a temp of 55 degree centrigrade

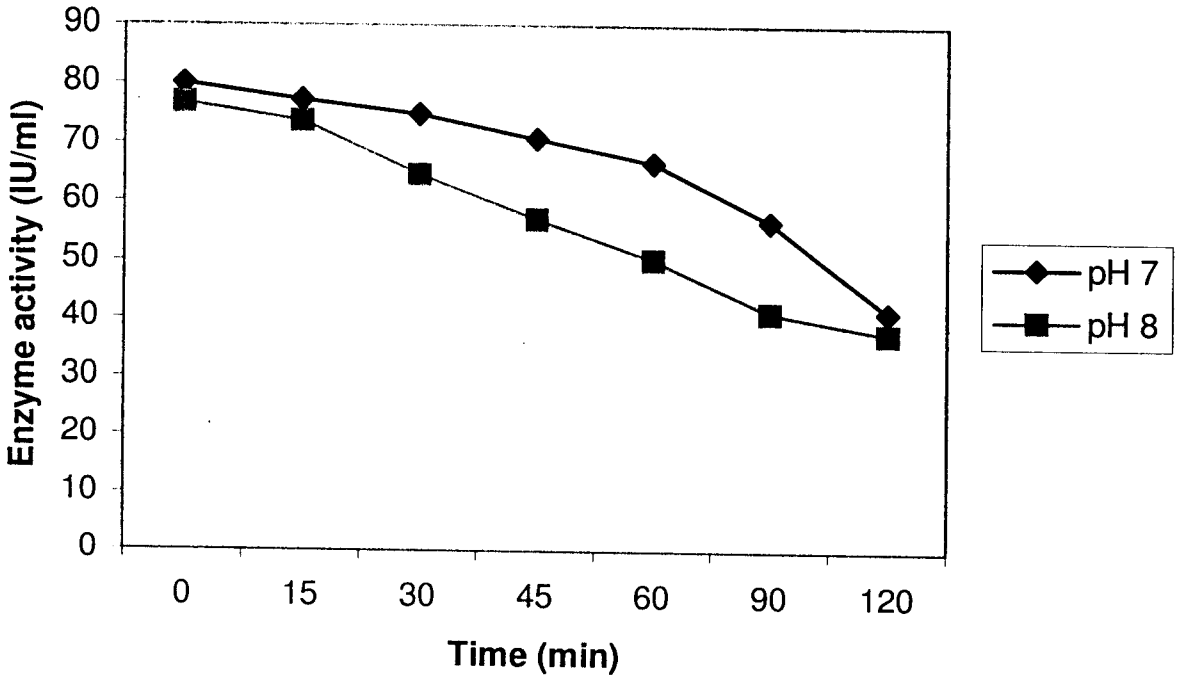
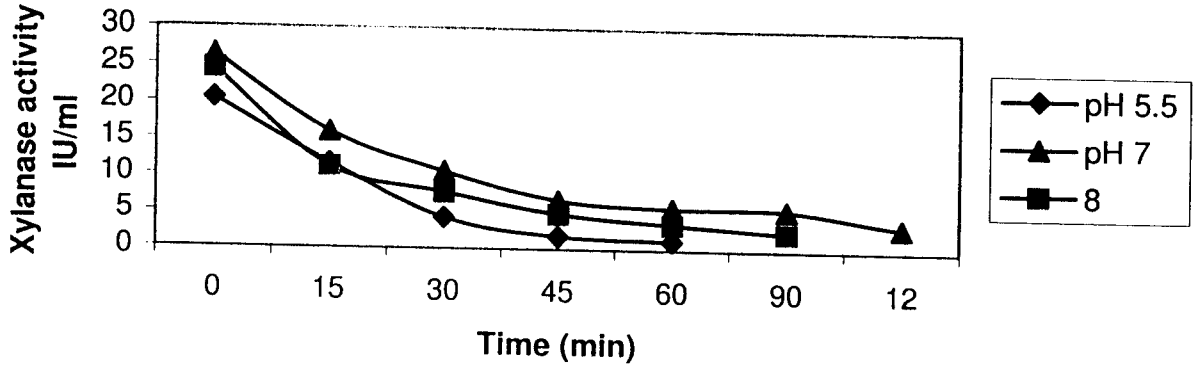


Fig 15: Enzyme (Bacillus) stability at three different pH values and at a temp of 55 degree centrigrade



Debranching enzyme study :

The xylosidic linkages of lignocellulose are not all equivalent and equally accessible to xylanolytic enzymes. Moreover, the accessibility of some linkages changes during the course of hydrolysis which necessities the production of an array of enzymes each with specialized functions. Synthesis of multiple enzymes is one of the strategies, which a microorganism adopts to achieve superior xylan hydrolysis.

The main enzymes responsible for the hydrolysis of xylan backbone are endo-β-xylanase, β-xylosidases, and exo-β-xylanases. Besides the main chain cleaving enzymes some side chain cleaving enzymes play an important role in xylan hydrolysis. Depending on the source of xylan, side chain cleaving activities include acetyl esterase, α-L-arabino furanosidase and α-D-glucuronidase. Many of these enzymes have been shown to act synergistically for xylan hydrolysis.

In the present study, xylan debranching enzymes has also been determined as means to characterize the enzymes. From this study it can be concluded that both of these enzymes differ in thermostability, alkaline stability and also in debranching activities. Due to this differences it is expected to have different responses of these enzymes towards different pulps.

Table 3.6 : Debranching enzyme activity of enzyme samples

Activity Iu/ml	*Bacillus* enzyme	Culture 2
β-xylosidase	0.198	0.290
α-L-arabino furanosidase	1.38	0.301
α-D-glucuronidase	0.198	0.297
acetyl esterase	4.24	3.86

From the results (Table 3.6) it can be seen that *Bacillus* enzyme does contain proportionately higher α-L-arabino furanosidase activity. An another important aspect of this study is that both the enzymes contain higher proportion of acetyl esterase activity.

Effect of incubation time and buffer concentration on enzyme assay:

The enzyme assay protocol for *Bacillus* and isolate number 2 enzymes have been adopted from the literatures. However there are no standard protocols has been followed by various investigators. As it has been mentioned in the chapter 1 that incubation time reported varies from 5 min to 2 hours. So, there is need for studying the effect of incubation time on enzyme assay. Although temperature of incubation is also vary in different reported literature but in these study optimum temperature has been chosen as the incubation temperature. Xylanase enzyme of isolate number 2 from a batch fermentation has been taken for this study. The effect of incubation time has been studied at 70^0C (optimum temperature) with 1% Birchwood xylan and time has been varied from 5 min to 30 min. The result (Table 3.7) shows that with increase in incubation time enzyme activity decreased but upto 15 min , decrease in enzyme activity is marginal. This can be a explained interm of thermal stability of enzyme. So, in all further studies 5 min incubation time has been chosen at 70^0C for enzyme of isolate number 2.

Table 3.7 : Effect of incubation time on enzyme (Isolate no 2) assay

Time of incubation in min	Enzyme activity IU/ml
5	12.69
15	11.89
30	8.54

Similarly the effect of buffer concentration on enzyme assay has also been studied in the present study. Generally 50 mM phosphate buffer has been used by various investigators but there are also other reported values. So, in this study enzyme assay has been carried out at 50mM and 20 mM phosphate buffer of pH 7. In these studies concentrated enzyme has been taken. From the results (Table 3.8) it can be concluded that there is rarely any difference in enzyme activity for both the enzymes (Bacillus and Isolate no 2) at two different buffer concentrations.

Table 3.8 : Effect of buffer concentration on enzyme assay

Enzyme	Enzyme activity IU/ml
Bacillus enzyme with 20mM Buffer	194.9
Bacillus enzyme with 50 mM buffer	199.5
Isolate no 2 enzyme with 20 mM buffer	173.4
Isolate no 2 enzyme with 50 mM buffer	168.68

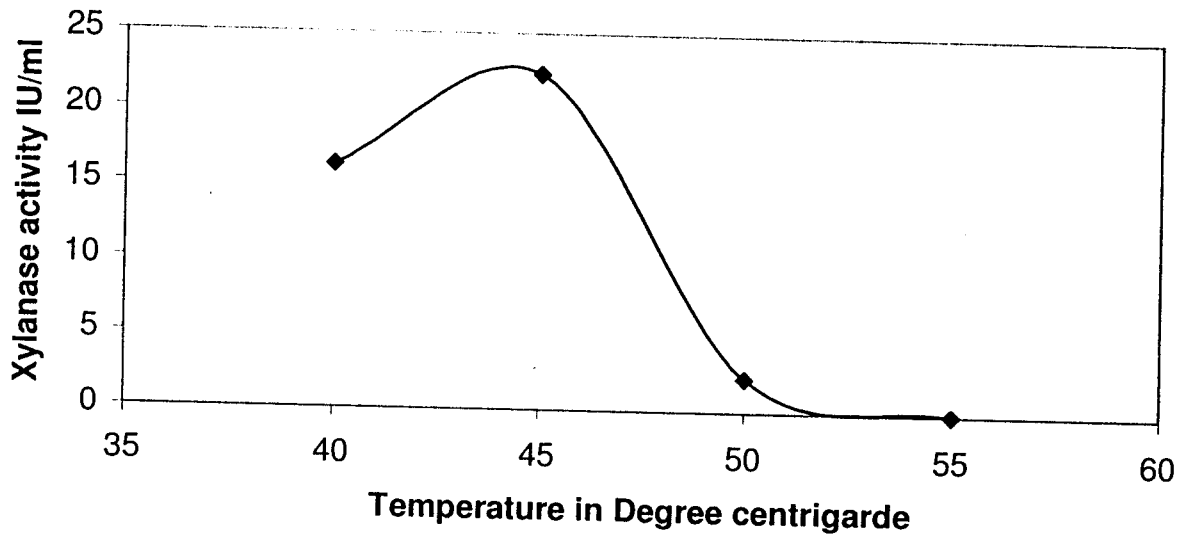
3.4 Optimization of enzyme production in shake flask and fermentor level:

In the present work specifically two enzymes have been chosen for further optimization of enzyme production. The major objectives of this study are choosing suitable substrate, optimum substrate concentration, optimum pH, temperature etc. In the first part of this section, optimization study related to *Bacillus* enzyme will be described.

Optimization of growth temperature for Bacillus:

Initially, a suitable medium was chosen based on the literature reports. Medium described by Yang *et al.* 1995 for alkaliphilic *Bacillus* sp. has been chosen for this study. The medium composition has been described in the materials and methods section. In case of Birch wood xylan, wheat straw powder has been chosen in the present study as carbon source. However in the latter stage it was found that wheat straw is the most suitable carbon source for xylanase enzyme production for this specific culture. Temperature optimization study for this culture has been carried out in shake flask culture at various temperatures (40-55^{0}C). After an incubation period of 24 hr, cell free broth has been used for enzyme assay. From the results (Fig 17) it can be seen that maximum enzyme activity has been obtained at temperature of 45 ^{O}C which can be assumed as optimum temperature.

Fig 17 :Optimization of temperature for enzyme production using Bacillus culture



Optimization of pH for enzyme production:

Initial pH of medium has been adjusted at various pH ranging from 6 to 8. This optimization study has been carried out in shake flasks without any pH control. As a result, final pH of the medium was far away from the initial pH. Although, final pH in all cases are in the range of 8.5-9, but maximum enzyme activity was obtained at a pH value of 7-7.5. After pH value of 7.5 enzyme activity decreased significantly (Table 3.9). So, from the above mentioned studies optimum pH and temperature for enzyme production using *Bacillus* culture has been found to be 7 and 45^{O} C respectively.

Table 3.9 : pH optimization for enzyme production using Bacillus culture

Initial pH	Time (hr)	Xylanase activity (Iu/ml)	Final pH
6	24	3.21	8.72
6.5	24	11.64	8.77
7	24	14.32	8.86
7.5	24	13.44	8.89
8	24	4.52	8.90

Selection of proper carbon source for enzyme production:

In case of inducible enzyme like xylanase choice of suitable carbon source is an essential part in optimizing the enzyme production process. The production of xylanase enzyme with various carbohydrates as carbon source (sucrose, lactose, CMC, cellulose, solka floc, xylose, glucose) has been investigated at the shake flask level and the concentration of all these cases has been kept constant at

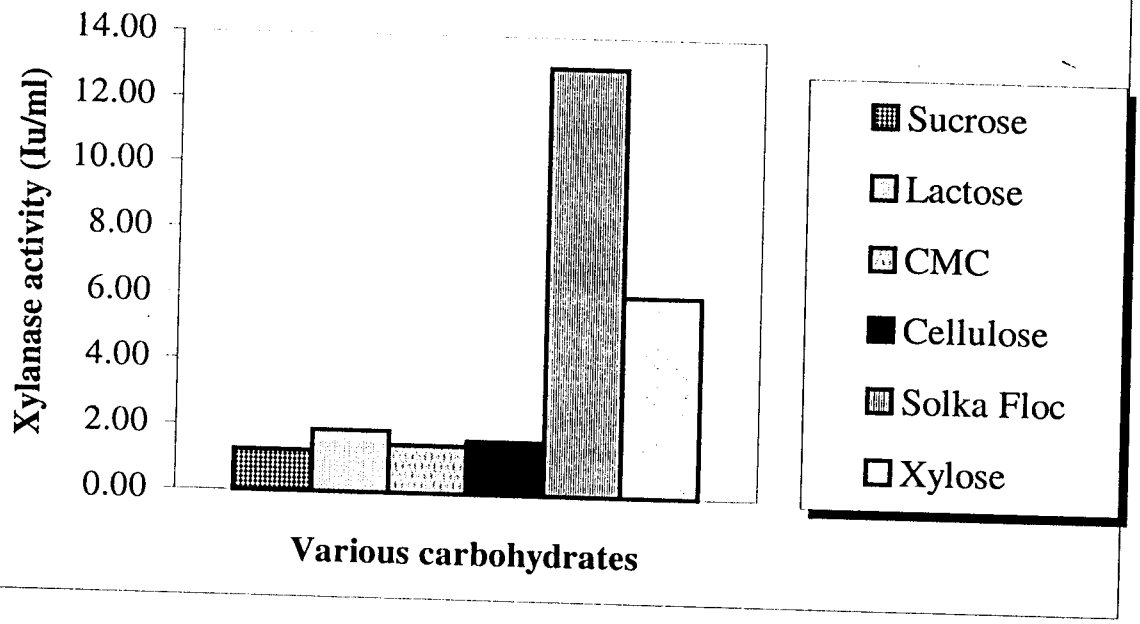
1%. The incubation temperature of 45^{O}C has been maintained in the shake flasks. From the results (Fig 18) it can be seen that maximum enzyme production has been obtained with 1% solka floc followed by 1% xylose. However in case of glucose, enzyme production was insignificant. This study thus shows that xylose acts as an inducer for xylanase enzyme production.

Besides these carbohydrate sources, rice straw powder has also been used as a carbon source for enzyme production. In comparison to wheat straw, the production of enzyme is significantly less in case of rice straw (Table 3.10). Activity of enzyme in case of rice straw is almost half of activity with wheat straw.

Table 3.10: Xylanase enzyme production in different agroresidues

Substrate	Time (hr)	Xylanase activity (Iu/ml)
Wheat straw	24	14.16
Rice straw	24	7.358

Figure 18 : Bacillus Xylanase production in various carbohydrates



As, activity of enzyme with wheat straw is maximum so further attempt has been made to isolate xylan from wheat straw and use as a carbon source for enzyme production. Isolation of wheat straw xylan has been described in the materials and methods section. For comparative study, 1% wheat straw, 1% Wheat straw pulp, 1% wheat straw xylan and 1% Sigma Birch wood xylan has been used for xylanase enzyme production using *Bacillus* culture. Maximum xylanase enzyme production has been observed with 1% wheat straw xylan and enzyme activity is five times the activity with 1% wheat straw (Table 3.11). However, the effect of wheat straw pulp and Birch wood xylan on the enzyme production is almost insignificant.

Table 3.11 : Xylanase enzyme production using *Bacillus* culture

Substrate	Concentration	Enzyme activity (IU/ml)
Wheat straw pulp	1%	25.04
Wheat straw xylan	1%	98.7
Wheat straw	1%	21.3
Sigma Birchwood xylan	1%	24.2

Optimization of substrate concentration:

As it has been observed that wheat straw xylan supports maximum xylanase enzyme production so this can be the most suitable carbon enzyme production. However, the cost of wheat straw xylan may be limiting factor for economic production of xylanase enzyme. So It has been planned to increase the xylanase enzyme production by increasing the wheat straw concentration. To study the

effect of wheat straw concentration on xylanase enzyme production, shake flask study has been carried out with different concentrations of wheat straw powder.

Table 3.12 : Effect of wheat straw concentration on enzyme production

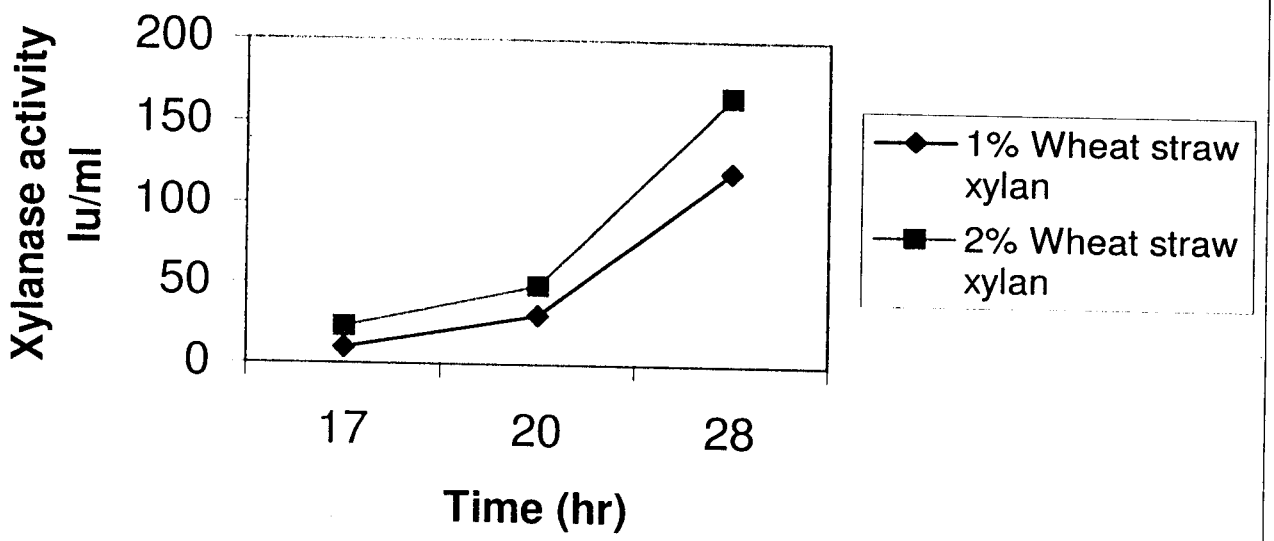
Concentration of wheat straw powder	Enzyme activity IU/ml
1%	15.6
2%	34
3%	45.2
4%	34.2

From the results (Table 3.12) it can be concluded that enzyme activity increased with increase in concentration of wheat straw powder till 3% thereafter enzyme activity decreased. So, wheat straw concentration of 3% seems to be optimum.

The concentration of wheat straw xylan used in the previous study was 1% and the effect of higher wheat straw xylan on enzyme production has also been studied. Shake flask study has been carried out with 1% and 2% wheat straw xylan and incubated for a longer time. Time profile of enzyme activity for the cases has been shown in the Fig 19

From the result it can seen that with increase in wheat straw xylan concentration enzyme activity increased to 166 IU/ml. However, enzyme activity increment is not proportional to the increase in wheat straw xylan concentration.

Fig 19: Bacillus Enzyme production profile at various wheat straw xylan conc



Optimization of enzyme production for isolate number 2:

Temperature optimization for enzyme production has been carried out in shake flask level with M9 medium (media used for isolation and screening) at various temperatures (35-70^{O}C). Wheat straw powder has been chosen as carbon source. From the results (Fig 20) it can be concluded that 45^{O}C seems to be the optimum temperature. In this culture incubation time was 48 hrs which has been earlier optimized.

Although enzyme from this isolate has an optimum activity at a temperature of 70^{O}C but its optimum temperature for enzyme production is 45^{O}C.

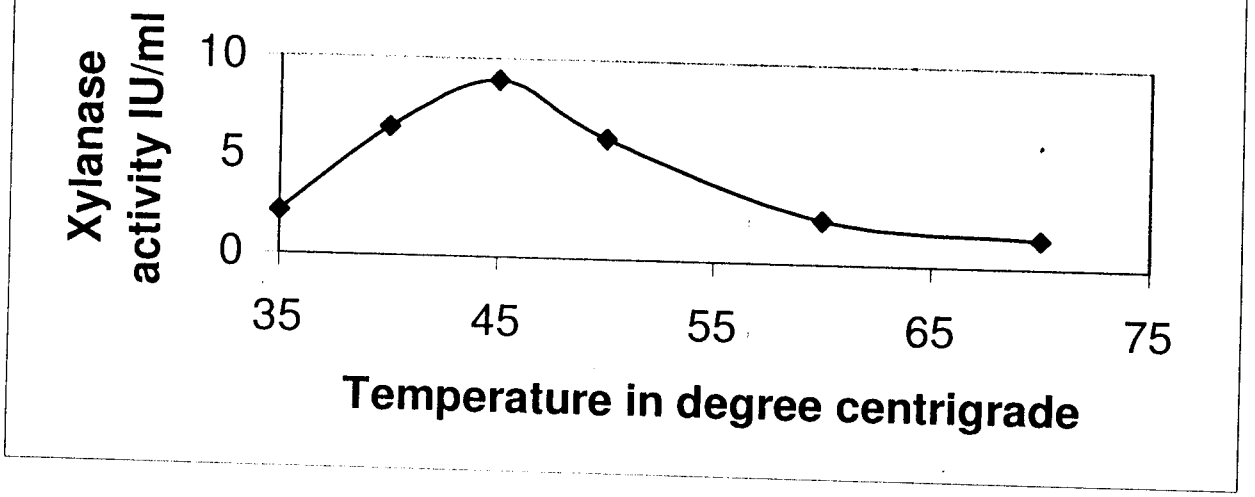
Optimization of enzyme production at various pH has similarly been carried out in shake flask level with M9 medium.

Table 3.13 : Effect of pH on enzyme production

Initial pH	Xylanase activity at pH 7 IU/ml
8.5	4.39
9	5.2
9.5	5.24
10	6.36
10.5	6.72
11	9.1

From the results (Table 3.13) it can be seen that enzyme activity increases with increase in pH. It was difficult to find out the optimum from present sets of data. It has been planned to carry out the optimization study in the reactor under controlled pH and temperature conditions.

Fig 20: Temperature optimization for Isolate number 2



Selection of proper carbon source is an important step in optimizing the enzyme production process. In an attempt to choose the most suitable carbon source, wheat straw, Birch wood xylan, Solka floc, corncob and rice straw has been used to produce the enzyme.

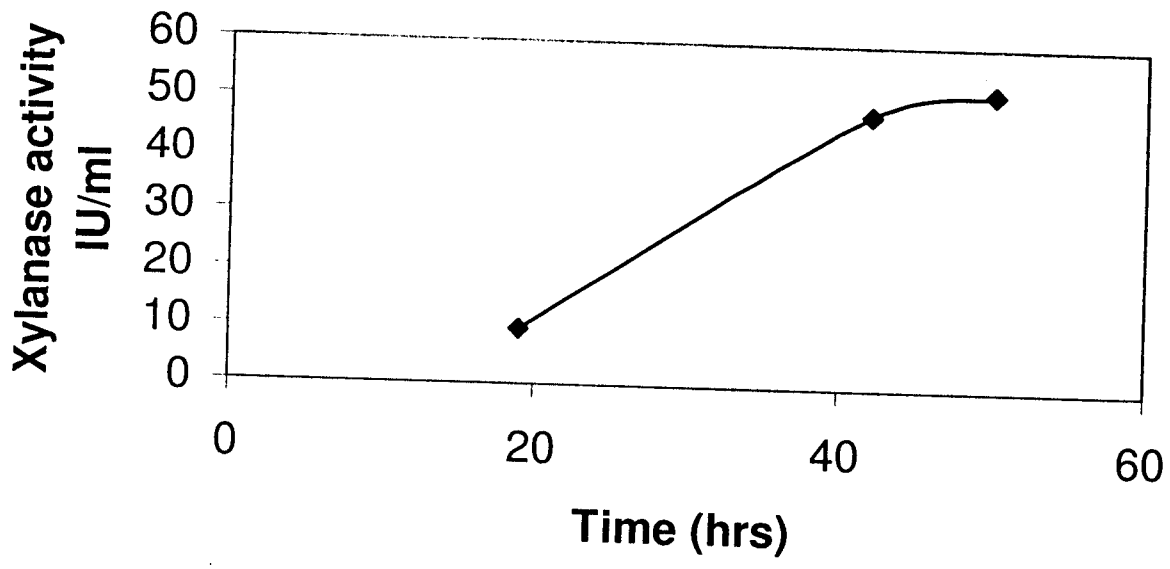
Table 3.14: Effect of various carbon sources on xylanase enzyme production by Isolate number 2.

Substrate	Xylanase activity at pH 11 IU/ml
Wheat straw	2.95
Birchwood xylan	2.57
Wheat bran	3.35
Solka floc	0
Corn cob	2.01
Rice straw	1.70

The result (Table 3.14) indicates that wheat straw and wheat bran is the preferred carbon source for xylanase enzyme production. However, corn cob and birch wood xylan can also be used for enzyme production.

Although birchwood xylan seems to be not very effective for enhanced enzyme production but an attempt has been made to improve the enzyme production by using wheat straw xylan as the carbon source. In the initial experiments, only 1% wheat straw xylan has been selected for enzyme production. Time profile of enzyme production with 1% wheat straw xylan has been shown in Fig 21. The result shows that xylanase enzyme activity of 52 IU/ml has been achieved within 50 hrs, which is significantly higher than 1% wheat straw medium.

Fig 21: Xylanase enzyme production profile of isolate number 2 in wheat straw xylan



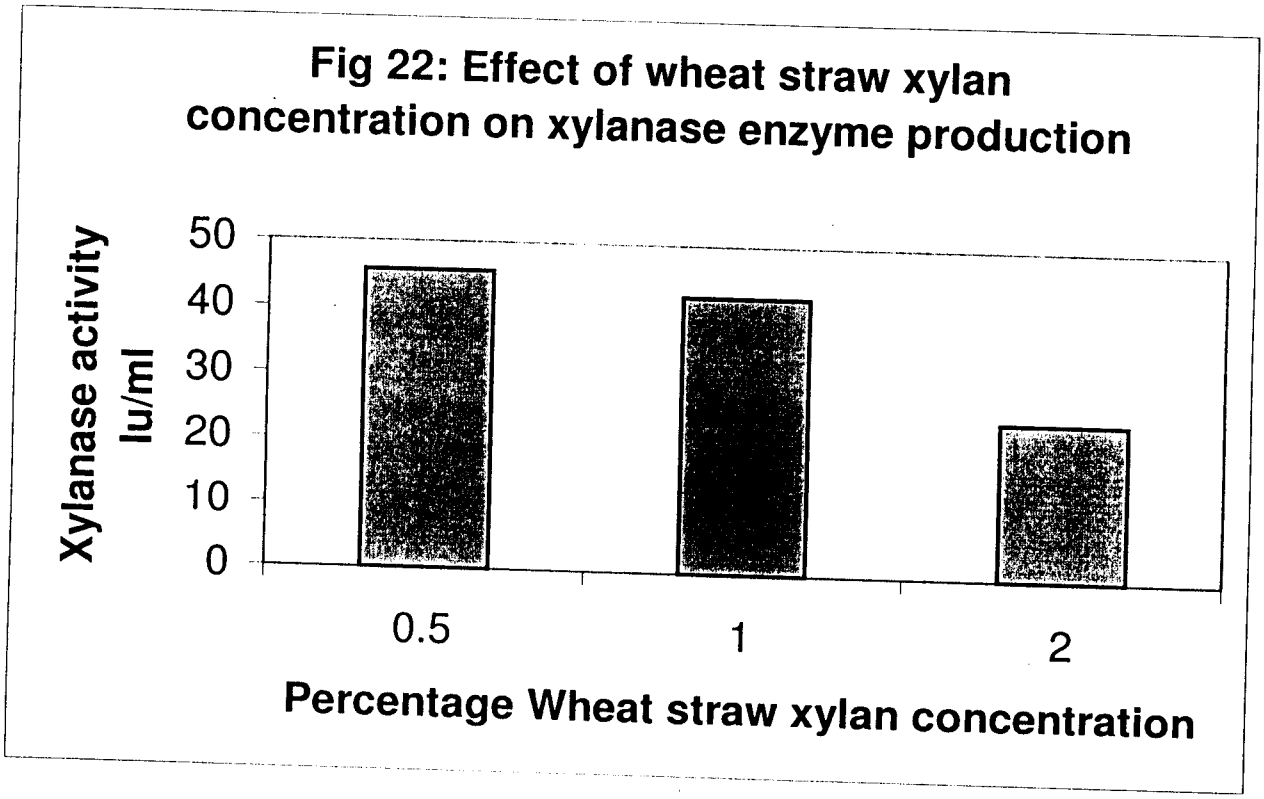


Fig 22: Effect of wheat straw xylan concentration on xylanase enzyme production
Xylanase activity lu/ml
50
40
30
20
10
0
0.5
1
2
Percentage Wheat straw xylan concentration

The effect of wheat straw xylan concentration on xylanase enzyme production has been studied for isolate number 2 in shake flask with substrate concentration ranging from 0.5% to 2%. The result (Fig 22) shows that with increase in xylan concentration enzyme production has decreased which is in contrast to the observation made with *Bacillus* culture.

Fermentation study: The production enzyme in the fermentor under controlled conditions has been carried out with working volume of 4 lit. A 10% v/v inoculum has been used for this study. The detail about the inoculum media composition has been described in the previous chapter. A 8-10 hr grown culture was used as inoculum for the present study. The pH was controlled by the automatic addition of 2(N) NaOH and 2(N) HCl. The pH and temperature of the fermentation has been maintained at 7 and 45^{O}C respectively. Sampling has been done at regular interval of time and cell free broth has been used for enzyme activity determination. From the result (Fig 23) it can be concluded that maximum enzyme production occurred at around 24 hrs thereafter activity decreased sharply. Time of harvesting is a crucial factor in *Bacillus* culture as it seems protease contamination probably responsible for sharp decrease in enzyme activity.

As in shake flask studies it has been observed that 1%wheat straw xylan improves the xylanase enzyme production so a batch studies has been carried out in the fermentor with pH and temperature control. In this case fermentation has been carried out beyond 24 hrs. One of the critical factor for the xylanase

enzyme production is the time of harvesting. Besides, maximum enzyme production occurs in a very short period of time. In this study, maximum enzyme (120 IU/ml) production occurred at 26 hr and thereafter it decreased to 57.5 IU/ml in 42 hrs (Fig 24) .

To improve the enzyme production a fed batch fermentation has been planned with 0.25% Wheat straw xylan as intermittent feed. Initially, 1% wheat straw powder has been used to start batch fermentation thereafter 0.25% of 100ml wheat straw xylan has been added after 14 hrs of fermentation. The activity after 20 hrs almost reached a value of 42IU/ml. Table 3.15 shows that comparative advantage of fed batch fermentation over batch fermentation.

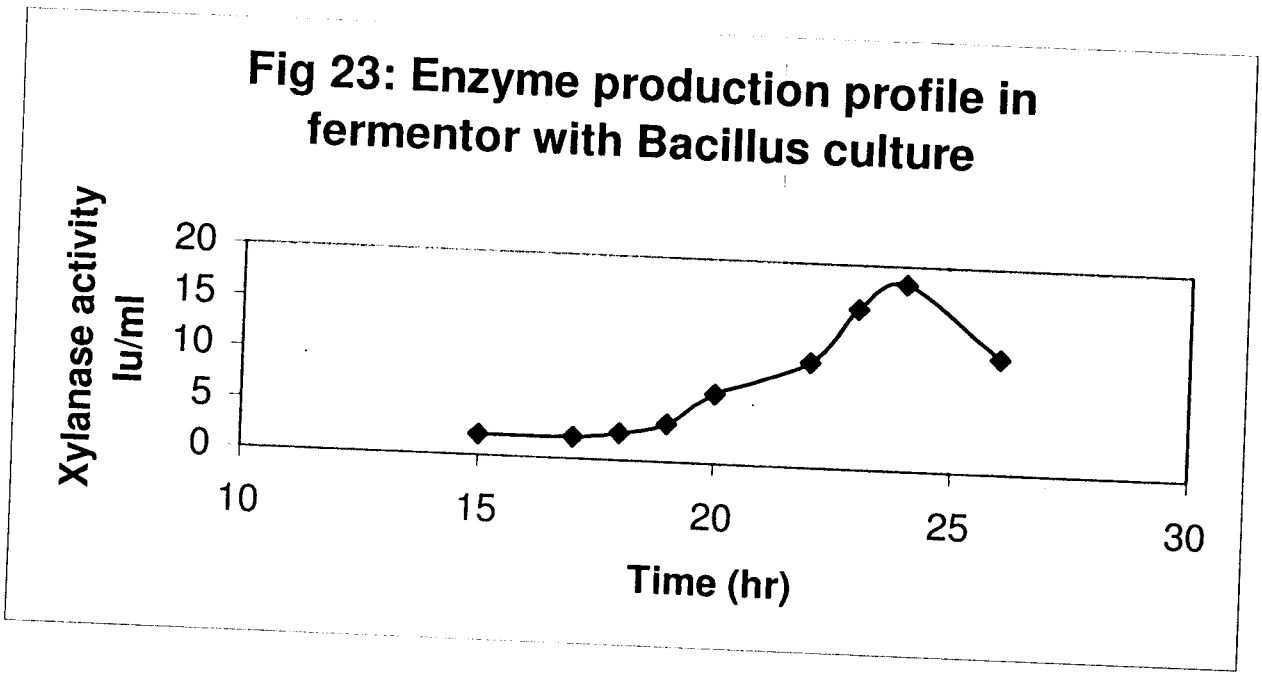


Fig 23: Enzyme production profile in fermentor with Bacillus culture
Xylanase activity lu/ml
20
15
10
5
0
10
15
20
25
30
Time (hr)

Fig 24: Bacillus enzyme production with 1% wheat straw xylan

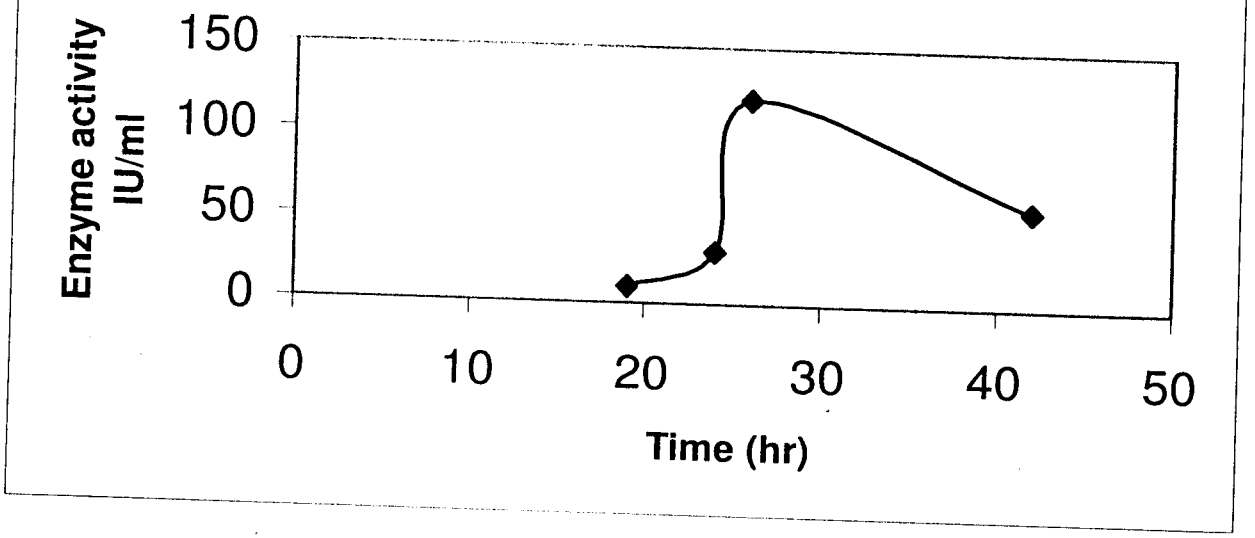


Table 3.15: Comparative study of Fed batch and Batch fermentation

	Enzyme activity Iu/ml
Batch operation with 1%wheat straw powder	18 IU/ml
Fed batch operation with 0.25% wheat straw xylan	42 IU/ml

Batch fermentation studies with isolate number 2:

In shake flask studies with isolate number 2, M9 medium has been used where there was no organic nitrogen source. The maximum enzyme production has been occurred within 48 hrs. To increase the productivity of enzyme production process, enriched medium containing yeast extract and peptone has been tried with isolate number 2. It has been observed (Fig 25) that in shake flask studies similar level of enzyme production has been achieved in enriched medium within a shorter period of time (24-30 hrs). So, all the fermentation studies have been carried out with enriched medium containing 5g/l of yeast extract and 5g/l peptone.

As in shake flask studies it was not possible to optimize the fermentation pH so pH optimization has been carried out in the fermentor under pH and temperature controlled conditions. Temperature of the fermentation was controlled at 45^0C. Agitation and aeration has been used as tool for maintaining Do above 30%. The control of pH has been achieved by automatic addition of 2(N) HCl and 2(N) NaOH. In this study enzyme assay has been carried out at 55^0C , p[illegible] for 15 min

incubation time using 1% Birchwood xylan. As the pulp treatment has been carried out at 50-55^{0}C so enzyme incubation temperature has also been changed. The pH range of 7-10 has been chosen for pH optimization studies. From the result (Fig 26) it can be seen that pH 8 is the optimum pH for enzyme production and also within 24-30 hrs maximum enzyme production occurs.

However in case of isolate number 2, decrease in enzyme activity beyond 24 hr is minimal which is in contrast to the observation made with *Bacillus* culture. Besides enzyme production starts even at early stage of fermentation which was not observed in *Bacillus culture.* This indicates that probably in case of isolate number enzyme production occurs at during growth phase whereas in case of *Bacillus* culture growth occurs at late exponential phase.

As in shake flask studies it was observed that wheat bran is a better carbon source as compared to wheat straw so a batch fermentation has been carried out at pH 8 using 1% wheat bran. Time profile of enzyme production (Fig 27) shows that higher enzyme activity (15.84 IU/ml) was obtained with 1% wheat bran.

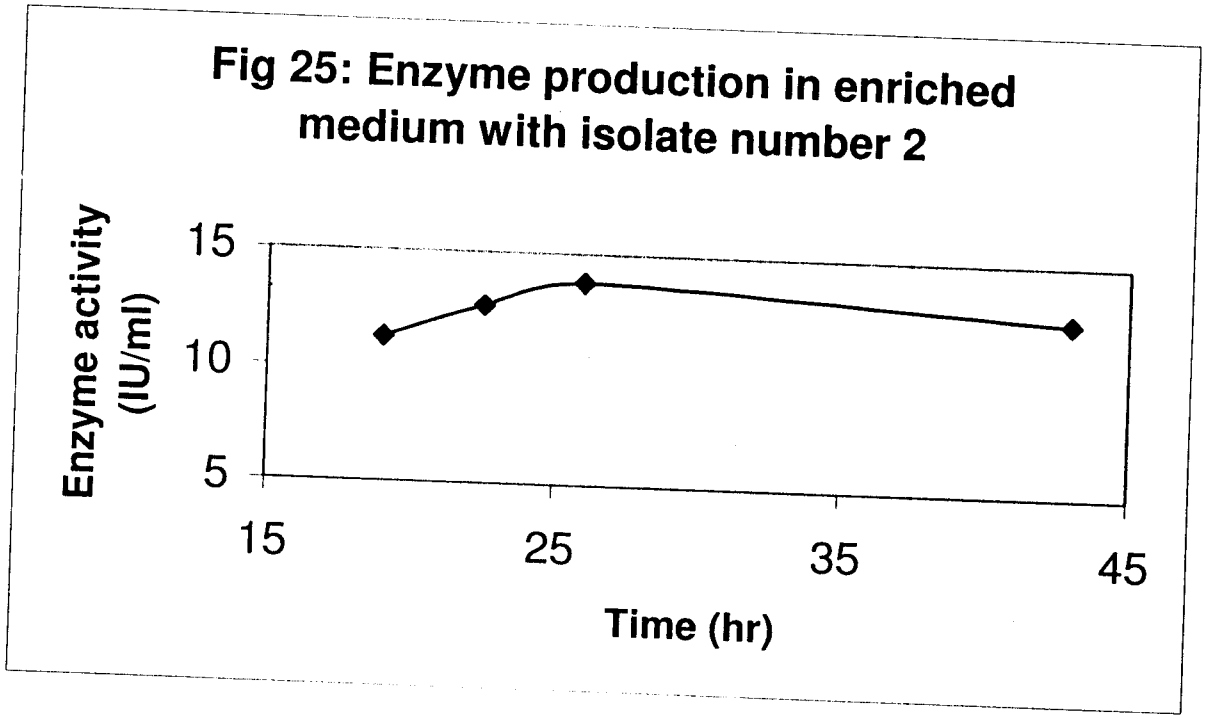


Fig 25: Enzyme production in enriched medium with isolate number 2
Enzyme activity (IU/ml)
15
10
5
15
25
35
45
Time (hr)

Fig 26: pH optimization for enzyme production using isolate number 2

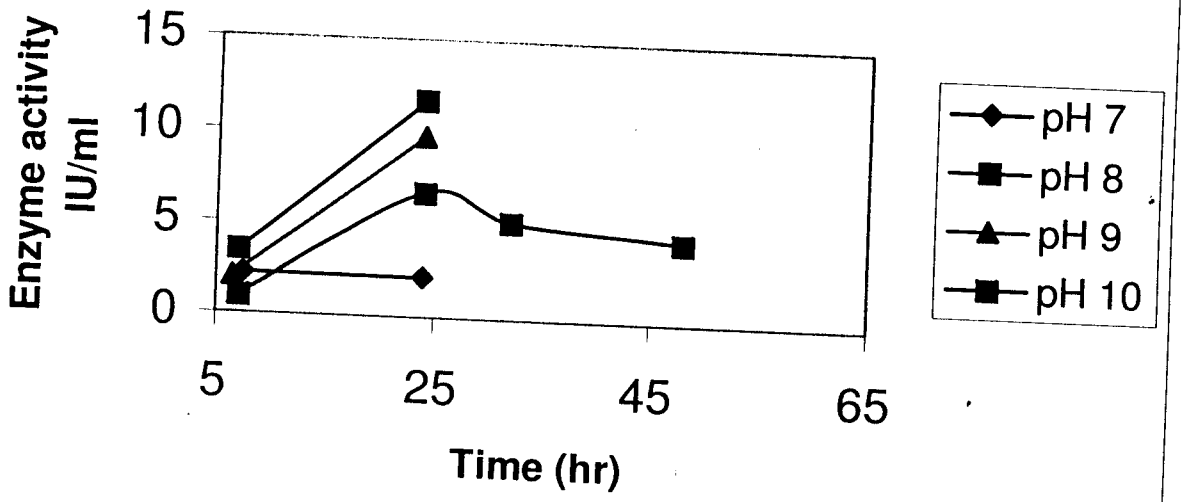
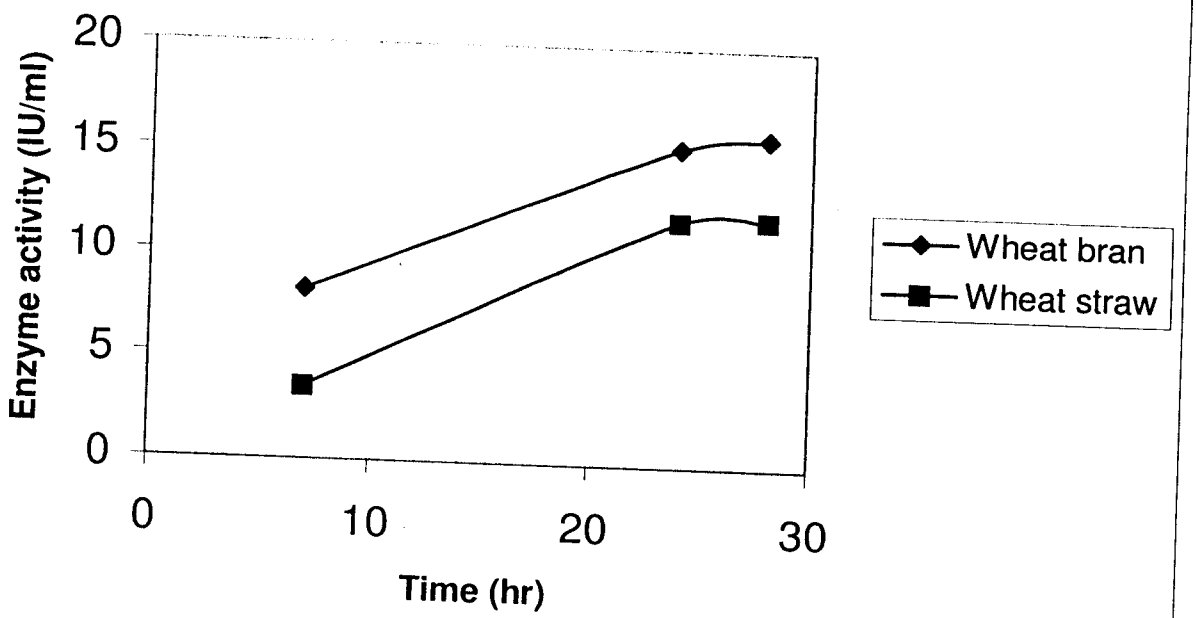


Fig 27: Enzyme production profile for isolate number in two different substrates



3.5 : Enzyme concentration in ultra filtration membrane:

Concentration of enzyme is a usual practice in commercial enzyme production. This can be achieved either by ammonium sulphate precipitation or by ultrafiltration in tangential membrane system. Although ammonium sulphate precipitation is usually used before chromatographic purification due to its ability to partially purify the crude enzyme. However, in commercial scale enzyme concentration can be economically achieved by ultrafiltration. So, in the present study, enzyme concentration has been attempted with Sartorius mini module tangential system with 10kD ultrafiltration membrane (polysulphone). To achieve sufficient permeate flux in ultrafiltration system, a peristaltic pump has been used to feed cell free broth (after centrifugation of fermentation broth at 10,000 rpm, 4^0C for 20 min) to the membrane system at a flow rate of 40-50 lit/hr. Inlet pressure of the membrane was initially 2.5 Bar and retentate outlet pressure was adjusted at 1 Bar by regulating a valve in the retentate line. During concentration of enzyme, broth has been kept in a chilling water bath maintained at 4^0C. This prevents the deactivation of enzyme due to heat generation. After concentrating the enzyme both permeate and retentate (enzyme concentrate) are collected and membrane was washed with 100 ml 50 mM phosphate buffer (pH7) and this was also collected for enzyme assay. From the enzyme assay of permeate, retentate and membrane wash it can be seen enzymes has been concentrated almost 20 times. However in both the enzymes, permeate contains enzyme activity but in case of *Bacillus* enzyme permeate activity is much higher (Table 3.16). Also some amount of enzyme has also been lost as a membrane wash. In case of

Bacillus enzyme, however, membrane with low molecular cutoff should be taken. Polysulphone membrane used in the present study is hydrophobic in nature which probably adsorbs proteins. So, to improve the enzyme concentration process a hydrophilic membrane is preferable.

Table3.16: Enzyme concentration in ultrafiltration membrane

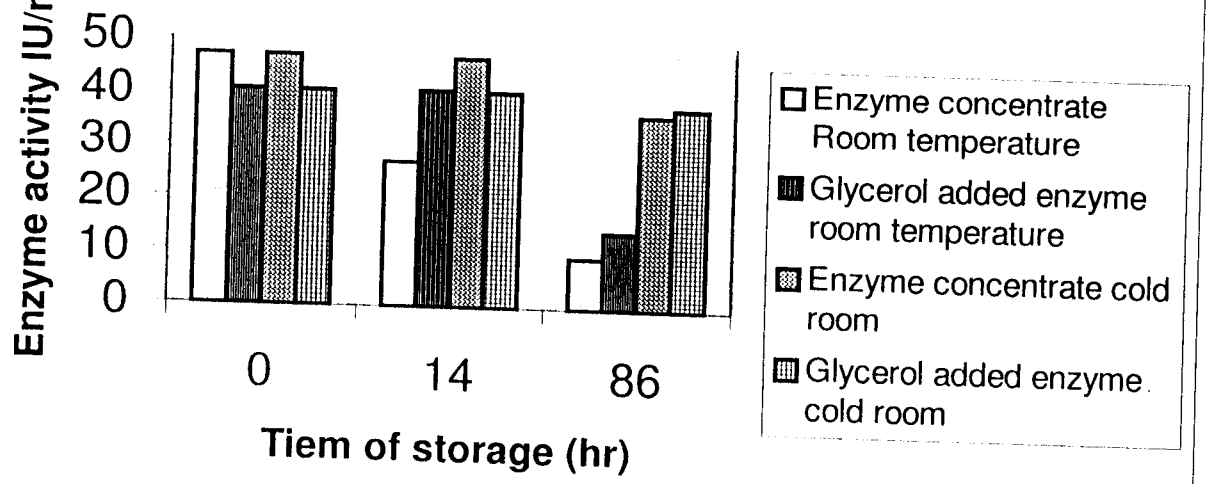
	Bacillus enzyme activity IU/ml	Isolate number no 2 enzyme activity
Enzyme	15.11	12.8
Enzyme concentrate	395.2	225.17
Membrane wash	9.41	9.12
Permeate	7.18	0.198

3.6 Storage stability of enzyme:

Storage stability of any enzyme is an important criterion for successful commercialization. Although xylanase enzyme can be stored at cold room ($<10^0$C) for a longer duration of but addition of various additives can be beneficial for enzyme stabilization. In the present study, a known stabilizer like glycerol has been used upto a concentration of 20-25% (v/v). The beneficial effect of glycerol on enzyme storage stability has been studied for 86 hrs. *Bacillus* enzyme concentrate and glycerol added *Bacillus* enzyme concentrate has been kept at room temperature and at cold room and their activity has been measured at certain interval of time. From the results (Fig 28) it can be seen that at room temperature enzyme gets easily deactivated and lost 45% of its activity within 14

hrs whereas glycerol added enzyme was stable at room temperature for the same duration of time. In case of cold room storage, enzyme concentrate and glycerol added enzyme retain its activity for 14 hrs. However after 86 hrs of storage it was observed that even in cold room storage both enzyme concentrate and glycerol added enzyme concentrate lost activity . In case of glycerol added enzyme loss of activity was 7% whereas 23% activity loss was occurred for enzyme concentrate in cold room storage. In case of room temperature storage growth of unwanted organism was observed for both the case and enzyme activity loss was drastic after 86 hrs of storage. So it can be concluded, glycerol can stabilize enzyme at room temperature for a shorter period of time and cold room storage with glycerol addition can improve the enzyme storage stability for a longer duration of time.

Fig 28: Enzyme storage stability



3.7 Purification of crude xylanase:

Purified xylanase is necessary for various biochemical studies as well as in determining amino acid sequence. Various purification protocol has been followed for extracellular enzyme and a combination of methods has been followed. In the initial stage, concentration of enzyme is necessary which can be achieved either by precipitation or by ultrafiltration. Precipitation can be achieved either by solvents or ammonium sulphate. In the initial stage, Bacillus enzyme has been used for precipitation study and various percentage of ammonium sulphate has been added to find out the effect on purification. After precipitation of one fraction supernatant was used for next fraction. Precipitate of each fraction is dissolved in same volume of buffer. Amount of ammonium sulphate dissolved in different fraction was calculated from the formula given below.

$X= 5.33*(S2-S1)/(100-0.3*s2)$

Where x =amount of ammonium sulphate added for 1L solution.

S2=Final concentration of solution in %

S1= Initial concentration of solution in %

And the amount of ammonium sulphate dissolved in 10ml of crude enzyme solution is as follows

0-30% fraction : 1.75 gm

3-60% fraction : 1.81 gm

60-90% fraction 2.19 gm

90-100% fraction 0.761 gm

After fractionation of enzyme, each fraction has been analyzed for xylanase activity and protein concentration. The results of which has been presented in the following table **3.17**.

Fraction	Activity IU/ml	Protein mg/ml	Specific activity Iu/mg protein
0- 30 precipitate	4.36	0.0534	81.7
0-30 Supernatant	2.53	0.293	8.6
30-60 precipitate	10.1	0.156	64.6
30-60 Supernatant	1.17	0.21	5.56
60-90 precipitate	2.03	0.146	13.93
60-90 Supernatant	1.3	0.154	8.47
90-100 precipitate	1.68	0.035	47.69
90-100 Supernatant	1.41	0.158	8.9
Crude enzyme	7.618	0.090	2.81

From the result it can be seen that 0-30 fraction gave highest purification factor followed by 30-60 fraction. This study thus shows that ammonium sulphate precipitation is not only useful for concentration but also removes impurities. Ammonium sulphate of 60% saturated can be used for concentrating and preliminary purification.

Gel filtration: In purification of xylanase enzyme gel filtration is a standard method followed by various investigators and in this study, Sephadex G100 (fractionation range 4-100kD) has been used in glass column (Pharmacia

16/100). Inner diameter of the column was 16mm and mobile phase was delivered by a peristaltic pump at a flow rate 0.6 ml/min. Pre swollen gel is taken and washed with Milli Q water for 4-5 times and fines are decanted. The gel matrix mixed with Sodium phosphate buffer (0.05 M, pH7) and the slurry was degassed and kept at 4^0C for 20 mins. A 50 times concentrated (by ultrafiltration) 2ml enzyme sample has been loaded into the column. A fraction collector has been used where fraction of 3ml of eluant has been collected continuously. Each fraction has been further analyzed for enzyme assay and protein concentration by measuring absorbance at 280 nm. The absorbance and enzyme activity of each fraction has been shown in the following tables 3.18 and 3.19 and in Fig 29. As it can be seen that fraction 36 to 58 contains maximum amount of xylanase enzyme so this fraction has been pooled in the three different pools.

Pool A: Fraction no 36-40

Pool B: Fraction no 40-46

Pool C: Fraction no 46-58

Table 3.18 : Absorbance at 280 nm of different gel filtered fractions

Abosrbance at 280 nm	Fraction number	Absorbance at 280 nm	Fraction number
0.03	1	4	46
0.073	2	2.451	47
0.051	3	2.422	48
0.023	4	2.301	49
0.042	5	2.144	50
0.062	6	2.027	51
0.05	7	2.212	52
0.029	8	2.301	53
0.042	9	2.334	54
0.025	10	2.382	55
0.046	11	1.75	56
0.067	12	1.437	57
0.023	13	1.106	58
0.021	14	0.805	59
0.024	15	0.587	60
0.034	16	0.457	61
0.036	17	0.376	62
0.335	18	0.32	63
1.349	19	0.275	64
1.621	20	0.237	65
1.092	21	0.208	66
1.021	22	0.18	67
0.916	23	0.154	68
0.514	24	0.131	69
0.368	25	0.11	70
0.321	26	0.093	71
0.322	27	0.082	72
0.337	28	0.074	73
0.381	29	0.068	74
0.427	30		
0.783	31		
0.675	32		
0.578	33		
0.512	34		
0.458	35		
1.441	36		
1.324	37		
1.179	38		
1.064	39		
0.922	40		
2	41		
1.872	42		
1.747	43		
1.637	44		
1.54	45		

Table 3.19: Xylanase of gel filtered fractions

Fraction number	Xylanase activity IU/ml	Fraction number	Xylanase activity IU/ml
1	0	41	5.762
2	0	42	6.715
3	0	43	7.634
4	0	44	7.217
5	0	45	8.051
6	0	46	1.85
7	0	47	4.372
8	0	48	4.53
9	0	49	4.859
10	0	50	5.436
11	0	51	3.917
12	0	52	3.579
13	0	53	3.784
14	0	54	5.81
15	0	55	5.179
16	0	56	1.564
17	0.078	57	1.36
18	0.087	58	0.299
19	0.172	59	0.255
20	0.213	60	0.218
21	0.169	61	0.278
22	0.172	62	0.237
23	0.17	63	0.226
24	0.126	64	0.202
25	0.087	65	0.229
26	0.24	66	0.289
27	0.116	67	0.239
28	0.302	68	0.208
29	0.567	69	0.225
30	0.345	70	0.237
31	0.64	71	0.228
32	0.623	72	0.256
33	0.541	73	0.22
34	0.377	74	0.22
35	0.369		
36	7.89		
37	8.189		
38	4.993		
39	2.185		
40	1.659		

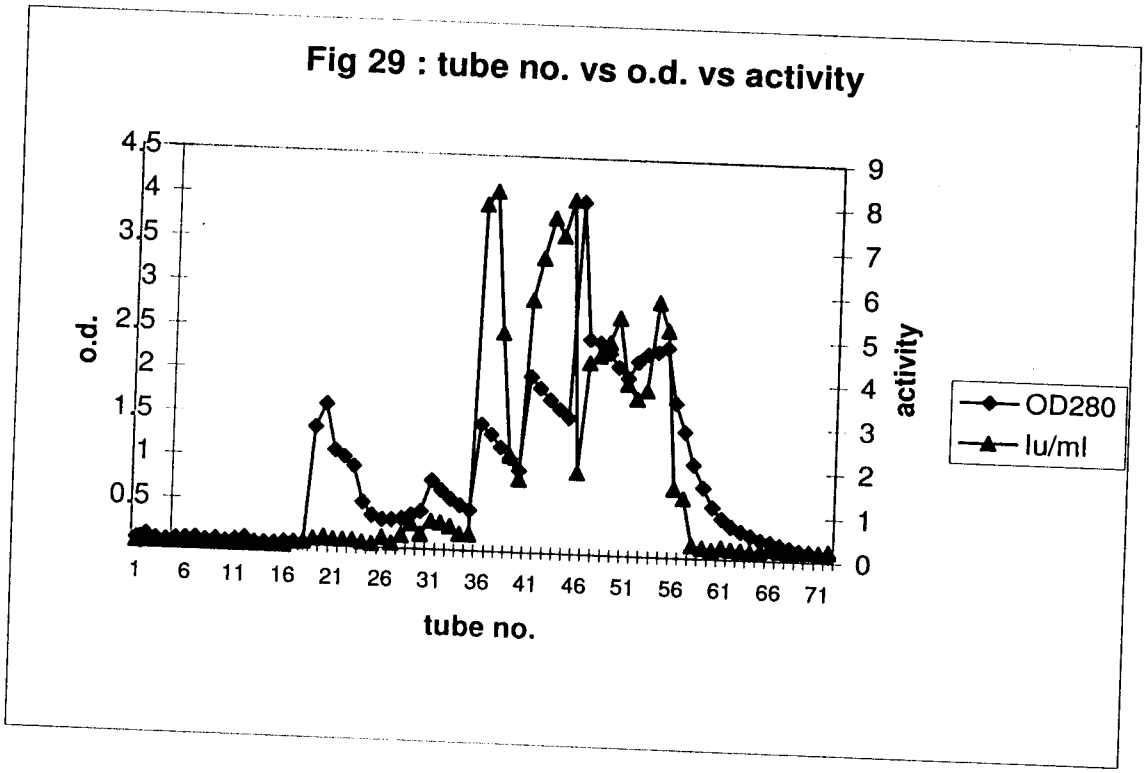


Fig 29 : tube no. vs o.d. vs activity
o.d.
activity
tube no.
OD280
lu/ml
4.5
4
3.5
3
2.5
2
1.5
1
0.5
9
8
7
6
5
4
3
2
1
0
1 6 11 16 21 26 31 36 41 46 51 56 61 66 71

All the three pools have been further analyzed for protein concentration and enzyme activity. The results (Table 3.20) are as follows.

Table 3.20: Enzyme assay and protein estimation of different pools

Pool	**Enzyme activity IU/ml**	**Protein conc mg/ml**	**Specific activity IU/mg protein**
A	4.98	0.18	26.99
B	5.55	0.33	16.51
C	3.85	0.33	11.48
Crude enzyme	393.95	25.85	15.23

Table 3.21: Balance table for gel filtration:

Pool	Activity IU/ml	Protein mg/ml	Volume ml	Total activity	Total protein
A	4.98	0.184	15	74.80	2.77
B	3.55	0.335	21	116.68	7.07
C	3.85	0.335	36	138.67	12.07
Crude enzyme	393.95	25.85	2	787.91	51.70

From the results (Table 3.20) it can be seen that POOL A has better purification than any other poll but still it is fur less than the 0-30 fraction ammonium sulphate precipitate. Besides it can also be seen that almost 58-60% loss of enzyme activity and protein during gel filtration (Table 3.21). However, these pools can be

further purified using ion-exchange chromatography in combination with other chromatography system.

3.8 :Evaluation of enzymes as a pre-bleaching agent:

The effective use of xylanase as a pre-bleaching agent has already been reported in various published literatures (Chinnaraj *et.al.* 1994, Paice *et.al.* 1992, Presley *et.al.* 1998, Jimenez et. al. 1996, Sivaswamy 1998, Viceena *et.al.* 1995, Suurnakki *et.al.* 1995, Shah *et.al.* 2000, Bajpai and Bajpai 1997). Although most of the scientific literatures dealt with hardwood Kraft pulp but application of xylanase enzyme on nonwoody pulp has also been reported. The bleach boosting effect of various xylanase enzymes has been reported for various pulps and the probable mechanism of action has also been proposed. Still the identification of most critical parameters affecting the enhanced bleaching of pulp was not possible due to the complexity of pulping and bleaching process of various raw materials. Some of the important parameters of enzyme have been identified for this purpose and they are high thermal stability, low molecular weight and higher pH optimum etc. As pH of the pulp generally lies in the alkaline range so the production of enzyme from an alkanophilic organism is most desirable. Thermostability of enzyme is also an important parameter in pulp treatment as pulps are generally treated after the pulping process, which is a high temperature process. Low molecular weight enzyme favor faster diffusion in the pulp matrix resulting in enhanced removal of hemicullose. Literature reports also indicate that efficiency of xylanase enzyme depends on the type of pulp and method of pulping.

In the present study, two xylanase enzyme producing organisms were chosen which have been isolated in our laboratory from local sources using minimal medium containing xylan as the only carbon source. Cultures were further purified by using enrichment culture technique and finally cultivated in liquid culture for enzyme production. Once the enzyme has been produced it has been further analyzed for its suitability. Among a large number of cultures, two cultures have been selected for the enzyme production and have been used in the present study. Enzyme production has been optimized in the shake flask culture and finally produced in the fermentor under pH and temperature controlled conditions. Enzymes obtained from fermentation have been used to characterize it. The parameters evaluated for the enzyme are activity profiles at different temperature and pH and thermal stability. Activity of *Bacillus* enzyme and Culture 2 enzyme against different pH (Fig 13) shows that both the enzyme has optimum activity at a pH of 7 but *Culture 2 enzyme* is active at a much broader pH range (6-11) whereas *Bacillus enzyme* is active at a narrow pH range (6-9.5). Temperature activity profiles of both the enzymes (Fig 12) also indicate that both of these are active in thermophillic range (50–80^{O}C). However, *Culture 2 enzyme* has optimum activity at a temperature of 70 ^{O}C, which is significantly higher than the optimum temperature of *Culture 2 enzyme* (55^{O}C). Another important parameter for enzymatic treatment of pulp is minimal cellulase contamination in xylanase enzyme preparation. In the present study enzyme concentrate used have cellulase activity but it is insignificant as compared to xylanase activity (*Bacillus enzyme*: Xylanase activity 330 IU/ml FPU: 0.12 IU/ml, *Culture 2*

enzyme: xylanase activity 94 IU/ml, FPU 0.21 IU/ml). So from the point of view of suitability of enzyme as a prebleaching agent both the enzymes seems to be suited better.

Evaluation of bleaching efficiency of the enzymes produced:

Three different pulps produced from wheat straw, rice straw and jute were treated with the two enzymes at a temperature of 55°C and at two different pH values of 7.0 and 8.5. The water extracts or pulp filtrates released from the treated and control pulps (without enzyme) were then analyzed for lignin, color and reducing sugars (as xylose) release (Fig 30,31,32). As far as lignin-release is concerned, a higher amount of lignin was released in the filtrates of enzymatically treated pulps as compared to their respective controls (without enzyme treatment). This gives a preliminary indication of the effect of enzyme. Similarly a higher amount of color and reducing sugars could be observed for all the treated pulps. Analysis of pulp filtrates also demonstrates that for *Bacillus enzyme* an initial pH of the pulp doesn't have significant influence on reducing sugar release. Maximum affect of *Culture 2 enzyme* was observed in case of rice straw pulp and significant amount lignin and color released at pH 8.5. In case of jute pulp maximum lignin was released at pH 7 with *Culture 2 enzyme*. So, type of pulp does have a significant effect for the enzymes. However, any generalizations on the effect of pH and type of pulp can not be made from this study.

The treated and control pulps (without enzyme treatment) were then bleached with hydrogen Peroxide after an EDTA treatment. The bleached pulps were used

to make hand sheets for analyzing optical and strength properties (Fig 33,34,35). The efficacy of the enzyme has been evaluated in terms of brightness gain in enzyme treated pulp over the control. A gain in brightness of the treated pulp could be easily observed. A maximum gain of 5.5 points was obtained in the case of rice straw pulp treated with *Culture 2 enzyme* at pH 7. Similar gain was there with rice straw pulp at pH 8.5 with *Bacillus enzyme.* The result also shows that *Bacillus enzyme* is significantly better than *Culture 2 enzyme* that is contrary to the conclusion made from enzyme-characterization studies. Some of the important observations for *Bacillus enzyme* are higher brightness gain at higher pH for wheat straw and rice straw pulp. *Culture 2 enzyme* however seems to be more effective with rice straw pulp and the effect of pH is insignificant. This study does indicate that type of pulp is an important parameter and usual characteristics of enzyme like high optimum pH and thermo stability are not only the sole criteria for enhanced bleaching of pulp. From the strength properties of enzyme treated pulp effect of enzyme on pulp strength can be determined. The result indicates that there is rarely any decrease in strength of pulp except jute pulp where there was marginal decrease in tensile index for both the enzyme. So the result indicates that minimal cellulase contamination in xylanase enzyme doesn't have any adverse effect on pulp.

Jimanz *et. al.*1996 have reported on biobleaching of wheat straw pulp and they have reported a brightness gain of 2.4 points for enzyme-peroxide bleaching and 3 points gain in case of enzyme-peroxide-active chlorine bleaching. Although in Kraft pulp enzymatic treatment prior to usual bleaching result in usually 2-4

points brightness gain but highest brightness gain of 7.9 point was also reported by Paice *et.al.* 1992. Effect of enzymatic prebleaching on bagasse and hardwood kraft pulp also reported by Chinnaraj *et.al.* 1999 and they have obtained a brightness gain of 2.5-4 points for bagasse pulp. Number of published literature on enzymatic treatment of nonconventational pulp like wheat straw, jute etc are very few. In this context the present work provides a preliminary idea on this area. This study also shows that proper understanding of bleach boosting effect of enzyme needs to be considered not only from conventional parameters but also from isoenzyme activity of enzyme and pulp chemical structure.

The present work suggests that the effect of enzyme varies remarkably with the type of pulp. As it is clear that both the enzymes affect different pulps in different manner. On one hand, *Bacillus* enzyme is showing good results in all the three pulps at both the pH values with a significantly large gain of 5.1 points in the rice straw pulp as compared to its control. On the other hand, *Culture 2 enzyme* shows an equivalent effect on the rice straw pulp only while there is only a mild gain in wheat straw pulp at pH 7.0 and no gain in the case of jute pulp. The pulping procedure, which has been used here, may also have an important effect on the bleaching potential of the enzyme. Since there is not much change in the pH of the pulp throughout the pulping process, the redeposition of xylan may not be significant and perhaps the enzyme is directly attacking the lignin-xylan complexes and thereby enabling an easy accessibility of lignin to the bleaching agents.

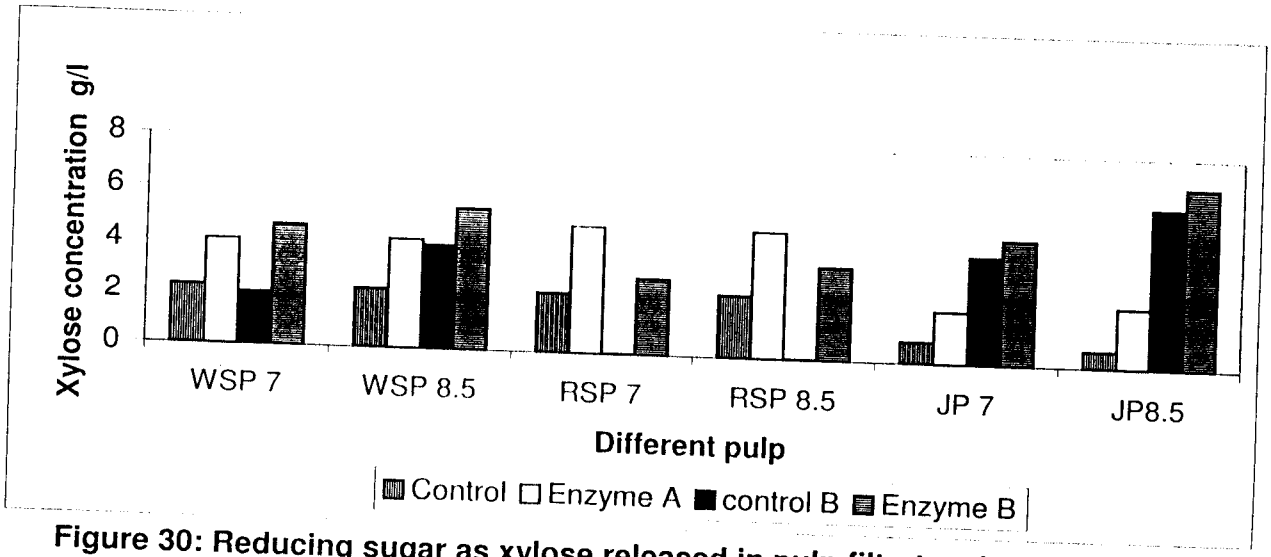


Figure 30: Reducing sugar as xylose released in pulp filtrates due to enzyme treatment of three different pulps

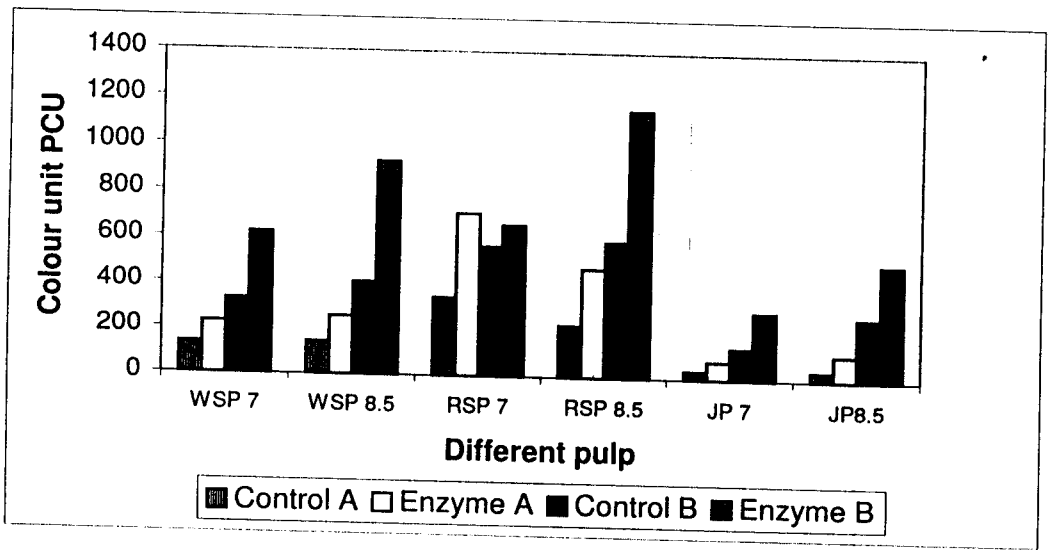


Figure 31: Color released in pulp filtrates due to enzyme treatment of three different pulps

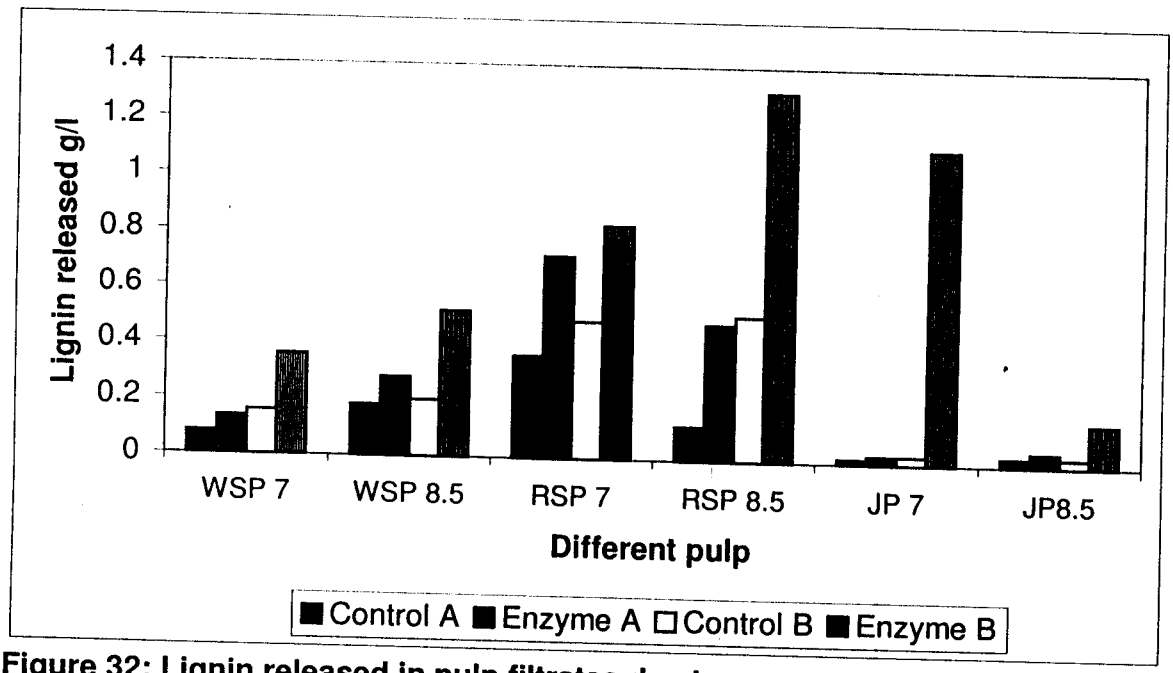


Figure 32: Lignin released in pulp filtrates due to enzyme treatment of three different pulps

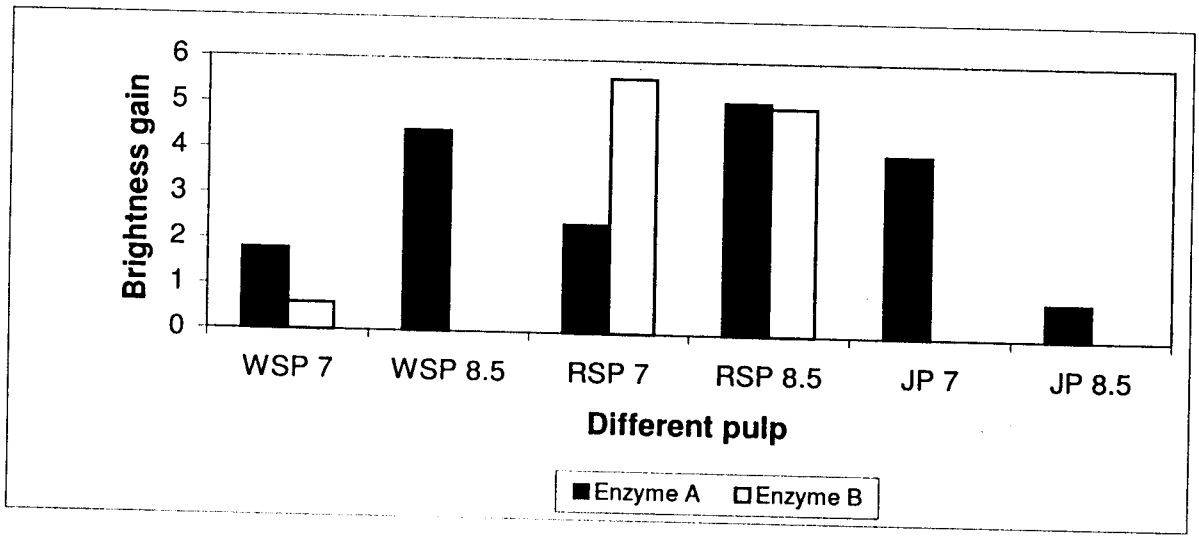


Figure 33: Brightness gain in enzyme treated pulps

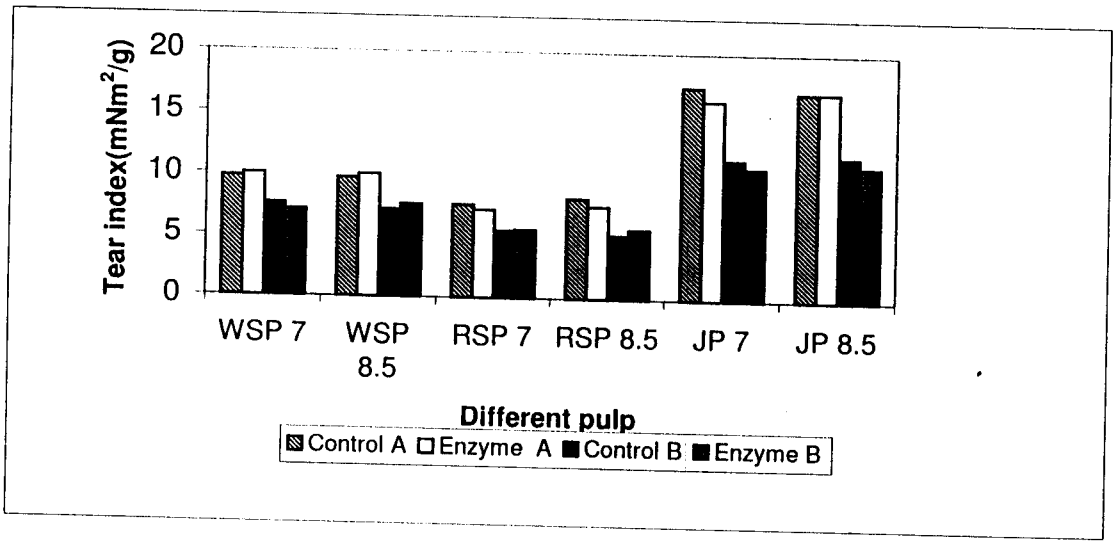


Figure 34: Effect of enzyme treatment on tear index of pulp

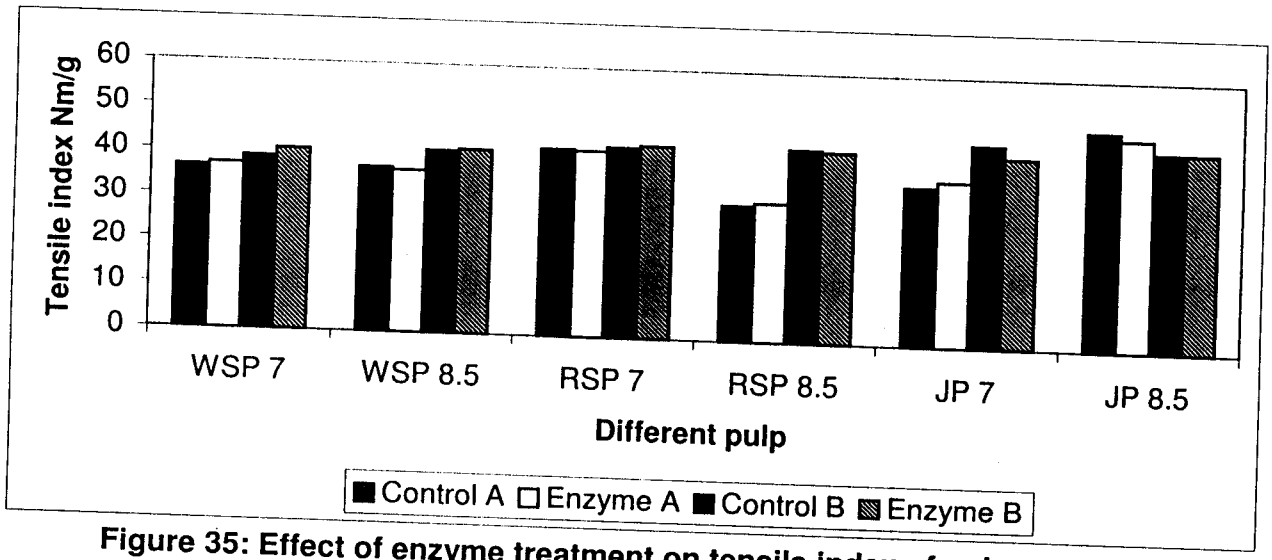


Figure 35: Effect of enzyme treatment on tensile index of pulp

EVALUATION REPORT OF ENZYMES CARRIED OUT BY CPPRI, SAHARANPUR

STUDIES ON EVALUATION OF ENZYMES RECEIVED FROM BIRLA INSTITUTE OF SCIENTIFIC RESEARCH AS PREBLEACHING AGENTS

Pulp Source : Hardwood : Bamboo (9:1) pulp from wood based mill (Kappa number – 21)

Enzyme Details

No. Of Enzymes Received : 2

Enzyme Details : 1. Bacillus enzyme
2. Culture 2 enzyme

Nature Of Enzyme : Xylanase

Sample Received On : 26/06/2002

Enzyme	Enzyme Activity , IU/Ml (Oat Spelt Xylan) Considered For Enzymatic Prebleaching
1. Bacillus enzyme	170
2. Culture 2 enzyme	240

TABLE- 1

ENZYME PRE-TREATMENT CONDITIONS OF KRAFT EUCALYPTUS PULP			
Particulars	**Control pulp**	**BISR enzyme treated pulp**	
		Bacillus enzyme	**Culture 2 enzyme**
Enzyme Dose , IU/G	0	7	7
Pulp Consistency, %	10	10	10
Temperature, °C	50	50	50
Treatment Time, (Hrs)	1.5	1.5	1.5
Ph	7.0-7.2	7.0-7.2	7.0 -7.2

TABLE –2

CHARACTERIZATION OF PULP & PULP FILTRATES BEFORE & AFTER ENZYME TREATMENT			
Particulars	**Control pulp**	**Enzyme treated pulps**	
		Bacillus Enzyme	**Culture 2 Enzyme**
Kappa number	20.2	19.1	18.6
Brightness, % ISO	26.86	27.41	27.8
CED Viscosity cm^3/gm	645	648	682
Reducing Sugars (as Xylose), Kg/tp	10.31	12.22	12.71

TABLE – 3

IMPACT OF ENZYMATIC PREBLEACHING ON CONVENTIONAL BLEACHING OF HARD WOOD PULPS

Particulars	Control	Bleaching sequence - CEH			
		Enzyme treated pulps			
		Bacillus Enzyme		Culture 2 Enzyme	
		ET1	ET2	ET1	ET2
Chlorination Stage					
Chlorine Applied , %	4.2	4.2	3.57	4.2	3.57
Alkali Extraction Stage					
Alkali Applied , %	1.80	1.80	1.80	1.80	1.80
Ce Brightness	40.95	43.0	38.8	44.73	40.45
Hypo Stage					
Hypo Applied , %	2.5	2.5	2.5	2.5	2.5
Brightness, % ISO	79.8	80.7	79.33	82.0	80.4

ET - Enzyme treated Pulps

TABLE – 4

STRENGTH PROPERTIES BEFORE & AFTER ENZYME TREATMENT

Particulars	Control Pulp	Enzyme Treated Pulp			
		Bacillus enzyme		Culture 2 enzyme	
		ET1	ET2	ET1	ET2
Strength Properties					
Freeness, CSF (ml)	240	250	270	240	255
Burst Index, k Pa.m^2/g	4.5	4.4	5.10	4.60	4.65
Tensile Index, Nm/g	70.0	71.5	70.5	73.0	76.5
Tear Index , mN.m^2/g	5.2	4.75	5.10	4.50	5.10
Optical Properties					
Opacity, %	77.56	79.71	81.38	79.86	81.06

ET - Enzyme treated Pulps

EVALUATION OF XYLANASE ENZYMES OF BIRLA INSTITUTE OF SCIENTIFIC RESEARCH AS PREBLEACHING AGENT

Table 1: Xylanase enzyme activity

Enzyme code	Substrate	Enzyme activity IU/ml
Enzyme 1	Oat spelt xylan	43.74
	Birchwood xylan	76.39
Enzyme 2	Oat spelt xylan	26.97
	Birchwood xylan	37.43

Table 2: Enzyme pretreatment conditions of enzymatic pretreatment of Kraft Eucalyptus pulp using xylanases received from BISR

Particulars	Control pulp	BISR enzyme treated pulp	
		Enzyme 1	Enzyme 2
Enzyme dose IU/g	0	10	11
Pulp consistency	10	10	10
Temperature ^{0}C	50	50	50
Treatment time (h)	2	2	2
PH	6.5-7.2	6.5-7.2	6.5-7.2

Table3 : Characteristics of unbleached pulp before and after enzyme treatment			
Particulars	**Control pulp**	**Enzyme 1**	**Enzyme 2**
Kappa no of pulp	19.35	18.21	18.33
Brightness %	24	25.4	25.2
CED Viscosity cm^3/gm	625.6	630.5	558.3
Analysis of pulp filtrates before and after enzyme treatment			
Particulars	**Control pulp**	**Enzyme 1**	**Enzyme 2**
Lignin $_{280}$ Kg/tp	2.22	4.05	5.02
$Colour_{465}$ Kg/tp	19.31	34.99	44.14
Reducing sugars (as xylose) kg/tp	1.93	5.29	8.11

Table 4: Bleaching of pulp using conventional CEH sequence before and after enzyme treatment

Particulars	Control pulp	Enzyme 1 treated pulp	Enzyme 2 treated pulp
		ET 1	ET1
Chlorination stage			
% Chlorine applied	3.4	3.4	3.4
Alkali extraction stage			
% Alkali applied	1.8	1.8	1.8
Hypo stage			
% Hypo applied	2.5	2.5	2.5
Brightness % ISO	79	80.9	80.9

Table 5: Strength Properties before and after enzyme treatment

Particulars	Control pulp	Enzyme treated pulp	
		Enzyme 1 ET1	Enzyme 2 ET2
Freeness, CSF	280	255	265
Appraent index g/m^3	0.8	0.8	0.78
Brust Index K $Pa.m^2/g$	3.9	3.65	4
Tensile index Nm/g	66	55	58
Tear index mN m^2/g	5.15	5.4	5.5
Optical properties			
Final pulp brightness % ISO	79	80.9	80.9
Opacity %	77.6	78	76.8

ET: Pulp treated with xylanase

ET1: Enzyme treated pulp with same chlorine dose

Table 6: Bleaching of pulp using conventional CEH sequence before and after enzyme treatment

Particulars	Control pulp	Enzyme 1 treated pulp ET 2	Enzyme 2 treated pulp ET2
Chlorination stage			
% Chlorine applied	3.4	2.9	2.9
Alkali extraction stage			
% Alkali applied	1.8	1.8	1.8
Hypo stage			
% Hypo applied	2.5	2.5	2.5
Brightness % ISO	79	80.4	79.4

Table 7: Strength Properties before and after enzyme treatment

Particulars	Control pulp	Enzyme treated pulp	
		Enzyme 1 ET1	Enzyme 2 ET2
Freeness, CSF	280	240	230
Appraent index g/m^3	0.8	0.83	0.84
Brust Index K $Pa.m^2/g$	3.9	4.5	4.3
Tensile index Nm/g	66	68	60.5
Tear index mN m^2/g	5.15	5.8	5.8
Optical properties			
Final pulp brightness % ISO	79	80.9	79.4
Opacity %	77.6	70.83	73.84

ET: Pulp treated with xylanase

ET2: Enzyme treated pulp with less chlorine dose

Summary of results

Xylanase as a prebleaching agent has been commercially demonstrated in many European countries. Efforts in India in commercializing this technology have faced with certain setback due to non-availability of appropriate enzymes. However, isolation of suitable cultures from local sources with the ability to produce xylanase enzyme has resulted in intense research on this area in various Indian universities and Institutes. In this project, an effort has been made to isolate thermophilic culture with the ability to produce xylanase enzyme and its application as prebleaching agent in pulp bleaching. Major objectives of this project were to use alkali and thermostable xylanase enzyme for pulp treatment. During implementation of the project, number of sources have been utilized for isolation of xylanase producing cultures. After number of attempts, two isolates have been identified as suitable for biobleaching applications. One of the cultures has been identified as *Bacillus* culture and other isolate is yet to be identified. Sequence similarity matching of this isolate didn't provide any specific identification. Both of these cultures have been used to produce xylanase enzyme and enzyme produced has been characterized. Some of the observations made for these cultures are as follows:

- Enzyme produced from *Bacillus* culture has an optimum activity at a pH of 7 and optimum temperature of 55^0C. However the enzyme is active over the pH range of 6-9.5.

- Enzyme produced from isolate number 2 has also optimum pH of 7 and optimum temperature of 70^0C. However, this enzyme is active over a broader pH range of 6-11.
- Stability of both enzymes have been studied at optimum temperature and pH. It has been observed that enzyme from isolate number 2 has higher thermal stability and pH stability. At 55^0C (usually the pulp treatment temp), *Bacillus* enzyme lost 85% of its activity within 2 hrs whereas enzyme from culture no 2 retained 50% of its activity after 2hrs.
- Debranching enzyme activity of both enzyme samples have been carried out and it was observed that acetyl esterase activity was proportionately higher for both the enzymes.
- Effect of buffer concentration on enzyme assay was minimal for both the enzyme and incubation time obviously effects the enzyme assay.
- Optimization of enzyme production using *Bacillus* and culture no 2 have been carried out in shake flask level. Optimum temperature for enzyme production was 45^0C for both the cultures and optimum pH was 7 for *Bacillus* culture.
- Among the various carbon sources tested, wheat straw powder seems to be better for *Bacillus* culture and xylan isolated from wheat straw favors xylanase production.
- Maximum xylanase enzyme production of 166 IU/ml was observed with *Bacillus* culture using 2% wheat straw Xylan.
- Similarly, xylanase enzyme production with culture 2 has been studied and maximum activity of 52 IU/ml was achieved with 1% wheat straw xylan.

- In case of *Bacillus* culture, increasing wheat straw concentration (upto 3%) resulted in higher enzyme production but reverse trends has been observed with culture 2.
- Fed batch fermentation with *Bacillus* culture has resulted in higher enzyme activity as compared to batch fermentation.
- pH optimization for enzyme production in case of culture 2 has been carried out in the fermentor and optimum value was found to be 8.
- Production time for enzyme has been reduced from 48 hrs to 30 hrs by using enriched medium for culture no 2.
- In case of culture no 2, wheat bran found to be a better carbon source for enzyme production as compared to Wheat straw.
- In case of *Bacillus* culture, time of harvesting is a critical factor as fermentation beyond 24 hrs results in significant enzyme activity decrease.
- As it was observed that *Bacillus* enzyme is relatively less stable so an attempt has been made to enhance storage stability by adding glycerol (20-25%) to the enzyme sample. Addition of glycerol has improved storage stability of enzyme in cold room whereas, at room temperature effect was not significant.
- Preliminary purification of crude enzyme using ammonium sulphate precipitation and gel chromatography has been carried out and it was observed that maximum purification was obtained with ammonium sulphate precipitation (forty fold purification).
- Both the enzymes have been used to treat three different agro based pulps (jute straw, wheat straw and rice straw) at two different pH values (7 and 8.5).

Pulps were made by open hot digestion method using 8% NaOH. All these pulps were subjected to EDTA and peroxide bleaching.

- A maximum gain of 5.5 points was obtained in the case of rice straw pulp treated with *Culture 2 enzyme* at pH 7. Similar gain was there with rice straw pulp at pH 8.5 with *Bacillus enzyme.*
- This study also shows that *Bacillus enzyme* is significantly better than *Culture 2 enzyme* as a prebleaching agent.
- Some of the important observations for *Bacillus enzyme* are higher brightness gain at higher pH for wheat straw and rice straw pulp. *Culture 2 enzyme* however seems to be more effective with rice straw pulp and the effect of pH was insignificant.
- Both the enzymes produced have also been evaluated in CPPRI, Saharanpur on hardwood pulp with conventional CEH sequences. Both the enzymes were found to have bleach-boosting effects. Although in the initial stage evaluation, strength was affected due to probably higher doses but in the next stage evaluation, it was observed that both enzymes have minimal cellulase contamination. Besides strength properties of pulp were also marginally improved after enzyme treatment with a corresponding increase in CED viscosity of enzyme treated pulp.

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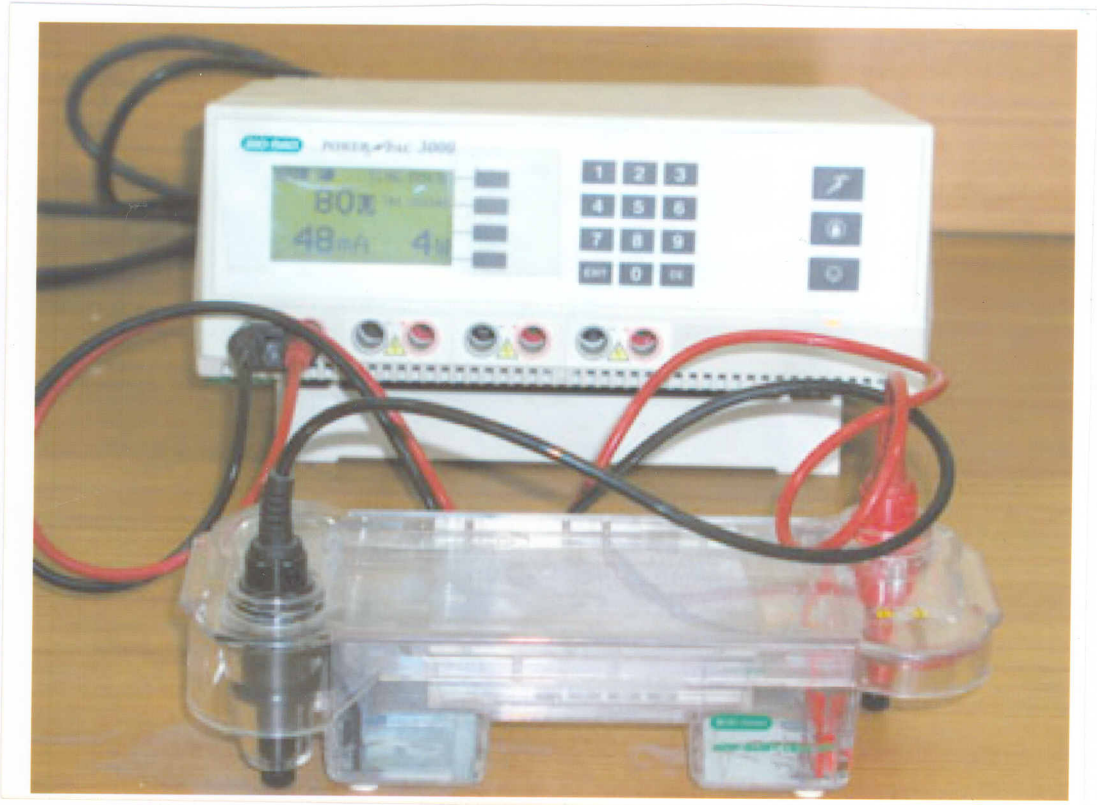
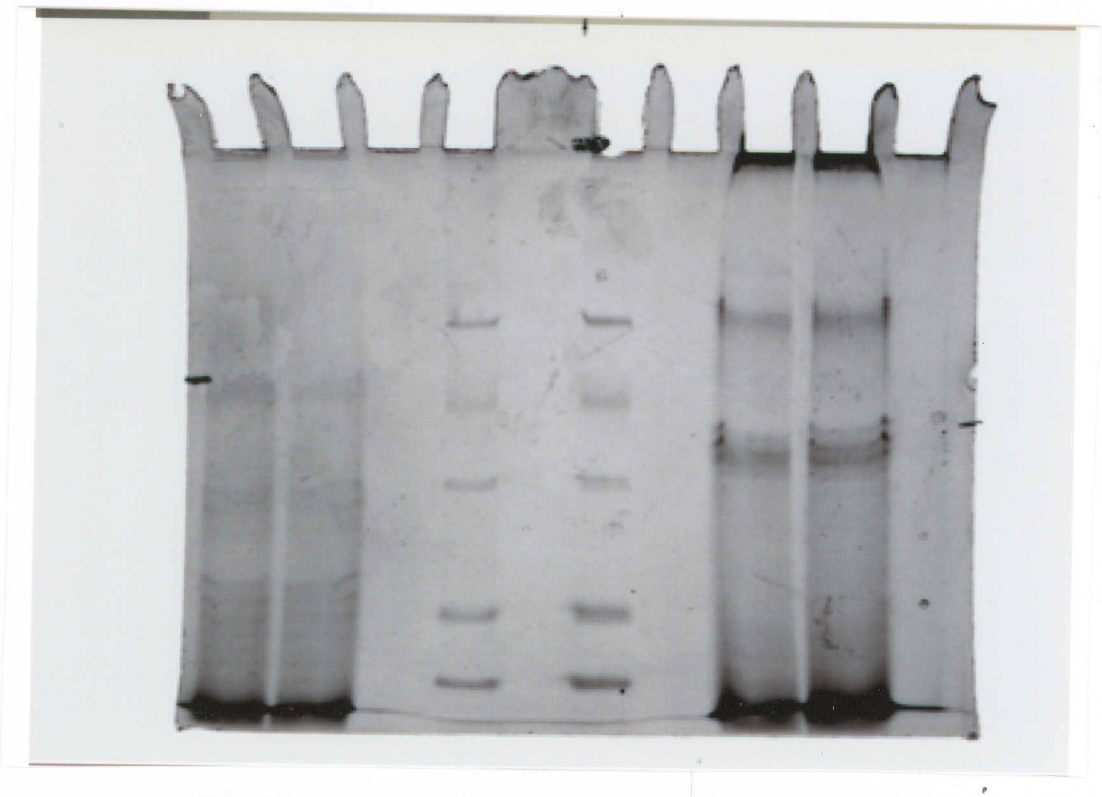
APPENDIX

FERMENTOR SETUP FOR XYLANASE PRODUCTION



BIORAD GEL ELECTROPHORESIS SYSTEM



SDS-GEL ELECTROPHORESIS OF BOTH ENZYMES