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PURIFICATION AND CHARACTERZATION OF XYLANASE FROM FUNGAL ISOLATES AND ITS APPLICATION IN PAPER AND PULP INDUSTRIES

A Project Report

Submitted by

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BONAFIDE CERTIFICATE

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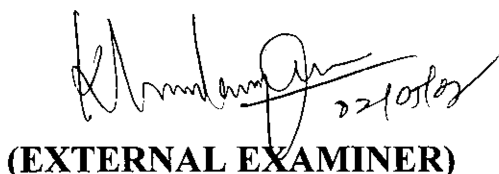

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ABSTRACT

The number of possible applications of microbial xylanase in the paper and pulp industry is generally increasing and several are approaching commercial use. However, the properties of commercial xylanase make them unsuitable for the real process of paper and pulp bleaching.

The fungus was isolated from soil. Xylanase activity was found in the culture filtrate. Optimum pH and temperature of purified xylanase enzyme was found to be 6.0 and 55°C respectively. The xylanase enzyme retains 30% of its original activity after 1 hr at 60°C and lost 100% of its original activity. EDTA, 1,10 Phenanthroline & ß-mercaptoethanol reduced the enzyme activity. The enzymes were purified to homogeneity as conformed by non-denaturing PAGE. The molecular weight of the purified enzyme was found to be 23 kDa. Application of xylanase to reduce the amount of bleach chemicals to achieve brightness in pulp & paper industry was tested.

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1. INTRODUCTION

1. INTRODUCTION

Enzymes are biological catalysts produced by all living things. Xylanase deconstructs plant structural material by breaking down hemicelluloses, a major component of the plant cell wall. Plant cell walls are necessary to prevent dehydration and maintain physical integrity. They also act as physical barrier to attack by plant pathogens. In nature, some plant consumers or pathogens use xylanase to digest or attack plants. Many microorganisms produce xylanase, but mammals do not. Some herbivorous insects and crustaceans also produce xylanase.

The xylanase enzyme molecular weights vary from source to source. Xylanase consists of 190 amino acids. Xylanases belong to the glucanase enzyme family and are characterized by their ability to break down various xylans to produce short-chain xylo-oligosaccharides. Xylanase readily crystallizes in ammonium sulfate and sodium/potassium phosphate buffer pH 3.5 to 9.0. Xylanase can also be crystallized with other salts, polymers, and organic solvents. Xylanase solubility increases with increasing temperature in moderate concentrations of ammonium sulfate. Xylanase solubility in phosphate buffer pH 9 decreases in the temperature range of 0°C to 10°C. But remains constant in the range of 10 through 37°C. Xylanase has been extracted from many different fungi and bacteria. It is commonly used in animal feeds, paper production, and food production.

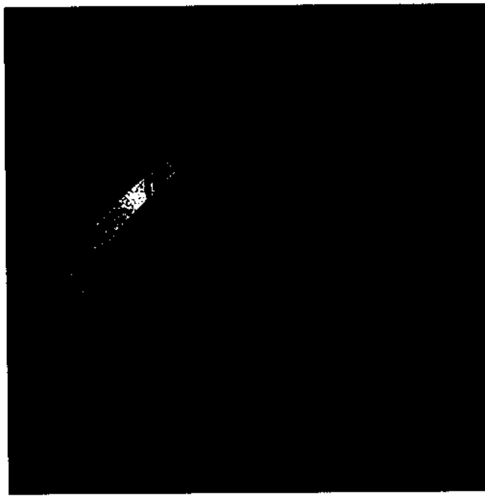

Figure 1.1. Structure of xylanase

1.1. Mechanism of action

Xylanase enzymes hydrolyze the xylan polymer that exists within pulp fibers. Xylans are intimately linked to cellulose and lignin, thus it follows that disruption of the xylan backbone affect their separation during bleaching. Xylanase was also shown to increase fiber wall swelling and in turn increase the speed of diffusion through the walls. Since it is believed that extraction of depolymerized lignin from pulp is a diffusion-limited process, xylanase treatment ultimately improves the extraction of lignin from pulp. Thus, for enzyme-treated pulp, subsequent bleaching stages are more efficient, and higher brightness can be expected. Other work suggests that if lignin covalently bound to xylan was made smaller by enzyme use, it would be more easily extracted (Biela, P. 1985).

Another hypothesis that came from research was that xylanase enzymes catalyze the hydrolysis of xylan that has reprecipitated on the fibers during alkaline pulping. Removal of this xylan was thought to remove a physical barrier preventing the extraction of residual lignin. However, recent work has shown that pulp prepared under condition that

prevents xylan reprecipitation also responds well to the xylanase bleach boosting effect.

1.2. Xylanase has proved useful in many ways:

1.2.1. Bio bleaching paper pulp: The use of xylanase leads to a reduction in organo-chlorine pollutants such as dioxin from the paper making process. In addition, chlorine-free bleaching (such as peroxide or ozone bleaching) can achieve brighter results with the xylanase. Because xylanase does not harm cellulose, the strength of the paper product is not adversely affected.

1.2.2. Improving animal feed: Adding xylanase stimulates growth rates by improving digestibility, which also improves the quality of the animal litter. Xylanase thins out the gut contents and allows increased nutrient absorption and increased diffusion of pancreatic enzymes in the digesta. It also changes hemi cellulose to sugars so that nutrients formerly trapped within the cell walls are released.

1.2.3. Improving silage (or enhanced fermentative composting): Treatment of forages with xylanase (along with cellulase) results in better quality silage and improves the subsequent rate of plant cell wall digestion by ruminants. There is a considerable amount of sugar sequestered in the xylan of plant biomass. In addition to converting hemicelluloses to nutritive sugars that the cow or other ruminant can digest, xylanase also produces compound that may be a nutritive for the ruminal microflora.

1.3. Practical uses of xylanase

The earliest U.S. Patent for a method of xylanase production was issued in 1979 for an enzyme mixture used as an animal feed additive for dairy cattle. Xylanase has since proven useful in many ways:

1.3.1. Bio bleaching paper pulp

Paper producers need retain cellulose while removing the lignin from paper pulp. The classic way to perform this operation is to add chlorine-based bleaches to the pulp. Xylanase breaks the hemicelluloses chains that are responsible for the close adherence of lignin to the cellulose network. There is thus a reduced need for bleach to remove the loosened lignin. When the bleach used is chlorine-based, the use of xylanase leads to a reduction in organo-chlorine, pollutants such as dioxin from the paper making process. In addition, chlorine-free bleaching (such as peroxide or ozone bleaching) can achieve brighter results with the addition of xylanase. Because xylanase does not harm cellulose, the strength of the paper product is not adversely affected.

1.3.2. Improving animal feed

Adding xylanase stimulates growth rates by improving digestibility, which also improves the quality of the anima litter. For example, chicken feed based on wheat, rye, and many other grains is incompletely digested without added enzymes. These grains tend to be too viscous in the chicken′s intestine for complete digestion. Xylanase thins out the gut contents and allows increased nutrient absorption and increased diffusion of pancreatic enzymes in the digesta. It also changes hemicellulose to sugar so that nutrients formerly trapped within the cell walls are released. The chickens get sufficient energy from less feed. The barn is cleaner because the feed is more thoroughly digested so the chicken waste is drier and less sticky. In addition, chicken eggs are cleaner because the excrement in the laying area is drier. In a sense, the addition of xylanase to animal feed pre-digests that feed.

1.3.3. Making bread fluffier and keeping it fresh longer

Added xylanase modifies wheat flour arabinoxylans and can result in a loaf with more than 10% greater volume. Crumb softness after storage is also improved.

1.3.4. Increasing juice yield from fruits or vegetables

Xylanase aids in the maceration (chewing up) process. In addition, added xylanase can reduce the viscosity of the juice, improving its filterability.

1.3.5. Extracting more fermentable sugar from barley for making beer, as well as processing the spent barley for animal feed. In both cases, xylanase has the ability to break hemicelluloses down into sugars. In addition, added xylanase can reduce the viscosity of the brewing liquid, improving its filterability.

1.3.6. Improving silage (or enhanced fermentative composting):

Treatment of forages with xylanase (along with cellulose) results in better quality silage and improves the subsequent rate of plant cell wall digestion by ruminants. There is a considerable amount of sugar sequestered in the xylan of plant biomass. In addition to converting hemicellulose to nutritive sugar that the cow or other ruminant can digest, xylanase also produces compounds that may be a nutritive source for the ruminal microflora.

1.3.7. Improve degradability of plant waste material (for instance, agricultural wastes) thereby reducing organic waste disposal in landfill sites.

1.3.8. Improve the cleaning ability of detergents that are especially effective in cleaning fruit and vegetable soils and grass stains.

1.3.9. Fuel-alcohol production

Xylanase decreases the viscosity of the mash and prevents fouling problems in distilling equipment.

1.3.10. Improve the extraction of oil from oil-rich plant material such as corn-oil from corn embryos.

1.3.11. Improve retting of flax fibers:

Retting is the decomposition of the outer stem of the flax plant necessary before the fibers are processed into linen.

1.3.12. Enzyme use can lower the bleaching cost.

1.4. Enzyme benefits

Following the first application of xylanase in pulp bleaching, other studies have demonstrated the benefits of enzymes in the bleaching of softwood, and hardwood pulps. These benefits can be exploited in the bleach plant in several different ways, which have been described in a recent survey of xylanase use in Canadian mills.

1.4.1. Brightness gain

Xylanase improve bleaching chemical efficiency, leading to higher pulp brightness. This benefit is particularly attractive for mills with shortened bleaching sequences needing a high final brightness.

1.4.2. Chemical savings

If the xylanase increase brightness in the bleach plant, the mill can cut chemical use and still attain the original target. Saving money with lower Chemical use is the primary driving force for mills to adopt enzymes. At the time of this writing, typical net savings range between US $ 1 to $3 per ton of pulp.

1.4.3. AOX and dioxin reduction

Using enzymes to reduce chlorine dioxide charge can also reduce AOX in the mill's effluent. This has been documented in laboratory and mill situations Mills still using chlorine can often reduce chlorine charge below the threshold level where chlorinated dioxin formation begins.

1.4.4. ClO_2 limitation

The ability to generate an adequate supply of chlorine dioxin many be a bottleneck. Enzymes can effectively make more ClO_2 available since less is used per ton, leading to increased production.

1.4.5. Other benefits

Two Scandinavian mills are using enzymes to eliminate the DO stage completely, while maintaining brightness targets. This has allowed the mills to send bleach filtrates prior to D1 stages back to recovery and resulted in partial closure of their water loop.

Xylanase can reduce the use of caustic soda, hydrogen peroxide, ozone and other chemicals such as activated oxygen. Therefore, enzymes can be used for total chlorine free (TCF) bleaching and, occasionally a bleach sequence can be modified or stages eliminated.

1.5. Mill operation: The most conventional method is to add xylanase to the brownstock pulp to the high-density (HD) tower.

The enzymes reaction takes place in the tower and the treated pulp then passes into the bleach plant. Various ways to add enzymes have been used, including: spraying on the decker pulp mat; adding to either the decker repulper or discharge chute; adding into the stock of medium consistency pump leading to the HD tower; and adding directly into the HD tower. Xylanase has also been added later in the bleaching sequence, rather than to brownstock pulp (Fischeri *et al.*, 2004).

The latest generations of alkali-tolerant enzymes require little, if any, addition of acid to adjust the pH. Earlier generations of enzymes had pH optima ranging from 5 to 6.5 and required acid addition to brownstock pulp. Instances of corrosion problems were seen when acid was incorrectly applied. New xylanase have higher pH optima and function optimally without pH adjustment.

The acid of preference by far has been sulfuric acid. However, with the development of alkaline xylanase, non-corrosive dioxide is an excellent choice and also improves washer performance. The addition of acid prior to the DO stage in ECF bleaching has also been shown to improve the performance of the DO stage. This is because the higher acidity in the stage prevents decomposition of chlorine dioxide to chlorate, and because the chemistry of delignification with dioxide favors and acidic environment. During the benefit of acid alone should be established.

Typical sites of acidification are indicated. Acid added to the low consistency pulp to the washer vat provides the benefit of reducing pitch deposits; however, acid charges here tend to be much higher due to the large volume that must be treated. Acid can also be added on the washer shower bars washer or in the repulper discharge section. Experience has shown that prevention of corrosion must be a priority.

Retention in higher-density towers has generally needed to be greater than one hour. However, new thermo-tolerant enzymes now permit higher brownstock thus shortening retention requirements.

1.6. Xylanase application at high pH

Various enzymes have been available for almost ten years. Many trials have been conducted over the years but few have translated into full-time application on the mill level. In the last year, new high-temperature and high pH enzymes have been developed at considerably lower cost. This enzyme allows enzymes use with minimal or no acidification of the brownstock pulp. The following mill data shows the application of this enzyme (Ecopulp TX-200C) (Kohli *et al.*, 2001).

Country	**Mill**	**Applications**
Finland	Stora Enso	ClO_2l*
	Veitsiluoto Kemi	Eliminate Do stages
	Stora Enso	ClO_2l*
	Imatra	
	Stora Enso	ClO_2l*
	Enocell	
	Stora Enso	TCF
	Kemijarvi	
	UPM-Kymmene	ClO_2l*
	Wisaforest	
	UPM-Kymmene	ClO_2l*
	Kuusanniemi	
Sweden	Mill A	Eliminate Do stages
	Mill B	ClO_2l*
	Mill C	ClO_2l*
North America	Eastern Canadian SWD	ClO_2l*

	Central Canadian SWD/HWD	$ClO_2$1*
	Western Canadian SWD	$ClO_2$1* & NaOH
1* chlorine dioxide reduction; NaOH reduction		

1.7. Applications

1.7.1. Drinks

Enzymes have many applications in drink industry. The use of chymosin in cheese making to coagulate milk protein was already discussed. Another enzyme use in milk industry is beta-galactosidase or lactase, with splits milk-sugar lactose into glucose and galactose. This process is used for milk products that are consumed by lactose intolerant consumers.

Enzymes are used also in fruit juice manufacturing. Fruit cell wall needs to be broken down to improve juice liberation. Pectin's are polymeric substances in fruit lamella and cell walls. They are closely related to polysaccharides. The cell wall contains also hemicelluloses and cellulose. Addition of pectinase, xylanase and celluase improve the liberation of the juice from the pulp. pectinases and amylases are used in juice clarification.

Brewing is an enzymatic process. Malting is a process. This increases the enzyme level in the grain. In the mashing process the enzymes are librated and they hydrolyze the starch into soluble fermentable sugars like maltose, which is a glucose disaccharide. Additional enzymes can be used to help the starch hydrolysis (typically alpha-amylases), solve filtration problems caused by beta-glucans present in malt (beta- glucanase), hydrolyze proteins (neutral proteinase), and

control haze during maturation, filtration and storage (papain, alpha-amylase and beta-glucanase).

Similarly enzymes are widely used in wine production to obtain a better extraction of the necessary components and thus improving the yield. Enzymes hydrolyse the high molecular weight substances like pectin.

1.7.2. Animal feed

Intensive study to use enzymes in animal feed started in early 80s. The first commercial success was addition of beta- glucanase into barley based feed diets. Barley contains beta-glucan, which causes high viscosity in the chicken gut. The net effect of enzyme usage in feed has been increased animal weight gain with the same amount of barley resulting in increased feed conversion ratio. Finnfeeds International was the pioneer in animal feed enzymes.

Enzymes were tested later also in wheat-based diets. xylanase enzymes were found to be the most effective ones in this case. Addition of xylanase to wheat-based broiler feed had increased the available metabolizable energy 7-10% in various studies. xylanases are nowadays routinely used in feed formulations. Figure 2 shows the three-dimensional structure of Trichoderma xylanase. Usually a feed-enzyme preparation is a multi enzyme cocktail containing glucanases, xylanases, proteinases and amylases. Enzyme addition reduces viscosity, which increases absorption of nutrients, liberatates nutrients either by hydrolysis of non-degradable fibers or by liberating nutrients blocked by these fibers, and reduces the amount of faeces.

Another type of important feed enzyme is phytase marketed e.g. by DSM in the Netherlands. Phytase is a phosphoesterase which liberates

phosphate from phytic acid which is a common compound in plant based feed materials. The net effect is reduced phosphorous in faces resulting in reduced environmental pollution. The use of phytase reduces the need to add phosphorous to feed diet.

Enzymes have become an important aspect of animal feed industry. In addition to poultry, enzymes are used in pig feeds and turkey feeds. They are added as enzyme premixes (enzyme-flour mixture) during the feed manufacturing process, which involves extrusion of wet feed mass in high temperature (80-90°C). Therefore the feed enzymes need to be thermo tolerant during the feed manufacturing and operative in the animal body temperature.

1.7.3 Baking:

Similar fiber materials are used in baking than in animal feed. It is therefore conceivable that enzymes also affect the baking process. Alpha-amylases have been most widely studied in connection with improved bread quality and increased shelf life. Both fungal and bacterial amylases are used. Over dosage may lead to sticky dough so the added amount needs to be carefully controlled.

One of the motivations to study the effect of enzymes on dough and bread qualities comes from the pressure to reduce other additives. In addition to starch, flour typically contains minor amounts of cellulose, glucans and hemicelluloses like arabinoxylan and arabinogalactan. There is evidence that the use of xylanases decreases the water absorption and thus reduces the amount of added water needed in baking. This leads to more stable dough. Especially xylanases are used in whole meal rye baking and dry crisps common in Scandinavia.

1.7.4. Pulp and Paper

Intensive studies have been carried out during the last twenty years to apply many different enzymes in pulp and paper industry. A real excitement started with the discovery of lignin degrading peroxidases in the early 80S. In spite extensive research no oxidative enzymes are applied in pulp and paper industry. The major application is the use of xylanases in pulp bleaching. Xylanases liberate lignin fragments by hydrolyzing residual xylan. This reduces considerably the need for chlorine based bleaching chemicals. Other minor enzyme applications in pulp production include the use of enzymes to remove fine particles from pulp. This facilitates water removal.

In the use of secondary (recycled) cellulose fiber removal of ink is important. The fiber is diluted 1% concentration with water, flocculation surfactants and ink solvents added and the mixture is aerated. The ink particles float to the surface. There are reports that this process is facilitated by addition of cellulose enzymes.

In paper making enzymes are used especially in modification of starch, which is used as an important additive. Starch improves the strength, stiffness and erasability of paper. The starch suspension must have a certain viscosity, which is achieved by adding amylase enzymes in a controlled process.

Pitch is a sticky substance present mainly in softwoods. It is composed of lipids. It is a special problem when mechanical pulps of red pine are used as a raw material. Pitch causes problems in paper machines and can be removed by lipases.

2. LITERATURE REVIEW

2. LITERATURE REVIEW

One of the first applications of enzymes to modify pulp properties was reported by Paice and Jurasek in 1984. In this instance, a crude mixture of degradative enzymes, including xylanase, was applied to dissolving pulp to remove xylan. The resulting increase in alpha cellulose content was expected to allow the pulp to be derivatized to higher value products.

Although no significant reductions in hemicelluloses were observed, a subsequent study by Finnish workers demonstrated that a similar enzymes treatment surprisingly reduced the requirement of chemical needed to bleach the pulp. Other work showed that the xylanase in the crude enzyme mixture was responsible for the pre-bleaching effect.

The Enso Gutzeit mill in Imatra, Finland conducted the first mil trial which was reported in 1989. A reduction in chlorine used for bleaching allowed the AOX level to be lowered by over 50%. More notable was that the level of chlorinated dioxins formed was reduced below the detection threshold. The positive results were tempered, however, by a loss in pulp viscosity and yield. This problem was due to the presence of cellulases which contaminated the early enzyme preparations.

Many of species of fungi known to produce xylanase include *Aspergillus (*Chantasingh *et al*.,1994*), Disporotrichum, penicillium (*Tanaka *et al*.,1999*),Neurospora, Fusarium, Melanocarpus (*Saraswat *et al*.,1998*), Talaromyces(*Tuohy *et al*,1999*), Coniothyrium*, etc.A number of species of bacteria, some from extreme environments which makes them more suitable for industrial environments. Transgenic

(recombinant) bacteria, fungi, or yeast transformed with genes from other microorganisms.

Chantasingh *et al.,* (1994) have reported on cloning, expression, and characterization of xylanase 10 from *Aspergillus terreus* (BCC129) in *Pichia pastoris.* Ghareib *et al.,* (1994), has reported on purification and general properties of xylanase from *Aspergillus terreus.*

Tahir *et al.,* (1987) have reported on specific characterization of substrate and inhibitor binding sites of a glycosyl hydrolase family 11 xylanase from *Aspergillus niger.* Tyagi *et al.,* (2001) has reported on immobilization of *Aspergillus niger* xylanase on magnetic latex beads.

Gawande *et al.,* (1995) has worked on preparation, characterization and application of *Aspergillus sp.* Xylanase immobilized on Endragit S-100. Gouda *et al.,* (1998) have reported on catalytic properties of the immobilization *Aspergillus tamarii* xylanase.

Berrin *et al.*, (1999) have reported their work on high-level production of recombinant fungal endo-beta-1, 4-xylanase in the methylotrophic yeast *Pichia pastori.* Koseki, *et al.*, (1995) have reported on *Aspergillus oryzae* acetyl xylan esterase: molecular cloning and characteristics of recombinant enzyme expressed in *Pichia pastoris.*

Fierens *et al.*, (2002) have reportd on high-level expression, purification, and characterization of recombinant wheat xylanase inhibitor secreted by the yeast *Pichia pastoris.*

Yangnd *et al.*, (1993) have worked on high-level expressiong of an extreme-thermostable xylanase from *Thermotoga maritime* MSB8 in *Escherichia coli* and *Pichia pastoris.* Boonyapakron *et al.*, (2001), have published a paper on cloning and expression of xylanase 10 from *Cryptovalsa mangrovei* (BCC7197) in *Pichia pastoris.* Optimized

expression of thermostable xylanase from *Thermomyces lanuginosus* in *Pichia pastoris*.

Tanaka *et al.*, (1994) have worked on purification and properties of a family-10 xylanase from *Aurebasidium pullulans*. Suzuki *et al.*, (2000) have worked on cloning, expression, and characterization of a family 52 beta-xylosidase gene (xysB) of a multiple-xylanase-producing bacterium, *Aeromonas caviae*. Tanaka *et al.*, (`1999) have published their work on purification and properties of an extracellular endo-1, 4-beta- xylanase from *Penicillium citrinum* and characterization of the encoding gene.

Inagaki *et al.*, (1996) have reported on gene cloning and characterization of an acidic xylanase from *Acidobacterum capsulatum*.

Ramchuran *et al.*, (2000) have published and article on the methylotrophic yeast *Pichia pastoris* as a host of the expression and production of thermostable exlanase from the bacterium *Rhodothermus marinus*.

Ohta *et al.*, (1998) have worked on purification and characterization of an acidophilic xylanase from *Aureobasidium pullulans* and sequence analysis of the encoding gene.

Roy *et al.*, (1999) have reported on immobilization of xylan-degrading enzymews from *Melanocarpus albomyces*. Saraswat *et al.*, (1998) has worked on preparation, characterization and substrate specificities of xylanase isoenxymes from *Melanocarpus albomyces* IIS68.

Duarte *et al.*, (1996) have worked on xylan-hydrolyzing enzyme system from *Bacillus pumilus* CBMAI 0008 and its effects on Eucalyptus grandis gradis kraft pulp for pulp bleaching improvement.

Bocchini *et al.*, (1997) have reported on effect of *Bacillus circulans* D1 thermostable xylanase on biobleaching of eucalyptus kraft pulp.

Tuohy *et al.*, (1999) have worked on the xylan-degrading enzyme system of *Talaromyces emersonii* novel enzymes with activity against aryl beta-D-xylosides and unsubstituted xylans.

3. MATERIALS AND METHODS

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3.1. Reagents preparation

3.1.1. 1% xylan solution

1 g of xylan is dissolved in 100 ml of distilled water, warm if necessary and filtrate.

3.1.2. DNSA solution

1g dinitrosalycylic acid (DNSA) & 1% (w/v) NaOH dissolved in 100ml distilled water.

3.2. Organism

A mold strain of fungus was isolated from soil and cultured on Czapek-dox agar.The composition of Czapek-dox agar:(g/L) glucose,10.0; KH_2PO_4,1.0; KCl,0.5; $NaNO_3$,2.0; $MgSO_4.7H_2O$,5.0; $FeSO_4.7H_2O$,0.001; agar, 25.0.The pH was maintained at 5.6- 6.7.

3.3. Preparation of crude enzyme

The medium was taken in a 500ml Erlenmeyer flask and was inoculated with 3 days old culture and the flask was kept at orbital shaker for 6 days at 120 rpm and 28°C. Then fungal mycelia were removed by filtration through Whatman filter paper No.1 and filtrate was as a source of enzyme.

3.4. Enzyme Assay

Enzyme assays performed using 1 ml of 1% xylan as substrate and 0.6 ml of the crude enzyme extract, this mixture kept at 55°C for 15 minute, and 1 ml of DNSA (DiNitro Salicylic acid) was added to arrest the reaction. To the blank 1 ml DNSA was added to the tubes before the addition of enzymes. Then tubes were kept at boiling water bath for 5 min & absorbance was measured at 540 nm. One unit of enzyme activity

is defined as the quantity of enzyme hydrolyzing 1µmol of substrate per minute.

3.5. Characterization

3.5.1. Optimum pH

To determine the optimum pH of the enzyme, buffers of varying from pH 3.5 to pH 7.5. Acetate buffers (pH 3.5 to 5.5, 0.2 M) and phosphate buffers (pH 6.0 to 7.5, 0.2 M) (Kohli *et al.*, 2001) were used.

3.5.2. Optimum Temperature

To determine the optimum temperature of the enzyme, enzyme was assayed at pH 6.0 in various temperatures ranging from 10°C to 80°C. (Nascimento *et al.*, 2002).

3.5.3. pH Stability

pH stability tests were carryout by incubating a mixture of 3 ml enzyme & 5ml buffers, at pH 7 and pH 8 respectively.

3.5.4. Temperature Stability

Temperature stability tests was performed by measuring the absorbance at intervals of 15 min for enzyme placed in varying temperatures ranging from 60°C to 70°C for a total of 1h.

3.5.5. Effect of metal ions and some reagents

The effect of various metal ions and reagents on the enzyme activity was studied by adding various meal ions (Ag+, Hg2+, K+, Fe, Zn, Ca, Cu and Mg) reagents (EDTA, 1,10-phenanthroline, SDS, PMSF, n- bromosuccinamide and mercaptoehanol along with the reaction mixture and the assay was carried out.

3.6. Purification

3.6.1. Acetone precipitation

Chilled acetone (-20°C) was added slowly to the enzyme extract (730 ml) with constant shaking and the mixture was kept at 4°C. Then the mixture was centrifuged at 5000 rpm for 15 mins and the supernatant was discarded. The tubes were placed in an invertical position till there is no acetone smell, because trace amount of acetone will not allow the precipitate to dissolve in buffer. The precipitate was dissolved in phosphate buffer (0.2 M, pH 6.0), the content was centrifuged & the enzyme activity was carryout in the supernatant.

3.6.2. Dialysis

Dialysis bag was filled with acetone-precipitated enzyme. Dialysis bag immersed in 25 mM phosphate buffer (pH 6.0).The dialysis bag was kept at 4°C with intermittent change in buffer for every 12 h. The content of dialysis bag was centrifuged to remove precipitates, if any, and the enzyme activity was carryout in the supernatant.

3.6.3. Ultra filtration

The dialyzed acetone precipitated enzyme was filtered through a 50KDa Omega ultra filtration membrane in a PALL ultra filtration unit, the retentate and filtrate was collected separately and was checked for xylanase activity.

3.6.4. Ion exchange chromatography

3.6.4.1. Regeneration of the DEAE-Sepharose column

DEAE -Sepharose column (2.3 x 12.2cm) was regenerated by washing with 250ml of Tris-HCI buffer (0.1M, pH 8.3) containing 0.5M Nacl followed by passing 250 ml of sodium acetate buffer (0.1M, pH 4.5) containing 0.5 NaC1. The regenerated DEAE-Sepharose was

equilibrated with 200ml of phosphate buffer (25mM, pH6.0). DEAE-Sepharose was kept at 4°C.

3.6.4.2. DEAE-Sepharose chromatography

The Ultra filtered enzyme was loaded on to a DEAE-Sepharose column (2.3 x 12.2 cm) equilibrated with the same buffer. The enzyme bound to DEAE-Sepharose was eluted with a linear salt gradient 0.5M NaC1 in equilibration buffer (25mM, PH 6.0). Fractions were collected at a flow rate of 51.4ml/hr using collector. All the fractions were checked for protein (A_{280}) and xylanase activity (A_{540}). The fractions having maximum activity were pooled.

3.6.5. Polyacrylamide gel electrophoresis (PAGE)

Protein purity and molecular mass estimation was performed by native PAGE. PAGE was carried out by the method as described by Laemmli. Non-denaturing PAGE (native PAGE) was carried out without SDS.

3.7. Application

3.7. 1. Pulp and paper industry

Xylanase was used in the pulp paper industry to decrease the amount of chlorine used during the bleaching process during the treatment. The enzyme trial was carried out at two different concentrations. The over all process in this treatment involves 4 steps.

3.7. 2. Enzyme treatment

The pulp was weighed and was treated with the xylanase enzyme produced at two different concentrations. 3xu units of the enzyme have to be added and so after calculations. 2.14 ml and 4.28 ml were added along with the pulp. The pulp was packed along with the enzyme **and** certain amount of water to maintain the water level as constant and was

incubated in a water bath at 60°C for 1 hour. Untreated pulp was packed with water and marked as control.

3.7.3. Chlorine treatment

The after incubating with enzyme, the pulp was taken down to the washing area and is washed thoroughly to remove the enzyme by using 1 bucket of water. 3% was to be added and by doing the calculations, 948 ml of chlorine was added in both the enzyme treated as well as the control. Now once again water was added and packed and was kept at room temperature for a period of 90 minutes.

3.7. 4. Peroxide treatment

The pulp was taken back for washing to remove the chlorine from the pulp by using 1 bucket of water. In this stage, peroxide and NaOH has to be added and packed again and should be incubated at 70°C for a period of 90 minutes. By calculating, 19.3 ml of peroxide and 45 ml of NaOH was added.

3.7. 5. Hypo treatment

The pulp is washed again with 1 bucket of water to remove the peroxide and was treated now with 2% hypo and 0.2% NaOH. By calculations, 95.55 ml of hypo solution and 4.25 ml of NaOH solution was added and packed. The packets were incubated at 45 C for a period of 120 minutes.

3.7.6. To determine the strength of chemicals used

The amount of Chlorine, peroxide and Hypo to be added are calculated by doing certain titration procedure to find the strength of the following chemicals.

3.7.6.1. Chlorines strength

Titration was done to determine the strength of chlorine.

- Burette solution: Sodium thio sulphate.
- Conical flask: 25 ml chlorine+10ml of 10% KI and starch as indicator.

3.7.6.2. Peroxide strength

Titration was done to determine the strength of peroxide.

- Burette solution: Sodium thio sulphate.
- Conical flask: 2 ml peroxide +5 ml of ammonium sulphate +4 drops of ammonium molybdate and starch as indicator.

3.7.6.3. Hypo strength

Titration was done to determine the strength of hypo solution.

- Burette solution: Sodium thio sulphate.
- Conical flask: 10 ml of hypo +10ml of acetic acid +10ml of 10% KI+ Starch as indicator.

4. RESULTS AND DISCUSSIONS

4. RESULTS AND DISCUSSION

4.1. Xylanase assay

Xylanase assay was performed by DNS method. The activity of enzyme was found to be 1.347 U/ml.

4.2. Estimation of protein

Protein content of the sample was estimated by Lowry's method. The concentration was found to be 30 mg.

4.3. Characterization of Xylanase

4.3.1. Optimum pH

Xylanase activity was detected in the czapek dox medium, xylanase activity was found to vary with pH. The effect of pH on enzyme activity is shown in figure. xylanase activity carry out in a pH range between 4.5 to 7.5, optimum was pH 6.0. The xylanase enzyme was found to be not stable at pH-7 and above even after incubation for a period of one hr.

pH	Relative activity (%)
4.5	0.07
5.0	1.48
5.5	7.05
6.0	100
6.5	8.83
7.0	1.26
7.5	0.519

Table 4.3.1. Determination of optimum pH

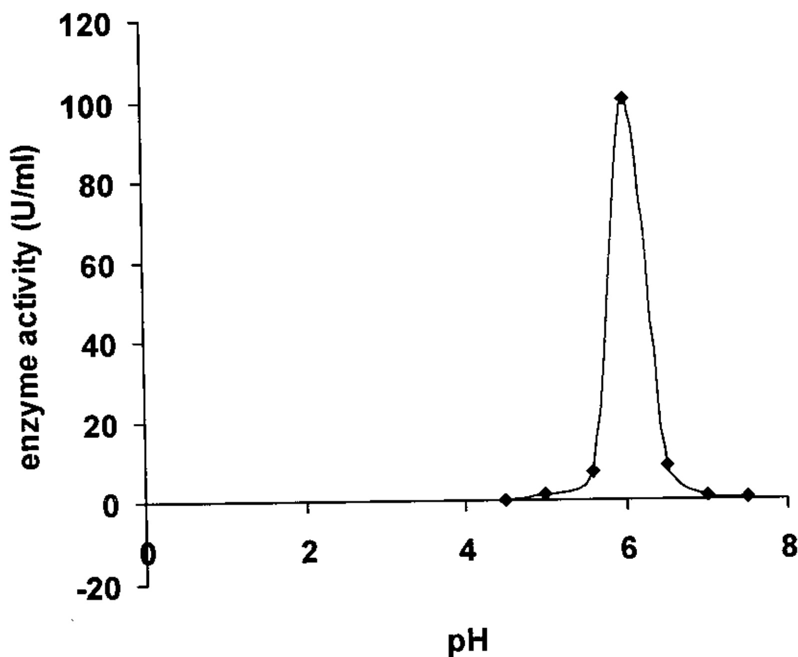

Figure 4.3.1. Determination of optimum pH

4.3.2. Optimum temperature

Optimum temperature of the enzyme, varied temperatures from 10°C to 80°C was used & recorded the absorbance readings. The results indicated that 55°C was the optimum temperature for enzyme activity.

Temperature (°C)	Relative activity (%)
10	35.48
15	56.98
20	62.66
25	73.75
30	74.53

35	75.31
40	84.54
45	89.52
50	93.47
55	100
60	96.42
65	86.10
70	47.28
75	40.60
80	30.67

Table 4.3.2. Determination of optimum temperature

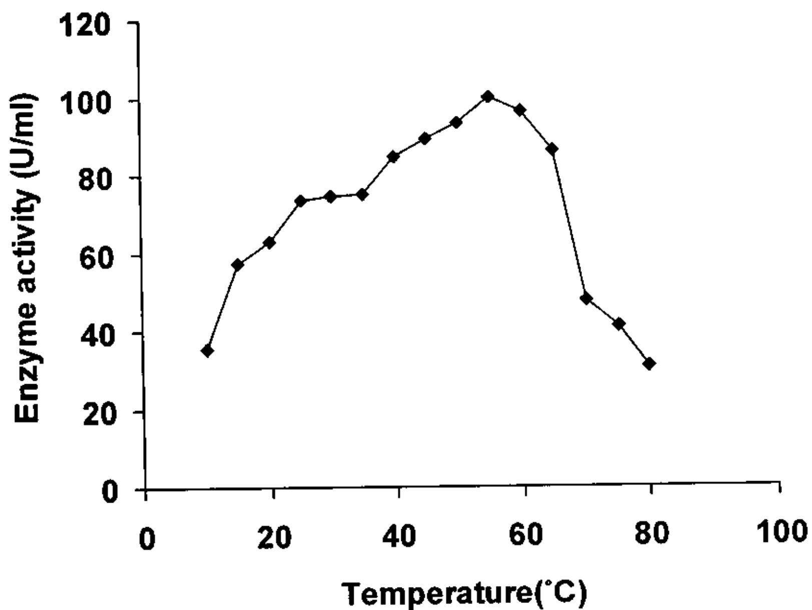

Figure 4.3.2. Determination of optimum temperature

4.3.3. Temperature stability

Temperature stability tests were done by measuring the absorbance at 15 min time intervals by placing the enzyme at 60°C and 70°C for 1h. The enzyme was thermo stable, i.e., 30% of its original activity is retained at the end of 1 h at 60°C, which proves its suitability in paper and pulp industry.

Time (min)	Enzyme activity (U/ml)
0	38.77
15	20
30	15
45	10.55
60	8.33

Table 4.3.3.1. Temperature stability at 60°C

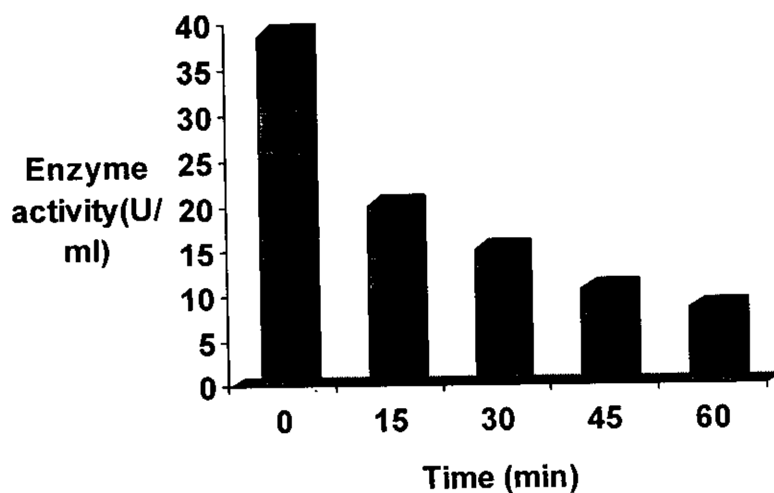

Figure 4.3.3.1.Temperature stability at 60°C

Time (min)	Enzyme activity (U/ml)
0	38.88
15	2.66
30	1.11
45	0.55
60	0

Table 4.3.3.2. Temperature stability at 70°C

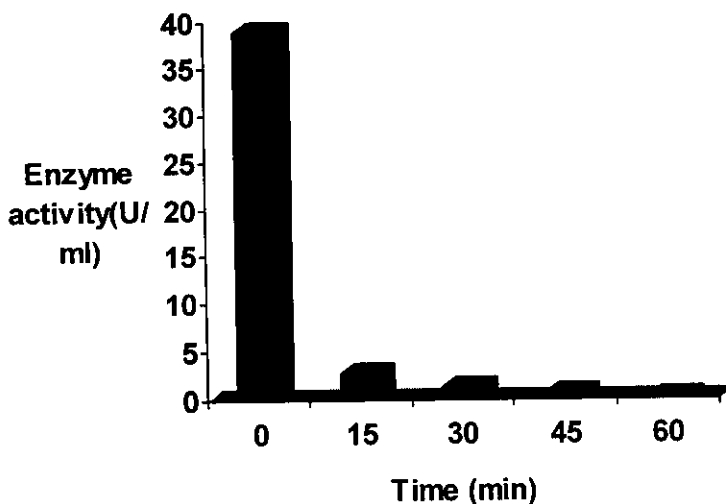

Figure 4.3.3.2. Temperature stability at 70°C

4.3.4. Effect of metal ions

The graph 4.1.5 shows the effect of metal ions on xylanase activity. Among the cations tested, heavy metal ion, Cu^{2+} strongly inhibited the enzyme activity to about 69%, where as other cations such as

K^+, Ca^{2+} and Hg^{2+} increased the enzyme activity to about 34%, 20% and 100% respectively.

Metal ion	Enzyme activity
Control	23.33
KC1	15.55
$CaC1_2$	20.55
$ZnSO_4$	13.88
$CuSO_4$	7.22
$HgC1_2$	0
$AgNo_3$	0
$CdCl_2$	10.55
$FeCl_3$	32.22

Table 4.3.4. Effect of metal ions

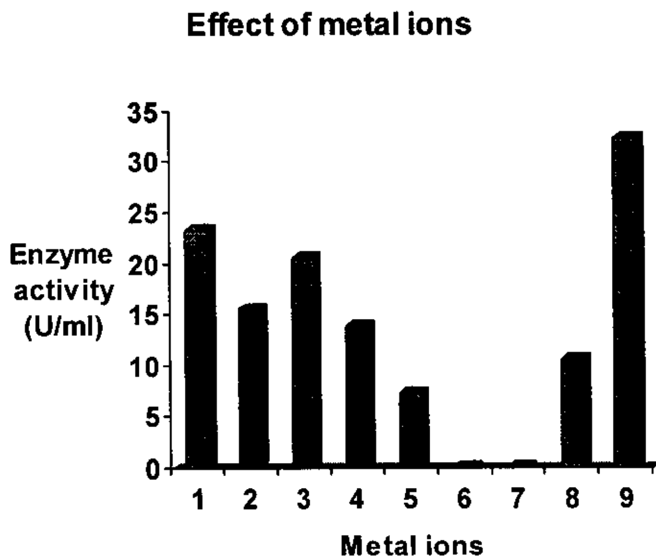

Figure 4.3.4. Effect of metal ions

1-Control , 2-KCl, 3-Cacl$_2$, 4-ZnSO$_4$, 5-CuSO$_4$, 6-HgCl$_2$, 7-AgNo$_3$, 8-CdCl$_3$, 9-FeCl$_3$.

4.3.5. Effect of reagents

The graph 4.1.6 shows the effect of reagents on xylanase activity. Among the reagents tested PMSF increased the enzyme activity to about 1.5 times. The reagents such as EDTA, 1, 10 phenanthroline, SDS, PMSF and ß-Mercaptoethanol inhibited the enzyme activity.

Reagents	Enzyme activity (U/ml)
Control	23.33
1,10-Phenanthroline	14.44
PMSF	31.11
N-Bromosuccinimide	0.55

Table 4.3.5 Effect reagents

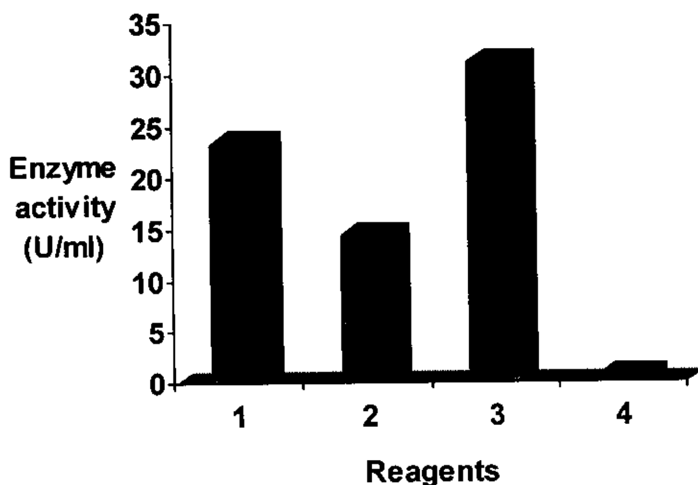

Figure 4.3.5 Effect of reagents

1- Control 3- PMSF

2- 1, 10-phenanthroline 4- N-bromosuccinimide

4.4. Purification

The xylanase was purified to homogeneity and achieved by combination of acetone precipitation, dialysis, ultrafiltration, and ion-exchange chromatography on DEAE-Sepharose.

4.4.1. Acetone precipitation

The enzyme was purified by acetone precipitation. The purification fold and yield was estimated. The enzyme was purified to 1.026 fold and the yield was 0.00023%.

4.4.2. Ultrafiltration

The filtrate and retentate collected after ultrafiltration of the dialysed enzyme was subjected to xylanase assay. The xylanase activity was found in the filtrate, thus confirming the fact that the enzyme is less than 50 KDa. The purification fold and was estimated. The enzyme was purified to 3.846 fold and the yield was 0.013%. Ultra filtrated protein sample was run in native PAGE and the assay revealed xylanase activity in the first section of the gel.

4.4.3. Ion Exchange chromatography

Xylanase activity was observed in fraction numbers 5-15. The active fractions were pooled and lyophilized to reduce its volume. The purification fold and yield were estimated. Enzyme was purified to 0.519 and the yield was 0.0010%.

4.4.4. Native PAGE

After three purification steps, native PAGE of the final enzyme preparation showed single band. The molecular weight of purified xylanase by native PAGE was estimated to be 23kDa.

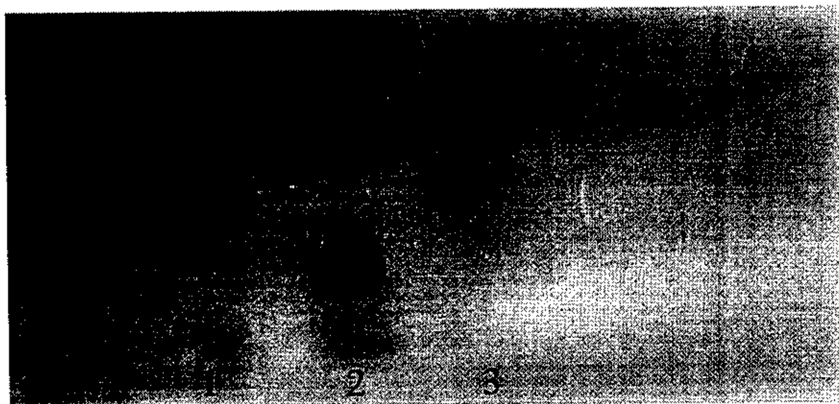

Figure 4.4.4. Native PAGE

1&3 - Samples

2 - Molecular marker

Enzyme fraction	Volume (ml)	Protein Conc (mg)	Activity (Units)	Specific activity (U/mg)	Purification Fold	Yield %
Crude	730	328	23116	70.37	1	100
Acetone Precipitation	232	23.2	1675.5	72.22	1.026	0.00023
Ultrafiltration	32	6.02	1672.22	277.77	3.846	0.013
Ion exchange Chromatography	28	1.4	202.22	144.44	0.519	0.0010

Table 4.4. Purification Chart

4.5 Application

4.5.1. Pulp and paper industry

Xylanase was used in the pulp and paper industry to decrease the amount of chlorine used for the bleaching of the pulp. In this experiment we kept the amount of chlorine as constant and increased the amount of enzyme used in order to find whether there is any change in the final brightness (in %) of the treated pulp. We used two different concentrations of enzyme for this study. The result were found to be convincing and it is tabulated as follows,

Treatment step	Control	Enzyme trial 1	Enzyme trial 2
Enzyme	20.51	20.24	20.03
Chlorine	29.19	29.26	28.66
Peroxide	64.34	64.86	65.31
Hypo	79.15	80.28	81.78

Table 4.5.1. Brightness comparison in the pulp

5. CONCLUSION

5. CONCLUSION

Xylanase activity was found in culture filtrate. Optimum pH and temperature of the enzyme was found to be pH 6.0 and 55°C. Enzyme was thermal stable, i.e., 30% of its original activity is retained at the end of 1 hr at 60°C. Enzyme was not stable in pH above 7.0. There was no cellulase enzyme in the culture filtrate. Effect of metal ions and reagents on enzyme also studied. Thus the xylanase molecular weight found to be approximately 23 kDa. Xylanase enzyme was useful in paper & pulp industry to reduce the quantity of chemicals used to achieve brightness of pulp.

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