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CHARACTERIZATION AND PURIFICATION OF CELLULASE-FREE XYLANASE FROM *Bacillus megaterium* AND ITS APPLICATION IN PULP AND PAPER INDUSTRY

A PROJECT REPORT

Submitted by

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BONAFIDE CERTIFICATE

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The report of the project work submitted by the above students in partial fulfillment for the award of Bachelor of Engineering degree in Industrial Biotechnology of Anna University were evaluated and confirmed to be report of the work done by the above students.

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ABSTRACT

The number of possible applications of microbial xylanases in the pulp and paper industry is gradually increasing and several are approaching commercial use. However, the properties of commercial xylanases make them unsuitable for the real process of pulp and paper bleaching. The industry needs xylanase free of cellulase, function for a long time, at least 2 hours at temperatures over 60°C and which are active at a pH higher than 8.0. Newly isolated microbes, which produce cellulase-free xylanases and are easily grown using low-cost substrate were investigated.

In the project work, we produced cellulase-free xylanase from *Bacillus megaterim* by using low-cost rice bran as the substrate. The properties of the cellulase-free xylanase enzyme produced matched the following industrial requirements: active and stable at temperatures of 50-80°C for a period of 2 hours, active at alkaline pH (pH 7-9). The enzyme was purified and their application in pulp and paper industry to reduce the amount of chlorine used in the bleaching of the pulp was tested. Its application was proved to be effective and has increased the final brightness of the treated pulp.

ABSTRACT

Chapter 1
INTRODUCTION

1. INTRODUCTION

Enzymes are biological catalysts produced by all living things. The enzyme named xylanase deconstructs plant structural material by breaking down hemicellulose, a major component of the plant cell wall. Plant cell walls are necessary to prevent dehydration and maintain physical integrity. They also act as a 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 (Endo-1,4-Beta-xylanase, or XYNII, EC 3.2.1.8) from has a pI of 9.0 and is produced by fermentation. Xylanase consists of 190 amino acids and has a molecular weight of 21 kDa. 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 across 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 pH 9 decreases in the temperature range of 0 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.

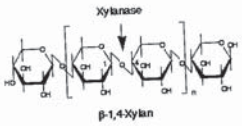
Figure 1.1 Structure of xylanase

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 affects 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 enyzme use, it would be more easily extracted.

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 conditions that prevent xylan reprecipitation also responds well to the xylanase bleach boosting effect.



Xylanase has proved useful in many ways:
Biobleaching 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 addition of xylanase. Because xylanase does not harm cellulose, the strength of the paper product is not adversely affected.

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 hemicellulose to sugars so that nutrients formerly trapped within the cell walls are released.

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 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

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:

- **Biobleaching paper pulp:** Paper producers need to 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 hemicellulose 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.
- **Improving animal feed:** Adding xylanase stimulates growth rates by improving digestibility, which also improves the quality of the animal

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 sugars 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.

- **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.
- **Aiding in separation of wheat or other cereal gluten from starch.**
- **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.
- **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 hemicellulose down into sugars. In addition, added xylanase can reduce the viscosity of the brewing liquid, improving its filterability.
- **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 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.

- **Improve degradability of plant waste material** (for instance, agricultural wastes) thereby reducing organic waste disposal in landfill sites.
- **Improve the cleaning ability of detergents** that are especially effective in cleaning fruit and vegetable soils and grass stains.
- **Fuel-alcohol production:** Xylanase decreases the viscosity of the mash and prevents fouling problems in distilling equipment.
- **Improve the extraction of oil** from oil-rich plant material such as corn-oil from corn embryos.
- **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.

Practical Sources of Xylanase

- Many of species of fungi. Genera known to produce xylanase include *Aspergillus, Disporotrichum, Penicillium, Neurospora, Fusarium, Neocallimastix, Trichoderma, Coniothyrium*, etc.
- A number of species of bacteria, some from extreme environments (hot, alkaline, etc.) which makes them more suitable for industrial environments.

- Transgenic (recombinant) bacteria, fungi, or yeast transformed with genes from other microorganisms.
- Transgenic *Brassica napus* (canola) has also been invented. The meal produced from this canola can be used as an animal feed supplement (Canola meal is the protein-rich residue left after the production of canola oil).

Enzyme Use Can Lower Bleaching Costs, Aid ECF Conversions

Interest in using xylanase enzymes for pulp bleaching has jumped dramatically in 1999. In the US, promulgation of the Cluster Rules has pressured mills to complete conversion to elemental chlorine free (ECF) bleaching in an economic way. In addition, weak markets for many pulp and paper products have forced mills to lower their bleaching costs. Fortunately, the use of enzymes can address both needs. Most full-time applications focus on cost reductions using O_2-ECF bleaching. Recent developments involve use of enzymes to eliminate the first chlorine dioxide stage and thereby help reduce water usage. In this article, a background of enzyme use will be presented along with current mill situations that highlight the benefits of enzyme use.

Historical perspective

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 xylanases, 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 enzyme treatment surprisingly reduced the requirement of chemicals 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 mill 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.

As a result of this work, much of the development efforts at the industrial scale have focused on the removal of cellulase contamination. Cellulase-free preparations are now available. Xylanases are produced using microbes that are either naturally free of cellulase-producing ability or have been mutated or genetically engineered to eliminate the cellulases. The most significant advancement is development of xylanases such as Ecopulp TX-200C that function in alkaline pHs and at high temperatures. Advances in production technology have drastically reduced production cost, making enzymes a sensible economic choice in bleaching. Enzymes are now available to work in all possible bleach plant scenarios.

Benefits of enzyme use

Following the first application of xylanases 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 a bleach plant in several different ways, which have been described in a recent survey of xylanase use in Canadian mills.

Brightness Gain

Xylanases 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.

Chemical savings

If the xylanases 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.

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.

ClO_2 limitation.

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

Other Benefits.

Two Scandinavian mills are using enzymes to eliminate the D0 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.

MILL OPERATION: The most conventional method is to add xylanase to the brownstock pulp prior to the high-density (HD) tower.

The enzyme 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.

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 xylanases 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 xylanases, non-corrosive carbon dioxide is an excellent choice and also improves washer performance. The addition of acid prior to the D0 stage in ECF bleaching has also been shown to improve the performance of the D0 stage. This is because the higher acidity in the stage prevents decomposition of chlorine dioxide to chlorate, and because the chemistry of delignification with chlorine dioxide favors an acidic environment. During trials the benefit of acid alone should be established.

Typical sites of acidification are also indicated. Acid added to the low consistency pulp prior 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 high-density towers has generally needed to be greater than one hour. However, new thermo-tolerant enzymes now permit higher brownstock thus shortening retention requirements.

Mill experiences: Xylanase use in bleach plants is more common in Canada than the U.S. because of more stringent AOX levels, and thus a greater percentage of Canadian mills also produce ECF pulp. Reducing ECF cost is the main benefit of xylanases. A similar drive to reduce bleaching costs and AOX is occurring now that the guidelines for U.S. Cluster Rules have been implemented. Mill-based applications and benefits are discussed below.

Increased Brightness. A Western Canadian market pulp mill, producing 770 to 880 tonnes per day of softwood pulp using coastal woods, wanted higher brightness. The mill employed a DEoWD sequence with the water soak stage taking place in an unused hypo tower. Typical brightness produced was 86% ISO.

Spraying a dilute solution of SO_2, dissolved in water, onto the pulp mat of the decker to achieve a 6.2 pH during the enzyme trial acidified the pulp. The enzyme was diluted and hosed into the repulper section of the decker and the pulp was then pumped to the HD tower at temperatures between 55°C to 58°C.

For example, an increase in pulp brightness was obtained using xylanase. On average, an additional 1.5% ISO brightness points were achieved using enzymes for the duration of the two-week trial. Use of enzymes in this simple application provides help to mills experiencing problems with brightness and who are looking to penetrate higher-brightness markets.

Chlorine Dioxide Reduction

Xylanase enzyme was applied at an Eastern Canadian mill to reduce chlorine dioxide use and its inherent costs. This mill produced 992 tonnes per day of softwood market pulp of >90% ISO brightness using MCC pulping and a DEoDEpD sequence. The pulp was acidified to pH 6 to 7 with dilute sulfuric acid sprayed over the repulper of the decker. The enzyme was diluted and hosed into the discharge chute of the decker. Pulp was pumped using a medium consistency (MC) pump to the HD tower with a retention time of one to two hours at 59°C to 61°C. A control test period without enzyme was conducted for two weeks prior to enzyme addition. When enzyme was applied, an increase in the DEo brightness signaled that the enzyme was working. At this point, chlorine dioxide application in the Do stage was cut. The mill operated under these conditions for one week and was able to maintain the >90% ISO target brightness as well as strength properties. The reduction in chlorine dioxide, expressed as a reduction in kappa factor, can be seen.

During the enzyme application period, the kappa factor was reduced from an average of 0.165 to 0.12-0.13. The mill was able to reduce actual chlorine dioxide use by 11 lb per ton (5.5 kg per metric ton). This estimation was made using pre-trial control data gathered over a three-month period.

The reduction in chlorine dioxide use was also expected to reduce the AOX released in the final mill effluent. In a later trial, AOX discharges into the local river were monitored. An average of >0.35 kg/mt of AOX was measured during a one-month control period prior to enzyme addition. AOX in effluents released during the xylanase application period ranged between

0.08 kg/mt and 0.23 kg/mt and returned to pre-trial levels after the enzyme was turned off.

In a similar application, a Western Canadian mill that has used enzymes since 1992 has recently reported a savings of 7.8% in bleaching chemical costs. ClO2 use was reduced 8.2% and caustic soda use 21%. Enzyme use lowers the kappa factor, which reduces the acidity of the pulp carried into extraction stages, leading to lower caustic soda use in extraction.

In another recent example, a Finnish mill produced up to 600 tpd of softwood pulp bleached using an ECF sequence. This mill has been running full-time on xylanase for the last two years. Enzyme is applied at 55°C for two hours on brown stock batch pulp. As can be seen in Figure 5, the mill trial resulted in a 23% reduction in chlorine dioxide (expressed as total available chlorine) application and experienced better overall brightness. This mill currently reports a 15% chlorine dioxide savings. Several of the half-dozen mills running full-time in Finland also have bulk storage facilities for enzymes. The majority of these also use oxygen delignification.

Xylanase Applications at High pH

Recently, a high pH tolerant xylanase has been developed. This enzyme allows enzyme use with minimal or no acidification of the brownstock pulp. The following mill data shows the application of this enzyme (Ecopulp TX-200C).

No acidification of the northern softwood brownstock pulp was done prior to the bleach plant. Thus, the pH for the enzyme reaction in the high-

density tank was 10.0 to 10.3 with a retention of about one hour at 65°C. A reduction in the bleaching kappa factor of approximately 15% was achieved.

Current Use

As is the case for most new technologies, the rate of adoption of enzymes has been relatively slow. 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. Thus, interest has been rekindled as is shown by a significant increase in the number of trials and planned trials that are taking place. In the case of Ecopulp TX-200C, which has been available in Scandinavia for a year and half, there are nine mills using the enzyme.

In most instances, enzymes are applied to reduce chlorine dioxide use. However, two mills are using enzymes to eliminate the first bleaching stage and thereby recycle filtrates from the first two stages.

Presently there are three Canadian users and there is a large list of mills in Canada and the United States preparing for trials.

Country	Mill	Application
Finland	Stora Enso,	$ClO_2$1*
	Veitsiluoto Kemi	Eliminate Do stage
	Stora Enso,	$ClO_2$1*
	Imatra	
	Stora Enso,	$ClO_2$1*
	Enocell	
	Stora Enso,	TCF
	Kemijarvi	
	UPM-Kymmene,	$ClO_2$1*
	Wisaforest	
	UPM-Kymmene,	$ClO_2$1*
	Kuusanniemi	
Sweden	Mill A	Eliminate Do stage
	Mill B	$ClO_2$1*
	Mill C	$ClO_2$1*
North Am.	Eastern Canadian SWD	$ClO_2$1*
	Central Canadian SWD/HWD	$ClO_2$1*
	Western Canadian SWD	$ClO_2$1* & NaOH
1* chlorine dioxide reduction; NaOH reduction		

Applications

(a) Drinks:

Enzymes have many applications in drink industry. The use of chymosin in cheese making to coagulate milk protein was already discussed. Another enzyme used in milk industry is beta-galactosidase or lactase, which 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. Pectins 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 cellulase 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, which increases the enzyme levels in the grain. In the mashing process the enzymes are liberated and they hydrolyse 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-glucanases), hydrolyse 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.

(b)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 has 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 a Trichoderma xylanase. Usually a feed-enzyme preparation is a multienzyme cocktail containing glucanases, xylanases, proteinases and amylases. Enzyme addition reduces viscosity, which increases absorption of nutrients, liberatates nutrients either by hydrolysis of non-degradable fibres or by liberating nutrients blocked by these fibres, 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 faeces resulting in reduced environmental pollution. The use of phytase reduces the need to add phosphorus to the 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.

(c) Baking:

Similar fibre 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. Overdosage 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.

(d) 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 of 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 hydrolysing 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 fibre the removal of ink is important. The fibre is diluted to 1% concentration with water, flocculating 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 cellulase 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.

Chapter 2
REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

1. Chantasingh D, Pootanakit K, Champreda V, Kanokratana P, Eurwilaichitra have reported on Cloning, expression, and characterization of a xylanase 10 from *Aspergillus terreus (BCC129) in Pichia pastoris*.

2. Berrin JG, Williamson G, Puigserver A, Chaix JC, McLauchlan WR, JugeN. have reported their work on High-level production of recombinant fungal endo-beta-1,4-xylanase in the methylotrophic yeast *Pichia pastoris*.

3. Tanaka H, Muguruma M, Ohta K.have worked on Purification and properties of a family-10 xylanase from Aureobasidium pullulans ATCC 20524 and characterization of the encoding gene.

4. Koseki T, Miwa Y, Akao T, Akita O, Hashizume K. have done a work on Aspergillus oryzae acetyl xylan esterase: molecular cloning and characteristics of recombinant enzyme expressed in *Pichia pastoris*.

5. Fierens K, Geudens N, Brijs K, Courtin CM, Gebruers K, Robben J, Van Campenhout S, Volckaert G, Delcour JA, have reportd on High-level expression, purification, and characterization of recombinant wheat xylanase inhibitor TAXI-I secreted by the yeast *Pichia pastoris*.

6. Tanaka H, Nakamura T, Hayashi S, Ohta K, 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.

7. Lee CC, Wong DW, Robertson GH, have worked on Cloning and characterization of the xyn11A gene from *Lentinula edodes*.

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Chapter 3

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Reagents preparation

Tris buffer

A- 0.2M solution of Tris was prepared by dissolving 24.22 g of Tris in minimal amount of distilled water and making up to 1000 ml with distilled water.

B- 0.2N HCl solution was prepared by adding 1.72 ml of concentrated HCl in minimal amount of distilled water and making up to 1000 ml with distilled water.

pH 9: 50 ml of A and 14.2 ml were taken and diluted up to 200 ml.

1% xylan solution

1 g of xylan is dissolved in 100 ml of distilled water.

40% Rochelles' salt

40 g of Sodium Potassium tartarate is dissolved by constant stirring in 40 ml of distilled water and then made up to 100ml with distilled water.

DNSA solution

DNSA is prepared by stirring 1g DNSA, 200mg Crystalline Phenol and 50mg Sodium Sulphite in 100 ml of 1% Sodium hydroxide solution.

3.2. Culture

Bacillus megaterium was obtained from departmental culture collection center.. The culture was used for all our activities.

We inoculated the organism into 10% xylan solution in order to isolate the enzyme. But we obtained only very poor results, i.e. activity of enzyme. So, we tried inoculating the organism into varying concentrations of rice bran solution, which was effective.

We opted for rice bran since it is a good substrate for xylanase. Using rice bran had certain advantages, like being a very effective growth medium and also being very cheap and hence cost-effective.

Extraction of crude enzyme

The inoculated conical flask was taken and the broth was decanted into the centrifuge tube and was spun at 5000 rpm for 20 minutes. The supernatant was collected as the crude enzyme in a clean beaker.

Optimum Concentration of Rice Bran

We used different concentrations of rice bran varying from 1% to 15%, of which 10% was found to be the optimum concentration, for growth.

3.3. Characterization

3.3.1. Assays

We performed assays on all the cultures from all concentrations using 1% xylan as substrate and using 0.1 ml of the crude enzyme extract, along with 1 ml of DNS (Di Nitro Salicylic acid) as the colouring agent. We recorded an optimum absorbance of 0.656 at 540 nm for 10% concentration of rice bran for *B. megaterium*.

3.3.2. Optimum pH

To determine the optimum pH of the enzyme, we used buffers of varying pH ranging from pH 2.6 to pH 10.6. Of all these buffers, we determined that the optimum pH are 2.6, 5 and 9, having absorbance values of 0.386, 0.398 and 0.412, for just 0.1 ml of crude enzyme extract.

3.3.3. Optimum Temperature

To determine the optimum temperature of the enzyme, we varied the temperature during the assay from 15°C to 70°C. We obtained the maximum absorbance for the temperature of 45°C. We recorded an absorbance of 0.376 at 45°C.

3.3.4. pH Stability

We performed pH stability tests by measuring the absorbance at intervals of half hours for a mixture of 1.5 ml of pH 6 buffer and 1.5 ml of pH 10 buffer, by using buffers of pH 5 and pH 9 respectively for the assay. We found our enzyme to be stable even after two hours. It retained nearly half its activity.

3.3.5. Temperature Stability

We performed temperature stability tests by measuring the absorbance at intervals of half hours for enzyme placed in varying temperatures ranging from 20°C to 70°C for a total of two hours. The enzyme was active even after two hours at high temperatures, which proves that the enzyme is thermostable.

3.3.6. Effect of metal ions and some reagents

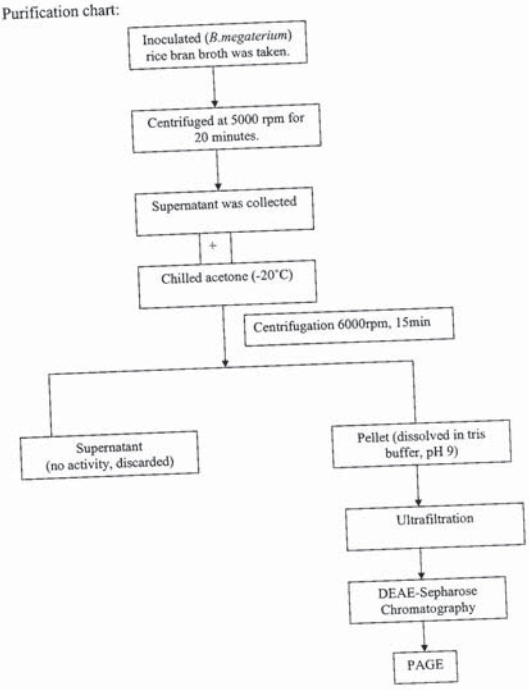
The effect of various metal ions and reagents on the crude enzyme activity was studied by adding various meal ions (Ag^{2+}, Hg^{2+}, K^{+}, Fe^{2+}, Zn^{2+}, Ca^{2+}, Cu^{2+} and Mg^{2+}) and reagents (EDTA, 1,10-phenanthroline, SDS, PMSF, n-bromosuccinamide and mercaptoehanol) along with the reaction mixture and the assay was carried out.

3.3.7. Effect of varying substrate concentration

Varying the concentrations of xylan from 100μL to 800μL performed the xylanase assay. The absorbance was measured at 540nm and readings were tabulated.

The xylan solution was prepared (1g in 100mL). The degradation of xylan by xylanase was checked by DNS method. Varying concentrations of xylan solution (100μL to 800μL) was treated with 0.1ml of appropriately diluted enzyme. This was incubated at 40°C for 15 min in a water bath. 1mL of DNS reagent was added and kept in boiling water bath for 5 min (Dubois et.al., 1951) and to that 1mL of 40% of Rochelle salt (sodium potassium tartarate) solution was added and the color developed was measured spectrophotometrically at 540nm.

Purification chart:



3.4. Purification

3.4.1. Acetone precipitation

To the enzyme extract (55 ml) chilled acetone (-20°C) was added slowly with constant shaking and the mixture was kept at 4°C for 6 h. At the end of 6 h, the mixture was centrifuged at 6000 rpm for 15 min and the supernatant was discarded. The precipitate was dissolved in acetate buffer (0.2M, pH 4.6).

3.4.2. Ultrafiltration

The acetone precipitated enzyme was filtered with a 50KDa Omega ultrafiltration membrane in a PALL ultrafiltration unit. The retentate and filtrate was collected separately and was checked for xylanase activity.

3.4.3. Ion exchange chromatography

Regeneration of the DEAE-Sepharose column

DEAE-Sephacel in the column (2.3 x 12 cm) was regenerated by washing with 250ml of Tris-HCl 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 0.5M NaCl. The regenerated DEAE-Sephacel was equilibrated with 500 ml of Tris-HCl buffer (0.05M, pH 7.5). DEAE-Sephacel was kept at 4 C after its use in Tris-HCl buffer containing 0.02% NaN_3.

DEAE-Sepharose chromatography

The Ultra filtered enzyme was loaded on to a DEAE-Sephacel column (2.3 x 12 cm) equilibrated with Tris-HCl buffer (0.05M, pH 7.5) and the column was washed with the same buffer. The enzyme bound to SEAE-

Sephacel was eluted with a linear salt gradient 0.5M NaCl in equilibration buffer (50mM, pH 7.5). Fractions were collected at a flow rate of 29ml/h using collector. All the fractions were checked for protein (A_{280}) and Xylanase activity (A_{540}). The fractions having maximum activity were pooled.

3.4.4. Polyacrylamide gel electrophoresis (PAGE)

PAGE was used to analyze the proteins present in the samples according to the method of Lammli (1970). Non-denaturing PAGE (native PAGE) was carried out without SDS.

(a). Monomer solution

The solution containing 29.2g of acrylamide and 0.8g of N,N-methylenebisacrylamide in a final volume of 100 ml made in distilled water. The solution was filtered through Whatman no.1 filter paper and stored at 4°C

(b). 1.875M Tris-buffer, pH 8.8

22.7g OF Tris dissolved in distilled water. The pH was adjusted to 8.8 with HCl and the volume was made up to 100 ml with distilled water. The solution was stored in refrigerator.

(c) 1.25M Tris-HCl buffer, pH 6.8

15.25 g of Tris was dissolved in distilled water. The pH was adjusted to 6.8 with 1N HCl and the volume of the solution was made up to 100 ml and stored in the refrigerator.

(d) Ammonium persulphate, 1% (w/v)

Ammonium persulphate 100 mg was dissolved in distilled water and the volume made up to 10 ml. Freshly prepared ammonium sulphate was used every time.

(e) Electrophoresis buffer (10x)

Tris base 30g, glycine 144g were dissolved in distilled water and the volume made up to 1000ml and stored at room temperature.

(f) Running gel (12%)

Acrylamide (30%)	40.0 ml
Tris-HCl buffer (1.875M, pH 8.8)	20.0 ml
Distilled water	34.0 ml
Ammonium persulphate (1%)	5.0 ml

The mixture was degassed, 12 µl of TEMED was added. The solution was poured into cassette kept in the gel casting apparatus. The solution was over layered with distilled water using a syringe with out disturbing the surface and was allowed to polymerize.

(g) Stacking solution

Acrylamide	12.0 ml
Tris-HCl buffer (1.25M, pH 6.8)	3.0 ml
Distilled water	13.2 ml
Ammonium persulphate (1%)	1.5 ml

After degassing the mixture, 12 µl of TEMED was added. The over layered water on running gel was poured into the cassette over the running gel. A comb was introduced into the spacer gel and was allowed to polymerize in the presence of light. After polymerization the comb was removed from the cassette and the gel was inserted into the gasket of electrophoresis apparatus. Electrophoresis was performed at room temperature and constant current of 50 mA. When the bromophenol blue dye stopped 1 cm from the bottom of the gel, electrophoresis was stopped. The gel was removed from the glass plates and was stained.

Assay

One part of the gel was equilibriated with acetate buffer (0.2M , pH4.6) and was cut into uniform pieces. Each piece was checked for α-galactosidase activity.

Coomassie brilliant blue staining

The gel was soaked in staining solution. After allowing it to stain for 1 hour the gel was immersed in the destaining solution. Destaining was stopped when the gel background was colorless.

- Staining solution

1g Coomassie brilliant blue in a solution containing 40 ml methanol, 10 ml glacial acetic acid and 50 ml distilled water.

- Destaining solution

Solution containing 40ml methanol, 10ml glacial acetic acid and 50 ml distilled water.

Silver staining

- The gel was transferred into a container containing washing solution and was washed for ten minutes.
- The washing solution was then discarded and the gel was rinsed with distilled water for two minutes.
- The gel was soaked in sodium thiosulphate for 1 to 2 minutes.
- Then it was washed twice with distilled water for 2 minutes.
- The gel was silver nitrate solution for 10 minutes with accompanied shaking.
- Then it was washed twice distilled with water for 2 minutes.
- Then it was soaked in developing solution with intermittent shaking till dark brown color band appears.
- When sufficient intensity of band developed, the reaction is stopped by adding stopper solution.

Reagents

Washing solution:

1mL of formaldehyde,40 mL methanol and 60 mL of distilled water was mixed together.

Sodium thiosulphate

200mg of sodium thiosulphate was dissolved in 1000 mL of distilled water.

Silver nitrate solution (0.1%):

0.1 g of silver nitrate was dissolved and the volume was made upto 100 mL with distilled water.

Developing solution

3g of sodium carbonate was dissolved in 80 mL of distilled water and to this 1 mL of above sodium thiosulphate solution and 1mL of formaldehyde was added and the volume was made upto 100mL with distilled water.

Stopper solution:

5% acetic acid solution(5 mL of glacial acetic acid made upto 100 mL with distilled water).

BSA calibration curve

A standard protein curve was constructed by the method described by Lowry et al(1951).Bovine serum albumin(BSA)0.1mg/ml was used as standard protein. Different concentrations of BSA solutions were pipetted out in the test tube and the total volume was made upto 1mL with distilled water. 2.1mL of alkaline copper reagent was added to each tube, mixed thoroughly and allowed to stand at room temperature for10min. Then to each tube 0.2mL of 1N Folins-Ciocalteau's reagent was added with immediate mixing. The extinction was read after 20min incubation against appropriate reagent blank at 660 nm in a ELICO spectrophotometer. A standard graph was constructed.

Alkaline copper reagent

Solution A: 1% copper sulphate

In a clean dry 100 ml standard flask, weigh 1g of copper sulphate and made up to the mark with distilled water.

Solution B: 1% sodium potassium tartarate

In a clean dry 100 ml standard flask, weigh 1g of sodium potassium tartarate and made up to the mark with distilled water.

Solution C: 2% sodium carbonate in 0.1N sodium hydroxide

In a clean dry 10 ml standard flask, weigh 2g of sodium carbonate and made up to the mark with 0.1N sodium hydroxide.

Mix 1 ml of solution A and 1 ml of Solution B and 98 ml of solution C, to form alkaline copper reagent.

Folin-Ciocalteau reagent

The composition of folin-ciocalteau reagent is 100 ml of sodium tungstate, 25g of sodium molybdate, 500 ml of distilled water, 50ml of 65% phosphoric acid, 100 ml of concentrated hydrochloric acid.

The commercial reagent was mixed with equal volume of distilled water, the reagent should be prepared freshly.

Xylose calibration curve

A standard calibration curve of xylose was constructed. A standard 1% xylose solution was prepared, different volumes of xylose solution 0.2, 0.4, 0.6, 0.8 and 1 ml was taken in a series of test tubes and the volume was made up to 2 ml with distilled water to each tube. To each tube, 1 ml of DNS is added. The tubes are now kept for incubation at boiling water bath for a period of 5 minutes. The tubes are removed and 1 ml of Rochelle salt is added and the absorbance is measured at 540 nm. A graph was drawn to obtain the standard xylose curve.

Table 3.1 BSA Calibration curve

Reagents	B	S_1	S_2	S_3	S_4	S_5
Volume of working std (ml)	0.0	0.2	0.4	0.6	0.8	1.0
Concentration(μg)	0	20	40	60	80	100
Volume of distilled water(ml)	1.0	0.8	0.6	0.4	0.2	0.0
Volume of alkaline Cu reagent(ml)	2.1	2.1	2.1	2.1	2.1	2.1
Incubate at room temperature for 10 min						
Volume of folin's reagent(ml)	0.2	0.2	0.2	0.2	0.2	0.2
Incubate at room temperature for 20 min						
Absorbance at 660nm.	0.0	0.071	0.148	0.230	0.321	0.406

Table 3.2 Xylose calibration table

Reagents	B	S_1	S_2	S_3	S_4	S_5
Volume of xylose (ml)	0.0	0.2	0.4	0.6	0.8	1.0
Concentration (μg)	0.0	200	400	600	800	1000
Volume of distilled water (ml)	2.0	1.8	1.6	1.4	1.2	1.0
Volume of DNSA (ml)	1.0	1.0	1.0	1.0	1.0	1.0
Incubate in boiling water bath for 5 min						
OD at 540nm	0.0	0.331	0.996	1.526	1.909	> 2

3.5. Immobilization

Immobilization in calcium alginate

2.5% sodium alginate solution was prepared and to that 2ml of crude enzyme extract was added and mixed well. It was left undisturbed for 30 minutes. The calcium alginate beads developed when sodium alginate

solution was added to excess of 0.2M calcium chloride solution. The beads were used as enzyme source for xylanase assay.

Immobilization in agar

2.5% agar solution was prepared and to that 2mL of crude enzyme extract was added and mixed well. It was poured onto a glass plate and left undisturbed for 20 minutes, after which it was cut into pieces of uniform size and used as enzyme source for xylanase assay.

3.6. Application

Pulp and paper industry

Xylanase was used in the pulp and 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.

(a) 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 was marked as control.

(b) Chlorine treatment

After incubating with the 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.

(c) 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.

(d) 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.

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.

(a) Chlorine strength

Titration was done to determine the strength of chlorine.

Burette solution: Sodium thio sulphate.

Conical flask: 25 ml of chlorine + 10 ml of 10% KI and starch as indicator.

(b) Thio strength

Titration was done to determine the strength of thio.

Burette solution: Sodium thio sulphate.

Conical flask: 10 ml of 10% potassium dichromate + 3 ml of concentrated HCl + 10 ml of 10% KI + D.M. water and starch as indicator.

(c) 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.

(d) Hypo strength

Titration was done to determine the strength of hypo solution.

Burette solution: Sodium thio sulphate

Conical flask: 10 ml of hypo + 10 ml of acetic acid + 10 ml of 10% KI + D.M. water and starch as indicator.

Chapter 4
RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

4.1 Characterization

Xylanase activity was detected in the rice bran broth inoculated with *B.megaterium*.

As expected, Xylanase activity was found to vary with pH .The effect of pH on enzyme activity is shown in figure. The cell free extracts exhibited xylanase activity in a pH range between 2.2 to 10.7, there were three optimum pH's, 3, 5 and 9. Xylanase from *Bacillus megaterium* B6 ATCC 5194 (Ray RRand nanda G) showed an optimum pH of 7.5. Xylanase from *Fusarium oxysporum* (Ramesh Chander Kuhad et al,1997)showed an optimum pH 3.5.

Xylanase from *B.meagterium* had an optimum temperature at 45°C. Xylanase from *Bacillus megaterium* B6 ATCC 5194 (Ray RRand nanda G) showed an optimum temperature of 85 C. Xylanase from from *Fusarium oxysporum* (Ramesh Chander Kuhad et al,1997)showed an optimum temperature of 30 C.

The xylanase enzyme was found to be stable at pH- 6 and 10 even after incubation for a period of two hours.

The xylanase activity was found to be stable at temperatures 60°C and 70°C for a period of two hours, hence it is a thermostable enzyme.

The graph 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 85%, where as other cations such as K^{+}, $Ca2^{+}$ and Hg^{2+} increased the enzyme activity to about 10%, 80% and 60% respectively.

The graph shows the effect of reagents on xylanase activity. Among the reagents tested n- bromosuccinamide increased the enzyme activity to about 3 times. The reagents such as EDTA and 1, 10 phenanthroline, SDS, PMSF and Mercaptoethanol inhibited the enzyme activity.

Table 4.1 Determination of optimum temperature

Temperature °C	Enzyme activity (U/ml)
15	80
20	83
25	87
30	90
35	93
40	107
45	110
50	100
55	93
60	89
65	30
70	27

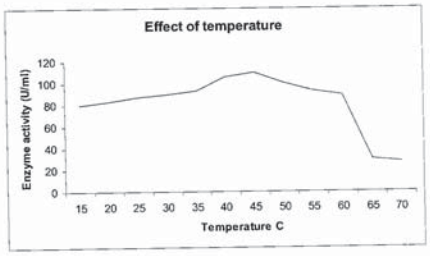


Figure 4.1 Determination of optimum temperature

Table 4.2 Determination of optimum pH

pH	Enzyme activity (U/ml)
2.2	23
2.6	110
3.0	100
3.5	33
4.0	30
4.5	80
5.0	100
5.5	70
6.0	50
6.5	53
7.0	57
7.5	73
8.0	75
8.5	88
9.0	107
9.6	67
10.0	53
10.7	23

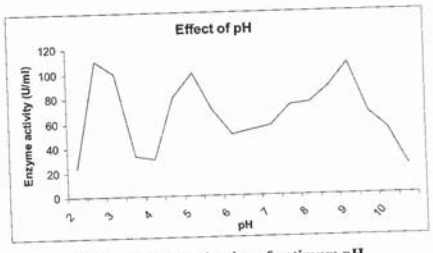


Figure 4.2 Determination of optimum pH

Table 4.3 Temperature stability at 60°C

Time (min)	Enzyme activity (U/ml)
0	43
30	35
60	33
90	30
120	27

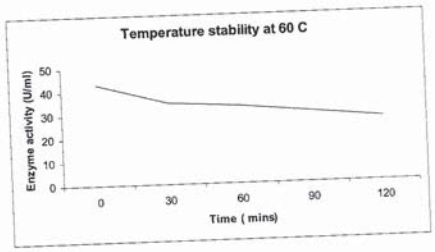


Figure 4.3 Temperature stability at 60°C

Table 4.4 Temperature stability at 70°C

Time (min)	Enzyme activity (U/ml)
0	63
30	60
60	53
90	47
120	43

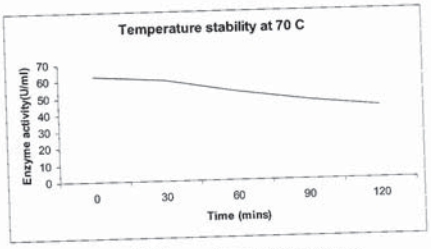


Figure 4.4 Temperature stability at 70°C

Table 4.5 Determination of optimum medium concentration

% of medium	Enzyme activity (U/ml)
1	13
2.5	27
5	53
7.5	60
10	90
12.5	76
15	70
20	63

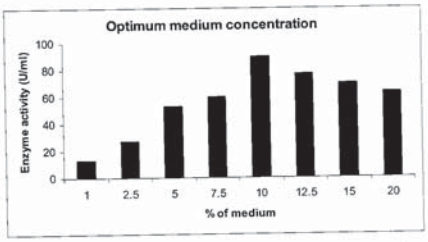


Figure 4.5 Determination of optimum medium concentration

Table 4.6 pH stability at 6

Time (min)	Enzyme activity (U/ml)
0	47
30	37
60	32
90	27
120	21

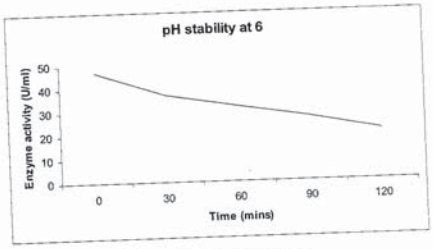


Figure 4.6 pH stability at 6

Table 4.7 pH stability at 10

Time (min)	Enzyme activity (U/ml)
0	83
30	73
60	67
90	60
120	52

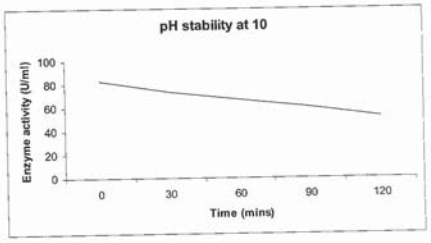


Figure 4.7 pH stability at 10

Table 4.8 Effect of metal ions

Metal ion	Enzyme activity (U/ml)
Control	85
KCl	93
$CaCl_2$	147
$ZnSO_4$	70
$CuSO_4$	13
$HgCl_2$	107

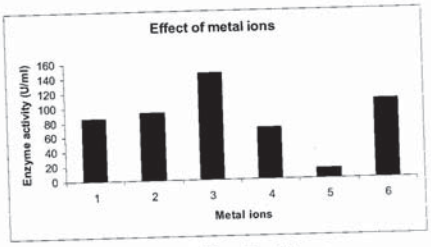


Figure 4.7 Effect of metal ions

1- control
2- KCl
3- $CaCl_2$
4- $ZnSO_4$
5- $CuSO_4$
6- $HgCl_2$

Table 4.8 Effect of reagents

Reagents	Enzyme activity (U/ml)
Control	93
1,10-phenanthroline	87
PMSF	80
SDS	67
n-bromosuccinimde	333
Mercaptoethanol (1%)	37
Mercaptoethanol (5%)	20
Mercaptoethanol (10%)	13

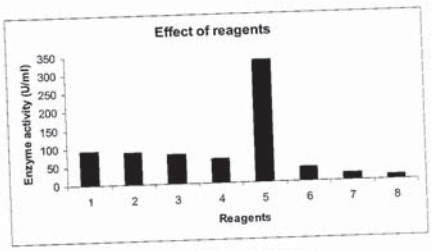


Figure 4.8 Effect of reagents

1- control
2- 1,10-phenanthroline
3- PMSF
4- SDS
5- N-bromosuccinimide
6- Mercaptoethanol (1%)
7- Mercaptoethanol (5%)
8- Mercaptoethanol (10%)

4.2 Purification

The xylanase was partially purified; this was achieved by combination of acetone precipitation, ultrafiltration, and ion exchange chromatography on DEAE-Sepharose.

The acetone precipitated protein was run in a native PAGE and the assay revealed xylanase activity in the first section of the gel. With reference to the protein marker (PMW-H), the molecular weight of the protein was found to be approximately greater than 50KDa.

Ultrafiltration

The filtrate and retentate collected after ultrafiltration of the acetone precipitated enzyme was subjected to xylanase assay. The xylanase activity was found only in the retentate and not in the filtrate, thus confirming the fact that the enzyme is greater than 50 KDa.

Ion Exchange Chromatography:

Xylanase activity was observed in fraction numbers 2 - 24. The active fractions were found to be 15, 18 and19.

Native PAGE

After three purification steps, native PAGE of the final enzyme preparation showed three band. The molecular weight of partially purified xylanase by native PAGE was estimated to be 60kDa.

Figure 4.9. Native PAGE

Table 4.10 Purification chart

Steps	Volume (ml)	Protein conc (mg)	Activity (Units)	Specific activity (U/mg)	Purification fold	Yield %
Crude	900	2.6	100.34	38.59	1	100
Acetone precipitation	100	1.9	120.66	63.16	1.64	13.66
Ultrafiltration	45	0.8	143.33	179.16	2.82	53.45
Ion exchange chromatography	40	0.03	33.33	1111	6.20	20.67

4.3 Immobilization

The xylanase immobilized in 2.5% calcium alginate beads retained 73% activity of free enzyme respectively. The xylanase immobilized in 2.5% of agar retained 81% of free enzyme activity.

4.4 Application

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,

Table 4.11 Brightness comparison in the pulp

Treatment step	Control	Enzyme trial 1	Enzyme trial 2
Enzyme	20.51	20.24	20.03
Chlorine	29.19	29.16	28.66
Peroxide	64.34	64.86	65.31
Hypo	79.15	80.28	81.78

Chapter 5

CONCLUSION

5. CONCLUSION

Thus the optimum temperature and pH was determined to be 45°C and 9 respectively. Its application in the reduction of chlorine during the bleaching of the pulp was proved to be very effective. The enzyme was partially purified. Further more trials with varying the concentrations of enzyme can be studied. As well as the growth of microbes by adding additional nitrogen sources in the rice bran medium can also be studied.

Chapter 6

BIBLOGRAPHY

6. BIBLIOGRAPHY

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