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PRODUCTION, PURIFICATION AND CHARACTERIZATION OF THERMO-ALKALI STABLE XYLANASE FROM *Bacillus brevis*

P-3088

PROJECT REPORT

Submitted by

KRISHNANAND.N

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Department of Biotechnology

PROJECT WORK -PHASE II

MAY 2010

This is to certify that the project entitled " **PRODUCTION, PURIFICATION AND CHARACTERIZATION OF THERMO-ALKALI STABLE XYLANASE FROM *Bacillus brevis*"** is the bonafide record of project work done by **N.KRISHNANAND Register No: 0820203004** of M.Tech during the year 2009-2010.

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DECLARATION

I affirm that the project work titled **"Production, Purification and Characterization of Thermo-Alkali Stable Xylanase from *Bacillus brevis*"** being submitted in partial fulfilment for the award of **M.Tech. (Biotechnology)** is the original work carried out by me. It has not formed the part of any other project work submitted for award of any degree or diploma, either in this or any other University.

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I certify that the declaration made by the above candidate is true.

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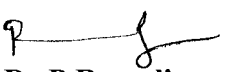

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KRISHNANAND.N

DEDICATED TO MY DEAR PARENTS

&

TO MY BELOVED SISTER

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ABSTRACT

A strain of *Bacillus brevis* producing thermo-alkali stable xylanase was isolated from soil sample collected from agriculture fields of Polavakkalipalayam, Gobichettipalayam taluk of Erode district, India. The highest production of xylanase was observed in cultures grown for 3 days in 6% wheat straw with 2% arabinose as supplementary carbon source and 4% urea as nitrogen source, at 40°C and pH 10.5, with shaking at 130 rpm. Xylanase was purified upto 17.1 fold by ultra filtration and DEAE-Sephadex. The purified enzyme had an apparent molecular weight of 60 kDa. Optimal pH and temperature values for the enzyme activity were about 9.0 and 60°C, respectively. The enzyme was stable at 50–60°C at pH 9. Metal ions $FeSO_4$ and $MgSO_4$ increased the activity by two fold, while $CuSO_4$, $HgCl_2$, $ZnCl_2$ inhibited the enzyme activity completely.

Key words: *Bacillus brevis,* wheat straw, Xylanase, Arabinose, Thermo-alkali stable

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LIST OF ABBREVIATIONS

ABBREVIATION	EXPLANATION
mg	Milligram
ml	Millitre
h	Hour
L	Litre
Min	Minutes
M	Molar
nm	Nanometer
EDTA	Ethylenediamine tetracetic acid
M	Molar
Nm	Nanometer
AHM	Akiba Horikoshi medium
Dbb	Dry Bacterial Bran

INTRODUCTION

1. INTRODUCTION

Enzymes are distinct biological polymers that catalyze the chemical reactions and convert substrates to particular products. They are specific in function and speed up reactions by providing alternative pathways of lower activation energy without being consumed. These are the fundamental elements for biochemical processes and utilized in a number of food processing industries (Haq *et al.*, 2006).

The manipulation of biotechnological techniques have played an important role in the recent advances occurred in Paper, Animal feed, Baking industries etc. India is an agricultural country; resultantly agro-industrial wastes and by-products are in abundance here. Wheat bran, sugar cane bagasse, corn cobs, rice bran etc. are some of the prominent waste materials from the allied food industries. These waste materials, if not handled properly, certainly are a source of environmental pollution (GOP, 2001).

Producers and stack holders are taking interest to utilize even a bit of resources to cope with the economic cost of finished products. The processing units generating agro-waste materials/by-products are struggling hard for their conversion into value added products. The agricultural waste materials if manipulated properly can play a significant role in the economic uplift of a state. There is an increasing gist to utilize such neglected materials in the production of enzymes which can be employed further in food processing (Mohammadi *et al.*, 2006; Okafor *et al.*, 2007).

Various organisms have different potential for the synthesis of enzymes; Bacteria are the most common source of hemicellulases like xylanases, β-mannase and glucanases. Over the years, a number of organisms including the

and Gammel, 2001), *PaeniBacillus xylanisolvens* (Saowapar *et al.,* 2010) and *Bacillus pumilus* (Rashid, 1999) have been exploited for xylanase production. However, in the present era, *Bacillus* sp. has been reported as the most potent organism for xylanase biosynthesis. *Bacillus* is a genus of gram-positive rod-shaped bacteria and a member of the division Firmicutes. *Bacillus* species can be obligate aerobes or facultative anaerobes, spore forming and test positive for the enzyme catalase. It includes both free-living and pathogenic species.

Xylans; the substrates for xylanases are polysaccharides composed of β-1,4 linked xylopyranose units. They are highly branched and in firm association with other polymers. Acid hydrolysis of xylans is a rapid process for the production of xylose but a number of toxic and undesirable substances are also produced (Jackson and Hoseney, 1986). Xylan is widely distributed in plant cell walls and forms a main part of the hemicellulose fraction. In some higher plants and agricultural wastes, xylan constitutes from 20-40% of the dry weight. Xylan together with hemicellulose forms the second most abundant renewable polysaccharide in the biosphere. It has been estimated that 500 million tons of such materials could be annually available from the residues of major crops. Xylan has a complex structure consisting of ß-1,4-linked xylose residues in the backbone, to which short side chains of o-acetyl, α-L-arabinofuranosyl, D - α-glucuronic and phenolic acid residues are attached. Xylan structure is variable, ranging from linear 1,4-β-linked polyxylose chains to highly branched heteropolysaccharides. α-L-Arabinofuranosyl (AFase) residues constitute monomeric or oligomeric side chains on the xylose or galactose backbones in xylans, arabinoxylans and arabinogalactans at O-3 positions or sometimes at both the O-2 and O-3 positions, whereas α-1,5-linked arabinofuranosyl residues form the backbone of arabinans.

Nowadays, the demand for chemical free food products is increasing rapidly over the globe and biotechnology can play a significant role to meet the

Xylanase is an extracellular enzyme which hydrolyses β-1,4 D-xylosidic linkages of highly polymerized and substituted β-1,4 linked D-xylobiose, xylotriose and glucucoronosyl residues. The enzyme holds potential for the degradation of plant cell wall materials (Kulkarni *et al.,* 1999; Omar *et al.,* 2008). 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 sulphate and sodium/potassium phosphate buffer pH 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 sulphate. Xylanase solubility in phosphate buffer pH 9 decreases in the temperature range of 0°C to 10°C. But remains constant in the pH range of 10 through 37°C. Xylanase has been extracted from many different fungi, bacteria and actinomycetes. It is commonly used in animal feeds, paper production, and food production.

Xylanases are important in the bioconversion of hemicelluloses into their constituent sugars. Xylanase enzymes hydrolyze the xylan polymer that exists within pulp fibres. 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 depolymerised lignin from pulp is a diffusion-limited process, xylanase treatment ultimately improves the extraction of lignin from pulp.

Microbial xylanases (β-1,4 D-xylan xylanohydrolase, EC 3.2.1.8) are being used in various industries including food, feed, textile and paper processing industries. In food and feed, they liberate the nutrients by hydrolyzing the nondegradable hemicellulose fibers thus make the nutrients

Xylanase due to their multidimensional role in fermentation processes have gained immense importance. Sugars like xylose, xylobiose and xylooligomers can be prepared by the enzymatic hydrolysis of xylan. The depolymerization action of xylanase results in the conversion of polymeric substances into xylo-oligosaccharides and xylose (Subramaniyan and Prema, 1998; Bajpai, 1999; Omar *et al.*, 2008).

Presently, efforts are being made to increase the production of hydrolytic enzymes by fermenting the agricultural waste materials through biotechnological approaches. Fermentation process gives promising yield of enzymes and is an economical method due to low cost and accessibility of waste materials as substrates. Most of the enzymes are being synthesized by utilizing the submerged fermentation technique. Agricultural waste materials can be used as substrates that provide carbon and mineral nutrients to the organisms under the controlled conditions i.e. pH of the culture medium and temperature of incubation. Generally the organisms produce the extra cellular enzyme which is collected and purified (Ali *et al.*, 2002; Skowronek and Fiedurek, 2006).

Microbial xylanases have commercial applications in agriculture, industry and human food production. Xylanases are useful in bioconversion of lignocellulosics to fuel and chemicals, to improve silage for better digestion by ruminants, to improve quality of detergent, and also used for clarification of fruit juices, in flour improvements for bakery products and in controlling environmental hazards through bio pulping. Xylanases used in pulp pre-bleaching process remove the hemicelluloses, which bind to the pulp. The hydrolysis of pulp bound hemicelluloses releases the lignin in the pulp, reducing the amount of chlorine required for conventional chemical bleaching and minimizing the toxic, chloroorganic waste.

Therefore, xylanases from alkalophilic bacteria and actinomycetes have

Thermostable xylanases active at alkaline pH are of great interest for application in the pulp and paper industry to decrease the consumption of chlorine chemicals. Xylanases have been reported from bacteria, Fungi and actinomycetes. However, large-scale cultivation of fungi and actinomycetes is often difficult because of their slow generation time, coproduction of highly viscous polymers, and poor oxygen transfer. *Bacillus* sp. is used more extensively than other bacteria in industrial fermentations, since they secrete most of their enzymes.

Other applications of xylanases include improvement of silage by 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. 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. In fuel-alcohol production xylanase decreases the viscosity of the mash and prevents fouling problems in distilling equipment. In improve the extraction of oil from oil-rich plant material such as corn-oil from corn embryos. In improving retting of flax fibres by the decomposition of the outer stem of the flax plant necessary before the fibres are processed into linen.

The aim of the present study is to isolate alkalophilic and thermophilic bacterial strains, and produce, purify and characterize the cellulase free alkali and thermostable xylanase.

OBJECTIVES

2. OBJECTIVES

†To isolate and screen bacteria producing alkalistable and thermostable xylanase

†To optimize production parameters for xylanase production

†To purify the xylanase enzyme

†To characterize the xylanase enzyme

REVIEW OF LITERATURE

3. REVIEW OF LITERATURE

Paper & pulp, Animal feed, Baking industries, with the advancement of biotechnology, has undergone a lot of improvements during the last few years. Its processes become more efficient and the expectations of industrialists befall higher to achieve the best possible products in economical way.

Generally, the use of chemical additives in the flour to improve bread-making performance and the use of chlorine chemicals in pulp and paper industry might have some side effects in environment as well as in human health. Currently, the enzymes are replacing the chemical additives in numerous food applications. The use of enzymes has become a need of the time, because they promote effects similar to those of chemical additives with the advantage of being considered as safe natural additives (Penstone, 1996).

Agro-based wastes or by-products are generated every year over the globe in huge amounts that are potent threat as environmental pollutants. Food industry is contributing a significant share in producing agro-based biological wastes. Fermentation processes are playing a major role in the removal of these contaminants by exploiting the wide adaptability of microorganisms to degrade such compounds.

The present project has been planned to produce, purify and characterize xylanase and to study its application in pulp and paper industry. The literature pertaining to different aspects of current studies has been summarized under the following sets;

3.1 Xylans: an overview

3.2 Xylanases

3.5 Xylanase application

3.1 Xylans: an overview

Xylan is the major type of hemicellulose and its hydrolysis depends on two classes of enzymes. The Endoxylanases (EC 3.2.1.8) cleave the xylan backbone into smaller parts like oligosaccharides that are further degraded to xylose by xylosidases (EC 3.2.1.37). During the last few decades, great interest has been developed in xylan and its hydrolytic enzymatic complex, for application in bread production, supplement in animal feed, preparation of drinks, textiles, bleaching of cellulose pulp, ethanol and xylitol production.

The plant cell wall is a composite material in which cellulose, hemicellulose (mainly xylan) and lignin are closely joined together. Xylan is the second most abundant polysaccharide and major component in plant cell wall that consists of β-1, 4-linked xylopyranosyl residues (Puls, 1997). The structure of xylans found in cell walls of plants can differ greatly depending on their origin and different structures attached to the xylan backbone. Although most of the xylans have branched structures, however some linear polysaccharides have been isolated (De Vries and Visser, 2001). Lignin is bound to xylans by an ester linkage to 4-O-methyl-D-glucuronic acid residues. These linkages in lignocellulose can be disrupted by using various pretreatment methods that expose most of the polysaccharide components to enzymatic hydrolysis (Chang *et al..*, 1981). Selective hydrolysis of xylan has been observed when purified (Paice and Jurasek, 1984) and crude enzyme was applied in which cellulases were inhibited (Mora *et al.,* 1986). In all of these cases, complete removal of the xylosyl residues from the fibers was not achieved. The residual xylosyl residues may be inaccessible to xylanolytic enzymes due to the presence of substituents; modification of fiber synthesis and occurrence of xylans enclosed by other

mannan is selectively removed from delignified fiber, the residual cellulose becomes accessible to cellulase hydrolysis. However, a similar prehydrolysis of cellulose or mannan does not improve accessibility of xylan to xylanases. Selective removal of xylans increases accessibility of other polysaccharides due to increased fiber porosity that is positively correlated with cellulose hydrolysis in pretreated fibers (Grethlein, 1985; Wong *et al.,* 1988).

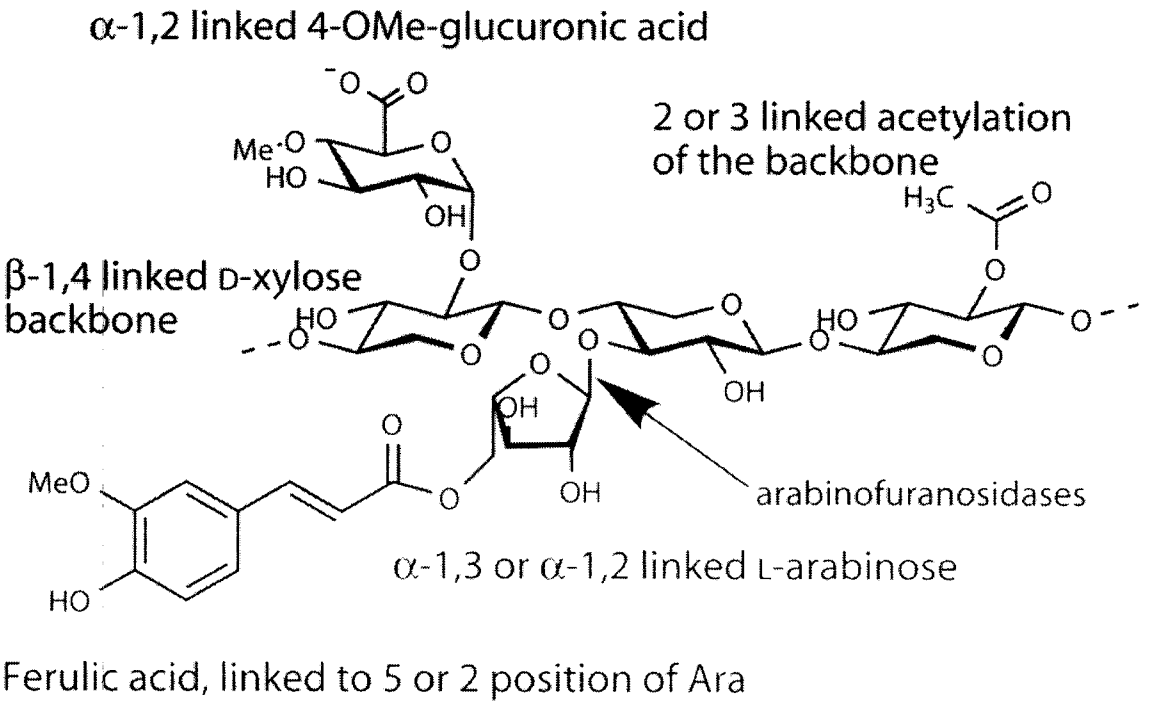


Figure 3.1.1: Xylan structure

In cereals, arabinoxylans are among the major non-starch polysaccharides. They constitute 4-8% of barley kernel and represent 25 and 70% of the cell wall polysaccharides of endosperm and aleurone layer, respectively. The arabinoxylans are partly water-soluble and result in highly viscous aqueous solution (Dervilly-Pinel *et al.,* 2001). Cereal xylans contain large quantities of L-arabinose and therefore, often referred as arabinoxylans

to the backbone of xylan via α-1, 2 or α-1, 3 linkage either as single residues or as short side chains. The side chains also contain xylose α-1, 2-linked to arabinose, and galactose, which can be either β-1, 5-linked to arabinose or α-1, 4-linked to xylose (De Vries and Visser, 2001).

The main component of non-starch polysaccharides in wheat flour are pentosans (mainly arabinoxylans, AX). Arabinoxylans occur as minor components of wheat grains (2-3%, dry basis); can be divided into soluble or water-extractable arabinoxylans (WE-AX) and insoluble or water-unextractable arabinoxylans (WU-AX). However, they play an important role in dough rheology and bread quality (Courtin and Delcour, 2002; McCleary, 1986). Numerous studies on the functional role of pentosans in dough development have been performed investigating their effect on bread properties (Biliaderis *et al.,* 1995; Maeda and Morita, 2003).

3.2 Xylanases

Xylanases are genetically single chain glycoproteins, ranging from 6-80 kDa, active between pH 4.5-6.5, at 40-60°C. Xylanases from different sources differ in their requirements for temperature, pH etc. for optimum functioning. The complete enzymatic hydrolysis of xylan into its constituent monosaccharides requires the synergistic action of a group of xylanolytic enzymes. This is due to the fact that xylans from different sources exhibit a significant variation in composition and structure (Hazlewood and Gilbert, 1993; Cesar and Mrsa, 1996; Latif *et al.,* 2006). The most important enzyme is endo-1,4-xylanase (EC 3.2.1.8) that initiates the conversion of xylan into xylooligosaccharides. Xylosidase, debranching enzymes (L-arabinofuranosidase and glucuronidase) and esterases (acetyl xylan esterase, feruloyl esterase) allow the complete degradation of the xylooligosaccharides to their monomeric

The heterogeneity and complexity of xylan has resulted in an abundance of diverse xylanases with varying specificities, primary sequences and structures. Wong *et al.* (1988) classified xylanases into two groups on the basis of their physicochemical properties: (i) having low molecular mass (<30 kDa) and basic pI, and (ii) having high molecular mass (>30 kDa) and acidic pI. However, many xylanases, in particular fungal xylanases, cannot be classified by this system. A more complete classification system has been introduced which allows the classification of not only xylanases, but also of glycosidases in general. This system has now become the standard means for the classification of these enzymes. It is based on primary structure comparison of the catalytic domains only and classifies the enzymes in families of related sequences (Henrissat and Coutinho, 2001).

Xylanases have been grouped into families F10 and G11 on the basis of amino acid sequence similarity and three-dimensional structure analysis.

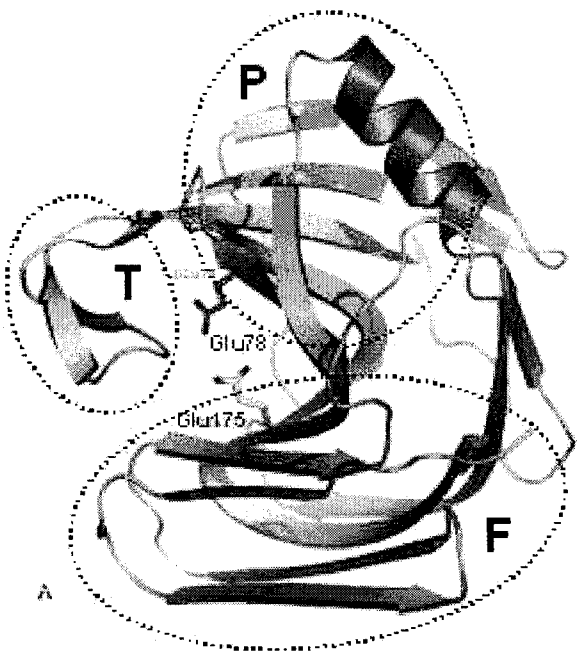


Figure 3.2.1: Xylanase structure

XynA) as determined by x-ray crystallography. The structure is typical of most G11 xylanases and, contains two twisted b-sheets forming a so called "jellyroll" fold which looks remarkably like a right hand, in which the individual b-strands thread back and forth to form the "finger" and "palm" domains (shown as the regions F and P in the diagram on the right). Residues lining the cleft formed between these two domains contribute to the substrate binding and active sites of the protein. An extended loop form the "thumb" domain (shown as T), which can open and close over the active site, so regulating the access of substrate to the catalytic region of the enzyme.

Different enzymes may be more effective in the hydrolysis of xylobiose, substituted xylo-oligosaccharides, xylosyl substituents or oligosaccharides containing xylosyl and other residues. The different forms may also have varying abilities to interact with xylanases in xylan hydrolysis (Reilly, 1981). Three apparent xylosidases have been classified as 'exoxylanases' because they have detectable activity on xylan. Two of these enzymes apparently lack transferase activity and one enzyme causes configuration inversion (initially yield α-D-xylose during hydrolysis), which is the characteristic used to distinguish between β-glucosidases and exoglucanases (Reilly, 1981; Eriksson and Wood, 1985). Furthermore, an exoglucanase from *Trichoderma viride* has been shown to attack xylan in an endwise fashion to initially yield xylobiose. Exoxylanases increase the rate of xylan hydrolysis by attacking large xylo-oligosaccharides that are released by endoxylanases and are ineffectively hydrolyzed by β-xylosidases. This form of cooperation would not be expected to increase the extent of hydrolysis unless other factors are involved e.g. accessibility of xylosidic linkages in short and/or branched xylo-oligosaccharides, reduction of product inhibitions, or amounts of extracellular β-xylosidases (Shikata and Nisizawa, 1975). Xylans are usually cleaved at unsubstituted regions to yield mixtures of unsubstituted xylo-oligomers, as well

groups by auxiliary enzymes creates new substrates for endoxylanase (EC 3.2.1.8) action (Si, 1997; Maheshwari *et al.,* 2000).

3.3 Xylanase production

The various biotechnological techniques like submerged and solid state fermentation are employed for xylanase biosynthesis (Cai *et al.,* 1998; Gawande and Kamat, 1999; Kansoh and Gammel, 2001). The submerged fermentation is most beneficial as compared to other techniques due to more nutrients availability, sufficient oxygen supply and less time required for the fermentation (Hoq *et al.,* 1994; Gomes *et al.,* 1994; Veluz *et al.,* 1999; Bim and Franco, 2000; Gouda, 2000). The production of microbial xylanases is preferred over plant and animal sources because of their availability, structural stability and easy genetic manipulation (Bilgrami and Pandy, 1992).

Xylanase activity was measured using oat spelt xylan as the substrate. To estimate the temperature, pH optima and heat stability, the activity was determined by carrying out the standard assay at several temperatures or pH values. After the incubating at different temperatures or pH's, the xylanase activity was measured under standard conditions (Coral and Çolak, 2000).

The effect of carbon and nitrogen sources and their concentrations on the production of xylanase by mutant strain of *Aspergillus niger* GCBMX-45, high xylanase activities were observed when starch was used as carbon source and ammonium sulphate was found to be best nitrogen source for optimum enzyme production. The production of enzyme reached maximum, 72 hours after inoculation (Yasser Bakri *et al.,* 2007).

The effect of aeration on xylanase production by *Bacillus* sp. I-1018 grown in batch cultures on xylan was investigated. Efficiency of aeration and

pressure (pO2). Growth and enzyme production were faster when K_La was increased (productivity in xylanase was up more than 50%). (P.L.Pham *et.a.l.*, 2004)

B Choudhury *et al,* (2005) studied the production of xylanase from *Bacillus coagulans* with respect to the environmental parameters, the carbon source and the concentration of carbon source at the shake flask level. Among the various carbon sources used, wheat straw powder favoured higher enzyme production. Xylan isolated from wheat straw gave higher enzyme production as compared to the birchwood xylan. Maximum enzyme activity of 165 IU/ml was obtained with 2% wheat straw xylan in a shake flask study. Improvement of xylanase production was achieved by increasing the wheat straw powder concentration up to 3%.

Bacillus pumilus ASH produced a high level of an extracellular and thermostable xylanase enzyme when grown using solid-state fermentation (SSF). Among a few easily available lignocellulosics tested, wheat bran was found to be the best substrate (5,300 U/g of dry bacterial bran). Maximum xylanase production was achieved in 72 h (5,824 U/g). Higher xylanase activity was obtained when wheat bran was moistened with deionized water (6,378 U/g) at a substrate-to-moisture ratio of 1:2.5 (w/v). The optimum temperature for xylanase production was found to be 37°C. The inoculum level of 15% was found to be the most suitable for maximum xylanase production (7,087 U/g). Addition of peptone stimulated enzyme production. The enzyme activity was slightly lower in SSF than in SmF but the ability of the organism to produce such a high level of xylanase at room temperature and with deionized water without addition of any mineral salts in SSF, could lead to substantial reduction in the overall cost of enzyme production. (B Battan *et al.*, 2006)

Later, M Saleem and companions (2002) isolated locally *Bacillus*

and kraft pulp at 2% concentration. The organism produced higher level of xylanase activity when bagasse was used as a carbon source followed by rice straw, wheat straw, wheat bran and kraft pulp. Xylanase activity was enhanced when different supplements were added in the bagasse medium. The maximal induction was observed when medium supplemented with 0.2% sucrose.

In another study, E I Emanuilova *et al.* (2000) investigated Xylanase production by newly isolated thermophilic alkali-tolerant *Bacillus* sp. strain SP and strain BC in batch and continuous cultures. Enzyme synthesis was inducible with both strains and was observed only in xylan-containing media. Xylan from oat spelt is a better inducer than xylan from birch for strain *Bacillus* sp. BC while such difference was not observed for strain SP. Compared with batch cultures xylanase production of both strains increased about two times and its rate became more than four times faster in continuous cultures at a dilution rate of 0.2 h^{-1}

A *Bacillus* species 2129 was tested for the xylanase production under submerged cultivation conditions (Mullai *et al.*, 2010). Maximum xylanase activities were achieved using oat as the substrate and by optimizing process conditions such as substrate concentration, pH and nitrogen source using statistically significant design of experiments, employing the response surface methodology (RSM) concept. Under optimized conditions there was an 8% increase in the enzyme activity and results from statistical approximation in the form of analysis of variance (ANOVA) shows that the squared effects of the variables were significant than both the main and interaction effects.

The literature discussed above describes the potential of *Bacillus* sp. to synthesize the appreciable activities of xylanase for its application in different processing industries.

3.4 Purification and characterization:

During enzyme production, in addition to the required enzyme, growth medium may have some undesirable metabolites of the micro-organisms which lead to lower enzyme activity. Purified enzymes exhibit higher activity, lesser risk of harmful substances and better application for the specific product. Moreover, characterization of enzyme is vital to achieve better performance in a particular application; as it provides information regarding suitable conditions for enzyme functioning.

Morales *et al.* (1993) observed three xylanases from *Bacillus polymyxa.* Xylanases X34C and X34E are closely related alkaline enzymes of 34 kDa and a pI of greater than 9.3, and xylanase X22 is a highly active enzyme of 22 kDa and a pI of 9.0. The enzymes were nondebranching endo-beta-xylanases. Xylose is a major product of xylan hydrolysis by xylanases X34C and X34E, but this is not an end product by xylanase X22. However, these enzymes do not hydrolyze xylobiose.

In another study, J Kiddinamoorthy *et al.* (2007) purified xylanase from *Bacillus* sp. GRE7 which had an apparent molecular weight of 42 kDa and showed optimum activity at 70°C and pH 7. The enzyme was stable at 60–80°C at pH 7 and pH 5–11 at 37°C. Metal ions Mn^{2+} and Co^{2+} increased activity by twofold, while Cu^{2+} and Fe^{2+} reduced activity by fivefold as compared to the control. At 60°C and pH 6, the Km for oat-spelt xylan was 2.23 mg ml−1 and V max was 296.8 IU mg−1 protein.

Later, A Blanco *et al.* (1995) purified Xylanase A to homogeneity from *Bacillus* sp. strain BP-23. The enzyme showed a molecular mass of 32 kDa and an isoelectric point of 9.3. Optimum temperature and pH for xylanase activity were 50°C and 5.5 respectively. Xylanase A was completely inhibited by N-bromosuccinimide. The main products of birchwood xylan hydrolysis were

An alkaline xylanase was purified from crude xylanase fermentation broth extracted in aqueous two phase system (ATPS) composed of 16% polyethylene glycol (PEG 6000) and 6.0% phosphate salt. A purification factor 57 and 41% yield of the enzyme activity were calculated for the system containing 16% PEG 6000, 8% K2HPO4 and 12% NaCl (Duarte *et al.*, 1999).

Damasco *et al.* (2002) produced cellulase free xylanase from Thermomyces lanuginosus in shake cultures by using corn cobs as carbon source and found that crude xylanase exhibited appreciable thermostability, retaining almost 50% of activity during 24 hours of incubation at 50°C and about 50% of activity was present at 60°C even after 4 hours of incubation.

Damasco *et al.* (2000) reported that xylanase was active in the broad range of pH and temperature, however the optimum pH and temperature were found as 6.0 and 75°C respectively.

Goulart *et al.* (2005) cultivated Rhizopus stolonifer on wheat bran to produce cellulase free xylanase. The purified xylanase exhibited optimum pH and temperature as 6.0 and 45°C respectively. Coelho and Carmona (2008) stated that xylanase exhibited high thermal stability in the pH range 4.5 to 10.5. Earlier Christakopoulos *et al.* (2007) demonstrated that xylanase II from the fungus *Fusarium oxysporum* F3 was stable at a temperature 44-55°C.

L Jung Yin *et al.* (2009) determined the molecular weight of xylanase produced by *Bacillus* sp. YJ6 strain as 19 kDa which had an optimal pH and temperature at 5.0 and 50 °C, respectively, and was stable at pH 5.0–9.0 or <50 °C. It was inhibited by Cu2+, Fe3+, Hg2+, phenylmethyl sulfonyl fluoride (PMSF), N-tosyl-l-phenylalanine chloromethyl ketone (TPCK), N-ethylmaleimide (NEM), and leupeptin but activated by K+, Na+, Co2+, Mg2+, β-mercaptoethanol (β-ME), and glutathione (GSH). The purified xylanase had high specificity to beechwood, birchwood, and oat spelt xylans.

3.5 Xylanase application

Xylanase has multiple applications in Paper, Animal feed & food industry; however much emphasis has been paid for its use in paper and pulp industry. A brief description of previous work done regarding the applications of xylanase is discussed below:

Xylanase treatment improves accessibility of bleaching chemicals to the pulp and decrease diffusion resistance to outward movement of the degraded lignin fragments from cell wall. SEM of hard sheets treatment with *Streptomyces chromofuscus* show cell wall swelling softening and collapse of the cell structure (Sachs *et al.,* 1990).

Nisson *et al.* have pointed out that with the xylanases available commercially at present, a pH adjustment of the incoming pulp from pH 10–11 to 6–8 is necessary for its optimal activity. From an industrial point of view, it is simple to adjust the pH but difficult and expensive to control temperature due to the cost of cooling. The ideal solution therefore would be to use enzymes with higher pH and temperature stability, which will make the large-scale operations more simple and cost effective. It is thus obvious that the focus of future developments will be on identifying xylanases with higher thermostability at high alkaline pH, and developing process technologies for commercial-scale manufacture of such enzymes. In this context, alkalophilic and alkalothermophilic *Bacillus* strains as well as xylanolytic thermophilic bacteria, viz. Dictyoglomus sp. may possess the right combination of gene pools which could be gainfully employed in future for developing the ideal strains suited for pulp biotechnology.

In commercial bread production, several strategies are employed to extend bread freshness such as formulation modifications, variation of production parameters and use of various production methods. The mostly used

shortening and enzymes. They interact with each other and it is difficult to estimate their specific effects on bread texture (Zobel and Kulp, 1996). Different enzymes are currently being used in bread making process for improving dough handling, freshness and shelf life (Haros *et al.,* 2002).

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 digestion (Timothy D *et al*, 2005)

Treatment with xylanases can improve the chemical extraction of lignin from pulp. Which would lead to a significant reduction in the amount of chemicals required for bleaching and, hence, in the levels of toxic chlorine compounds released into the environment (H. M. Rifaat *et al*, 2005).

Other applications of xylanases include improvement of silage by 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.

MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1. Sample Collection and Processing

Soil samples were collected using sterile spatula & air tight zip pouches from Polavakalipalayam, Gobichettipalayam taluk of Erode district, India. Soil sample was crushed, mixed thoroughly and sieved to get rid of large debris, and the sieved soil is used for isolation of *Bacillus* sp.

4.2. Bacterial Isolation

Soil samples of 1 g were suspended in 100 ml sterile distilled water then kept in a vortex mixture with intermittent vortexing for 5min. This suspension was allowed to settle then serially diluted upto 10^{-7}. 0.1ml of the diluted samples from 10^{-6} and 10^{-7} dilutions was plated on sterile Akiba-Horikoshi Medium agar (AHMA) using L-shaped glass rod and the inoculated plates were kept in an incubation chamber at 40°C ± 1°C for 2 days. Plated dilutions that gave 30 colonies were chosen for further isolation. Repeated streaking on AHMA plates gave purified bacterial colonies that showed different morphological appearance.

Those isolated organisms from the preliminary screening were cultured in Akiba-Horikoshi liquid media at pH 10 in Erlenmeyer flasks. After incubation on a rotary shaker (40°C, 130 rpm) for 3 days, the culture broth was centrifuged (10,000 rpm for 20 min) and the supernatant was collected for enzyme assay. Its ability to produce xylanase enzyme was further confirmed by the formation of orange coloured digestion halos on birchwood xylan agar plates when treated with Congo red and washed with 1 M NaCl. The strain *B.brevis*, which was isolated from soil collected from Polavakalipalayam dominated, zone and showed highest production of xylanase. The strain was maintained on Akiba

4.3. Preparation of inoculum

The medium for inoculation was prepared; pH 10 was maintained at 40°C and sterilized by autoclaving. The high yielding xylanase culture from the AHMA plate was transferred to the inoculation medium in 250 ml conical flask by using inoculation loop under aseptic conditions. The inoculated medium was incubated at 40°C in an orbital shaker at 130 rpm for 3 days.

4.4. Enzyme production

After 72 hours of incubation, 5% of the inoculum was added to each fermentation flask (250 ml) for xylanase synthesis. The optimization of various culture conditions like pH, temperature of incubation, carbon sources & their concentration level and period of fermentation was carried out during the study.

4.5. ENZYME ASSAY

4.5.1. Buffer Preparation

0.1 M Tris-HCl buffer was used. Preparation procedure is given in Appendix

4.5.2. Crude Enzyme

After specific interval of incubation, the biomass from the experimental flasks was filtered through Whatman filter paper No.1. The filtrate was centrifuged at 10,000rpm for 15 minutes at 10°C in the centrifuge. The supernatant thus was used as a source of enzyme and stored at refrigerated temperature in sterilized glass bottles.

4.5.3. 1% Xylan Solution

1 g of xylan Birchwood xylan, (Sigma-Aldrich Co., USA), was melted

and filtered using a filter paper. The filtrate is then used as substrate and stored at 4°C in a refrigerator.

4.5.4. DNS Solution

Different ingredients used for the preparation of DNS solution are given in Appendix. After dissolving the above ingredients, the solution was filtered through ordinary filter paper and stored at room temperature in an amber colored bottle to avoid photo oxidation.

4.5.5. Xylanase Assay Procedure

Xylanase assay was carried out using 2ml of buffer, 1 ml of xylan solution and 1 ml of enzyme, the mixture was incubated at 60°C for 15 min. Then, 1ml of DNS was added. The tubes were kept in a boiling water bath exactly for 5min and the optical density was measured at 540 nm and cooled in ice water. Enzyme activity was expressed as IU/mL.

4.5.6. Enzyme activity

The xylanase hydrolyzes the polymer xylan into the xylose monomers. The free xylose units produced as a result of xylanase activity; react with 3-5 dinitrosalicylic acid (DNS) reagent and form a colored complex that is measured by spectrophotometer at wavelength 540 nm. Greater the amount of xylose produced, darker will be the color of the enzyme-xylose complex and more will be light absorbed. The optical density was measured at 540 nm. One unit of xylanase activity was described as the amount of enzyme producing 1μmol of reducing sugar in 1mL medium in 1 min under standard test conditions.

4.5.7. Standard curve of xylose

Seven different concentrations (0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 mg/mL) of xylose were prepared in distilled water. Two ml of the each

also prepared (2.0 ml each of distilled water and DNS solution). Absorbance was determined at 540nm and a graph was drawn by plotting absorbance against concentration.

4.5.8. Estimation of protein

Protein content was estimated by method of Lowry *et al.* (1951) by comparing with BSA (Bovine Serum Albumin, Himedia, Mumbai, India) standard curve.

4.6 OPTIMIZATION OF PRODUCTION PARAMETERS

4.6.1 Production of Xylanase using various carbon sources

Effect of various carbon sources on the xylanase production was assessed by culturing the isolate in the Akiba-Horikoshi medium (pH 9.0) at 40°C. Either of xylan, xylose, arabionse, glucose, sucrose, Lactose, Maltose, Raffinose and Ribose was used as carbon source (2 %, w/v) individually in liquid medium, while other carbon sources such as rice bran, rice husk, wheat bran, bagassae were used in the SSF culture medium. After 72 h of the culture growth xylanase activity was estimated by the procedure as described above (4.5.5). Further studies were also carried out to find the optimum concentration of the best carbon source from the above studies.

4.6.2. Production of Xylanase using various nitrogen sources

Different nitrogen sources (2%, w/v) such as ammonium nitrate, ammonium sulphate, beef extract, casein, meat extract, potassium nitrate, urea and yeast extract were tested for xylanase production. Further studies were also carried out to find the optimum concentration of the best nitrogen source from the above studies. The enzyme activity was determined by the procedure as

4.6.3. Effect of pH

Xylanase biosynthesis was carried out at different pH values (6, 7, 8, 9, 10, 11 & 12) using mentioned carbon and nitrogen sources at optimum concentration to find out the optimum pH level for enzyme production.

4.6.4. Effect of Temperature

For optimization of temperature, the production of xylanase was performed at different temperatures (25, 30, 35, 40 and 45°C).

4.6.5. Effect of Incubation time

To find out the optimum time required for maximum xylanase activity, samples were harvested at different time intervals i.e. 24, 48, 72 and 96 hours.

4.6.6. Large scale production of xylanase

Based on the results of optimized parameters like fermentation time, incubation temperature, media pH, carbon source, nitrogen source and its concentration; the xylanase was produced at large scale for further studies including purification, characterization and application in paper and pulp industry.

4.7 PURIFICATION

4.7.1. Ammonium sulphate precipitation

The crude enzyme was purified from the culture supernatant fluid using ammonium sulphate. For the purpose, 35% and 75% of ammonium sulphate saturation were used for the precipitation of enzymes. The respective levels were mixed to a volume of crude enzyme filtrate and kept at 4°C for one to two hours with continuous stirring. Then the mixture was kept overnight. The precipitates

pellets were resuspended in Tris-HCl buffer (50mM, pH 9.0) and dialyzed at 4°C.

4.7.2. Dialysis

After precipitation, the ammonium sulphate present in the enzyme solution was removed by subjecting the solution to dialysis in Tris-HCl buffer (50mM, pH 9.0) at 4°C with intermittent change in buffer for every 12 hours. The content of dialysis bag was centrifuged to remove precipitates, if any. The dialyzed sample was then collected and analyzed for xylanase activity. The optimum xylanase activity at a specific concentration of ammonium sulphate reflects the best concentration to attain maximum enzyme recovery.

4.7.3. Ultra filtration

The dialyzed ammonium sulphate precipitated enzyme with optimum xylanase activity (35% saturation) was concentrated using 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.

4.7.4. Ion exchange chromatography

4.7.4.1. Regeneration of the DEAE-Sephadex column

DEAE-Sephadex column (2.3 x 12.2cm) was regenerated by washing with 250ml of Tris-HCI buffer (0.1M, pH 9.0) containing 0.5M NaCl followed by passing 300ml of sodium acetate buffer (0.1M, pH 4.5) containing 0.5 NaC1. The regenerated DEAE-Sephadex was equilibrated with 400ml of Tris-HCI buffer (25mM, pH9.0). DEAE-Sephadex was kept at 4°C.

4.7.4.2. DEAE-Sephadex chromatography

The Ultra filtered enzyme was loaded on to a DEAE-Sephadex column (2.3 x 12.2 cm) equilibrated with the same buffer. The enzyme bound to DEAE-

collector. All the fractions were checked for protein (A_{280}) and xylanase activity (A540). The fractions having maximum activity were pooled, dialyzed and lyophilized.

4.7.5. Molecular weight determination

The molecular mass & purity of the purified xylanase was estimated by SDS PAGE electrophoresis. SDS-PAGE (12%) was performed as described by Laemmli using medium range (14.3 to 97.4 kDa) molecular weight markers (Bangalore Genei Pvt., India). Proteins were visualized by staining with Coomassie brilliant blue. Non-denaturing PAGE (Native PAGE) was also carried out without SDS.

4.7.6. Activity staining

Activity staining for xylanase was carried out using Native PAGE electrophoresis. After electrophoresis, the gel with separated proteins was washed using 100mM Tris-HCl buffer (pH 9.0). Washed gel was then laid over a pre-cast 0.5% (w/v) agar gel containing 0.5% (w/v) birchwood xylan in 100mM Tris-HCl buffer (pH 9.0). Agar plates were incubated for 10 min at 40°C temperature. The polyacrylamide gel was removed while the agar gel was immersed in 5.0% (w/v) Congo red dye for 30 min and washed with 1.0% (w/v) NaCl to visualize zones of clearance corresponding to xylanase activity.

4.8. CHARACTERIZATION

Characterization of xylanase was performed following the method described by Annamalai *et al.* (2009).

4.8.1. Effect of pH on xylanase activity and stability

The relative xylanase activity using 1% (w/v) birchwood xylan was determined at various pH. The pH range used varied from 4 to 11. Four different

Glycine-NaOH buffer was used for pH from 9-11. pH stability was determined by incubating the enzyme at pH 8.0, 9.0 and 10.0 for 60 min and the residual activity was found for every 30 min.

4.8.2. Effect of Temperature on xylanase activity and stability

The optimal temperature for the xylanase enzyme was obtained by assaying the enzyme activity at different temperatures (10°C to 80°C) using a water bath. At each temperature, the enzyme was incubated for 5 minutes and then assayed. In order to assess the stability, the enzyme solution (5 ml) was incubated at 50 °C, 60 °C, and 70°C for 60min using a water bath maintained at the respective temperature. Sub-Samples were removed at definite time intervals over the period of incubation. The residual enzyme activity was measured following the procedure described above.

4.8.3. Effect of Metal ion concentration

Effect of metal ion on enzyme activity was determined by incubating the enzyme with different metals (1mM and 10mM) for 1 h at 60°C temperature. Residual activity was measured using enzyme assay described above. The activity of enzyme without any metal assayed was considered as control and the activity was taken as 100%.

RESULTS AND DISCUSSION

5. RESULTS AND DISCUSSION

The aim of present project was to produce and purify thermo-alkali stable xylanase enzyme using indigenous agricultural wastes/by products like wheat bran, corn cobs and sugar cane bagasse as carbon source for the bacteria *Bacillus brevis* to carry out the submerged fermentation. The culture conditions were optimized to achieve highest xylanase activity. Then xylanase was subjected to purification and characterization for optimum efficiency. Therefore, the purified xylanase was investigated for efficacy studies in pulp bleaching. The findings of the above mentioned aspects are discussed below in detail.

5.1. Culture and growth conditions

The culture was isolated locally from a sediment sample collected from the Polavakalipalayam, Gobichettipalayam taluk of Erode district, India. Culture showed good growth at 40 °C ± 1 °C in Akiba-Horikoshi medium of pH 10 supplemented with 0.5 % birchwood xylan. Its ability to produce xylanase enzyme was further confirmed when it formed orange digestion halos on birchwood xylan plates, when treated with congo red and washed with 1M NaCl.

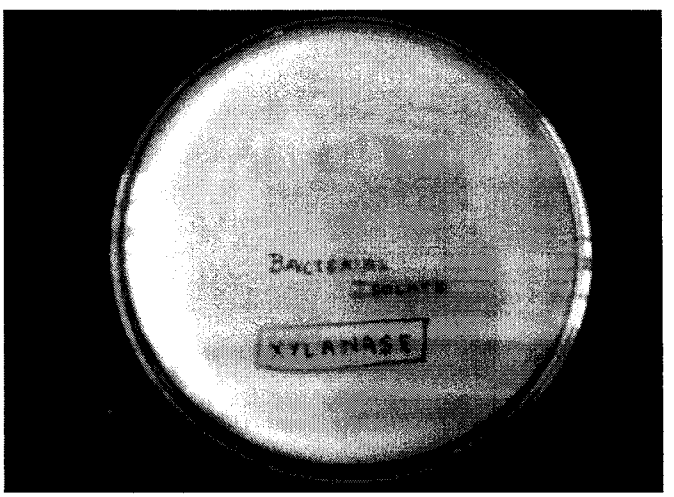


Figure 5.1.1: Bacteria isolated on Akiba-Horikoshi medium containing agar plate

5.2. Production

Bacillus brevis was used for the xylanase biosynthesis and various conditions including incubation temperature, pH of medium, incubation time, inoculum size types and concentration of carbon & nitrogen sources were optimized.

5.2.1. Effect of carbon sources on xylanase activity

Xylanase was synthesized by *Bacillus brevis* using various sugars and agricultural wastes as carbon source (2%).The maximum xylanase production was observed on arabinose followed by ribose and xylose. Whereas the xylanase production using other agricultural residues, it was seen that wheat straw gives better yield compared to others. There are limited numbers of studies on this subject in literature, among which most of them were carried out using agro wastes. In a study carried out with *Bacillus* sp., it was concluded that arabinose showed induction effects with concentration of 0.5% and 1.0% in the presence of wheat bran and corn cob as carbon source respectively (Gupta *et al.*, 2008). In my experimental results its revealed that arabinose (2% w/v) along with wheat straw (6% w/v) as carbon source yields maximum production efficiency of xylanase. Since xylan is costly for large-scale production of xylanases, lignocellulosic materials can be used as cost-effective substrates for xylanase production (Haltrich *et al.*, 1996; Beg *et al.*, 2000)

SOURCE (2%) (w/v)	XYLANASE ACTIVITY/ (U/mL)
Control (*Glucose*)	19
Arabinose	44.5
Fructose	11
Guargum	15
Lactose	22.5
Maltose	23.5
Raffinose	22
Ribose	40.5
Starch	16.5
Sucrose	14

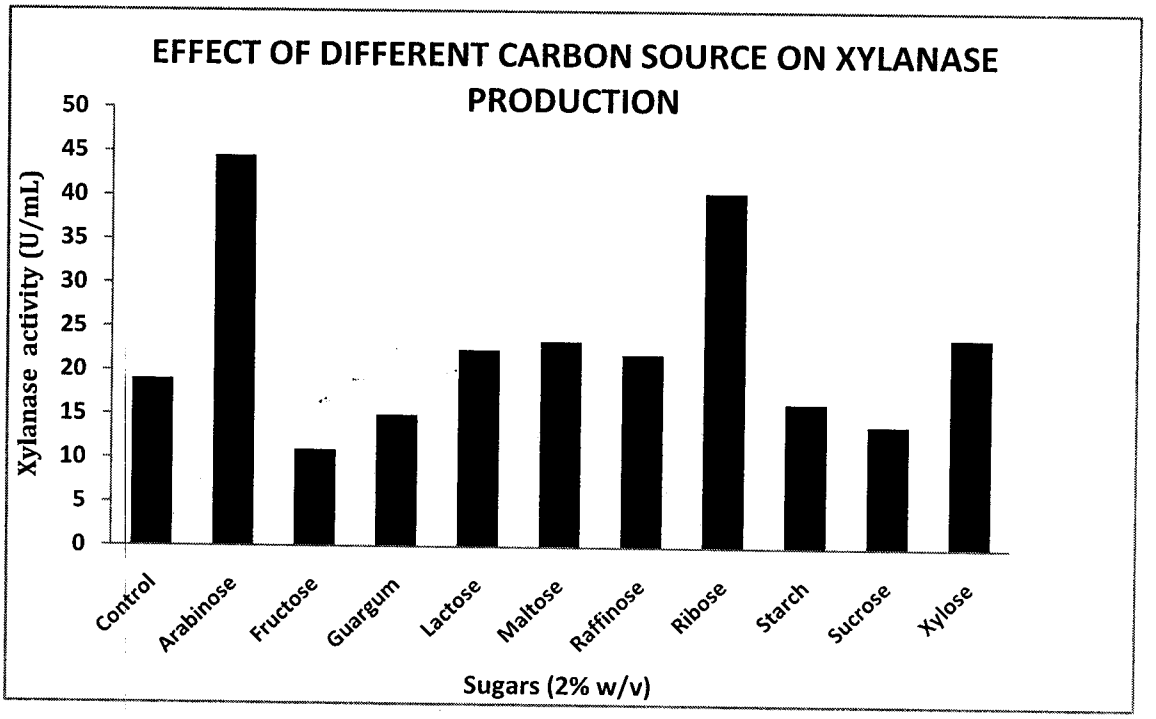


Figure 5.2.1.1: Effect of different carbon source on xylanase production

ARABINOSE (%) (w/v)	XYLANASE ACTIVITY U/mL
2	50.8
4	43.3
6	39.3
8	34.8
10	32.3

Table 5.2.1.2: Varied concentrations of arabinose influencing xylanase production

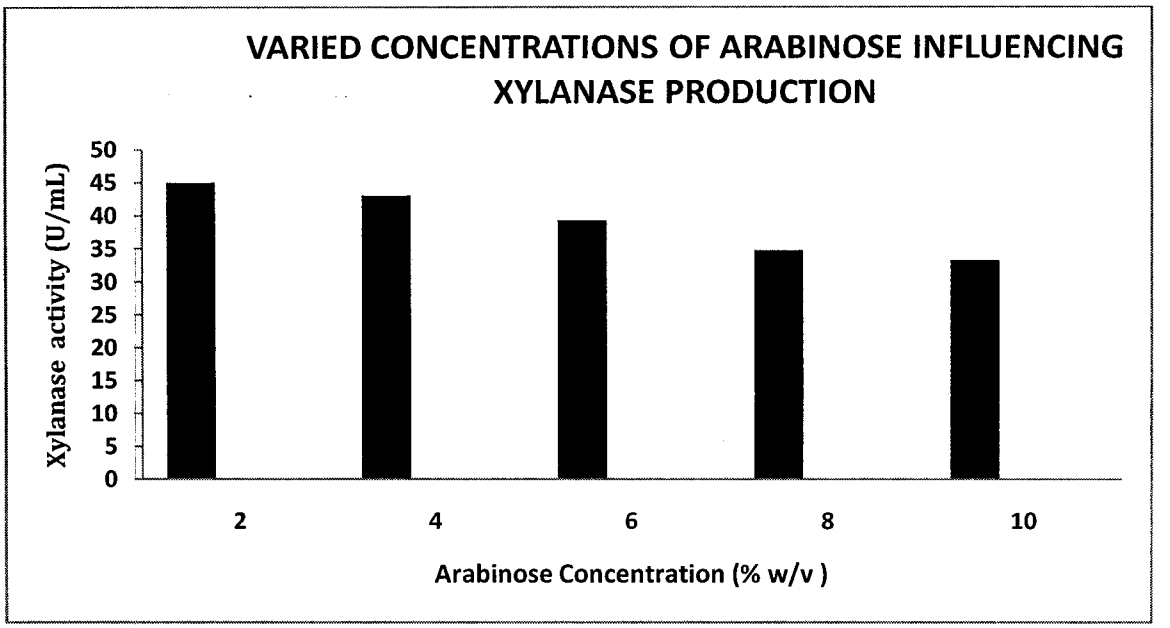


Figure 5.2.1.2: Varied concentrations of arabinose influencing xylanase production

CARBON SOURCE (2% w/v)	XYLANASE ACTIVITY (U/mL)
Rice bran	9.6
Eichornia stem Wheat bran	11.7
Lemon peel	5.7
Rice Bran	16.44
Sugarcane Bagasse	21
Wheat straw	28
Control	40

Table 5.2.1.3: Effect of different carbon source on xylanase production

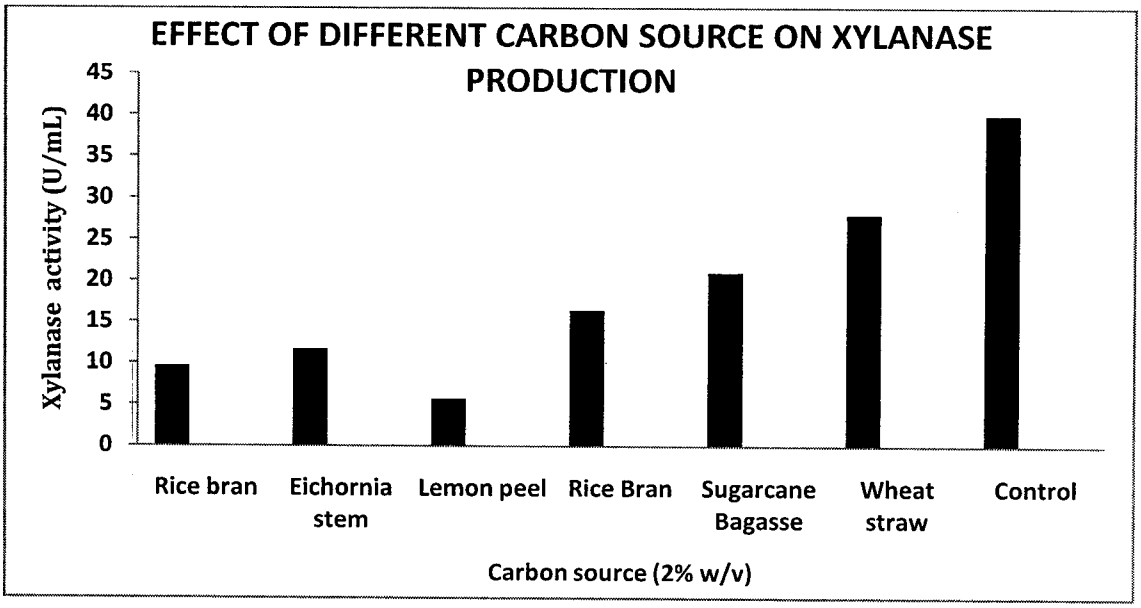


Figure 5.2.1.3: Effect of different carbon source on xylanase production

WHEAT STRAW (% w/v)	XYLANASE ACTIVITY U/mL
2	50.8
4	43.3
6	39.3
8	34.8
10	32.3

Table 5.2.1.4: Varied concentrations of wheat straw influencing xylanase production

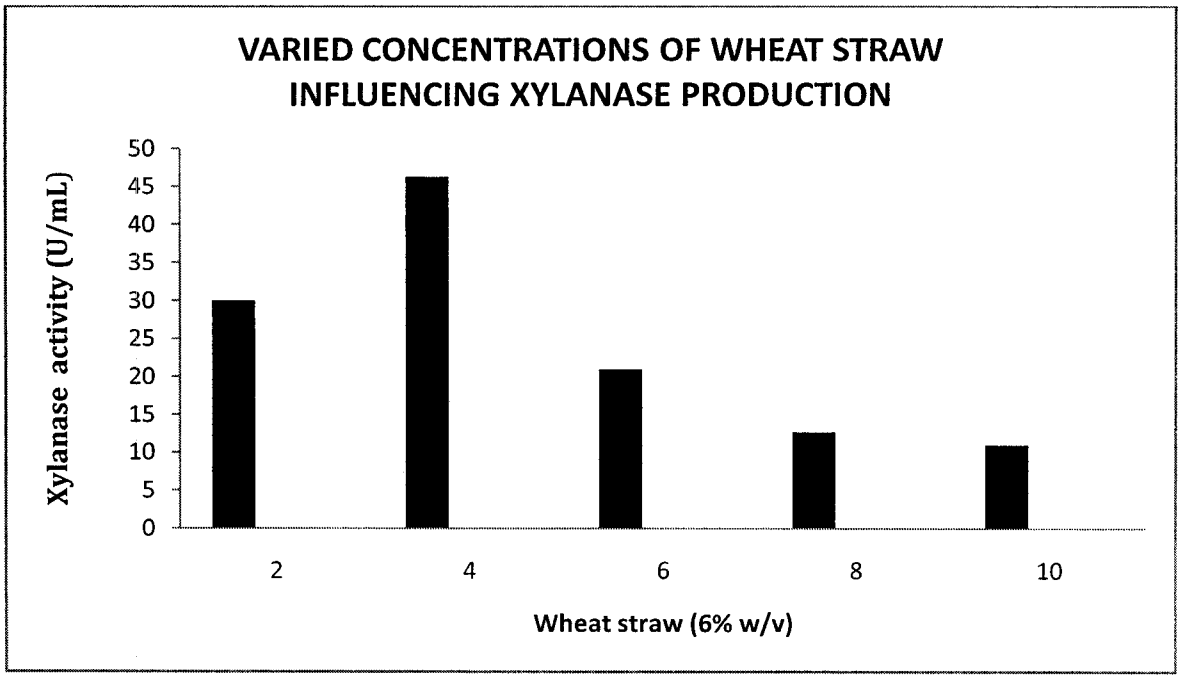


Figure 5.2.1.4: Varied concentrations of wheat straw influencing xylanase production

5.2.2. Effect of Nitrogen sources on xylanase activity

In this analysis, the effect of nitrogen source was tested by replacing Yeast extract in the medium with other organic and inorganic nitrogen sources. (At 2%, w/v) such as ammonium nitrate, ammonium sulphate, beef extract, casein, meat extract, potassium nitrate and urea. *Bacillus* brevis responded differently to all inorganic and organic sources. Organic nitrogen sources were better than the inorganic ones. This may be attributed to the fact that the organism was isolated from soil where organic nitrogen is used as the nitrogen fertilizer, so it assimilates organic nitrogen sources more efficiently as compared to inorganic ones. Out of organic nitrogen sources, Urea was the best nitrogen source followed by casein and incase of inorganic nitrogen source potassium nitrate showed better enzyme productivity. The results showed the highest yield of xylanase was 51.25 U/mL with 4% (w/v) urea. Moreover, in a study aimed at optimization of cultural and nutritional parameters for enhanced production of

SOURCE (2%) (w/v)	XYLANASE ACTIVITY/(U/mL)
Control	23.2
Ammonium chloride	5
Ammonium nitrate	4
Ammonium sulphate	4
Beef extract	16.5
Casein	41.5
Meat extract	15.5
Potassium nitrate	36.5
Urea	40.5
Yeast extract	25

Table 5.2.2.1: Effect of different nitrogen sources on xylanase production

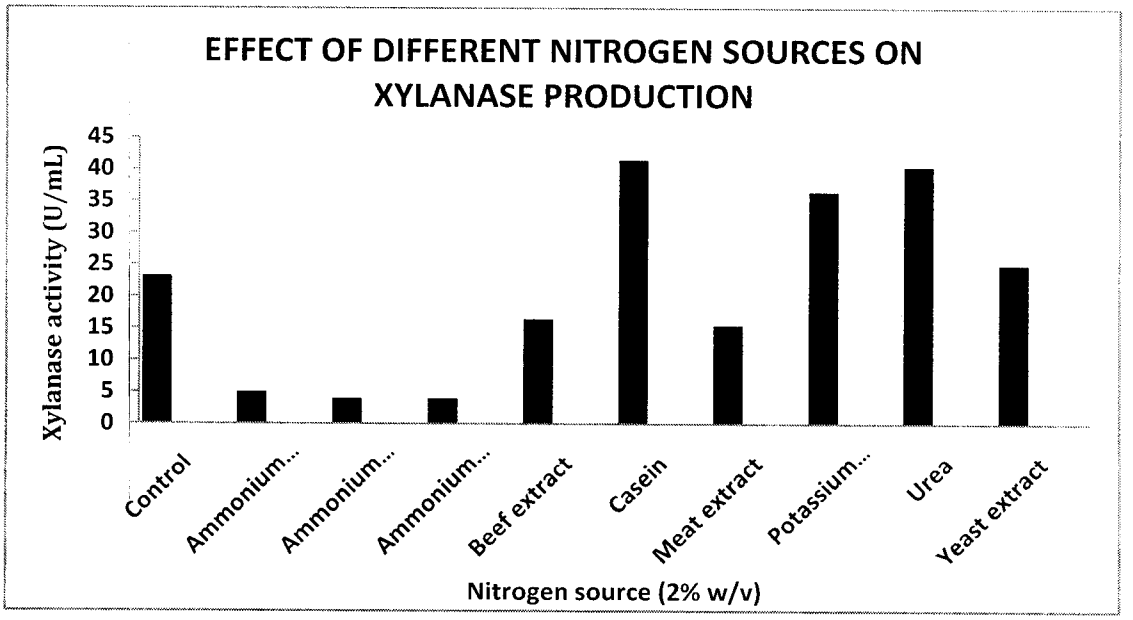


Figure 5.2.2.1: Effect of different nitrogen sources on xylanase production

UREA (%) (w/v)	XYLANASE ACTIVITY U/mL
2	49.75
4	51.25
6	43.25
8	36.6
10	28.4

Table 5.2.2.2: Varied concentrations of urea influencing xylanase production

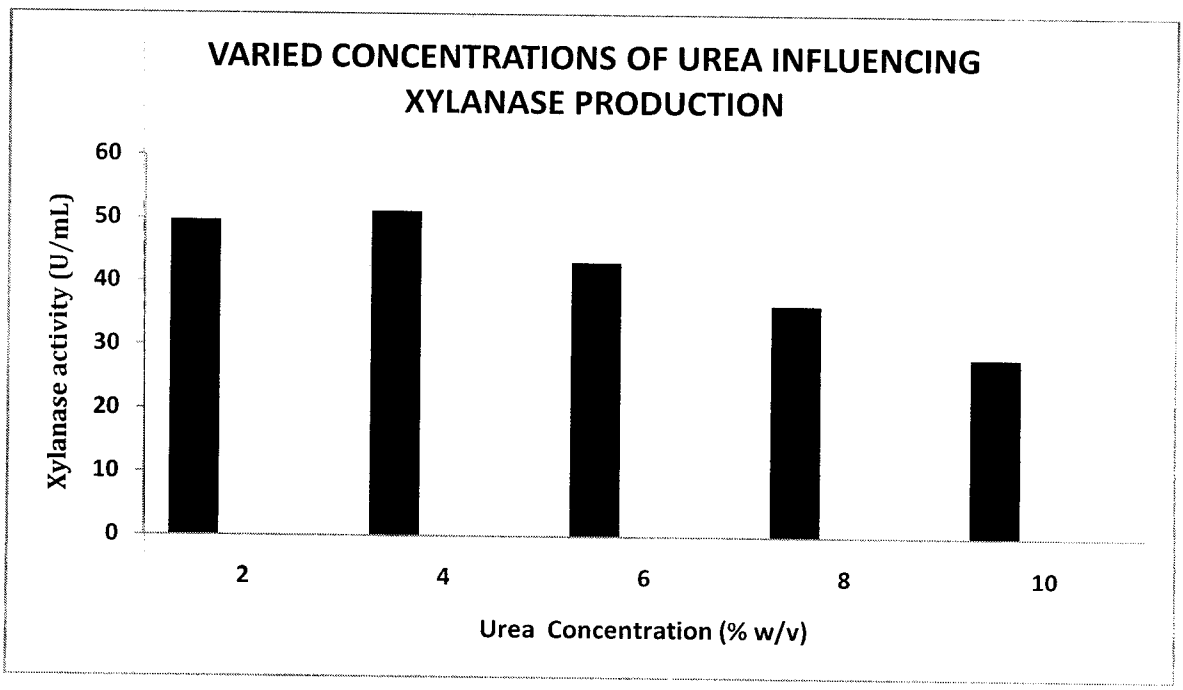


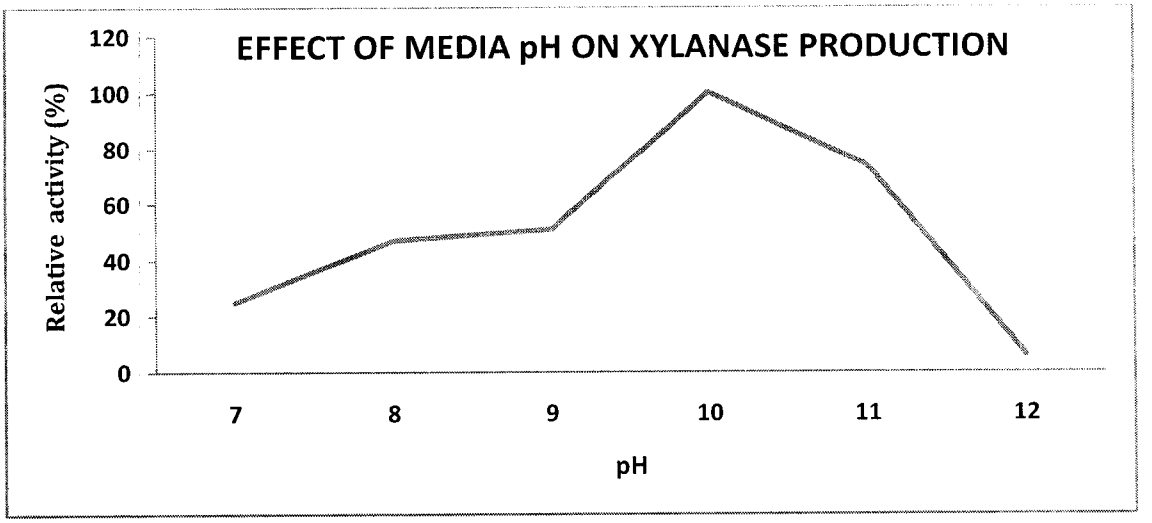
Figure 5.2.2.2: Varied concentrations of urea influencing xylanase production

5.2.3. Effect of medium pH on xylanase activity

Xylanase production by this bacterium was greatly affected by the media pH; with pH 10 the extracellular xylanase productivity was high. Till date only few bacterial xylanase was reported. Sa-Pereira *et al* (2007) reported that xylanase from *Bacillus* subtilis, when grown on SSF using oat spelt xylan, showed maximum activity at pH 6.0. Similarly xylanase isolated from B. coagulans BL69 grown on soybean residue showed a pH optimum of 7.0 (Heck *et al*, 2005)

pH	RELATIVE ACTIVITY (%)
7	25
8	47
9	51
10	100
11	74
12	6

Table 5.2.3.1: Effect of media pH on xylanase production

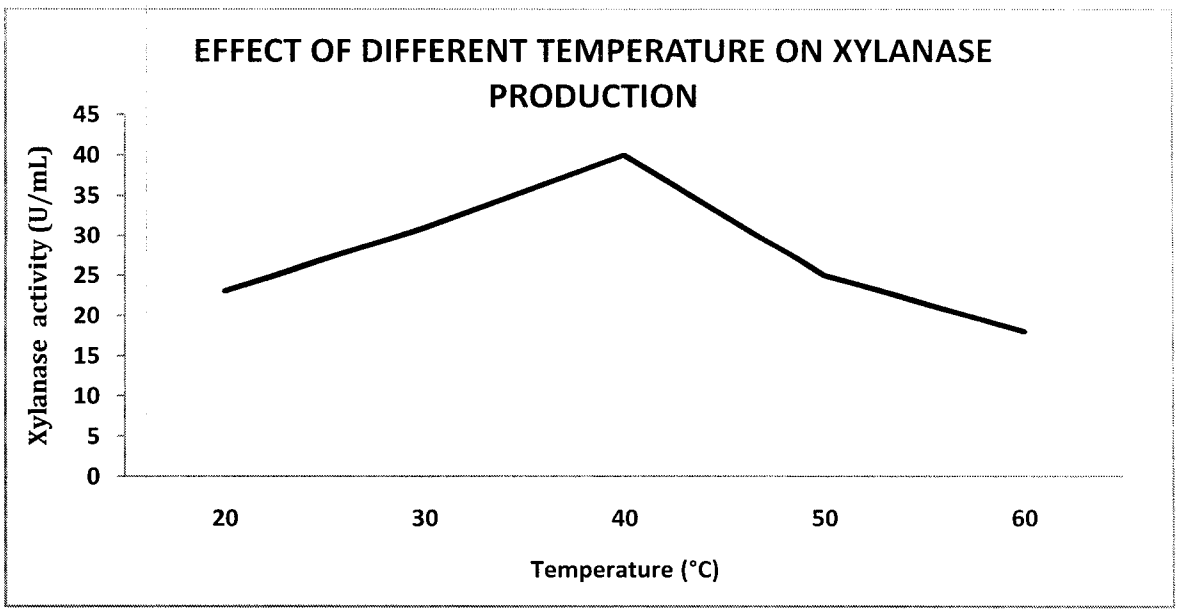


5.2.4. Effect of medium Temperature on xylanase activity

The highest xylanase productivity was recorded at 40°C with 130 rpm. Adriana *et al.* reported that highest xylanase activity was verified at 25°C, while the maximum value of specific activity was obtained at 30°C, respectively by *P.sclerotiorum*. Limited number of studies on this subject, among which most of them were carried out using fungi.

TEMPERATURE (°C)	XYLANASE ACTIVITY (U/mL)
20	23
30	31
40	40
50	25
60	18

Table 5.2.4.1: Effect of different temperature on xylanase production

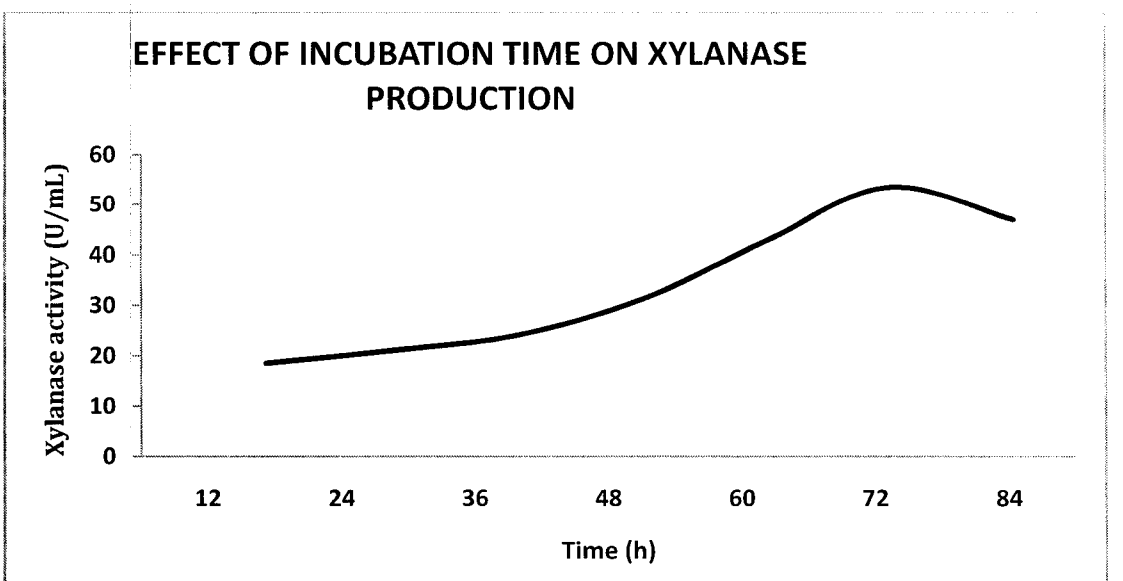


5.2.5. Effect of incubation time on xylanase production:

The maximum xylanase production was obtained at 72h at 40°C with 130 rpm. In a study with *Bacillus subtilis*producing xylanase the highest activity was obtained after 14 h of fermentation at 50°C (Saleem *et al.*, 2002). Li *et al*, reported that for *A.awamori* the cultivation time required to achieve maximum xylanase productivity is at 103.7 h

TIME (h)	XYLANASE ACTIVITY U/mL
12	18.5
24	21
36	24
48	31
60	42.5
72	53.5
84	47

Table 5.2.5.1: Effect of incubation time on xylanase activity



5.3. Purification

The enzyme was isolated and purified; all purification steps were carried out at 4°C. The extracellular xylanase was purified to homogeneity by a four step procedure from the culture filtrate when grown on wheat bran. A summary of purification procedures is as follows and finally concluded with a purification table.

5.3.1. Purification by precipitation

By ammonium sulfate precipitation method, specific activity increased upon adding 35% ammonium sulfate and increased further after dialyzed. Recovery of 82% was obtained as shown in Table. Precipitation by acetone and ethanol were also conducted but both methods gave worse results in comparison to precipitation by ammonium sulfate (data not shown).

5.3.2. Purification by ion exchange chromatography

Xylanase enzyme was produced by the culture in the wheat bran (carbon source) containing optimized medium. It was further purified by a four-step procedure (Ammonium sulphate precipitation, Ultra filtration, Dialysis & Ion Exchange chromatography). The enzyme was eluted as a blunt peak when passed through DEAE Sephadex chromatographic column. The enzyme was further loaded onto the anion exchange columns and eluted fraction were collected and analyzed. Two major peak and three minor protein peaks were seen. Each fraction was assayed for xylanase activity where the maximum xylanase activity was recorded, of these one major protein peak and one minor peak showed xylanase activity at fraction 22 & 27respectively. The fractions showing higher activity alone pooled out, dialyzed and lyophilized.

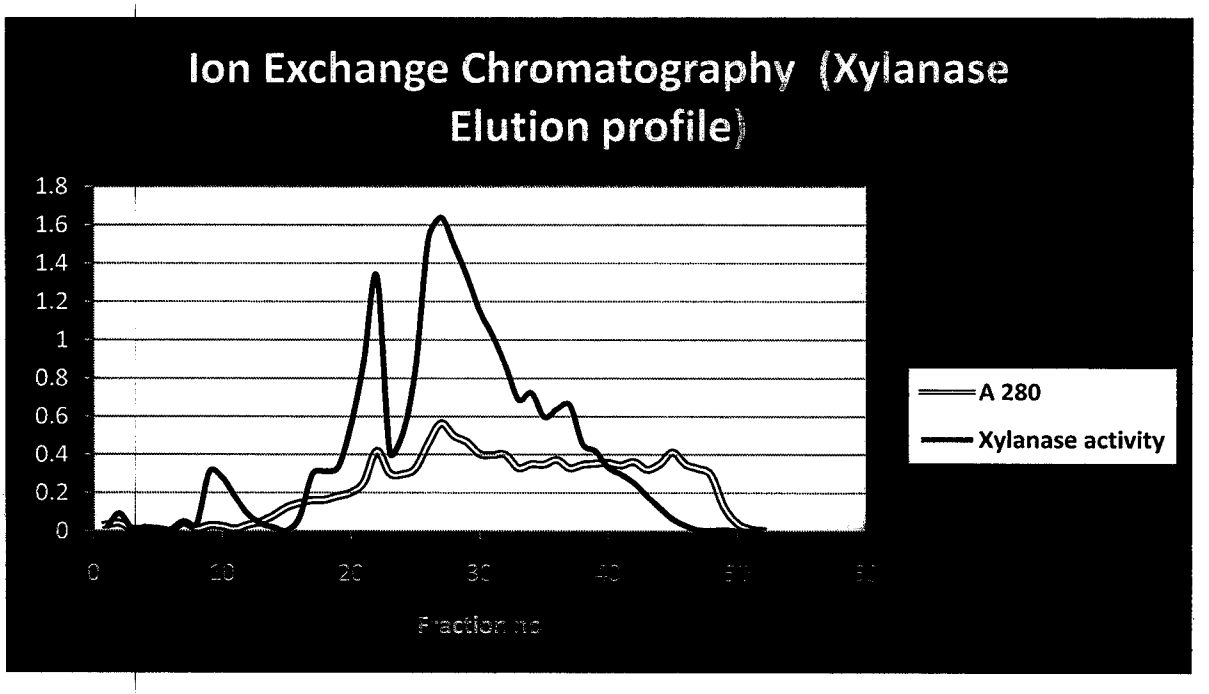
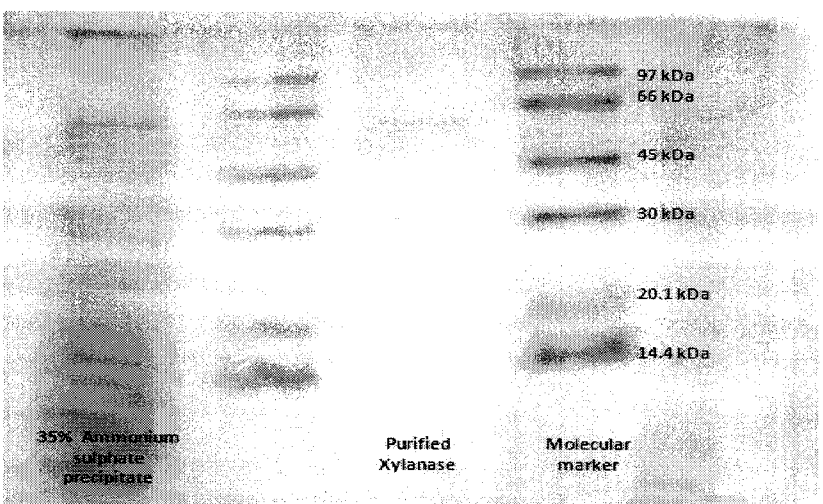


Figure 5.3.2.1: Elution profile of xylanase on DEAE-Sephadex

5.3.3. Molecular weight determination and zymogram analysis

The purified enzyme showed a single protein band on SDS-PAGE. The molecular mass of denatured xylanase was estimated from the relative mobility of protein marker adjacent to the purified sample a~60 kDa. Zymogram of xylanase enzyme showed a prominent activity band corresponding to ~60 kDa in the lane.



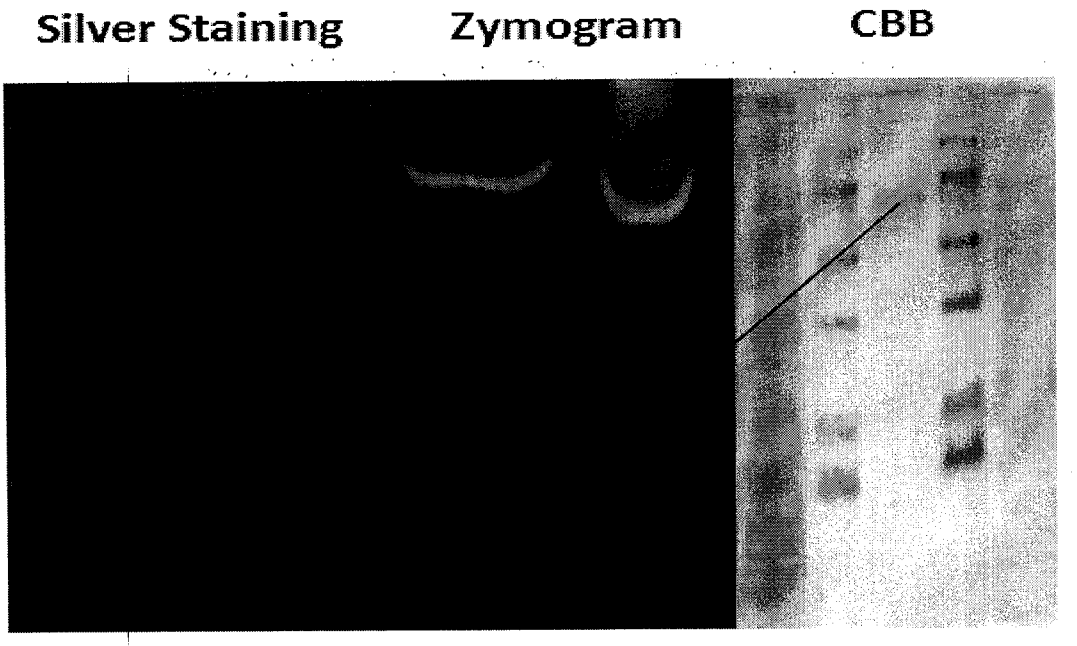


Figure 5.3.3.2: Prominent activity band corresponding to ~ 60 kDa, in Silver staining, Activity staining and Coomassie Brilliant Blue staining

PURIFICATION STEP	TOTAL ACTIVITY (U)	TOTAL PROTEIN CONTENT (mg)	SPECIFIC ACTIVITY ($U\ mg^{-1}$)	FOLD PURITY	% YEILD
CRUDE	20000	100	200	1	100
35% AMMONIUM SULPHATE SATURATION	1740	1.8	966	4.83	8.7
ION EXCHANGE CHROMATOGRAPHY	650	.19	3421	17.1	3.25

Table 5.3.3.1: Purification of Xylanase from *Bacillus brevis*

5.4. Characterization

The enzyme was isolated, purified and characterized; all characterization

xylanase purified upto ultrafiltration (partially purified xylanase) was subjected to characterization studies.

5.4.1. Effect of pH on xylanase activity and stability

The enzyme activity under the influence of different pH values indicated that the enzyme exhibited maximum relative activity at pH 9.0 (41U/ml). The graphical representation shows that with the increase in pH, the activity of the enzyme did not show a much decrease. But when the pH was decreased below pH 7.5, a steep fall in enzyme activity was observed. In case of pH stability of the enzyme, xylanase retained 83% of its original activity even after incubating the enzyme in pH 9 buffer at 50°C for 60 min. While at pH 10 and pH 8 a gradual decrease in enzyme activity was observed at end of 60 minutes. In a recent research by Jing *et al.*(2009), its reported that xylanase produced by an alkaliphilic *Bacillus pumilus* had an optimum pH of 8.0–9.0, and showed good stability after incubated at pH 9.0 for 120 min.

pH	RELATIVE ACTIVITY,%
5	13.9
5.5	18
6	34.9
6.5	47.9
7	68
7.5	81.9
8	86
8.5	86.9
9	100
9.5	95
10	91.9
10.5	90.1
11	80

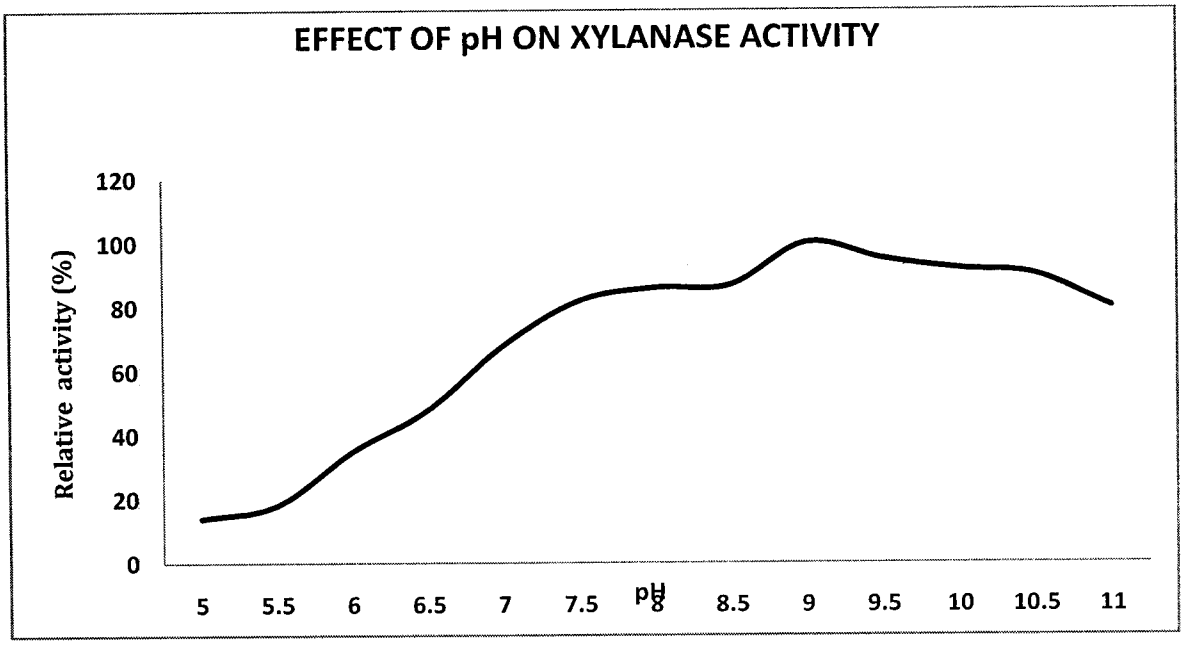


Figure 5.4.1.1: Effect of pH on xylanase activity

TIME (Min)	pH- 8 RESIDUAL ACTIVITY (%)	pH- 9 RESIDUAL ACTIVITY (%)	pH- 10 RESIDUAL ACTIVITY (%)
0	100	100	100
30	66	97	80
60	20	59	46

Table 5.4.1.2: Effect of pH on xylanase stability

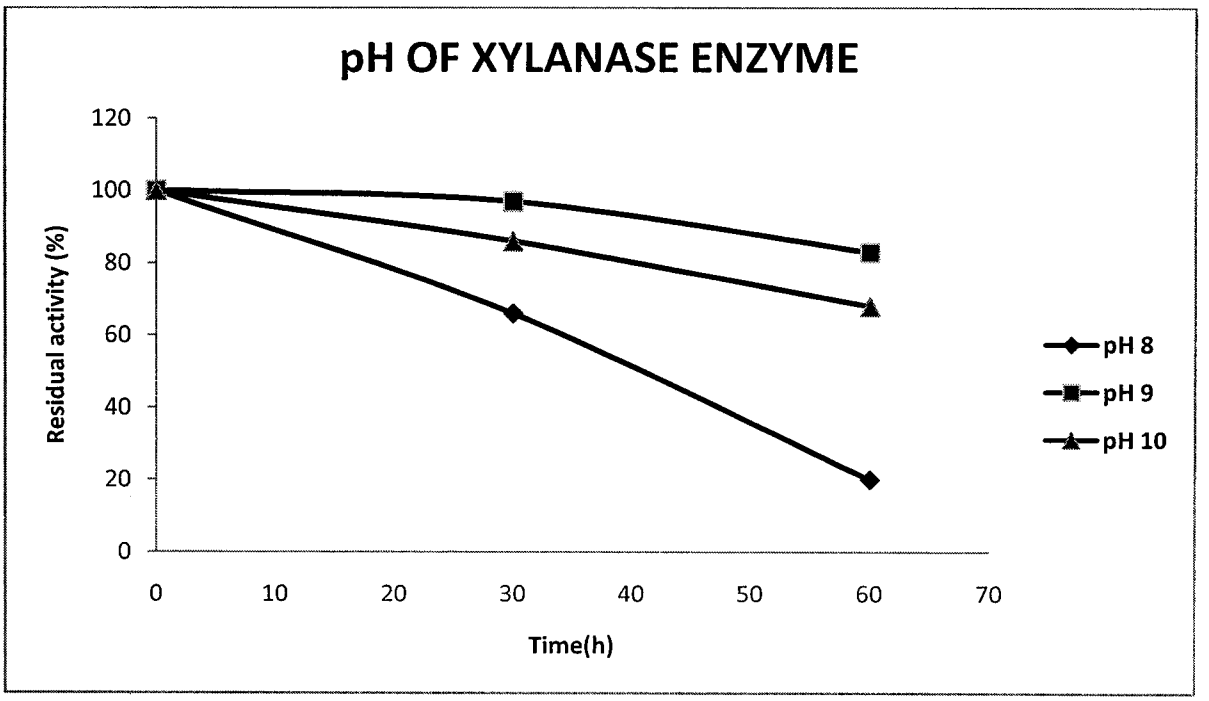


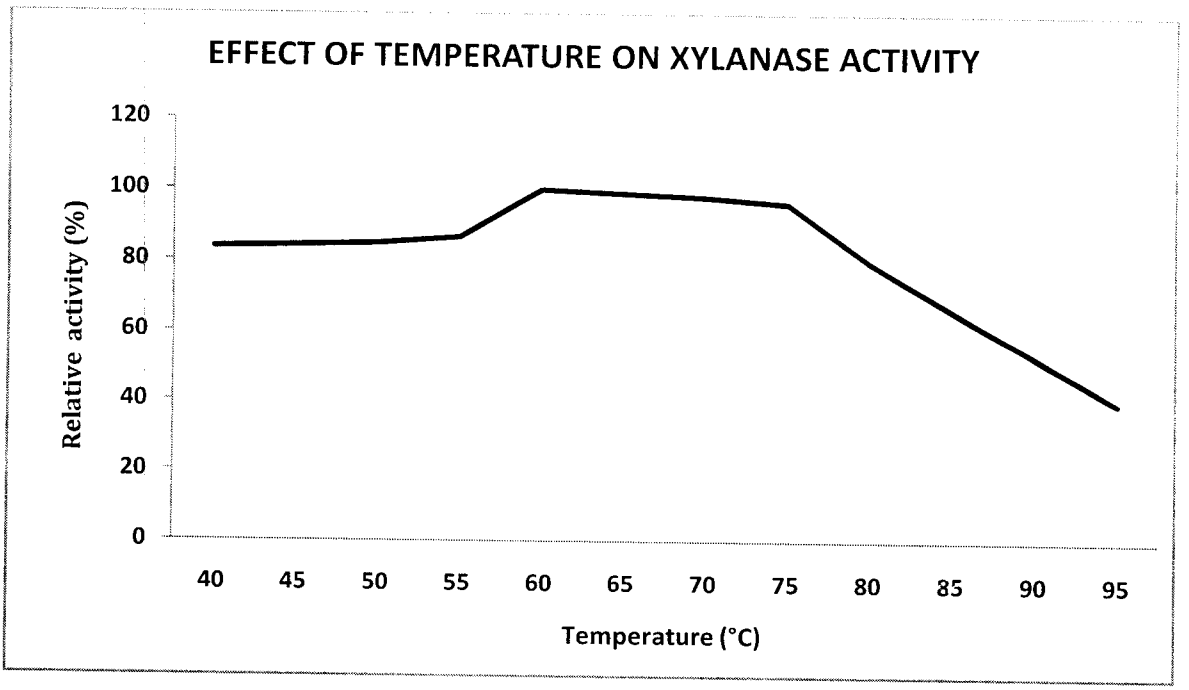
Figure 5.4.1.2: Effect of pH on xylanase stability

5.4.2. Effect of temperature on xylanase activity and stability

It is observed that when enzyme assay was performed at various temperatures, the xylanase activity increased with rise in temperature up to 60°C and exhibited maximum activity. However, further increase in temperature caused a sudden decrease in activity after 75°C. Minimum activity was observed at 100°C, the highest temperature studied in the present study. While moving on to stability the enzyme exhibited a activity of 72% and 68% at 50 and 60°C respectively. The results of current work are in close conformity to the findings of Annamalai *et al.* (2009), they reported that the purified xylanase from the bacteria *Bacillus subtilis* exhibited highest activity at 55°C. Likewise, Daniela *et al.* (2007), and Marta (2000), calculated optimum activity and stability of xylanase at 55°C and 60°C.

TEMPERATURE (°C)	RELATIVE ACTIVITY,%
40	83.7
45	84.2
50	84.8
55	86.6
60	100
65	99
70	98
75	96.2
80	79.7
85	66.3
90	52.9
95	39.8

Table 5.4.2.1: Effect of temperature on xylanase activity



TIME (Min)	RESIDUAL ACTIVITY (%) AT 50°C	RESIDUAL ACTIVITY (%) AT 60°C	RESIDUAL ACTIVITY (%) AT 70°C
0	100	100	100
30	83	82	52
60	72	68	14

Table 5.4.2.2: Effect of temperature on xylanase stability

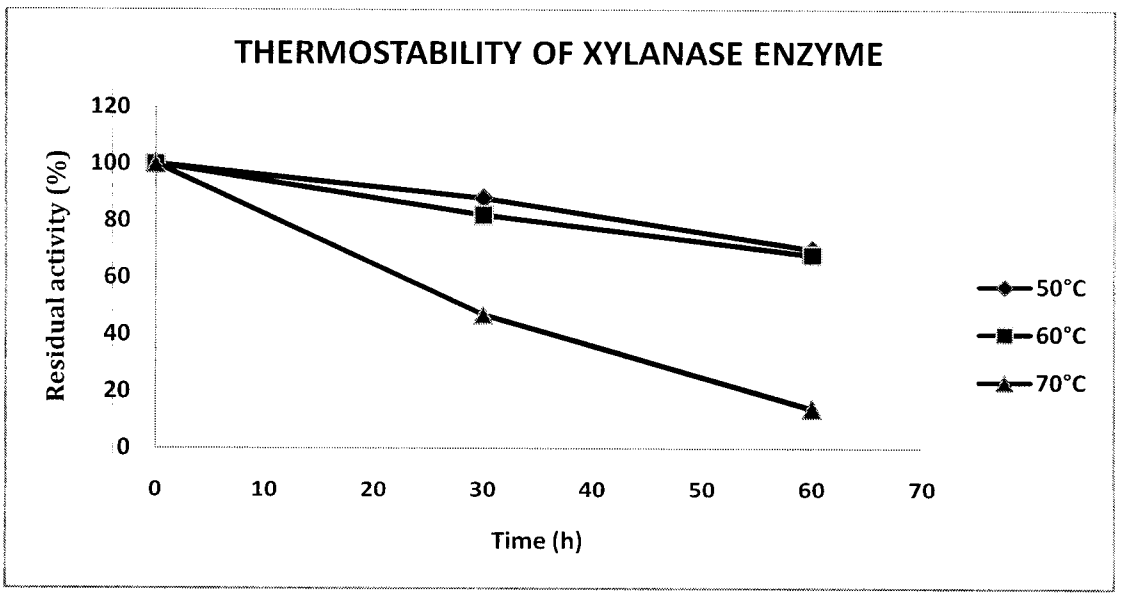


Figure 5.4.2.2: Effect of temperature on xylanase stability

5.4.3. Effect of Metal ion concentration

In the study of metal ions on enzyme activity, the enzyme activity was enhanced by $FeSO_4$ and $MgsO_4$ in both 1 and/10mM concentrations. While $CaCl_2$, $ZnSO_4$ and $MnSO_4$ showed slight inhibition on enzyme activity. In contrast $CuSO_4$, $HgCl_2$, $ZnCl_2$ showed complete / severe inhibition. . The present results were closely related to the results of Annamalai *et al.*(2009), its seen that $MgSO_4$ showed enhancing property in xylanase at both 1 and/ 10mM concentration. EDTA, showed moderate inhibition and finally $HgCl_2$ at 1o mM concentration showed complete inhibition

METAL ION	RESIDUAL ACTIVITY (%)	
	1mM	10mM
Control	100	100
$CaCl_2$	90	92
$CuSO_4$	2	0
$FeCl_2$	54	41
$FeSo_4$	118	134
$HgCl_2$	0	0
$MgSO_4$	102	126
$MnSO_4$	61	81
$ZnCl_2$	12	0
$ZnSO_4$	73	28

Table 5.4.3.1: Effect of Metal ion concentration

CONCLUSION

6. CONCLUSION

The characteristics of the culture are similar to those described for Bacillus sp in the Bergey's Manual of Systematic Bacteriology. Based on these characteristics the culture was tentatively identified as *Bacillus sp,* and was deposited with the Tamilnadu Agriculture University, Coimbatore, India. *Bacillus brevis* used in the present study is novel, as to the best of my knowledge; there are no published reports on the production of xylanase enzyme by this culture.

Furthermore, the outstanding feature of this culture is that it produced reasonably good amounts of alkalophilic and thermophilic xylanase. The amount of enzyme produced and the time required for the maximum production of enzyme was differed with varied agriculture residues, sugars as carbon source and various organic and inorganic substances as nitrogen sources. The enzyme production on utilizing 6% wheat straw, 2% arabinose, 4% urea at 40°C and pH 10 with shaking at 130 rpm showed highest xylanase productivity of 53.5U/ml was observed.

The extracellular xylanase was purified to homogeneity by a four step procedure (Ammonium sulphate precipitation, Ultra filtration, Dialysis & Ion Exchange chromatography) from the culture filtrate when grown on wheat bran. Finally a 17.1 fold purified xylanase with a molecular weight of ~60 kDa was obtained.

Xylanase enzyme from *Bacillus brevis* showed highest activity at high pH 9.0 and temperature 60°C. The enzyme was stable at 50–60°C at pH 9 and pH 8–10 at 60°C for 1hour. Metal ions $FeSO_4$ and $MgSO_4$ increased the xylanase activity by twofold, in contrast $CuSO_4$, $HgCl_2$, $ZnCl_2$ showed complete/ severe inhibition of the enzyme activity when compared to the control.

In summary, Bacillus brevis produced a novel thermoalkalophilic xylanase when grown on SmF using wheat bran as a substrate. These properties make this enzyme potentially very effective for applications in paper and pulp industry.

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8. APPENDIX

8.1. Appendix -1:

8.1.1. Media Composition (Akiba Horikoshi medium):

COMPONENTS	g/l
Polypeptone	10
Yeast extract	5
KH_2PO_4	1
$MgSO_4.7H_2O$	0.2
Brich wood xylan	5
NaCl	3
Na_2Co_3	10

Note: Sodium carbonate was sterilized separately and added to the medium separately.

8.1.2. Tris-HCl Buffer:

Stock solutions:

A: 0.2M solution of Tris (hydroxymethyl) aminomethane (24.2g in 1000mL).

B: 0.2N HCl

50mL of A, 5 mL of B, diluted to a total of 200mL to attain pH 9.0.