PRODUCTION, PURIFICATION AND APPLICATION OF XYLANASE FROM BACTERIAL SPECIES ISOLATED FROM SEA WATER

A PROJECT REPORT

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BONAFIDE CERTIFICATE

Certified that this project report **"Production, Purification and Applications of xylanase from bacterial species isolated from sea water"** is the bonafide work of **R. ARTHI (REG NO : 0810204004)** and **A.V.M. EZHILARSI (REG NO : 0810204010)** who carried out the project work under my supervision.

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ABSTRACT

Hemicellulolytic microorganisms play a significant role in nature by recycling hemicellulose, one of the main components of plant polysaccharides. Xylanases (EC 3.2.1.8) catalyze the hydrolysis of xylan, the major constituent of hemicelluloses. Alkali-thermostable xylanase producing *Bacillus* sp was isolated from sea water (near Calicut). Different medium parameters for the production of xylanase were studied. Fructose (5%) and sodium nitrate (0.5%) as carbon and nitrogen source supported maximum enzyme secretion. Alkali-thermostable xylanase was partially purified by ammonium sulphate precipitation followed by anion exchange chromatography. Optimum pH and temperature for xylanase activity was found to be 7.5 and 60°C, respectively. Thermal stability and pH stability studies were also studied. Alkali-thermostable properties of xylanase from *Bacillus* sp can be exploited for its biotechnological applications in paper and pulp industry, baking industry and animal feed industry

Keywords: Alkali-thermostable xylanase, *Bacillus* sp.

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LIST OF ABBREVIATIONS

ABBREVIATIONS	EXPLANATIONS
µg	Microgram
µl	Microlitre
mg	Milligram
h	Hour
M	Molar
ml	Millilitre
l	Litre
mM	Millimolar
min	Minutes

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.

Most of the microorganisms isolated from soil or wastes or composting waste material are capable of producing a spectrum of cell wall degrading enzymes (Badhan *et al*., 2007).

Complete degradation of complex heteropolysaccharides requires the action of several main-chain and side-chain cleaving enzymes such as endoxylanases (endo-1,4β- xylanase), β-xylosidase (xylan-1,4β-xylosidase), α-glucuronidase (α-glucosiduronase), α-arabinofuranosidase (α-L- arabinofuranosidase), acetylxylan esterase (Choi *et al*., 2000) . Endo- β-1, 4 xylanases can catalyse the hydrolysis of the backbone of xylan to produce xylooligosaccharides, which in turn can be converted to xylose by β- xylosidase (Zhang *et al*., 2007).

Xylanase (E.C. 3.2.1.8) catalyzes the hydrolysis of xylopyranosyl linkages of β-1,4-xylan, the major hemicellulosic polysaccharide of hardwood. Xylanase is produced by a variety of microorganisms including bacteria , fungi , and actinomycetes. Xylan-degrading enzymes have attracted considerable application in industrial processes such as improving the digestibility of animal feed stocks, biobleaching of pulp, textile industry, production of xylo-oligosaccharides, waste-water treatment, texture improvement in bakery products, clarification of juices and wine, debarking process and bio-conversion of lignocellulosic waste into useful economical products such as ethanol, single cell protein, sugar syrups and liquid and gaseous fuels.

Members of the genus *Bacillus* produce a great variety of extracellular enzymes. Among them, xylanase has received significant industrial importance. In general, the fermentation profile of an organism is affected by nutritional and physiological factors such as carbon source, nitrogen source, additives, pH of the media and incubation temperature. Alkalistable and thermostable xylanase produced by *Bacillus sp.* find its application in biobleaching in paper and pulp industry. In the present investigation an attempt is made to partly purify the enzyme and characterize the enzyme for its application in paper and pulp industry.

Xylan is the main carbohydrate found in the hemicellulosic fraction of plant tissues and accounts for one third of all renewable organic carbon available on earth. Xylanase, the major component of an enzymatic consortium, acts in nature by depolymerizing xylan molecules into monomeric pentosan units that are used by bacterial and fungal populations as a primary carbon source. Xylanase producers have been isolated from all ecological niches where plant material is deposited, and microorganisms often contain multiple loci encoding overlapping xylanolytic functions. The numerical excess of genes and the extensive sharing of structural features within beta-glycanase families suggests that extensive gene duplication and conversion events have occurred during xylanase evolution. Hydrolysis of beta-glycosidic linkages is sponsored by a

general acid catalytic reaction common to all glycanases, whereas substrate recognition is specified by subsites that interact with adjacent glycosyl units. Under natural conditions xylanases are inducible by the products of their own action and subject to carbon catabolite repression.

Bleaching paper pulps with xylanases is the first successful commercial application for these enzymes. The recovery of cellulosic textile fibers is the next logical application and bioconversion of biomass into fuels and chemicals, remains the ultimate target. Recent developments have shown that metabolic pathways can be transferred from one organism to another and proteins can be modified to gain conformational stability, suggesting that naturally occurring systems can be custom engineered to the situation in the fermentation tank. Thus, biotechnologies developed to transform biomass into marketable products that gradually substitute materials derived from non-renewable resources are becoming commercially worthwhile.

1.1 OBJECTIVES

- To isolate and screen bacteria producing alkalistable and thermostable xylanase,
- To optimize production parameters for xylanase production, and
- To purify and characterize the xylanase enzyme.

LITERATURE REVIEW

2. REVIEW OF LITERATURE

Various organisms have different potential for the synthesis of enzymes; Bacteria are the most common source of hemicellulase like xylanases, mannanases, and glucanases. Over the years , a number of organisms including the strains of *Butyrivibrio fibrisolvens* (Sewell *et al.,*1988), *Trichosporon cutaneum* (Liu *et al.,*1998) *Aspergillus nidulans* (Pinaga *et.al.,*1994; Ganga *et al.,*1998) have been exploited for xylanase production.

Xylanase has proven useful in many ways including biobleaching of 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.

Paper and pulp, animal feed, and baking industries, with the advancement of biotechnology, has undergone a lot of improvements during the last few years. Its presence become more efficient and the expectation of industrialists become higher to achieve the best possible products in economical way and the use of enzymes has become the 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).

2.1 XYLANS

The plant cell wall is a composite material in which cellulose, hemicelluloses (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 in cell walls of plants can differ greatly depending on the origin and different structures attached to the xylan backbone. Although most of the xylan structures have branched structures, but some of the linear polysaccharides have also been isolated (De Vries and Visser, 2001) Lignin is bound to xylans by an ester linkage to 4-O-methyl-D-glucoronic acid residues. These linkages in lignocelluloses can be disrupted by using various methods that expose most of the polysaccharide components to enzymatic hydrolysis (Chang *et al*.,1980).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). These observations suggested that cellulose id protected from celluloses due to xylan and mannan (Sinner *et al.,*1979) Selective removal of xylans increases accessibility of other polysaccharides due to increased porosity which is correlated with cellulose hydrolysis in pretreated fibres.

The main component of non starch polysaccharides in wheat flour are pentosans (arabinoxyalans). They occur as minor component of wheat grains(2-35,dry basis), can be divided into soluble or water-extractable arabinoxylans and insoluble or water-unextractable arabinoxylans. They play an important role in dough rheology and bread quality (Courtin and Delcour, 2002; McCleary,1986).

2.2 XYLANASE

Xylanase are generally single chain glycoproteins, ranging from 6-80 kDa, active between pH 4-6.5, at 40-60°C. Xylanase from different sources differ in their requirements for temperature, pH, etc., for optimum functioning. The Complete hydrolysis of xylan into its constituent monosaccharides requires the 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 β-1,4-xylanase that indicates The conversion Of xylan into xylooligosaccharides. Xylosidase and esterases allow the complete degradation of xylooligosaccharides o their monomeric constituents (Jeffries, 1996; Biely *et al.,*1997; Subramanian and Prema,1998).

The heterogenicity and complexity of xylan has resulted in an abundance of diverse xylanases with varying specifications, primary sequences and structures. Wong *et al.,* 1988 classified xylanase into two groups on the basis of their physiochemical properties: i) having low molecular mass (<30 kDa) and basic pI and ii) having high molecular mass(>30 kDa) and acidic pI. However mant fungal xylanase can not be classified based on this system. A more complete classification system was introduced which allows the classification of not only xylanases, but also of glycisidases 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).

Xylanase have been grouped into families F10 and G11 on the basis of aminoacid sequence similarity and three dimentional structures. Different enzymes may be more effective in the hydrolysis of xylobiose substituted xylooligosaccharides, xylosyl substituents or oligosaccharides containing xylosyl and other residues. The different forms may also have varying abilities to interact with xylanases in xylan hydrolysis (Reiley, 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 cause configuration inversion (initially yield α-D-xylose during hydrolysis), which is the characteristic used to distinguish between β-glucosidases and exoglucanases (Erikkson and Wood,1985). Exoxylanases increase the rate of xylan hydrolysis by attacking large xylooligosaccharides that are released by

endoxylanases and are inefficiently hydrolysed by β-xylosidases. This form of cooperation would not be expected to increase the extent of hydrolysis unless other factors are involved like accessibility of xylosidic linkages in short and branched xylooligosaccharides, 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 as short and long chain substituted xylo-oligomers. Removal of the substituents groups by auxillary enzymes creates new substrates for endoxylanases action (Si, 1997; Maheshwari, *et al.,* 2000).

2.3 XYLANASE PRODUCTION

The Various biotechnolohical techniques like submerged and solid state fermentation are employed foe xylanase biosynthesis (Cai *et al.,*1998; Gawande and Kamat,1999; Kanosh 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). The production of microbial xylanases is preferred overplant 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, heat stability, the activity was determined by carrying out the standard assay at several temperatures of pH ranges. After incubation at different temperature and pH ranges, the xylanase activity was measured under standard conditions (Coral and Colak, 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 the best nitrogen source for optimum enzyme production. The production of enzyme reached maximum after 72 hours of incubation.

Bataillon *et al*. (1998) found that a new thermophilic strain of *Bacillus* SPS-0 which produces thermostable xylanases was isolated from a hot spring in Portugal. Xylanase production

(50 nkat/ml) was observed in the presence of wheat bran arabinoxylan as source of carbon. The temperature and pH for optimum activity were 75°C and 6–9, respectively.

In a study conducted by Sevanan Murugan *et al.,*2011, saw dust was used as substrate for xylanase production from *Arthrobacter* sp. MTCC 6915. They optimized different parameters like pH, temperature, carbon source, nitrogen source, period of incubation, etc. Xylanase production was found to be optimum at an incubation period of 96 hrs (117.0U/mL), temperature 30°C and pH 9.0. The results showed that the xylanase production was found to be higher in the presence of carboxymethylcellulose and dextrose. It was also observed that peptone and beef extract supported maximum xylanase production. The enzyme was characterized and found to be fairly active at pH 9 and temperature 60°C. Even in the present study, a major difference in the production temperature (30°C) and optimal temperature (60°C) of the enzyme activity was observed.

The effect of various factors like growth conditions, incubation times, temperature, agitation rate and initial pH of medium that affect xylanase production by *Aspergillus carneus* M34 were studied (Fang *et al.,* 2007) sequentially with the help of classical "change-one-factor-at-a-time" method. Then Response Surface Methodology (RSM) was used to find the significance of these factors. The optimal cultivation conditions predicted from canonical analysis of this model were achieved by incubation at 35.08^{0}C with an agitation rate of 111.9 rpm and an initial pH of 5.16. Xylanase activity of 22.2 U/mL was verified using the predicted optimal conditions and confirmed the fitness and applicability of the model. The optimal temperature and pH of the crude xylanase activity was observed at 60^{0}C and acidic pH, respectively.

The effect of different carbon sources like Oat spelt xylan and other three agro-wastes were studied by Okafar *et al.* (2007) for the production of xylanase from wild-type *Aspergillus niger* ANL301, newly isolated from wood-waste. Highest xylanase activity of 6.47 units/mL was obtained at 96 h in media containing wheat bran as sole carbon source. The maximum specific xylanase activities were 3.86, 3.37, 5.69, and 9.36 units/ mg protein for sawdust, sugarcane pulp, wheat bran and oat spelt xylan, respectively. Hence it was concluded that wheat bran holds good for the low cost production of xylanase at high level.

Michael Michelin (2008) investigated the effect of substrates like xylan, wheat bran and liquor and found that the best conditions for xylanase production were observed when the

microorganism was cultivated in birchwood xylan for 6 days. And it was also found that the combination of both wheat bran and autohydrolysis liquor gave the better results.

The effect of different substrates for production of xylanases on different agro-residues was evaluated in solid state fermentation for xylanase production by a thermotolerent *Bacillus* isolate. Various fermentation parameters were optimized for enhanced endoxylanase production under solid state fermentation (SSF). Maximum enzyme production of 74.96±5.2 U/gds took place at 45°C with corn cob (CC) and mineral salt solution (MSS) after 72 h, at pH 6.0 and particle size of 500 µm.

Meenakshi *et al.*(2008) investigated the cultural and nutritional conditions that are required for the enhanced production of xylanase by a local soil isolate of *Trichoderma viride*, using various lignocellulosic substrates in submerged culture fermentation. Of the lignocellulosics used, maize straw (5%) was the best inducer for xylanase production. The highest activity achieved was between 14 to 17 days of fermentation. Sodium nitrate was the best nitrogen source. Maximum xylanase production was achieved with initial medium pH of 3.5-4.0 and incubation temperature of 25ºC.

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. Among the 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. Improvement of xylan production was achieved by increasing the wheat straw powder concentration to 3%.

Later, Saleem *et al.*(2002) isolated *Bacillus subtillis* producing significant level of xylanase activity in the presence of different carbon sources. However, *Bacillus subtillis* performed well when grown on pretreated natural substrates such as rice straw, wheat straw, bagasse, wheat bran and kraft pulp at 2% concentration. The organism produced higher level of xylanase activity when bagasse was used as carbon source followed by rice straw, wheat straw, wheat bran and kraft pulp. The maximum induction was observed when medium supplemented with 0.2% sucrose.

Judith *et al.,*2001 investigated the effect of different Nitrogen sources and the C:N ratio on xylanase production by *Aspergillus awamori* in experiments carried out in solid-state

fermentation. High extracellular xylanolytic activity was observed on cultivation of *A. awamori* on milled sugarcane bagasse and organic nitrogen sources (45 IU/mL for endoxylanase and 3.5 IU/mL for β-xylosidase).

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 concentrations, pH and nitrogen source using statistically significant design of experiments, employing response surface methodology 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.

Seyis and Aksoz. (2005) studied The effect of some natural on the production of xylanase from *Trichoderma harzianum 1073 D3* and maximum activity has been observed on melon peel (26.5 U/mg of protein) followed by apple pomace and hazelnut shell. Also found that molasses could be used as an additional carbon source as it decreased the production time approximately by 50 % and replacement of peptone with cotton leaf and soybean residue wastes as nitrogen sources will be a cost effective method.

Yang *et al* (1995) studied that *Bacillus* sp (V1-4) isolated from hardwood kraft pulp was found to be capable of growing in diluted kraft black liquor at pH 11.5 and produced 49 IU of xylanase when cultivated in alkaline medium at pH 9. Maximal enzyme activity was obtained by cultivation in a defined alkaline medium with 2% birchwood xylan and 1% corn steep liquor at pH 9, but high enzyme production was also obtained on wheat bran. Biobleaching studies showed that the enzyme would brighten both hardwood and softwood kraft pulp and release chromophores at pH 7 and 9. Because of the alkaline nature of kraft pulp this enzyme could be used for prebleaching with minimal pH adjustment.

Various fermentation parameters were optimized by Uma Gupta and Rita kar, 2008 for enhanced endoxylanase production under solid state fermentation (SSF). Maximum enzyme production of 74.96±5.2 U/gds took place at 45^{0}C with corn cob (CC) and mineral salt solution (MSS) after 72 h, at pH 6.0 and particle size of 500 µm.

The production of extracellular xylanase by a newly isolated thermophilic fungus, *Paecilomyces themophila* J18, on the lignocellulosic Materials (wheat straw) was studied in solid-state fermentation (SSF) by Yang *et al* (2006). The optimum temperature for maximum xylanase activity was found to be 50^0C and production was enhanced by optimizing the particle size of wheat straw, nitrogen source, initial moisture level, growth temperature and initial pH of the culture medium. Under the optimized conditions, yield as high as 18580 U/g of carbon source of xylanase was achieved. The xylanase exhibited remarkable stability and retained more than 50% of its original activity at 70^0C for 4 hrs at pH 7.0-8.0.

The production of xylanases from *Aspergillus niveus, A. niger, and A. ochraceus* under solid-state fermentation using agro-industrial residues as substrates was studied by Betini *et al.* (2009). They found that the Enzyme production was improved when a mixture of wheat bran and yeast extract or peptone is used and maximum activity was obtained when incubated at the pH of 5.5 for 1 or 2 h, at 55^0C. They also investigated the use of xylanases to reduce the amount of chlorine compounds used in cellulose pulp treatment

Subramanian and Prema (2000) have isolated and characterized several xylanase-producing cultures, one of which (an alkalophilic *Bacillus* SSP-34) produced more than 100 IU ml^{-1} of xylanase activity. The SSP-34 xylanases have optimum activity at 50°C in a pH range 6-8.

Bacillus circulans AB 16 was able to produce 50 IU/ml of xylanase, with negligible cellulase activity when grown on untreated wheat straw. The pH optimum of the crude enzyme was 6±7 with a temperature optimum of 80°C. The enzyme showed high pH and thermal stability retaining 100% activity at 60°C, pH 8 and 9 after 2.5 h of incubation.(Ashita and Sunil,2000)

Enzyme production by a new mesophilic *Streptomyces* isolate was investigated which grew optimally on 1% (w/v) xylan and 10% (w/v) wheat bran at pH 7 and 37°C. The pH and temperature optima of the crude xylanase activity were 5.5 and 65°C respectively (Uma and Chandra, 2000).

Bacillus pumilus ASH produced a high level of an extracellular and thermostable xylanase enzyme when grown using solid-state fermentation and the optimum temperature for xylanase production was found to be 37°C (Battan *et al*., 2007)

Pang and Ibrahim (2005) identified *Aspergillus niger* to be the potential producer of xylanase via a solid state fermentation system (SSF) using palm kernel cake (PKC) as substrate and also found that the supplementation of additional carbon and nitrogen sources like xylose and Sodium Nitrate in the PKC medium could enhance enzyme productivity.

Boonlue *et al.,* found a genetically new isolate *Scytalidium thermophilum* Af101-3, to be the best isolate that produced higher xylanase and corncob decomposing activity than the other strains isolated in Japanese soil at 50°C. This enzyme was purified and characterized. Pang *et al.*,2006 also investigated the production of xylanase by a local isolate *Trichoderma* spp. FETL c3-2 via solid state fermentation system using sugar cane baggase and palm kernel cake as substrates with additional carbon and nitrogen sources of dextrin(4%) and tryptone(6%). These modifications has resulted in an increase in xylanase productivity by 180% and growth by 40% compared to the basal SSF system.

In another study by Sushil Nagar *et al.* (2010), it has been found that *Bacillus pumilis* SV-85S make use of cheap and easily available agro-residue wheat bran. The enzyme production in optimized conditions was enhanced to 2995.20±200 IU/ml, which was 9.91 fold higher than the activity under unoptimized basal medium (302.21 IU/ml) the effect of different media components on production was found using response surface methodology.

The production of an alkali-stable xylanase from haloalkalophilic Staphylococcus sp. SG-13 was studied by Gupta *et al.*(2001) and found that it has dual pH optima(7.5 and 9.2) under submerged fermentation using agro based residues like as wheat bran, sugarcane bagasse, corncobs and poplar wood, they witnessed an improvement in the xylanase yield by five-fold as compared to xylose and xylan. In the biphasic growth system the prime substrate, wheat bran (1% w/v), resulted in maximum xylanase production of 4525 U/L (pH 7.5) and 4540 U/L (pH 9.2) at an agar: broth ratio of 4.0 after 48 h of incubation at 37 °C under static conditions.

The effect of aeration on xylanase production in batch cultures using *Bacillus sp.*I-1018 on xylan was investigated. Efficiency of aeration and agitation was evaluated through K_La coefficient, and the consumption of oxygen by bacteria was evaluated by measuring the dissolved oxygen pressure. Growth and enzyme enzyme production were faster when K_La was increased (Pham *et al.,* 2004). Ancharida Svarachorn (1999) has made a comparative study on using different Substrated for the production of xylanase from *Aspergillus fumigatus* strain 4-45-1F, and he found that rice straw was found to be the best substrate with the maximum xylanase production of 16.8× 104 units/g rice straw) at 40°C with the pH of 4.5.

The production of xylanase by the fungus *Penicillium sclerotiorum* under submerged cultivation was investigated by Adriana Knob and Eleonora Cano Carmona, 2008 where Oat spelts xylan and wheat bran were found to be the best inducers of xylanase activity. The optimum temperature and pH for maximum activity was 50ºC and 4.5. The enzyme was stable at 40ºC, with a half-life of 72 min and when it was incubated at 45 and 50ºC, the half-life was 8 min and shorter than 4 min, respectively.

The factors for improvement of xylanase production by *Scleratoria sclerotiorum* was investigated by Olfa Ellouze *et al.*(2008) using factorial design. They optimized the culture medium, various concentrations of different inducers for the enhanced production of xylanase and found that there is an increase in xylanase production of upto 1.6 fold. A study was conducted by Bharathidhasan *et al.* (2009) to assess the potency of different commercial feed enzyme preparations available in the market by in vitro and the xylanase activity (IU/g) was estimated to maximum of 241.35.

2.4 MODE OF ACTION OF XYLANASES

The complete enzymatic hydrolysis of xylan into its constituent monocarbohydrates requires the synergistic action of group of xylanolytic enzymes. This is due to the fact that xylans from different sources exhibit a significant variation in composition and structure (Coughlan and Hazzlewood, 1993; Jeffries, 1996). The most important enzyme in the hydrolysis of xylan is endo-1,4-β- xylanase that initiates the conversion of xylan into xylo- oligosaccharides. β-xylosidase , debranching enzymes (α-L-arabinofuranosidase and α-glucuronidase) and esterases (acetyl xylan esterase, feruloyl esterase and ρ-coumaroyl esterase) allow the complete degradation of the xylo-oligosaccharides to their monomeric constituents (Wong *et al*.,1988).

2.5 CHARACTERIZATION AND PURIFICATION

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 pI of 9.0. The enzyme were non debranching endo β-xylanases. Xylose is a major product of xylan hydrolysis by xylanases X34C and X34E, but this is not an end product by xylanase X22 and these do not hydrolyse xylobiose.

Kiddanamoorthy *et al.*,(2007) purified xylanase from *Bacillus sp.* GRE7 which had an apparent molecular weight of 42 kDa and showed optimum activity at 70^0C and pH 7. The enzyme was stable at 60-80^0C at ph7 and pH 5-11 at 37^0C. At 60^0C and pH 6, the Km for oat spelt xylan was 2.23 mg/ml and V_{max} was 296.8 IU/mg protein. Coral *et al.* (2002) studied the properties of thermostable xylanase isolated from an *Aspergillus niger* wild type strain in a liquid Czapek-Dox medium, containing oat spelts xylan as the sole carbon source. The molecular mass of the enzyme was estimated to be about 36 kDa by SDS PAGE gel electrophoresis. The optimum pH and temperature for maximum activity was found to be 7.5 and 60°C, respectively. The enzyme remained stable up to 100°C, yet lost about 50% of its activity after 15 min at this temperature.

Blanco *et al.*(1995) purified xylanse A to homogeneity from *Bacillus* sp. strainBP-23. The enzyme showed a molecular weigh of 32 kDa and an isoelectric point of 9.3. Optimum temperature and pH for xylanase were 50^0C and 5.5 respectively. Xylanase A was completely inhibited by N-bromosuccinimide. The main products of birchwood xylan hydrolysis were xylotetraose and xylobiose. 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% phosphate salt. A purification factor 57 and 41% yield of the enzyme activity were calculated for the system containing 16% PEG 6000, 8% K_2HPO_4 and 12% NaCl (Duarte *et al.,* 1999).

Damasco *et al.,* (2000) reported that xylanase was active in the broad range of pH and temperature, however the optimum pH and temperature was found to be 6 and 75^0C, respectively. Damasco *et al.* (2002) produced cellulose 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 hr of incubation at 50^0C and about 50% of activity at 60^0C even after 4 hrs of incubation.

2.6 APPLICATION OF XYLANASE

Xylanase has multiple applications in paper, animal feed and food industry. More emphasis has been paid for its use in paper and pulp industry. Xylanase treatment improves accessibility of bleaching chemicals to the pulp and decrease diffusion resistance to outward movement of the degraded lignin fragments fron cell wall. SEM of hard sheets treatment with *Streptomyces chromofuscus* show cell wall swelling softening and collapse of the cell structure.

Angayarkanni *et al.*,(2006) studied the application of three fungal enzymes produced by *Aspergillus indicus, Aspergillus flavus* and *Aspergillus niveus* in the pretreatment of hardwood kraft pulp prior to conventional alkali extraction and conventional chlorine extraction sequence (EDED process) normally used for bleaching of pulp and found that when the enzyme pretreated pulp is subjected to EDED process, there is a maximum reduction in kappa number of 6.7, 7.2 and 7.1 and a maximum increase in brightness of 41.28, 41.06 and 41.07 ISO units were observed in case of *A. indicus, A. flavus* and *A. niveus*, respectively.

Three commercially available xylanases were tested by Rafael Vicuna *et al.,* 1997, for the bleaching of Kraft pulps from *Eucaliptus globulus* and *Eucaliptus nitens* and also found that Enzyme-treated pulps required slightly higher beating to reach the same freeness than the controls. Neeta Kulkarni *et al.*(1999) suggested that the use of xylanase enzymes could greatly improve the overall economics of processing lignocellulosic materials for the generation of liquid fuels and chemicals and recently cellulase-free xylanases have received great attention in the development of environmentally friendly technologies in the paper and pulp industry.

Damiano *et al.*,(2003) investigated the efficiency of an extracellular alkali-tolerant xylanase produced by alkalophilic *Bacillus licheniformis* 77-2 with negligible cellulose activity in medium containing corn straw. The effectiveness of crude xylanase on treatment of eucalyptus kraft pulp was evaluated. A biobleaching experiment was carried out to compare the chlorine saving with pulp treated and untreated by the enzyme and found that 30% less ClO_2 was required for brightness for treated samples in comparison to the enzymatically untreated samples

Adding xylanase stimulates growth rates by improving digestibility, which also improves the quality of the animal litter. Chicken food, rye and many other grains are incompletely digested without added enzymes. These grains tend to be viscous in the chicken's intestine for

complete digestion. Xylanase thins out the gut contents and allows increased nutrient absorption and increased nutrient diffusion of pancreatic enzymes in the digestion.

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 the levels of toxic chlorine compounds released in the environment is also reduced (Rifaat *et al.*,2005).

MATERIALS AND METHODS

3.MATERIALS AND METHODS

The potent bacterial isolate was identified as *Bacillus* sp.

3.1 CHARACTERIZATION OF XYLANASE ENZYME

3.1.1 Effect of pH on xylanase activity

To determine the optimum pH of the enzyme, assay was carried out using buffers of varying pH ranging from 3.5 to 9.0. Acetate buffer (pH 3.5 to 5.5, 0.2M), and phosphate buffer (pH 6.0 to 8.0, 0.2M) and Tris-HCl buffer (8.5 & 9.0, 0.2M).

3.1.2 Effect of temperature on xylanase activity

To determine the optimum temperature of the enzyme, enzyme was assayed at pH 7.5 in various temperatures ranging from 10ºC to 70ºC.

3.1.3 pH Stability

pH stability tests were carried out by incubating a mixture of 10 ml buffer and 5 ml enzyme at pH 7.5 and pH 8. At particular time interval, an aliquot of enzyme was withdrawn and the residual enzyme activity was checked under optimal conditions.

3.1.4 Thermal stability

Temperature stability tests were performed by measuring the absorbance at intervals of 30 mins for enzyme placed in varying temperature ranging from 60ºC to 70ºC for a total of 2 hrs.

3.2 OPTIMIZATION OF MEDIA COMPOSITION

3.2.1 Effect of Carbon source

Enzyme production was carried out using the carbon sources like lactose, starch, glucose, maltose, sucrose, fructose, galactose, cellulose, xylose, arabinose, melibose, raffinose, and birchwood xylan. Further, the effect of the selected carbon source on enzyme production was investigated at its different concentrations.

3.2.2 Effect of Nitrogen source

Enzyme production was monitored using beef extract, ammonium sulphate, potassium nitrate, urea, sodium nitrate and soyabean meal. Further, effect of the selected nitrogen source on enzyme production was investigated at its different concentrations.

3.2.3 Effect of different media components

Enzyme production was monitored using the major medium components such as $MgCl_2$, $MgSO_4$ and NaCl. Effect of these components on enzyme production was investigated at its different concentration.

3.3 PARTIAL PURIFICATION OF ENZYME

3.3.1 Acetone Precipitation

An equal amount of chilled acetone [-20^{0}C] was added slowly with stirring to the enzyme extract and the mixture was kept at 4ºC. Then the mixture was centrifuged at 10000 rpm for 15 mins and the supernatant was discarded. The tubes were placed in an inverted position till there is no acetone smell, because trace amount of acetone will not allow the precipitate to dissolve in

buffer. The precipitate was dissolved in minimum amount of buffer, the content was centrifuged and the resulting supernatant was carried was used as source enzyme.

3.3.2 Dialysis

Dialysis bag was filled with acetone-precipitated enzyme. Dialysis bag was immersed in 25 mM phosphate buffer (pH 7.5) and dialysis was carried out at 4°C with intermittent change in buffer for every 12 h. The content of dialysis bag was then centrifuged to remove precipitates, if any, and the enzyme activity was carryout in the supernatant.

RESULTS AND DISCUSSIONS

4.RESULTS AND DISCUSSION

4.1 OPTIMUM CARBON SOURCE

Among different carbon sources, fructose [5%, w/v] supported highest xylanase production is shown in Table 4.1.

Table 4.1: Effect of different carbon source on enzyme activity

Carbon Source	Enzyme Activity(U/ml)
Sucrose	1.332
Fructose	55.50
Xylose	58.61
Arabinose	57.72
Maltose	23.74
Melibose	39.07
Starch	0.88
Cellulose	1.33
Raffinose	3.10
Lactose	42.18
Galactose	35.08
Glucose	45.29

4.2 OPTIMUM NITROGEN SOURCE

Among different nitrogen sources used, sodium nitrate (0.5%) supported highest xylanase production is shown in Table 4.2.

Table 4.2 :Effect of different nitrogen sources on enzyme activity

Nitrogen Sources	Enzyme activity(U/ml)

Beef extract	37.5
Urea	30.45
Ammonium sulphate	1.33
Potasium nitrate	20
Sodium nitrate	58.3
Soyabean meal	42.89

4.3 OPTIMUM CONCENTRATION OF MAJOR MEDIA COMPONENTS

Assay was carried out with varying concentration of $Mgcl_2$, $MgSO_4$ and NaCl. Xylanase activity was found to be maximum at the concentration of $MgSO_4$ (2%), $MgCl_2$ (0.5g/l), and NaCl (10g/l).

4.4 CHARACTERIZATION XYLANASE

4.4.1 Optimum pH

Extracellular xylanase activity was detected in MH medium and the enzyme activity was found to vary with pH. The effect of pH on enzyme activity is shown in figure 3 and xylanase showed its optimal activity at pH 7.5. The production of an alkali-stable xylanase from haloalkalophilic Staphylococcus sp. SG-13 was studied by Gupta *et al.*(2001), he found that the maximum xylanase activity was obtained at the pH of 7.5.

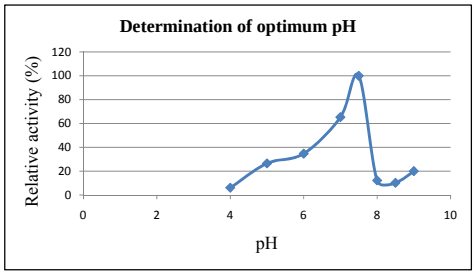

Fig 4.1:Effect of pH on enzyme activity

4.4.2 Optimum temperature

It is evident from Figure 4 that indicated that xylanase enzyme was optimally active at 60°C. Xylanase from *Bacillus pumilus* showed 65°C as its optimum temperature (Uma and Chandra, 2000).

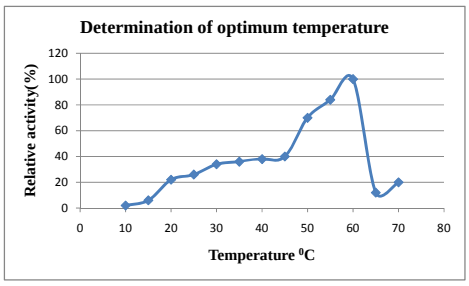

Fig 4.2: Effect of temperature on enzyme activity

4.4.3 Thermal stability of xylanase

Thermal stability tests were done by measuring the absorbance at 15 min time intervals by placing the enzyme at 60°C and 70°C for 1h. The enzyme was thermo stable, i.e., 30% of its original activity is retained at the end of 1 h at 60°C, which proves its suitability in paper and pulp industry. *Bacillus circulans* AB 16 produce xylanase and the pH optimum of the crude enzyme was 6±7 with a temperature optimum of 80°C. The enzyme showed high thermal stability retaining 100% activity at 60°C.(Ashita and Sunil,2000)

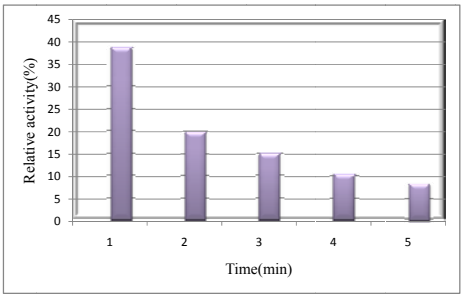

Fig 4.3: Thermal stability of xylanase

4.4.4 Purification

The xylanase was purified by acetone precipitation. The purification fold and yield was estimated(Table 4.3).

Table 4.3 Purification chart for xylanase enzyme

Enzyme Fraction	Volume (ml)	Total protein concentration (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification (Fold)	Yield (%)
Crude	580	598	318.03	0.531	1	100
Acetone precipitation	50	66.6	32	0.51	0.96	0.656

CONCLUSION

5. CONCLUSION

Thus the Bacterial isolates from sea water produce xylanase. The optimum pH, temperature and thermal stability were characterized.The optimum media components required for maximum activity were found.the enzyme was partially purified using Acetone preciciptation and this Alkali-thermostable xylanase from *Bacillus* sp can be exploited for its biotechnological applications in paper and pulp industry, baking industry and animal feed industry.

6.APPENDICES

6.1 Appendix-1

Akiba Horikoshi medim composed of the following:

Polypeptone	10g/L
Yeast Extract	5 g/L
KH_2PO_4	1 g/L
$MgSO_4$. $7H_2O$	0.2 g/L
Birchwood Xylan	5 g/L
NaCl	3 g/L
Na_2CO_3	10 g/L

The MH medium used in the production studies composed of :

NaCl	56 g/L
$MgCl_2$	7 g/L
$CaCl_2$	0.36 g/L
$MgSO_4$	9.6 g/L
KCl	2 g/L
$NaHCO_3$	0.06 g/L
Protease peptone	5 g/L
Yeast extract	10 g/L
Glucose	1 g/L

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7. REFERENCES

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