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Isolation, Production and Characterization of Xylanase from *Bacillus* sp. isolated form soil samples

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Abstract

Xylanases have wide application in various industries such as beverages, paper and bleaching, effluent treatments etc. of which their role in paper and pulp industries in most important. Un bleached pulp is usually alkaline in mature and has high temperature (50 - 60˚C). The use of a thermo alkaline Xylanase would greatly reduce the need for temperature and pH adjustment. Hence the present study was carried out with an objective to isolate thermo alkaline Xylanase producing *Bacillus sp* which can be used in pulp industries. *Bacillus sp* were isolated from local garden soil. The isolated strains of *Bacillus* were screened for the production of Xylanase. Out of the 10 strains screened one strains (BS - 2), was selected and used for further studies. The strain was mutated with UV light and the mutated colonies were screened for xylanase production. Out of the different mutants screened, Mutant I was found to produce more xylanase when compared to wild strain. The condition for maximum production of xylanase was standardized. It was found that maximum xylanase production occurred after 48 hr of incubation at 55°C and at pH 9.0. In order to determine the suitable carbon of nitrogen required for optimum xylanase production, different carbon and nitrogen sources were incorporated in the medium. Maximum xylanase production occurred when lactose, and potassium nitrite were incorporated in the medium. Cheaper carbon sources like Wheat bran and black gram residues were also tested for the ability to act as carbon source, it was found that both these were equally effective for enzyme production, next to lactose. The thermal and pH stability of the isolated xylanase enzyme was determined by incubating the enzyme in different temperatures and in different pH. The xylanase isolated from mutant strain was stable for up to 30 mins at 70°C at pH 9

Introduction

The study of enzymes is a subject of special interest because it lies just on the border line where the biological and physical sciences melt. Life depends on the complex network of chemical reactions brought about by specific enzymes and modifications of the enzyme may have far reaching consequences for the living organisms. Enzymes are protein catalysts synthesized by living systems and are important in synthetic as well as degradative process (Krishnamenon and Rao, 1992). Enzymes are among the most important products obtained for human needs through microbial sources. A large number of industrial processes in the areas of industrial, environmental and food biotechnology utilize enzymes at some stage or the other. The list of major enzymes that are used in various industries is given in Table – 1.

The catalytic properties of enzymes have been utilized in a wide range of industrial processes for over fifty year. Currently a number of new enzyme applications within the pulp and paper industry are under development. A large number of microorganisms including bacteria, yeast and fungi produce different group of enzymes that are being put into use in the paper and pulp industry.

Xylan is a major component of hemicellulose, and after cellulose it is the second most abundant polysaccharide in nature. It is found in large amounts in wood and plant material. Since Xylan is a widely distributed in plant cell walls the effective utilization of xylan-containing materials by enzymatic or microbiological procedures is considered on interesting subject (Ernst *et al*., 1990). Xylanases from various microorganisms including fungi and yeasts have been

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isolated and characterized but those from bacteria are relatively few (Ratto *et al*., 1993; Roger Bernier *et al*., 1983).

Although many bacteria and fungi have been studied for xylanase production only a few are alkalophiles. In many microorganisms xylanase activity has generally been found in association with cellulases, β - glucosidase or other enzymes (Sjostram, 1993). Plant cell walls, the major reservoirs of fixed carbon in nature, have 3 major polymers: cellulose, hemicellulose, and lignin. Among these carbon sources, xylan is mainly found in secondary walls of plants (Akihiko *et al*., 2001).

Xylan, a β-1,4-glycoside linked polymer of D- xylose is a major component of forest and agricultural materials such as hardwood, grain straw, corn cobs, and grasses.(Wilkie,1979; Robert *et al*.,1988). Xylan can be enzymatically hydrolyzed to xylose (Dekker,1976) and converted into economically valuable products such as xylulose(Wang *et al*.,1980), xylitol and ethanol (Jeffries,1981; Schneider,1981) Xylanases are generally quite small proteins, with molecular weights ranging from 15,000 to 30,000 although higher molecular weight xylanases have occasionally been isolated (Robert *et al*., 1988; Dekker,1976).

Pentosan is one of the commonest materials in agricultural wastes, in some cases accounting for 30% of the dry weight. The primary component of Pentosan is xylan a polymer of Xylose containing β - 1- 4- xylosidic linkages. Because of its natural abundance, xylan like cellulose is potentially a good fermentation substrate for production of feedstruff. Attempts have been made to obtain xylose from xylan by xylan hydrolysis or by use of microbial xylanase obtained by a combination of both (Watanalai *et al*., 1983).

Xylanolytic enzymes and their mode of action

For complete hydrolysis of xylan, many xylanolytic microorganisms often synthesize the multiple groups of xylanolytic enzymes for cooperative actions. These enzymes include endo β-1, 4- xylanases, β- xylosidase and enzymes which leave side chain sugar from the xylan backbone such as α - arabinofuranosidases and acetyl esterases.

Occurrence of xylanases Xylanases are produced by a vast variety of prokaryotes and eukaryotes including bacteria, fungi, protozoa, insect snails and germinating plant seeds. The extensively studied bacterial producers include the species of *Aeromonas, Agrobacterium, Bacillus, Dictyoglomus, Nocardia, Pseudomonas, Streptomyces, Thermotoga* and *Xanthomonas* sp.

Although many fungi are Xylanase producers, their large scale cultivation is often difficult because of slow generation time, co-production of highly viscous polymers and poor oxygen transfer. *Bacillus* sp. are used more extensively than other bacteria in industrial fermentations since they excrete most of their enzymes (Roger *et al*., 1983). Cost of enzyme production which is one of the major factor determining the economics of a biocatalytic process, can be reduced by finding optimal conditions for enzyme production, isolation of hyper enzyme producing strains and by inducing mutants (Aravind kumar *et al*., 1994).

Suitability of xylanases in Pre-bleaching of Paper pulp

Xylanases are useful in a number of applications in the paper and pulp industry. Since the most common pulping reactions and recycled fibre processes operate in alkaline pH and the bioreactor temperature rises during pulping operations, the obvious need for the hour is procurement or isolation of thermophilic microbe producing thermo-alkalostable xylanases, that are more robust in terms of temperature and pH tolerance.

Thermostability

The advantage of employing a thermostable enzyme are immense. Although fungi produce higher quantities of xylanases than bacteria, the xylanases from thermophilic eubacteria and archaebacteria have requisite half-lives at relatively higher temperatures. Mostly thermophilic microbes have been found suitable for treatment of pulp for the simple reason that xylanases secreted by them are frequently quite thermostable. Xylanases with a half-life of a few minutes upto 90min at 80° C. The xylanases of an extremely thermophilic was reported to have half life of more than 20 min at 105° C (Akihiko *et al*., 2001).

Alkaline stability

Many alkali stable xylanases from alkalophiles have also been reported. A good alkaline xylanase producing thermophilic *Bacillus* sp. was studied by (Cley *et al*. 2000), an alkaline xylanase produced by *Bacillus* sp. was isolated from an alkaline lake. Out of thermostability and alkalistability, even of one kind of tolerance is naturally present in the microbe, the other one may be acquired by enzyme engineering.

Application of xylanases

Xylanases have application in various industries, such as food and beverages, paper pulp bleaching, effluent treatments etc, of which their role in paper and pulp industries is the most important. Xylanases have also gained increasing attention because of their application in pre-bleaching of kraft pulps fiber modification, the extraction of coffee, plant oils, and the improvement of the nutritional properties of agriculture silage and grain feed. Xylanase have a number of biotechnological applications in clouding its use in pulp and paper industry. In pulp bleaching xylanase degrades the xylan interrupting lignin

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– carbohydrate bonds in the fiber pores, there by enchancing the free flow of bleaching chemicals into the fiber (Cleys *et al*., 1980; Akihiko *et al*., 2001).

Especially, cellulase – free xylanase finds extensive use in pulp and paper industry where in only xylan is to be removed from the cellulase without affecting its fiber length. The pulp and paper industries generally use CI_2 and ClO_2 for bleaching, and this leads to the release of chlorinated organic compounds, which are toxic , mutagenic as well as persistent (Cleys *et al*., 1980).

Unbleached pulp is usually alkaline in nature and has slightly high temperature (50-60° C) due to pulping process. The advantages of using thermo alkaline xylanase for pulp bioleaching would be to greatly reduce the need for temperature and pH adjustment, thus offering enough technical and environmental advantages. Xylanases from thermophilic micro organisms have high temperature optima. Various investigators stressed the importance of isolation of microbes through traditional screening methods for novel enzymes (Cheetham, 1987; Steele, 1991).

Collection of Samples

The soil samples were collected from areas in and around Pudukkottai.

Isolation

Serial Dilution

One gram of soil was collected from various sites and added to 10ml of sterile distilled water and mixed well (10^{-1}). 1ml of the sample was pipetted out from 10^{-1} tubes onto 9ml of sterile distilled water taken in another tube. The sample was then serially diluted upto 10^{-6}. 1ml of sample from $10^{-3,\ -4\ -5.}$ dilutions were poured in separate sterile petri plates containing nutrient agar and incubated at 37˚ for 2days.The colonies formed were isolated and streaked on Akiba and Horikoshi medium and incubated at 37˚C for 2days to isolate xylanase producing organisms.

Xylan clearing assay

Colonies were evaluated for xylan hydrolysis by the production of clearing zones on xylan plates by utilizing birchwood zylan as an inducer. After staining with cango red dye (1% aqueous soltution) for 30 minutes,the gel was destained with 2M NaCl.

Identification

Colony morphology

After incubation the colonies on the plates were observed according to the colour and structure of the colonies in order to identify whether they belonged to genus *Bacillus*.

Nutrient broth culture

The isolated bacterial strains were inoculated into sterile nutrient broth and incubated at 50°C for 24 to 48 hours. Sample was taken from the cultures, stained, observed under microscope and subjected to various biochemical test for further differentiation and characterization.

Gram staining

A smear of the isolated bacterial strain was made on a clean glass slide using sterile technique. The smear was allowed to air dry and then heat fixed. Crystal violet was added over the smear and allowed to stand for 1 min and then washed with tap water. Gram's Iodine was applied as mordant and left to stand for 1 ml. The slide was again washed in the tap water. Decolourizing agent (alcohol) was added drop by drop until the dye stops draining from the smear, finally saffranin (Counter stain) was added and allowed to stand for 45 secs and washed with tap water. The slide was then examined under a microscope.

Motility

A loop-full of the isolated bacterial strain was tested for motility using hanging drop method. A small drop of the bacterial culture was aseptically placed on the middle of a coverslip using a sterile loop. Vaseline was applied on the four corners of the cover slip inverted over cavity slide and observed under an oil immersion objective.

Spore Staining

A loop-full of 3-4 day old culture was transferred aseptically on a slide and a smear was prepared. The slide was air-dried and heat fixed. One drop of malachite green was added and the slide was passed over flame for 3-4 mins.The slide was then washed in tap water and stained with saffranin. After washing with tap water the slide was observed under a oil immersion objective in a microscope.

Biochemical Tests
Catalase

A loopful of the isolated bacterial strain was taken in a glass slide and 2 drops of 37 of H_2O_2 solution was added on the culture. The slide was observed for the formation of air bubbles, which indicate a positive reaction.

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Starch hydrolysis

Procedure

Starch agar was prepared and poured into sterile petriplates. After solidification the bacterial culture was streaked on the starch agar surface. The plats were incubated at 37°C for 24 – 48 hrs. After incubation the starch agar plates culture, were flooded with grams iodine solution and allowed for 30 secs. The cultures were observed for the development of clear zone.

Gelatin Hydrolysis

Gelatin agar medium was prepared and sterilized. The gelatin agar medium was poured into sterile petriplates and after solidification the isolated bacterial strain was streaked on the gelatin agar surface. The plates were incubated at 37°C for 4 to 7 days. After incubation the plates were placed in a refrigerator at 4°C for 15 mins and observed for any clear zone around the line for growth.

Indole test

To determine the ability of the *Bacillus sp*.to degrade the amino acid tryptophan.Tryptone water is prepared and sterlized and inoculated separately with the five strains of *Bacillus sp* under sterile conditions. The tubes were then placed at 37^0C for 24 to 48 hours. Kovac's reagent is added for testing and observed for the formation of red colour ring formation.Formation of red colour ring indicates indole positive and in the absence shows negative.

Methyl red test

The MR- VP broth was prepared and sterilized. The isolated bacterial strains were inoculated into MR-VP broth. The tubes were incubated at 37^0C for 24 - 48hours. After incubation, few drops of methyl red was added and the tubes were shaken, the tubes were observed for red colour formation

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Voges Proskauer Test:

The MR VP broth was prepared and sterilized .The isolated bacterial strains were incubated at 37°C for 24 – 48 hrs. after incubation few drops, Barritts reagent was added and the tubes were observed for red colour formation.

Citrate utilization test

Simmons Citrate agar was prepared sterilized nad kept as slants. The five strains were streaked separately into the test tubes. The tubes were incubated for 24h. at 37^oC. The uninoculated medium serves as the control. The change in the colour from green to blue shows the positive result and in the absence it shows only green. This test is done to differentiate among enteric organisms on the basis of their ability to erment citrte as a sole carbon source.

Catalase

A loopful of the isolated bacterial strain culture was taken in a glass slide and 3% of H2O2 solution was added on the culture. The slide was observed for the formation of air bubbles which indicate a positive reaction.

Media And Cultivation

Akiba and horikoshi medium was prepared, the pH of the medium was adjusted to 7 with sterile 1N NaOH or HCl. The medium was dispensed in sterile conical flasks (25 ml) was inoculated with 1% of 24 hrs old culture of the different strains of Bacillus, the flasks were incubated for 48 hrs at 37°C.

Xylanase assay (Takashi Nanmori *et al.*, 1990)

Enzyme solution (0.5ml) was added to 2 xylan suspension (0.5ml) in 100mM Tris-Hcl buffer (pH 7.0) and the mixtures were incubated at 55°C for 30 min. After incubation the mixtures were cooled rapidly on ice water, the insoluble xylan was removed by centrifugation. To the resulting supernatant (0.5ml), 1 ml of 3, 5 dinitrosalicylate (0.5%) solution was added and the mixture was cooked in boiling water. Colour development was measured using a spectrophotometer at 535 nm. The enzyme activity was expressed as μmol of xylose released per ml/min.

Strain improvement through UV

In order to achieve the maximum xylanase production, the isolated maximum xylanse producing *Bacillus* strain (BS I) was mutated for strain improvement. Nutrient agar medium was prepared, sterilized and poured into sterile petriplates. Then the plates were swabbed with the efficient strain and the plate was exposed to UV light at a height of about 15cm for different time intervals (5,10 and 15min). The mutant colonies were individually streaked in xylan containing medium and zone formation was measured.

Optimising conditions for maximum Xylanase prodcution

Time Vs Production

After the screening process was over the Xylanase producing *Bacillus strains* were inoculated separately in the sterilized modified Akiba and Horikoshi's medium. The medium was kept at 50^0C in Orbiteck scientific shaker at 200rpm. Then the medium was taken periodically under sterile conditions and the xylanase activity in the medium was measured spectrophotometrically.

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Effect of different pH on xylanase production

In order to determine the optimum pH required for the xylanase production, modified Akiba and Horikoshi medium was prepared, sterilized and 25ml of the medium was dispensed into different sterile conical flaks. The pH of the medium was adjusted between 6-9 using sterile 10% Sodium carbonate and 1N HCl. The flasks were inoculated with 1% 24h old seed culture. The flasks were incubated for 72 hours at 50^0C in an Orbiteck shaker at 200rpm. The xylanase production in different flasks were determined spectrophotometrically.

Effect of different temperature on xylanase production

In order to determine the favourable temperature required for xylanase production, modified Akiba and Horikoshi medium was prepared, sterilized and the pH was adjusted to 9 using sterile 10% Sodium carbonate and 25ml of the medium was dispensed into different sterile conical flasks and the flasks were inoculated with 1% 24 hour old seed culture and were incubated at different temperature from 40^0C - 70^0C for 72 hours. The xylanse production in different flasks were determined spectrophotometrically.

Effect of different carbon source on xylanase production

In order to determine the best carbon source for maximum xylanase production, by *Bacillus* species different carbon sources -Dextrose, Lactose, Sucrose, black gram residue, wheat bran, were added to the medium instead of xylan. The pH of the medium was maintained alkaline and it was inoculated with a loopful of 24 hour old culture. Then the flasks were incubated at 37^0C for 48h in an Orbiteck shaker at 200rpm. The xylanase production in different flasks were determined spectrophotometrically.

Effect of different nitrogen source on xylanase production

In order to determine the best nitrogen source for maximum xylanase production, by the *Bacillus* species, peptone was replaced with different nitrogen sources, which were sterilized separately and added to the medium. The pH was maintained alkaline and it was inoculated with a culture of 24h old culture. The flasks were incubated at 37^0C for 48h in an Orbiteck shaker at 200rpm. The xylanase production in different flasks were determined spectrophotometrically.

Enzyme characterization.

pH Stability of the enzyme

The pH stability of the enzyme was determined by incubating different pH Tris acetate (pH 6.0 - 10.0). The purified enzyme was incubated in the buffer for 30min at 50^0C and then assayed for the residual activity for determining its pH stability.

Thermal Stability of the enzyme

To determine the thermal stability of the xylanase enzyme to 0.1ml of the enzyme, 2ml Tris HCl buffer (pH 9) was added. These preparation were taken in a series of test tubes and incubated at different temperatures, from 40^0C to 70^0C for 30min.

Xylan binding Assay (Irwin *et al*., 1994; Ratana Khanokchai *et al.,* 1999)

Xylanase binding assays were performed by the modified method of Irwin *et al*. The binding assay was conducted by adding 0.5 ml of the culture supernatant to 1% of insoluble xylan in 1.0 ml of 100mM.Tris HCl buffer (pH 9) in 1.5 ml Eppendorff tube. Samples were shaken at intervals at 4°C for 30 min before being subjected to centrifugation. The amount of enzyme remaining in the supernatant was determined by the standard xylanase – assay method. The activity lost from the supernatant was assumed to be the activity bound.

Zymogram Analysis (Ratanakhanokchai *et al*., 1999)

The culture supernatant in the sample application buffer was boiled for 3 min and subjected to electrophoresis on a SDS 10% Polyacrylamide gel containing 0.1% xylan. After electrophoresis the gel was soaked in 25% (v/v) isopropanol with gentle shaking to remove the SDS and renature the protein in the gel. The gel was then washed four times for 30 min at 4°C in 0.1 M acetate buffer (pH 5.5). After further incubation for 60 min at 50°C, the gel was soaked in 0.1% cangored solution for 30 min at room temperature and washed with 1 M NaCl, until excess dye was removed from the active band. After the gel was submerged in 0.5% acetic acid, the background turned dark blue and the activity bands were observed as clear colourless areas.

Results and Discussion

Totally 20 organisms were isolated from the various samples and tested for their xylanase activity by growing them in modified Akiba and Horikoshi medium. It was found that 10 organisms *viz.* (BS-1 to BS-10) had the capacity to produce xylanase .All the isolates were alkalophilic, aerobic, endospore forming. They were motile, gram positive bacteria. All the strains hydrolysed starch. The strains were negative to gelatin hydrolysis, Indole, citrate, MR and VP test (Table-2). These results indicates that the isolated strains belong to the genus *Bacillus* according to the Bergey`s manual of determinative bacteriology (Claus *et al.,* 1986). Various investigators have reported the isolation of *Bacillus sp*. from different habitats. A thermoalkalophilic xylanase isolated from a hot spring in Portugal, soil (Battaillon *et al.*,1998; Balakrishnan *et al*.,1992).

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The isolated strains were grown in modified Akiba and Horikoshi medium (pH-7) and incubated at 37°C for 48 hrs and samples were collected for the assay of xylanase. Maximum xylanase activity was observed in strain BS – 2 followed by BS – 1, where as the activity was comparatively lower in the other strains (Table – 3 and Fig.1). Based on the above results the potent xylanase producing strain BS – 2 was selected for further studies regarding the time at which maximum production occurred and optimum pH and temperature required for the maximum production.

Xylanase production from *Bacillus sp* BS – 2 began after 24hrs and increased exponentially upto 48 hrs. The maximum production was obtained at pH 8 . The number of cells also increased with time, maximum growth was observed between 24 – 48 hrs (Table -4). The enzyme synthesis and secretion is related to cell growth, incubation period and pH of the medium.

UV Mutation

Strain improvement plays a key role in the commercial development of microbial fermentation processes. As a rule the wild strains usually produce limited quantities of the desired enzyme to be useful for commercial application (Glazer *et al* ., 1995). However, in most cases, by adopting simple selection methods, such as spreading of the culture on specific media, it is possible to pick mutant colonies that show a substantial increase in yield. Conventional physical and chemical mutagens are used for producing high yielding strains.

In the present study the maximum Xylanase producing strain (BS – 2) was mutated using UV. After exposure to UV for different time intervals the different mutated colonies were individually picked up and streaked in modified Akhiba and Horikoshi medium to observe for maximum clear Zone formation. Out of the different mutated colonies that were screened maximum clear Zone formation was observed for Mutant – II that was produced from wild BS – 2 after exposure to UV for 10 minutes (Table - 5). The Mutant II produced nearly 10% more enzyme than the wild strain. The results are in accordance with earlier reports on wild type *B. polymyxa* which produced higher levels of a alkaline protease on modified Reese's medium (Manjeet *et al.,* 1998; Meenu Madan *et al.,* 2000).

Time Vs Production

The isolated strains were grown in modified Akiba and Horikoshi medium and incubated at 37^0C for 72 h. Samples were collected at different time intervals and growth and xylanse activity were determined. The number of cells increased with time and maximum growth was observed after 48h of incubation. The xylanse production in all the three strains began after 12h of incubation and increased exponentially upto 48 h and there after it declined (Fig. 2).

The enzyme synthesis and secretion is related to cell growth, incubation period and pH of the medium (Manjeet *et al.,*1998). In the present study optimum xylanase production occurred after 48 h of incubation. The results were in accordance with that observed for *Bacillus stearothermophilus* by Alexander Khasin *et al.,* (1993). They reported that xylanase production began at 12h increased sharply and reached maximum value after 48h and there after it declined. However, the results contradicts with that observed for *Flavobacterium* sp. by Aravind Kumar Bhatt *et al.,* (1994). They reported that xylanase production began after 12 h and increased exponentially upto 72h, after which their was a decline.

Temperature Vs Xylanase Production

Temperature is yet another critical parameter that has to be controlled and varied from organism to organism. The mechanism of temperature control of enzyme production is not well understood. However, studies by Frankern *et al*., (1986) have shown that a link existed between enzyme synthesis and energy metabolism in *Bacilli*, which was controlled by temperature and oxygen uptake. The *Bacillus* strains subjected to varied temperatures (30 –80°C) released maximum xylanase at 40°C. However, an abrupt decline in enzyme yield was observed at temperatures above and below this range (Table 6 and Fig. 3). This behavior of the present bacterial strain is similar to the usual response of the mesophilic organisms where the metabolic activities get slowed down below the optimum and the temperatures above the optimum result in a denaturation of certain essential enzymes involved in various metabolic pathways. Our results are in accordance with several earlier reports for *Bacillus subtilis* (Jurasek and Paice, 1988) and for *Aeromonas hydrophila* (Araki and Kitamikado, 1988).

pH Vs xylanase production

The important characteristic of most alkalopilic microorganisms is their strong dependence on the extracellular pH for cell growth and enzyme production. In order to optimize the pH for maximum xylanase production modified Akiba and Horikoshi medium was prepared and the pH was adjusted to 6 - 10 using dilute acid or alkali. Maximum xylanase production in mutant was observed when the organism was grown at pH 8 and minimum in pH 10 (Table 7 and Fig. 4). The results obtained are similar to those reported earlier for *Bacillus subtilis* (Jurasek and Paice, 1988) and several other bacterial isolates.

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Carbon source

Increased yields of xylanase has been reported by several workers who used different sugars sources (Ashbah F. Qureshy *et al.,* 2002). In order to determine the suitable carbon source for maximum xylanase production, different carbon sources were incorporated into the medium by replacing xylan. Lactose and Xylan were found to be the best inducer of xylanase among the solubale substrates, while amongst the agricultural residue wheat bran was the best inducer followed by black gram residue (Table- 8 and Fig. 5). Very low level of induction was seen with sucrose and dextrose. High xylanase activity has also been reported in cultures grown on wheat bran and corn stalk and has been attributed to poor accessibility of xylan, which causes slow enzymatic release of xylan, which causes slow enzymatic release of xylan resulting in high xylanse production (Dobozi *et al.,* 1992; Shendye *et al.,* 1994). The fact that xylanase could be formed in the absence of xylan indicates towards the constitutive nature of the enzyme as has been reported in *Cellulomonas fimi* (Khanna and Gauri, 1993) and *Clostridium stercorarium* (Berenger *et al.,* 1985). On the basis of maximum alkaline protease production, the different carbon sources, irrespective of the incubation period can be rated in the following order:

Nitrogen source

In most microorganisms, both inorganic and organic forms of nitrogen are metabolized to produce amino acids, nucleic acids, proteins, and cell wall components. The xylanase and protease comprise of nearly 15.6 per cent nitrogen and its production is dependent on the availability of both carbon and nitrogen sources in the medium (Kole *et al.,* 1988). Although complex nitrogen sources are usually used for xylanase production the requirement for a specific nitrogen supplement differs from organism to organism. To study the effect of nitrogen source on induction and secretion of xylanase, peptone in the medium was replaced with different nitrogen sources. Maximum xylanase production was observed with KNO_3 (12.3 U mL^{-1}) and minimum with Urea (2.1 UmL^{-1}) in Mutant strain I (Table 9 and Fig. 6). On the basis of maximum xylanase production, the different nitrogen source irrespective of the incubation period can be rated in the following order. KNO_3 > $(NH_4)_2$ SO_4 > Casein > Beef extract > Urea.

In their study Archana and Satyanarayana (1998) reported that, among nitrogen sources, organic compounds were better than inorganic ones, except for diammonium hydrogen phosphate. However, the present study contradicts the above results as xylanase produced was increased by the use of inorganic source of nitrogen and supports the findings of Frankena *et al*., (1986), who reported that inorganic sources are the best inducers of xylanase.

Enzyme Characterization

Thermal Stability

In order to determine the thermal stability of the enzyme, xylanase enzyme isolated from wild and mutant strains were incubated at different temperatures ranging from 40°C to 70°C. Unlike other mesophilic bacterial xylanases, which are maximally active around 40°C and rapidly inactivated at higher temperature, the xylanase enzyme isolated from the Mutant I was fully stable and showed 100% activity at 50°C. There after, the activity declined and was 82.5% and 42.5% of the original activity at 60^0C and 70^0C (Table – 10 and Fig. 7). However, there was a drastic decline in the xylanase activity of wild strain at the same temperatures. Thermostable xylanase have been reported earlier from *Bacillus licheniformis* A99 (Archana and Sathyanarayana, 1998), *B. stearothermophilus strain* (Takashi Nanmori *et al.,* 1990) and from many other bacteria.

pH Stability

The pH range at which the isolated xylanases were stable was determined by incubating in buffers of different pH ranging from 6–10. The xylanase enzyme isolated from Mutant was quite stable upto pH 9 and retained almost full activity (Table 11 and Fig. 8). Decline in activity was observed with increase in pH and at pH 10.0 the enzyme retained nearly 36% of its original activity. Where as the xylanase isolated from the wild strain was stable only upto pH 7 and there after their activity declined (Fig. 8). Most xylanases known today are active at acidic pH (Alexander Khasin *et al.,* 1993) or neutral pHs (Marul *et al.,* 1985). Recently, however, several alkaline-tolerant xylanases are in the offing and are being characterized (Tsujibo *et al.,* 1990; Akiba *et al.,* 1988; Asbah *et al.* 2002).

Xylan Binding

Binding of xylanase to insoluble substances like cellulose and xylan is more important in the use of xylanase in pulp bleaching process (Ratanakhanokchai *et al.* 1998). Xylan binding assay was carried out by incubating the xylanase enzyme with insoluble xylan for 30 min. and removing the bound xylan-xylanase complex and measuring the residual activity of the supernatant. It was found that the mutant I showed a decline in the activity, indicating that it was able to bind with insoluble xylan (Table- 12). The present results is supported by the findings of Ratanakhanokchai *et al.,* (1998), who isolated two forms of xylanase from alkalophilic *Bacillus* sp. Strain K1.

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Table–1.List of Industrial important enzymes, their sources and their applications

S. No	Enzymes	Sources	Substrates	Applications
1	-amylase	Fungal Bacterial	Starch	Liquefaction to dextrains, alcohol production, proper volume in baked goods
2	amylase	Plant	Starch	Maltose production, proper volume in baked goods
3	Anthocyanase	Fungal	Anthocynine Glycosidde	Decolourization of juice / wine
4	Catalase	Fungal, mammals	Hydrogen peroxide	Milk sterilization cheese making
5	Cellulase	Fungal	Cellulose	Ethanol production
6	Glucoamylase	Fungal	Dextrin	Dextrin degradation to glucose
7	Glucose isomerase	Bacterial	Glucose	High fructose syrup
8	Glucose oxidase	Fungal	Glucose oxygen	Flavour and colour.
9	Invertase	Yeast	Sucrose	Production of invert sugar sugar confectionary
10	Lactose	Fungal yeast	Lactose	Lactose hydrolysis in cheese whey
11	Lipase	Fungal, Bacterial Animal goat, calf lamb throat	Lipid	Cheese ripening
12	Lipooxidase	Plant, Bacterial	Lipid Carotein	Sausage curing, Bleaching agent in baking
13	Naringinase	Fungal	Naringin glycoside	Debittening of juice
14	Pectinase(polygalacturonase)	Fungal,Bacterial	Protein	Wine/fruit juice clarification viscosity reduction in fruit processing
15	Proteases	Fungal, Plant Fungal bacterial calf stomach (rennin)	Protein Casein	Meat tenderizes Cheese production, Dough production Beerhaze Removal Soyasauce preparation
16	Xylanase	Fungal Bacterial	Xylan	Paper and Pulp industry, Fruit juice clarification

Table- 2. Biochemical Characteristics of the isolated strains

Sl.No.	Characteristics	Isolated strains (BS1-10)
1	Colony	Round with entire margin.
2	Pigment	No
3.	Gram reaction	Positive
4.	Shape	Rods
5.	Arrangement	Single in chains
6	Growth at 30^0C	Good
7	Growth at 50^0C	Little
8	Optimum pH for growth	5-9.
12	Starch hydrolysis	Positive
14	Gelatin liquefaction	Negative
15	Citrate Utilizaion	Negative
16	Catalase	Positive
17	Methyl red	Negative
18	Indole	Negative
19	Voges Proskauer	Negative

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Table – 3. Xylanase production by the isolated strains of *Bacillus* sp.

S. No.	Name of the strain	Xylanase activity ($U\ ml^{-1}$)
1	BS –1	14.29
2	BS –2	16.35
3	BS –3	12.85
4	BS –4	9.9
5	BS –5	8.3
6	BS –6	6.1
7	BS –7	9.2
8	BS –8	11.0
9	BS –9	7.2
10	BS –10	12.6

Table – 4. Growth of Xylanase producing wild and mutant strains of *Bacillus* sp

.

S. No.	Incubation Time (h)	Wild Strain	Mutant I	Mutant II
1	24	0.045	0.067	0.041
2	48	0.088	0.084	0.080
3	72	0.050	0.066	0.049

Table – 4.1. Effect of incubation time on the Xylanase production ($U\ ml^{-1}$) in wild and mutant strains of *Bacillus* sp

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S. No.	Incubation Time (h)	Wild Strain	Mutant I	Mutant II
1	24	6.42	9.6	5.6
2	48	12.6	22.6	20.4
3	72	7.1	18.4	17.0

Table – 5. Xylanase production by the wild and mutant strains of *Bacillus* sp.

S. No.	Name of the strain	Xylanase activity
1	Wild	++
2	Mutant I	++++
3	Mutant II	+++
4	Mutant III	+
5	Mutant IV	+
6	Mutant V	++
7	Mutant VI	++
8	Mutant VII	++

Table –6. Effect of Incubation temperature on the Xylanase production ($U\ ml^{-1}$) in wild and mutant strains of Bacillus sp. Assay was done after 48 h of incubation.

S. No.	Temperature (°C)	Wild Strain	Mutant I	Mutant II
1	40	14.2	17.3	16.0
2	50	21.7	40.0	25.6
3	60	15.6	33.8	18.7
4	70	9.9	15.9	12.0

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Table – 7. Effect of pH on Xylanase production (U ml^{-1}) in wild and mutant strains of *Bacillus* sp. Assay was done after 48 h of incubation.

S. No.	pH	**Wild Strain**	**Mutant I**	**Mutant II**
1	6	11.4	8.6	6.3
2	7	7.14	9.4	7.0
3	8	19.8	14.1	13.1
4	9	19.4	9.6	15.6

Table – 8. Effect of different carbon sources on the Xylanase production (U ml^{-1}) in wild and mutant strains of *Bacillus* sp. Assay was done after 48 h of incubation.

S. No.	Carbon source	**Wild Strain**	**Mutant I**	**Mutant II**
1	Wheat bran	8.0	16.2	13.8
2	Black gram residue	7.0	13.4	11.8
3	Lactose	8.28	17.6	9.8
4	Sucrose	3.14	4.0	6.0
5	Dextrose	2.28	3.5	3.2

Table – 9. Effect of different nitrogen sources on the Xylanase production (U ml^{-1}) in wild and mutant strains of *Bacillus* sp. Assay was done after 48 h of incubation.

S. No.	**Nitrogen source**	**Wild Strain**	**Mutant I**	**Mutant II**
1	KNO_3	4.86	12.3	10.3
2	$(NH_4)_2SO_4$	2.0	6.8	5.6
3	Urea	0.71	2.1	1.4
4	Casein	1.86	4.3	2.6
5	Beef Extract	1.0	2.3	1.8

Table – 10. Temperature stability of the xylanase isolated from wild and mutant strains of *Bacillus* sp. Assay was done after 30 min. of incubation.

S. No.	Temperature (°C)	**Wild Strain**	**Mutant I**	**Mutant II**
1	40	16.4	17.3	16.8
2	50	21.7	40.0	26.8
3	60	15.6	33.1	19.4
4	70	9.9	24.3	16.6

Table – 11. pH stability of the xylanase isolated from wild and mutant strains of *Bacillus* sp. Assay was done after 30 min. of incubation.

S. No.	pH	**Wild Strain**	**Mutant I**	**Mutant II**
1	6	13.8	16.8	14.4
2	7	12.8	19.4	16.6
3	8	9.3	24.3	18.3
4	9	6.0	14.0	8.14
5	10	4.6	8.5	4.28

Table – 12. Xylan binding assay of the xylanase isolated from wild and mutant strains of *Bacillus* sp. Assay was done after 30 min. of incubation.

S. No.	**Organism**	**Control**	**Xylan Binding**
1	Wild strain	22.1	22.8
2	Mutant I	26.2	20.0
3	Mutant II	24.1	21.7

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Zymogram Analysis

Zymogram analysis of the isolated xylanase enzyme was performed according to the method of Ratanakhanokchai *et al.* (1998). The zymogram analysis showed a single active xylanase, as detected by the Congo red staining (Fig. 9), indicating that their was a single major extracellular enzyme that is being produced by this bacterium. The results are in contradiction to the results observed by Ratanakhanokchai *et al.* (1998), who observed two bands, indicating two major forms of extracellular xylanases from *Bacillus* sp. Strain K1.

References

Akihiko Kosugi,Koichiro Murashima and Roy H.D.O.(2001) Characterization of xylanolytic enzymes in *Clostridium*,*Cellulomonas* : Expression of xylanase activity dependent on growth siubstrates.J.Bacteriol.183,34:7037-7043.

Archana,A.Satyananayana .T.(118).Cellulase –free xylanase production by Thermophilic bacteria *Bacillus licheniformis* A99.Ind.J.Microbiol.38:135-139.

Arvind kumar Bhatt,Tek Chand Bhalla,Hari , OM ,Agarwal and Sharma.N.(1994).Characterization of xylanolytic activity of *Flavobacterium* sp. Isolated from forest soil.Ind.J.Microbiol.34(2):119-123.

Asbah,F.Qreshy,Khan L.A., and Khannas(2002).Cloning ,regulation and purification of cellulose –free xylanase from *Bacillus circulans*.Teri-42.Ind.J.Microbiol.42:35-41.

Ernst Luthi,Nila Bhana Jasmat,and Peter L.Berguist.(1990). Xylanase from the extremely thermophilic bacterium "*Caldocellum saccharolyticum*" over expression of the gene in *Escherichia coli* and characterization of the gene product.Appl.Environ.Microbiol,56,(9):2677-2683.

Frankena J, Koningstein G.M, van Verseveld H.W and Stouthamer A.H, (1986). Effect of different limitations in chemostat cultures on growth and production of extracellular protease by *Bacillus licheniformis*, *Appl Microbiol Biotechnol,* **241**, 06.

Fuji Uchina and Toshihiko Nakane(1981).A thermophilic xylanase from a thermophilic acidophilic *Bacillus* sp.Agri.Biol.Chem.45(5) : 1121-1127.

Hiroshi Honda,Toshiaki Kudo and Kokl Horikoshi.(1985).Molecular cloning and expression of the xylanase gene of Alkaliophilic *Bacillius* sp. Strain C-125 in Escherichia coli.J.Bacteriol.161(2):784-785.

Jean-Francois Berenger,Chantal Frixon, Jacueline Bigliardi and Nichole Creuzet(1985).Production ,purification and properties of thermostable xylanase from *Clostridium stercoraium*.Can.J.Microbiol.31:635-643.

Johanna Buchert,Marjatta Ranua,Anne Kamtelinenand Liisa Viikari.(1992).The role of two *Trichoderma reessei* xylanases in the bleaching of pine kraft pulp.Appl.Microbiol.Biotechnol. 825-829.

Kaisa Poutanen,Marjaana Ratta,Jurgesh Puls,and Liisa Viikari(1987).Evaluation of different microbial xylanolytic systems.J.Biotechnol.6:49-60.

Khanok Ratanakhanokchai,Khin Lay Kyu and Morakot Tantichroen(1999).Purification and properties of a xylan binding endoxylanase from Alkaliophilic *Bacillu*s p. Strain K-1 Appl.Environ.Microbiol.65(2):694-703.

Kluepfel.D,Shareck F., Mondou F.,and Morosoli R.(1986).Characterization of cellulose and xylanase activities of *Streptomyces lividans*.Appl.Microbiol.Biotechnol.24:230-234.

Koki Horikoshi and Yoko Atsukawa .(1973).Xylanase produced by Alkalophilic *Bacillus* No.C-59-2.Agr.Biol.Chem.37(9)2097-2103.

Marajanna ratta ,Indra M Mathrani ,Birgitte,Ahring,Liisa Viikari(1994).Application of thermostable xylanase of *Dictyoglomus* Sp in enzymatic treatment of Kraft pulps.Appl.Microbiol.Biotechnol.41:130-133.

Paice M.G., Bernier R.Jr. and Jurasek L.(1998).Viscosity-enhaancing bleaching of Hard wood kraft pulp with xylanase from a cloned gene.235-239.

Ratto M, Poutanem K., Viikari L.(1992).Production of xylanolytic enzymes by an alkalitolerant *Bacillus circulans* strain.Appl.Microbiol.Biotechnol.37:470-473.

Robert C.A .Yang,Roger Mc Kenzie .C.,Doris Bilous,Verner L.Seigy,and

Roger Bernier .JR.,Michael Desrochers,Lubomir Jurasek and Michael G.Paice.(1983).Isolation and characterization of a xylanase from *Bacillus subtilis*.Appl.Environ. Microbiol.46(2) 511-514.

Saran A.Narang.(1988).Molecular cloning and expression of a xylanase gene from *Bacillus polymyxa* in *Escherichia coli* .Appl.Environ.Microbiol.54(4):1023-1029.

Sirin A.I.Adham,Ana B.Campelo,Angelina Ramos, and Jose A.Gil.(2001).Construction of a xylanase producing strain of *Brevibacterium lactofermentum* by stable integration of an engineered xys A gene from Streptomyces halstidii.JM8.Appl. Environ.Microbiol.67(12):5425-5430.

Sridevi Rajaram and Ajit Varma(1990).Production and characterization of xylanase from *Bacillus thermoalkalophilus* grown on agricultural wastes.Appl.Microbiol.Biotechnol.34:141-144.

Watanalai Panbangred,Atsuhiko Shinmyo,Shinichi,Kinoshita and Hirosuke Okada(1983).Purification and properties of Endoxylanase produced by Bacillus purilis,.Agri.Biol.Chem.47(5):957-963.

Watoru O kazaki,Terhiko Akiba Koki Horikoshi, and Ryoich Akahoshi.(1984).Production and properties of two types of xylanases from Alkalophilic thermophilic Bacillus sp.Appl.Microbiol.Biotechnol.19:335-340.

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