



Original Research Article

Production and Purification of alkali stable xylanase from *Bacillus* sp.

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A B S T R A C T

Keywords

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An alkaliphilic bacterium, *Bacillus* sp. Strain AP4 produces extracellular xylanolytic enzymes such as xylanases, is a stable in alkaline state. Total thirty bacterial strains screened by Congo red staining followed by spore and gram staining, culture conditions such as time, carbon and nitrogen sources also optimized for better yield. The extracellular enzyme was purified by ammonium sulfate precipitation, Ion exchange chromatography. The enzyme activity is stable at pH 9 and along with at pH 11 and was also found to be moderately acid stable at pH6, optimal temperature of 40^oC. The metal ions such as ca and Zn increased the residual enzyme activity where as Pb, Hg and Mn Strongly inhibits the enzyme activity.

Introduction

Xylan is a major structural polysaccharide of plant-cell walls and a second most abundant in nature after cellulose. Structurally it is a linear β -(1, 4)-D-xylose backbone and substituted with different side chains especially α -D-glucuronosyl and α -L-arabinosyl units (Adriana Knob et al 2013). Due to its structural complexity, plenty of enzymes required to degrade it. Xylanases are the enzymes which can degrade xylan backbone to xylooligosaccharides (N. Kulkarni., 1999 and T. Collins., 2005).

The endo- β -1, 4-xylanase (EC 3.2.1.8), β -xylosidase (EC3.2.1.37) (J.-x. Feng.,2010), are the main xylanases, which attracting increasing attention for its potential applications in a number of biotechnological processes, such as bleaching of cellulose pulp in paper manufacturing, and pretreatment of lignocelluloses in the utilization of biomass (Belien et al. 2006).

Xylanases have been reported from bacteria, fungi and actinomycetes. Among

bacteria *Bacillus*, *Aeromonas*, *Bacteriodes*, *Cellulomonas*, *Microbacter*, *Rumnicoccus*, *Paenibacillus* and *Streptomyces* have been reported as xylanase producers (Rapp and Wagner, 1986).

Most of researchers have targeted on using residues from agro and food industry, thereby restricting socioeconomics related to environment pollution. These residues (which contain nearly 20-30% hemicellulosic material) are used for production of xylanase by microorganisms (Milagres *et al*, 2004). The most commonly used substrates are rice bran, corn crop, rumen, sorghum straw and cassava peel (Wang *et.al.* 2003, Sonia *et.al.* 2005).

Amount of nitrogen also plays important role in enhancing the rate of enzyme production. Ammonium nitrate, sodium nitrate, ammonium sulphate have been used essentially as nitrogen source (Abdel-Sater and Elsaid, 2001). Microorganisms produce different types of products in different growth stages, so harvesting product from culture media after a particular time interval is necessary for getting higher yield of product (Sanghi *et.al.* 2008, Nagar *et.al.* 2010).

Most of industrial processes are run in harsh and hostile conditions and the reported xylanases don't withstand such an extreme of pH and temperature besides having a high cost of production. This paper reports on the production, purification and characterization of xylanase from *Bacillus* sp. capable of utilizing wheat bran and peptone as carbon and nitrogen as growth substrates at pH 9 along with stimulatory effect of zinc chloride and calcium on purified enzyme.

Materials and Methods

Primary screening for xylanase producing microorganism

35 bacterial strains are used in this study are available in cultural repository of the lab, was sub cultured in nutrient agar and the final PH was adjusted to 9. Now actively growing culture was inoculated in a Petri plate which contains oat spelt xylan 0.5%, mineral salt solution 1.0% (NH_4NO_3 1%, KH_2PO_4 0.5%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1%, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.01%, NaCl 0.01% $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 0.01%), agar 2% at pH 9 for 48hrs. culture plates were stained with Congo red (0.1%) for 10mins and destain with 1M NaCl solution for 10 minutes. Remove the excess stain by repeating destaining process for 3 times. Positive clones were observed with a clear zone around the culture spot. Gram staining and spore staining was carried out for further screening of bacterial strains by using gram staining kit (Himedia), schaeffer and fulto's spore staining kit (Himedia) respectively.

Xylanase enzymatic activity essay

To 10ml of production media, a loopful of the culture was inoculated and incubated for 24 hours at room temperature at 150rpm. This was treated as preinoculum and transferred into 150ml conical flask containing 90ml of the medium and incubated for 24 hours. Enzyme was extracted from inoculated media by centrifuging at 7,500 rpm for 15 minutes at 40°C. The cell free extract of media thus obtained was used to estimate reducing sugar released and soluble protein estimation.

Xylanase activity was determined at 40°C using 0.5 % oat spelt xylan (w/v) in Tris buffer pH 9. After 5 and 10 min of incubation, the reaction was interrupted by the addition of 3, 5-dinitrosalicylic acid (DNS), and the reducing sugars released were quantified (G. L. Miller., 1959), using xylose as standard. One unit of enzyme activity was defined as the amount of enzyme capable of releasing 1 µmol of reducing sugar per min, under assay conditions. Specific activity was expressed as unit per milligram of protein. All enzymatic assays were developed in triplicate, and the results are presented through mean values.

Total protein was determined by modified Bradford method (J. J. Sedmak., 1977), using bovine serum albumin (BSA) as standard.

Optimization of culture stipulation for xylanases production

Selected strains were inoculated in a production media with oat spelt as a carbon source and pH was adjusted to 9.0. The inoculated flasks were incubated at 37°C under static rotation for proper aeration. The samples were collected at different time intervals such as 24h, 48h, 72h, 96h, and 120h. Xylanase activity was measured a previously mentioned.

Optimization of Carbon and nitrogen sources and in combination

Effect of carbon and nitrogen source on *Bacillus* was studied by supplementing 6 different crude carbon sources such as wheat bran, rice husk, saw dust, wood waste, maize bran and rice bran and 6 nitrogen sources of urea, yeast extract, casein, Mustard oil cake, soybean meal and peptone were tested. 1% of carbon and

nitrogen sources was used separately for the screening of best source and finally optimized the carbon and nitrogen source together. Samples were collected at different time intervals for the enzymatic activity analysis.

Purification of Xylanase enzyme

Ammonium sulphate precipitaton

The crude enzyme fraction (500 ml) was treated with ammonium sulphate precipitation (0-85%). Enzyme was precipitated by centrifugation at 10 000 x g for 10 min and dissolved the precipitate in Tris-Cl buffer, pH 9.0 and dialyzed overnight against the same buffer. Protein content was determined by Folin reaction (Lowry et. al., 1951) as standard and xylanase activity was determined by DNS method.

Ion exchange column chromatography

Matrix selection

The protein samples obtained by 85% ammonium sulphate saturation were tested with two different matrix, Carboxy methyl cellulose (CMC) and Diethylaminoethyl cellulose DEAE (1 mg each). Each matrix was taken into three different ependrofs with different buffer pH of 7, 8 and 9 (500ul), were incubated at 4°C for 30 mins. Ependrofs were spinned 3 times by changing the buffer. Now add 25ul of enzyme and 300ul of buffer in ependrof tube and incubate for 30 min at 4°C. Supernatant was collected by centrifuging at 5000g for 10mins and enzyme activity was measured.

CarboxyMethyl Cellulose column chromatography

Carboxymethyl cellulose column was

prepared by making a slurry of CMC with Tris Cl Buffer (pH 9). The column length was 15 cm, now it was equilibrated with Tris Cl buffer (pH 9) 2 -3 times for 30 mins each. 2 ml of enzyme was added into the column and incubated for 40 mins. Elution was done with 30 ml of 0.2M NaCl, 0.5 M NaCl, 0.75 M NaCl & 1 M NaCl in succession. Protein content and enzyme activity of each fraction was measured as mention above.

pH and thermal stability of enzyme fraction

Purified enzyme was diluted (1:10 v/v) with Tris buffer and thermal stability was tested by incubating the diluted enzyme at different temperatures (40⁰C to 100⁰C) for 10 to 30 mins and pH stability was also analysed by incubating the enzyme with different pH ranges for 10 to 30 mins. Now enzyme activity was checked by essay method.

Effect of Metal ions on Xylanase activity

Eight metal ions viz. Mn²⁺, ZnCl, Ca²⁺, Pb²⁺ and Hg²⁺ were used to study the effect of metal ions on enzyme. 0.5µmoles/µl of metal ion solution was added in an enzyme assay mixture and the essay was carried out to check the activity of an enzyme against metal ions.

Confirmatory Assay

Confirmatory assay was done to cross check the enzyme activity. 10ul of partially purified enzyme was used as a control and enzyme treated with proteinase K for 4hrs was used as a test. In this method Xylan agar plates were prepared and a small hole was made with the help of borer. Now test and control samples were added into the wells and incubated for 24hrs.

Results and Discussion

Primary screening for xylanase producing microorganism

Total thirty five strains were screened by Congo red staining. When the culture was grown in a media contain Xylan, Bacillus produce the enzyme Xylanases and convert xylan to xylose. It can be used as a carbon source for the growth. It was clearly observed as a clear zone formation around the culture when stained with Congo red showed Clear zone formation. Out of these strains three strains known as AP4, AS11 and F shown significant zone formation. From the results it appeared that the proposed technique was simple and quantified enzyme activity as already reported (Samanta et al., 2011). The further experiment was carried out by gram and spore staining. Gram Positive, long rod shaped Bacilli were observed and Clear spores were observed during the process of spore staining under microscope at 100X magnification. Bacillus is gram positive, rod shaped bacteria and it can produce spores.

Time profile for xylanase production

The time course of xylanase production was investigated and maximum production was observed. For 24hrs(0.376U/ml) after 24hrs the enzyme activity was reduced, probably due to increase in toxic unwanted wastes and depletion of nutrients in the media, which leads to decreased growth and enzyme activity. The organism was cultivated in production medium with oat spelt xylan as C -source and medium pH-9. The samples were drawn after 24h, 48h, 72h, 96h, 120h and xylanase production was assayed.

Selection of the organism

The isolates were screened as positive clones by Gram, Spore and Congo red staining are used for the further analysis. Out of which, three clones known as AP4, AS11 and F shown significant zone formation, based on the three parameters (Time course, Temperature and pH) best isolate was screened and AP4 shown a larger zone compared with others and selected as a potent clone for further characterization.

Optimization of carbon source in the production medium

Agro waste such as Wheat bran, saw dust, Rice bran, wood waste, Rice husk and maize bran was used as a carbon source. As per the (Oliveira et al., 2006) agricultural waste evaluated in submerged fermentation by *Penicillium janthinellum* and oat bran found to be as a best inducer of xylanase. From the optimized solid state fermentation with different carbon source, the results showed that high level of enzyme activity in wheat bran after 48hrs of incubation. Wheat bran shown to be the best carbon source for the extra cellular enzyme production. But remaining carbon source inhibits the enzyme activity.

Optimization of nitrogen source

Six sources were tested for best xylanase production such as urea, yeast extract, peptone, caesine, mustard oil cake, soya bean meal, but out of these peptone (0.62 IU/ml) and casein (0.765 IU/ml) showed maximum xylanase activity. Previous studies reported that organic nitrogen sources have been found to increase the xylanase production in *Bacillus* species (Battan B et al., 2007)

Optimization of best carbon and

nitrogen source

Xylanase production was determined using best carbon and nitrogen sources. The combinations used were wheat bran + casein and wheat bran + peptone. Wheat bran +peptone (0.99 IU/ml) showed maximum xylanase production after 48 hrs.

Purification of xylanase

The major *Bacillus species* xylanase was purified by protein precipitation with ammonium sulfate and Ion exchange chromatography. High enzyme proportion (about 84%) was observed in the 85% ammonium sulfate saturation precipitant. Purification was done by ammonium sulphate precipitation followed by dialysis. After that Ion-exchange chromatography followed by ammonium sulphate precipitation & dialysis was done. CMC at pH 9 showed least enzyme activity so it was used for the purification. In ammonium sulphate precipitation, maximum xylanase activity was obtained in 85% saturation. The pellet was dissolved in 1.5ml of phosphate buffer (pH-9) and 3ml of that sample was dialysed for 24h. During dialysis, buffer was changed 3-4 times. In Ion exchange chromatography two types of matrix such as CMC and DEAE were used to increase the enzyme activity. Purification details have been listed in Table 1 and 2.

Effect of temperature and pH on purified enzyme activity

The thermal stability of the purified enzyme was determined by varying the temperature with a constant pH at 7. The enzyme activity was measure for 10mins and 30mins at different temperature range from 20⁰C to 100⁰C. The residual activity

was measured by Xylan standard. It was observed that the enzyme activity retained for 10 minutes and optimal activity is at 40°C. In terms of pH stability, it was found most stable at pH-9, after that the stability decreased and then increased again at pH11. Reports show that microbial xylanases are usually stable over a wide range from pH 3 to the pH10 (Kulkarni N et al., 1999). Enzyme was found to be moderately acid stable (at pH6) but only for 10 min.

Effect of metal ions on xylanase activity

The effect of metal ions on enzyme activity was investigated. Metal ions such as Mn²⁺, ZnCl₂, Ca²⁺, Pb²⁺ and Hg²⁺ were tested. The increase was maximum in case of ZnCl₂(0.24 IU/ml) followed by Ca²⁺ (0.175 IU/ml). Khasin *et.al.* (1993) suggested that xylanase activity was strongly inhibited by

Figure.1 Optimization of Time interval

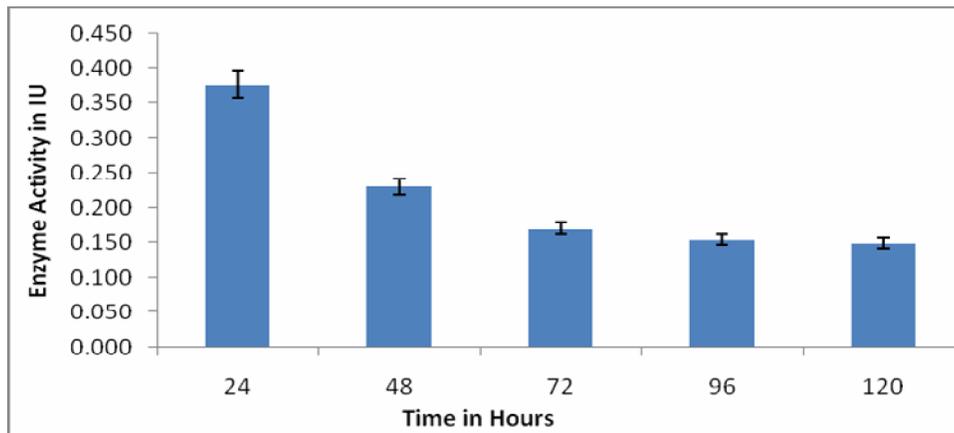


Figure.2 Screening of the best strain based on the pH, Temperature and Time.

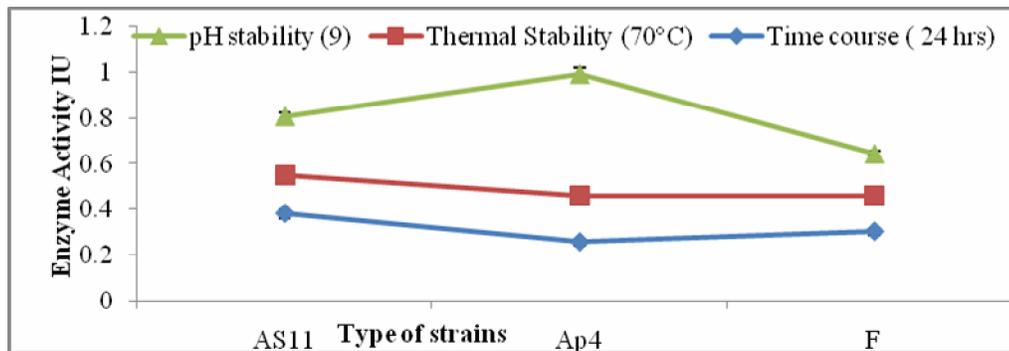


Figure.3 Optimization of Carbon source

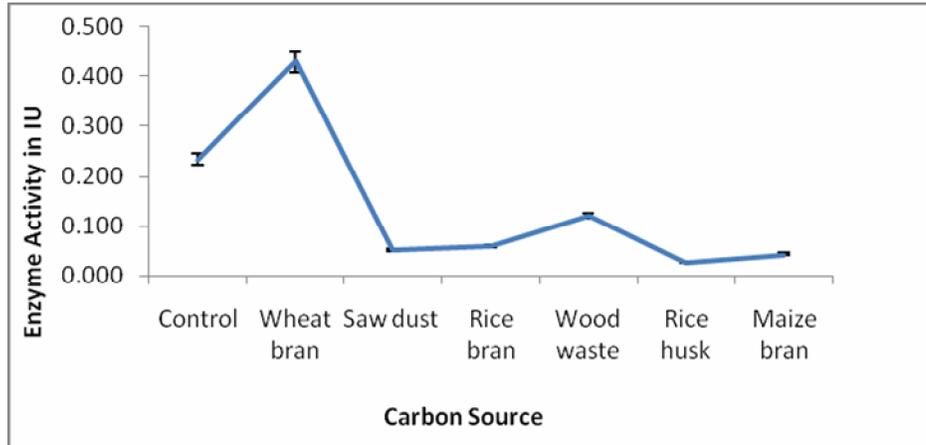


Figure.4 Optimization of Nitrogen Source

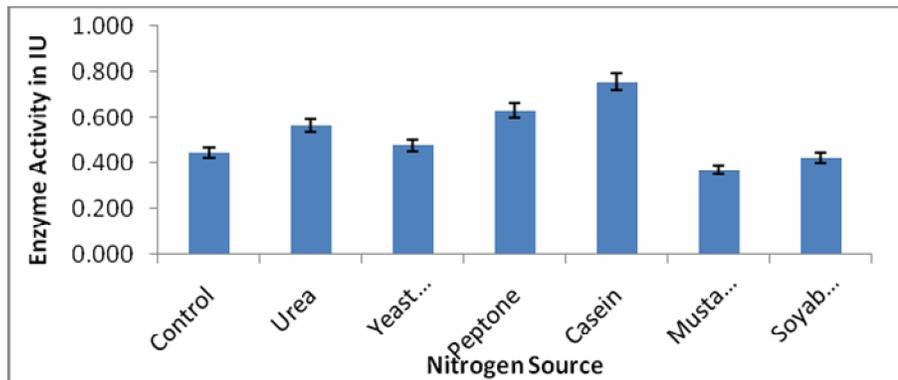


Figure.5 Optimization of best C+ N source.

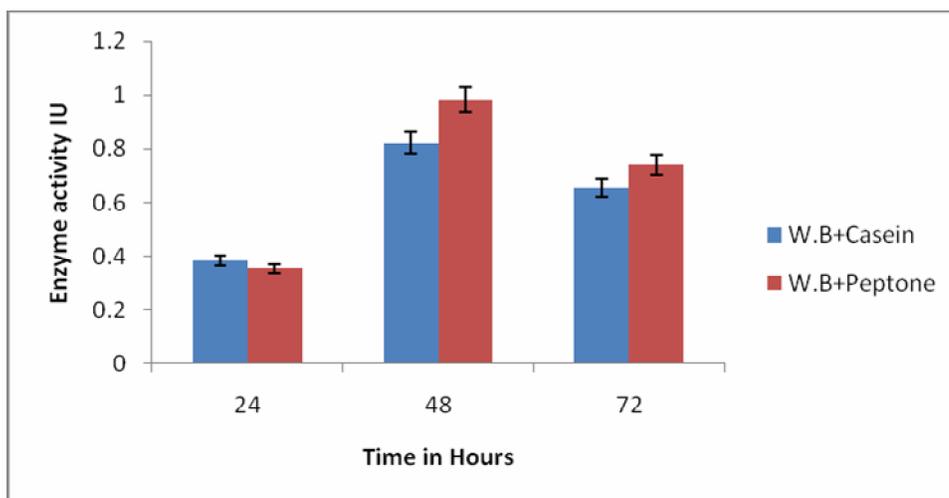


Table.1 Selection of matrix for enzyme purification

Type of Matrix	Enzyme Activity (IU) pH 7	Enzyme Activity (IU) pH8	Enzyme Activity (IU) pH9
CarboxymethylCellulose	0.05±0.093	0.028±0.021	0.01±0.004
DEAE	0.04±0.006	0.054±0.017	0.048±0.019

Table.2 Purification of xylanase from Bacillus sp.

	Volume(ml)	Activity(U/ml)	Total activity (U)	Protein(µg/ml)	Total protein (µg)	Specific activity	Purification fold (PF)
Crude extract	459	0.075	34.425	3.3	1514.7	22.73	1
Ammonium sulphate precipitation (60- 85%)& dialysis	3	0.187	0.561	4.6	13.8	40.65	1.788
After Ion - exchange chromatography (0- 90% A S P) & dialysis	5	0.125	0.625	2.7	13.5	46.29	2.036

Figure.6 Thermal stability of purified enzyme

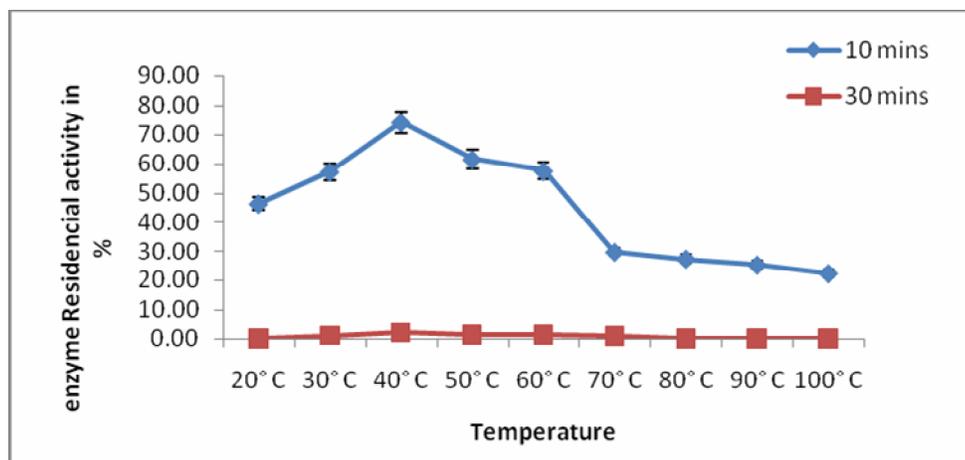


Figure.7 pH stability of Purified enzyme

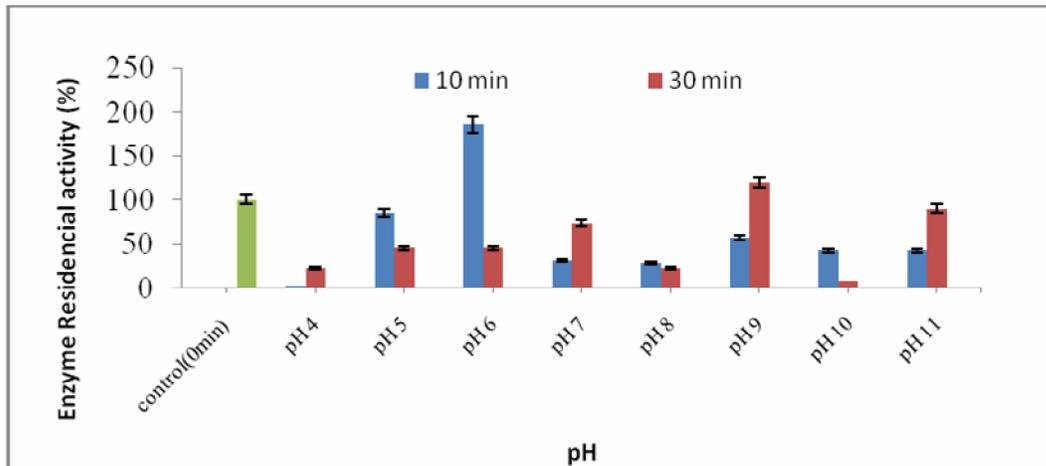
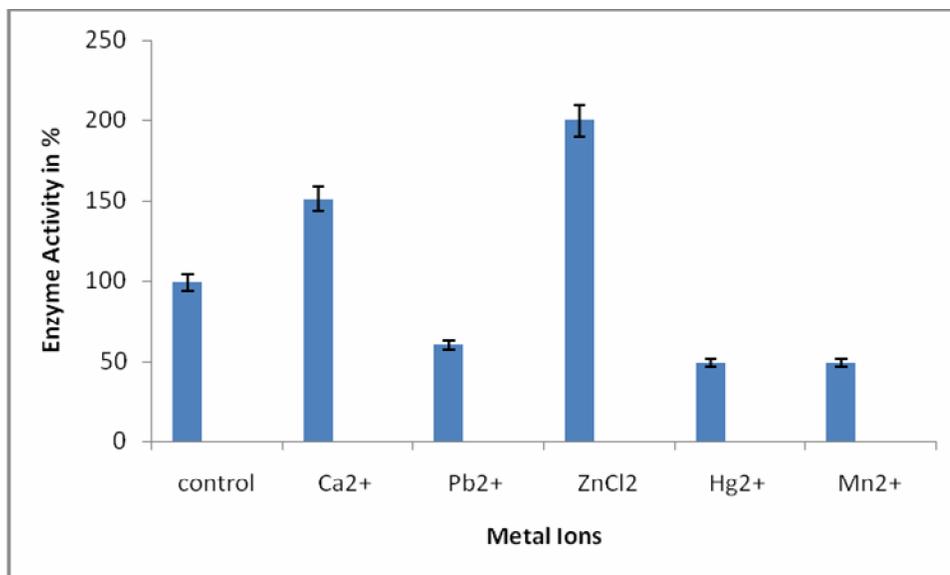


Figure.8 Effect of metal ions on xylanase activity



Hg²⁺ and Cd²⁺ in case of xylanase isolated from *Bacillus* and reported that sulphhydryl groups of cysteine residue in or close to the active site of the enzyme (Khandeparkar and Bhosle.,2006b). (Qinnghe *et.al.* 2004) tested different metal ions on xylanase produced by *Pleurotus ostreatus* and found no significant effect on xylanase activity.

Plate assay

Zone formation in case of control confirmed the presence of enzyme in the purified protein whereas no zone formation in test indicated the enzyme was degraded by proteinase K

There are number of bacteria reported for producing alkali stable xylanase (Annamalai et. al., 2009, Prakash et. al., 2009, Sanghi et. al., 2008). These have been found to be useful in the paperpulp industry. Pollution caused by chloro-organo compounds in the paperpulp industry due to usage of chlorine for bleaching possess a significant health hazard (Buchert et al., 1994). The alkali stable xylanase have been found to be useful in reducing that pollution(Buchert et. al., 1994, Bajpai et. al., 1999). The utilization of low-cost raw materials, for example, wheat bran led to a reduction in the culture medium cost for xylanase production, which usually ranges from 25% to 50% of the total production cost. The bacteria when observed under microscope appeared long rod shaped bacilli and stained gram positive. The bacteria gave large white colonies with undulate margins, circular form and flat elevation when streaked on NA plates. The optimum time for production was 48 h when crude nitrogen & carbon source was used. Wheat bran and peptone were found to be best carbon and nitrogen source when provided individually. The optimum pH for enzyme production was 9 but a significant amount of enzyme production was also observed at pH 6 and 11. It showed maximum stability at pH 9 indicating that the enzyme was alkalistable as well as moderately acidstable. The enzyme showed activity from 40°C - 80°C but only upto 10 minutes of incubation. Therefore, the use of raw materials wheat bran and peptone and alkali stable nature of the enzyme found to be cost effective from industrial perspective by reported *Bacillus* sp. and can be regarded as economically attractive. The purified enzyme activity can be increased further by the use of calcium and zinc chloride.

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