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PURIFICATION AND CHARACTERIZATION OF XYLANASE FROM *B. LICHENIFORMIS* STRAIN JS

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ABSTRACT: Second only to cellulose in natural abundance, xylan is a major component of the hemicellulose fraction in the plant cell walls. The most abundant form of xylan is heteroxylan, which comprises xylose residues in the backbone with acetyl, arabinosyl and glucuronosyl residues as substituents (Sunna A,1997). Its enzymatic hydrolysis requires endo-1,4-xylanase (-1,4-d-xylan that cleaves glycosidic bonds to produce xylooligosaccharides and β-1,4-xylosidase (β-1,4-D-xyloside xylohydrolase, EC 3.2.1.37), responsible for the final breakdown of small xylooligosaccharides into xylose (Biely P 1993). Xylanase (EC. 3.2.1.8; 1,4- β -D-xylanase, xylanohydrolase) can hydrolyze β-1,4-glycosidic linkages of the xylan backbone to produce short chain xylooligosaccharides of various lengths-hence, the crucial enzyme component of microbial xylanolytic systems .

KEYWORDS: Purification, xylanase, *B. licheniformis*, arabinosyl, cellulose.

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1.INTRODUCTION

Second only to cellulose in natural abundance, xylan is a major component of the hemicellulose fraction in the plant cell walls. The most abundant form of xylan is heteroxylan, which comprises xylose residues in the backbone with acetyl, arabinosyl and glucuronosyl residues as substituents (Sunna A, 1997). Its enzymatic hydrolysis requires endo-1,4-xylanase (_-1,4-d-xylan xylanohydrolase, EC 3.2.1.8), that cleaves glycosidic bonds to produce xylooligosaccharides and β-1,4-xylosidase (β-1,4-D-xyloside xylohydrolase, EC 3.2.1.37), responsible for the final breakdown of small xylooligosaccharides into xylose (Biely P 1993). Xylanase (EC. 3.2.1.8; 1,4- β -D-xylanase, xylanohydrolase) can hydrolyze β-1,4-glycosidic linkages of the xylan backbone to produce short chain xylooligosaccharides of various lengths-hence, the crucial enzyme component of microbial xylanolytic systems (Sunna A 1997). Xylanases have attracted considerable research interest because of their potential industrial applications. One of the exciting applications of xylanases is the production of xylo- oligosaccharides (Beg QK 2001). Recently, interest in xylanase (1,4-b-D-xylan xylanohydrolase, EC3.2.1.8) has increased markedly due to its huge potential industrial applications. The use of cellulase-free and thermostable xylanases is particularly important in the pulping and bleaching processes (Subramaniyan and Prema, 2002). Xylitol can be produced from the products of hydrolysis and used as a sweetener or used in the food industry as a thickener and a fat substitute. Xylobiose has also been shown to have prebiotic properties (Cannio R 2004). In the biofuel industry, xylanases can be used to improve delignification and isolation of cellulose from the hemicellulose matrix (Beg QK, 2001).

2. MATERIALS AND METHODS

Xylanase production

The *Bacillus licheniformis* strain JS has been screened for the xylanase production on the mineral salt agar medium containing NaNO3 - 0.3 gm, K2HPO4 - 0.1gm, KCl -0.5 gm, MgSO4.7H2O - 0.5gm, FeSO4.7H2O- 0.01gm and 0.1% Birchwood xylan as carbon source per 1000ml distilled water having pH-7.2. The plate was incubated at 37 °C and observed for zone of hydrolysis around the growth of organism. The production of xylanase was carried out by using *B. licheniformis* strain JS. The 24 hrs fresh culture was inoculated into a 100 ml mineral salt medium which was same as that of used for the screening of xylanase production. The flasks were incubated at 37°C for 72 hrs. Production of xylanase was also carried out by replacing agricultural waste like Rice husk and Wheat Bran with moistening agent such as distilled water and mineral salt medium. The protein was determined by the Lowry method using standard graph of Bovine Serum Albumin as standard protein.

Purification of xylanase

The purification of enzyme was carried out at 4 °C. The growth was inoculated in the medium containing the xylan as carbon source. After 48 hr of incubation the cell free extract was collected by centrifugation at 5000 rpm for 15 min. This was then precipitated by ammonium sulphate precipitation. The precipitate was collected by centrifugation at 8000 rpm for 20 min at 4 °C, dissolved in sodium phosphate buffer (pH-7.0) and dialyzed against same buffer overnight. This was further purified by the DEAE-cellulose ion exchange column chromatography and column was eluted with the NaCl gradient of 0.1M to 1.0 M concentration, and the fraction of 5ml was collected at the flow rate of 0.5 ml per min. All fractions were checked for their protein content by measuring absorbance 280nm with a spectrophotometer.

Characterization of purified xylanase Effect of pH on xylanase activity

The effect of pH on enzyme activity was studied for a pH range from 4.0 to 9.0, using 50Mm Sodium acetate buffer(pH 4.0- 5.0), sodium- phosphate buffer (pH 6.0- 7.0), Tris-HCl buffer (pH 6.0) Glycine- NaOH buffer (pH 9.0). The enzyme activity was carried out in various buffers having pH ranging from 4.0 to 9.0 at 37°C.

Effect of temperature on xylanase activity

The effect of temperature on xylanase activity was carried out at different temperature such as 10 to 70 °C. In that the enzyme assay was carried between 10 to 70 °C and the % relative activity was measured according to method described in the enzyme assay section.

Effect of metal ions on xylanase activity

The purified xylanase was pre-incubated for 1hr with metal ions like KCl, $MnSO_4$, $MgSO_4$, HgCl2, $ZnSO_4$, $COCl_2$ and $CuSO_4$ using their water soluble forms in 50 mM phosphate buffer having pH-7.4. The % residual activity were measured.

SDS-PAGE

Purity of the fractions, showing xylanase activity, was checked by SDS-PAGE by the method of Laemmli et al (1970). The bands were visualized by Coommassie Briliant blue staining. The molecular weight of xylanase were determined by comparison with standard molecular marker proteins (Phosphorylase b 98 kDa, Bovine Serum Albumin 66 kDa, Ovalalbumin 43 kDa,Carbonic Anhydrase 29 kDa, Soyabean Trypsin Inhibitor 20 kDa).

Immobilization of xylanase

The enzyme was immobilized in calcium alginate beads according to the method of Ates and Mehmetoglu (1997). Four ml of sodium alginate solution (3.75%) was mixed with 1 ml enzyme solution (2mg/ml) to a homogenous final alginate concentration of 3%. The mixture was extruded

drop by drop using a sterile hypodermic syringe needle into 0.2M CaCl2 solution at 4°C to form beads. The beads were allowed to harden in the CaCl2 solution for 2 hr. The resulting spherical beads were washed with sterile distilled water. The beads were stored in 25 mM sodium phosphate buffer (pH-7.6) at 4°C.

Analysis of hydrolyzed product:

The hydrolysed products of xylan were analyzed by thin layer chromatography. The TLC analysis was carried out using ethyl acetate, isopropanol (65:35) as mobile phase and products were detected by spraying aniline- diphenylamine reagent.

3. RESULTS AND DISCUSSION

A] Screening of xylanase producers:

The organism was selected on the basis of their ability to hydrolyse xylan. The zone of hydrolysis was observed around the colony utilizing the xylan. It was observed from the experiments that the Bacillus licheniformis strain JS were able to utilize the xylan from the surrounding the medium. This organism was used for further production of xlanase.

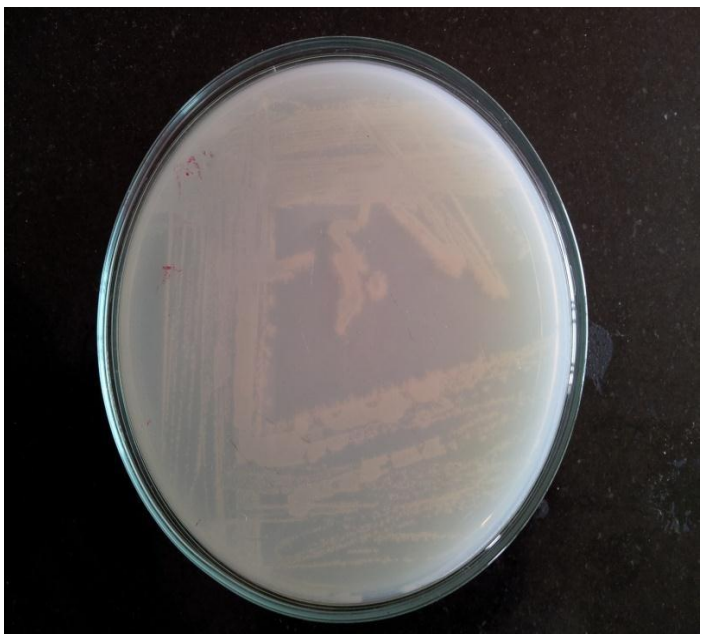

Fig 1. Hydrolysis of xylan from medium by *Bacillus licheniformis* strain JS

B] Production of xylanase:

The production of xylanase was carried out by using *Bacillus licheniformis* strain JS. The 24 hrs fresh culture of the *Bacillus licheniformis* strain JS was inoculated into a mineral based medium containing pure xylan. The production of xylanase was also carried out by using agricultural waste like Rice husk and Wheat bran with distilled water and minimal salt solution.

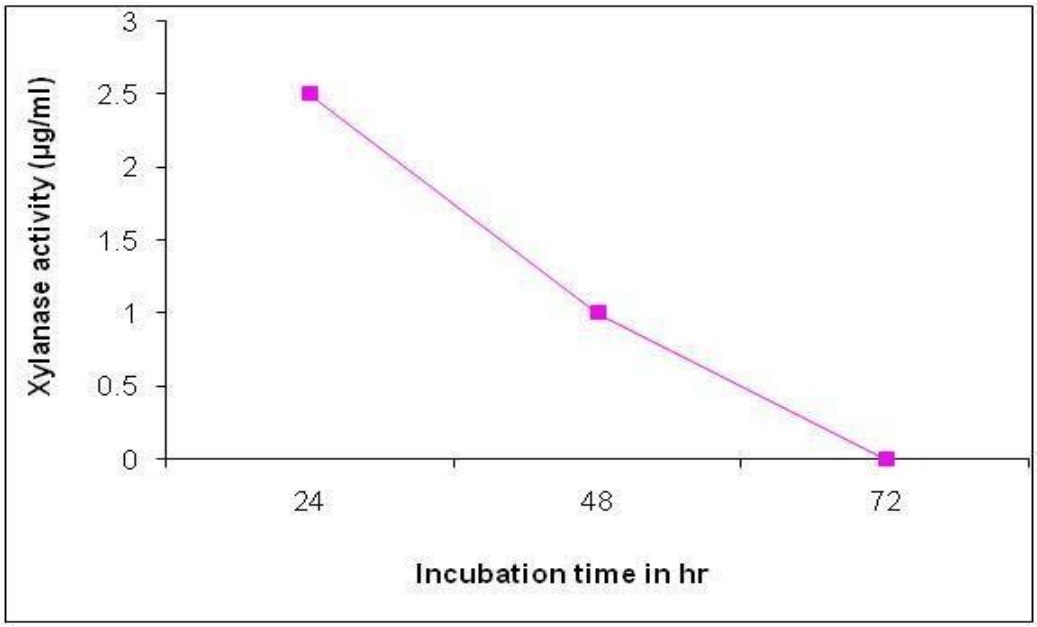

Fig 2. xylanase production in edium containing pure xylan.

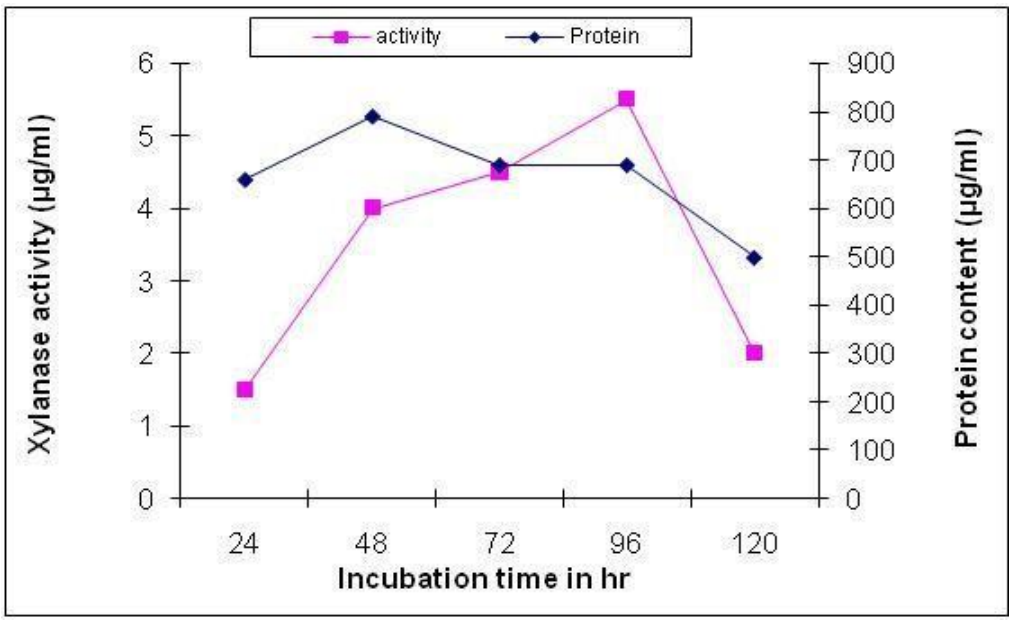

Fig 3. Xylanase production in medium containing Rice husk and minimal salt solution.

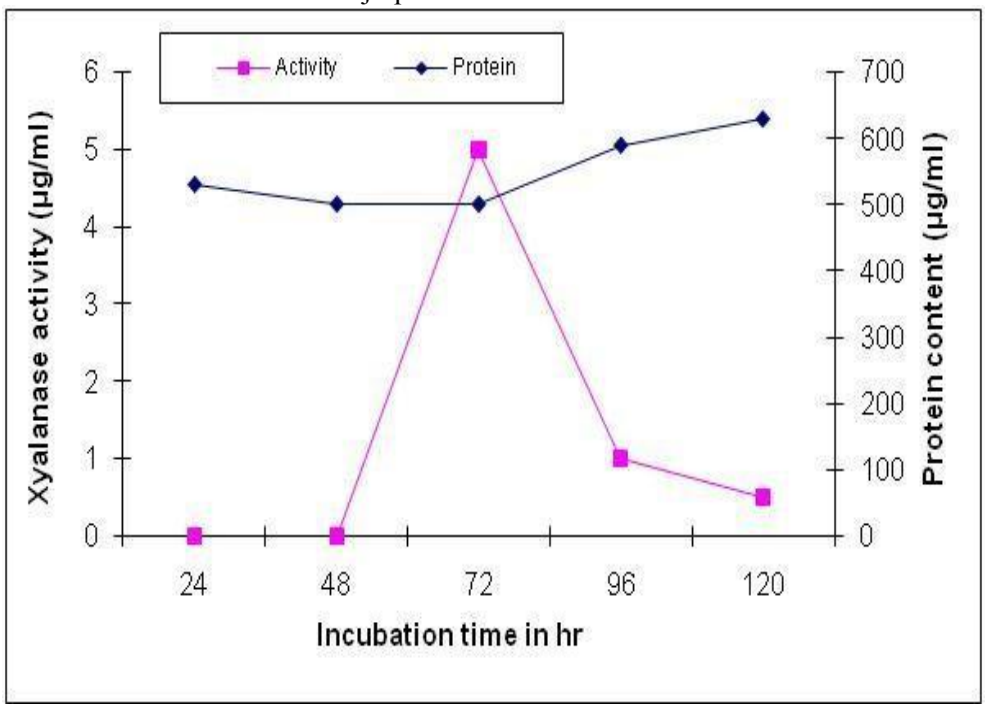

Fig 4. Xylanase production in medium containing Rice husk and DW.

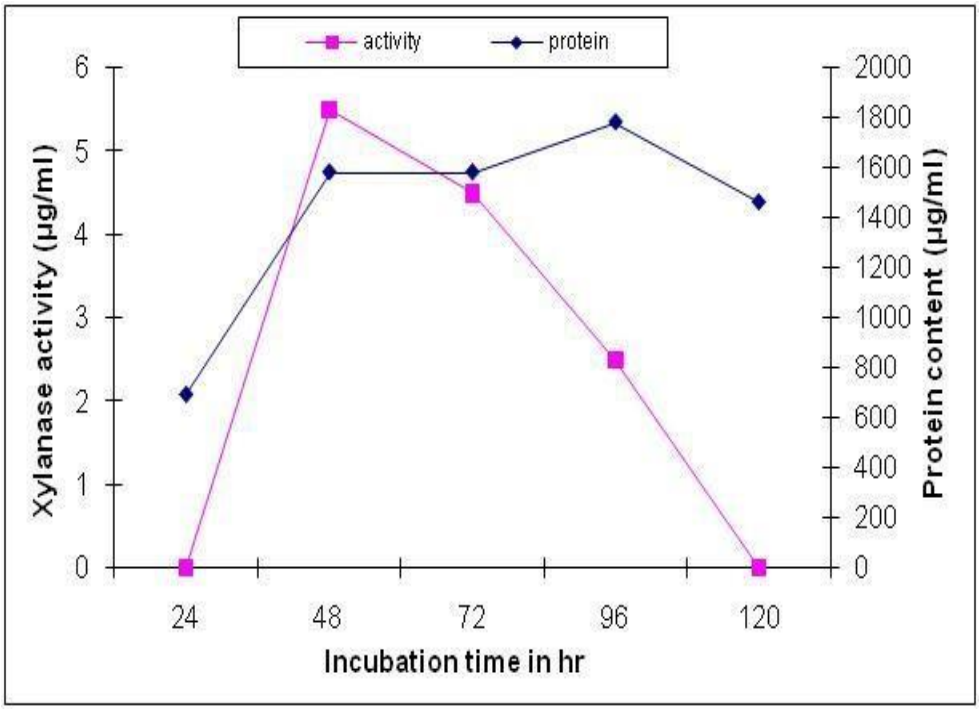

Fig 5. Xylanase production in medium containing Wheat bran and DW.

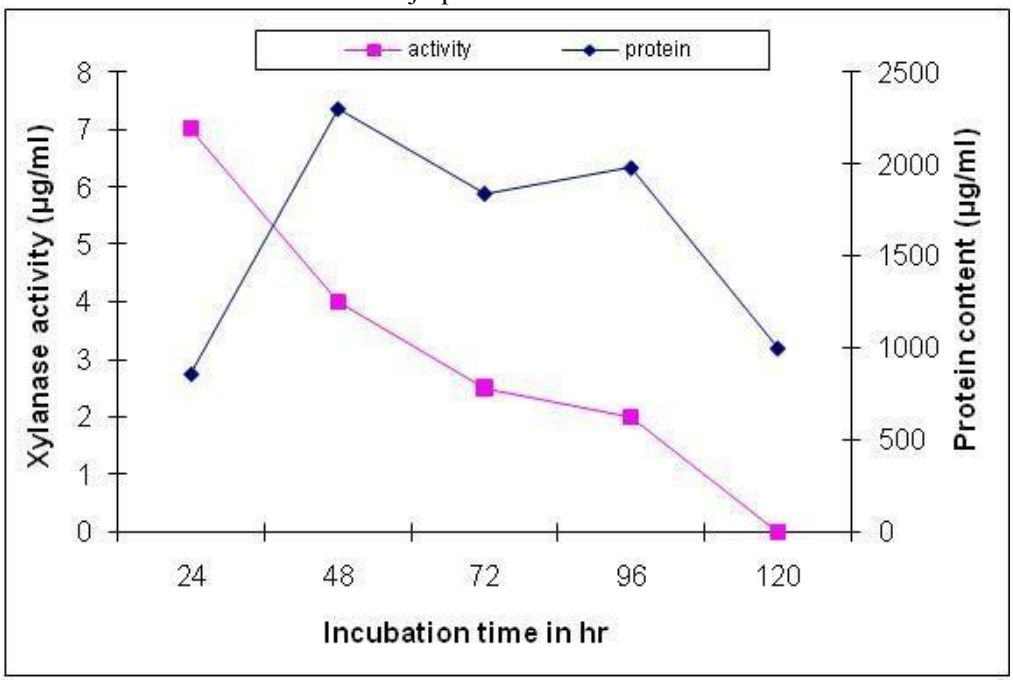

Fig 6. Xylanase production in medium containing Wheat bran and minimal salt solution.

C) Purification by Ion Exchange Chromatography:

Purification of xylanase of Bacillus licheniformis strain JS was carried out by the DEAE-cellulose column chromatography. The column was packed with activated DEAE- cellulose equilibrated with 50 mM sodium phosphate buffer. The height of column was 20 cm with the 2.5cm diameter. The protein was eluted with the 0.1 to 1.0 M NaCl gradient. The 100 fractions were collected having 5ml volume of each fraction with the flow rate of 1ml min-1. All the steps were carried out at 4 to 8ºC. It was found that the xylanase was not eluted by the NaCl gradient 0.1M to 1.0M, but it was eluted when the column was washed with 1.5 M NaCl.

D) Characterization of Xylanase

1) Effect of pH on enzyme activity:

In the effect of pH on enzyme activity the xylanase was found to be active between the pH 6.0 to 9.0. The optimum pH of the enzyme was found to be 7.0. It indicates the xylanase of B. licheniformis is neutrophilic in nature.

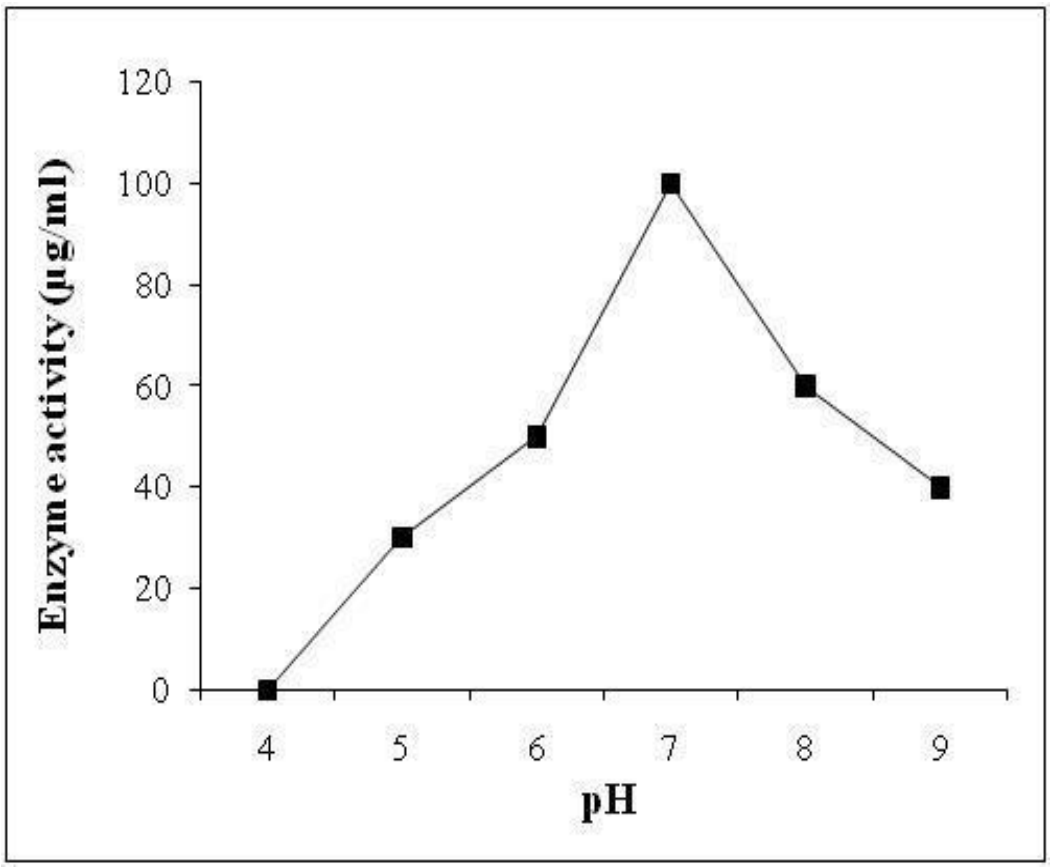

Fig 7. Effect of pH on xylanase activity

3) Effect of Metal ions on Enzyme activity:

The effect of metal ions on enzyme activity was carried out in presence of various salts of metal ions such as KCL, $MnSO_4$, $MgSO_4$, Hgcl2, $ZnSO_4$, $CoCl_2$ and CuSO4. It was found that the activity of xylanase was increased in presence of KCl, whereas 80 % activity inhibited in presence of $MgSO_4$, HgCl2, and completely inhibited by $MnSO_4$, $ZnSO_4$, and $CoCl_2$, as shown in Fig-8. Therefore was acts as activator of xylanase purified from the *B. licheniformis.*

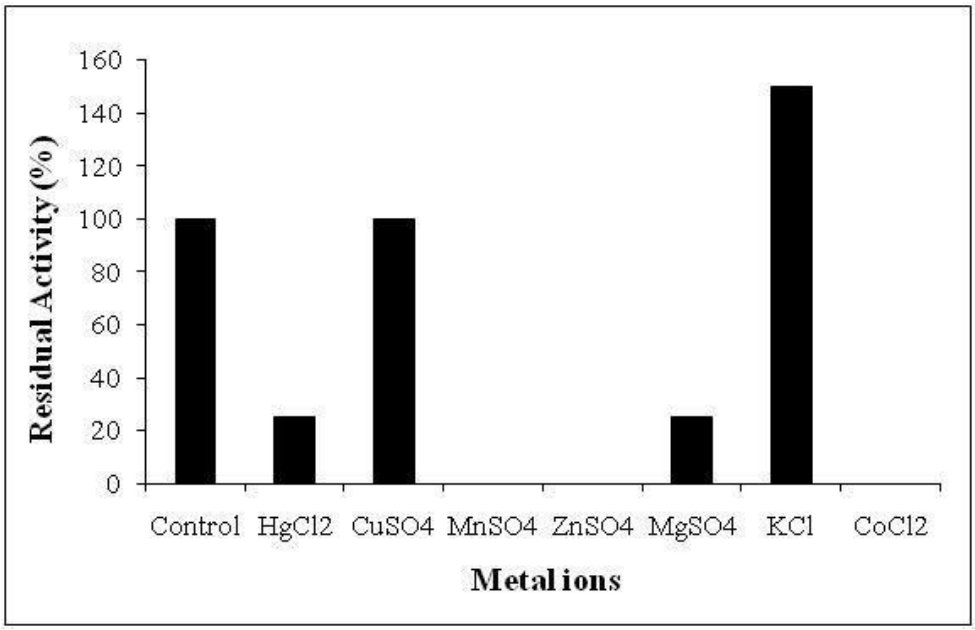

Fig. 8 Effect of metal ions on xylanase activity

E] SDS-PAGE Analysis

The purified xyalanase enzyme was analysed SDS-PAGE for the molecular weight determination. It was found that the enzyme was found to be approximately 66 kDa with single polypeptide chain, which indicates the enzyme was purified by the ion exchange column chromatography.

G) Application of Xylanase

1) Immobilisation of xylanase enzyme:

To analyse the different products formed after the hydrolysis of xylan. TLC was carried out. It was seen that xylan was hyrolysed into xylobiose and xylotriose i.e. xylo-oligosaccharides were produced, as shown in fig-9.

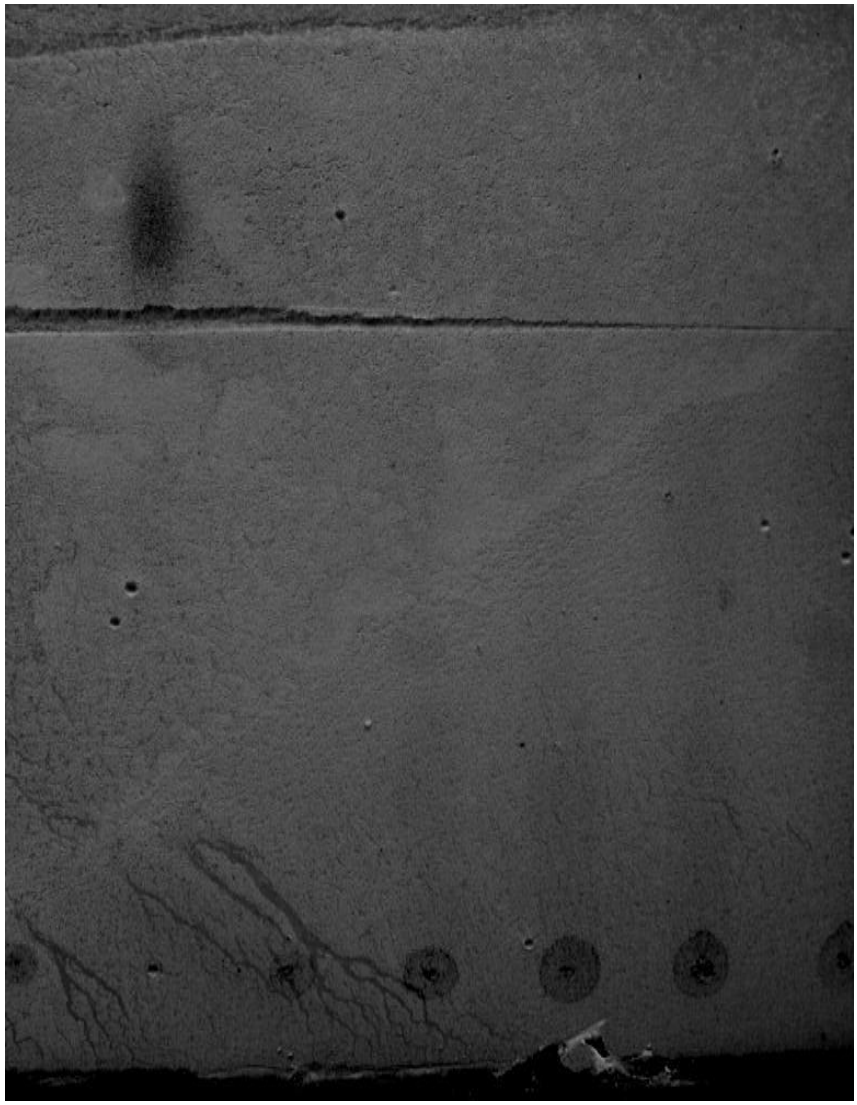

Fig 9. TLC plate showing hydrolysed products of xylan.

2) To study the effect of wheat bran and its hydrolysed product on the germination of *Vigna aconitifolia* was carried out. It was found that in presence of hydrolyzed product of wheat bran which was treated by xylanase, the germination rate of *Vigna aconitifolia* was higher than control and in presence of wheat bran.

Figure 10 Effect of xylooligosaccharides on the germination of Vigna aconitifolia.

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