PURIFICATION AND KINETIC STUDY OF XYLANASES FROM *LYSINIBACILLUS BORONITOLERANS* ISOLATED FROM SOIL

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Abstract: Hydrolysis of xylan, the chief type of hemicellulose is achieved by group of xylanolytic enzymes endo-1, 4-β-xylanase and β-xylosidase among other such enzyme complex. These enzymes are mainly produced by fungi, bacteria. Xylanase finds applications in animal feed, manufacture of bread, beverages, textiles, bleaching cellulose pulp, ethanol and xylitol production. Xylanase depolymerizes xylan molecules into xylose units, a primary carbon source for bacteria and fungi. In this study, a bacterial isolate was obtained from soil exhibiting good extracellular xylanase activity. The bacterium identified was *Lysinibacillus boronitolerans*. Xylanases produced were partially purified and maximum activity was obtained at 60% ammonium salt concentration. The enzyme kinetics and properties were studied by DNSA method. The maximal enzyme activity was demonstrated at 55˚C and pH 5.0. Crude extract fractionated by ammonium sulphate precipitation had a specific activity of 1.6 U/mg of protein. Xylanase demonstrated Km value 6.0 mg ml-1 and Vmax of 213.14 µmol/ml/min. Molecular weight of xylanase was found to be 40 KD as determined by SDS-PAGE. Thus the present study reveals that the bacterial strain *Lysinibacillus boronitolerans,* a potent xylanase producer could be of potential use in industry.

Keywords: Extra-cellular xylanases, *Lysinibacillus boronitolerans,* kinetic study, Industrial application.

Introduction: Xylanases (endo-1,4-β-D-xylan xylanohydrolase) are extracellular enzymes that degrade the xylan backbone into small oligomers (xylose). They are produced by microorganisms such as bacteria, mycorrhizic fungi and some yeasts[1][2]. Theycan be classified as endo- and exo-xylanases[3]. Exo-xylanases (ß-D-xylopyranosidase)are sometimes referred to as extracellular xylanases. *Bacillus* species secrete appreciable levels of extracellular xylanases[4]. Xylanases have wide range of industrial and environmental applications. They are in demand for paper and pulp industries, extraction of coffee, plant oils, starch and used in combination with pectinase and cellulasefor clarification of fruit juices[5]. The enzymes also finds applications in textile industry for enhancement of fiber quality[6][7][8].

The present study deals with isolation of potent xylanase producing organism/s from soil and partial purification of the extracted enzymes. The study also includes kinetics of the above enzyme and its characterization.

Materials And Methods:

Collection and Enrichment of soil samples: Fifty soil samples were collected from areas in and around the sugar-cane growing fields in Nasik, Katraj (Pune) and from the forest regions of Matheran and Sanjay Gandhi National Park (Mumbai). They were used for isolation of xylanolytic organisms. For enrichment, the nutritional and environmental conditions of xylanolytic organisms were taken into consideration. One gram of soil sample was inoculated in 100 ml sterile modified Macbeth's

brothcontaining (g/l) K_2HPO_4, 2; $CaCO_3$, 2; $MgSO_4$, 2; Na_2CO_3, 2; $(NH4)_2SO_4$, 2 and 1%(w/v) xylanas the main source of carbon, pH 7.0, and incubated at room temperature on a rotary shakerfor 3-4 days. These enriched soil samples were further used for isolation of xylanolytic organisms.

Isolation of xylanolytic organisms: Xylanolytic organisms were isolated from other natural populations by using selective medium containing xylan as major source of carbon. In the following study, loopful of culture from enriched broth was inoculated on sterile xylan agar plates [composition of the medium (g/l) (NH4)2SO4, 1.0; MgSO4, 2.0; K2HPO4, 2.0; CaCl2, 2.0; yeast extract, 1.0; xylan, 10; Agar 15, pH 7.0] and were incubated at room temperature for 2-3 days[9][10]. Seven xylanolyticisolates (growing on selective medium where xylan was the major source of carbon) were obtained. They were studied macroscopically as well as microscopically and labelled as HC-2, HC-3, HC-4, HC-7, HC-8, HC-10 and HC-11. The isolates were sub cultured on nutrient agar slants and stored at 4˚C for further studies.

Qualitative determination of xylanolytic activity:

Qualitative estimation of xylanolytic activity was confirmed by the 'Dye diffusion from a hemicellulose-dye complex' technique. A (soluble) xylan was bound to the dye bromothymol blue to form the substrate BTB-xylan giving yellow coloured plates[11][12]. The isolated cultures were spot inoculated on these sterile xylan agar plates incorporated with bromothymol blue and incubated at room temperature for 3 days. The plates were checked for blue coloured zone around the colonies after every 24 hours. These zones were measured and isolates showing comparatively bigger blue coloured zone were selected.

Quantitative estimation of xylanolytic activity Xylanase activity was detected quantitatively by estimating total amount of reducing sugar (xylose) released from xylan [12][13]. 1 ml of the above bacterial suspensions (adjusted to 0.1 O.D at 540 nm) was inoculated in 10 ml of 1% (w/v) xylan (prepared in 50 mM citrate phosphate buffer) and incubated at R.T for 48 hours under shaker conditions. Aliquots were taken at 0, 24 and 48 hours. The amount of reducing sugar formed was determined by p - bromoaniline method using 1% (w/v) xylose as standard. Based on the experimental observations isolate HC-2 was found to be a potent xylanase producing culture and was selected for further enzyme study.

Identification of the isolate HC-2:

The isolate HC-2 was identified on the basis of its colony appearance, morphology, Gram character and biochemical characters using Bergey's manual of determinative bacteriology, 9th edition and confirmed by 16S rRNA gene analysis[14][15]. Isolate HC-2 was identified as *Lysinibacillus boronitolerans* and was used for further enzyme studies.

Enzyme production and purification: Xylanase is an extracellular enzyme produced by xylanolytic strains. In the following study xylanase enzyme was produced using isolated *Lysinibacillus boronitolerans*culture and the conditions required to obtain high yield were optimised. DNSA method of sugar determination was followed throughout the study for estimation of reducing sugar produced at every optimisation step.

Inoculum preparation [16]:
The inoculum was prepared by growing *Lysinibacillus boronitolerans* in 250 ml Erlenmeyer flask containing 50 ml of sterile nutrient broth incorporated with 1% xylan. The system was incubated on a rotary shaker set to 120 rpm at RT for 24 hours.

Enzyme production [16]:

Xylanase production was carried out by inoculating 5 ml of 24 hour old inoculum (adjusted to O.D 0.1 at 540 nm) in 500ml of sterile nutrient broth incorporated with 1% (W/V) xylan as the carbon source. This set up was incubated for 48 hours on a rotary shaker set to 120 rpm at RT. After 48 hours the biomass was separated by centrifugation (10,000 rpm/10mins) at 4˚C. The supernatant was collected for further studies.

Purification of xyalanses[17]:

In the present study a two-step purification process was carried out to prepare crude extract of enzyme.

Purification by ammonium sulphate precipitation:

Purification of enzyme was done by salting out technique using ammonium sulphate for precipitation. Different fractions of the precipitate (10-100%) were obtained from the cell free supernatant and tested for xylanase activity. Fraction showing maximum xylanase activity was subjected to dialysis.

Purification by dialysis:

Dialysis of crude enzyme for removal of salts was done using standard HiMedia make dialysis bagsThe precipitate obtained was dissolved in 50mM Phosphate buffer (pH 8.0) and was dialyzed in 12 KD membrane against same buffer. This partially purified enzyme was used for further enzyme studies.

Determination of enzyme activity:

Xylanase activity was detected using Okeke & Obi method[18].The reaction mixture contained 0.5 ml of the above crude enzyme and 0.5 ml of 1% (w/v) xylan in 50 mM citrate phosphate buffer (pH 5.5). The system was incubated at RT for 30 mins. The release of reducing sugars was estimated by DNSA method[19] and the xylanase activity was calculated using xylose standard curve. One unit of enzyme activity was defined as the amount of enzyme required for releasing 1 mol of reducing group per minute per ml.

$$\text{Enzyme Units} = \frac{\text{'X' x } 10^{6}}{\text{Molecular weight of xylose x Time in min. x amt. of enzyme}}$$

Where, X = Reducing sugar content of enzyme obtained from the standard xylose curve

Study of Enzyme kinetics:

The activity of xylanase was determined at varying temperature, pH and substrate concentrations. The molecular weight of the enzyme was also found out.

Effect of Ph:

The optimum pH for xylanase activity was determined from the enzyme-substrate reactionsset up at range of pH value (Na-Citrate buffer pH 4, 5, 6 and Phosphate buffer pH 7 and 8) at 37ºC for 30 min.

Effect of temperature:

The optimum temperature for xylanase activity was determined in this phase. For this, the xylanase enzyme extract and substrate xylan were incubated at a range of temperatures: 4ºC, R.T, 37ºC, 55ºC and 100ºC for 30 mins maintaining optimum pH constant at 5.0

Effect of substrate concentrations:

Activity of partially purified xylanase was determined at a range of concentrations of xylan between 2.0 mg/ml – 20 mg/ml at optimum pH and temperature, obtained in the earlier experiments. The Michaelis – Menten constant Km and Vmax values for partially purified xylanase were also determined.

Protein Measurements:

Protein content was determined using Folin – Lowry's method. 1 mg/ml Bovine serum albumin was used as a standard. The results were used to calculate specific activity which was calculated

as Enzyme units/ml /Protein conc. mg/ml.

Molecular weight determination:

Molecular weight of xylanase enzyme was determined by Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis technique.10% of acrylamide gel stained with Coomassie Brilliant Blue R-250 was used. Standard molecular weight marker(HiMedia) was used to compare and determine the molecular weight of the above enzyme.

Results And Discussion:

Isolation, Identification and Estimation of Xylanolytic Activity:

Isolation of xylanolytic organisms was done using sterile xylan agar plates incorporated with bromothymol blue indicator. Xylanolytic activity was determined qualitatively by the size of blue coloured zone around the colony which indicates the presence of xylanolytic enzymes. Isolate HC-2 produced a larger zone compared to other isolates. Quantitative estimations of xylanolytic activity is depicted in Figure 1. It can be seen from the figure that isolate HC-2 showed higher xylanolytic activity and hence was used in further studies. Identification of the bacteria was done based on biochemical studies and 16S rRNA gene analysis. The isolate HC-2 was identified as *Lysinibacillus boronitolerans* and was used in the further phases of study.

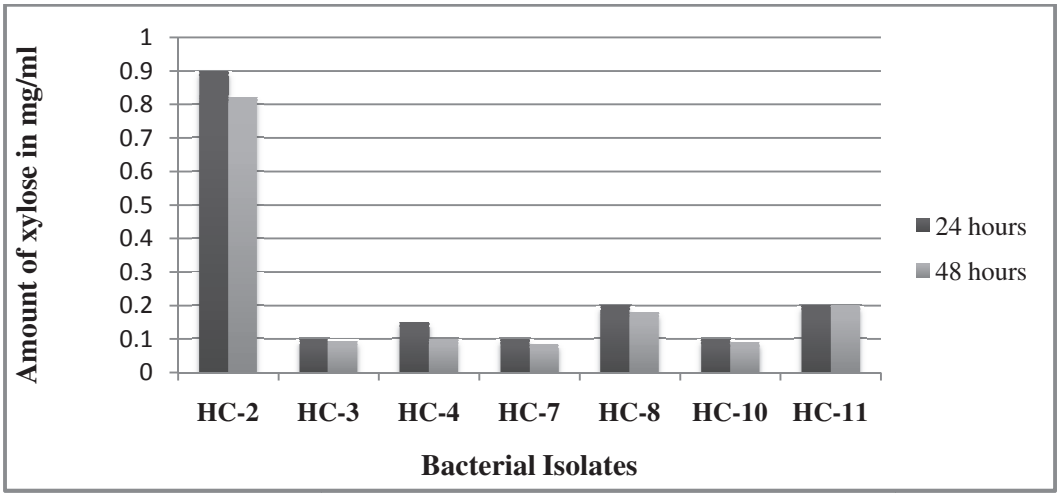

Figure 1: Quantitative estimation of hemicellulolytic activity of the isolates

Enzyme production and assay:

Xylanase production was carried out by the isolated xylanolytic strain *Lysinibacillus boronitolerans* in an Erlenmeyer flask, under conditions stated in Materials and methods. Xylanases were then subjected to a twostep purification process to obtain crude enzyme..The enzyme activity was determined by Okeke & Obi method using the standard xylose curve which is shown in Figure 2. The sugar concentration produced by the enzyme was found to be 0.21 mg/ml. The enzyme units were then calculated and it was as 84.44U/ml.

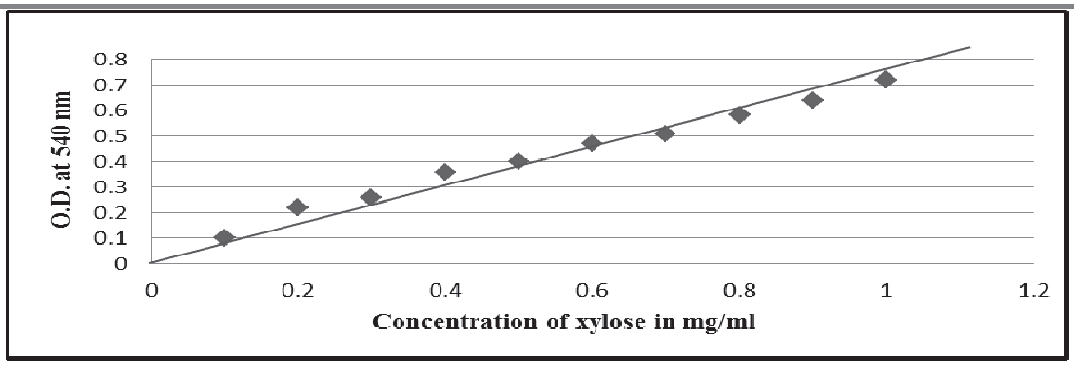

Figure 2: Estimation of xylanase activity by Okeke & Obi method

Enzyme Kinetics: The results of the effect of pH conditions on enzyme substrate reactions are depicted in Figure 3. Maximum xylanase activity was found at pH 5.0, suggesting acidic nature of enzyme.It can also be seen that at pH 8.0 xylanase activitydecreased rapidly reaching insignificant levels.Similar results were reported by Polizeli et al.[2]. Effects of temperature on substrate reactions are presented in Figure 4. The maximum activity of enzyme was observed at 55ºC. Xylanases from *Bacillus* sp. are usually effective at a temperature range between 50-80°C[20][21].

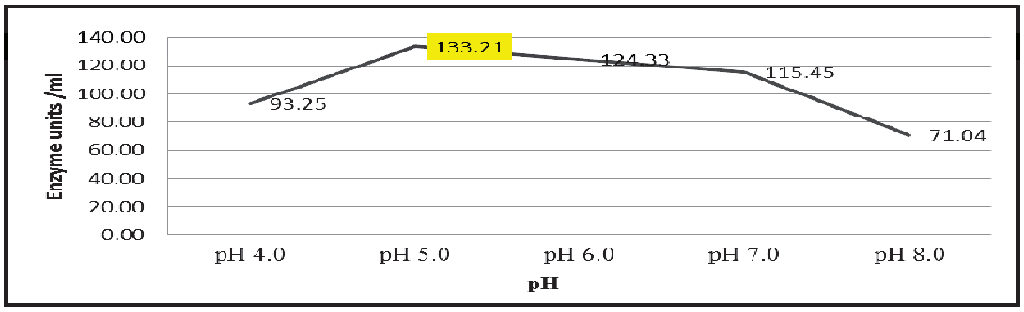

Figure 3: Effect of pH on xylanase activity

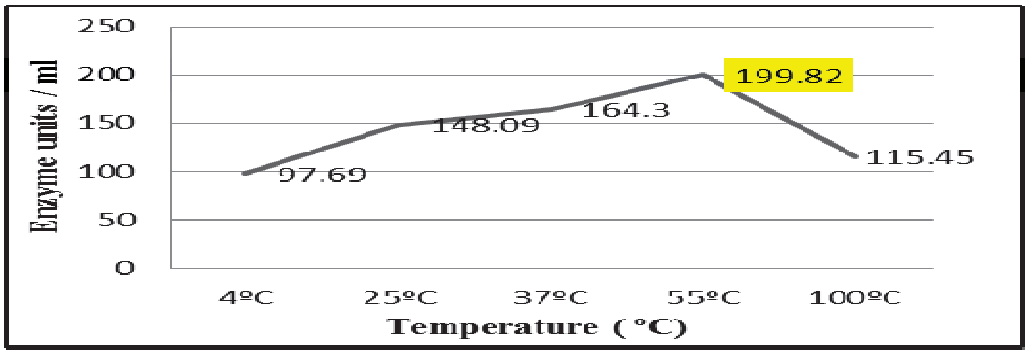

Figure 4: Effect of Temperature on xylanase activity

Substrate concentration is one of the most important factors which determine the velocity of enzyme reactions. The activity of partially purified xylanase was checked at various substrate concentrations ranging between 2.0 mg – 20 mg xylan/ml of buffer. As shown in Figure 5, the enzyme activity of xylanase was found increasing correspondingly with increase in substrate concentration.

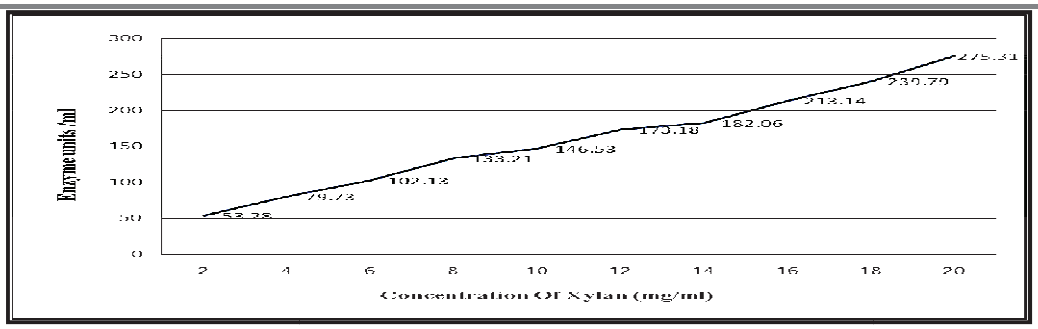

Figure 5: Effect of substrate concentrations on xylanase activity

Based on the Michaelis-Menton graphshown in Figure 6, the Km valuewas calculated as 6 mg/ml and the Vmax of partially purified xylanase was 213.14µmol/ml/min.The enzyme xylanase required a lower substrate concentration to reach Vmax, thus the xylanases appear to show higher catalytic power, and potentially could have higher technology efficiency.

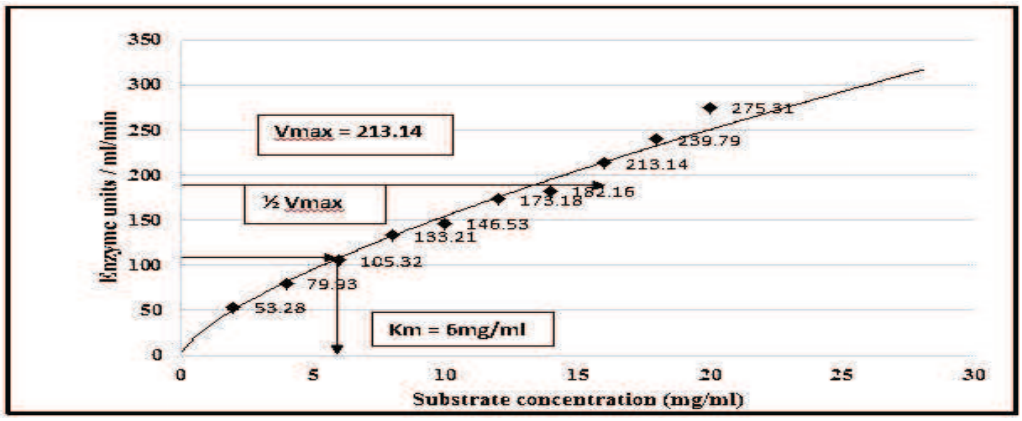

Figure 6: Kinetic parameters of xylanases

Estimation of protein content of enzyme: Protein content of enzyme estimated by Folin – Lowry's method was 52.5 mg/ml. Specific activity of xylanase was found to be 1.6 U.

Molecular weight determination of enzyme: SDS-PAGE followed by Coomassie Brilliant Blue R-250 staining method revealed single band of enzyme. Molecular weight of purified xylanase enzyme was found to be 40 KD.

Conclusion:

A potent xylanase producing bacterium was isolated from soil and cultured in a medium containing xylan as the substrate. The bacterium was identified as *Lysinibacillus boronitolerans.*Enzyme kinetics study was carried out to determine optimum pH, temperature and substrate concentration for enzyme activity. A broad range of acidic to alkaline activity was observed for the xylanase enzyme which could have potential application in industries. Further studies to enhance enzyme yield, quality and efficacy, as well as scale-up could be accomplished based on the current findings.

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