Characterization of thermoalkalophilic xylanase isolated from Enterobacter isolates

Anjana Sharma*, Rajesh Pujari and Pratibha Patel
Bacteriology Laboratory, Department of P G Studies and Research in Biological Sciences
R D University, Jabalpur 482 001 India

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Thermoalkalophilic xylanase was isolated from Enterobacter spp. with significant activity. Maximum enzyme activity was observed in isolate BGCC#259 (E. cloacae), i.e., 0.056 IU/mL at 24 h, while the highest biomass was observed at 36 h of incubation. Hydrolysis study revealed complete degradation of xylan to xylose after 12 h of incubation. The molecular mass of the xylanase was ~43 kDa. The enzyme was characterized at varied range of pH and temperature, and it was found that all five Enterobacter isolates had a pH and temperature optima of 8.0 and 80°C, respectively. At 80°C, xylanase from isolate BGCC#254 (E. cloacae) retained 100% activity, while BGCC#259 retained more than 90% activity after 24 h.

Keywords: Characterization, Enterobacter, enzyme activity, thermoalkalophilic, xylanase

Introduction

Biodegradation of xylan, the major component of hemicellulose, is a complex process that requires the coordinated action of several enzymes. Xylanases cleave internal linkages on the β-1,4-xylose backbone. Xylanases are widely used in industries as additives for animal feed to enhance its digestion and diminish the health problem due to undigested plant fibre; in production of ethanol, aroma and fruit juices; in baking, including textile and paper and pulp industries.

Cellulase-free xylanases active at high temperature and pH are gaining importance in pulp and paper technology as alternatives to the use of toxic chlorinated compounds. If xylanases are to be used for industrial applications, the enzyme must be thermophilic and alkalophilic in nature. However, most of the xylanases known to date are optimally active at temperatures below 50°C and are active in acidic or neutral pH. Only a few xylanases reported to be active and stable at alkaline pH and high temperature. Therefore, there is a need to search for new sources of xylanases and their characterization, especially those with extremophilic properties.

In view of the above, present investigation was undertaken to isolate and characterize thermoalkalophilic xylanase from Enterobacter isolates.

Materials and Methods

Bacterial Strains

Fifty seven Enterobacter isolates used in this study were obtained from Bacterial Germplasm Collection Centre (BGCC), Bacteriology Laboratory, Department of Biological Sciences, Rani Durgavati University, Jabalpur (MP), India and the reference strains (MTCC 509, MTCC 621, MTCC 659 and MTCC 111) were obtained from the Microbial Type Culture Centre (MTCC), Institute of Microbial Technology, Chandigarh, India.

Screening of Enterobacter Isolates for Xylanase Production

An enrichment technique involving xylan as a sole source of carbon was used to isolate and screen xylan-utilizing bacteria. Screening of bacterial isolates for xylanase activity was performed by following the congo red assay method. After 48 h of incubation period at 37°C, the plates were stained with 1% congo red and destained with 1 M NaCl. Xylanase-positive isolates were identified by a zone of clearing around the isolates.

Potent xylanase producing cultures were grown in Basal Salt Solution (BSS) containing 0.5% xylan (w/v) as sole carbon source at 40°C and pH 8.0 for 72 h. The composition of the medium (w/v) was as follows: NaCl, 30 g; KCl, 0.75 g; MgSO₄, 7 g;
NH₄Cl, 1 g; K₂HPO₄ (10%), 7 mL; KH₂PO₄ (10%), 3 mL; trace metal solution, 1 mL; distilled water, 1000 mL; the trace metal solution had the following composition: H₂BO₃, 2.85 g; MnCl₂·7H₂O, 1.80 g; FeSO₄·7H₂O, 2.49 g; Na-tartarate, 1.77 g; CuCl₂, 0.03 g; ZnCl₂, 0.02 g; CoCl₂, 0.04 g; Na₂MoO₄·2H₂O, 0.02 g; distilled water, 1000 mL.

Production of Enzyme
Pre-culture grown for above 48 h (A₄₅₀nm 0.6) was used to inoculate 100 mL of BSS medium (pH 8.0) in 500 mL Erlenmeyer flask and incubated in a shaker incubator (100 rpm) at 40°C. Samples were collected at 12 h interval for over a period of 72 h. Growth was monitored spectrophotometrically by measuring O. D. at 540 nm. Cell broth was centrifuged at 10,000 g and at 4°C for 10 min. The supernatant was then served as a crude enzyme extract.

Xylanase Assay
Xylanase activity was assayed using 1% birchwood xylan as substrate dissolved in 50 mM glycine-NaOH buffer (pH 9.0). The reaction mixture containing 10 µg of the crude enzyme and 2.5 mg of substrate, was incubated for 10 min at 100°C. After incubation, the amount of reducing sugars liberated was determined using 3,5-dinitrosalicylic acid (DNS) method. The absorbance of the reference samples (substrate solution incubated without enzyme and diluted enzyme in buffer) was deduced from the values of the test samples. One unit of xylanase activity was expressed as µmol of reducing sugars (xylose equivalent) released in 1 min under above conditions.

Protein Estimation
Protein was estimated following the method of bicinchoninic acid with Bovine Serum Albumin (BSA) as standard.

Optimization of Culture Media for Xylanase Production
Different concentrations of yeast extract (0-3 mg/mL), phosphate (potassium phosphate, 0-3 mg/mL), nitrogen (ammonium chloride, 0-3 mg/mL) and peptone (0-3 mg/mL) were supplemented in the culture medium to optimize xylanase production. Xylanase activity was analyzed after growing the culture for 48 h, wherein concentration of one of the nutrients was varied in culture medium.

Hydrolysis Studies
To 50 mL of xylan suspension (1% of birchwood xylan in 50 mM glycine-NaOH buffer, pH 9.0), 0.45 mg of xylanase enzyme (90 U/mL) was added and incubated at 70°C. Samples (5 mL) were removed at 12 h interval upto 72 h. The unused xylan from the sample was precipitated using isopropanol. The precipitate was removed by centrifugation at 10,000 g and at 4°C for 10 min. The supernatant was analyzed by thin layer chromatography (TLC) to detect hydrolyzed products using a mixture of acetone:n-butanol:water (8:1:1 v/v) as a solvent system.

Molecular Mass Determination
The molecular mass of xylanase was determined by SDS-PAGE as described by Laemmli using medium range (14.3-97.4 kDa) molecular weight markers (Bangalore Genie Pvt., India)

Effect of Temperature on Xylanase Activity
The optimal temperature for xylanase activity was obtained by assaying the residual enzyme activity at different temperatures (40-100°C). Effect of temperature on enzyme activity was determined by incubating the crude enzyme extract along with the substrate for 10 min, i.e., 40-100°C with the regular increase of 10°C at respective temperature. The residual enzyme activity was measured at 3 h interval upto 24 h using DNS method.

Effect of pH on Xylanase Activity
The relative xylanase activity using 1% (w/v) birchwood xylan was determined at pH 4.0 to 10.0. Citrate buffer (0.05 M) was used for pH 4-6, phosphate buffer for pH 6-8 and glycine-NaOH buffer for pH 8-10. The residual enzyme activity was estimated at 3 h interval upto 24 h.

Results
Of 57 Enterobacter isolates screened for xylanase activity, 5 isolates, viz., BGCC#215, 250, 254, 259 (E. cloacae) and BGCC#243 (E. aerogens), showed significant xylanase activity (Fig. 1). Isolate BGCC#259 showed maximum xylanase activity of 0.056 IU/mL at 24 h of incubation, while BGCC#215 showed maximum activity of 0.052 IU/mL and BGCC#250 showed lowest enzyme activity of 0.037 IU/mL at 36 h of incubation. Isolates BGCC# 243 and BGCC#254 also showed reasonable level of enzyme activity of 0.047 and 0.043 IU/mL, respectively at 36 h of incubation (Fig. 1).

Optimization of Growth Medium for Xylanase Production
Enterobacter spp. showed the highest xylanase production when the growth medium was
supplemented with yeast extract (2 mg/mL), potassium phosphate (1 mg/mL), ammonium chloride (1 mg/mL) and peptone (1 mg/mL) at 40°C after 48 h of incubation (Fig. 2).

**Analysis of Hydrolytic Products**

In the early stages of the reaction, a large number of intermediate products were obtained but there was no trace of xylose until 11-11.5 h of reaction time. After 12 h of incubation, xylose was detected as one of the hydrolysis products. These results indicated that initially xylanase cleaved the substrate to liberate xylo-oligosaccharides and then the resulting oligosaccharides were probably cleaved to form xylose (Fig. 3).

**Molecular Mass Determination**

The enzyme showed a single protein band on SDS-PAGE. The molecular mass of denatured xylanase, estimated from the relative mobility of standard proteins on SDS-PAGE was ~43 kDa (Fig. 4).

**Effect of Temperature on Xylanase Activity**

The temperature optima of the xylanase enzyme was 80°C with a maximum enzyme activity of 0.064 IU/mL, retaining 80% enzyme activity for BGCC#215, while BGCC#254 and BGCC#259 showed maximum enzyme activity of 0.043 IU/mL and 0.060 IU/mL, respectively, retaining 100% and more than 90% activity at the same temperature. For BGCC#243 and BGCC#250, maximum enzyme activity of 0.058 and 0.044 IU/mL was observed, respectively retaining 80% and 84% of activity (Fig. 5).

**Effect of pH on Xylanase Activity**

The optimum pH for xylanase activity was found to be 8.0 with a maximum enzyme activity of 0.042 IU/mL, retaining 80% of activity for BGCC#215 and a minimum enzyme activity of 0.032 IU/mL for BGCC#254 that retained ~75% of activity at the same pH. At pH 8.0, BGCC#250, BGCC#259 and BGCC#243 showed enzyme activity of 0.033, 0.040 and 0.037 IU/mL, respectively retaining 90%, 70% and 78% activity (Fig. 6).

**Discussion**

Fifty seven isolates of *Enterobacter* spp. were screened for xylanase production and only five isolates were selected for further characterization on the basis of maximum clear halo orange digestion zone on plates containing birchwood xylan as carbon

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**Fig. 1**—Effect of incubation time on the growth and activity of enzyme xylanase

**Fig. 2**—Effect of yeast extract (a), phosphate (b), ammonium chloride (c) and peptone (d) on the production of xylanase from *Enterobacter* spp.
source. The present study demonstrates the significant thermoalkalophilic xylanase activity in *Enterobacter* spp. The optimization of production medium is a very useful tool to attain high levels of enzyme activity.

Xylanase enzyme from *Enterobacter* spp. showed highest activity at pH 8.0 and 80°C temperature. It was observed that the enzyme activity was directly correlated with the growth of *Enterobacter* spp., except in the case of isolate BGCC#259 (*E. cloacae*), where the highest enzyme activity was found at 24 h, while the growth was maximum after 36 h. Till date, only few bacterial xylanase was reported. Sa-Pereira et al.\(^{16}\) reported that xylanase from *Bacillus subtilis*, when grown on SSF using oat spelt xylan, showed maximum activity at pH 6.0. Similarly xylanase isolated from *B. coagulans* BL69 grown on soybean residue showed a pH optimum of 7.6\(^{17}\).

In the hydrolysis studies the presence of xylose was detected at 12 h of incubation. The abundance of xylose increased over the period of immersion. These results indicated that initially xylanase cleaved the substrate to liberate xylo-oligosaccharides and then the resulting oligosaccharides were probably cleaved to form xylose.

In the present study, the molecular weight of crude xylanase enzyme from *Enterobacter* isolates was found to be ~43 kDa as compared with the standard. Xylanase obtained from *Arthrobacter* spp. was reported to be of ~20 kDa\(^{18}\) and that from *Bacillus* sp. strain K-1 of 23 kDa\(^{19}\). In case of *Bacillus* sp. 41M, the molecular mass of xylanase was found to be 36 kDa\(^{20}\), while xylanase isolated from *Bacillus subtilis* had a high molecular weight of 340 kDa. The multienzyme complex produced by *Paenibacillus curdlanolyticus* B-6 showed a molecular mass of >1,400 kDa\(^{16}\).

It can be concluded that the 5 isolates of *Enterobacter* spp., viz., BGCC#215, BGCC#250, BGCC#254, BGCC#259 (*E. cloacae*) and BGCC#243 (*E. aerogenes*), produced a novel thermoalkalophilic xylanase when grown on basal salt solution using birchwood xylan as a substrate. Xylanase activity was enhanced by optimizing the concentrations of four of the medium components. Activity of xylanase was highest when medium was supplemented with yeast (2 mg/mL), ammonium chloride (1 mg/mL),
phosphate (1 mg/mL) and peptone (1 mg/mL). TLC studies concluded that oligosaccharides were formed as a result of hydrolysis of xylan (as a substrate). The molecular mass of xylanase produced by Enterobacter isolates was ~43 kDa. Xylanase produced was active at pH 8.0 and 80°C temperature. Xylanase from Enterobacter spp. possessing these properties has better potential for industrial applications.

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References