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CHARACTERIZATION OF XYLANASE FROM A XYLANOLYTIC-THERMOPHILIC BACTERIUM ISOLATED FROM GUNUNG PANCAR HOT SPRING, WEST JAVA

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ABSTRACT

A xylanolytic-thermophilic bacterium (IT-08) was isolated from Gunung Pancar Hot Spring after two days of enrichment in Modified Thermus Medium (MTM) supplemented with 0.5% oat spelt xylan. 16S-rRNA sequence analysis indicated that IT-08 resembles *Bacilus thermoleovorans*, a species of thermophilic bacteria. When grown on xylan containing media, IT-08 produces a thermoactive xylanase. Xylanase from IT-08 was active at temperatures between 40 and 100°C, at pH values between 4.0 and 9.0 with optimum values obtained at 80°C and pH 6.0, respectively. SDS-PAGE and zymogram analysis demonstrated that a crude xylanase complex of IT-08 comprised two active bands with molecular masses of 78 and 60 kDa.

Keywords: xylanase / xylanolytic-thermophilic bacteria

INTRODUCTION

Xylan, a major component of plant cell wall hemicellulose, is composed of a backbone of β-1,4-linked xylose units with arabinose and acetate residues (Millward-Sadler *et al.* 1994). For complete hydrolysis of xylan, many xylanolytic microorganisms often synthesize the multiple groups of xylanolytic enzymes for cooperative actions (Kelett *et al.* 1990). These enzymes include endo-β-1,4-xylanases (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37), and enzymes that cleave side chain sugars from the xylan backbone such as α-arabinofuranosidases (EC 3.2.1.55) and acetyl esterases (EC 3.1.1.6).

Kraft pulping, a process widely used in paper manufacture, removes about 95% of the lignin by alkaline sulfate cooking. The remaining lignin gives the pulp a brown color that is removed in a multistage bleaching process using a variety of agents. Currently there is concern about the environmental impact of some of the compounds used in the process, particularly chlorine and chlorine dioxide. Enzymes, including xylanases, have been shown to reduce the amount of chlorine required to achieve comparable levels of paper brightness (Viikari *et al.* 1994). However, for xylanase to be used, xylanase pretreatment must be done at a high temperature and under alkaline conditions. Thermostable xylanases capable of acting at a high pH are, therefore, of great importance.

MATERIALS AND METHODS

Isolation and growth conditions

Isolate IT-08 was obtained from Gunung Pancar Hot Spring, Citeurep, Bogor. Enrichment was performed at 70°C in liquid Modified Thermus Medium or MTM containing: 100 mg/l nitrilotriacetic acid, 60 mg/l $CaCl_2$, 60 mg/l Na_2SO_4, 100 mg/l $MgSO_4$, 8 mg/l NaCl, 100 mg/l KNO_3, 700 mg/l $(NH_4)_2SO_4$, 111 mg/l Na_2HPO_4, 0.28 mg/l $FeSO_4$, 20 µl/l molibdate 1% 100 µl/l Sistrom vitamins and minerals stock, and supplemented with 5 g/l oat spelt xylan and 1 g/l yeast extract. For solid agar plates the medium was supplemented with 20 g/l agar. The pH of the medium was adjusted to 7.0. After enrichment, the culture was streaked on agar plates to obtain pure colonies.

Enzyme Production and Characterization

The inoculum was grown on 25 ml MTM supplemented with 0.25% yeast extract and 0.5% tryptone and incubated at 60°C for 8 hours. One ml of inoculum was added to 100 ml MTM supplemented with 0.25% yeast extract, 0.1% tryptone and 0.5% oat spelt xylan and placed on a shaker at 170 rpm at a temperature of 60°C. The enzyme was harvested after 14 hours of cultivation. The reaction mixture for enzymatic hydrolysis comprised: 250 µl 0.5% oat spelt xylan, 250µl crude enzyme (supernatant) and 500 µl buffer Tris-Cl 0.1M pH 7.5. Xylanase activity was measured after 30 min at temperatures ranging from 40 to 100°C. Enzyme activity was assayed at pH values between 4.0 and 10.0 at optimal temperature using various buffers (citrate-phosphate buffer for pH 4,5,6; phosphate buffer for pH 7,8; Tris-Cl buffer for pH 9; and glycine-NaOH buffer for pH 10.0). The influence of divalent cations on enzyme activity was assayed by adding 1 and 5 mM of the following: $CaCl_2$, $MgCl_2$, $ZnCl_2$, $CoCl_2$, $FeSO_4$, $NiCl_2$ and $CuSO_4$.

Thermostability was determined by incubating the enzyme solution in 50 mM phosphate buffer at pH 6.0 without substrate at 70 and 80°C. Aliquots were removed at different time intervals and immediately cooled on ice. Residual enzyme activity was assayed under standard conditions.

Electrophoresis

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and zymogram staining were performed as described by Sunna *et al.* (1997a). A suspension of oat spelt xylan (final concentration, 1g/l) was incorporated into the gels before polymerization. After electrophoresis the gels were washed in Triton X-100 (25g/l) for 60 min at room temperature and then incubated at 60°C for 2 hours in 50 mM sodium phosphate buffer, pH 6.0. The gels were stained with Congo red (1 g/l) for 30 min and destained with 1 M NaCl. Distinct bands with xylanase activity could be observed after the addition of 1 N HCl. The Low-molecular-mass calibration kit of Pharmacia was used as a protein molecular standard and stained with Coomassie brilliant blue R-250.

16S rRNA sequence determination and analysis

Genomic DNA extraction was performed using the ISOQUICK DNA extraction kit. PCR-mediated amplification of the 16S rRNA was performed as described by Marchesi *et al.* (1998) employing GeneAmp PCR System 2400 (Perkin Elmer). The PCR product was cloned into pGEM-T Easy Vector (Promega). Sequencing of the 16S rRNA was done using the ABI Prism 377 Automated DNA sequencer. The Cluster analysis was conducted according to a program provided by European Bioinformatics Institute (http://www.ebi.ac.uk) and the phylogenetic tree was constructed using Treecon (Van de Peer and De Wachter 1993). The distance estimation was determined according to jukes and Cantor (1969).

RESULTS AND DISCUSSION

One aerobic thermophilic strain (IT-08) was isolated from Gunung Pancar Hot Spring, Bogor. The result of 16S rRNA sequencing of isolate IT-08 showed that this isolate was closely related to *Bacillus thermoleovorans* (99.6% for 16S DNA sequence similarity). According to Wayne *et al.* (1987) the level of total DNA-DNA similarity for species boundaries was suggested to be around 70%. Sunna *et al.* (1997b) suggested that *B. thermoleovorans*, *B. kaustophilus* and *B. thermocatenulatus* should be combined into one species, namely *B. thermoleovorans*, which also includes the strains *B. caldolyticus*, *B. caldovelox* and *B. caldotenax*. All the bacillus strains share more than 99% in 16S rRNA sequence similarity. The constructed phylogenetic tree for IT-08 is shown in Figure 1.

Xylanase from IT-08 was active over a temperature range of 40 to 100°C (Figure 2) upon incubation for 30 min at pH 7.5. The optimal temperature is 80°C. The enzyme was active at a pH range of 4.0 to 9.0 and showed maximum activity at pH 6.0. At pH 7.0 to 9.0, it still demonstrated more than 80% activity (Figure 3). By comparison, xylanases obtained from *Bacillus stearothermophilus* T-6 (Khasin *et al.* 1993), *B. thermoalkalophilus* (Sridevi and Varma 1990), *Bacillus sp.* Strain TAR-1 (Nakamura *et al.* 1994), *B. thermoleovorans* K-3d and *B. flavothermus* LB3A (Sunna *et al.* 1997a) showed optimal activity at temperatures between 70 and 80°C at pH values of 6.0 – 7.0. The broad range of temperatures and pH values for enzyme activity is very useful to industrial application. Unfortunately, the enzyme

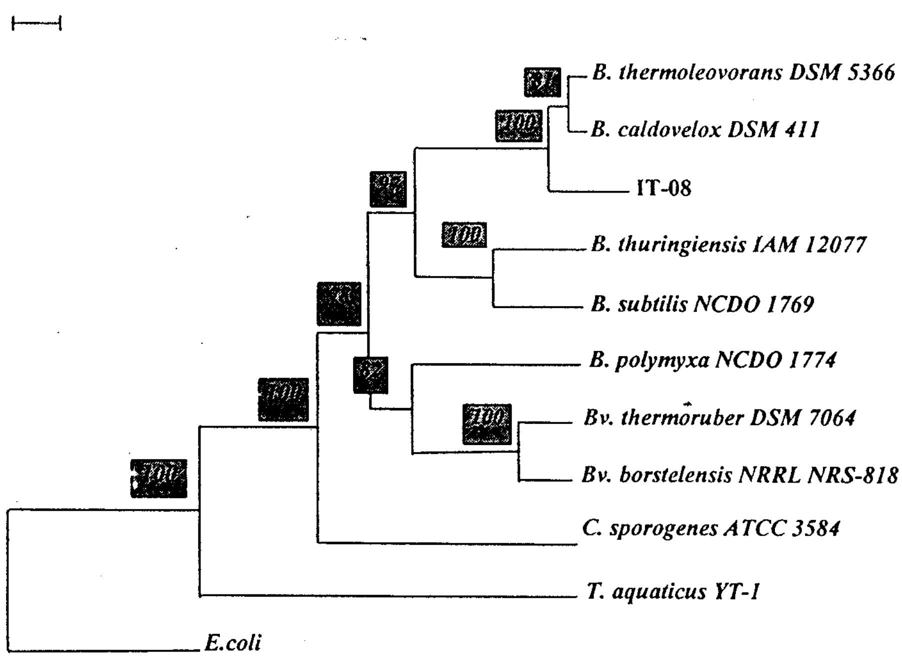

Figure 1. Phylogenetic tree construction for the IT-08 isolate

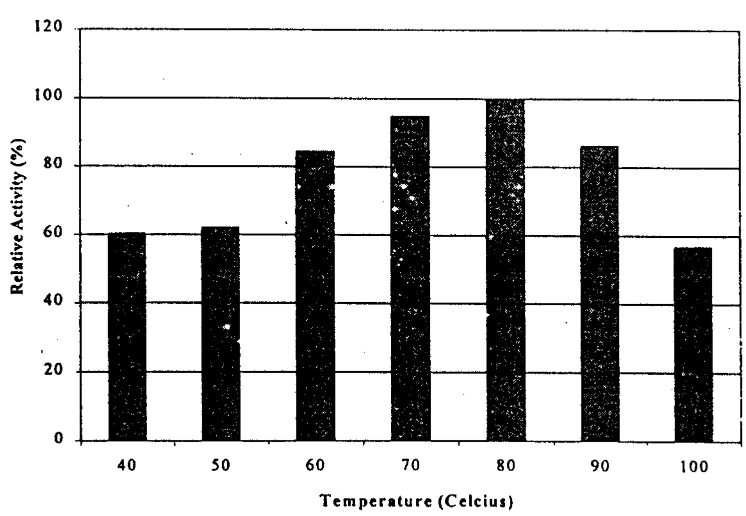

Figure 2. Influence of temperature on xylanase activity

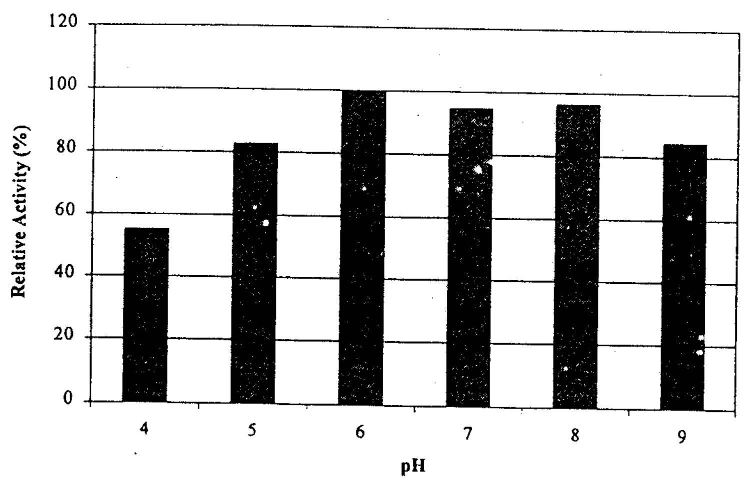

Figure 3. Influence of pH on xylanase activity

produced by IT-08 was not active at pH 10.0, required for application in commercial pulp bleaching.

Figure 4 shows that addition of cations Mg^{2+} and Ca^{2+} enhances enzyme activity. Treatment with Mg^{2+} increased enzyme activity by 40% and with Ca^{2+} about 22%. The results suggest that Mg^{2+} is a cofactor for IT-08's xylanase activity. On the other hand, other cations such as Cu^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+}, and Fe^{2+} and EDTA decrease enzyme activity. Addition of Fe^{2+} completely inhibited enzyme activity. EDTA treatment suggests that this enzyme has a metal cofactor requirement because administration of 1 and 5 mM EDTA lowers enzyme activity to approximately 40 and 68%, respectively.

Thermal stability of the enzyme was determined in the absence of substrate at 70 and 80°C. Xylanase produced by IT-08 was relatively stable after incubation at 70°C for at least 24 hours. At 80°C the xylanase showed a half-life value of 90 min (Figure 5). By comparison, xylanase of *Bacillus thermoleovorans* K-3d only has a half-life value of 18 min at 80°C (Sunna *et al.* 1997a) and *Thermotoga* sp., that has an optimum temperature for growth at 105°C, has a half-life value of 90 min at 95°C. Thermostability is important to certain industrial processes that require analyses at high temperature. Enzymes with low thermostability contribute to the increase in cost required for enzyme preparation.

The crude enzyme produced by isolate IT-08 consisted of two isozymes with molecular mass of about 78 and 60 kDa, respectively (Figure 6). The presence of two isozymes indicates that at least two xylanase-encoded genes are present in IT-08. Bacterial xylanase usually consists of a single subunit and many bacteria produce two xylanase isozymes (Kulkarni *et al.* 1999).

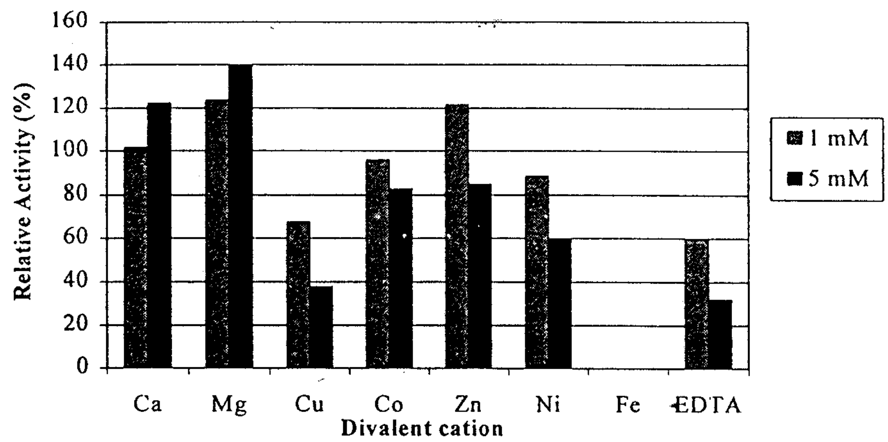

Figure 4. Xylanase activity in the presence of divalent cation and EDTA above the activity of xylanase in Tris buffer without added cofactors

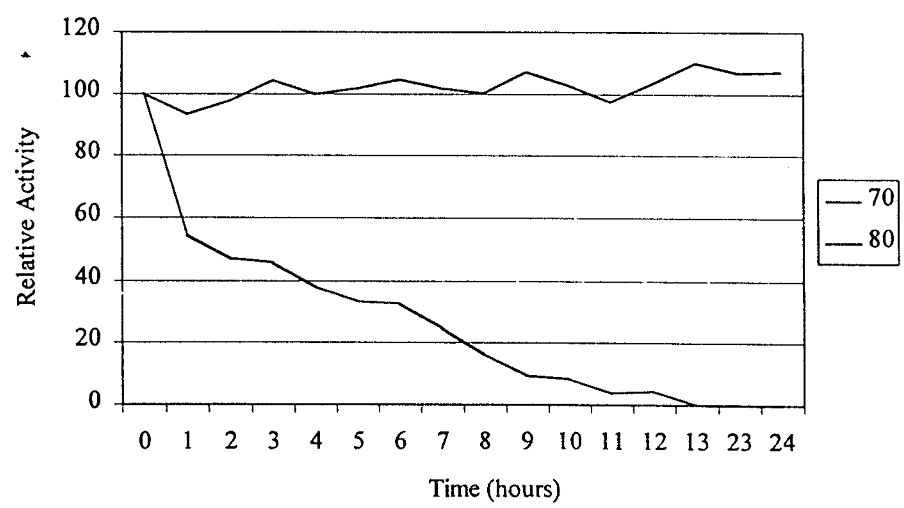

Figure 5. Thermostability of the xylanase from IT-08 isolate at 70 and 80°C

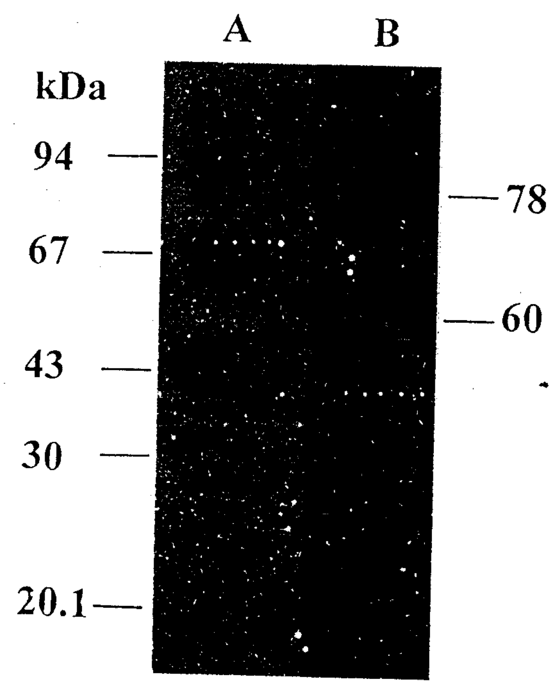

Figure 6. Zymogram of crude xylanase from IT-08 isolate. Lane A indicates the molecular size standard used in this study.

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