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ISOLATION, PARTIAL PURIFICATION AND CHARACTERIZATION OF THERMOSTABLE XYLANASE FROM THERMOPHILIC *ANOXYBACILLUS* SP. ISOLATED FROM HOT SPRINGS

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ABSTRACT

Isolation, partial purification and characterization of thermostable xylanase from thermophilic *Anoxybacillus* isolated from hot springs located in Afyonkarahisar were performed. The bacterium was determined to be bacilli (rods) shape, gram-positive and movable. Optimum conditions for bacterial growth and enzyme production were determined to be 55ºC, pH 7.0 and 48 hours. Maximum anzyme activity was reached at 60ºC and pH 7.0. Partial purification of xylanase enzyme was performed with precipitation of 80% ammonium sulfate and dialysis procedures (1.2-fold and 35% recovery, respectively). The molecular weight of xylanase was ~34 kDa, as estimated by SDS-PAGE. Partially purified anzyme was observed to maintain its stability after 2 hours in 50ºC and 60ºC and after 1 hour in 70ºC temperature. It was determined that enzyme activity was maintained 100% at the end of the1-hour period at pH 6.0, 7.0 and 8.0. A particularly strong inhibition was observed with the SDS (83%) effect caused by detergents used on enzyme inhibition.

KEYWORDS:
Anoxybacillus sp., xylanase, partial purification, isolation

INTRODUCTION

The interest in xylanolytic enzymes obtained from microbial sources has increased considerably in recent years. Xylan, base component of the hemicellulosic part of cell walls, is a heteropolymer formed with β-1,4-D-xylopranosyl or oligosaccharides and monosaccharide. Among xylanolytic enzymes xylanases are the most abundant ones. Many bacteria and fungi need xylanases in order to digest xylan. Therefore pathogens and saprophytes produce cell wall-degrading enzymes [1].

Thermophilic bacteria have been identified as capable of living at high temperature, can be anaerobic or facultative anaerobic depending on oxygen demands and have either rod, disc or elliptic morphologies. Since thermophilic microorganisms are heat-resistant, they produce some advantages [2]. Higher temperatures in microbiological fermentation increase the resolution and diffusion capacity of many compounds and decrease the viscosity of the medium. If the temperature is high enough, some volatile products are removed preventing cell growth by evaporation [3]. Because of their natural structure, thermostable enzymes are basically isolated from the thermophilic organisms used in many commercial areas [4]. For example, many thermostable enzymes are used in industries which involve the production of detergents, food, feed, starch, textile, leather, paper and medicine etc. Thermostable enzymes show high tolerance to different denaturing conditions. Thermostable enzymes also prevent the formation of environmental pollutants which are biologically hardly degradable and insoluble [5].

Xylanase (1,4-β-D-xylan xylanohydrolase; E.C. 3.2.1.8) is commercially used in the fruitessence, paper, food and animal feed industries. In the fruit and paper industries, xylanase enzymes increase the whiteness of paper and decrease the amount of compounds contained and chlorine in the process [6]. Particularly in Western Europe and North America, Xylanases are substantially used in bark removal, paint removal of recycled fibers and purification of cellulose for the preparation of paper solutions. Many commercial xylanases are produced by *Bacillus*, *Trichoderma, Aspergillus, Penicilium, Aureobasidium* and *Talaromyces* spp [7].

Members belonging to the *Anoxybacillus* species were previously categorised under the *Bacillus* species. This species was first described by Pikuta et al [8]. It has been determined that all anoxybacillus species defined are thermophile, gram-positive, spore forming bacilli shape bacteria [9].

Because of the thermophile properties of the bacterium isolated from the hot spring in Afyonkarahisar, the enzymes produced by thermophiles have a longer shelf life and the

catalyzing of biochemical reactions in high temperatures well as the production, partial purification and characterization of this bacterium were all performed.

MATERIALS AND METHODS

Microorganisms and Culture conditions. In this study, *Anoxybacillus* sp. isolated from a hot spring in Afyonkarahisar was used. The organism was identified by biochemical tests and 16S rRNA sequence. The 16S rRNA sequence analyses of isolates obtained were conducted by Ref-Gen (METU Technocity/Ankara). *Anoxybacillus* sp was produced in Nutrient Broth (NB; peptone 5.0, meat extract 3.0) medium containing 120 rpm shaker at pH 7.0, 55ºC temperature and 0.5% xylan for 48 hours. Supernatant was used to measure xylanase activity. Bacterial growth was measured to be 460 nm in the spectrophotometer.

Biochemical Tests of *Anoxybacillus* sp. Isolated bacteria were produced in nutrient agar containing 0.5% xylan and were tested biochemically.

Determination of Protein Amount. Determination of Protein amount was made according to the Lowry method [10].

Determination of Xylanase Activity. Determination of xylanase enzyme activity was performed according to the dinitrosalicylic acid (DNS) method [11]. For activity determination, enzyme solution and 0.5% xylan solution was incubated at 55ºC for 45 minutes. Then, in order to stop the reaction, 3.5 DNS was added and it was placed in boiling water for 5 minutes. Spectrophotometric measurements were made at 535 nm.

Effect of temperature, pH and incubation time on bacteria and xylanase production. By preparing 25 ml culture media in a 100ml conical flask, 2ml bacteria cultivation was performed. In order to determine the optimum values of bacteria and enzyme production between 25-80ºC temperature ranges, it was kept in a 120 rpm water bath shaker and absorbance measurement was performed in spectrophotometer.

In the prepared NB medium containing 0.5% xylan, enzyme and bacteria production were performed by increasing pH from 4.0 to 10.0 by 0.5 increments.

For the effect of incubation time on microbial growth and enzyme production; bacteria is produced in a NB cultivation medium (pH 7.0) and 55ºC, absorbance measurements were performed by taking samples every 4 hours.

Effect of Temperature and pH on Xylanase Activity. As enzyme while investigating the effect of temperature and pH on xylanase activity, the supernatant obtained by centrifuging bacterial cultivation culture in NB cultivation medium was used.

To determine the effects of temperature; xylanase activity was measured at temperatures from 35ºC to 85ºC byin 5ºC increments and relative enzyme activity was determined.

Xylan (0.5%) was prepared in 50 mM buffers at different pHs. A citric acid buffer, Tris-HCl buffer and carbonate/bicarbonate buffer were used for pH 4.0–6.0, 7.0–9.0, and10.0–11.0, respectively. Relative enzyme activity was measured under standard assay conditions.

Partial Purification of the Enzyme. 350 ml NB cultivation medium (pH 7.0) prepared in 1000ml flasks and autoclaved. Then by the cultivation of bacteria, they were incubated in the temperature (55ºC) optimum for bacteria growth. At the end of 48 hours incubation, the cultivation medium was centrifuged for 15 minutes at 10 000 rpm refrigerated centrifuge. In enzyme purification, two subsequent procedures were performed. These procedures are precipitation with ammonium sulfate (80%) and dialysis.

Electrophoresis. The molecular weight of the purified α-amylase was estimated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli [12]. The purified enzyme was loaded onto 1 mm thick 10% polyacrylamide gel together with molecular size markers. After completion of electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250.

The Effect of Temperature and pH on Xylanase Stability. In order to determine the temperature stability of xylanase enzyme, incubation was performed between 15-120 minutes by only using enzyme at 50ºC, 60ºC and 70ºC. After pre-incubation, to determine what level of enzyme activity remained and enzyme substrate mixture, the samples were left for incubation at the optimum temperature where enzyme showed activities. In order to determine the pH stability of xylanase enzyme obtained from partial purification, 0.1 M citric acid, 0.1 M. Tris-HCI and 0.1 M carbonate / bicarbonate were prepared. The enzyme was left for pre-incubation for 180 minutes. After pre-incubation, the remaining activity determination were measured under experimental conditions by adding substrate.

The Effect of Detergents on Partially Purified Enzyme Activity. In order to investigate the effect of some detergents on purified enzyme

TABLE 1
Morphological, Physological and Biochemical Tests

Characteristics	*Anoxybacillus* sp.
Aerobic growth	+
Gram painting	+
Spore forming	+
Cell form	rod shape
Pigmentation	white
Temperature range (°C)	30-70
Optimum temperature (°C)	55
pH range	3.0-10.0
Optimum pH	7
Hemolysis	-
Motility	+
Hydrolysis of:	
Starch	+
Caseine	-
Gelatine	-
Activity of:	
Lipase	+
Urease	+
Catalase	++
□-galactosidase	++
Indole	+
Phosphatase	+

+, positive result or growth; –, negative result or no growth.

activities, 0.5% SDS, Tween-40, Tween-80 and TritonX-100 were used. These detergents were prepared in 0.1 M pH 7.0 Tris-HCI buffers. Prepared detergents were left for pre-incubation with the enzyme for 30 minutes. After this step, the remaining enzyme activity determination was obtained by adding substrate.

RESULTS

Biochemical and Molecular Identification. As a result of the biochemical tests performed, features of isolates such as being Gram positive, bacilli (rods) shape and produced xylanase enzyme in xylan media were determined (Table 1).

The isolate from the spring water was confirmed to be a member of the genus *Anoxybacillus*. 16S rDNA gene product with approximately 1418 bp was sequenced for the isolated microorganism. As a result of 16S rRNA analysis, a comparison of the 16S rDNA sequence of this strain with other related bacteria shows that the 16S rDNA sequence of this strain has a high level of similarity with *Anoxybacillus*. The phylogenetic position of the rDNA sequences was determined by the construction of a phylogenetic tree (Fig. 1). The 16S rRNA sequence of this strain (1418 nucleotid sequences) is given below

The Effect of Temperature, pH and Incubation Time on Bacteria and Xylanase Production. In order to investigate the effect of the temperature on bacterial growth and enzyme production, they were left for incubation in NB cultivation media from 25°C to 80°C by 5°C increment. Maximum bacterial growth and enzyme production were determined to be at 55°C (Fig. 1). At various pHs, it was left in order to examine bacterial growth and enzyme production. At the end of this process, maximum bacterial growth and xylanase production were observed at pH 7.0 (Fig. 2). In order to determine the effect of various incubation periods on enzyme productions and bacterial growth, they were incubated between 20-60 hours at 7.0 pH and 55°C. Maximum bacterial growth and enzyme production after incubation were determined to occur with at 48-hour periods (Fig. 3).

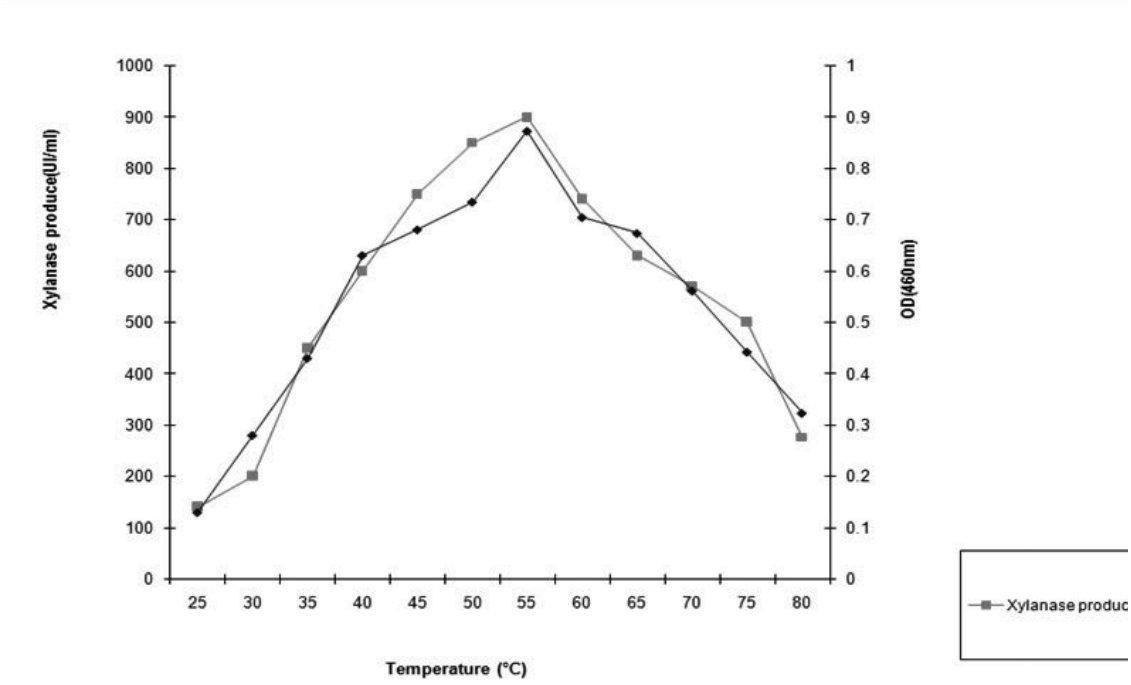

FIGURE 1
Effect of temperature on bacterial growth and enzyme activity

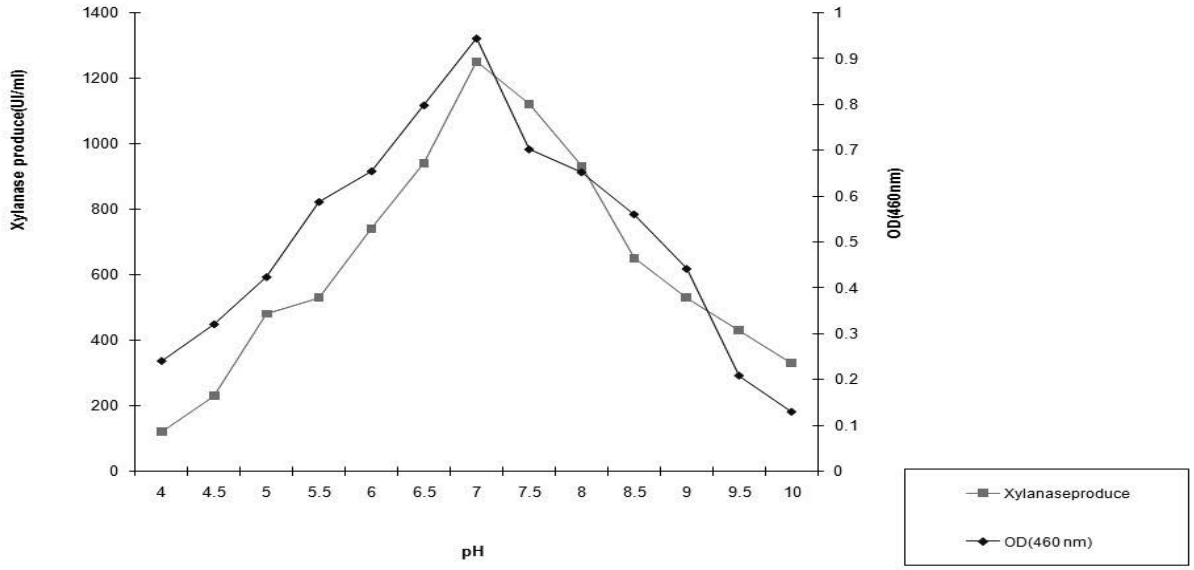

FIGURE 2
Effect of pH on bacterial growth and enzyme activity

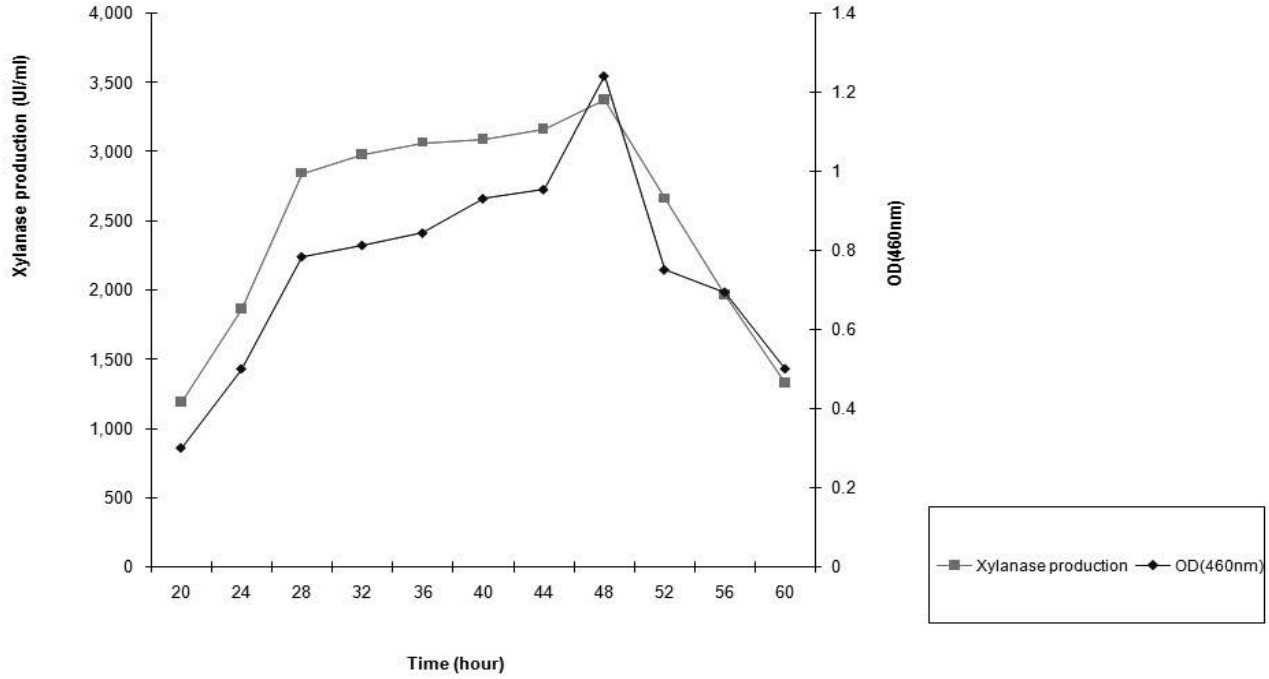

FIGURE 3
Effect of time on bacterial growth and enzyme production

Temperature and pH effect on Enzyme Activity. From the top liquid obtained after 48 hours when the maximum xylanase secreted, produced under optimum conditions, in the temperature determination in 35-85ºC, the maximum temperature activities of the enzyme were determined in 60ºC (Fig. 4). In the same environmental conditions, xylanase activity was determined to be the maximum at pH 7.0 in the analyzed performed between 4-11.0 pH (Fig. 5).

pH and Temperature on Xylanase Stability. Partially purified enzyme was observed to preserve its stability at 50ºC and 60ºC after 2 hours. In 70ºC however, it was observed to preserve its activity 100% after 1 hour and lost its activity after 2 hours (Fig. 6). While enzyme activity was determined to be stable after an hour at pH 6.0, 7.0 and 8.0, it preserved 78% at pH 6.0, 70% at pH 7.0 and 71% at pH 8.0 (Fig. 7).

Detergent Effect on Xylanase Activity. All applied detergents were determined to inhibit the enzyme (Fig.8). When the effect of detergent on partially purified enzyme was examined, the remaining enzyme amounts of depending on control were determined to be; 17% SDS, 73% Tween-80, 72% TritonX-100.

Partial purification of the xylanase. The enzyme was purified using 80% ammonium sulfate precipitation and dialysis**,** with a 1.2-fold and 35% recovery.

Molecular mass determination. The molecular weight of partial purified from *Anoxybacillus* sp. was ~34 kDa, as estimated by SDS-PAGE (Fig.9).

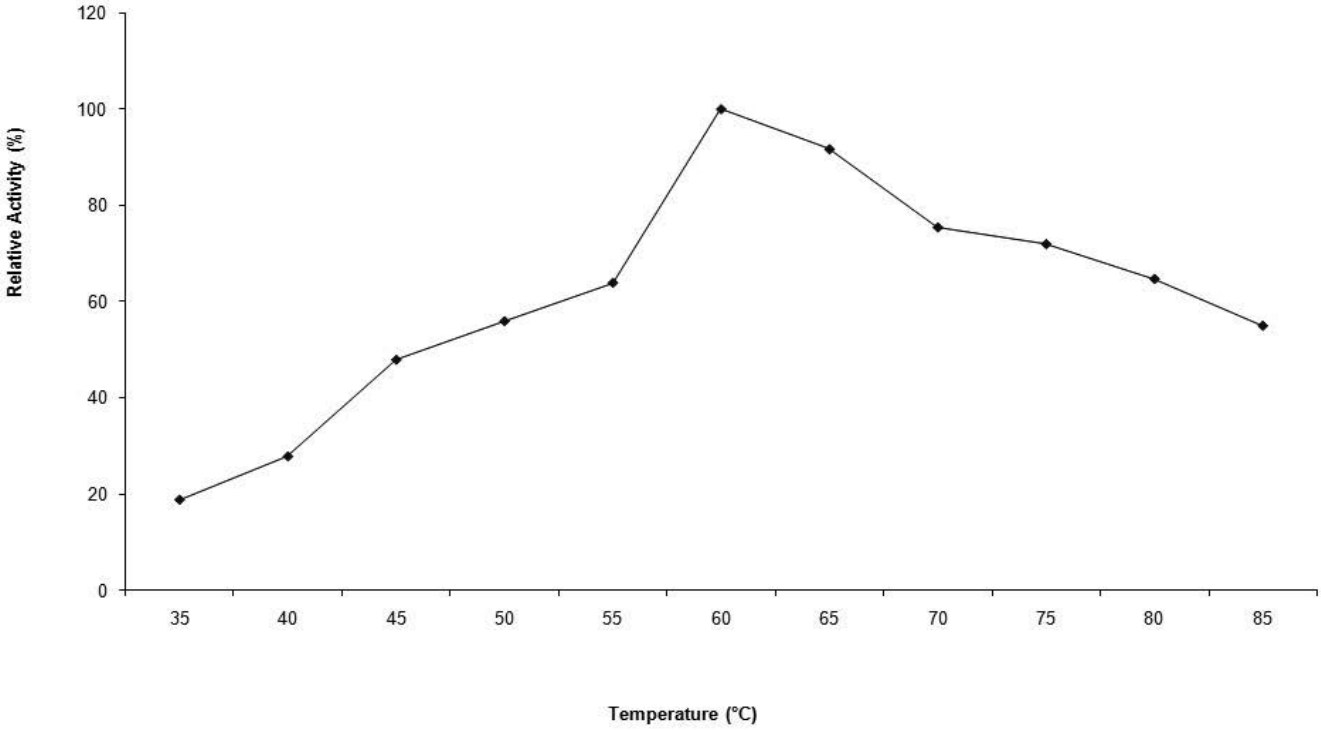

FIGURE 4
Effect of temperature on xylanase activity

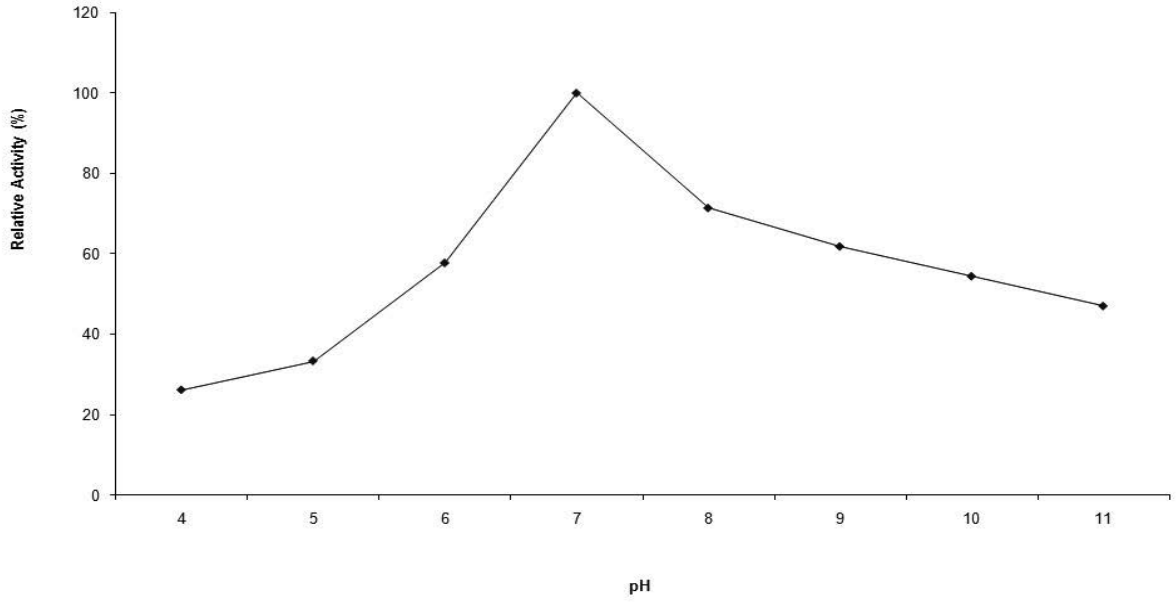

FIGURE 5
Effect of pH on xylanase activity

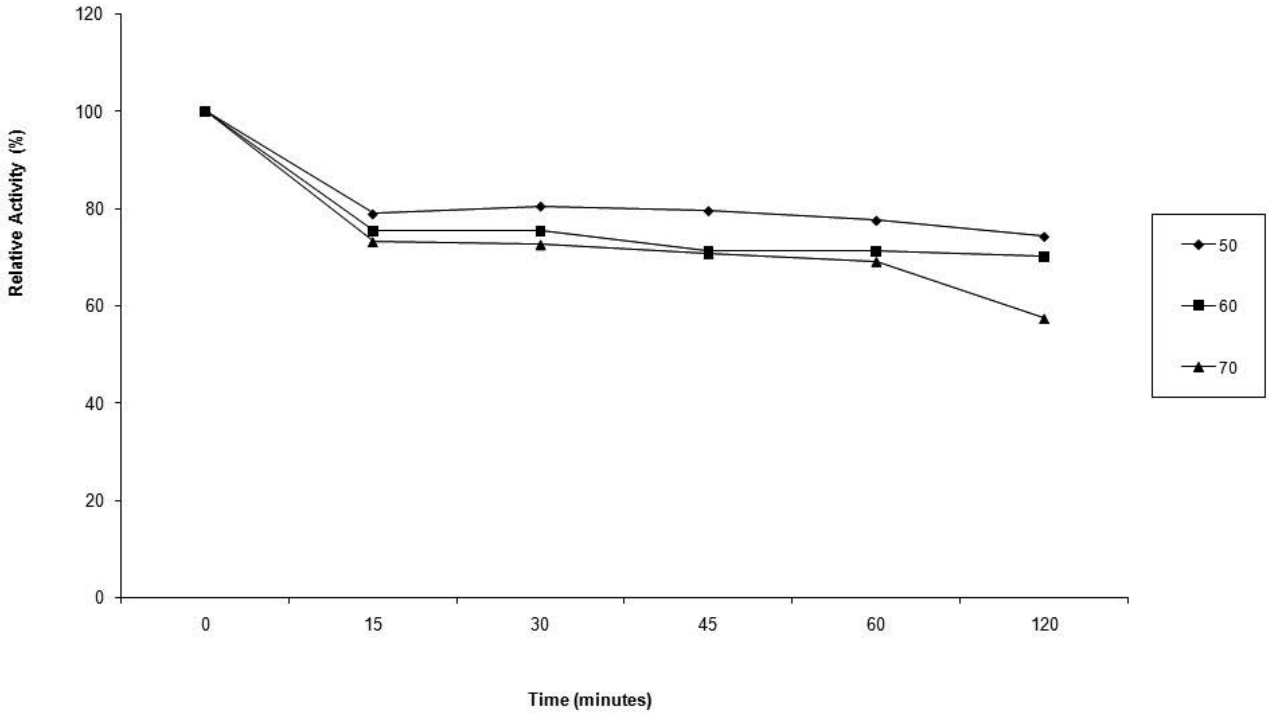

FIGURE 6
Effect of temperature on xylanase stability

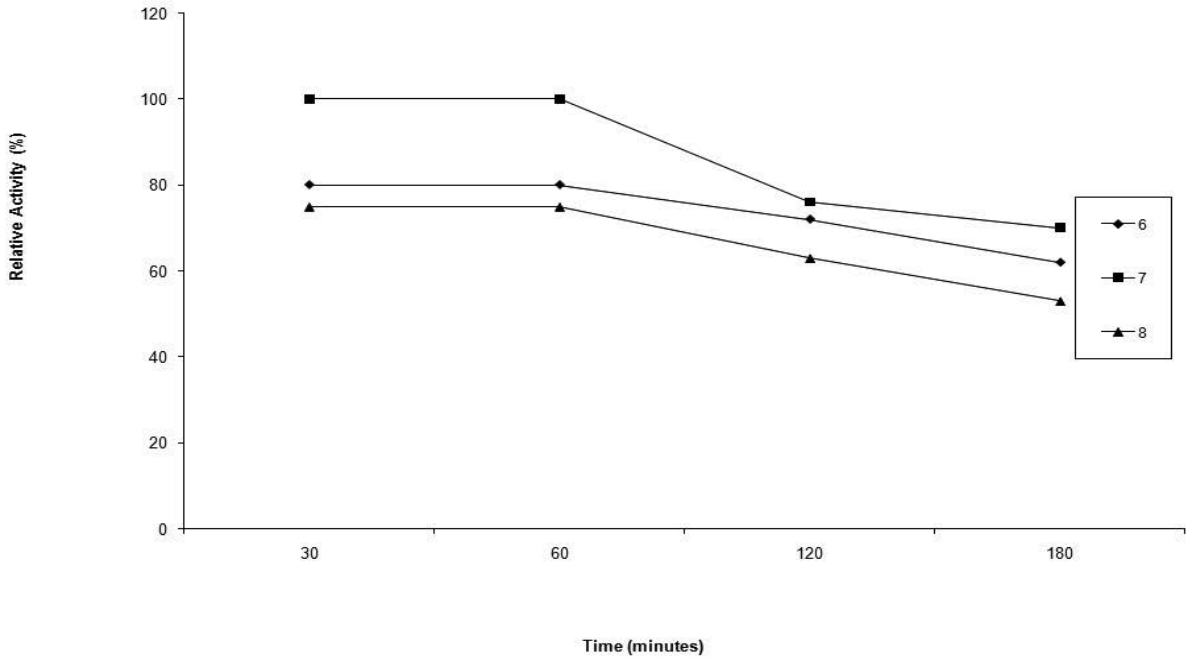

FIGURE 7
Effect of pH on xylanase stability

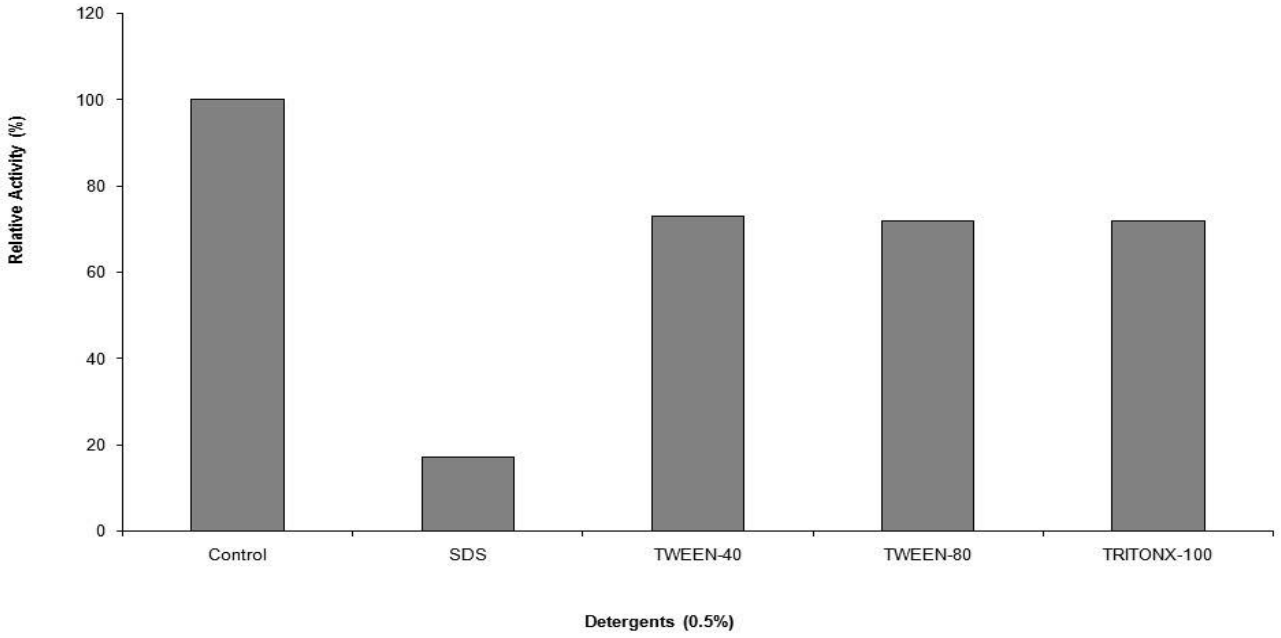

FIGURE 8
Effect of detergents on xylanase activity

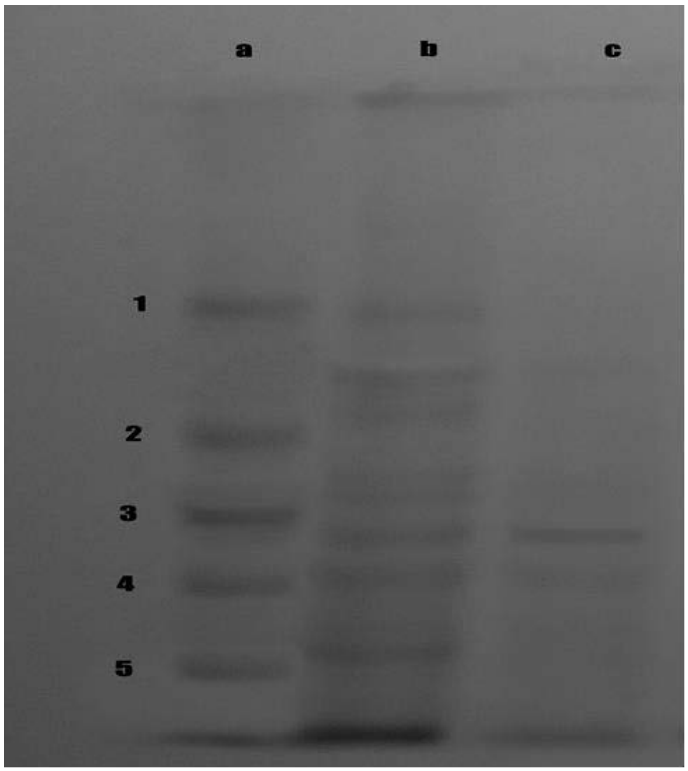

FIGURE 9
SDS-PAGE analysis of partial purified xylanase *Anoxybacillus sp.* Bovine serum albumine (66 000), albumin egg (45 000), glyceraldehyde-phosphate dehydrogenase (36 000), carbonic anhydrase (29000), trypsin inhibitor (20 100) (lane 1); xylanase crude extract (lane 2); dialyse (lane 3)

DISCUSSION AND CONCLUSIONS

Many xylanases from the genus *Bacillus* had been reported. For production of *Anoxybacillus amylolyticus* sp isolated from Antarctica, the optimum temperature, pH were determined to be 61ºC and 5.6, respectively, for *Bacillus pumilus* ASH production, optimum temperature was found to be 55ºC [9,13]. In previous studies, the highest xylanase production in *Bacillus circulans* WL-2, *Bacillus thermoleovorans* K-3d, *Geobacillus thermoleovorans* was obtained at pH 7.0 [14-16]. It was shown at the end of 96 hours period that enzyme production reaches to its maximum at pH 8.0 and 37ºC in *Bacillus pumilus*, pH 6.8 and 80ºC in *Bacillus amyloliquefaciens*, pH 6.5-7.0 in *Chaetomium thermophile* NIBGE xylanase [13,17,18]. Yuan et al. were found the maximum enzyme activity at pH 7.0 and 50ºC in their study on *Bacillus subtilis* [19]. Sa'-Pereira et al. [20]. were determined the optimum temperature and pH for xylanase enzyme obtained from *Bacillus subtilis* as 60ºC and 6.0, respectively. They have determined the optimal temperature and pH level for xylanase produced from Poorna and Prema [21] *Bacillus pumilus* as 50ºC and 6.5-7.0. Lv et al. [22]. reported that termostable xylanase obtained from EMSD5 shows optimum activity at 50ºC and at 6.8 pH. The highest enzyme activity were measured 60ºC and pH 7.0 in *Bacillus circulans* and 55ºC and pH 5.0 in *Aspergillus niger* US368 [23,24]. Although the xylanase from *B. stearothermophilus* T-6 had good thermostability at 65ºC, it showed high activity within a relatively narrow range (more than 80% of maximal activity at pH 5.5–pH 7.5) [25].

It was reported that xylanase obtained from *Bacillus* sp. kept its activities after 2 hours at 30-50ºC, lost its activity by 10% at 60ºC, 11% at 70ºC and 29% at 100ºC [26]. By contrast xylanase isolated from *Bacillus* sp. SPS-0 was reported to be stable for 4 hours at 70ºC and pH 6.0 [27]. and xylanase of *Bacillus flavothermus* LB3A stayed stable for 2 hours at 70ºC [15].

It was observed from previous studies that SDS has an inhibitory effect on the production of *Bacillus circulans* BL53, *Bacillus pumilus*, *Bacillus pumilus* ASH xylanases [23, 21, 13]. Similarly, it was shown that enzyme production was inhibited by Tween 20 treatment. In Sayari's [24] work, while only 20%of the SDS effect of xylanase from *Aspergillus niger* US368 was preserved, an increase was observed with the amounts of 33.32% Tween-80 and 26.64% TritonX-100.

Many xylanases produced by the *Bacillus* genus are in the range of 22–45 kDa [28,29]. Molecular masses for different xylanase have been reported: 38.8 kDa for *Anoxybacillus* sp. E2 [30]; 45 kDa for *Bacillus thermantarcticus* [31], ~ 20 kDa for *Arthrobacter* sp. MTCC 5214 [32], 38 kDa for *Bacillus circulans* BL53 [33].

This study describes the partial purification and characterization of the thermostable □-xylanase produced by thermophilic *Anoxybacillus* sp. Thermostabilization of a purified enzyme shows that it is usable biotechnologically because it is highly tolerant against diverse denaturing agents within the medium**.**

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