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Partial Purification and Biochemical Characterization of Cellulase from *Bacillus pumilus* ND8 Isolated from Garden Waste

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Abstract: Cellulose, one of the most abundant carbohydrates on Earth, is a promising candidate for the production of second-generation biofuels such as ethanol and various everyday products. This polysaccharide is degraded by the enzyme cellulase, which is usually produced by microorganisms. Microbial cellulases are widely used in various industries (such as textiles, detergents, pharmaceuticals, food and paper).

In the present study, cellulase enzyme was partially purified from *Bacillus pumilus* ND8 strain isolated from garden waste and the pH and temperature values at which the enzyme showed optimum activity were determined. *B. pumilus* ND8 strain exhibited a cellulase activity of 8.6 U/mL as a result of partial purification and the protein concentration of the enzyme was measured as 6.72 mg/mL. The pH and temperature values at which the partially purified cellulase showed optimum activity were pH 5.5 and 60 °C, respectively. K_m and V_{max} values of the enzyme were determined as 0.81 mM and 14.2 µmol/min, respectively. In conclusion, cellulase purified from *B. pumilus* ND8 strain was found to possess unique properties that make it suitable for industrial applications.

Bahçe Atıklarından İzole Edilen *Bacillus pumilus* ND8'den Selülazın Kısmi Saflaştırılması ve Biyokimyasal Karakterizasyonu

Anahtar Kelimeler
Bacillus pumilus, Selülaz, Kısmi saflaştırma

Öz: Dünyada bol miktarda bulunan karbonhidratlardan biri olan selüloz, etanol gibi ikinci nesil biyoyakıtların ve çeşitli günlük ürünlerin üretimi için umut verici bir adaydır. Bu polisakkarit, genellikle mikroorganizmalar tarafından üretilen selülaz enzimi tarafından parçalanır. Mikrobiyal selülazlar çeşitli endüstrilerde (tekstil, deterjan, ilaç, gıda ve kağıt gibi) yaygın olarak kullanılmaktadır.

Mevcut çalışmada, bahçe atıklarından izole edilen *Bacillus pumilus* ND8 suşundan selülaz enzimi kısmi olarak saflaştırıldı ve enzimin optimum aktivite gösterdiği pH ve sıcaklık değerleri belirlendi. *B. pumilus* ND8 suşu kısmi saflaştırma sonucunda 8.6 U/mL'lik bir selülaz aktivitesi sergiledi ve enzimin protein konsantrasyonu 6.72 mg/mL olarak ölçüldü. Kısmi olarak saflaştırılan selülazın optimum aktivite gösterdiği pH ve sıcaklık değerleri sırasıyla pH 5.5 ve 60 °C olarak bulundu. Enzimin K_m ve V_{max} değerleri ise sırasıyla 0.81 mM ve 14.2 µmol/min olarak belirlendi. Sonuç olarak, *B. pumilus* ND8 suşundan saflaştırılan selülazın endüstriyel uygulamalarda kullanılmasını uygun kılan kendine özgü niteliklere sahip olduğu tespit edilmiştir.

1. INTRODUCTION

The continued increase in global industrialization has led researchers in various industrial fields (textiles, animal feed, paper, detergents, food, etc.) to search for solutions that are economically viable and do not cause environmental damage [1]. Cellulose is the most abundant renewable energy source in the world, easily and cheaply available for the production of different biotechnologically important products [2].

Cellulose is the main component of the plant cell wall and is a polysaccharide formed by D-glucose units linked by β-1,4 bonds [1,3,4]. Cellulose is mainly broken down by the enzyme cellulase, which is widely produced by microorganisms [1,5]. Cellulases efficiently hydrolyze cellulose into glucose units through the synergistic effects of endo-β-1,4-glucanase, β-D-glucosidase and cellobiohydrolase enzymes [4,6,7]. Known for their fast growth rates compared to fungi, bacteria have become a preferred choice for industrial enzyme production due to their ability to produce high purity cellulase under a variety of growth media [8,9].

Cellulases are of great interest due to their wide-ranging applications in various industries (detergent, food, textile, paper, feed, feed, leather industry, etc.) [6,10,11,12]. They also play important roles in fiber modification, biomass fermentation and pharmaceutical development [6,13,14]. The widespread use of these enzymes in various industries requires the discovery of robust enzymes that can operate efficiently at high temperature and pH levels [6,11].

In this study, which was designed to add a new cellulase enzyme to the cellulase enzymes used industrially in various fields, isolation and identification of cellulotic bacteria from garden wastes and partial purification and characterization of cellulase enzyme from *B. pumilus* showing the highest activity were carried out.

2. MATERIAL AND METHOD

2.1. Bacterial Strains

The garden waste used in the study was collected from Atatürk University campus. Samples were collected with a sterile spatula and collected in a sterile ziplock bag. 0.1 g samples were suspended in physiological saline (9 mL) by vortexing for 2 min. A 10x dilution series was made and 1 mL of each dilution was transferred to Nutrient Agar (NA). Petri dishes were incubated at 28 °C for 72 hours. According to their morphological characteristics (shape, size and color), 60 bacterial colonies were selected and inoculated into NA by drawing method. After incubation at 28 °C for 2 days, they were stored at -80 °C in 50% glycerol stocks.

2.2. Molecular Identification by 16S rRNA Gene Sequencing

EurX GeneMATRIX Bacterial & Yeast DNA isolation kit (Poland) was used for DNA isolation of the strains. The 16S rRNA gene was amplified from the genomic DNA of the strain by polymerase chain reaction (PCR) using universal primers (27F 5' AGAGTTTGATCMTGGCTCAG 3' and 1492R 5' TACGGYTACCTTTGTTACGACTT 3'). Sequence analysis of the strains was outsourced to BM Lab and the strains were identified by comparing the sequence results in the NCBI database.

2.3. Assay of Cellulase Activity

Dinitrosalicylic acid (DNS) method was used to determine the cellulase activity of bacterial strains. 0.5 mL of enzyme solution and 0.5 mL of substrate were incubated at 37 °C for 30 minutes. Then 1 mL of DNS solution was added. The experiments were carried out in 3 repetitions and the results are given as the average of the three repetitions. The mixture was boiled for 5 min and after cooling, enzyme activity was determined by measuring at OD540 nm [15].

2.4. Purification of Cellulase

Partial purification of cellulase enzyme was carried out according to Dikbaş et al. [16] with minor modifications. *B. pumilus* ND8 strain grown in CMC broth at 35 °C for 2 days was centrifuged (8000 rpm at 4°C for 10 min). Cellulase was partially purified in the range of 0-80% ammonium sulfate. The partially purified enzyme was dissolved in 0.1 M sodium acetate buffer with pH 5.5 and stored at +4 °C.

2.5. Protein Determination

Protein concentration was determined using bovine serum albumin as a standard according to Bradford [17] and color change was measured spectrophotometrically at 595 nm.

2.6. Effect of pH on The Activity of Purified Cellulase

Substrate solutions were prepared using sodium acetate (pH 2.0-3.0), sodium citrate (pH 4.0-5.0-6.0), Tris-HCl (pH 7.0-8.0-9.0) and sodium carbonate (pH 10.0-11.0) buffers to determine the pH at which cellulase showed optimum activity. The experiments were carried out in 3 repetitions and the results are given as the average of the three repetitions. The pH at which the cellulase enzyme showed the highest activity was determined spectrophotometrically at 540 nm [16].

2.7. Effect of Temperature on The Activity of Purified Cellulase

To determine the optimum temperature at which cellulase showed the highest activity, reactions were carried out in the range of 10-90 °C with temperature increments of 10 °C. The experiments were carried out in 3 repetitions and the results are given as the average of the three repetitions. The optimum temperature of the enzyme was determined by measuring activity in a spectrophotometer (540 nm) at each temperature range [16].

2.8. Determination of K_m and V_{max} Values

Cellulase activity was measured at different substrate concentrations under optimum conditions and K_m and V_{max} values were determined by drawing Lineweaver-Burk graph [18].

3. RESULTS

3.1. Determination of cellulase activity of strains

Seven strains isolated from garden waste were tested for cellulase activity and strain ND8 showed the highest cellulase activity with a value of 43 U/mL (Figure 1). This strain was identified as *B. pumilus* in NCBI (Accession number: PP940105).

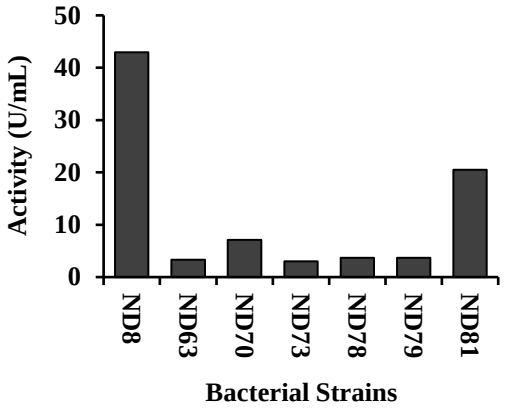

Figure 1. Cellulase activity results of seven different strains

3.2. Partial Purification Results of Cellulase

The cellulase enzyme partially purified by ammonium sulfate precipitation from the isolated and identified *B. pumilus* ND8 strain showed the best activity in the purification range 0-20% with an activity of 8.6 U/mL (Figure 2). The protein concentration in the range 0-20% was measured as 6.72 mg/mL.

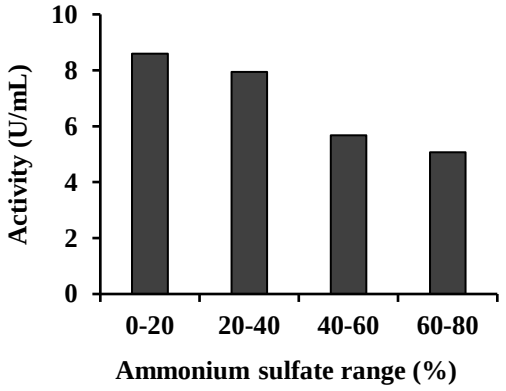

Figure 2. Cellulase activity of ammonium sulfate precipitation intervals

3.3. Optimum pH Results of Cellulase Enzyme

The pH value at which the cellulase enzyme partially purified from *B. pumilus* ND8 strain showed optimum activity was determined as pH 5.5. Cellulase showed an activity of 10.1 U/mL at pH 5.5 (Figure 3).

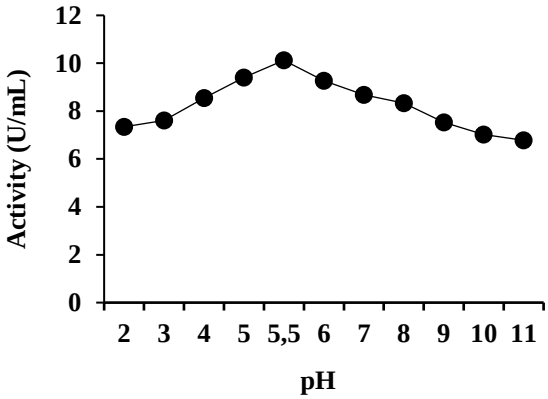

Figure 3. Effect of pH on activity of cellulase enzyme

3.4. Optimum Temperature Results of Cellulase Enzyme

The optimum temperature at which the cellulase enzyme partially purified from *B. pumilus* ND8 showed optimum activity was determined to be 60 °C and it showed 14.1 U/mL activity at this temperature (Figure 4).

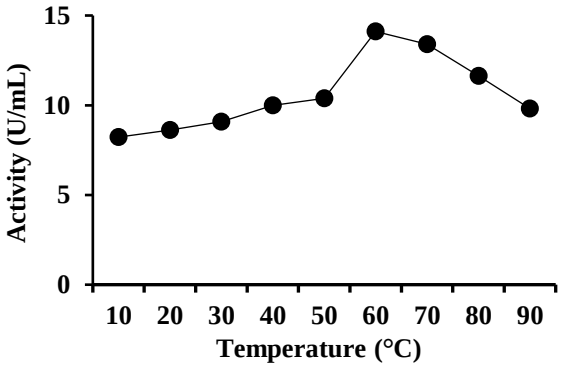

Figure 4. Effect of temperature on activity of cellulase enzyme

3.5. K_m and V_{max} Values

When the partially purified cellulase from *B. pumilus* ND8 was tested against carboxylmethyl cellulose, the enzyme exhibited a K_m value of 0.81 mM and a V_{max} value of 14.2 µmol/min (Figure 5).

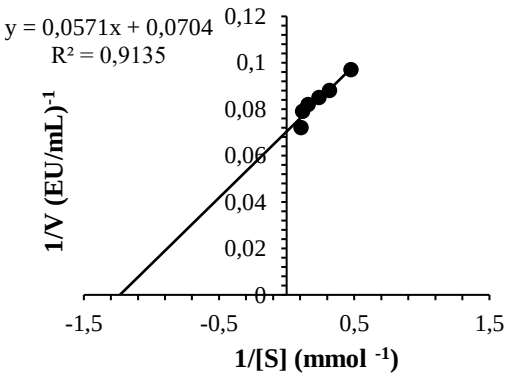

Figure 5. Graph for determining K_m and V_{max} values of cellulase enzyme for carboxylmethyl cellulose

4. DISCUSSION AND CONCLUSION

Cellulases are evaluated as potential biocatalysts by various industries worldwide due to their capacity to hydrolyze cellulose [19]. Due to their industrial importance, interest in the isolation of new bacterial strains that have higher catalytic activity, produce stable cellulase under variable conditions, and are easy to modify and optimize is increasing day by day [20]. Numerous studies have documented the discovery of bacterial cellulases from different environments (soil, water, etc.) and highlighted their potential for industrial use [21,22,23,24,25]. In this study, 7 bacteria isolated from garden waste were tested for cellulase production, and the strain showing the highest activity was identified as *B. pumilus* ND8. The cellulase enzyme from this strain was partially purified and some of its biochemical properties were determined.

The ammonium sulfate precipitation range in which *B. pumilus* ND8 strain showed the highest cellulase activity was determined as 0-20 and cellulase was purified in this range. The activity of the purified enzyme was determined as 8.6 U/mL. It was observed that *B. pumilus* ND8 strain had a better cellulase activity than other cellulase producing bacteria in the literature (*Aneurinibacillus aneurinilyticus* BKT-9 [21], *Pseudomonas* sp. D1-PT [26], *Bacillus* sp. T2-D2 [26], *Arthrobacter woluwensis* TDS9 [23].

A wide pH range is required for enzymes to be used in different application areas [27]. In the study, the pH value at which cellulase showed optimum activity was determined as 5.5 and it was observed that it did not completely lose its activity in the pH 2-11 range. These results are in agreement with *B. subtilis* CD001 (pH 5.0), *B. subtilis* subsp. *subtilis* JJBS300 (pH 5.0) and *B. licheniformis* PANG L (pH 5.0) cellulase, which show optimum activity under acidic conditions [28,29,30]. However, there are reports in the literature on the production of alkaline and neutral cellulases by *Bacillus* species [27,31,32,33]. As a result, the optimum pH for cellulase activity is enzyme specific and may vary depending on the type and source of cellulase.
It was determined that partially purified cellulase was thermostable and showed its optimum activity at 60 °C. Results are consistent with *B. subtilis* BC1 (60 °C) and *B. pumilus* EWBCM1 (50 °C) isolated from the gut of *Zeuzera pyrina* and *Eudrilus eugeniae* [31,32]. In addition, *B. subtilis* CD001 isolated from cow manure and *B. subtilis* F3 strain isolated from hot spring water showed optimum cellulase activity at 60 °C and 50 °C, respectively. [29,33]. The thermostability of purified cellulase will give it advantages such as high desirability in various industries, competitiveness, longer enzyme lifetime and versatility in applications.

The kinetic parameters of the purified enzyme were determined as K_m (0.81 mM) and V_{max} (14.2 µmol/min), respectively. Considering the results, *B. pumilus* ND8 cellulase has a high affinity for its substrate. This suggests that it may be more effective in the hydrolysis of cellulase [9].

In conclusion, *B. pumilus* ND8 isolated from garden waste was able to produce a cellulase with thermostable and acidic properties. Further studies are needed to better characterize the usefulness of this bacterium, which has an important industrial role, and the secondary metabolites it produces in different fields (food, feed, textile, soil improvement, etc.).

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