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Isolation and Characterisatıon of Acidic Cellulase Enzyme from Native *Bacillus* Sp. S-41 Strain

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Abstract

Acidic cellulase producing *Bacillus* sp. S-41was screened from soil samples in Ardicli village, Tarsus, Turkey and was optimized for the production of extracellular cellulolytic enzyme (CMCase). Using SDS-PAGE one band is found with molecular mass 53.7 kDa. The partially purified enzyme showed optimally activity at pH 4.0 (average 88%) and 50°C and it was stable from pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 after incubating at 37 °C for 24 hour 82%, 96%, 94%, 92%, 90%, 81% and 70%, respectively. Enzyme retained its original activity at 50, 60 and 100°C for 60 min 100%, 93%, 70%, respectively. While the enzyme activity was increased in the presence of (1% v/v) β-merkaptoethanol 145% , EDTA (5mM) 64%, $CaCl_2$ (5mM) 58%, $ZnCl_2$ (5mM) 59%, Na-sulfite (5 mM) 60%, 1,10-phenantroline (5mM) 53%, $MnCl_2$ PMSF (3mM) 55%, TritonX100 (1% v/v) 61%, SDS (1% v/v) 60% and Urea (8M) 62% were inhibited respectively. According to these results, Bacillus sp. S-41 cellulase enzyme showed acid active and stable, meso-thermophile (between at 40-90 °C, 88% active), highly thermostable and metallo-enzyme properties. Therefore it has the potentials to use in cellulose, beverage, prebiotic, feed and food industries areas, bioremediation applications and second generation bioethanol production.

Keyword: Acidic cellulase, *Bacillus*, Thermostable, pH stable

INTRODUCTION

Microorganisms are considered to be an excellent source of enzymes due to their biochemical diversity and suitability for genetic manipulations [1]. Today, about 90% of the enzymes used in industry are produced by fermentation using microorganisms and approximately 75% of the enzymes used in the industry are hydrolytic enzymes [2]. Cellulose enzyme is expected to increase significantly in the enzyme market because cellulosic materials are converted to sugars and converted into bio-based products such as bio-ethanol [3]. *Bacillus* family bacteria are commonly found in soil, animal faeces and plant products. They are attractive industrial organisms for many reasons such as their general safety and the ability of the proteins they synthesize to secrete to the external environment. When the production of toxic or allergen compounds such as aflatoxin in mushrooms is taken into consideration, gram-positive bacteria, especially Bacillus species are preferred in industrial enzyme production [4]. Cellulose is the most abundant renewable biopolymer in nature and is commonly found in plant cell walls. It is formed by the binding of glucose units with oz-1,4 glycosidic bonds. Cellulase enzymes endocellulose or endo-β-1, 4-D glucanase (EC 3.2.1.4), exocellulose or exo-β-1,4-D glucanase (EC 3.2.1.91) and endo β-glucosidase (EC 3.2.1.21) were divided into three main groups. Endocellulase and exocellulose breaks down cellulose to oligosaccharides and glucose, and glucosidase breaks down cellobiose into glucose monomers [5].

MATERIALS AND METHODS

Isolation of *Bacillus* sp. and Culture Conditions

1 g of composted bovine feces was taken and suspended in 4.5 mL sterile distilled water and incubated at 80 ° C for 10 minutes. Spore-forming bacteria were grown at a temperature of 24 h 37 ° C by smearing into CMC agar by diluting to a single colony level. The colony was determined as Bacillus sp. strain S-41 after gram staining, movement, spore formation and morphological analysis, biochemical tests. The strain has been used in enzyme production and characterization studies [6].

Enzyme Production

For the production of cellulase enzyme, the medium containing Na_2HPO_4 x $7H_2O$ (1.18 g / L), KH_2PO_4 (0.9 g/L), $NaNO_3$ (1 g /L), KCl (0.5 g/L), $MgSO_4x7H_2O$ (0.5 g/L), yeast extract (0.5 g/L), peptone (0.5 g/L) and CMC (10 g/L) was inoculated with an overnight culture and incubated at 37 °C for 24 hours at 24 rpm [7].

Partial Purification of Enzyme

The culture was centrifuged at 6000 rpm and + 4 °C for 20 minutes to remove the bacteria. 80% of the sample volume from 96% ethanol was added the supernatant and incubated at -33 °C for 24 hours. The sample was precipitated for 20 minutes at 8000 rpm and + 4 °C to obtain the enzyme and was used in analysis in 100 mM Na-phosphate buffer (pH 7.0) [8].

Enzyme Assay

To determine cellulase activity; 0.5 mL of enzyme and 0.5 mL of CMC (prepared in 100 mM Na-Phosphate buffer) (1% w/v) were mixed and incubated at 37 °C for 60 minutes. The reaction was stopped by adding 1 mL 3, 5-dinithrosalicylic acid (DNS) to the medium and measured with spectrophotometer [9].

Determination of the optimum temperature and pH of the enzyme

In order to determine the optimal temperature of the enzyme, standard trials were performed between 20, 30, 40, 50, 60, 70, 80, 90 and 100 ° C. To determine the pH range (pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0), different buffer systems giving the most effective pH were used. These include Citrate-phosphate buffer (pH 3.0-5.0); Sodium phosphate buffer (pH 6.0-8.0); Glycine-NaOH buffer (pH 9.0-10.0) and Borax-NaOH buffer (pH 11.0-13.0) [8].

Determination of the temperature and pH stability of the enzyme

The enzyme (0.5 mL) was incubated for 30 minutes at temperatures between 20, 30, 40, 50, 60, 70, 80, 90 and 100 °C to determine the resistance of the enzyme to temperature. After that, the enzyme immediately was taken up on ice. The cooled enzyme samples were mixed with 0.5 mL of substrate and incubated at optimum temperature for 60 minutes. Reducing sugar content was determined by DNS method. The enzyme was incubated for 24 hours in solutions pH 3.0-9.0 to determine pH stability. 0.5 mL of each pH enzyme sample was taken and mixed with 0.5 mL of substrate and standard activity was determined [8].

SDS-PAGE Zymogram Analysis

For zymogram analysis and molecular weight determination, the enzyme was separated at 20 mA in 10% SDS-PAGE containing 0.1% CMC. The gel was stained with Coomassie Brillant Blue (CBB) R 250 for 1 hour and protein bands were made visible. To determine the cellulase activity in the gel, the gel was incubated for 1 hour in 50 mM Na_2HPO_4 + 50mM NaH_2PO_4 + 40% isopropanol solution (pH: 7.2), and for 1 hour in 50 mM Na_2HPO_4 + 50mM NaH_2PO_4 (pH: 7.2) solution, and for 1 night 50 mM Na_2HPO_4 + 50mM NaH_2PO_4 + 5mM Beta-mercaptoethanol + 1mM EDTA (pH: 7.2) solution at + 4 °C. At the end of this period, the gel was wrapped with stretch film, incubated for 4-5 hours at 37 °C and the zone (yellow hydrolysis) was determined [10].

Effect of Inhibitors and Chelators on Enzyme Activity

To determine the effect of various metal ions and chelating agents on enzyme activity, different concentrations of metal ions, inhibitors, detergents and chelating agents were mixed with the enzyme and pre-incubated for 30 minutes and then standard activity determination was performed [11].

RESULTS AND DISCUSSION

Bacillus S-41 strain was isolated from bovine faeces and it was found that the enzyme synthesis was taken place in solid medium at temperatures between 20-60 °C and optimum synthesis was at 55 °C. It was determined that the largest (30 mm) hydrolysis zone was formed at pH 6.0 when different pH values were tested (Figure 1A). When 50 µL of the partially purified enzyme suspension was applied to the solid medium pH 7.0, a transparent zone was obtained at 30 °C (Figure 1B)

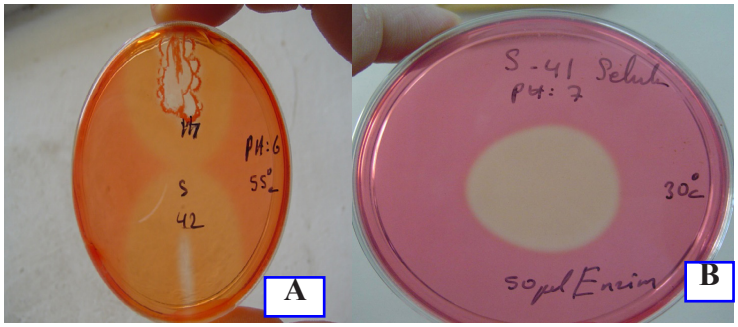

Figure 1. Cellulase Enzyme Synthesis of *Bacillus* sp. S-41 Strain in Solid Medium (A) and Activity of Enzyme Suspension in Solid Medium (B).

The enzyme obtained by partial purification was tested for standard enzyme activity at different temperatures and pH values. The optimum temperature at which the enzyme showed activity was found to be 50 °C (30-90, average 84%) and the optimum pH 4.0 (average 88% between 3.0-7.0) (Figure 2 and 3). Li et al. [12] stated that cellulase enzyme activity in the range of pH 4.5-6.5. In the literature, there are many cellulase enzymes with different optimum activity temperatures. Some of them are acidophilic with optimum pH and optimum activity temperatures are 65-70 ºC. Aygan [7] was found that halophile endoglucanase enzyme has an optimum activity at 50ºC. Mawadza et al. [13] isolated cellulase enzyme has been found to have optimum activity in the range of pH 6.0-8.0. Therefore, S-41 enzyme is thermophilic and acidic.

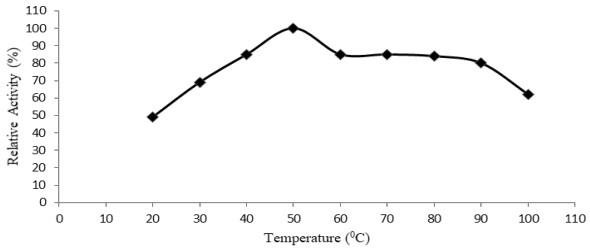

Figure 2. Optimum activity temperature of S-41 cellulase enzyme

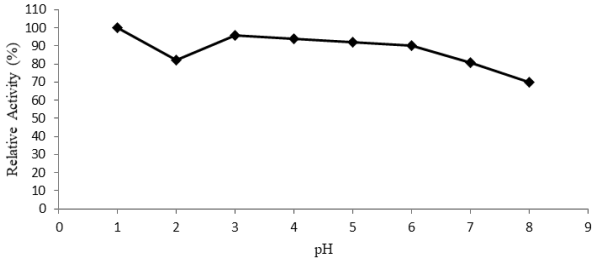

Figure 3. Optimum activity pH values of S-41cellulase enzyme

After 24 hours pre-incubation at 37 °C at pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0, the cellulase enzyme was stable 82%, 96%, 94%, 92%, 90%, 81% and 70% respectively. Enzyme average rate of 88% was stable. Cellulase enzyme retained its original activity at 50, 60 and 100°C for 60 minutes 100%, 93% 70%, respectively (Figure 4 and 5).

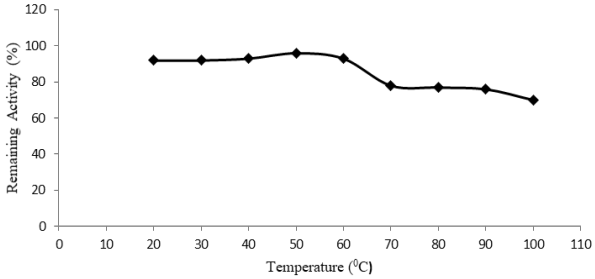

Figure 4. Thermal stability of S-41 cellulase enzyme at various temperatures

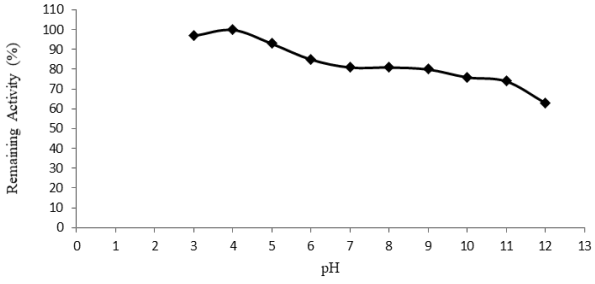

Figure 5. pH Stability of the S-41cellulose enzyme at various pH Values

In literature; cellulase enzyme pH 5.0 and 50ºC when incubated for 1 hour 100% of the original activity is preserved, pH 6.0 has been observed to decrease activity to 55% [14]. It was found that endoglucanase enzyme, which has an optimum activity temperature of 50ºC, maintained its original activity completely at 20ºC after 10 minutes of incubation, activity decreased to 90% at 40ºC, 50% at 50ºC and 30% at 100ºC [15]. S-41 enzyme is highly thermostable and pH stable.

After 60 minutes preincubation at 37 °C, the enzyme (5 mM 1-10) Phenantroline 53.5%, (1% w/v) Triton X-100 61.5%, (5 mM) $CaCl_2$ 58% and (5 mM) Na -Sulfide inhibited 60%, (5 mM) EDTA 64%, (5 mM) $ZnCl_2$ 59%, (3 mM) PMSF 55%, (1% v/v) SDS 60% and (8M) urea 61%. In contrast, activity (1% v/v) increased 145% in β-mercaptoethanol (Table 1). Li et al. [12] found that 5% SDS inhibits cellulase activity by 64%, while Triton X-100 activates 127%. It is reported in the literature that metal ions such as Zn^+ and K^+ inhibit enzyme activity at different rates (23% and 60%, respectively), and inhibition by Zn^{+2} and 8 M urea is indicative of thermostability [16]. S-41 cellulase enzyme has metallo and serine protease properties.

Table 1. Effect of inhibitor, chelator, detergent and metal ions on the enzyme activity

Inhibitor	Residual Enzyme Activity (%)
Control	100
EDTA	36.0
$CaCl_2$	42.0
PMSF	45.0
B-Mercaptoethanol	145.0
SDS	40.0
Triton X-100	38.5
Na2SO3	40.0
NaCl	48.5
ZnCl2	41.0
1,10 phenantroline	46.5
Urea	39.0

Zymogram analysis with SDS-Polyacrylamide Gel Electrophoresis (PAGE) with CMC revealed a single activity band of 53.7 kDa (Figure 6). In the literature, the majority of endoglucanase enzymes have been reported to vary between 35-85 kDa molecular weights [17]. In another source thermos-acidophilic endoglucanase (Ba-EGA) enzyme was found to have a molecular weight of 67 kDa [12]. Endo et al. [18] was found that the endoglucanase enzyme isolated from the Basillus sp. KSM-N252 strain had a molecular weight of 50 kDa.

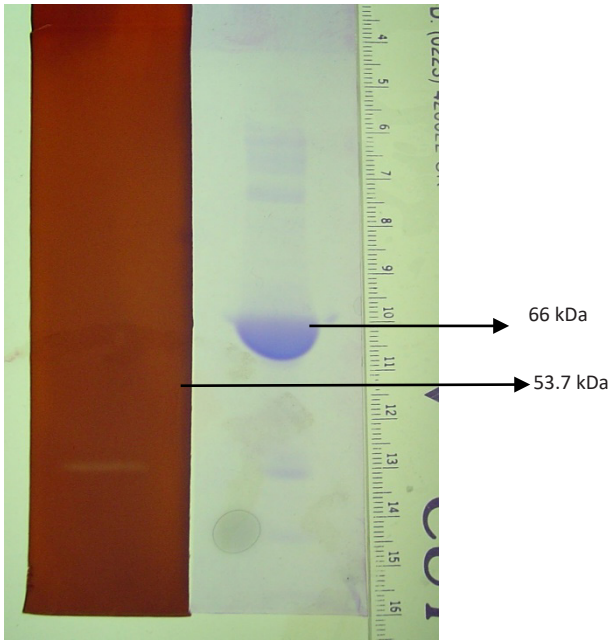

Figure 6. SDS-PAGE Zymogram analysis

CONCLUSION

Isolated cellulase enzyme is acidic, thermophile (average 87% at 50-90 °C), pH stable (average 86% at pH 3-9), highly thermostable (average 85% at 20-100 °C), metallo-enzyme (inhibited by 64% of EDTA) and significant serine structures (55% inhibition with PMSF). Because of these properties the enzyme can be utilized as followers;

1. Production of prebiotic oligosaccharides from cellulosic material
2. Use of cellulase in feed production to increase digestibility of rumen and monogastric feeds
3. Production of bioethanol alternative to fossil fuels by using cellulosic material as an alternative / renewable energy source and its use in fuels
4. Utilization of food products, various beverages, nutrients and silage production
5. It has the potential to be used in many different industrial fields as detergent additive.

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