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Isolation and Characterization of a Novel Acidophilic, Halotolerant and Cold-adaptive Cellulase (CMCase) from a Native Isolate *Bacillus* sp.

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Abstract
A cold-adaptive acidic cellulase enzyme was produced by native *Bacillus* sp. TG-1 from Adana-TURKEY and the cellulase producing bacteria and the enzyme was characterized. In SDS-PAGE analysis the enzyme showed single bond of 52.4 kDa. The cellulase TG-1 had a pH optimum of 4.6 and displayed maximum activity at 45ºC. The enzyme was stable at pH ranging from 4.0 to 9.0 with more than 85% of original activity for 24 h. Also, the enzyme showed 100% stability at 20-40ºC for 60 min and exhibited more than 63% of maximum activity in different NaCl concentrations. The enzyme showed up to 70% stability against detergents and chelators, while it was strongly inhibited by Urea, H_2O_2, PMSF and 1,10-Phenanthroline and induced by β-mercaptoethanol and $MnCl_2$. TLC analyses of the hydrolysis of Carboxymethyl cellulose (CMC) by the enzyme, revealed the presence of maltose and etc.
Keywords: *Bacillus* sp, Cold–adaptive cellulase, detergent, energy saving

INTRODUCTION

Cellulose, the most abundant renewable material in the world, is important raw material in sugars, biofuels and other industries [1]. Hence, there is a considerable economic interest to develop processes for the effective treatment and utilization of cellulosic waste as an inexpensive carbon source. Cellulases provide a key opportunity for exploiting the tremendous benefits of biomass utilization [2]. Several cellulase producing fungi such as *Aspergillus, Rhizopus*, and *Trichoderma* species [3] and bacteria such as *Bacillus, Clostridium, Cellulomonas, Thermomonospora, Ruminococcus, Bacteroides, Erwinia*, and *Acetivibrio* species [4] have been identified. The growth rate of bacteria are usually higher than fungi, also, enzyme production by bacteria is greater. Accordingly, the isolation and characterization of novel cellulose hydrolyzing enzymes from bacteria considerably attracts attention of many researchers [2]. Also, the habitat of bacteria covers different environmental niches, which favors the existence of versatile strains such as thermophiles, psychrophiles, alkaliphiles, and acidophiles [5].

It is estimated that approximately 20% of the >1 billion US dollars of the world's sale of industrial enzymes consists of cellulases, hemicellulases and pectinases. Since the production of cellulase enzyme is a major process and economically viable, much work has been done on the production of cellulases from lignocellulosics [6].

Cellulolytic enzymes isolated from environments with extreme temperatures and pH values are receiving a lot of interest as these enzymes are expected to be better adapted to the conditions of industrial processes, such as low temperature washing, biopolishing of cotton-based fabric to remove fuzz at low temperature, finishing denims by stonewashing using cellulase at low pH and low temperature in textile industry [7]. This research was aimed to produce and characterize a cold-adaptive acidic cellulase enzyme by native *Bacillus* sp. TG-1 from Adana-TURKEY

MATERIALS AND METHODS

Isolation and identification of cellulolytic bacterial strain

Soil samples were collected from different regions of Adana-Turkey and were serially diluted using sterile distilled water. The diluted samples were plated on nutrient agar plates by spread plate method. The isolated colonies were further purified using streak plate technique and screened for Cellulase production. The screening was done by streaking the isolated colonies on screening medium (CMC (Carboxymethyl cellulose) 1%, yeast extract 0.5%, $(NH4)_2SO_4$ 0.05%, KH_2PO_4 0.26%, Na_2HPO_4 0.44%, Agar 1.6%). Its pH was adjusted to 5.0 with 1M HCl. [5]. After 24 hours incubation the plates were flooded with 0.1% Congo red solution and left undisturbed for 15 minutes. To visualize clear zones formed by cellulase positive strains the plates were destained using 1M NaCl solution. Positive and better zone producing strain was selected for characterization and enzyme production. Also the selected strain was characterized by morphological and biochemical tests [8, 9].

Optimization of medium composition

Culture conditions such as different temperatures, pHs, carbon and nitrogen sources, salt concentrations, and substrate (CMC) concentrations were optimized for enzyme production. For this purpose production medium containing different carbon sources (fructose, glucose, maltose and sucrose) and different nitrogen sources (beef extract, yeast extract, tryptone, casein and peptone), different NaCl concentrations (0.1–1%, with increments of 0,1%), different CMC concentration (0.1–1%, with increments of 0,1%) and incubated under different temperature (0–50°C, with increments of 5°C) and pH (3.0–9.0 with increments of one unit) were analyzed for enzyme production by the selected *Bacillus* strain [10, 11].

Enzyme production

The isolated strain was cultivated aerobically in CMC containing medium (casein pepton 0.8%, yeast extract 0.4%, NaCl 0.45%, CMC 0.4%; and pH 5.0) at 15°C for 72 hours in a shaker incubator at 170 rpm. The culture was centrifuged at 8,000 rpm for 20 min using a refrigerated centrifuge; the supernatant was subjected to partially purification by ethanol precipitation, then the partially purified enzyme used for enzyme assays and further characterization [12].

Enzyme assay

CMCase (cellulase) activity was assayed by adding 0.5 mL of enzyme solution to 0.5 mL CMC (1% gr/ml of 0.1 M Glisine-NaOH buffer, pH 5.0) and incubating at 20°C for 60 min. The reaction was stopped by the addition of 1 mL of DNS (3,5-Dinitrosalicylic acid) reagent and its OD_{540} was measured in UV-visible spectrophotometer [13].

Influence of pH and temperature on the enzyme activity and stability

The optimum pH for activity of the enzyme was determined using different pH buffers: 0,01M citrate phosphate buffer (pH 3.0-5.0), 0,01M sodium phosphate buffer (pH 6.0-8.0) and 0.01M glycine buffer (pH 8.0-10.0). And the optimum temperature was tested at different temperatures 10°C -50ºC. For measurement of thermal stability, the enzyme was pre-incubated at temperatures 10-80°C for 60 min also, in order to determine of pH stability, the enzyme was pre-incubated at pH 3.0-10.0 at room temperature for 24 h. After pre-incubation the remaining activity of the enzyme was determined under optimum enzyme activity conditions [14].

Effect of different NaCl concentrations on enzyme stability

Effect of NaCl concentrations on enzyme activity was determined by pre-incubating the enzyme in buffers including 3, 5, 7.5, 10, 15, 20, 25 and 30% of NaCl at 25°C for 24 h and remaining activity of the enzyme was determined under optimum assay conditions [15].

Effect of metal ions, surfactants, chelating agents and inhibitors on enzyme activity

The effects of metal ions, surfactants, chelating agents and inhibitors on cellulolytic activity of the enzyme were determined by pre-incubating the enzyme with EDTA (5mM), $CaCl_2$ (5mM), $ZnCl_2$ (5mM), $MgCl_2$ (5mM), PMSF (3mM), Tween20 (0.8mM), Tween80 (0.8mM) 1,10-phenontroline (5mM), SDS (34mM), Urea (8M), TritonX-100 (16mM), 2-Mercaptoethanol (5mM) and H_2O_2 (29mM) for 60 min at room temperature before adding the substrate. Afterwards, residual activity of the enzyme was measured at optimum activity assay conditions. The control (without metal ions, surfactants, chelating agents and inhibitors) was considered as having 100% activity [14].

Determination of molecular weight of the partial purified enzyme and zymogram analysis

To determine of molecular weight and active bond, the enzyme was electrophrosed by using SDS-PAGE (10%) including CMC (0.1%) and molecular weight markers (Sigma SDS6H2). After electrophoresis the gel was cut into two pieces, one (having marker bands) was stained with 0.1% Coomassie Blue R250 and destained in methanol-acetic acid-water solution (1:1:8), and the other piece (having protein bands) was subjected to renaturation solutions.

Renaturation of the enzyme carried out by incubation of the gel in solution A containing 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2), isopropanol 40% for 1 h and solution B containing 50 mM Na_2HPO_4, 50mM Na_2HPO_4 (pH 7.2) for 1 h, respectively. After that the gel incubated in solution C containing 50 mM Na_2HPO_4, 50 mM Na_2HPO_4 (pH 7.2), 5 mM β-mercaptoethanol and 1 mM EDTA at 4°C for overnight. Then, the gel was incubated with substrate at 20°C for 5-6 h. After that it was stained with 1% Congo Red solution for 15 min and the activity band was detected after destaining the gel in 1M NaCl solution for 15 min [16].

Chromatography of the end products of the isolated enzyme hydrolysis

The enzyme was incubated with CMC (2%) at 20°C for 2h. The end products were analyzed (10 µL) by thin layer chromatography. After conducting the products with chloroform-acetic acid-distilled water (6:7:1, v/v/v), the spots were visualized by spraying aniline (1%, v/v), diphenilamine (1%, w/v), orthophosphoric acid (10%, v/v) in aceton and baking in oven at 120°C for 45 min [17].

RESULTS and DISCUSSION

Isolation of cellulolytic bacteria and culture conditions

The selected, best and hight cellulase producing strain was gram positive, rod shaped, aerobic and spore forming bacterial strain. According to the results of various morphological and biochemical tests, it was identified as *Bacillus* sp. TG-1. The strain optimally synthesized cellulase enzyme at 15ºC, pH value of 5.0 and incubation time of 72 h. Optimal concentrations of the production medium components were determined as casein pepton 0.8%, yeast extract 0.4%, NaCl 0.45%, CMC 0.4%.

Zymogram analysis

The partial purified cellulase showed single active band on zymogram analysis with molecular masses of 52.4 kDa (Fig. 1). This finding is consistent with those of monomeric cellulases reported from some *Bacillus* species [18, 19, 20].

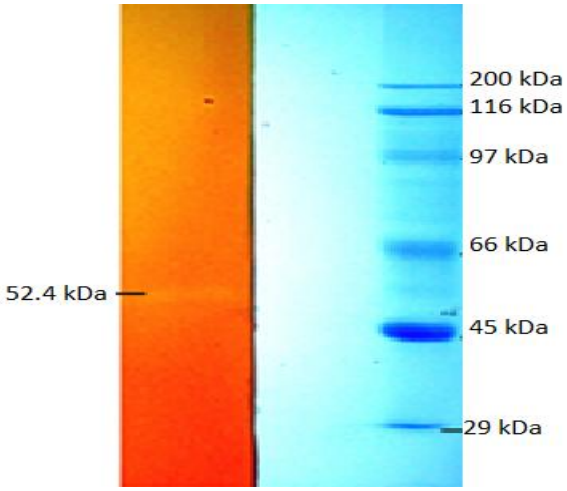

Figure 1. Zymogram analysis of the partially purified TG-1 cellulase enzyme

Effect of temperature and pH on the enzyme activity and stability

Analyses of pH effect on TG-1 cellulase enzyme showed that the enzyme had optimum activity at pH 4.6 and it conserved 85% of its maximum activity at pH 4.0-9.0 for 24 hour,(Fig.2) before, similar finding had been reported by Mawadza et al. [21]. The enzyme displayed maximum activity at 45ºC. Also, The enzyme was stable from 20 to 40ºC with 100 % activity and more than 44% of its original activity was observed after preheating at 60ºC for 60 min. These results are in accordance with the reports of Li and Fan for acidophilic cellulase [22, 23]. The results showed that the TG-1 cellulase enzyme was a novel and wonderful lignocellulose-degrading enzyme with important economic advantages.

Effect of various NaCl concentrations, metal ions, and other additives on enzyme activity

The enzyme conserved more than 63 % of original activity after pre-incubating in different concentrations of NaCl (from 3% to 30%) at room temperature for 60 min (Fig. 3).

This finding is similar to the result of cellulase produced by *Bacillus* sp. C14 which maintained 75% of its original activity in different NaCl concentrations from 3 to 30 % after 1 h incubation at 50ºC [12]. The Cellulase TG-1 was strongly inhibited in the presence of 8M Urea, 29mM H_2O_2, 5mM PMSF and 1, 10-Phenanthroline up to 60, 77, 71, and 67%, respectively (Fig. 4). It is widely accepted that urea and guanidine act as protein denaturants by breaking intramolecular hydrogen bonds [24]. Loss of catalytic activity in the presence of urea is thought to occur by elimination of bonds contributing to the tertiary structure of enzyme molecules. Subsequent restoration of structural and catalytic properties by removal of the denaturant, "reversible denaturation," is usually interpreted as evidence of the reformation of the ruptured hydrogen bonds

Inactivation by chemical oxidation has been reported previously for α-amylase from B. subtilis [25], the AC-7 amylase showed 20% of its activity after treatment with 1% H_2O_2. Inhibition by PMSF (a serine modifier) revealed that serine residues played an important role in its catalytic function [26].

The TG-1 enzyme conserved its activity in the presence of 5mM EDTA, $ZnCl_2$, $MgCl_2$, $CaCl_2$, 34mM SDS, 16mM Triton-X-100, 0.8mM Tween-20 and Tween-80 up to 79, 72, 76, 82, 69, 75, 76 and 76 %, respectively after pre-incubation at room temperature for 60 min. Different metal ions and EDTA (a chelator) decreased activity of the enzyme , while $MnCl_2$ increased its activity. Consequently it is possible that the enzyme is a metaloenzyme and it has need to $MnCl_2$ for activity. The non-ionic detergents (%0.1 (v/v)) such as Triton X-100, Tween 20, and Tween 80 and SDS slightly decreased the enzyme activity. Detergents could interact with the hydrophobic group of amino acids, resulting in the decreased enzyme activity [27]. However, the activity was increased in the presence of 127mM β-mercaptoethanol and 5mM $MnCl_2$ up to 53, 94 %, respectively.

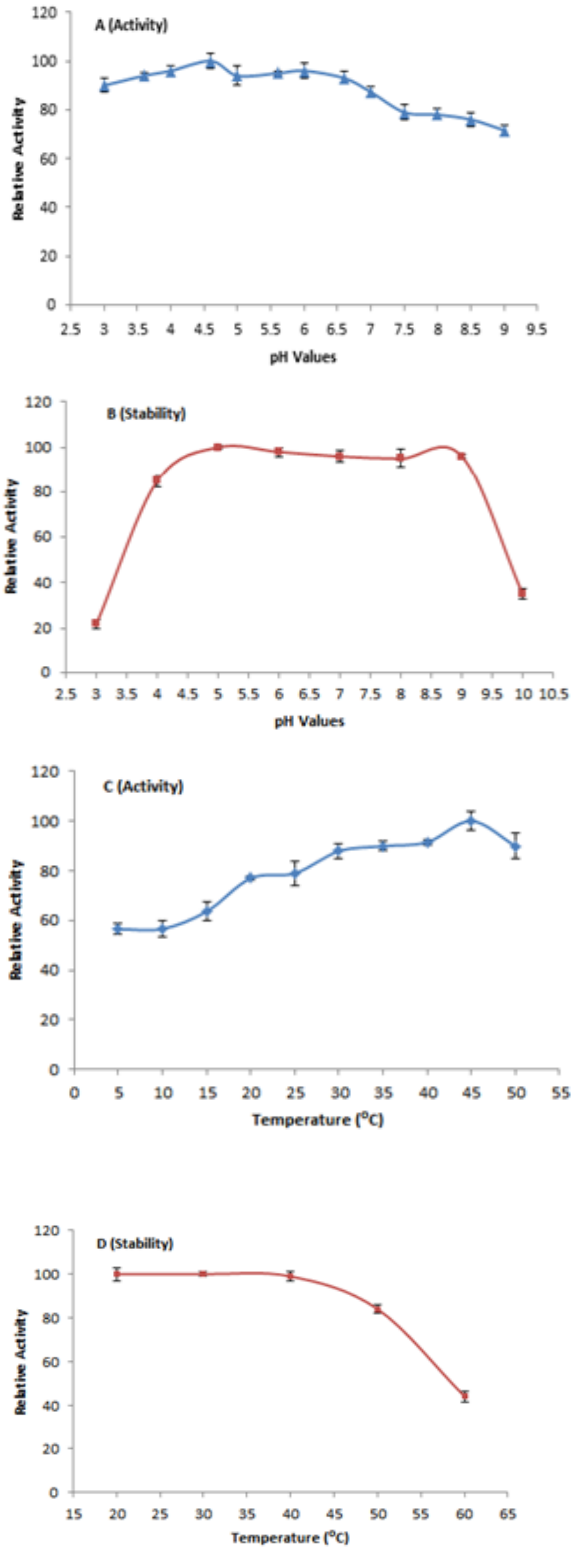

Figure 2. Effect of pH values on (A) activity and (B) stability of the cellulase. And effect of temperature on (C) activity and (D) stability of the enzyme. Relative activity was defined as the percentage of activity detected with respect to the maximum enzyme activity. For determining of the stability, the activity of the enzyme without any pretreatment was taken as 100 %.

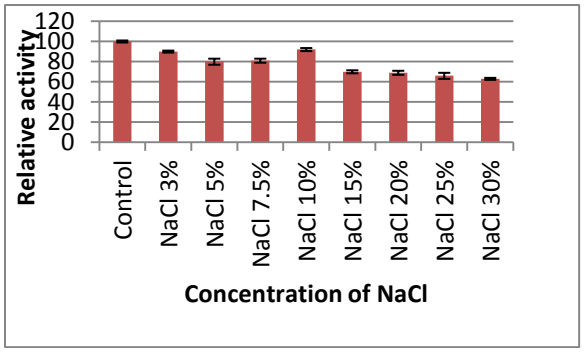

Figure 3. Effect of NaCl concentration on the enzyme activity.

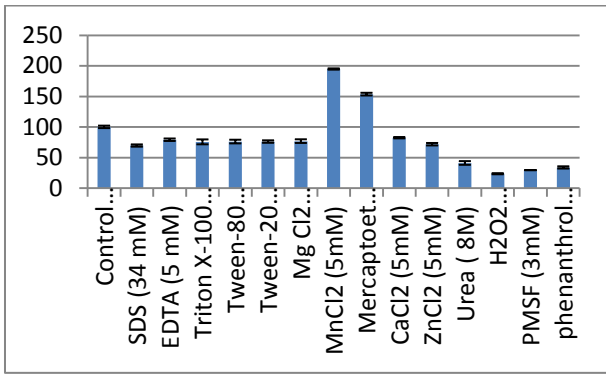

Figure 4. Effect of some chemicals on the enzyme activity.

Chromatographic analysis of the end products of CMC hydrolysis

After 2 h incubation of TG-1 enzyme-substrate (CMC) reaction mixture the thin layer chromatography revealed the presence of maltose, maltotriose, etc. This result suggested that the TG-1 cellulase is a very good producer of maltose [14].

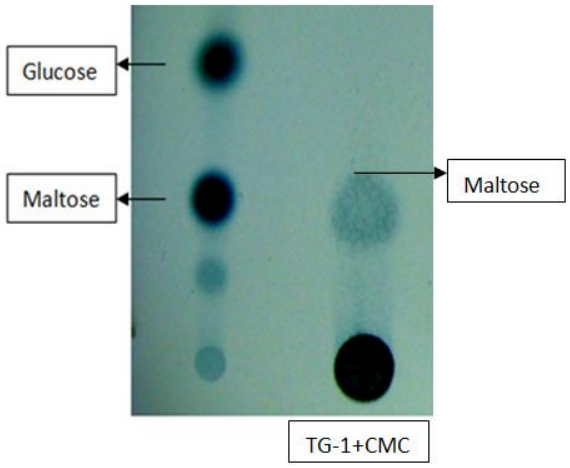

Figure 5. TLC analysis of products derived from TG-1-mediated hydrolysis of CMC.

CONCLUSION

In conclusion, the enzyme TG-1 is an acidophilic, halotolerant, cold-adaptive, detergent resistant and surfactant resistant enzyme. To days Cold-active detergents are in great demand in order to energy saving, so owing to the mentioned characteristics the isolated TG-1 cellulase enzyme can be useful in detergent industries.

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