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Isolation and Characterization of Thermostable, Alkaline, Detergent and H_2O_2 Resistant Cellulase (CMCase) from a Novel Strain *Bacillus sp*. CY8 Isolate

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Abstract

A thermostable carboxymethyl cellulase producing *Bacillus* sp. CY8 was isolated from soil. The enzyme was produced and partially purified. The enzyme showed a single band with molecular weight of 49.4 kDa in SDS-PAGE analysis. The enzyme showed its optimal activity at pH 10.0 and 80°C. More than 78% of original activity of the enzyme was observed after pre-incubation at pH 7.0-12.0 at room temperature for 24 h. It conserved more than 90% of its original activity after pre-incubation at temperature ranging from 30 to 110°C for 60 min. The enzyme activity was induced at 3-30% concentrations of NaCl. In the presence of 5mM EDTA, $CaCl_2$, $ZnCl_2$, $MgCl_2$ and 1,10-phenantrolin, 3mM PMSF, 0.1% tween20 and tween80, 1% SDS, tritonX-100 and β-mercaptoethanol, 0.1% H_2O_2, 8M urea, it remained 63, 76, 69, 67, 68, 70, 60, 68, 75, 67, 83, 97 and 65% of its original activity, respectively. According to TLC analysis, CMC was hydrolyzed into maltose, glucose and other oligosaccharides by CY8 CMCase. High thermostability, pH stability, detergent and antioxidant stability of *Bacillus* sp. CY8 CMCase make This Enzyme Useful In Textile, Detergent And Other Industries.

Key Words: *Bacillus* sp, thermostable, CMCase, textile, H_2O_2

INTRODUCTION

Cellulose which is the most polymeric and renewable component of plant biomass is hydrolyzed into soluble sugars by cellulases. Cellulases are accountable to hydrolysis of the β-1,4 glucosidic bonds in cellulose. This cellulolytic activity occurs by the synergistic effect of three major components; endo-β-glucanase (EC 3.2.1.4), exo-β-glucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). Cellulases are used in different industrial applications, such as in textile industry for bio-polishing for fabrics and producing stonewashed look of denims, in animal feed industry for improving the nutritional quality and digestibility, in food industry for processing of fruit juices and in baking, in detergent industry for improving fabric softness and brightness and removing the soil from cotton fibers [3], [4]. In addition to these applications, cellulases are used in bio-ethanol production from various lignocellulosic substrates [5].

Alkaliphilic *Bacillus* specieses are known to secrete various alkaline hydrolitic extracellular enzymes, such as cellulases, amylases, proteases and pectinases, which have industrial importance. However, *Bacillus* enzymes show high activity and stability under extreme alkaline environments [6], [7].

This research was aimed to isolate and characterize of alkaliphilic and thermophilic cellulase enzyme from *Bacillus* sp. CY8 strain in Adana, Turkey.

MATERIALS AND METHODS

Isolation of bacterial strains and culture conditions

Several soil samples were collected from Adana, Turkey. Each sample was incubated at 80°C for 10 min for selection gram-positive spore forming bacteria, *Bacillus* sp. The isolates were screened for cellulase production on CMC-LB agar medium plates containing per litre 10g tryptone, 5g yeast extract, 5g NaCl, 6g CMC, 15g agar agar. The pH was adjusted to 10.0 with 3% NaOH. Cellulase producing isolates were selected by staining with solution of 0.1% Congo Red [8], [9].

Enzyme Production

It was cultivated aerobically in CMC-LB broth (pH 10.0), containing per litre 10g tryptone, 5g yeast extract, 5g NaCl, 6g CMC, at 55°C for 26 h in a shaker incubator at 190 rpm. The culture was centrifuged at 8,000 rpm for 20 min. The supernatant was used for the enzyme assays [9].

Enzyme assay

CMCase activity was assayed by adding 0.5 mL of enzyme to 0.5 mL CMC (1% v/v) in 0.1 M Glisine-NaOH buffer (pH 10.0) and incubating at 80°C for 60 min. The reaction was stopped by the addition of 1 mL of 3,5-dinitrosalicylic acid reagent and the absorbance was read at 540 nm against blank using spectrophotometer [9].

Determination of pH and temperature effects on the enzyme activity and stability

The optimum temperature was tested at different temperatures 30–110°C. The optimum pH for activity of enzyme was determined using following pH buffers: Na–phosphate buffer (pH 6.0–8.0), glycine–NaOH buffer (pH 9.0–10.0) and borax–NaOH buffer (pH 11.0–12.0) [10]. For measurement of thermal stability, the enzyme was pre-incubated at temperatures between 30-100°C for 60 min at optimum pH. To determine pH stability, the enzyme was pre-incubated at 55°C for 24 h at pH 7.0 to 12.0. The remaining CMCase activity was detected under standart enzyme assay conditions [9].

Effect of various NaCl concentrations on enzyme activity

Effect of NaCl on the enzyme activity was studied by using various concentration of NaCl such as 3, 5, 7.5, 10, 15, 20, 25 and 30% at reaction mixture. The related enzyme assay was carried out at optimum condition [10], [11].

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 were determined by pre-incubating the enzyme at EDTA (5mM), $CaCl_2$ (5mM), $ZnCl_2$ (5mM), $MgCl_2$ (5mM), PMSF (3mM), tween20 (0,1%), tween80 (0.1%), 1,10-phenantroline (5mM), SDS (1%), urea (8M), tritonX-100 (1%), β-mercaptoethanol (1%) and H_2O_2 (0.1%) for 60 min at 55°C before adding substrate. Afterwards, the residual activities were measured at optimum condition. Control sample used in this part of research was considered as a sample without any pretreatmented and having 100% activity [5], [12].

SDS PAGE and Zimogram analysis

SDS-PAGE (10%) including CMC (0.1%) as a substrate and molecular weight markers (Sigma SDS6H2, 29.000, 45.000, 66.000, 97.000, 116.000, 200.000 Da) were used for the determination of molecular mass and zymogram analysis. After electrophoresis the gel was cut into two pieces, one (having marker bands) was stained with 0.1% Coomassie Blue R250 and detected by destaining the gel in methanol-acetic asid-water solution (1:1:8), other (having protein bands) was subjected to renaturation solutions before incubation for 5-6 h at 55°C [10], [12]. For zymogram of cellulase activity, SDS was removed by washing the gel at room temperature in renaturation solutions containing 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2), isopropanol 40% for 1 h and 50 mM Na_2HPO_4, 50mM Na_2HPO_4 (pH 7.2) for 1 h, respectively. Renaturation of the gel was carried out by keeping the gel in a solution 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. After incubation, the gel was stained with 1% Congo Red solution for 15 min and the activity bands were detected after destaining the gel in 1M NaCl solution for 15 min [3].

Chromatography of the end products of CMC hydrolysis

CMCase was incubated with CMC (2%) at 55°C for 2h. The end products were analyzed (15 µL) by thin layer chromatography. After treating the end products with chloroform-acetic asid-distilled water (6:7:1, v/v/v) solution, the spots were visualized by spraying aniline (1%, v/v), diphenilamine (1%, w/v), orthophosphoric acid (10%, v/v) in acetone and baking in oven at 120°C for 45 min [9], [13].

RESULTS AND DISCUSSION

The cellulase producing *Bacillus* sp. CY8 was isolated from soil samples near the cow manure and the plant waste mixture. The CY8 strain was a gram positive, rod shaped, spore forming and an aerobic bacterium. Based on the results of morphological and biochemical tests, CY8 strain was identified as belonging to the genus *Bacillus*. Isolation of bacteria and optimization of the culture conditions are major steps for a successful production of enzyme. However, the diameter of halo zones around the colonies showed enzyme production ability of bacteria (Fig. 1). Therefore, CMCase synthesis of *Bacillus* sp. CY8 observed at temperatures between 30-60 ºC with an optimum of 55ºC and at different pH values between 8.0-12.0 with an optimum pH 10.0 on solid medium including CMC in petri dishes. According to this results, the isolate CY8 is called typically alkaliphilic and thermophilic, as it grows and produce optimally at pH values above 8.0 and at high temperature [3].

Figure 1. Cellulase synthesis of *Bacillus* sp. CY8 on CMC agar plate

Molecular weight was determined by SDS-PAGE as described above. Analyses of the enzyme by SDS-PAGE revealed single band that showed cellulolytic activity. The molecular weight of this band was indicated as 49.4 kDa (Fig. 2). Similar result of this zymogram analyses was reported by [5], [7], [14], [15] and [16].

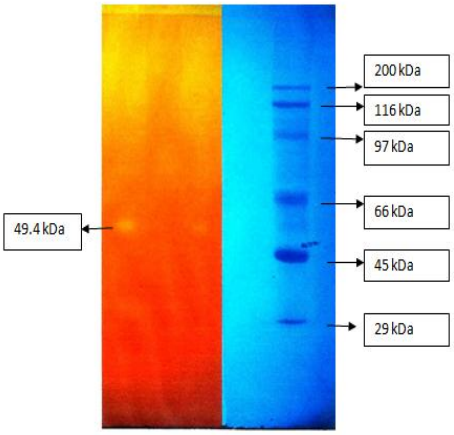

Figure 2. Analysis of CY8 cellulase by %10 homogenized SDS-PAGE. The sample and marker were subjected to SDS-PAGE and after the electrophoresis the gel was cut into two pieces. The first part of the gel (containing marker) was stained with Coomasie Brillant Blue and destained in methanol-acetic asid-water solution (1:1:8). Other piece (containing enzyme bands) was subjected to the renaturation solutions before incubation for 5-6 h at 55°C and the activity of enzyme revealed by Congo Red (0.1%).

CY8 CMCase was active over a broad range of pH 7.0-10.0 and it showed maximum activity at pH 10.0 (Fig. 3). Although, activity of the enzyme showed linear increase with increase in pH from 8.0 to 10.0, it was sharply decreased at pH 11.0 and 13.0. Similar observations were also made by other researches. Cellulase enzyme produced by *B. halodurans* CAS 1 exhibited optimum activity at pH 9.0 [11]. Also, cellulose enzyme from *Bacillus flexus* showed the optimum activity at pH 10.0 [17].

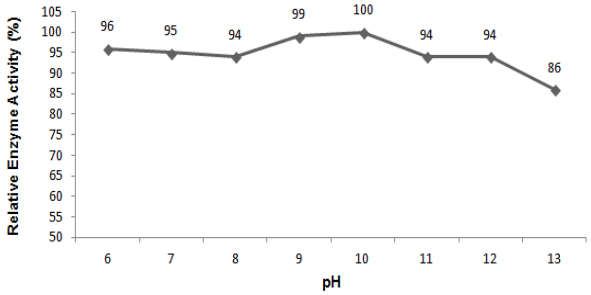

Figure 3. Effect of pH on the activity of *Bacillus* sp. CY8 cellulase

The pH stability of CMCase was determined by incubating at 55°C for 24 h in a range of pH 7.0-13.0 and then assaying the residual activity at 80°C for 60 min. The enzyme was stable between pH 7.0-12.0 with more than 73% activity. However it showed 59% activity in pH 13.0 (Fig. 4). So, this is evidence that the enzyme is an alkaline tolerant enzyme. The present study correlate well with earlier reports for alkaline extracellular cellulase enzyme reported from *Bacillus flexus* [17], that has shown its pH stability in the range of 8.0-12.0. The cellulase enzyme of marine bacterium *Bacillus aquimaris* was found to be stable in the broad range of pH (6.0-12.0). The enzyme retained 85% of its activity at incubation pH of 12.0 and reduced to 65% at pH 13.0 [18]. High activity and stability of CY8 cellulase at high tempratures and alkaline pH conditions shows that this enzyme could be useful for house-hold laundry detergent, textile, leather, agriculture, food, paper and pulp industries [11], [17], [19].

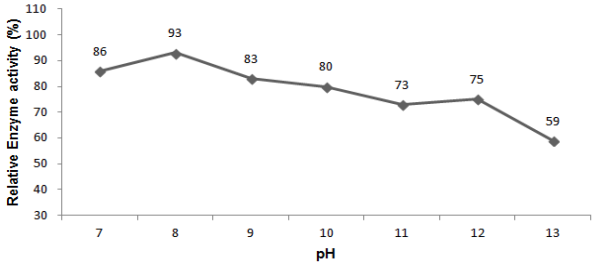

Figure 4. Effect of pH on the stability of *Bacillus* sp. CY8 cellulase.

CY8 CMCase was active over a broad range of 30-100°C and it showed maximum activity at 80°C (Fig. 5), which is similar to the optimum temperature reported for such enzymes from [20], [21]. And the optimum temperature of CY-8 cellulase was higher than the cellulase produced by *B. halodurans* CAS 1 [11] which exhibited optimum activity at 60°C. The optimum temperature was 65 and 70°C for the cellulases produced by two *Bacillus* strains, CH43 and HR68, respectively [19].

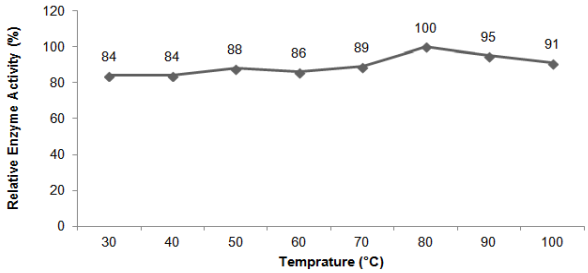

Figure 5. Effect of temperature on the activity of *Bacillus* sp. CY8 cellulase

The thermal stability of CMCase was determined by heating for 60 min at the temperature indicated in the range of 30-100°C and then assaying the residual activity at 80°C for 60 min. The enzyme was stable between 30-100°C with more than 88% of its activity (Fig. 6). The thermal stability of the carboxymethyl cellulase produced by *Bacillus pumilus* S124A was determined at various temperatures ranging from 20 to 90°C. More than 70% of the enzyme activity was maintained at temperatures ranging from 40 to 70°C. It was stable about 84.4% at 50°C [5]. According to the thermostability studies of the cellulase enzyme of *Bacillus halodurans* CAS 1, the enzyme was 100% stable up to 70°C and it retained more than 90% of its original activity at 80°C and 36% even at 95°C [11].

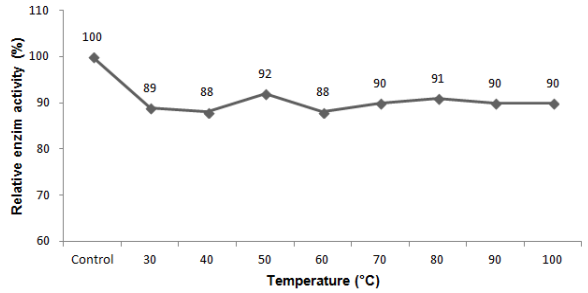

Figure 6. Effect of temperature on thermal stability of *Bacillus* sp. CY8

Figure 7 shows the effect of various metal ions, chealators, surfactants and inhibitors on the activity of CY8 cellulase. The presence of EDTA, $CaCl_2$, $ZnCl_2$, $MgCl_2$, PMSF, tween20, tween80, 1,10-phenantroline, SDS, urea, tritonX-100 and β-mercaptoethanol, CY8 CMCase was showed 63, 76, 68, 68, 59, 67, 69, 73, 75, 65, 67 and 83% residual activity, respectively. On the other hand, it was unaffected by H_2O_2 (97%). These findings are in accordance with the results of other scintists [7], [22]. The effects of different metal ions on CY8 Cellulase activity was studied at 5 mM concentration. $CaCl_2$, $ZnCl_2$ and $MgCl_2$ showed inhibitory effect, 24, 32, 32%, respectively. SDS (0.1%) decreased the enzyme activity about 35%. SDS could interact with the hydrophobic group of amino acids, resulting in the decreased enzyme activity [9], [23], [24]. The non-ionic detergents 0.1% (v/v) tritonX-100, tween20 and tween80 showed slightly inhibitory effect, 33, 33, 31%, respectively [9], [24]. 1,10-phenantroline and 5mM EDTA decreased the activity of CY8 cellulase, 37 and 32%, respectively. The major inhibition of these inhibitors suggested that the CY8 cellulase is a metalloprotein [9], [25]. However, the massive

inhibition observed against PMSF (0.1%) suggested that the enzyme do possesses modification of a serine residue (ser) at its active site [3], [25]. Additionally, it was not affected by H_2O_2 (97%) [9], [22].

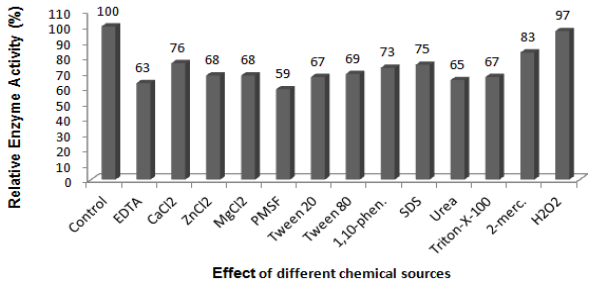

Figure 7. Effect of different chemical sources on the activity of *Bacillus* sp. CY8 cellulase

In this study CY8 enzyme activity was stimulated by various NaCl concentrations (3-30%) about average 26% (Fig. 8). Similar results have been reported by [26] and other researchers. *Bacillus aquimaris* cellulase retained nearly 100% of its activity in the presence of NaCl (10%) [18]. *Bacillus flexus* cellulase showed high tolerance towards high salt concentrations (NaCl 15%) [17].

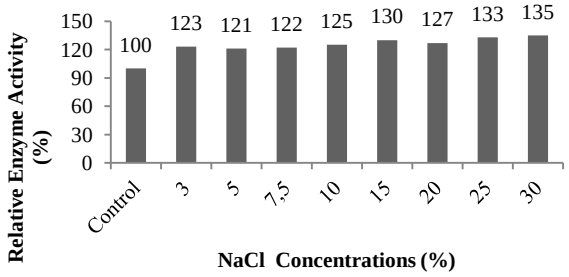

Figure 8. Effect of NaCl on the activity of *Bacillus* sp. CY8 cellulase

After 6 h incubation of enzyme-substrate mixture, the thin layer chromatography of the CMC hydrolysate revealed the presence of maltose, etc (Fig. 9). This result suggested that CY8 CMCase is a good producer of maltose [9], [13].

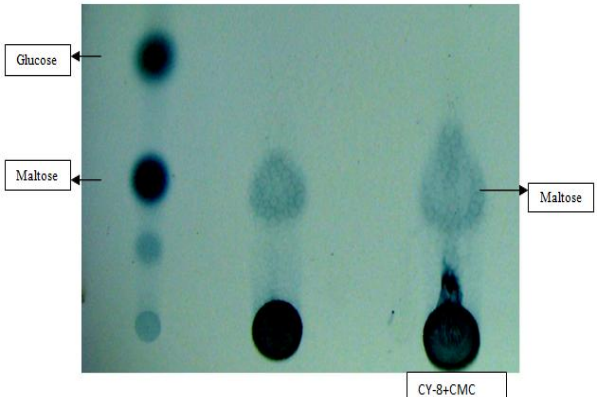

Figure 9. Thin Layer Chromotography (TLC) of CY8 cellulase

CONCLUSION

According to these results, *Bacillus* sp. CY8 cellulase shows high thermostability, pH stability, detergent and antioxidant stability. These properties make this cellulase useful in textile, detergent and other industrial applications.

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