

## Partial Purification and Characterization of Thermostable, Alkaline and Chelator-resistant Protease from a Newly Isolated *Bacillus* sp. CY7 and its potential applications in various industries

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### Abstract

A thermophile alkaline protease producing *Bacillus* sp. strains isolated from soil samples. Enzyme synthesis occurred at temperatures between 20°C and 60°C with an optimum of 55°C and between the pH range of 6.0-13.0 with an optimum pH of 10.0 on skimmilk agar plates. Analyses of molecular mass of the partially purified enzyme was carried out by SDS-PAGE which revealed two bands as 112 and 97 kDa. The enzyme was active in a broad temperature range between 30°C and 100°C, with an optimum at 70°C, and maximum activity was at pH 11.0. The enzyme also presented the alkaline-stable properties with a remaining activity around 94%, at pH 6.0-13.0 for 24 h, at 55°C. The enzyme activity was highly stable between 30-100°C with a remaining activity 91%, after pre-incubation for 1 h. Enzyme was exposed to various NaCl concentrations (3-30%), and the highest residual activity was determined in the presence of 3% NaCl (87%). While after being exposed with  $\beta$ -mercaptoethanol (1%), PMSF (3mM), Tween20 (0.1%), Tween80 (0.1%), SDS (1%), TritonX100 (1%), H<sub>2</sub>O<sub>2</sub> (1%), MnCl<sub>2</sub> (5mM), CaCl<sub>2</sub> (5mM), BaCl<sub>2</sub> (5mM), MgCl (5mM), FeCl<sub>2</sub> (5mM) and EDTA (5mM), the enzyme exhibited the following activities 105%, 101%, 107%, 119%, 72%, 99%, 98%, 98%, 99%, 98%, 97%, 97%, 96% and 99%, respectively.

According to these results, CY7 protease shows thermostable, high alkaline, alkali-stable and chelator resistant properties. Owing to its mentioned properties this protease is an ideal choice for application in detergent formulations, leather and textile industries.

**Keywords:** Alkali-Aggregate Reaction, Inhibitor, Slag, Silica Fume, Test Concrete Siliceous, Silica

## INTRODUCTION

Proteases, hydrolytic enzymes that cleave peptide bonds between amino acid residues, and particularly alkaline proteases, are the most commercialized and used enzymes in the world [1]. They have a variety of applications in detergent, food, pharmaceutical and leather industries, peptide synthesis and recovery of silver from used X-ray films [2]. Bacteria, moulds and yeasts are some of the microorganisms that produce proteases. Most of the commercial alkaline proteases were isolated from *Bacillus* species [3]. The reason for this is their high pH and temperature stability. Alkaline proteases belong to the group

of proteases, which have either a serine center or are of metallo-type, exhibiting a wide pH range of pH 6.0-13.0. Among these are the serine proteases with industrial importance [4]. Major application of proteases with high activity and stability in high alkaline range and high temperatures is in detergent industry, because the pH of laundry detergents is generally in the range of 9.0-12.0, accounting for about 35% of the total microbial enzymes sales [3].

This work describes the isolation of the CY7 strain, characterization and optimization of the CY7 protease and some properties of the enzyme activity of industrial interest.

## METHODS

### Organism and culture conditions

*Bacillus* sp. CY7 was isolated from soil samples. After the isolation of strain CY7, it was identified by various morphological and biochemical tests. This isolate was screened for protease production on skim milk agar plates at different temperatures (20-60°C) and pH values (6.0-13.0). Protease production was verified by the development of a clear zone surrounding bacterial colonies.

### Production and partial purification of enzyme

The production of alkaline protease by strain CY7 was investigated during 72 h of cultivation in the production medium containing casein (at 55°C). The sample was centrifuged at 8.000 rpm for 15 min at 4°C and cell-free supernatant of strain CY7 was used for partial purification of enzyme. Pre-chilled ethanol was slowly added to the supernatant to 80% (v/v) saturation and the mixture was left at -33°C for overnight. The precipitate was collected by centrifugation at 10.000 rpm for 20 min at 4°C, dissolved in 0.1 M sodium-phosphate buffer at pH 7.0.

### Enzyme assay

Protease activity was measured by the modified method of Sana et al (2006) using casein as a substrate. The reaction mixture containing 0.5 mL of Glycine-NaOH buffer (100mM, pH10.0), 0.5 mL of 2% casein solution and 1 mL of enzyme solution was incubated at 55°C for 1 h. The reaction was terminated by addition of 2 mL of 10% trichloroacetic acid solution and the non-hydrolyzed casein was removed by centrifugation at 10.000 rpm for 10 min. The acid-soluble material was estimated spectrophotometrically at 280 nm.

### Effect of temperature and pH on protease activity and stability

The enzyme assay was carried out at different temperatures, in the range of 30–110°C, to investigate the effect of temperature on enzyme activity [6]. The following buffers were used to study the effect of pH on enzyme activity: 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) [7]. The enzyme assay was performed at 55°C as described above. The thermal stability was studied by pre-incubating the enzymes at different temperatures; 30–100°C for 60 min. The residual activity was determined by incubating enzyme at optimum temperature for 60 min under standard conditions as mentioned above [2]. For the measurement of pH stability, enzyme was pre-incubated in buffers at different pH in the range of 6.0–13.0 for 1 h at 55°C. The remaining activity was determined at optimum temperature for 60 min under standard conditions [2]. The untreated enzyme was considered as a control (100%).

### Effect of different NaCl concentrations, metal ions, surfactants, chelating agents and inhibitors on enzyme activity

The enzyme was pre-incubated at optimum temperature in the presence of different concentrations of NaCl (3, 5, 7.5, 10, 15, 20, 25, 30%) for 60 min and the residual activity was measured under standard assay conditions [8]. The activity of the enzyme alone in Glycine-NaOH buffer (100 mM, pH 10.0) was taken to be 100% as a control.

The influence of various chemicals on protease activity was studied by pre-incubating the enzyme at 55°C for 60 min and then the residual activity was measured using casein as substrate at pH 10.0 and 70°C [9]. The activity of the enzyme (without any additives) was taken as 100%.

### SDS-PAGE and Zymogram analysis

SDS-PAGE was performed to determine the homogeneity and molecular mass of the purified protease by the method of Laemmli (1970) using 5% stacking gel and 10% resolving gel. The electrophoresis was performed with 25 mA and 15 mA constant currents. The molecular weight of the enzyme was estimated using a standard molecular weight marker (200, 116, 97, 66, 45, 29 kDa). Zymogram analysis was performed by incorporating skim milk (3%) into the separating gel before polymerization. After the electrophoresis, the gels were rinsed in 2.5% Triton X-100 for 45 min to remove SDS. The gel was then incubated in Glycine-NaOH buffer (100 mM, pH 10.0) overnight. Finally, the gel was stained with Coomassie brilliant blue R-250 and destained, and the clear zones indicated the presence of protease activity [9].

### Chromatographic analysis of the end products of casein hydrolysis

The enzyme was incubated with a 2% casein solution in Glycine-NaOH buffer (100 mM, pH 10.0) at 70°C for 2 h. The hydrolysis products of casein were submitted to thin-layer chromatography (TLC) with a silica gel 60 (F<sub>254</sub>, Merck) [11]. Tyrosine used as a standard for TLC. After developing the products with a solvent system composed of butanol/acetic acid/distilled water (3:1:1, v/v/v) [12], the spots were visualized by spraying TLC plate with a reagent containing 0.25% ninhydrin in acetone and baking it in an oven at 110°C for 15-20 min [13].

## RESULTS AND DISCUSSION

### Isolation of alkaline thermophilic *Bacillus* sp.

Strain CY7 was Gram positive, rod shaped, spor forming bacterium and aerobic. With the respect to this results of various morphological and biochemical characteristic, it was identified as belonging to the genus *Bacillus*. that grew and produced protease enzyme at pH 6.0-13.0 with an optimal of 10.0, and at 20-60°C with an

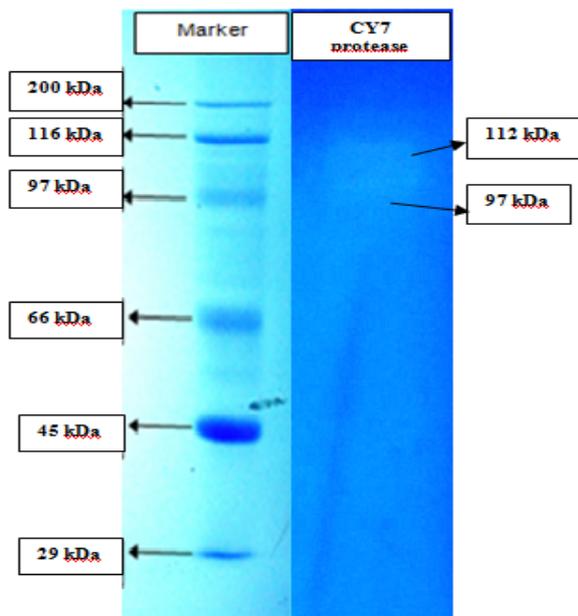
optimal temperature of 55°C on skim milk agar plates (Fig. 1). Alkaliphiles are defined as microbes growing optimally within pH 9-12, although the optimal pH varies depending on the growths conditions [14].



**Fig. 1.** Protease synthesis of *Bacillus* sp. CY7 on skim milk agar plate

**Determination of molecular mass**

Analyses of molecular mass of the partially purified enzyme was carried out by SDS-PAGE which revealed two bands as 112 and 97 kDa (Fig. 8). The similar results with CY7 protease zymogram analyses were reported by researchers. These results suggested that the enzyme have two subunits or dimeric structure [15].



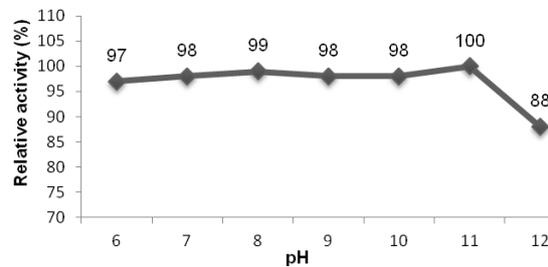
**Fig. 8.** SDS-PAGE and Zymogram analysis of CY7 protease

**Effect of temperature and pH on protease activity and stability**

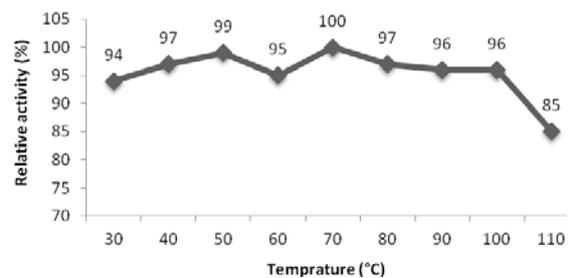
The enzyme was active in a broad temperature range between 30°C and 100°C, with an optimum at 70°C (Fig. 3), and maximum activity was at pH 11.0 (Fig. 2). Similarly, an optimum temperature of 70 and 80°C was reported for an alkaline protease from *Bacillus* sp. The optimum temperature of *Bacillus subtilis* PE-1 protease recorded was at 60°C for protease activity [7]. *Bacillus subtilis* PE-1 thermostable serine alkaline protease showed the highest protease activity at pH 10.0 using glycine-NaOH buffer [7]. The crude extracellular protease produced by the isolate had optimal activity at 65–70 and 70°C in the absence or presence of 2mM CaCl<sub>2</sub>, respectively [3]. An extracellular alkaline protease produced by *Bacillus licheniformis* AP-1 was optimally active at pH 11.0 and at 60°C [16].

It was almost completely stable from pH 6.0 to 13.0 for 60 min (about %91 residual activity, Fig. 4). CY7 can be classified as an alkaline protease. The similar results of this analyses were reported in literatures [17]. This is a very important characteristic for its eventual use in detergent formulations, because the pH of laundry detergents is generally in the range of 9.0-12.0 [17, 18].

The enzyme was extremely stable at 20 to 100 °C after more than 60 min incubation with casein substrate with a remaining activity 91% (Fig. 5). This stability might be an advantage for using CY7 protease in industrial application such as laundry detergent formulations for example [19].



**Fig. 2.** Effect of pH on the activity of *Bacillus* sp. CY7 protease



**Fig. 3.** Effect of temperature on the activity of *Bacillus* sp. CY7 protease

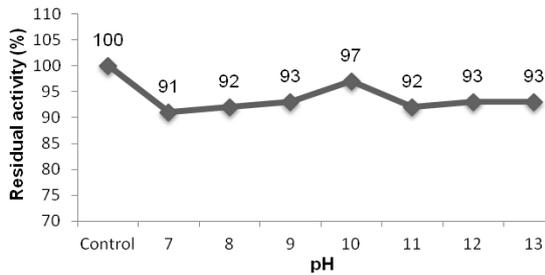


Fig. 4. Effect of pH on the stability of *Bacillus* sp. CY7 protease

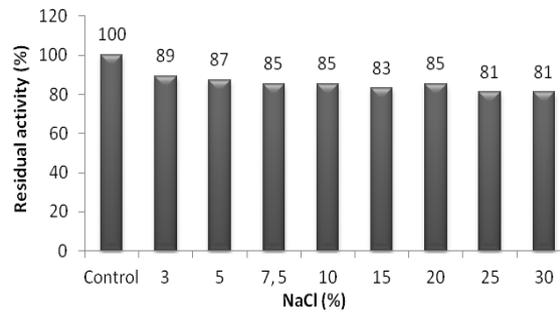


Fig. 6. Effect of NaCl and on enzyme activity of *Bacillus* sp. CY7 protease

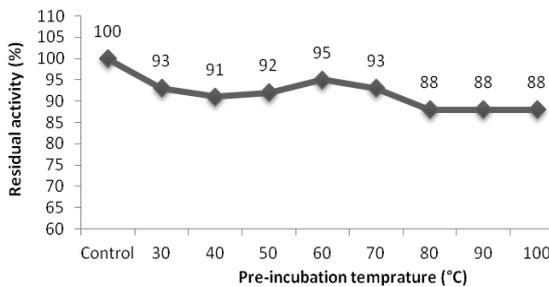


Fig. 5. Effect of temperature on the stability of *Bacillus* sp. CY7 protease

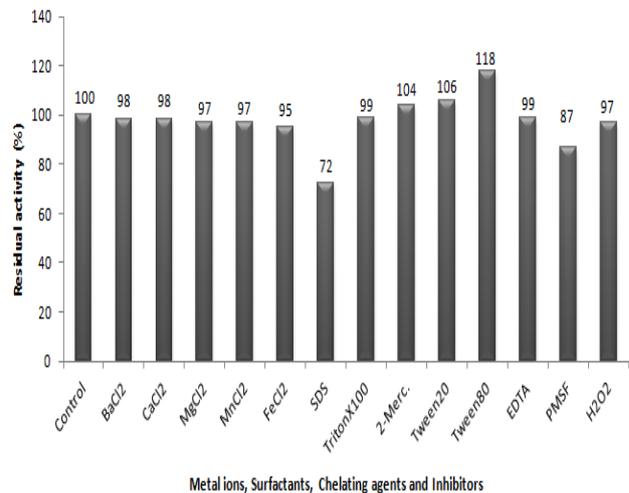


Fig. 7. Effect of various chemicals on enzyme activity of *Bacillus* sp. CY7 protease

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

The residual enzyme activity results have given in Fig. 6 and Fig. 7. The highest residual activity was shown in the presence of 3% concentration of NaCl [20]. While after being exposed with  $\beta$ -mercaptoethanol (1%), PMSF (3 mM), Tween20 (0.1%), Tween80 (0.1%), SDS (1%), TritonX100 (1%), H<sub>2</sub>O<sub>2</sub> (1%), MnCl<sub>2</sub> (5 mM), CaCl<sub>2</sub> (5 mM), BaCl<sub>2</sub> (5 mM), MgCl<sub>2</sub> (5 mM), FeCl<sub>2</sub> (5 mM) and EDTA (5 mM), the enzyme exhibited the following activities 105%, 87%, 107%, 119%, 72%, 99%, 98%, 98%, 99%, 98%, 97%, 97%, 96% and 99%, respectively. The CY7 protease activity was inhibited (13%) by PMSF (5mM). Thus, this result is thought that the enzyme do not possesses modification of a serine (ser) residue at the active site. Because PMSF is known as serine protein inhibitors [21, 22]. Incubation with 1% of Tween-20, Tween-80, and TritonX100 at 70°C for 1h, CY7 exhibited enhanced residual activities between 105-119 %. These results are consistent with those reported for alkaline proteases from *B. clausii*, *B. mojavensis* and *Bacillus* sp. [23, 24, 25, 26, 27, 28]. CY7 protease retained 72% and 99% of its original activity, respectively, even after incubation with 0.5% SDS and 1% H<sub>2</sub>O<sub>2</sub> at 72°C for 1 h. Proteases that use in detergent formulations should maintain activity in the presence of these surfactants and oxidants. These results showed that our enzyme is a good source for detergent additive [29, 30].

**Chromatographic analysis of the end products of casein hydrolysis**

Enzyme-substrate mixture was incubated for 2 h and then the mixture was submitted to TLC plate and incubated in a solvent system. According to the results of TLC analysis, the end products of casein are tyrosine, hydroxyproline and a large quantities of proline (the yellow spots) (Fig. 9) as mentioned by the other researchers [13].

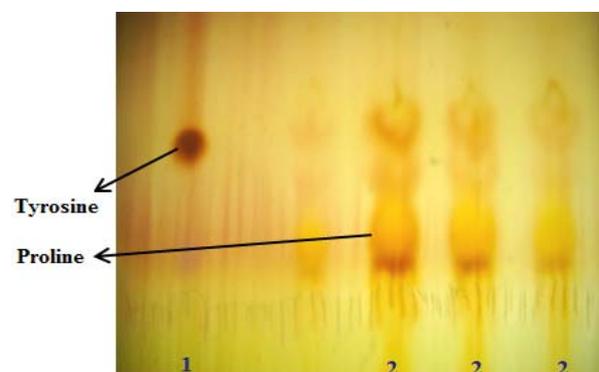


Fig. 9. Thin layer chromatography analysis of CY7 protease. Lane 1, tyrosine; lane 2, products of hydrolysed casein

## CONCLUSION

According to these results, *Bacillus* sp. CY7 protease shows thermostable, high alkaline, alkali-stable and chelator resistant properties. Owing to its mentioned properties this protease is an ideal choice for application in detergent formulations, leather and textile industries.

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