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Optimization of Production and Factors Affecting the Stability of a New Protease Produced by a Thermohaloalkali Tolerant *Halobacillus* Strain

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The optimized conditions for the production of a protease produced by a thermohaloalkali tolerant *Halobacillus* strain were determined. The results showed that the optimum temperature for enzyme activity was 65ºC and the highest proteolytic activity was obtained at 10% NaCl. Also, the enzyme exhibited proteolytic activity over a broad pH range from 5 to 13 and the highest activity achieved in the range from 9 to 12. However, casein, glycerol and glucose as carbon sources supported the highest growth, but protease production was not supported by them similarly and the highest protease activity was obtained with galactose. The highest protease activity was obtained also with NH_4Cl as a nitrogen source. On the other hand, the maximum protease stability was obtained at 30ºC and remained stable till 65ºC after which the stability decreased gradually and beyond 75ºC the activity decreased sharply. Surprisingly, the stability of the protease decreased with the increase of NaCl concentrations. The stability of the protease was maximum at pH 7 and remained highly stable up to pH 11. These results clearly indicated the thermohaloalkali tolerant nature of the enzyme.

Key words: Protease, production optimization, stability, thermohaloalkali tolerant, *Halobacillus* sp.

Enzymes play very important roles in the environment and serve as very powerful tools for sustainable development in a variety of industries and biotechnological applications. They are applied in detergent, textiles, baking, brewing, starch, animal food, leather, and pulp industries. Several substrates can only be degraded or converted by enzymes which perform under extreme conditions [1].

Recent research has focused on the identification of extremozymes relevant for industrial biocatalysis. Demirjian et al.[2] recorded many of the most interesting enzyme classes for use in industrial biotransformations that are active or stable under extreme conditions.

Proteases are used in a range of biotechnological applications which is widely acknowledged. Many extremophilic proteases have been studied because of their utility in the laundry detergent industry[3]. In this regard, the inherent stability of thermostable proteases at elevated temperatures and in the presence of organic solvents and denaturing agents enables their use in processes that restrict the use of conventional enzymes [4].

A remarkable obstacle for the industrial applications of extremophilic proteases is that the alkaline proteases are usually stable in highly

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alkaline conditions but unstable at high temperatures. On the other hand, proteases that are generally stable in thermal conditions are unstable at alkaline pH [5]. Therefore, proteases that are stable at different extreme conditions are of industrial interest.

In this paper, optimizing the production conditions of a protease enzyme and studying the effects of different factors on its stability was carried out. In a previous study, the production, purification and characterization of this protease were reported [6].

MATERIALS AND METHODS

Bacterial strain

Isolation of the thermohaloalkali tolerant strain B300 on Sato agar medium [7] adjusted at pH 10 and amended with 10% NaCl after incubation at 55°C for 3 days; and identification of it phylogenetically to *Halobacillus* sp. was reported in a previous study [6].

Optimization of culture conditions for protease production

Effect of temperature, NaCl and pH

The production medium and growth conditions were the same as described previously [6]. In brief, the culture was grown in 500 ml flasks containing 100 ml of Czapek dox broth [8] supplemented with 10% NaCl and the pH was adjusted to 10 as the production medium. The bacterial culture was grown for 24 h at 55°C on a shaking incubator at 150 rpm. For optimization of the culture conditions for protease production, the bacterial culture was grown at different temperatures, NaCl concentrations and pH values. This was carried out by incubating the production flasks at 30, 40, 50, 55, 60, 65, 70, 75, 80, 85 and 90°C with initial pH of 10 and 10% NaCl. For the effect of NaCl, the production medium was prepared with different NaCl concentrations (0, 5, 10, 15, 20, 25, and 30%), while the pH value was 10 and incubated 65°C. The effect of the initial pH of the medium was tested by growing the producing strain in medium supplemented with 10% NaCl at different initial pH values (5 to 13) and the flasks were incubated at 65°C. The growth and protease production were monitored every 4 h by measuring the optical density of the broth at 600 nm and the enzyme activity was measured by a modification of the method described by Johnvesly and Naik [9].

Effect of different carbon and nitrogen sources

The effect of different carbon and nitrogen sources on growth and protease production was tested. L-arabinose, casein, D-fructose, D-galactose, D-glucose, glycerol, D-lactose, maltose, mannitol, raffinose, ribose, D-sorbitol, starch and sodium citrate were used a sole carbon source instead of sucrose in the Czapek dox broth. Organic and inorganic nitrogen sources including ammonium acetate, ammonium chloride, ammonium formate, ammonium nitrate, ammonium sulphate, beef extract, casein, gelatin, peptone, potassium nitrate, urea and yeast extract were tested as sole nitrogen source instead of sodium nitrate originally in the Czapek dox broth. All media were supplemented with 10% NaCl, the initial pH was adjusted to 10 and then the cultures were incubated at 50°C. The growth and protease production were monitored as described above.

Effect of temperature on stability of the protease

For determination of thermostability, 0.1 ml of the pure enzyme was pre-incubated with 0.9 ml of sodium phosphate buffer (pH 7) for ten minutes at temperature in the range 30-90°C. After incubation, the remaining activity was measured by standard procedure [9].

Effect of salinity on stability of the protease

The effect of NaCl on stability of the protease was determined by incubating the purified enzyme at various NaCl concentrations (0, 5, 10, 15, 15, 20, 25 and 30%) at pH 10 and 65°C for 24 hours. Then, protease activity was determined by the method mentioned above.

Effect of pH on stability of the protease

The effect of pH value on stability of the protease enzyme was determined by pre-incubating the pure enzyme in different buffers (citrate-phosphate, pH 5-6; sodium phosphate, pH 7.0; Tris-HCl, pH 8.0; and glycine-NaOH, pH 9-13) for ten hours at 65°C. Then, the remaining activity was measured.

RESULTS AND DISCUSSION

Temperature plays an important role in enzyme production. The optimum temperature varies according to the kind of microorganism, substrate used as well as the environmental conditions [10]. According to Fig.1, the optimum

temperature for enzyme activity was found to be 65ºC. No activity was recorded at 90ºC. This means that the protease under study can be regarded as a thermophilic or thermotolerant enzyme.

Similar to most halophilic/halotolerant enzymes, the results showed that the protease activity was highly reduced in the absence of NaCl (Fig. 2). The highest proteolytic activity was obtained at 10% NaCl. Also, high protease activity was obtained under high salt conditions. This may be due to the adaptation of the extracellular protease to the high salt conditions in the environment of the producing organism. The requirement of high salt concentration for the activity and stability of the enzymes of halotolerant and halophilic microorganisms was recorded by many researchers [10, 11, 12].

Also, pH plays an important role on enzyme production. Several researchers [11, 13] discussed the effect of pH value on the production of enzymes by various organisms. The protease under test exhibited proteolytic activity over a wide pH range from 5 to 13, while the higher activity occurred in the range from 9 to 12 (Fig. 3). This indicated the alkaliphilic nature of the enzyme. The optimum pH range between 9 and 11 for protease production is common among alkaliphilic and haloalkaliphilic organisms [10, 14, 15].

Protease activity was not detected at temperature less than 30ºC, or when there was no NaCl and at low pH value less than 5 in the reaction mixture. Maximum protease activity (100%) was obtained with 10% NaCl, pH 10-11 and at 65ºC. These results clearly indicated the thermohaloalkali tolerant nature of the enzyme.

On the other hand, the effect of carbon and nitrogen sources on growth and protease production was also studied. It was found that casein, glycerol and glucose supported the highest growth, but protease production was not supported by them at the same level. The results revealed that glucose greatly repressed the production of the protease enzyme (Fig. 4). Similar effects of glucose on the production of alkaline

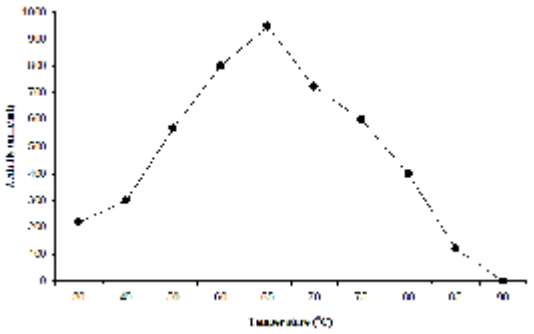

Fig. 1. Effect of temperature values on protease activity

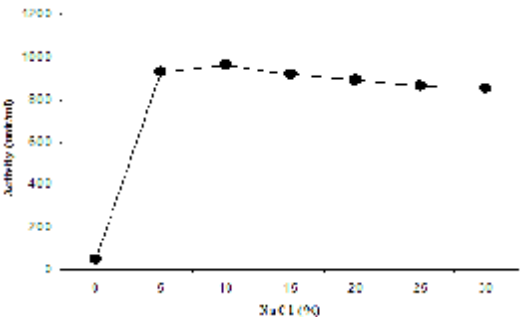

Fig. 2. Effect of different NaCl concentrations on protease activity

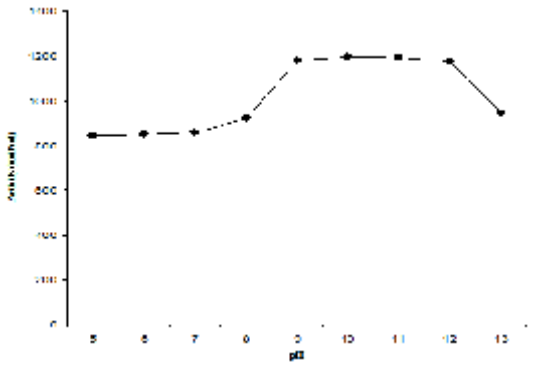

Fig. 3. Effect of different pH values on protease activity

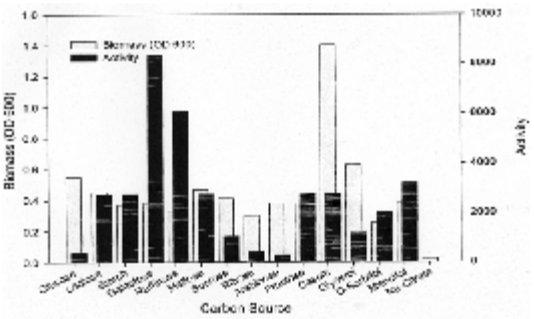

Fig. 4. Effect of different carbon sources on growth and protease activity

and thermo-stable proteases were also observed by other researchers [9]. The highest protease activity was obtained with galactose and raffinose, while no protease activity was recorded in case of sodium citrate.

Results in Fig. 5 showed that organic nitrogen sources in the medium as yeast extract, casein, beef extract, peptone and gelatin enhanced the highest growth but not the enzyme production. The highest protease activity was reached in case of NH_4Cl followed by ammonium acetate. Similar results were observed by [16]. These findings agree to some extent with the phenomenon of repression of growth, but not enzyme production by ammonium which was reported by other workers[17].

The optimization studies of the protease production revealed that the most suitable production medium for this enzyme should contain galactose (3%), ammonium chloride (0.3%) and 10% NaCl with initial pH of 10.

Figure (6) showed that the maximum protease stability in relation to temperature was obtained at 30°C and remained stable till 65°C after which the stability decreased gradually and beyond 75°C the activity decreased sharply. Similarly, Ferrero et al. [17] reported a thermostable protease from *B. licheriformis* MIR 29 showing 100% stability up to 70°C.

It was found that the stability of the protease enzyme decreased with the increase of NaCl concentrations (Fig. 7). This result may be due to the effect of using casein as a substrate in the enzyme assay. Similar results were observed by a halophilic serine proteinase produced by *Halobacillus* sp. SR5-3 [12].

Figure (8) revealed that the protease enzyme exhibited stability over a broad pH range from 5 to 13. The stability of the protease under study was maximum at pH 7 and remained highly stable up to pH 11 confirming that the enzyme is alkali stable. The protease stability was slightly low at the pH values 5, 6, 12 and 13. These results are in agreement with the earlier observations on protease from *Bacillus alcalophilus* sub sp.

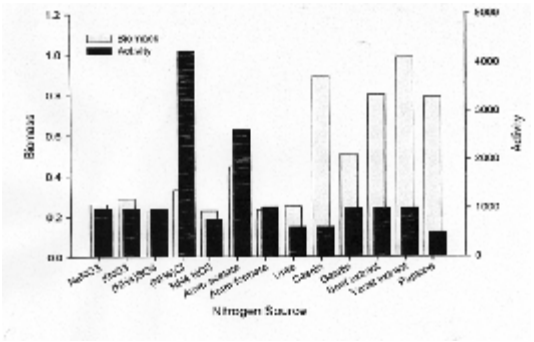

Fig. 5. Effect of different nitrogen sources on growth and protease activity

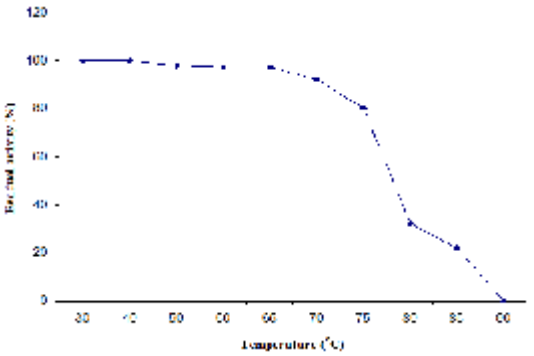

Fig. 6. Effect of temperature on protease stability

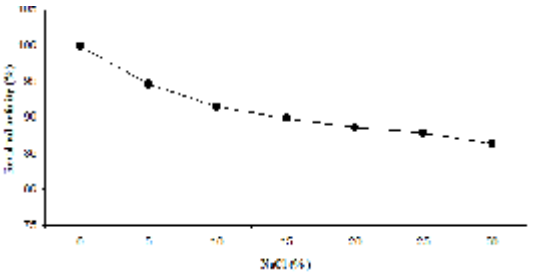

Fig. 7. Effect of different NaCl concentrations on protease stability

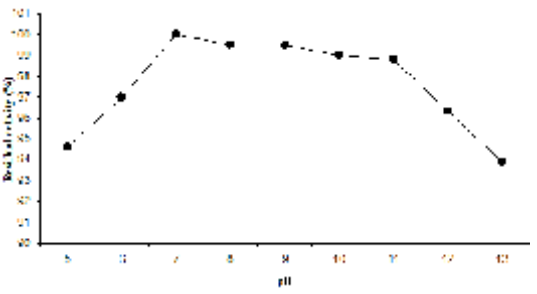

Fig. 8. Effect of different pH values on protease stability

halodurans that showed stability over a pH range of 5–10 [18]. Also, the alkaline protease isolated and purified from *B. licheniformis* has a wide pH range 4-12 with maximum stability at pH 10 [19].

The present results clearly indicated the thermohaloalkali tolerant nature of the protease enzyme under study. The ability of this enzyme to work and remain stable at these harsh conditions is very important and suitable for the industrial applications of the enzymes. Therefore, further studies on its application at large scale is required.

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