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Optimization of Metalloprotease Enzyme Extracted from Marine *B.thuringiensis* Strain 1257 Metabolism

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Abstract: ***Marine water contains large numbers of different species of microorganisms particularly, bacteria, which is considered to be a biological factory for producing protease enzymes that offer great biotechnological, industrial and pharmaceutical applications. At this work we isolated a hyper protease producer bacterium B.thuringiensis strain 1257 from the Mediterranean Sea water and a purified protease from strain 1257. The protease was identified as metalloprotease. Metalloprotease was partially purified by ammonium sulfate followed by application on Sephadex G-75 column. Gel filtration step resulted in more than 40 times fold purification of the purified enzyme. The enzyme activity was inhibited by EDTA (almost 80%) at 15 mM concentration. Optimum temperature for enzyme activity was 60 °C. It also exhibited a broad pH activity range (6-9) with an optimum pH of 8.***

Keywords: Marine Microbial Protease, Metalloprotease, *B. Thuringiensis*, EDTA.

1. Introduction

Continuity of survival on the surface of the globe depends on the surrounding environmental conditions and the ability to adapt. The seas and oceans are one of the main sections of the Earth's surface, as they constitute approximately 75% of the Earth's surface and contain most forms of life and ecosystems, including microorganisms [1]. Marine water is a great source of microorganisms which have superior and distinctive properties, these superior features have been acquired due to their ability to adapt to difficult marine environmental conditions such as salinity, PH, low temperatures and all other marine environmental influences [2]. Therefore, the molecules and enzymes extracted from these microorganisms have unique properties make them have large biotechnological, industrial and pharmaceutical importance.

Industrial and medical enzyme industries are also expected to expand in developing countries where there is several governmental attention towards the expansion in the industrial sector. With pharmaceutical companies having difficulty bringing new small-molecule drugs to market, biotechnology will be the cheap and clean solution, helping to sustain demand for research and biotechnology enzymes. The world market for enzymes reached $7 billion in 2013 [3]. The most demanded industrial enzymes are nucleases, polymerases, lipases, amylases, and proteases.

Proteases are a group of proteolytic enzymes that can hydrolyze proteins into small particles called amino acids [4, 5]. They could be useful in the treatment of wounds and injuries as they can hydrolyze the proteins secreted during swelling and cause pain, also they play a major role in many biological actions inside and outside the living cells like regulation of the fate, localization, and activation of many proteins, modulating protein-protein interactions, creating new bioactive molecules, contribution to the cellular information process, regulation and controlling molecular signals processes. Due to this wide biological importance proteases have great pharmaceutical and industrial applications [6]. They could be extracted from, plants and microorganisms like fungi and bacteria. One of these proteolytic enzymes is the metalloproteases.

Metalloprotease enzymes (metallo, metal) are members of proteases that contain a metal ion at their active site. These enzymes act as catalysts in the hydrolysis of peptide bonds [7]. Zinc metalloprotease is one of the most common members of this class of enzymes, it contains a zinc ion (Zn2+) in its active site [8]. Other transition metals have been found at the active sites of different metalloproteases such as Co2+ and Mn2+. It was found that Co2+ and Mn2+ could be used to restore function in zinc-metalloproteases in which the Zn2+ core has been removed [9]. Generally, metal ions are bound in a nearly tetrahedral conformation at the active site. The metal core mainly consists of three amino acid ligands, together with one water molecule which is used for hydrolysis [10].

There are two major subclasses of metalloproteases: metalloendopeptidases and metalloexopeptidases. Each subclass is named according to the region of hydrolysis in the targeted protein at which the reaction takes place. Like other proteases, they play key roles in many normal biological processes, these abnormal activities have been implicated in many diseases such as arthritis, cancer, cardiovascular diseases, nephritis, disorders in the central nervous system, fibrosis, and infection [11]. Early studies showed that metalloproteases also have an important role in immunity [12]. They also have fundamental roles in the activity of inflammatory cells [13]. On an industrial scale,

microorganisms are the most important sources of metalloproteases production.

The selection of the right organism plays a key role in the high yield of desirable enzymes. For the production of enzymes for industrial use, isolation and characterization of new promising strains using cheap carbon and nitrogen source is a continuous process [14]. Metalloproteases have been identified in both gram-positive and gram-negative pathogens (so, they are certainly not unique to pathogenic species. However, there is a great interest in those isolated from pathogenic bacteria [15]. In modern studies, many extracellular metalloproteases have been extracted from marine bacteria [16-19]. Furthermore, some of these enzymes have a promising industrial application [20-23], while biotechnological applications are still needed for more studies on these enzymes extracted from marine microorganisms [5].

At this research we aim to study the ability of screening marine bacteria with the ability of producing hydrolytic and gelatinolytic enzymes with unique characters in order to be useful in biotechnological applications.

2. Materials and Methods

2.1 Collecting of marine water samples and rapid screening for protease hyper producer bacteria

The marine water samples were collected from different areas along the coast of the Mediterranean Sea in the Syrian cities of Lattakia and Tartus, during the period from 15 July to 20 August 2013. Marine samples were then filtered to collect sediments. These sediments then were treated with 20 ml sterile manufactured seawater. Then dilutions with the concentrations from 10^{-1} to 10^{-4} of the sediments were spread on solid enrichment media [24] consisting of 10 g peptone, 5 g yeast extract, 15 g agar per liter, and sterilized seawater (pH 7.5), then supplemented with 1.5% casein for detecting the ability to produce proteases. The screening plates were then incubated at 30°C for 2 days until a clear zone around the colonies appeared meaning of producing proteolytic enzymes. Then these colonies with a clear hydrolytic zone were more purified by repeat streaking on screening plates using a rapid preliminary test called the Tooth picking technique, a type of a patching technique [25]. Gelatin plates were prepared by adding 2% (w/w) gelatin into a selective medium containing 5 g yeast extract, 15 g agar, and sterilized seawater. Strains that made a clear hydrolysis zone on the screening medium were more streaked on the gelatin plates using the Tooth picking technique and incubated at 30°C for 2 days to detect if they can form hydrolytic zones around their colonies.

2.2 Sequencing of the 1257 strain

Strain 1257 was cultured in a medium composed of 0.5% yeast extract, 1% peptone, and sterilized seawater (pH 7.5) with shaking at 30°C for 24 h. The DNA of strain 1257 was extracted by a bacterial DNA extraction Kit (Sigma Aldrich) and sequenced. Sequence data were processed with the genedoc sequence editor (www.nrbsc.org/gfx/genedoc/) to obtain the sequence. Then the sequence was compared with previously published sequences from GenBank using a BLAST search.

2.3 Monitoring growth and proteolytic activity

The activity of extracellular metalloprotease was monitored throughout the growth of the bacterial strain *B. thuringiensis* 1257. Cells were activated on PA (1% Peptone, 0.5% Yeast extract, sterilized marine water pH 7.5 and 1.5% Agar) plates at 30°C, then fresh cells were used to inoculate 5 ml of PY (PA without solidified agent, agar) medium and were allowed to grow for 24 hours at 30°C. 100 ml of PY were inoculated with one ml of the above culture and the new culture was allowed to grow at 30°C with shaking to the indicated time. Growth was monitored by measuring the absorbance at 660 nm which was very much correlated with the number of viable cells. At the indicated time 5 ml of the growing culture was taken and centrifuged at 6, 000 rpm for 10 minutes. The supernatants were used as a crude enzyme to measure the activity of the metalloprotease.

2.4 Identification activity of metalloprotease enzyme

The activity of the metalloprotease enzyme was detected by two methods.

2.4.1 Spectrophotometric method

The activity of the protease was determined according to the method of Reichard et al., [26] with some modifications. Reaction mixture (2 ml) containing 10 mg casein, 0.9 mMTris-HCl buffer, pH 7.2, containing 100 µmole of 0.1 mM CaCl2 and 0.1 ml (or an appropriate dilution) of samples. The reaction was carried out at 30°C for 30 minutes then it was terminated by the addition of 2 ml 5% w/v trichloroacetic acid (TCA). Reactions were then kept on ice for 30 minutes after which they were centrifuged at 4000 rpm for 10 minutes. The absorbance of the TCA soluble fractions was measured at 280 nm. One unit of enzyme activity was equal to the amount of enzyme that liberates one micromole of tyrosine from casein per 30 minutes at 30°C.

2.4.2Electrochemical method

Three separate DNNS-based protamine-sensitive membrane electrodes were used simultaneously to monitor the initial decrease in protamine levels [27]. Experiments were performed by adding 5 µl of a 5 mg/ml protamine (Sigma Aldrich) solution to 1 ml of Tris working buffer (50 mMTris and 120 mMNaCl, pH 7.4) to yield a final concentration of 25 µg/ml protamine. After reaching a steady-state/ non-equilibrium response (3 min), 100 µl of a pre-incubated (5 min) sample mixture containing MP was added to the solution. This preincubated sample mixture was composed of centrifuged bacterial culture solution in Tris working buffer. The decrease in the EMF response toward protamine was monitored over a 5-min period by each of the sensors. A calibration plot for MP was constructed by graphing the initial rate of the potential decrease, in mV/min (average response of three sensors), vs MP activity, in IU/ml sample.

Then total protein was determined using the Bradford protein assay kit (SigmaAldrich).

2.4.3 Purification of the extracellular metalloprotease

The extracellular metalloprotease produced by *B. thuringiensis* 1257 was primarily purified using the ammonium sulfate (70% saturation) technique in an ice bath then it was collected by centrifugation at 6, 000 rpm, dissolved, and dialyzed overnight using 0.1 M Tris-HCl buffer, pH 7.2. Then the collected enzyme samples were further purified using a gel filtration (Sephadex G-75) column. The collected samples were then allowed to dialyze overnight in a dialysis tube. The protein content and the metalloprotease activity for the dialyzed enzyme samples at every step were determined as above.

2.5 Characterization of the purified metalloprotease

The optimum pH of the purified extracellular metalloprotease was determined over a pH range (4-12). The optimum temperature also was determined at the range of (30- 70) °C. The effect of EDTA disodium as a specific inhibitor on the activity of metalloprotease also was studied using different concentrations (1-15 mM).The effect of different metal ions at concentrations range (5-15mM) on activity also was studied.

3. Results

3.1 Screening of protease hyper producer bacteria *B.thuringiensis* 1257

Marine bacteria collected from the beach of the Mediterranean Sea were screened on PA plates containing casein substrate to identify their ability to produce protease hydrolytic enzymes. After cultivation, the screening PA casein plates at 30°C for 2 days number of colonies appeared on the screening plates and about 40% of colonies made a clear hydrolytic zone around them supposing that they can produce proteases. Utterly, 80 protease-producing isolates were obtained and only 30 isolates of them could grow on gelatin plates with a hydrolytic clear zone. One of these isolates was the 1257 isolate which was considered a protease hyper producer, as shown in figure 1, with high gelatinolytic activity when cultivated on gelatin plates at 30°C for 2 days as shown in figure 2. This isolate was selected for further studies. The DNA of the selected strain was extracted and sequenced. After comparing the sequence with the previous sequences on GenBank data, the results showed about 98% matching with *B. thuringiensis* Btk, as a result, we named this strain *B. thuringiensis* 1257 and used it for further studies.

Figure 1: The hydrolytic zone of 1257 strain after incubation at 30°C for 2 days on PA casein plates. The clear zone around the growing clones meaning the proteolytic activity.

Figure 2: The hydrolytic zone of 1257 strain after incubation for two days on gelatin plates at 30°C.

3.2 Monitoring enzyme activity during microbial growth curve

To optimize the whole process of production of extracellular proteases produced by *B. thuringiensis* was carried out on the synthetic medium PY at 30°C. PY was chosen as a protein rich medium to enhance enzyme production. The best results of enzyme production are shown in figure 3. Production starts early in the exponential phase, to help the microbe in performing all its anabolic processes. Production reached more than 2000 U/ml after three hours of incubation at 30°C.

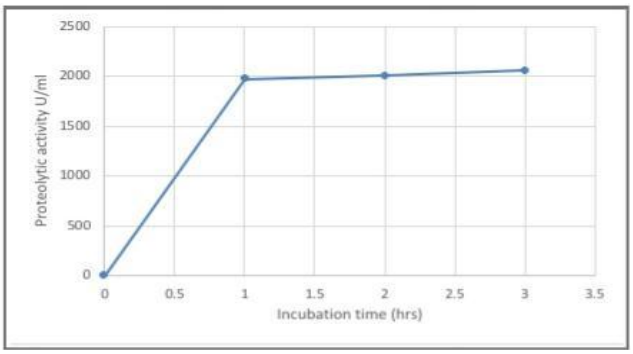

Figure 3: Prote-olytic activity of B. thuringiensis after incubation on PY medium at 30°C.

3.3 Spectrophotometric Identification of extracellular protease

To identify extracellular protease produced by *B. thuringiensis* partially purified enzyme was prepared by 70% ammonium sulfate precipitation. Enzyme activity was carried out according to the method of Reichard et al., with some modifications. Table 1 shows the activity of partially purified extracellular protease produced by *B. thuringiensis*. The table also shows the comparison of proteolytic activity after assaying with and without the chelating EDTA compound. Residual activity of the enzyme was dropped to almost one-third of its original value after the addition of 1mM EDTA to the reaction mixture. Moreover, the enzyme lost more than 75% of its original proteolytic activity at 10 mM EDTA concentration. These results categorized the extracellular protease produced by the microbe as a metalloprotease.

Table 1: Spectrophotometric identification of extracellular protease produced by *B.* thuringiensis .One unit of enzyme activity was equal to the amount of enzyme that liberates one micromole of tyrosine from casein per 30 minutes at 30°C.

mM EDTA added	Enzyme activity (U/ml)	Residual activity %
0	1176	100
1	430	36.6
5	310	26.4
10	270	23
15	236	20.1

3.4 Identification of extracellular metalloprotease enzyme

Assaying metalloprotease with electrochemical detectors, such as polymer membrane-based ion-selective electrodes offers many advantages over spectrophotometric methods, especially in cases where samples are highly colored or turbid like bacterial cultures [26]. Metalloprotease activity was detected by measuring the initial rate of decrease in the potentiometric response of the polycation-sensitive membrane electrode towards protamine degradation by the action of the enzyme.

Figure 4 shows the average potentiometric responses of tubular dinonylnaphthalenesulfonate (DNNS)-based protamine-sensitive membrane electrodes toward 25 µg/ml protamine and the effect of adding increased concentrations of metalloprotease. All measurements were compared to split samples that were measured with the spectrophotometric casein hydrolysis method.

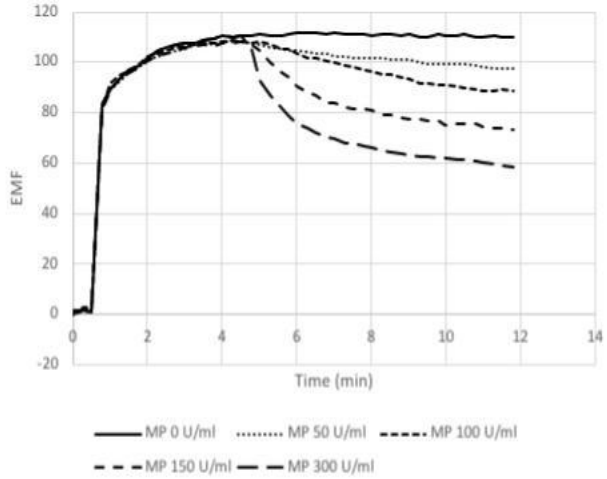

Figure 4: Potentiometric response of (DNNS)-based protamine-sensitive membrane electrodes towards 25 µg/ml protamine and subsequent addition of 100 µl of standard metalloprotease.

3.5 Characterization of the purified metalloprotease

When dealing with enzymes in the industrial or biotechnological fields some characteristics must be kept in mind (ex. pH, and temperature stabilities). The optimum pH and stability range of extracellular metalloprotease produced by *B. thuringiensis* at different buffer systems were examined. Figure 5 showed that the purified enzyme retained more than 50% of its activity over the pH range (6-9) with an optimum pH of 8. A very little decrease in the residual activity (less than 5 percent) was shown at pH 7.

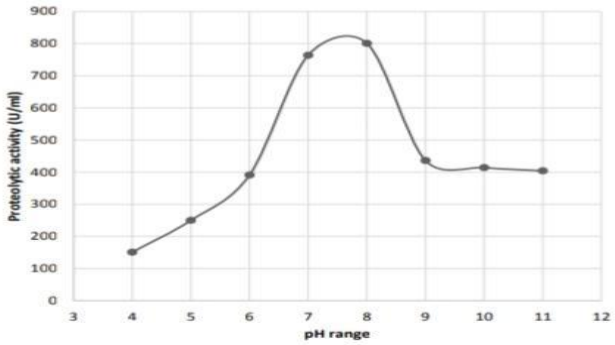

Figure 5: Effect of pH on the activity of extracellular metalloprotease produced by *B .Thuringiensis*. Enzyme activity was carried out as mentioned in the materials and methods.

The optimum temperature of the purified enzyme produced by *B. thuringiensis* was determined as described in Materials and Methods and investigated that the activity of purified enzyme from *B. thuringiensis* was sharply increased as the temperature increased after 40°C. The maximum proteolytic activity occurred at 60°C (as shown in figure 6). It was noticed that the proteolytic activity at 70°C was slightly low when compared to that obtained at 40°C. The proteolytic activity showed a wide range starting from 30°C and ending at 70°C, but it was dropped dramatically at 80°C.

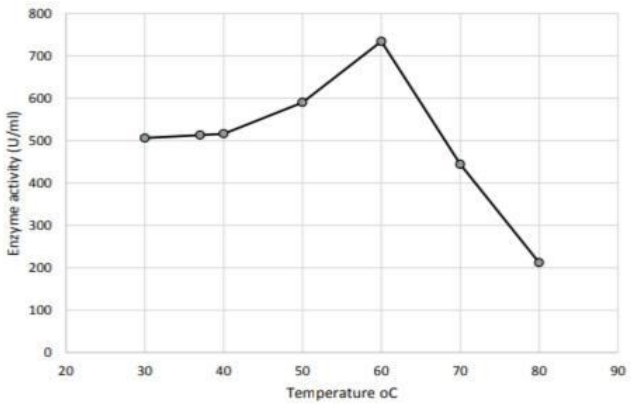

Figure 6: Effect of different temperature degrees on the residual activity of metalloprotease produced by *B. thuringiensis.*

The effect of disodium EDTA on the activity of extracellular metalloprotease also was obtained and showed that the residual activity of the purified enzyme was to almost dropped one fifth at 15 mM Disodium EDTA as in figure 7.

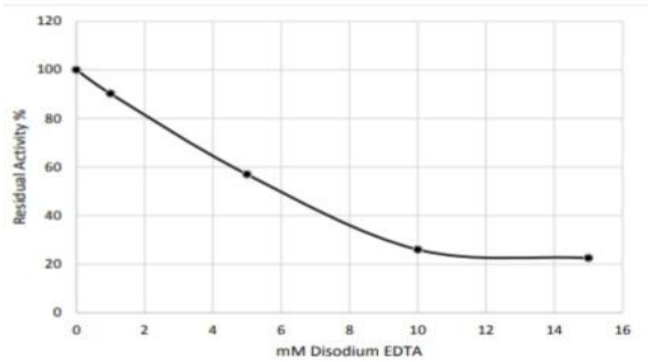

Figure 7: Effect of disodium EDTA salt on theactivity of extracellular metalloprotease produced by *B. thuringiensis.*

Also, the results of purification and activity of enzyme at every step of purification was measured and illustrated at table 2.

Table 2: Purification table of metalloprotease produced by *B. thuringiensis* cells.

Purification Step	Mg Protein/ ml	Metallo protease Units/ ml	Total Volume (ml)	Total Units	Specific activity	Fold purification
Call free supernatant	3.3	25.0	82.0	2050.0	7.6	1.0
Pellets after $(NH_4)_2SO_4$ ppt.	3.8	853.0	2.0	1706.0	222.1	29.3
Pellets after dialysis	2.2	841.0	2.0	1682.0	380.5	50.2
Gel filteration	1.2	567.0	2.0	1134.0	457.3	60.4

Table 3 showed the effect of salt concentration on the activity of the purified enzyme from *B. thuringiensis*. All tested divalent salts (except CoCl2) activated metalloprotease activity at 5 mM and 10 mM concentrations. ZnCl2 and MnCl2 showed the highest levels of enzyme activation at 5 mM concentration (194.9 and 162.6 percent residual activity respectively). In the case of ZnCl2 and StCl2, the enzyme retained more than 100 percent of its original activity at a 15 mM salt concentration. Otherwise, residual activity was dropped to more than half at a 15 mM concentration of other divalent salts. This offers a wide range of stability in case of exposure to contamination by salts.

Table 3: Effect of different salt concentration of some divalent salts onthe activity of the purified metalloprotease enzyme produced by *B. thuringiens.*

Divalent Salt	Residual Activity %		
	5mM	10 mM	15 mM
$MgCl_2$	113.1	111.6	46.4
$CoCl_2$	32.1	25.3	28.6
$ZnCl_2$	194.9	137.4	104.9
$StCl_2$	123.1	129.0	111.1
$MnCl_2$	162.6	127.7	44.2

4. Discussion

In this work, we isolated a hyper-protease-prodcing bacterial strain 1257 from the coast of the Mediterranean sea and identified it as *B. thuringiensis* strain depending on The 16S gene sequence which shows high similarity to *B. thuringiensis* Btk which was isolated from the soil [28]. It has not been reported if *B. thuringiensis* Btk has the ability to produce protease enzymes.

Here, we found that strain *B. thuringiensis* 1257 had the ability to secret a high yield of proteases compared to the other isolated strains. Therefore, we further purified and characterized the protease secreted by strain1257, which was identified as a metalloprotease based on its spectrophotometric and electrochemical test results. Both electrochemical and spectrophotometric enzymatic detection offered a robust and reliable method that can be used to assay for metalloprotease produced by *B.* thuringiensis. We found that *B. thuringiensis* 1257 metalloprotease showed high activity against casein and gelatin-like other M4 metalloproteases [29, 30].

Some metalloproteases offer perfect catalysis under critical conditions, such as at high temperatures, and in organic solvents, showing potential for biotechnological applications [31]. *B. thuringiens* 1257 metalloprotease also showed good thermo-stability and was stable in a range of pH 6.0–9.0. It also has good tolerance to detergents, some metallic ions in chloride salts, and organic solvents (Results not shown). These properties indicate its potential for industrial and biotechnological applications.

5. Conclusion

Due to the unique properties of particles and enzymes secreted from marine water, which make them have large biotechnological, industrial and pharmaceutical importance, and the extraction of marine enzymes is still developed.

This study aim to study the ability of secreting thermostable marine metalloprotease with specific characters from marine bacteria was isolatedfrom the Mediterranean Sea along the Syrian coast, which offered a great chance for evolving industrial and medical enzymeindustries in developing countries where there are several governmental attentions towards the expansionin the industrial sector.

The study recommend that more researches have to be carried out to expand the isolation of enzymes from saline bacteria with unique properties, and to study the possible biotechnological, industrial, therapeutic and also, environmental applications, according to the application of the idea of sustainable development and environment, especially in developing countries, as this provides a great

scientific and economic development in the field of therapeutic and enzyme industries in these countries.

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