Salt Loving Alkaline Protease from an Extreme Halophilic Strain Identified as *Halobacterium salinarum*

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Summary

This work included six research points summarized as follows

5.1. Screening and partial characterization of halophilic protease producing isolates

Ten different salty samples were collected from Brine, multicolor solar salt, saline soil, saline mud and raw salts (Amesal salt company located in Karun lake Fayoum, Egypt). To enrich halophilic protease producers, ten grams of solid samples or 10 ml of liquid samples were inoculated in liquid Sehgal & Gibbons medium supplemented with 2.5% skim milk powder (w/v) and 5% , 10, 15 and 25% NaCl and incubated at 40^{o}C for 2, 3, 3 and 7 days, respectively. Suitable dilutions of each treatment were tested for protease producers using skim milk agar supplemented with 5, 10, 15 and 25% salt to recognize enzyme producers from the halo-tolerant and extreme halophiles.

High efficient clear zone forming colonies were selected and purified. A total of 33 strains as protease producers were isolated and partial morphologically and physiologically characterized. On the basis of the growth on different salt concentrations, these isolates were divided into four groups: group (I) include 9 strains that could grow well at 25% NaCl and recognized as extremely halophiles; group (II) include 6 strains could grow well at 15% NaCl; group (III) include 9 strains could grow well at 10%; and group (IV) include 9 strains could grow well at 5 % NaCl. Among these isolates, 26 isolates were Gram-positive and 7 were gram-negative.

5.2. Taxonomical studies for the three selected strains

Based on the efficiency of enzyme production for all isolates, one isolate from each groups of salt concentration was selected as the most active halophilic, halotolerant or facultative protease producers. Strains HP25$^{(1)}$, HP10$^{(2)}$ and HP5$^{(1)}$ were selected as the most protease producers and were subjected to more detail characterization. Morphological characterization of the selected strains showed that colonies of HP5^{1}, HP10^{6} and HP25^{1} were circular, yellow and buttery; circular, white and buttery and punctiform, pink and Slimy, respectively. Cells of strain HP5^{1} and strain HP10^{6} were Gram-positive, motile rods and sporformers, while cells of strain HP25^{1} were Gram-negative, motile short rods and non-sporformers.

Biochemical and physiological characterization of selected strains was conducted by using the salty modified API 50CH and API 20E kits. The data of this experiment indicate that strain HP25(1), HP10(2) and HP5(1) able to utilize a narrow range of organic substrates. All three strains were positive for tryptophan desaminas and gelatinase. Strain HP25(1) was relatively more active than other strains in carbon source utilization and the enzyme activities. In addition, the ability of the three selected strains to grow on different of concentration of NaCl was determined. The data of these test showed that strain HP25(1) grew optimally at 25% NaCl and not able to grow at 15, 10, 5, and 0.0 % NaCl, indicating that this strain is an extremely halophile, while strain HP10[6] grew at 5, 10 and 15 % NaCl and not able to grow at 0% or 25% NaCl, indicating that this strain is moderately halophile. In addition, it was noticed that strain HP5[1]grew at a wide range of NaCl concentration, so it could be considered as facultative halophilic bacteria.

5.3. Phenotypic and genotypic characterization for the most promising strain HP25(1)

Among the three selected halophilic protease producers strains, strain HP25(1)T which isolated from raw salt sample was subjected to further identification in comparison with other most closely related and published species of the genus *Halobacterium*.

The data of this experiment clearly showed that, strain HP25(1) grow optimally at 3.5-5.1 M NaCl, indicating that this strain among the extremely halophilic archaea. Based on the similarity results of these strains, strain HP25(1) is significantly similar to *Hbt. salinarum* (DSM 3754) in most features, but they were phenotypically differed from other reference strains. Also, it was noticed that strain HP25(1) and *Hbt. salinarum* (DSM 3754) were differed in pigmentation and utilization of inulin and D-Mannose. Based on the 16s rDNA gene sequence similarity, strain HP25(1)T was related most closely to *Halobacterium salinarum* JQ015374 (100%), *Halobacterium piscisalsi* HPC1-2 (98%), *Halobacterium jilantaiense* strain NG4 (98%) and *Halobacterium noricense* strain A1 (97%). Finally, it could be concluded that strains HP5(1) and HP10 (6) were phenotypically characterized as the genus *Halobacillu*s sp., while strain HP25(1) was phenotypically and genotypically characterized as *Halobacterium salinarum*.

5.4. Optimization of the growth and secretion of the halophilic protease from strain HP25(1)

In this experiment, the fermentation period, effect of different salt concentrations (5, 10, 15, 20, 25 and 30%), pH values (5, 6, 7, 8, and 9) and temperature (25, 30, 35, 40, 45, 50 and 55

^{o}C) were studied. Bacterial growth and halophilic protease secretion of strain HP25[1] was monitored by counting of viable cells and by measuring the enzyme activity of cell-free supernatant.

The optimum fermentation period for growth and the secretion of halophilic protease from strain HP25[(1)] was after 4 days. At 15% salt the growth flourished and increased in parallel with the increase in salt concentration up to 30%, where maximum growth was $3.4x10^{9}$ CFU/ml. The maximum enzyme production (112.7 units/ml) was recorded in presence of 25% salt. The pH optimum for growth and secretion of the halophilic protease from strain HP25[(1)] was pH 7.0**.** The maximum growth was detected at 40^{o}C, but drastically decreased at 55^{o}C.

5.5. Production and purification of the halophilic protease from strain HP25[1]

In this research point, the extracellular halophilic protease was purified from two liter culture supernatant of HP25[1] grown in S-G medium containing 25 % NaCl and 2.5 % skim milk and incubation at 40°C for 4 days. The fermentation experiment was carried out in the mini bioreactor ADI 1030. At the end of the fermentation period, the liquor was centrifuged at 10000 rpm and the supernatant was collected for the enzyme purification.

The extracellular halophilic protease from strain HP25[1] was purified in three successive steps; ultrafiltration by Amicon, dialysis after ethanol precipitation and subsequently complete purification by gel filtration using superdex protease.

Purification of the halophilic protease by gel filtration resulted in specific activity 6350 unit/mg (167 fold) and 31 % yield.

5.6. Characterization of the halophilic protease from strain HP25[(1)]

In this experiment, the effect of different salt concentrations (0.0 - 30%), pH values (4 - 12) and temperature (5 -100 ^{o}C) on the halophilic protease of strain HP2[(1)] were studied. As well as the effect of various enzyme inhibitors on the activity of purified halophilic protease were conducted.

The results of this experiment showed that, the highest activity was recorded in the range of 10-20% salt with maximum activity at 15 %. In addition, this enzyme was active at broad pH ranges

(7.0-11.0) with maximum activity at pH 8.0. Also, The maximum activity of this enzyme was found at 60^{o}C. The molecular mass of the purified halophilic protease of strain HP25$^{(1)}$ was 21 ± 0.5 kDa and exhibited one single polypeptide. In the presence of SDS approximately the most activity was lost, followed by PMSF 87.5%, urea; 69.5%, EDTA; 66.8% and $FeCl_2$; 44.2 %. Inhibition of the purified halophilic protease by ethylenediamine tretraacetate (EDTA) indicated that this enzyme requires some metal ions as cofactor for its activity. Also, it was noticed that the activity of halophilic protease of strain HP25[1] was drastically inactivated by phenylmethylsulfonyl fluoride (PMSF), indicating that this enzyme could belong to the class of serine proteases. The β-mercaptoethanol which act as a reducing agent (0.1%) also caused a moderate inhibition (20 %) on the activity of the halophilic protease of strain HP25[1].

Objectives of this study

1. To isolate and screening of halophilic protease producing microorganisms from brine, multicolor solar salt, saline soil, saline mud and raw salts (Amesal salt company located in Karun lake Fayoum, Egypt).

2. To systematically identify the most promising selected strains.

3. To optimize the culture conditions for growth and protease production.

4. To purify and characterize the halophilic protease from the most promising strain.