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Isolation and Identification of Alkaline Protease Producing Alkaliphilic Bacteria from an Egyptian Soda Lake

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Abstract: Screening of water and soil samples collected from Wadi El-Natrun, an Egyptian soda lake, for alkaline proteases producing bacteria, resulted in isolation of 15 alkaline proteases producing alkaliphilic strains. WN-SK5 showed the highest enzyme production (61.0 U/ml) after 48h. This isolate was gram positive and was able to grow in the presence of NaCl up to 15 %. Growth was observed at 25 ºC, 37 ºC, 45 ºC and 55 ºC but no growth was seen at 60 ºC. It could grow at pH value from 8 to 11. No growth was detected at pH 7 after 48 h incubation at 55 ºC, which indicted this strain to be moderate halophilic thermophilic alkaliphiles. It was found that 16S-rDNA sequence of strain WN-SK5 had 97.35 % identity with the corresponding sequence of *Bacillus halodurans* (Acc. Nr. gb10172612). The crude alkaline protease showed reasonable activity at temperature range of 65 to 75 °C with maximum activity at 70 ºC and had a relatively wide pH range of activity between pH 8 to 11, with maximum enzyme activity at pH 10 in 50 mM Tris -HCl buffer maximum activity at 70 °C and pH 10.0, indicating the enzyme to be thermo- alkaline proteases.

Keywords: Alkaliphiles, alkaline proteases, Soda lake isolation, 16S rDNA

INTRODUCTION

Proteolytic enzymes are degradative enzymes which catalyze the cleavage of peptide bonds in other proteins. Currently, proteases are classified on the basis of three major criteria, type of reaction catalyzed, chemical nature of the catalytic site and evolutionary relationship with reference structure. Alkaline proteases are referring to proteolytic enzymes which work optimally in alkaline pH (1, 12). The vast diversity of proteases, in contrast to the specificity of their action, has attracted worldwide attention in attempts to exploit their physiological and biotechnological applications,[6,22, 12] e. g. food and feed industry, peptide synthesis, leather industry, management of industrial household waste, photographic industry, medical usage, silk gumming and detergents industry.

Alkaliphiles are defined as organisms that grow optimally at alkaline pH, with pH optima for growth being in excess of pH 8 (usually between 9 and 10), and some being capable of growing at pH > 11[9,14]. Although they were once considered to be curiosities, awareness of alkaliphiles has blossomed in recent years due to an interest in their physiological adaptation to high pH and their potential uses in biotechnological applications. Soda lakes and soda deserts represent the major types of naturally occurring highly alkaline environments, in which the indigenous microflora is subjected to number of extreme ecological pressures. They represent the most stable high pH environments on Earth, where large amounts of carbonate minerals can generate pH values >11.5. Soda lakes are widely distributed throughout the world; however as a result of their inaccessibility, few of such lakes have been explored from the microbiological point of view[9,18]. One of those environmental niches which have not been studied in details is the Wadi El-Natrun soda lakes in northern Egypt. The aims of this work were isolation of aerobic alkaliphilic bacteria from some Wadi Natrun soda lakes and screening for and alkaline protease producing alkaliphilic bacteria, characterization and identification of the interested strains and preliminary investigation of the alkaline protease.

MATERIAL AND METHODS

Soil and Water Samples: Soil and water samples were taken from Wadi Natrun in northern Egypt. Wadi Natrun extends in a northwest by southeast direction between latitudes 30º 15′ north and longitude 30º 30′ east. The bottom of the Wadi Natrun area is 23 m below sea-level and 38 m below the water-level of Rosetta branch of the Nile. The lowest part of the depression, encircled by contour zero, covers an area of about 272 km2[24].

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Soil and water samples were collected from the different locations of Wadi Natrun soda lakes: Hamara, Bani salama, Dawood, Elbida lakes. Samples were kept in sterile tubes in refrigerator, at 4 °C, and were transferred to the laboratory within few hours

Enrichment and Isolation of Aerobic Alkaline Protease Producing Alkaliphilic Bacteria: Isolation of alkaline protease producing alkaliphilic bacteria was carried out using rich alkaline agar medium containing skimmed milk (27, modified). Aliquots (100 µl) of different dilutions of soil suspensions and water samples were plated and incubated at 37 °C, 50 °C and 60 °C for three days. Formation of halo zone around the colonies, resulting from casein hydrolysis, was taken as evidence of proteolytic activity. These colonies were isolated and streaked in fresh plates until single uniform colonies were obtained. The medium contained (g/l): Skimmed milk 100, yeast extracts 10, Na_2CO_3 15, agar 20. Skimmed milk, Na_2CO_3 and the other constituents were autoclaved separately (at 121 °C for 20 min) and mixed after cooling.

Some Characterization of the Isolate: Classification of the isolates as gram positive or gram negative was done by Gram stain reaction[7] and KOH sensitivity test[11]. For Gram staining the Color Gram 2 kit of bioMérieux (Marcy l'Etoile, France) was used. For KOH sensitivity test a heavy mass of 24 h bacterial cultures were picked up and transferred to glass slide with 2-3 drops of 3 % (w/v) KOH solution and the cells suspensions were agitated rapidly with circular motion with toothpick for 15-30 seconds. The formation of a string (DNA) in 3 % (w/v) KOH indicates that the isolate is a gram negative organism. *E. coli* and *Bacillus megaterium* were used as gram negative and gram positive control, respectively.

The effect of temperature on growth was studied by plating out the cells on alkaline agar medium and incubated at different temperatures: 30 °C, 37 °C, 45 °C, 50 °C, 55 °C and 60 °C, respectively, for 48 h. To study the influence of salinity on cells growth, alkaline agar medium containing different concentrations of NaCl (w/v) 0 %, 5 %, 10 %, 15 % and 20 %, respectively, was inoculated with the cells and incubated at 37 °C for 48 h. For the alkaliphily test, the isolates were inoculated to agar medium adjusted at different pH values: 7, 8, 9, 10 and pH 11 and incubated at 37 °C for 48 h.

Analyses of 16S rDNA Gene Sequences: For the sequence analysis, bacterial genomic DNA was extracted and purified using a Wizard Genomic DNA Prep. Kit (Promega Co., Madison, USA). Two primers annealing at the 5 and 3 end of the 16S rDNA were 5 -GAGTTTGATCCTGGCTCAG-3 (positions 9–27 [*Escherichia coli* 16S rDNA numbering]) and 5 -AGAAA GGAGG TGATC CAGCC-3 (positions 1542–1525 [*E. coli* 16S rDNA numbering]), respectively. (Chun and Bae, 2000). PCR amplification was performed in a final reaction volume of 100 µl, and the reaction mixture contained each primer at a concentration of 0.5 µM, each deoxynucleoside triphosphate at a concentration of 200 µM, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, and 2.5 U of *Taq* DNA polymerase. The PCR reaction was run for 35 cycles in a DNA thermal cycler (Model No. 9700, Perkin–Elmer Co. Wellesley, USA). The following thermal profile was used for the PCR: denaturation at 94 °C for 1 min, primer annealing at 60 °C for 1 min and extension at 72 °C for 2 min. The final cycle included extension for 10 min at 72 °C to ensure full extension of the products. The amplified PCR products were then analyzed in a 1.0% (w/v) agarose gel, excised from the gel, and purified. The purified products were cloned into pGEM-T Easy vector (Promega Co., Madison, USA) and subsequently sequenced using ALF Red automated DNA sequencer (Pharmacia, Sweden).The 16S rDNA sequence of the isolate was aligned with those in the GenBank database. Multiple alignment of sequences and calculations of levels of sequence similarity were performed by using CLUSTAL W[26]. The neighbor-joining phylogenetic analysis was carried out with MEGA program[19].

Production of Alkaline Proteases: A loopful of culture from agar plate was inoculated into 50 ml-glass tube containing 5 ml of alkaline protease production medium, and incubated overnight at 180 rpm and 50 °C. This culture was then inoculated into 500 ml capacity Erlenmeyer flask containing 95 ml of the same medium and incubated at 50 °C for 48 h. Cells and insoluble materials were removed by centrifugation at 10 000 g for 10 min at 4 °C and the cell-free supernatant was filtered through a 0.45-µm pore-size membrane filter and was used as the source of crude alkaline protease enzyme. The production medium contained (g/l) (Jasvir *et al*. 1999, modified): Glucose 10, Peptone 5, yeast extract 5, K_2HPO_4 1, $MgSO_4$ 0.2, Na_2CO_3 15, pH 10.5. Na_2CO_3, glucose and the other constituents were autoclaved separately (at 121 °C for 20 min) and mixed after cooling.

Alkaline Protease Assay: Proteolytic activity was assayed by a modified method of Kunitz[20]. Samples containing 400 µl of 0.5 % (w/v) casein in 50 mM Tris –HCl buffer, pH 10, with 100 µl enzyme sample were incubated in a water bath at 50 °C for 20 min.

The enzyme reaction was terminated by addition of 500 µl of 10 % (w/v) trichloroacetic acid and was kept at room temperature for 10 min. The reaction mixture was centrifuged at 10 000 g for 10 min at 4 °C and the absorbance was measured against a blank (non-incubated sample) at 280 nm. One unit of proteases was defined as the amount of the enzyme releasing the equivalent of one µmol of tyrosine per minute under the defined assay conditions. Standard curve of tyrosine was done using 10, 20, 30, 40, 50, 60, 70, 80 and 100 mg/ml tyrosine in 50 mM glycine-NaOH buffer, pH 10,

Protein Determination: Protein concentration was determined according to the method described by Bradford[3]. One ml of Bradford reagent was added to 50 µl of sample and the extinction was measured after 5 min at 595 nm. Different concentrations of bovine serum albumin (BSA) were used as a protein standard: 10, 20, 40, 60, 80, and 100 µg/ ml distilled water. One ml of Bradford reagent was added to 50 µl BSA standard and the extinction was measured after 5 min at 595 nm.

Effect of Temperature on the Activity of the Crude Alkaline Protease: The crude alkaline protease was prepared as described above. The influence of temperature on the catalytic activity of the crude alkaline protease was determined by measuring the enzyme activity at temperatures range from 25 °C to 90 °C under the standard assay conditions.

Effect of pH on the Activity of the Crude Alkaline Protease: The influence of pH on the alkaline protease activity was determined by measuring the enzyme activity at varying pH values ranging from 5 to 12 at 70 ºC using different suitable buffers, 50 mM sodium acetate (pH 5.0 and 6.0), 50 mM sodium phosphate buffer (pH 7.0 and 8.0), 50 mM Na2HPO4-NaOH buffer (pH 9.0, 10.0 and 11.0) and 50 mM KCl-NaOH (pH 12), respectively.

RESULTS AND DISCUSSIONS

Isolation of Alkaline Protease Producing Alkaliphilic Bacteria: Soda lakes are characterized by the presence of a high concentration of sodium carbonate formed by evaporative concentration, and are also associated with varying degree of salinity and low concentration of both Mg^{2+} and Ca^{2+} ions[10,8,18]. Wadi EL-Natrun (Wadi: Arabic for Valley) and its alkaline inland saline lakes is an elongated depression about 90 km northwest of Cairo. Its average length is about 60 km and average width about 10 km. The bottom of the Wadi Natrun area is below sea-level and below the water-level of Rosetta branch of the Nile[24] The features of Wadi Natrun area created an ecosystem which considers as rich sources for isolation of different extremophiles, including halophilic, alkaliphilic in addition to thermoalakiliphilic microorganisms, due to the sun-heated alkaline soil of this area which are not well studied up to date[24,17]. Isolation of alkaline protease producing alkaliphilic bacteria was carried out using rich alkaline agar medium containing skimmed

Fig. 1: Screening of alkaliphilic bacteria for alkaline proteases production. Isolate are streaked on alkaline agar plate containing skimmed milk, incubated for 72 h at 55 °C. The clear zone indicated the hydrolysis of casein as a result of alkaline protease production

Table 1: Production of alkaline protease by different isolated strains. Isolates were grown in alkaline medium, pH 10.4, at 50 ºC in shaking incubator at 180 rpm for 48 h; W for Wadi, N for Natrun, SK for skimmed milk.

Strains	Alkaline protease activity (U/ml)	
	24 h	48 h
WN- SK1	15.15	19.18
WN SK2	5.51	10.12
WN-SK3	5.11	0.91
WN-SK4	22.15	29.11
WN-SK5	50.16	61.00
WN-SK6	41.12	31.51
WN-SK7	38.91	39.15
WN-SK8	0.90	3.50
WN-SK9	11.11	19.50
WN-SK10	8.88	16.11
WN-SK11	19.11	3.05
WN-SK12	6.55	19.12
WN-SK13	31.15	19.12
WN-SK14	6.16	11.15
WN-SK15	19.11	15.12

milk. Formation of clear zone around colonies was considered as indication of alkaline protease production (Figure). 15 isolates, showing large clear zone around their colonies, were isolated. It has been established that there is not necessarily good correlation between zones of clearing around colonies on milk-agar plates and levels of proteinase activity[5]. Therefore, all the positive isolates were cultivated in the alkaline production medium and the proteolytic activity was measured. The results shown in Table 1 indicated that some isolates with considerable proteolytic activity could be isolated, WN-SK5, WN-SK6 and WN-SK7. The strain WN-SK5 showing the highest alkaline protease activity (61.0 U/ml) was selected for further characterization.

Characterization and Identification of the Alkaline Protease Producing Strains: The alkaline protease producing isolate, termed WN-SK5, showed white mucoide colonies with circular margin. Cells grown in liquid medium for 24 h showed non-motile long rod-shaped cells under light microscope. This isolate was gram positive using KOH sensitivity test. Strain WN-SK5 was able to grow in the presence of NaCl up to 15 %. Growth was observed at 25 ºC, 37 ºC, 45 ºC and 55 ºC but no growth was seen at 60 ºC after 48 h at pH 10.5. It could grow at pH value from 8 to 11. No growth was detected at pH 7 after 48 h incubation at 55 ºC which indicted this strain to be moderate halophilic thermophilic alkaliphiles[14].

Identification of the Strain WN-SK5 by 16S-rDNA Sequencing: Genomic DNA of the strains was isolated and the PCR amplified 16S-rDNA was cloned into *E. coli* and sequenced as already described in material and method section. By using six primers (three forward and three reverse), 1461 bp of the 16S-rDNA were sequenced with minimum mistakes. The 16S-rDNA sequence was aligned with the known 16S-rDNA sequences of other bacteria. It was found that 16S-rDNA sequence of strain WN-SK5 had 97.35 % identity with the corresponding sequence of *Bacillus halodurans* (Acc. Nr. gb10172612). Considering the exactly identified bases, there were 14 differences between the base sequences of strain WN-SK5 and *B. halodurans*. One base differences were found to be in the universal region (U5), six in semivariable regions (S 1, 2, 3, 5 and 6) and the other seven differences were found to be in the variable regions V1 and V5, but mainly in V6[21] . The difference of 2.65 % is the sum of these well identified mismatches and other unspecific. For differentiation of a unique species the study has to continue in more detail. An alkaliphilic bacterium, strain C-125 (JCM9153), isolated in 1977,

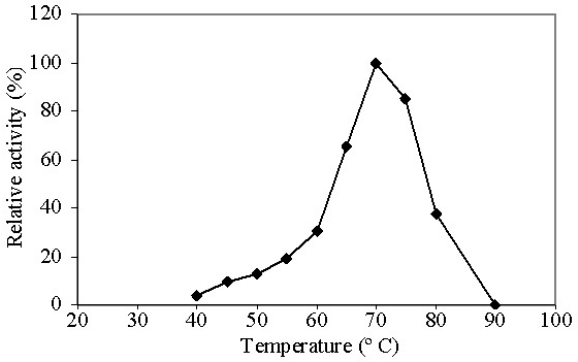

Fig. 2: Effect of temperature on the activity of crude alkaline protease from B. halodurans SK5. The alkaline protease activity was measured using 0.5 % casein Tris -HCl buffer, pH 10, at different temperatures. Standard deviations of the relative activities were in the range of 1.0-3.5 %.

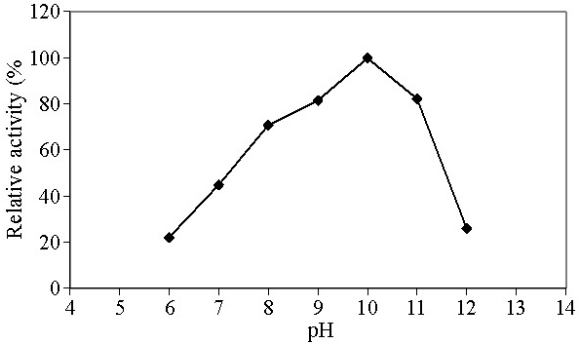

Fig. 3: Effect of pH on the activity of B. halodurans WN-SK5 alkaline protease. Activity was measured under the standard assay conditions at various pH values. Standard deviations of the relative activities were in the range of 1-4 %.

was recently identified as *Bacillus halodurans* based on 16S-rDNA sequence and DNA-DNA hybridization[25]. It is reported as a β-galactosidase[16] and xylanase producer[13].

Effect of Temperature on the Activity of the Crude Alkaline Protease from *B. Halodurans* WN-SK5: Cell-free supernatant was prepared as described in material and methods section and was filtered through a 0.45-µm pore-size membrane filter and used as the source of crude alkaline protease. For determination of the optimum temperature of the crude alkaline protease, the enzyme activity was measured at different temperatures at pH 10. The results illustrated graphically in Figure 2 indicated that the crude alkaline protease showed reasonable activity at temperature

range of 65 to 75 °C with maximum activity at 70 ºC. A rapid decrease of enzyme activity was detected above 80 ºC and the enzyme was completely inactivated at 90 ºC.

Effect of pH on Activity of Crude Alkaline Protease from *B. Halodurans* WN-SK5: The effect of different pH values of the reaction mixture on the activity of the crude alkaline protease was investigated in pH range from 5 to 11 at 70 ºC. It was found that the crude alkaline protease had a relatively wide pH range of activity between pH 8 to 11, with maximum enzyme activity at pH 10 in 50 mM Tris -HCl buffer (Fig. 3). These preliminary properties of the enzyme are considered to be interested in comparison to other alkaline proteases[15,12,2,23].

The results of this work indicated that the Wadi Natrun soda lakes, in Egypt, are a rich source of many alkaliphilic bacteria which could be a good source of many interested enzymes from the industrial point of view and further studies are recommended on this soda lakes including study of microbial biodiversity and the biotechnological potent of the isolated strains.

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