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Isolation and Screening of Proteolytic Bacteria from Sandy Soil in Sadat City, Egypt

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ABSTRACT

Proteases are one of the most studied microbial group of enzymes for their importance at commercial, industrial, pharmaceutical and also diagnostic fields. The aim of this work was the isolation of proteolytic bacteria from soil and characterization of their proteolytic activity. Two effective protease producing bacterial isolates have been obtained from soil from Sadat City and evaluated its extracellular protease production properties by exhibiting a clear zone on media containing skimmed milk casein as a substrate for the protease enzyme. The strains have been identified according to basic biochemical tests using Bergey's Manual of Systematic Bacteriology as *Bacillus* sp. Scanning electron microscopy revealed morphology of Cells of Bacillus colonies tended to be longer than in liquid culture and irregular in shape and to give the appearance of branching. The 16s rRNA gene pcr and sequencing were used for species identification of the 2 isolates. And by analysis of the sequence results using the BLASTn, it revealed that one isolate (Ash1) was identified as *bacillus cereus* and the other (Ash2) was *bacillus subtilis*. The enzyme production reached its maximum level at pH 8.0 at 37ºC incubation temperature, under aerated environment, suggesting the enzyme produced is alkaline in nature. The *Bacillus cereus* strain Ash1 and *B. subtilis* Ash2 showed maximum growth at 37ºC with alkaline protease production at pH 8.0 and at 150 rpm. This study reveals the importance of genus bacillus in protease production among the bacterial population in soil, although the narrow scale this study was applied on, but it has to be studied on larger scales in Egypt, an issue that will provide many beneficial information for various fields particularly the industrial sectors.

Keywords: *Bacillus* sp, Protease, soil, 16s rRNA

INTRODUCTION

Actually, all forms of life on earth including animals, plants and microbes need protease enzyme for their physiological performance. Microbes are ubiquitous and live in familiar settings such as Soil, water, food, and animal intestines, as well as in extreme settings like rocks, cold or hot areas, and deep-sea vents (Padmapriya *et al.,* 2012). An up-to-date classification of evolutionary super families of protease is found in the MEROPS database.

Proteases are classified, in this database, firstly by 'clan' (superfamily) according to structure, mechanism and catalytic residue order. Proteases within each 'clan' are classified into families based on sequence similarity, each family may contain many hundreds of related proteases (e.g. elastase, trypsin, streptogrisin and thrombin within the S1 family). Currently there are more than 50 clans. Alternatively, proteases may be classified according to the optimum pH at which they are active: Acid proteases,

Neutral proteases which are involved in type 1 hypersensitivity and basic proteases or alkaline proteases (Rao *et al.,* 1998). Protease constitutes nearly 60% of the world market of industrial enzymes. Many uses of free proteases like in dry cleaning, detergents, processing of meat products, cheese manufacturing, silver recovery from used photographic films, and certain medical treatments of inflammation and wounds (Fekadu Alemu, 2015). Bacterial Proteases are preferred for the rapid growth of bacteria, needless space, can be easily maintained and are accessible for genetic manipulations (Odu *et al.,* 2012). Currently, a large Proportion of commercially available proteases are derived from Bacillus strain (Sevine and Demirkan., 2011).

MATERIALS AND METHODS

Isolation of proteolytic bacteria from the soil

The sand and muddy soil samples were collected from Sadat City, Menofiya governorate, Egypt in sterile plastic bags. 1gm from each soil was weighted, suspended in 10ml of sterile saline solution. Serial dilutions were prepared from each one. 10µl from the diluted samples were plated by spread plate method on nutrient agar plates. Streak plate technique was used for further purification of the isolated colonies. After that the isolates were screened for protease production. The screening was done by cultivation of the i colonies on the isolation medium containing (g/l): 2 gm skimmed milk,1gm yeast extract, 1 gm Mg_2So_4 and 1.5 gm agar-agar.

Preservation of Isolates

All isolates were purified and stored in LB broth containing (g/l): (tryptone, 10; yeast extract, 5; sodium chloride, 10), and glycerol added to a final concentration of 25% and stored at -80°C (Sambrook *et al.,* 1989).

Screening for proteolytic activity using agar media containing skimmed milk (Gupta *et al.,* 2005)

Phenotypic and biochemical characterizations

The 2 isolates, which were chosen as the best for producing protease, were characterized on the basis of different biochemical and morphological parameters to tentatively identify them to the genus level. The parameters used were; Gram staining and Microscopic examination, Catalase, Oxidase and Nitrate reductase reactions. Colony morphological characterization was also employed.

Gram staining technique (Murray *et al.,* 1994)

Cultures grown on solid media for 24 hours were suspended on a microscopic slide with 2-5 µl of distilled water to a faint turbidity. Smear was prepared by spreading the drop with a lamel, it was then dried completely. The underside of the slide was passed three times over Bunsen flame to induce adherence.

Following treatments were performed:

Crystal violet staining reagent for 1 min. Washing under the tap water for some seconds gently. Staining in iodine mordant for 1 min. Washing under the tap water. Washing with 95% ethanol for 6 sec. Washing under the tap water. Staining in safranin counterstain for 30 sec. Washing under the tap water.

Finally, the slide was dried and the cells were observed under the light microscope. Purple cells considered as gram positive and pink coloured cells considered as gram negative (Murray *et al.,* 1994), cellular morphology was also determined.

Scanning Electron Microscope analysis (SEM):

Was used to examine the isolated strain morphology. The sample was prepared for SEM by transferring the strain to 1.5 ml of 3.5% glutaraldehyde solution in a clean eppendorff . The culture was then incubated at room temperature for 4 h followed by washing by phosphate buffer (100mM, pH 7.2). The culture was then dehydrated using alcohol gradient from 10 to 100%. Then, air drying of the dehydrated samples which fixed after that on the tubs using double adhesive tape. The sample was coated by a thin layer of gold using HUS-5GB Hitachi vacuum evaporator for 90 sec. The samples were then observed under scanning electron microscope (HitachiS-3000N, Japan) using various magnifications and acceleration voltage of 10.0 KV at AL-Azhar University in Cairo city.

DNA visualization after DNA extraction

The DNA was extracted by boiling method (Zhao et al. 2001), The yield of genomic DNA was visualized on 1% agarose gels. The needed amount of agarose was dissolved in

1x TAE buffer, melted and poured into the chamber of electrophoresis; about 1µl of 10% (v/v) loading buffer (60% glycerin, 0.3 % bromophenolblue and 0.3 % xylolcyanol) was added to the DNA samples (about 4µl), then loaded into the gel and the electrophoresis was performed at 20-100 Volt/cm in 1x TAE buffer. DNA was soaked in a dilute solution of ethidium bromide before visualization.

Polymerase Chain Reaction (PCR) amplifications

Amplification of 16S r RNA gene (*Srinivasan et al., 2015)*

The 16S r RNA genes of the isolates were amplified by Thermocycler (Biometra thermocycler, Germany) using the primer set: 27F (5'-GAGTTTGATC CTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). amplifications were usually performed on a ledom Touch Screen Thermal Cycler / PCR Model: A100/A200 (Hangzhou Long Gene Scientific Instruments Co., Ltd) in a final volume of 50 µl reaction mixture; 0.5 µM of each primer, 10X PCR buffer supplemented with 20 mM $MgCl_2$,10 mM of an equimolar d NTPs mix, 1.25 U of Taq DNA polymerase and as a template, 1µl of genomic DNA with a concentration between 0.1 to 10 ng. The Taq polymerase, d NTPs and PCR buffer were purchased from ThermoScintific.

Thermal cycling condition

The PCR amplification condition consisted of an initial denaturation at 94°C for 2 min, followed by 35 cycles (94°C denaturation for 30 sec, 55°C annealing for 30 sec, 72°C extension for 1min) and final elongation at 72°C for 5 min. The amplified 16S r RNA PCR product was sequenced using an automated sequencer at (Macrogen Inc., South Korea). The Sequence Similarity Search was done for the amplified 16S r RNA gene sequence using the online available search tool BLAST (http://www.ncbi.nlm.nih.gov/blast/). The unknown organisms were identified using the maximum aligned sequence through BLASTn available online (Ibrahim and Eldiwany, 2007).

Amplification of proteolytic (ash) gene.

DNA fragment encoding protease was amplified by PCR. Chromosomal DNA of *Bacillus cereus s*train was used as a template and two degenerative oligonucleotide primers: ash: F (5'ATGGTACATACATATTTAGGTGAG-3') and ash: R (5' CTAAACCGTCATCTTCCAACTAGATA A-3') were synthesized based on the nucleotide sequence of closest *Bacillus cereus* strain DSM13 (ATCC 14580) endoglucanase. The PCR was done using a final reaction volume of 50 ul (4 tubes). Amplification consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles (94°C denaturation 30 sec, 55°C annealing 30 sec, 72°C extension 1min) and final elongation at 72°C for 5 min. The amplified PCR products were then analyzed in a 1.0% (w/v) agarose gel, excised from the gel, purified and subsequently sequenced using Gene Jet Plasmid Miniprep Kit-Thermo Scientific).

Table (1): sequence of primers used to amplify the gene *ash*-and Oligonucleotide 16S rRNA genes.

Oligo name	Oligonucleotide sequence of primers (5'-3')
16S rRNA-27F	GAGTTTGATCCTGGCTCAG
16SrRNA-1492R	GGTTACCTTGTTACGACTT
ash-F	ATGGTACATACATATTTAGGTGGTGAG
ash-R	CTAAACCGTCATCTTCCAACTAGATAA

Agarose gel electrophoresis

PCR products were checked for integrity by gel electrophoresis. 1% agarose gel was dissolved in 1X TAE buffer, melted then poured into the chamber used for electrophoresis. A volume of 10% (v/v) loading buffer was added to the amplified DNA, then loaded onto the gel and the electrophoresis was performed at 50Volt/cm in 1X TAE buffer.

DNA was visualized by soaking in a dilute solution of ethidium bromide. DNA fragments were separated and their sizes examined and determined under UV light and used Gel Documentation System model 286-760 DigiDoc-ItTM for the imaging and documentation of agarose gels.

DNA extraction from agarose gel

The DNA extraction was processed by using Gene JET gel extraction kit (K0691) from Thermoscientific. Method (acc to the manufacturer):

After separation on agarose gels, excision of the DNA fragments of interest from the gel, weigting and placing into a 1.5 mL tube. Then equal volume of the Binding Buffer was added to the gel slice (volume: weight) (e.g., 100 µL of binding buffer added for every 100 mg of agarose gel).The mixture was transferred to the gene JET DNA purification column, then centrifugation of the column for 30-60 sec .The flow-through was discarded. An amount of 700 µl of Wash Buffer were added to the Gene JET DNA purification column and centrifuged for 30-60 sec. the flow-through was discarded and then the purification column was placed back into the collection tube and centrifuge empty column for 1 min. The gene JET DNA purification column was transferred into a clean 1.5 ml micro-centrifuge tube and 50 µl of elution buffer was added to the gene JET DNA purification column which was centrifuged for 1 minute to elute DNA.

RESULT AND DISCUSSION

Isolation of proteolytic bacteria

A total of twenty bacterial isolates were isolated from 30 collected soil samples from Sadat City, Menofiya Governorate, all isolates subjected to purification with streaking agar method and tested to production ability of protease enzyme. Two bacterial isolates (Ash1 and Ash 2) showed the production of protease.

Table (2); Biochemical and morphological characters

Characterization of selected isolates	Isolate Ash1	Isolate Ash2
Gram stain	+	+
Cell shape	Long- rod shape	Long- rod shape
Oxidase	-	-
Indol production	-	-
Methyl Red	-	-
Voges – Proskaeur	+	+
Gelatin hydrolysis	+	+
Growth at 65 C°	+	+
Anaerobic growth	-	+

Bacillus species are the predominant soil bacteria because of their highly resistant-endospore and production of essential antibiotics such as bacitracin (*Amin et al*., 2015). In this study, from the twenty bacterial isolates, only 2 isolates were found to be protease producers on media containing skimmed milk (20%). The two isolates were primarily identified as bacillus strains according to the morphological, gram staining and biochemical characters as shown in the table (2). And these results came in accordance with a previous study made in 2017 (*Hamza,* 2017) in Ethiopia and another study made in India by (Rupali. 2015) where one isolate was found to be the best protease producer from six tested soil isolates. Generally, the use of different microorganisms in production of enzymes not only has added many economic advantages, but also in recent years it has become the predominant mode for enzyme production (*Singh et al.*, 2015). Identification of both isolates using the 16s rRNA pcr and sequencing confirmed that the two isolates belong to genus Bacillus (the sequence alignment is available in the attached file).
Phylogenetic tree based on comparison of the 16S rRNA sequences of protease producing bacterial isolates, *Bacillus Cereus* strain Ash1 and *Bacillus Subtilis* strain Ash2 and some of the closest phylogenetic relatives using the CLUSTAL W 2.1 multiple sequence alignment software. The phylogenetic tree was constructed from evolutionary distances by using the neighbor-joining method of Mega 4 program package (Kumar *et al.,* 2004).The *B. cereus* strain Ash1 was found to have 77% identity with the strain *Bacillus cereus* MFS16 16S ribosomal RNA gene and 58% identity with both *B. anthracis* strain 58283 and *B.cereus* strain MI45, while the *B. subtilis* strain Ash2 was found to have 88% identity with *B. subtilis* strains; TAD21 and R241B as shown in the phylogenetic tree (figures 4 and 5).

Each organism or strain has its own special conditions that affect its growth and enzyme production. The isolation and characterization of new promising strains is a lifelong process. In the present study, among the different pH levels tested, the pH 8 was found to be optimum for both the test isolates for optimum growth and for protease production. These results came in accordance with studies made by (Shumi *et al.,* 2004); in Turkey by (Sevine and Demirkan., 2011); also (Asha and Palaniswamy, 2018) in India. The alkaline protease producing bacillus strains were found to produce the enzyme under a wide range of PH value (7-9) with the best growth was at PH 8.0 and hence, protease production.

Table (3): Effect of different PH values on growth and protease production.

Growth at PH	Result
5.0	-
6.0	-
7.0	+
8.0	++
9.0	++
12.0	-

CONCLUSION

Bacillus species had a special interest among researchers as a soil protease producing bacteria due to its predominance, resistant endospore and ability to produce protecting antibiotics, as bacitracin. So, bacillus species have been widely used in the detergent industry [28, 29]. The above results may provide some information about soil bacteria in Egypt for biotechnological industrial sectors that can benefit protease producing bacteria for many purposes particularly the biotechnological industrial sectors. Moreover, studies are needed for establishing a good data about the alkaline protease produced by soil bacteria regarding its optimum conditions affecting its maximal activity because the alkaline protease still one of the highly important enzymes in industrial fields.

Figure 1: clear zone around the colonies.

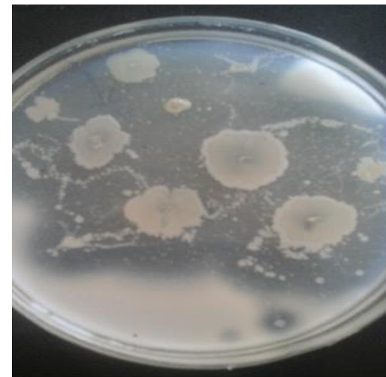

Figure 2. Precipitation of protease by ammonium sulphate; by adding ammonium sulphate drop by drop till full saturation then washing by tap water to remove the colony we notice that precipitation of protein on the media.

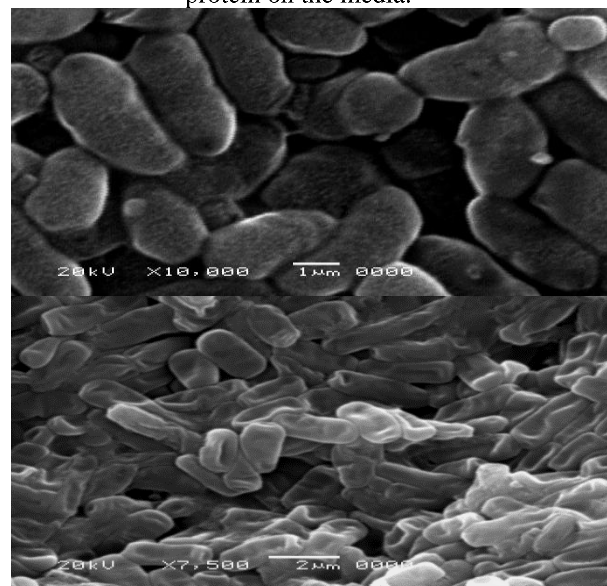

Figure 3. Scanning Electron Microscope analysis (SEM) showed the arrangement of individual cells within colonies. Cells of Bacillus colonies are long and irregular in shape and arranged in clumps to give the appearance of branching.

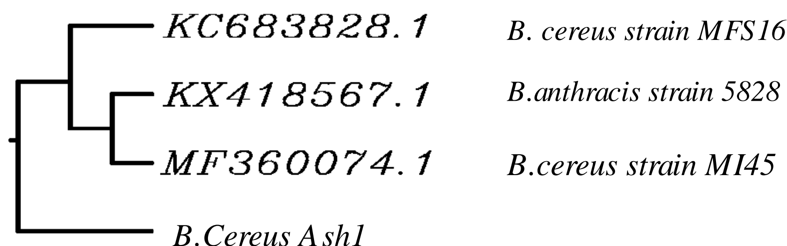

Figure 4. Phylogenetic tree of 16srRNA of Bacillus cereus strain and some of their closest phylogenetic relatives.

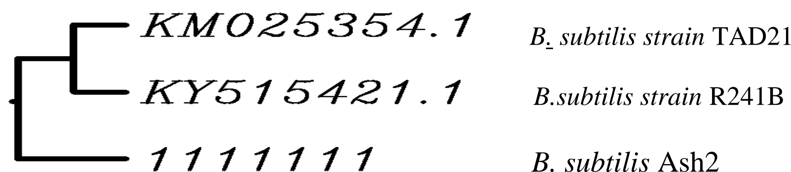

Figure 5. Phylogenetic tree of 16s rRNA of *Bacillus subtilis* strain and some of their closest phylogenetic relatives.

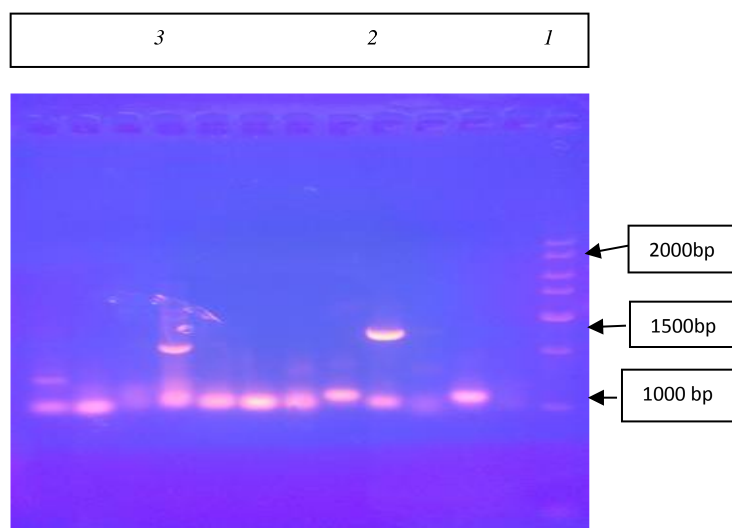

Figure 6: Gel electrophoresis of the PCR product from *B. Cereus* strain Ash1 and *B. subtilis* Ash2 using the primer set Ash-F and Ash-R for detection of protease gene.lane 1: one kb DNA size marker, lane 2: 1500 bp band of *B. Cereus* strain Ash1. Lane 3: 1500 bp band of *B. subtilis strain.*

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