

# BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF AN EGYPTIAN MARINE ISOLATE "*Alcaligenes faecalis*" PRODUCING THERMOSTABLE PROTEASES

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**M**icroorganisms constitute the major source of proteases, including both extracellular and intracellular ones (Tari *et al.*, 2006; Bommarius, 2015). Proteases have been isolated and purified from different sources, including, plant, animal, bacteria, fungi and viruses (Patel *et al.*, 2005). Thermostable protease enzymes are useful for numerous industrial processes by increasing the rate of reaction, giving a longer half-life to the enzyme and inhibiting microbial growth, thus reducing the possibility of microbial contamination (Rao *et al.*, 1998; Srinivasan *et al.*, 2009).

Furthermore, Scandurra *et al.* (2000) have devoted attention to thermophilic microorganisms as an enriched source of proteins to be studied, since these proteins were expected to exhibit unusual properties, mainly an outstanding resistance to physical stress, therefore it is useful to study its protein stability and thermotolerance from an academic point of view. As a result, the overall collected data will be essential for industrial manipulation as biocatalysts with an extended shelf-life (Scandurra *et*

*al.*, 2000). In addition, extracellular protease absorbs and utilizes hydrolytic products from proteinaceous substrates (Gupta *et al.*, 2002). The exopeptidases act only near the ends of the polypeptide chains. Based on their site of action at the C or N terminus, they are classified as carboxypeptidases and aminopeptidases, respectively (Rao *et al.*, 1998). Carboxypeptidases can be further divided into three major groups, serine carboxypeptidases, metallo-carboxy peptidases and cysteine (Ray, 2012). The carboxypeptidases acting at C terminals of the polypeptide chain can liberate one amino acid or a dipeptide. The present investigation aims to isolate thermophilic bacteria producing protease from Egypt; identify these isolates by studying their biochemical properties and 16S rRNA sequencing, then investigating the protease activity under some environmental stresses such as, temperature, pH, heavy metals, organic solvents, oxidant and EDTA. Also the genetic background of the identified selected isolate protease genes was analyzed through bioinformatics database.

## MATERIALS AND METHODS

### 1. Bacterial strain source

Water samples were collected from South Sinai, Hamam pherareon, to isolate thermophilic bacteria; identification of selected isolate was done morphologically and biochemically by El-Eskafy (2015).

#### 1.1. Molecular identification of the bacterial isolate

Pure isolate was characterized morphologically using scanning electron microscope (SEM) and based on the criteria of Bergey's Manual of Systematic Bacteriology by El-Eskafy (2015). 16S rRNA gene of the new isolate was amplified using universal 16S rRNA primers Bact 27F (5'AGAGTTTGATC(A/C)TGGCTCAG-3') and Bact 1492R (5'-TACGG(C/T)TACCTTGTTACGACTT-3'). Bacterial culture was sent to SolGent Company, South Korea for 16S rRNA gene sequencing. The following molecular techniques were done as follow: DNA extraction and isolation was done using SolGent purification bead. Prior to sequencing, the ribosomal rRNA gene (also referred to as rDNA) was amplified using the polymerase chain reaction (PCR) technique in which two universal primers 27F and 1492R were incorporated in the reaction mixture. The reaction conditions were, initial denaturation step at 94°C for 3 min, after which the Taq polymerase was added, and the reaction continued for the next 35 cycles of 45 sec of denaturation at 94°C, 60 sec for the annealing at

50°C, and the extension at 72°C for 90 sec. Final extension was done for 10 min at 72°C, after which the reaction hold at 4°C. Sequencing was done using the ABI3730XL platform. The analysis of the sequences and the phylogeny tree was done using BLAST suite (blastn), non-redundant nucleotide sequences (nr) database and phylogeny.fr site. (<http://phylogeny.lirmm.fr/phylo.cgi/index.cgi>) (Phylip, 1989 and 2000).

### 2. Crude enzyme extraction and proteolytic assay

The isolate was grown in selected medium containing; yeast extract 0.5% (w/v), peptone 1.0% (w/v), glucose 0.5 g/l, Na<sub>2</sub>HPO<sub>4</sub> 0.4 g/l, Na<sub>2</sub>CO<sub>3</sub> 0.085 g/l, ZnSO<sub>4</sub> 0.02 g/l, CaCl<sub>2</sub> 0.02 g/l, MgSO<sub>4</sub> 0.02 g/l, incubated at 50°C for 50 h, centrifuged at 14000 rpm for 30 min at 4°C. Crude enzymes were assayed for proteolytic activity in triplicate as described by Guangrong *et al.* (2006) using casein as the substrate.

#### 2.1. Protease assay

The proteolytic activity of the enzyme was assayed in triplicate as described by Guangrong *et al.* (2006) using casein as a substrate; initially a mixture of 400 µl casein solutions (2% (w/v) in 50 mM Tris-HCl buffer with pH 7.2) and 100 µl of crude enzymes were added to a tube. The reaction was carried out at 65°C in water bath (Memert, Germany) for 10 min and then terminated by the addition of 1

mL 10% trichloroacetic acid (w/v). The mixture was centrifuged at 14000 x g for 20 min. A 500  $\mu$ L supernatant was carefully removed to measure tyrosine content using a Folin-phenol method (Ledoux and Lamy, 1986). One unit of protease activity (U) was defined as the amount of enzyme that hydrolyzes casein to produce 1.0  $\mu$ mole of tyrosine per minute at 65°C.

### 2.2. Determination of total protein

Bacterial total protein was determined by diamond total protein kit using bovine serum albumin to calculate activity, and total protein was determined by the method of Lowry *et al.* (1951).

### 2.3. Effect of purification methods

Ammonium sulphate precipitate obtained at 60-90% saturation was dissolved in 0.02 M sodium phosphate buffer at pH 7 and dialyzed overnight with the same buffer (Mohamed *et al.*, 2013). Sediment formed was removed by centrifugation and the supernatant was loaded on previously equilibrated Sephadex G-100 column (31 $\times$ 16 cm). Column elution was performed by the same buffer with an increase in molarity from 0.02 M to 0.5 M of NaCl. Protease activity was assayed at pH 7 and the peaks obtained were tested for optimum pH. Fractions displaying maximum activity in the respective peak areas were pooled. A single peak of activity measured at the respective optimum pH (Table 1) was obtained in each case and constituted the purified enzyme (Al-Saman *et al.*, 2015).

### 2.4. Effect of different temperatures and pH values

The protease activity was determined using standard assay procedure after incubating the enzyme at temperatures ranged from 45°C to 95°C for 10 min (Akel *et al.*, 2009). The activity of the protease was measured at different pH values. The pH was adjusted using buffers such as, 50 mM sodium acetate (pH 3.8-4.8); 50 mM sodium phosphate (pH 5.0-6.8); Tris-HCl (pH 7.2-9.0) and 50 mM sodium carbonate (pH 9.2-10.8). The reaction was incubated at 65°C for 10 min and the enzyme activity was measured according to Akel *et al.* (2009).

### 2.5. Effect of some metal ions and EDTA

The effect of NaCl, CaCl<sub>2</sub>, MgSO<sub>4</sub>, FeCl<sub>3</sub>, MnSO<sub>4</sub> and CuSO<sub>4</sub> as sources of metals ions on the protease activity was investigated by the addition of the corresponding ions at a concentration of 5 mM to the reaction mixture. Purified enzyme preparation was pre incubated in 50 mM Tris-HCl buffer with pH 7.8 containing various Ethylene Diamine Tetra Acetic Acid (EDTA) concentrations ranging from 0 to 15 mM in the assay (Akel *et al.*, 2009). Crude and pure enzyme activity was measured at 65°C, under standard conditions.

### 2.6. Effect of some organic solvents

Three mL of crude protease enzyme were incubated with 1.0 mL of acetone, butanol and n-hexane individually as the organic solvent with constant shaking

at 150 rpm for 30 min (Gupta and Khare, 2006). The enzyme activity was measured after 30 min of incubation in 25% (v/v) of organic solvent according to Gupta and Khare (2006).

### **2.7. Effect of some surfactants and hydrogen peroxide**

The compatibility of protease with surfactants and an oxidizing agent was studied individually in the presence of 1% sodium dodecyl sulphate (SDS) as a surfactant, hydrogen peroxide (1%) as an oxidizing agent and Tween-80 as an emulsifier. The enzyme was incubated at 40°C with surfactant or oxidant for 30 min and the enzyme activity was measured according to Habib *et al.* (2011).

### **2.8. Data analysis of amino acid sequences of proteases genes**

All amino acid sequences and accession numbers were obtained from Gene bank NCBI website (<http://www.ncbi.nlm.nih.gov>) and listed in Table (2). SmartBlast site programs were used to analyze the protein data available at <http://blast.ncbi.nlm.nih.gov/smartblast>.

## **RESULTS AND DISCUSSION**

### **1. Isolation and screening of thermophilic protease producing bacteria**

Eighty four isolates from different water samples were isolated to select the thermostable protease producing bacteria. Primary selection of the isolates was according to the biggest clear zone using

casein method. Secondary selection was done based on their enzymes activity. A third selection based on the highest total protein was applied giving eight isolates, which was further screened for the highest specific protease activity, as a result, one isolate named as HFW-9081 was selected.

### **2. Identification of the isolated thermophilic bacteria**

#### **2.1. Morphological and biochemical characterization**

Morphological and biochemical characterization were done previously by (El-Eskafy 2015) and the results suggested that the isolate HFW-9081 belong to thermophilic microorganisms according to Bergey's manual of systematic bacteriology (Brenner *et al.*, 2005). The HFW-9081 cells studied by Electron Microscope (EM) showed short chain rod-shape cells (Fig. 1), chaining of cells obvious and ellipsoidal, HFW-9081 cells is non-spore former. Transmission electron micrographs of the HFW-9081 cells (Fig. 1) showed short rods or coccobacilli 0.2-1.0 to 0.5-2.6  $\mu\text{m}$ , occurring singly. Gram stain was negative; HFW-9081 was motile by peritrichious flagella. catalase, indole and gelatin hydrolysis tests were negative; HFW-9081 was able to utilize organic acids and amino acids as carbon sources. The sensitivity of the tested isolate to antibiotics was determined.

#### **2.2. Molecular identification using 16S rRNA gene sequencing**

Sequencing of 16S rRNA gene of the selected Egyptian marine isolate

HFW-9081 was performed; 16 S rRNA sequence using 27 F primer gave 1258 base pair (bp), while using the 1492 R primer gave 1248 bp. from the 1500bp fragment isolated by PCR. The search on the Gene bank nucleotide database using the blast-nt algorithm revealed significant matching (hi score and low e-value) and 99% identity with the gene sequence of the strain of *Alcaligenes faecalis*. Also the phylogenetic tree (Fig. 2) showed high genetic relationship between the Egyptian isolate HFW-9081 and the strain of *Alcaligenes faecalis* L48, which strongly prove that the Egyptian isolate can be identified molecularly as *Alcaligenes faecalis*, based on the 16srRNA (rDNA) nucleotide sequence and the phylo-tree analysis (Fig. 2). The complete taxonomy is Bacteria>Proteobacteria>Betaproteobacteria>Burkholderiales>Alcaligenaceae>*Alcaligenes*>*Alcaligenes faecalis*.

### 3. Determination of protease activity

The selected isolate were purified, and their proteolytic activities was evaluated by observing the hydrolysis of casein by measuring the clear zone for the tested isolates. The widest zone diameter of 2.5 cm was obtained for isolate HFW-9081 with protease activity of 236.4 U/mL at 65°C.

Thermostable protease was purified in 2-steps procedure involving ammonium sulfate (80%) fractionation followed by Sephadex G-100. Results obtained in Table (1) showed that both ammonium sulphate and sephadex G-100

methods enhanced the yield activity of *Alcaligenes faecalis* HFW-9081 to 125 and 121% and the specific activity to 458.9 and 590 U/mg, respectively, compared to cell free supernatant. In contrast, Asker *et al.* (2013) demonstrated that *Bacillus megatrium* protease possess a specific activity of 41.09 U/mg that was purified through protein precipitation using ammonium sulfate. However, the yield of the enzyme after purification was found to be low. This might be due to the autolysis of the enzyme in each purification step. Likewise, the thermoprotease activity of *Bacillus* sp. was reduced only by 1.7% after its purification in a 3-steps procedure, including ammonium sulfate precipitation, sephadex G-100 gel permeation followed by DEAE-ion exchange chromatography (Akel *et al.*, 2009).

### 4.2. The effect of temperature

Temperature was found to influence extracellular enzyme secretion; possibly by changing the physical properties of the cell membrane (Bahobil, 2011; Weng *et al.*, 2014). Maximum protease activity of 236.4 U/mL was observed at 65°C, and it was gradually decreased to reach 126.1 U/mL with increasing the temperature up to 95°C (Fig. 3). Results revealed that the enzyme had good activity between 55°C and 75°C; however it was affected markedly by increasing the temperature to 95°C. Overall, the protease enzyme obtained in our study was stable over a wide range of temperature and thus indicate its promising potential to be used in the detergent industries using hot or

cold wash cycles, and in other different biotechnological applications. Beena *et al.* (2012) explained the reduction in protease activity when exposed to high temperature by its probable thermal denaturation. In addition, Habib *et al.* (2011) reported that *Halobacterium* sp. produced protease with lower yield at 50°C. The overall results (Fig. 3) demonstrated that the strain under study had good enzyme activity between 55°C and 75°C. Thus, it can be classified as a thermophilic protease which is in agreement with Guangrong *et al.* (2006) and Asker *et al.* (2013) who studied *Bacillus megatrium* protease.

#### 4.3. The effect of pH

The effect of pH on protease activity was examined (Fig. 3) at different pH values of 3.5, 5, 6, 7, 8 and 9. Extracellular proteases produced by *Alcaligenes faecalis* can be separated into alkaline and/or neutral proteases (Sevinc and Demirkan, 2011). Maximum enzyme activity of 236.4 U/mL was observed at pH 7. However, the enzyme activity was greatly affected by lowering the pH to 3.5 and decreased to 32.73 U/mL (Fig. 3). Result showed that the protease remained active between pH 6 and pH 7. Moreover, the enzyme activity began to decrease sharply to 82.4 and 66.4 U/mL at pH 8 and pH 9, respectively (Fig. 3). Similar results were obtained for the enzymatic activity of different *Bacillus* spp., such as *B. subtilis* ITBCCB 148, *B. subtilis* HS08 and *B. subtilis* S17110 having their optimum pH at 7.5 (Guangrong *et al.*,

2006). However, pH 8.0 was the optimum for the enzyme activity of *B. cereus* KCTC 3674, thermophilic *B. cereus* SMIA2 and *B. cereus* BG1 (Nascimento and Martins, 2004; Ghorbel-Frikha *et al.*, 2005). Similarly, the optimum pH for protease activity was determined at pH 7.0 as reported by Sevinc and Demirkan (2011) who mentioned that it could be a neutral protease. Figure (3) showed that the protease enzyme possess activity in the pH range of 6.0-9.0 which is in agreement with Basu *et al.* (2007) and Merheb-Dini *et al.* (2009). Likewise, a proteolytic enzyme was produced by a strain of *Lactobacillus brevis* at optimum pH of 7.0, the enzyme is probably a neutral metalloprotease as reported by Amund *et al.* (1990). In contrast, Beena *et al.* (2012), Asker *et al.* (2013) and Habib *et al.* (2011) reported alkaline proteases activity from some *Bacillus* spp. and *Halobacterium* sp. with activity between pH 6-9 with gradual increase in their activity.

#### 4.4. Effect of various metal ions

Results obtained in Fig. (4) shows that all metal ions tested significantly decreased the activity as compared to the control. The toxic metal ions exert their toxicity by binding to a variety of organic ligands causing the denaturation of proteins including enzymes (Nascimento and Martins, 2004). The presence of CaCl<sub>2</sub> and MnSO<sub>4</sub> mostly affected the enzyme activity as shown in Fig. (4). Nevertheless, Habib *et al.* (2011) reported that only

NaCl and FeCl<sub>3</sub> stimulate the protease with 100% relative activity, while CaCl<sub>2</sub> retained 90% in *Halobacterium* sp. However, Nascimento and Martins (2004) reported that some metal ions protected the enzyme from the thermal denaturation and maintained its active conformation at the high temperature. In contrast, a maximum inhibition of about 40% with 1.0 mM Zn<sup>2+</sup> and Fe<sup>2+</sup> for the protease of *Pseudomonas* and *Burkholderia* was reported (Asker *et al.*, 2013).

#### 4.5. Effect of EDTA presence

Chelating agent as EDTA is a detergent additive; which functions as water softeners and also assists in the stain removal. Enzyme activity was 292.4 U/mL in the presence of 5 mM of EDTA; however it was decreased to 52.6 and 11.4 U/mL with 10 and 15 mM concentrations of EDTA, respectively (Fig. 4). The activity of the enzyme in presence of EDTA is advantageous for using the enzyme in the presence of detergent. Overall results (Fig. 4) are in agreement with Asker *et al.* (2013) who reported that 3 mM EDTA didn't affect the protease activity; however the activity was decreased to 20% when 4 mM EDTA was added. Similarly, Akel *et al.* (2009) reported that the protease activity of *Bacillus* HUTBS71 decreased to 70% in the presence of 1 mM EDTA.

#### 4.6. Effect of some organic solvents

The compatibility of the enzyme with different organic solvents was inves-

tigated. Results obtained in Fig. (5) showed that the enzyme activity was 185.4 U/mL and 207.4 U/mL with acetone and butanol, respectively. While the presence of N-hexane significantly decreased the enzyme activity to 28.4 U/mL. Similarly, Gupta and Khare, (2006) confirmed that low amount of protease was produced in the presence of heptane, hexane, cyclohexane and xylene by *P. aeruginosa*. Overall results (Fig. 5) are in agreement with Bahobil (2011) who reported that acetone reduces the relative protease activity of *Shewanella putrefaciens*-EGKSA21 to 40%. The results obtained can be attributed to the presence of organic solvents which might alters the catalytic process of enzyme by disruption of hydrogen bonds, hydrophobic interactions; and thus cause changes in the dynamics and conformation of the enzyme (Barberis *et al.*, 2006).

#### 4.7. Effect of some surfactants and hydrogen peroxide

Surfactants act as detergents, dispersants, emulsifiers, foaming and wetting agents. Surfactants are reported to improve the permeability of the cell membrane by increasing the uptake of nutrient into the organism and enhance the secretion of enzymes (Evans and Abdullahi, 2012). SDS decreased the protease activity to 122.4 U/mL as compared to control (Fig. 5). However, Tween-80 increased the activity 7.5 folds as compared to the control. Surfactants may also play a role in exposing the active sites and making them available for enzyme-substrate hy-

drophobic interaction (Evans and Abdullahi, 2012). Similarly, Tween-80 enhanced the relative enzyme activities between 105-112% for proteases from *B. clausii* and *B. mojavensis*, respectively (Rai *et al.*, 2010). On the other hand, the protease activity was reduced to 182.4 U/mL when H<sub>2</sub>O<sub>2</sub> was included in the medium.

### 5. *Bioinformatics and genetic background of proteases genes in Alcaligenes faecalis*

The genome sequences of *Alcaligenes faecalis* from Genebank showed four genes remarked as proteases, the phylogenetic tree for each protein dequences proves its genetic relationship and specified that these genes are *Alcaligenes faecalis* proteases with 97% to 100% identity (Fig. 6). Results obtained in Table (2) revealed that the analyses of amino acid sequences clarified that those genes are not gene copies or alleles for the same protease gene. Furthermore, the peptides family analysis by Smartblast for these genes protein sequences indicated that genes with accession numbers WP\_003804512 and WP\_003804510.1 contain 331 and 300 amino acids (AA), respectively and has one U32 family peptidase in C-terminal domain which is a common family peptide in proteases enzymes (Regar *et al.*, 2016). While gene with accession number WP\_059318132.1 has 388 AA and contains two peptidase families, namely, Trypsin-like peptidase domain, which includes trypsin-like peptidase domains.

The second one is PDZ\_serine\_protease, this domain of trypsin-like serine proteases, such as, DegP/HtrA and PDZ, are oligomeric proteins involved in heat-shock response. Moreover, the PDZ domain is common domain in thermostable proteases (Gupta *et al.*, 2008). In addition, the fourth gene with accession number WP\_063692761.1 is 308 AA and contains paraslipin (SPFH\_paraslipin and HflC) peptidase family, that function as a protease activity regulator, which have been identified in all the three domains of life (Hinderhofer *et al.*, 2009). Nevertheless, all the four genes did not contain S8 peptidase family commonly present in the majority of thermostable proteases (Rawlings and Barrett, 1993; Rawlings *et al.*, 2010). The variation in peptidase family provides the protease enzyme with many features making it able to remain active under various environmental stresses. The overall results explains how the thermostable enzyme from the Egyptian *Alcaligenes faecalis* strain HFW-9081 remain active at pH ranging from 3.5 to 9, temperature ranging from 45°C to 90°C, in presence of EDTA, selected organic solvents and even with different metal ions (Fig. 6 and Table 2).

Finally conclusions, Due to the growing market and potential uses of proteases, there is continuous interest in the isolation of new bacterial species that produce proteolytic enzymes with suitable properties for industrial applications, such as food, agriculture and detergent industries. In the present study, an Egyptian thermophilic bacteria producing protease

was isolated and identified from Hamam Freon region in Egypt, and could be used potentially for different industrial purposes. The isolated proteases showed considerable activity at wide temperature range of 55-90°C, with pH 6-8. The genome sequences of *Alcaligenes faecalis* in Gene bank showed four genes remarked as proteases, the phylogenetic tree for each gene proves the genetic relationship of these genes and specified that these genes are *Alcaligenes faecalis* proteases with 97 to 100% identity. However, more research is still needed for further characterization and optimization of genetic regulation of such proteases.

### SUMMARY

An Egyptian marine bacterium, isolated from Hamam Pheroon, South Sinai region was able to produce thermostable proteases, the isolate was identified morphologically, biochemically, and confirmed molecularly by 16S rRNA sequencing with 99% similarity to *Alcaligenes faecalis*. It exhibited optimum activity of 328.3 U/mg after ten min, incubation at 65°C and pH 7. Both ammonium sulphate and sephadex G-100 purification methods enhanced the yield of *Alcaligenes faecalis* strain HFW-9081 to 125 and 121% as well as the specific activity to 458.9 and 590 U/mg, respectively, compared to cell free supernatant. However, relative protease activity was reduced to 35.8% when H<sub>2</sub>O<sub>2</sub> was added. On the other hand, the activities increased 7.5 folds when Tween-80 was used as a surfactant. Genetic background of the

protease genes in *Alcaligenes faecalis* was analyzed using bioinformatics database for the proteases amino acids sequences in the desired bacteria; and it specified that *Alcaligenes faecalis* has four different protease genes; these genes encode for various peptidases family groups. The variation in the peptidase family groups provides the protease enzymes with many features making them able to remain active under various environmental stresses. The overall results showed promising thermostable proteases isolated from local marine Egyptian bacterium; that can be used potentially in many industrial applications.

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Table (1): Purification of proteases produced by *Alcaligenes faecalis*.

Purification Steps	<i>Alcaligenes faecalis</i> strain HFW-9081					
	Total protein (mg/mL)	Enzyme activity (U/mL)	Total enzyme activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Cell free supernatant	0.624	0.884	236.4	328.3	1.0	100
Ammonium sulfate (80%)	0.720	0.934	286.4	458.9	1.3	121
Sephadex G-100	0.504	0.945	297.4	590	1.7	125

Activity % = (activity of enzyme U/mL)/(activity of control U/mL)\*100.

Table (2): Genetic bioinformatics background for the proteases genes in *Alcaligenes faecalis*.

Protein/ Gene name	Accession number in NCBI	Amino Acid	Peptidase fami- ly	Strain	Reference
Protease (QWA_143 02)	WP_003804512	301AA	PrtC Peptidase family U32 family pepti- dase C- terminal do- main	<i>Alcaligenes faecalis</i> subsp. <i>faecalis</i> NCIB 8687	Regar <i>et al.</i> (2016)
Protease (2-alkenal reductase)	WP_059318132.1	388AA	Trypsin-like peptidase do- mains PDZ_serine protease	<i>Alcaligenes faecalis</i> subsp. <i>faecalis</i> NCIB 8687	<a href="http://www.ncbi.nlm.nih.gov">http://ww w.ncbi.nlm .nih.gov</a>
Protease ( <i>slp-2</i> )	WP_063692761.1	308AA	Paraslipin	<i>Alcaligenes faecalis</i> subsp. <i>faecalis</i> NCIB 8687	<a href="http://www.ncbi.nlm.nih.gov">http://ww w.ncbi.nlm .nih.gov</a>
Protease (QWA- 14297)	WP_003804510.1	300AA	U32 family peptidase	<i>Alcaligenes faecalis</i> subsp. <i>faecalis</i> NCIB 8687	Regar <i>et al.</i> (2016)

Fig. (1): A) Gram stain of isolate (HFW-9081) cells (x 100); (B and C) Transmission electron micrographs (bar 0.2  $\mu$ m) of isolate (HFW-9081) cells.

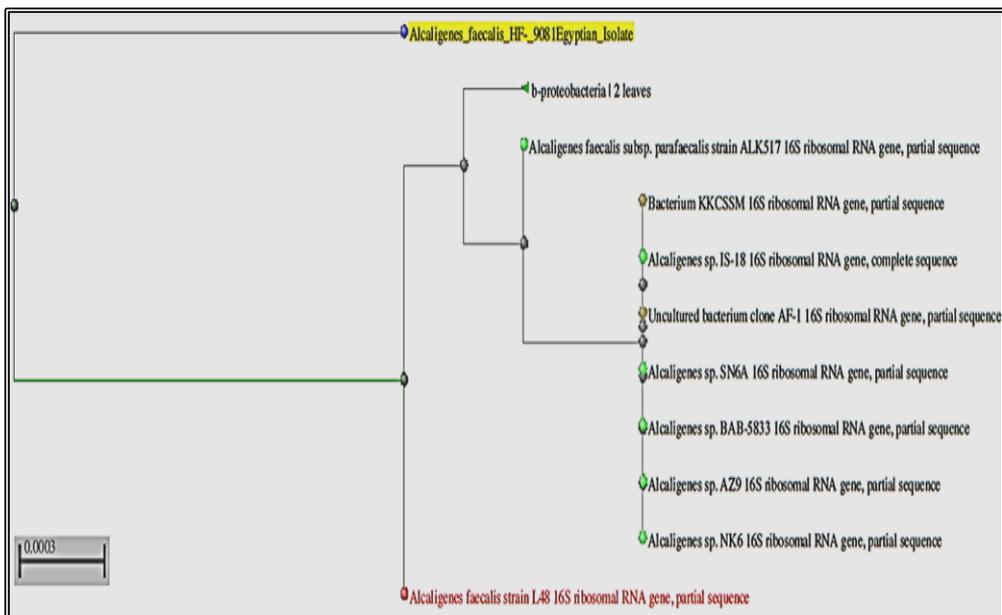
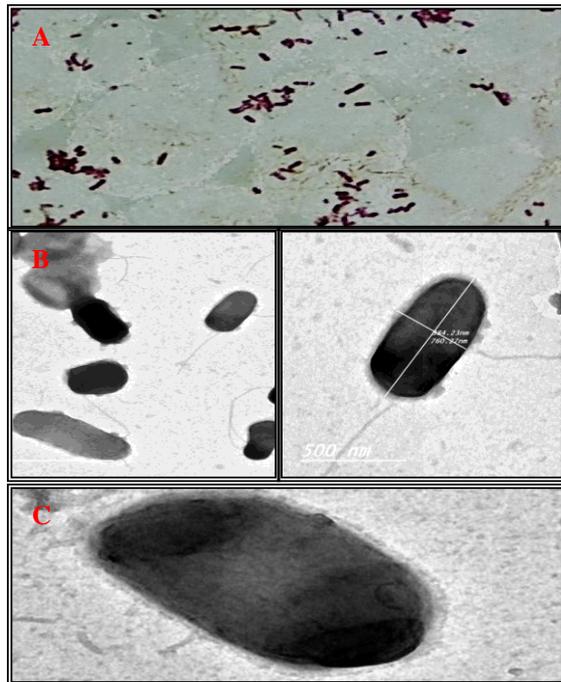


Fig. (2): The phylogenetic tree of the Egyptian isolate HFW-9081 indicating the genetic relationship with the standard strains of *Alcaligenes faecalis*.

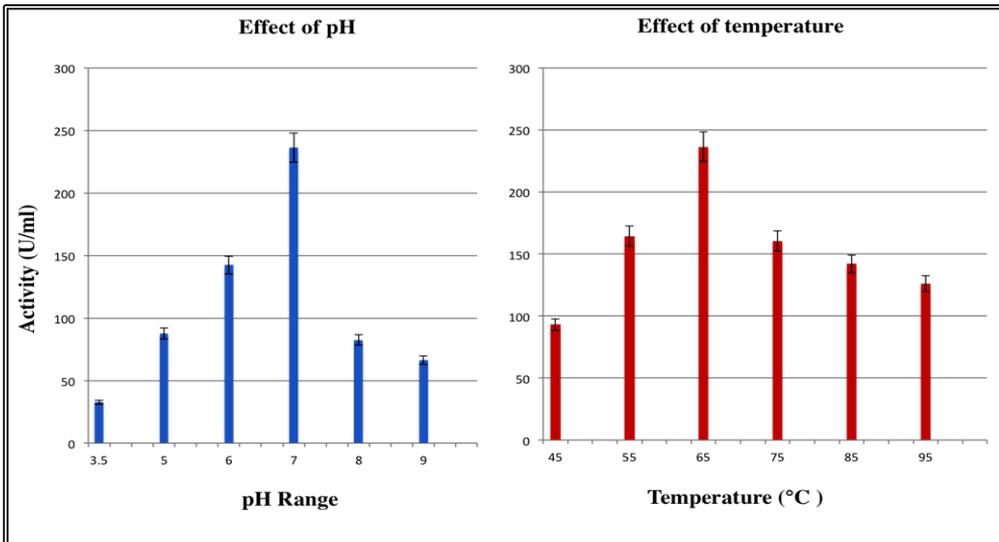


Fig. (3): Effect of different temperatures (right) and pH values (left) on the protease activity produced by Egyptian *Alcaligenes faecalis* strain HFW-9081.

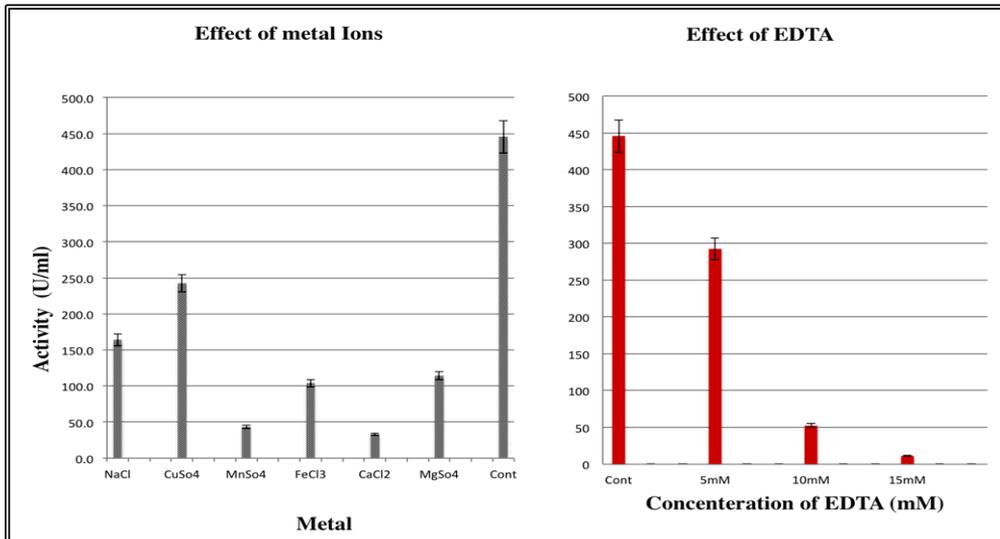


Fig. (4): Effect of different metal ions (left) and EDTA concentration (right) on the protease activity produced by Egyptian *Alcaligenes faecalis* strain HFW-9081; Cont: is control activity with no metal or EDTA added.

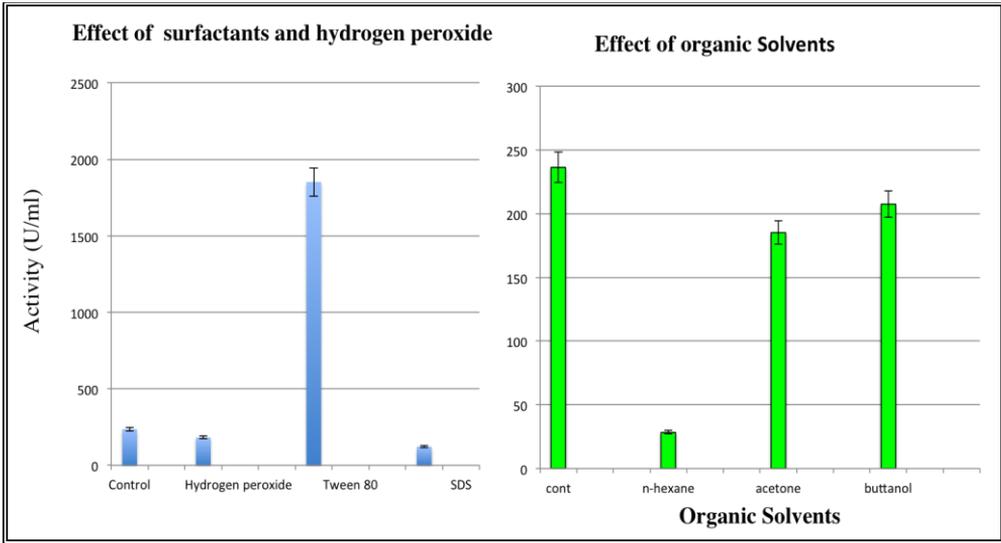


Fig. (5): Effect of selected organic solvents (right) and surfactants, hydrogen peroxide (left) on the protease activity produced by Egyptian *Alcaligenes faecalis* strain HFW-9081; Cont is control activity with no organic solvents or surfactant.

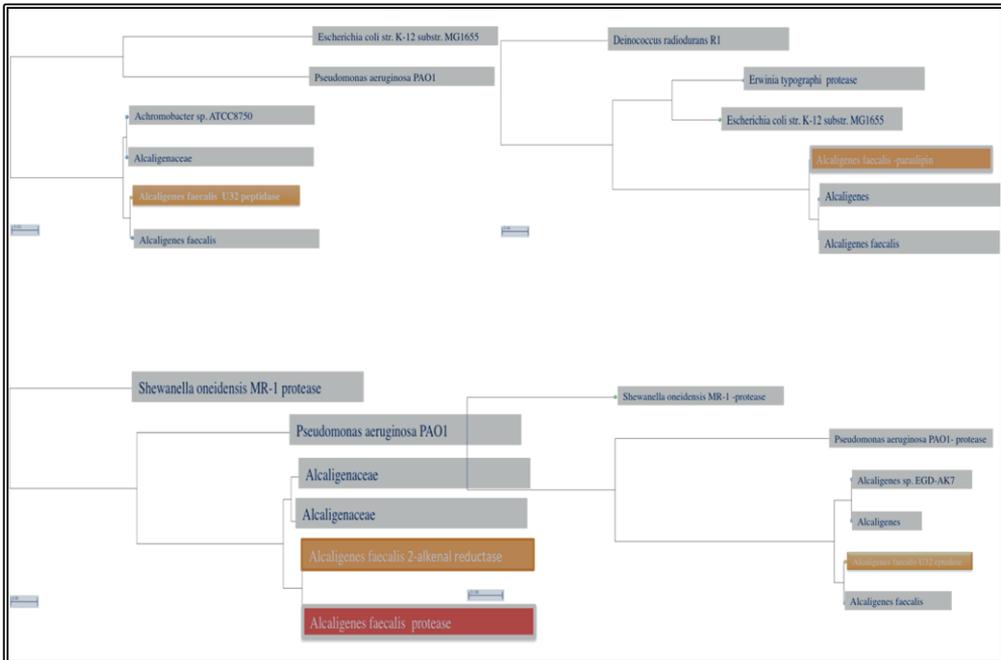


Fig. (6): Phylogenetic trees for the genetic relationship of four protease genes in *Alcaligenes faecalis*.