J.Agric.Chem.and Biotechn., Mansoura Univ.Vol. 7(8): 227 - 234 , 2016

Production of Thermostable Protease From"*Bacillus amyloliquefaciens":*An Egyptian Marine Isolate

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ABSTRACT

Proteases represent an essential group of enzymes that are widely produced and used industrially, thus the need for new microbial isolates with new features is of utmost necessity for industrial applications. A locally marine bacterium, isolated from Red Sea region in Egypt was able to produce thermostable proteases, the isolate was identified morphologically, biochemically, and confirmed molecularly by 16S rRNA sequencing with 98% similarity to *Bacillus amyloliquefaciens*. It exhibited optimum activity of 363.4 U/mLat 65°C and pH 7 for ten min .Both ammonium sulphate and Sephadex G-100 purification methods reduced the specific activity to 256.4 and 153.4 U/mL. However, the activity increased 3.8 folds when Tween-80 was used as surfactant. Genetic background of the protease genes in *Bacillus amyloliquefaciens*was analyzed using bioinformatics database for the proteases amino acids sequences in the desired bacteria; and it specified that *Bacillus amyloliquefaciens*has five different protease genes; these genes encode for various peptidase family groups. The variation in the peptidase family 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. The produced Enzyme showed good activity between 65°C and 85°C. While the addition of metal ions inhibited the enzyme activity.

Keywords: Thermophilic, Protease, Marine bacteria, *Bacillus amyloliquefaciens*, Peptidases family.

INTRODUCTION

Proteases represent an important group of enzymes that are used in various fields, covering a wide range of industrial applications, such as food, detergent, tannery, chemical and pharmaceutical industries (Li *et al*., 2007). Nowadays, microbial proteases dominate various commercial applications (Outtrup and Boye, 1990).In this respect, proteases produced by bacilli spp have been extensively studied and used industrially for a long time. However, a major requirement for its commercialization is its thermal stability, since thermal denaturation causes enzyme inactivation (Outtrup and Boye, 1990).In addition, for an enzyme to be used in detergents, it should be stable at high temperature and active in the presence of other detergent ingredients, such as surfactants, bleach activators, bleaching agents, fabric softeners and other formulation substances (Asker *et al*., 2013). As a result, there is a growing interest in isolating new proteases from thermophiles microbes, which are expected toproduce thermostable enzymes (Scandurra*et al*., 2000). Microorganisms constitute the major source of proteases, including both extracellular and intracellular ones (Bommarius, 2015). In general, bacilli produce two major types of proteases, alkaline protease and a metalloprotease or neutral protease (Vasantha*et al*., 1984).Based on their site of action at the C or N terminus, they are classified into carboxypeptidases and aminopeptidases, respectively (Rao*et al*., 1998). Furthermore, carboxypeptidasesare divided into three major groups, including serine carboxypeptidases, cysteine and metallo-carboxy peptidases (Ray, 2012). The present investigation aims to isolate thermophilic bacteria producing protease, identify the selected isolates, then investigating the protease activity under some stresses such as, temperature, pH, selected organic solvents, heavy metals, oxidizing agent and EDTA. Also the genetic background of the identified isolate protease genes will be analyzed through the bioinformatics database.

MATERIALS AND METHODS

1. Bacterial strain source.

Thermophilic water bacterial isolate was isolated from Red Sea and identified biochemically by El–Eskafy(2015).

Molecular identification of the bacterial Isolate.

Pure isolate was characterized morphologically using scan 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 Bact1492R 5‟-TACGG(C/T)TACCTTGTTACGACTT-3‟). Bacterial culture was sent to SolGent Company, South Korea for 16S rRNA gene sequencing. At the company DNA was extracted and isolated using SolGent purification bead. Prior to sequencing, the ribosomal rRNA gene (also referred to as rDNA) were amplified using the polymerase chain reaction (PCR) technique in which two universal primers 27F and 1492R were incorporated in the reaction mixture. 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_2HPO_4 0.4g/l, Na_2CO_3 0.085 g/l, $ZnSO_4$ 0.02g/l, $CaCl_2$ 0.02g/l, $MgSO_4$ 0.02g/l, incubated at 50 °C for 50 h, centrifuged at 14000 rpm for 30 min at 4°C. Cell free Extract was used as crude enzymes. were assayed for proteolytic activity in

triplicate using caseinas the substrate according to method described by Guangrong*et al.* (2006).

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 mMTris-HCl buffer with pH 7.2) and 100 µlof crude enzymeswere 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 µL supernatant was carefully removed to measure tyrosine content using a Folin-phenol method (Ledouxand Lamy,1986). One unit of protease activity (U) was defined as the amount of enzyme that hydrolyzes casein to produce 1.0 µmole of tyrosine per minute at 65°C.

Determination of total protein.

Total protein was determined by diamond total protein kit using bovine serum albumin, according to Lowry *et al*. (1951).

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 (31×16 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 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 respective optimum pH) was obtained in each case and constituted the purified enzyme according to Al-Samane*t al.* (2015).

Effect of different temperatures and pH

The thermostable activity was determined using standard assay procedure after incubating the enzyme at temperatures ranged from 45 °C to 95 °C for 10 min, according to (Akel*et al*., 2009) modified. The activity 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.

Effect of selected metal ions and EDTA

The effect of NaCl, $CaCl_2$, $MgSO_4$, $FeCl_3$, $MnSO_4$ and $CuSO_4$ as sources of metal 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 mMTris-HCl buffer with pH 7.8 containing various Ethylene Diamine Tetra Acetic Acid (EDTA) concentrations ranging from 0 to 15 mM (Akel*et al.,* 2009). Crude and pure enzyme activities were measured at 65 °C, under standard conditions.

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 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 toGupta and Khare (2006).

Effect of some surfactants and hydrogen peroxide

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

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 (4). SmartBlast site programs were used to analyze the protein data and peptide available at http://blast.ncbi.nlm.nih.gov/smartblast.

RESULTS AND DISCUSSION

1. Characterizationof the isolated thermophilic bacteria

Morphological and biochemical characterization by EL-Eskafy (2015) suggested that the isolate may belong to the thermophilic bacilli according to Bergey's manual of systematic bacteriology (Brenner *et al*., 2005)Morphological and cultural characterizations of RSW-8018 were studied by the examination of single creamy colonies. Colonies were large and vary in shape, from circular to irregular, with undulate and fimbriate edges; it has granular texture, sometimes smooth and moist colonies. Gram stain was positive; RSW-8018 was motile by peritrichious flagella. Indole, catalase and gelatine hydrolysis tests were positive; Cells studied by Scanning Electron Microscope (SEM) showed rod-shape cells, long chained with ellipsoidal, central and subterminal endospores (Figure 1). *Bacillus* cells often form chains and are motile, with peritrichious flagella (Brenner *et al*., 2005). Oval spores are central or paracentral in sporangia. Transmission electron micrographs of theRSW-8018 cells (Figure 1) showed rods 0.2-1.0 to 0.5-2.6 µm, occurring singly.RSW-8018 cellsis Gram positive, rod-shaped, motile, aerobic, grew at pH 5.5-8.0 with an optimal pH of 7.0 and at 30°C-60 °C with an optimal temperature of 50 °C(El-Eskafy 2015).

Molecular identification using 16S rRNA gene sequencing

Sequencing of 16S rRNA gene of the selected Egyptian marine isolate RSW-8018 was performed; 16 S rRNA sequence using 27 F primer gave 1243 base pair (bp) while using the 1492 R primer gave 1228 bp. The search on the Gene bank nucleotide database using

the blast-nt algorithm revealed significant matching (hi score and low e-value) and 98 % identity with the gene sequence of the strain of *Bacillus amyloliquefaciens*.

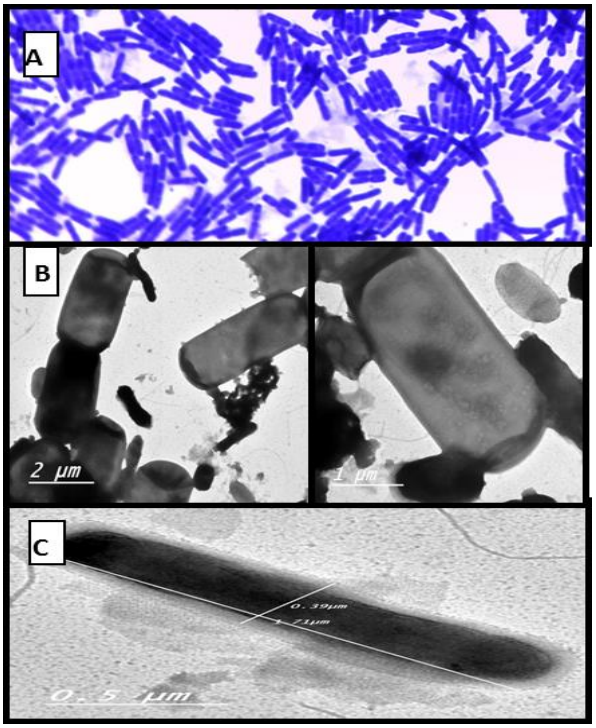

Figure (1).A)Gramstain of the isolate (RSW-8018) cells (x 100); (B,C) Transmission electron micrographs (bar 0.2 nm) of isolate (RSW-8018) cells.

Also the phylogenetic tree (Figure 2) showed high genetic relationship between the Egyptian isolate RSW-8018 and the strain of *Bacillus amyloliquefaciens* which strongly prove that the Egyptian isolate can be identified molecularly as *Bacillus amyloliquefaciens*, based on the 16srRNA (rDNA) nucleotide sequence and the phylo-tree analysis (Figure 2). Its complete taxonomy is:

Bacteria-Firmicutes-Bacilli-Bacillales-Bacillaceae-Bacillus-*Bacillus amyloliquefaciens.*

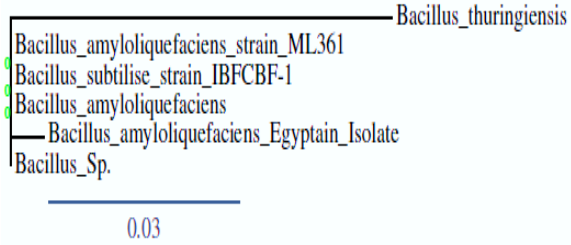

Figure (2).The phylogenetic tree of the Egyptian RSW-8018 indicating the genetic relationship with the standard strainsof*Bacillus amyloliquefaciens*.

2. Determination of protease activity

The selected isolates were purified, and their proteolytic activities were evaluated by observing the hydrolysis of casein by measuring the clear zone for the tested isolates. The widest zone diameter of 3.5 cm was obtained for *Bacillus amyloliquefaciens*, isolate RSW-8018 with enzyme activity of 363.4 U/mL. at pH7.

3. Factors affecting the protease activity

Effect of different purification methods

Thermostable protease was purified in 2-steps procedure involving ammonium sulfate (80%) precipitation followed by Sephadex G-100 fractionation, for the assessment of protease activity. Results obtained in Table (1) showed that both ammonium sulphate and Sephadex G-100 methods reduced the yield of *Bacillus amyloliquefaciens*RSW-8018 to 70.5 and 42.2% and the activity to 256.4 and 153.4 U/mL, respectively, compared to the cell free supernatant. Likewise, the thermoprotease activity of *Bacillus* sp. was reduced by 1.7% after purification in a 3-steps procedure, including ammonium sulfate precipitation, Sephadex G-100 gel permeation followed by DEAE-ion exchange chromatography (Jooet *al*., 2002; Akel*et al*., 2009). Similarly, Asker *et al*. (2013) demonstrated that *Bacillus megatrium* protease enzyme possess a specific activity of 41.09 U/mg after purification 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 (Asker *et al*., 2013)

Table (1).Purification of proteases produced by *Bacillus amyloliquefaciens*

	Bacillus amyloliquefaciensstrain RSW-8018					
Purification Steps	**Total Protein (mg/mL)**	**Enzyme Activity (U/mL)**	**Total Enzyme Activity (U)**	**Specific Activity (U/mg)**	**Purification Fold**	**Yield (%)**
Cell free supernatant	1.35	1.011	363.4	181.7	1.0	100
Ammonium sulfate (80%)	2.0	0.904	256.4	189.9	1.04	70.5
Sephadex G-100	0.80	0.801	153.4	191.7	1.10	42.2

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

The effect oftemperature

Maximum enzyme activity of 363.4 U/mL was observed at 65 ºC, and it was gradually decreased to reach 164.9 and 191.9 U/mL with increasing the temperature up to 85 °C and 95 °C, respectively (Figure 3). Results revealed that the enzyme had good activity between 65 °C and 85 °C; however, the activity was reduced markedly to almost one third by increasing the temperature to 95 °C. 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 (Figure 3) demonstrated that the strain under study had good enzyme activity between 65 °C and 85 °C. Therefore, it can be classified as a thermophilic-protease, data are in agreement with Asker *et al*. (2013).Therefore, results provide a promising enzyme that can be used in detergent industries using hot and/or cold wash cycles, and in other different biotechnological applications.

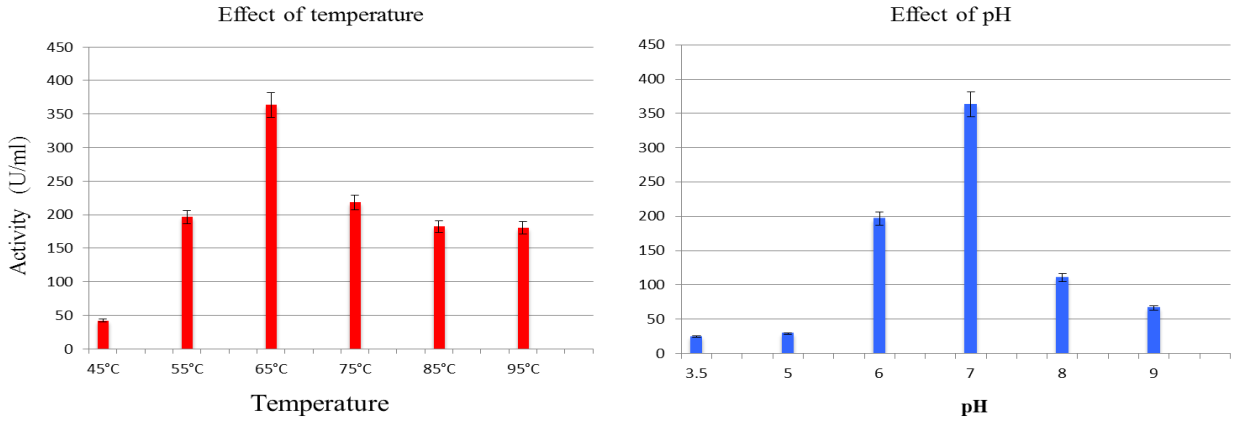

Figure (3).Effect of differenttemperatures (left) and pH values (right) on the protease activity produced by Egyptian *Bacillus amyloliquefaciens* strain RSW-8018.

The effect of pH

The effect of pH on protease activity was examined (Figure 3) at different pH values of 3.5, 5, 6, 7, 8 and 9. Maximum enzyme activity of 363.4 U/mL was observed at pH 7. However, the enzyme activity was significantly decreased to 22.4 U/mL by lowering the pH to 3.5 (Figure 3). Also results showed that the protease remained active between pH 6 and pH 7. 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).Nevertheless, the enzyme activity began to decrease sharply to 103.3 and 68.1 U/mL at pH 8 and pH 9, respectively (Figure 3). Similarly, the optimum pH for protease activity from *Bacillus* sp. was determined at pH 7.0 as reported bySevinc and Demirkan (2011) who mentioned that it could be a neutral protease. Likewise, the enzymatic activity of different *Bacillus* spp., such as *B. subtilis* ITBCCB 148, *B. subtilis* HS08 and *B. subtilis* S17110 wasoptimumat pH 7.5 (Joo*et al*., 2002; 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 (Kim *et al*., 2001; Nascimento and Martins, 2004; Ghorbel-Frikha*et al*., 2005). Figure (3) showed that the enzyme possess moderate activity in the pH range of 6-8 which is in agreement with Basu*et al*. (2008) and Merhep-Dini*et al*. (2009). In contrast, Beena*et al*. (2012), Asker *et al*. (2013) and Habib*et al*. (2011) reported alkaline proteases activity from some *Bacillus* spp. and *Halobaterium* sp. between pH 6-9 with gradual increase in their activity.

Effect of various metal ions

Results obtained in Figure (4) showed that all metal ions tested significantly decreased the activity as compared to the control. The presence of $MgSO_4$, NaCl and $CaCl_2$ mostly affected the enzyme activity as shown in Figure (4). Nevertheless,Habib*et al*. (2011) reported that only NaCl and $FeCl_3$ stimulate the protease with 100% relative activity, while $CaCl_2$ retained 90% in *Halobaterium* sp. However, Nascimento and Martins (2004) reported that some metal ions may protect the enzyme from the thermal denaturation and maintain its active conformation at the high temperature. In contrast, a maximum inhibition of about 40% with 1.0 mM Zn^{2+} and Fe^{2+} for the protease of *Pseudomonas* and *Burkholderia* was reported (Asker*et al*., 2013). 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).

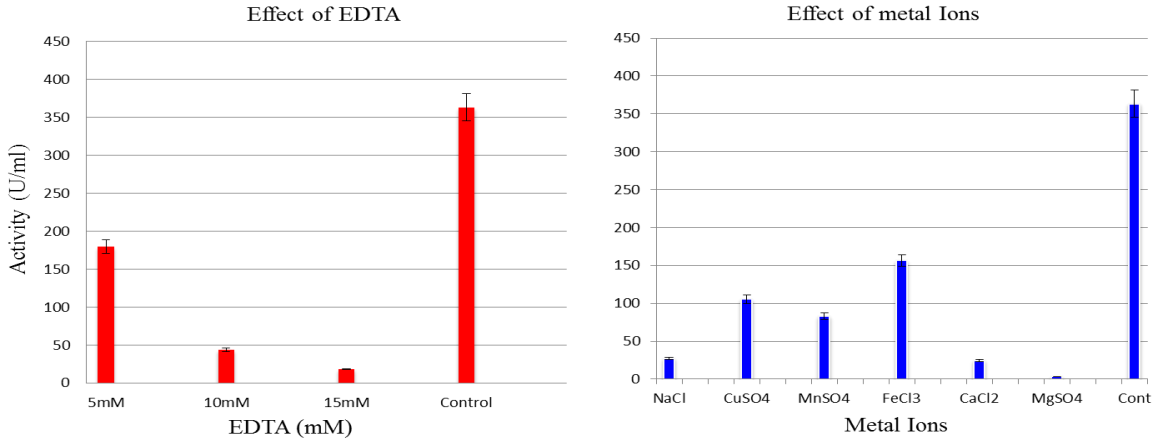

Figure (4).Effect of different metal ions (left) and EDTA concentrations (right) on the protease activity produced by Egyptian *Bacillus amyloliquefaciens*strain RSW-8018;Cont: is control activity with no metal ions or EDTA.

Effect of EDTA

Chelating agent as EDTA is a detergent additive which functions as water softener and also assists in the stain removal. Enzyme activity was 165.4 U/mL in presence of 5 mM of EDTA; however it was decreased to 43.9 and 18.4 U/mL with 10 and 15 mM concentrations of EDTA, respectively (Figure 4). Similarly, Akel*et al*. (2009) reported that the protease activity of *Bacillus* HUTBS71 decreased to 70% in the presence of 1 mM EDTA. The activity of the enzyme in presence of EDTA is advantageous for using the enzyme in the presence of detergent. Overall results (Figure 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.

Effect of organic solvents

Results obtained in Figure (5) showed that the enzyme activity was reduced to 52.4 and 88.4 U/mL with acetone and butanol, respectively. While, the enzyme activity significantly decreased to 23.4 U/mL in presence of N-hexane. Overall results (Figure 5) arein agreement with Bahobil (2011) who reported that acetone reduces the relative protease activity of *Shewanellaputrefaciens*-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).

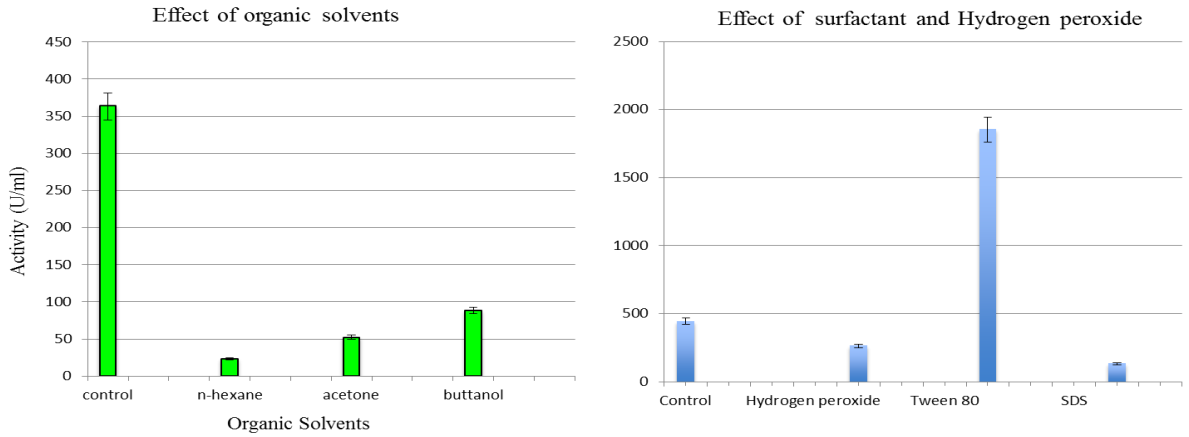

Figure (5).Effect of selected organic solvents (left) and surfactants, hydrogen peroxide (right) on the protease activity produced by Egyptian *Bacillus amyloliquefaciens*strain RSW-8018; Cont is control activity with no organic solvents or surfactant.

Effect of surfactants and hydrogen peroxide

Surfactants act as detergents, dispersants, emulsifiers, foaming and wetting agents. Results showed that SDS decreased the enzyme activity to 92.4 U/mL as compared to control (Figure 5). However, Tween-80 increased the activity 3.8 folds as compared to the control. Surfactants may also play a role in exposing the active sites and making them available for enzyme-substrate hydrophobic interactions (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).

Bioinformatics and genetic background of proteases genes in *Bacillus amyloliquefaciens*

Genetic background of protease genes in *Bacillus amyloliquefaciens* obtained from the bioinformatics database (*www.uniprot.org*) and gene bank reveals five different genes; classified as protease genes or subunits protein of proteases of *Bacillus amyloliquefaciens* with 98 to 100% identity to the (Table 2). However, the analyses of amino acid (AA) sequences clarified that those genes are not gene copies or alleles for the same protease gene. Furthermore, the peptides family analysis by Smart blast for these genes protein sequences indicated that the first gene *PrsW* with Accession WP_013352703.1 has 218 AA and contains protease prsW family; it is a M82 family of putative peptidases, possibly belonging to the MEROPS M79 family. *PrsW* may be responsible for site-1 cleavage of the RsiW anti-sigma factor, and it senses antimicrobial peptides that damage the cell membrane and other agents causing cell envelope stress (Odagaki*et al.*, 1999). The second protein gene subtilisin (*apr)* with accession WP_065981100 has 382 AA and belongs to S8 peptidase, members of the S8 peptidases and S35clan family, include endopeptidases, exopeptidases and tripeptidyl-peptidase. Also, some members of clan family contain disulfide bonds (Page and Di Cera, 2008). These enzymes could be intra- and extracellular, function at extreme temperatures and pH values. Subtilisin represents the highest commercially important proteolytic additive in detergents. Moreover, it is found in most *B. subtils* group as well as *Bacillus amyloliquefaciens*as reported byGupta *et al.* (2002). The third gene pyrrolidone-carboxylate peptidase (*pcp)* with accession WP_013350909.1 contains 215AA, it is apyroglutamyl peptidase (PGP) type I, also known as pyrrolidone carboxyl peptidase (*pcp*) type-I which is a protease enzyme responsible for cleaving pyroglutamate (pGlu) from the N-terminal end of specific proteins. The pGlu protein can be hydrolyzed only by PGP type-I

protease. PGPs are cysteine proteases with a Cys-His-Glu/Asp catalytic triad and type-I PGPs are found in different prokaryotes and eukaryotes(Marchler*et al*., 2014). However, its functional form is not clear if it is a monomer, a homodimer, or a homotetramer (Marchler*et al*., 2014). On the other hand, the fourth gene is zinc metalloprotease (*FtsH***)** with accession WP_061861769which has 639AA, and it is anextracellular protein. *FtsH* is the only membrane-bound ATP-dependent protease that is universally conserved in prokaryotes(Vasilyeva*et al*. 2002). It efficiently degrades only proteins that have a low thermodynamic stability. In *OenococcusoeniFtsH* is involved in the protection against environmental stress, and shows increased expression under heat or osmotic stress (Chen *et al.*, 2007). While, the fifth gene is *Lon serine* protease with accession WP_061582029.1.1 and has 744 AA; it contains two peptidases families Ch 61I subunit Ch1I of Mg-chelatase and peptidase S16. The *Lon serine* proteases hydrolyze ATP to degrade protein substrates in *Escherichia coli*, these proteases are involved in the turnover of intracellular proteins, and abnormal proteins that follow heat-shock. The active site for *Lon serine* protease resides in a C-terminal domain as reported by Vasilyeva*et al*. (2002). The variation in peptidase family provides the protease enzymes with many features making them able to remain active under various environmental stresses. Furthermore, the use of databases information can be applied to design selective primers for genetic markers that can be used to select microorganisms able to produce important proteins, such as thermostable proteases. The overall results explains how the thermostable enzyme from the Egyptian *Bacillus amyloliquefaciens*RSW-8018remain active at pH ranging from 3.5 to 9, temperature ranging from 45 °C to 90 °C, in the presence of EDTA, selected organic solvents and even with different metal ions (Table 2).

Table (2). Genetic bioinformatics background for the proteases genes in *Bacillus amyloliquefaciens*

Gene/Protein Name	Accesssion Number (in NCBI)	Amino Acid	Peptide Family	Strain	Reference
Protease (*PrsW)*	WP_013352703.1	218	M82 family	*Bacillus amyloliquefaciens*	http://www.ncbi.nlm.nih.gov
Subtilisin (*apr)*	WP_065981100.1	382	Peptidase S8	*Bacillus amyloliquefaciens*	http://www.ncbi.nlm.nih.gov
Pyrrolidone-carboxylate peptidase *(pcp)*	WP_013350909.1	215	Pyroglutamyl peptidase (PGP) (pcp) type I	*Bacillus amyloliquefaciens*	(Marchler*et al*., 2014)
Lonserine Protease	WP_061582029.1	774	ChlI: Subunit ChlI of Mg-chelatase Peptidase_S16 family M48	*Bacillus amyloliquefaciens*	http://www.ncbi.nlm.nih.gov
Zinc metalloprotease (*FtsH)*	WP_061861769.1	639	FtsH cell	*Bacillus amyloliquefaciens*	http://www.ncbi.nlm.nih.gov

CONCLUSION

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 bacterium producing protease was isolated from Red Sea in Egypt and identified, it 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 *Bacillus amyloliquefaciens* in Genebank showed five genes remarked as proteases, the phylogenetic tree for each gene proves the genetic relationship of these genes and specified that these genes are *Bacillus amyloliquefaciens* proteases with 98 to 100% identity. However, more research is still needed for further characterization and optimization of genetic regulation of such proteases.

Acknowledgements

Grateful and appreciate acknowledgement to the late Dr. AbdeenHamedElzanaty, the lecturer in Microbial Biotechnology Dept., Genetic Engineering and Biotechnology Institute, University of Sadat City, Egypt (*God Bless His Soul*); for suggesting the point for this investigation.

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انتاج بروتييز ثابت حرارياً من بكتريا الباسلس اميلولكيوفيشنس:عزلة مائيه مصرية

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تمثل انزيمات البروتييز مجموعة أساسية من الانزيمات التي يتم إنتاجها صناعيا على نطاق واسع ، وبالتالي فإن الحاجة إلى عزلة ميكروبية جديدة ذات مميزات انتاجية هي من الضرورة الملحة لاستخدمها في التطبيقات الصناعية وبناء عليه تم عزل بكتريا مائيه مصرية من احدي مناطق مياه البحر الاحمر لها القدرة علي انتاج بروتيز ثابت حرارياً. عرفت تلك العزله مسبقا باستخدام طرق التعريف البيوكيميائية واكدالميكروسكوب الالكتروني الماسح و التعريف علي المستوي الجزيئي باستخدام طريقة الـ 16S rRNA و التى اكدت وجود نسبة تماثل تساوى 98٪ مع بكتريا البسلاس اميلولكيوفيشنس. وقد حدد النشاط الأمثل لهذا الانزيم بـ 363.4 وحدة/مل بعد تحضين لمدة 10دقائق عند 65 درجة سليزيس و7 درجة حموض. واوضحت التجارب ان طريقة الاستخلاص اثرت بصورة واضحة على النشاط الانزيمى فقد ادى اسخدام كبريتات الامونيم و السيفاديكس ج-100 الى خفض النشاط النزيمى الى 256.4و153.4 وحدة/مل على التوالى . و قد ارتفع النشاط النزيمى فى وجود التلووين-80 كاحد مخلخلات التوتر السطحى الى 3.8 ضعف عن الكنترول.كما ادى استخدام فوق اكسيد الهدروجين الى خفض النشاط الانزيمى الى 130.4 وحدة/مل وقد ابدى الأنزيم نشاط ملحوظ تحت تباين من درجات الحموضة من 6 الى 8 درجة حموضة و كذلك تحت تباين حرارى من 55 الى 95 درجة سليزيس.كما اوضح تحليل البيانات الرقمية المتحصل عليها من قواعد البيانات و بنك الجينات ان الخلفية الوراثية لجنوم بكتريا البسلس اميلولكيوفيشنس. يحتوى فى الغالب على خمس جينات مختلفة تشفر لأنتاج انزيمات البروتييز(كاملة او لبعض تحت الوحدات الأنزيم) كما اكد تحليل تتابعات الاحماض الامينية للبروتينات الناتجه من تللك الجينات أنها تنتمى لعدد كبير من العائيلات الببتيدية والتى تختلف فى قدراتها الوظيفية و التحليلية و هو ما يفسر قدرة انزيم البروتييز المعزول من العزلة المصرية RSW-8018و المعرفة على انها بكتريا البسلس اميلولكيوفيشنس. على العمل تحت مدى واسع من تنوع الموثرات البيئية من حرارة و درجات حموضة وايونات المعادن وكذلك المذيبات العضوية مما يشير الى امكانية استخدامةفى العديد من الصناعات المختلفة كما اوضح استخدام قواعد البيانات الرقمية الى امكانية استخدام التحليل البيانى للجينات فى عمل دلائل•(واسمات وراثية) يمكن استخدامها فى الانتخاب السريع للكائنات الدقيقة المنتجة لمركبات ذات اهمية تطبيقة مثل انزيم البروتييز الثابت حرارياً.