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ISOLATION,SCREENING AND PRODUCTION OF EXTRACELLULARPROTEASEFROM THERMOPHILICBACTERIA

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ABSTRACT

Sixteen proteolytic thermophilic bacteria were isolated from soil at Fayoum Governorate. The isolates were screened for their proteolytic activity on skim milk agar medium; the diameter of hydrolysis zone was the measurements from the level of proteolytic activity. The most active isolates were selected for the fermentation experiments and the determination of their productivity in the submerged culture. The seven selected isolates were used in fermentation experiments and the proteolytic activity was determined after 48 hrs. The enzyme yields obtained in the fermentation medium were corresponding to the proteolytic level recorded by the diameter of hydrolysis zone on the skim milk agar medium. That indicates a positive relationship between the amount of the enzyme and its spreading in the skim milk agar medium. According to the results from the fermentation experiments, strains S-5, S-8 and S-9 which gave the highest enzymatic yields were chosen for studying the best environmental conditions for the enzyme production. The three isolates were identified based on morphological, biochemical and 16S rRNA gene sequencing analysis, isolates S-5 was identified as *Brevibacillus panacihumi*; isolates S-8 and S-9 were identified as *Bacillus aerius*. The environmental conditions such as pH, temperature and fermentation period were studied for the highest production of enzymatic yield. The results show that, the highest enzymatic yield was obtained in the fermentation medium at pH 7.0 for *Bacillus stearothermophilus* ATCC7953 and *Brevibacillus panacihumi* S-5 while at pH 8.0 for both *Bacillus aerius* S-8 and *Bacillus aerius* S-9. The fermentation temperatures at 40, 50 and 60 °C showedthat the

optimumincubation temperature for the enzyme synthesis was 50°C. However, the incubation period required for maximum accumulation of protease was 72 hrs.

INTODUCTION

Proteases are complex group of enzymes collectively known as peptidyl-peptide hydrolases and are responsible for hydrolysis of peptide bonds in a protein molecule (**Habib *et al.*, 2012).**

Proteases constitute a class of industrial enzymes, which alone form approximately 60-65% of the total world-wide enzyme production (**Rao *et al.*, 1998; Chu, 2007** and **Guangrong *et al.*, 2008**). They are used for various industrial applications, laundry detergents, leather preparation, and meat tenderization. Besides that, they are also used in pharmaceuticals, medical diagnosis and decomposition of gelatin on x-ray films as well as in textiles **(Joo *et al.*, 2002;** and **Patel *et al.*, 2005).** Proteases are also, used for the bioconversion of chitinous materials as a waste treatment alternative to the disposal of shellfish wastes (**Yang *et al.*, 2000** and **Manni *et al.*, 2010**). The extracted chitin and chatoyant may be used in many applications as some as cosmetics and water treatment (**Jellouli *et al.*, 2011).**

Proteases are widespread in nature, microbes serve as a preferred source of these enzymes because of their rapid growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications (**Josephine *et al.*, 2012).**

Mostly, all industrial processes are carried out at higher temperature, where normal enzyme becomes unstable (**Amann *et al.*, 1995** and **Egelseer *et al.*, 1995).** Therefore, there is an ample scape for searching thermostable proteases which can resist the changes in environmental conditions in which they are supposed to work in industries.

Bacilli are one of the most important producers of extracellular commercial proteases because of their high pH and temperature stabilities (**Periasamy, 2013**).

However, the current work was conducted to select some local strains of spore forming bacilli capable of producing a relatively more thermostable protease. Moreover it is to study the environmental factors affecting the productivity of protease such as pH of medium, temperature and incubation period .

MATERIALS AND METHODS

Microorganisms used:

a.Bacillus stearothermophilus ATCC7953

It was used as reference strain, it was kindly provided from Microbiological Resource Center (MERCIN) at Faculty of Agriculture, Aim Shamis University, Cairo, Egypt.

b.Microbial isolates:

Sixteen isolates of spore formes were isolated from enriched soil of private farm, at Fayoum Governorate, Egypt.

Isolation of proteolytic thermophilic bacteria:

50 gm. Samples from soil were mixed with casein at the rate of 20% of its weight, placed in Petri- dishes and incubated at 60°C for three weeks. During incubation sample was mixed with 10 ml. water of 3 day intervals to maintain the moisture at level suitable for the active growth of thermophilic microorganisms with the opportunity of increasing the number of proteolytic thermophilic. About one gram of enriched samples was added to 9.0 ml. sterile water up to10^{-7} pasteurized at 80°C for 15 min., the pasteurized samples were used for the preparation of serial dilutions. Three plates were inoculated with 1.0 ml. for each of the dilutions 10^{-4} to 10^{-7}ml. 10 ml. of skimmilk agar medium were poured in each plate. Plates were incubated at 60°C for 48 hrs. The colonies surrounded with a relatively large clear zone of casein hydrolysis were isolated and purified. The number of active proteolytic isolates were 16 isolates. The isolates were screened to their proteolytic activity according to the diameter of the clear zone of casein hydrolysis on skim milk agar medium. The 48 hrs.broth cultures were pasteurized; a loop was inoculated in 10 ml. sterile water and well mixed. One loop of the mixture was mixed with 10 ml. sterile skim milk agar medium, poured in Petri dish and incubated at 60°C for 72 hrs. The mean diameter of clear zones in each plate was daily recorded.

Identification of the active proteolytic thermophilic bacteria:

The isolates which gave the largest zones of hydrolysis were identified based on morphological characteristics by **Bergey's Manual of Systematic Bacteriology (1986)**and biochemical tests were then conducted using the AP150CHB system according to the manufacturer's protocols (Biomerieux).The identification was further confirmed by sequencing of the 16SrRNA and subsequent comparison of the sequence with those available in the Gen Bank nucleotide database by using the NCBI BLAST algorithm

(w.w.w.ncbi.nIm.nih.gov). The DNA was amplified by PCR using the 16SrRNA gene primers: 785F(GGATTAGATACCCBRGTAGTC), 907R (5´CCGTCAATTCMTTT-3´).

Maintenance of cultures:

The stoke cultures of the selected strains were maintained on skim milk agar slants, stored at 5°C and sub-cultured monthly.

Media used:

1-Skim milk agar (**Lelliott** and **Stead, 1987)**: It was used for testing hydrolysis of casein (caseinase test) for isolation. It composition is as follows: (g/L), yeast extract 2.5, casein 5.0, glucose 1.0, Skim milk solution 100ml. (10%), agar 15.0 and distilled water 1000ml. at pH 7.0

2- Nutrient broth (Difico, 1985): It was used for inoculum preparation. Its composition is as follows: (g/L), beef extract 3.0, peptone 5.0 and distilled water 1000ml. at pH 7.0

3-Modified Luria broth (Zamost *et al.*1990): It was used for protease production by four tested bacterial strains, the fermentation medium composition is as follows: (g/L), yeast extract 5.0, tryptone 10.0, $CaCl_2$ anhydrous 0.37, dextrin 10.0 and distilled water 1000ml. at pH 7.0

Fermentaion experiments:

1- Preparation of inoclum:

After an incubation period of 24 hrs. , cultures of the selected strains obtained on skim milk agar slants were suspended in sterilized distilled water. A1.0 ml. of culture was aseptically transferred to 250 ml. Erlenmyer flasks, each containing 100 ml. sterile inoculation medium. Flasks were incubated on a rotary shaker at 120 r. p. m. and 60 °C for 24 hours.

2- Fermentation:

Ten ml. of the inoculum were aseptically inoculated in 250 ml. Erlenmyer flasks containing 100 ml. of the fermentation medium and incubated on a rotary shaker of 120 r.p.m. for 48 hrs. at60°C. At the end of the experiment, the content of each flask was centrifuged at 4000 r.p.m. for 30 min. to obtain clear liquor, containing the enzyme, followed by the estimation of enzyme activity.

Factors affecting the production of proteolytic enzyme:

In order to study the effect of different factors on protease production, fermentation medium was modified to be suitable for studying the respective factors as follows:

1-Effect of pH:

The pH of the fermentation medium was adjusted the values of 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. At the end of the fermentation period the enzymatic activity was estimated.

2. Effect of incubation temperature:

Various incubation temperatures ,i.e.,40, 50 and 60 °C were tested to study the effect of temperature on the production of thermophilic proteolytic enzyme.

3- Effect of incubation period:

Fermentation experiments were carried out at 50°C for period of 24, 48,72, 96 and 120 hours. At the end of each period, the enzyme activity was determined.

Protease assay:

Protease activity was determined according to **Keay** and **Wildi, (1970**) and described by **Matta and Punj, (1998)** using tyrosine as standard. To 1.0 ml. of substrate (1.0% casein in 0.05 M Tris- HCl buffer, pH 7.5) was added 1.0 ml. of suitably diluted enzyme and the reaction mixture was incubated at 37°C for 10 min. The reaction was terminated by adding 2.0 ml. of 0.4 M trichloroacetic acid. The precipitated proteins were filtered through Whatman No.1 filter paper. To 1.0 ml. of filtrate, 5.0 ml. of 0.4 M sodium carbonate was added followed by 1.0 ml. Folin`s reagent. The solution was incubated at 37°C for 20 min. for colour development. The intensity of the blue colour developed was measured at 660 nm. Protease activity was expressed in units, where one unit was the amount of enzyme required to release 1.0 µm of tyrosine equivalent under assay conditions.

RESULTS AND DISCUSSION

Isolation and screening of proteolytic strains:

Thermophilic spore forming strains with proteolytic activity were isolated from soil. Selection of the proteolytic strains was carried out on skim milk agar; colonies surrounded by a clear zone of casein hydrolysis were isolated. The total of 16 isolates were purified and maintained on skim milk agar slants.

The purified isolates were screened for their proteolytic activity in order to select the highest productive strains for further studies. The proteolytic activity on skim milk agar medium was detected according to the diameter of the clear zone of casein hydrolysis. The data in Table (1) presents the daily mean diameter of the clear zones for each isolate. The results showed that the most active isolates were selected for the

fermentation experiments and the determination of their productively in the submerged culture.

The selected cultures were strains S-1, S-2, S-5, S-8, S-9, S-15 and S-16.The 7 selected isolates were used in the fermentation experiments and the protease activity was determined after 48 hours. Results in Fig. (1) showed that strains S-5, S-8 and S-9 were the most active. These three strains will be used for all next experiments. The enzymatic activity of the isolates in the fermentation medium was in most cases, corresponding to their activity on the skim milk agar medium (Table 1 and Fig.1).

Table (1) Mean diameters of the clear zones (mm) for each isolate.

No. of Isolates	Mean diameter (mm) of hydrolysis zone after			No. of Isolates	Mean diameter (mm) of hydrolysis zone after		
	24 hr.	48 hr.	72 hr.		24 hr.	48 hr.	72 hr.
S-1	7	14	24	S-9	8	20	29
S-2	8	17	25	S-10	2	6	13
S-3	2	7	9	S-11	2	6	14
S-4	2	4	11	S-12	2	7	12
S-5	7	21	30	S-13	2	7	16
S-6	2	5	12	S-14	2	8	12
S-7	4	8	14	S-15	8	17	28
S-8	7	18	29	S-16	9	17	26

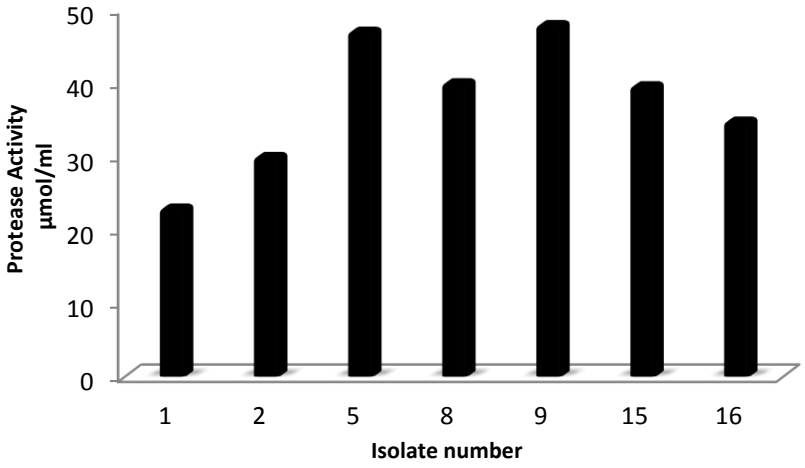

Fig. (1): Protease activity of the selected strains after 48 hrs.fermentation

Identification of thermophilicprotealotic strains:

Identification of the most active strains was performed according to the morphological and physiological characters are shown in Table (2). Based on phenotypically characteristics and on the analysis of 16S rRNA sequencing showed that S-5 gave 99% similarity to *Brevibacillus panacihumi*, while S-8 and S-9 had 99% similarity to *Bacillus aerius* (Fig.2 and Fig.3). Most of thermostable proteases were reported to be produced by *Bacillus sp.* Such as *Bacillus stearothermophilus* **(Razak *et al.*, 1993),***Bacillus subtilis* strain SH1 **(Ninghoujam and Kshetri, 2010),** *Bacillus cereus* FJ10 **(Jabeen and Qazi, 2011)** *and Bacillus licheniformis* **(Zilda*et al*., 2012)**. On the other hand, only few other types bacteria that were reported produce thermostable protease such as *Aquifexpyrophilus* **(Choi *et al.*, 1999)** and *Pseudomonas sp.* DR89 **(Asoodeh and Mussaabadi, 2012).**

Environmental conditions :

a- Effect of the pH value:

The fermentation medium was adjusted to various pH values 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 to study the effect of the pH of the medium on the production of the enzyme by the selected strains.

The obtained results are given in Fig. (4) show that the highest enzyme activity was obtained at pH 7.0 for *Bacillus stearothermophilus* ATCC7953 and *Brevibacillus panacihumi* S-5 while at pH 8.0 for both stains *Bacillus aerius* S-8 and *Bacillus aerius* S-9.Below or above this level showed decrease in the enzyme production.

These results are in-agreement with those reported by **EL- Sawah** and **Zin EL- Din, (2000)** and **Nayl (2004)**who found that maximum protease production by *Bacillus lichiniforms , Bacillus subtilis* M14 and *Bacillus coagulans* M53 was at pH 7.0. While, **Uyar *et al.* (2011)**and **Thakur** and **Tiwari (2013)** found that the optimum pH for protease production by *Bacillus cereus* CA15 and *Bacillus subtilis* Was 8.0.

On the other hand, **Vijayaraghavan *et al.* (2014)** showed that the *Bacillus cereus* strain AT was capable of producing protease in the pH range of 6-10. The production of protease was maximum at pH 9.0 and substantially decreased when it was above and below pH 9.0.

Table (2): Morphological and biochemical characteristics of the studied isolates.

Characteristic feature	Isolate S-5	Isolate S-8	Isolate S-9
Colony morphology	Irregular, smooth, flat, off white, opaque	Irregular, smooth, flat, off white to red edge, opaque	Irregular, smooth, flat, off white to red edge, opaque
Gram staining	Gram positive ,rod	Gram positive, rod	Gram positive, rod
Spore staining	Spore former	Spore former	Spore former
Hanging drop	Motile	Motile	Motile
Erythritol	-	-	-
D-Arabinose	-	-	-
L-Arabinose	-	+	+
D-Ribose	-	+	+
D-Xylose	-	+	+
L-Xylose	-	-	-
D-Aonitol	-	-	-
Methyl-β-D-Xylopyranoside	-	-	-
D-Galactose	-	-	-
D-Glucose	+	+	+
D-Fructose	-	+	-
D-Manose	-	+	-
L-Sorbose	-	-	-
L-Rhamnose	-	-	-
Dulciol	-	-	-
Inositol	-	-	-
D-Mannitol	-	+	-
D-Sorbitol	-	+	-
Methyl-α D-Mannopyranoside	-	-	-
Methyl-α Glucopyranoside	-	+	-
N-Acetyl-Glucosamine	-	-	-
Amygdalin	-	+	-
Arbutin	+	+	-
Esculin Ferric Citrate	+	+	+
Salicin	-	+	-
D-Cellobiose	-	+	+
D-Maltose	-	+	-
D-Lactose	-	+	-
D-Melibiose	-	-	-
D-Saccharose (sucrose)	-	-	-
D-Trehalose	-	-	-
Inulin	-	-	-
D-Melezitose	-	-	-
D-Raffinose	-	-	-
Amidon (Starch)	-	+	-
Glycogen	-	+	-
Xylitol	-	-	-
Gentibiose	-	-	-
D-Turanose	-	+	+
D-Lyxose	-	-	-
D-Tagtose	-	-	-
D-Fucose	-	-	-
L-Fucose	-	-	-
D-Arabitol	-	-	-
Potassium Gluconate	-	-	-

(+) Positive reaction (-) Negative reaction

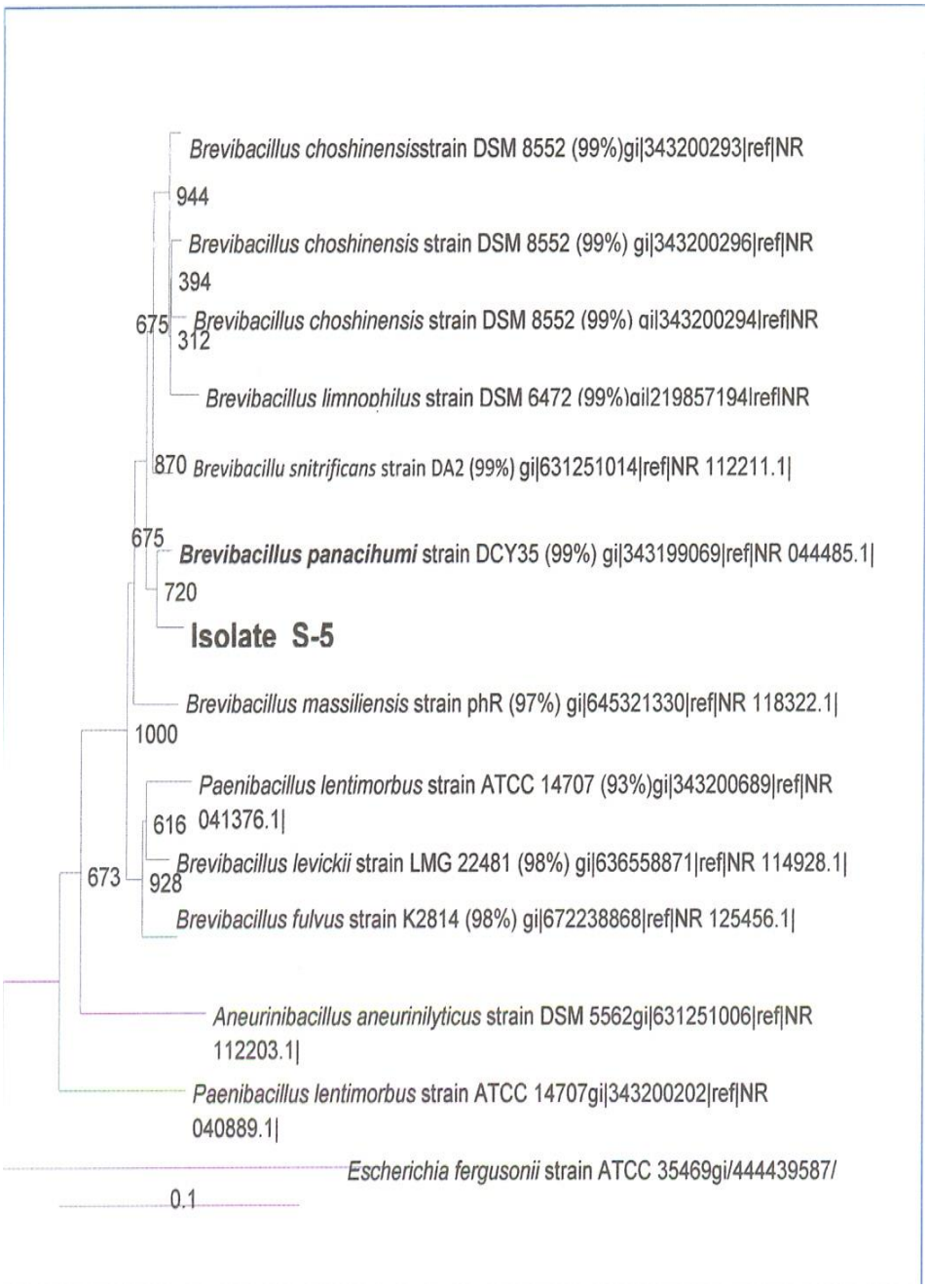

Fig.(2)Neighbor joining tree showing the estimated phylogenetic relationships of isolate S-5$^{T)}$ and the nearest members of bacteria. Accession numbers are given in parentheses and *Escherchia fergusonii* strain ATCC 35469gi/444439587/ used as out group. Bar: 0.01% sequence divergence.

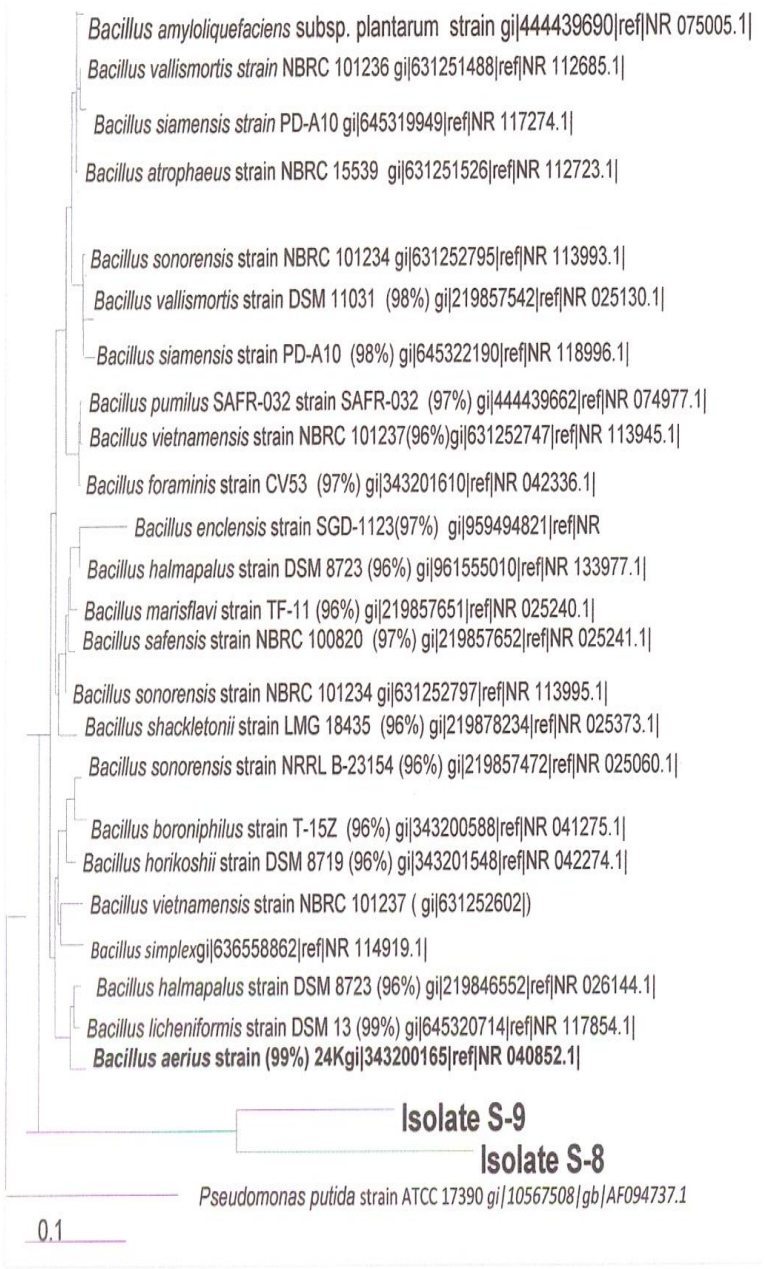

Fig. (3)Neighbor joining tree showing the estimated phylogenetic relationships of both isolate S-8 and S-9$^{(T)}$ and the nearest members of bacteria. Accession numbers are given in parentheses and *Pseudomonas putida* strain ATCC 17390 (*gi|10567508|gb|AF094737.1*) *used* as out group. Bar: 0.01% sequence divergence.

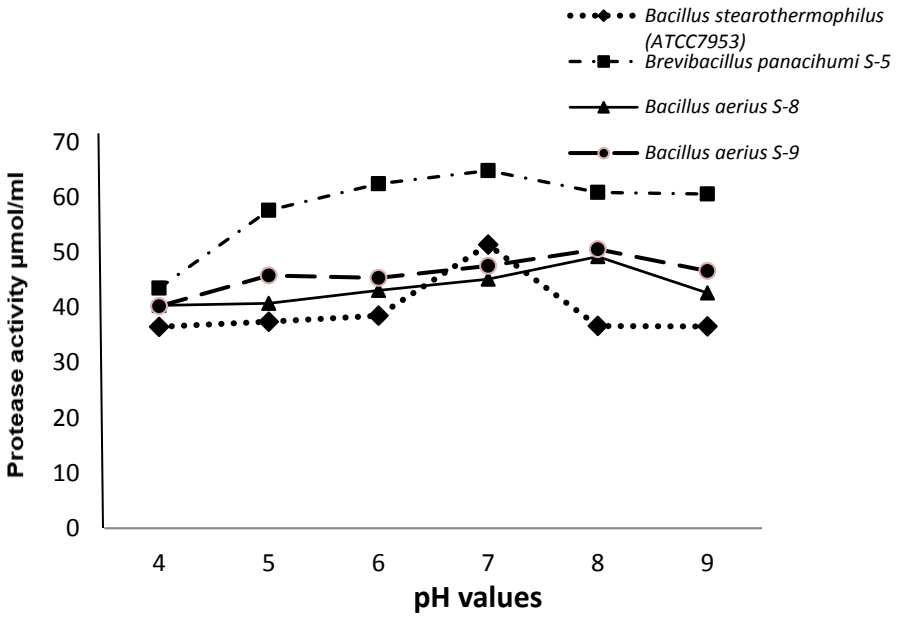

Fig. (4) Effect of pH medium on the production of protease by tested bacterial strains

b. Effect of incubation temperature:

The incubation temperature is one of the main factors affecting the secretion of protease especially in case of the thermostable protease. The fermentation experiments were achieved at incubation temperature of 40, 50, and 60 °C to detect the most favorable temperature for the maximum protease production.

Results in Table (3) indicate that 50°C was the optimum incubation temperature for the production of protease for all tested bacterial strains.

Table (3) Effect of incubation temperature on the production of Protease by tested bacterial strains.

Incubation temperature °C	*Bacillus stearothermophilus* ATCC7953		*Brevibacilluspanacihumi* S-5		*Bacillus aerius* S-8		*Bacillus aerius* S-9	
	Protease *Activity µmol/ml	*Activity %	Protease Activity µmol/ml	*Activity %	Protease Activity µmol/ml	*Activity %	Protease Activity µmol/ml	*Activity %
40	46.12	89.76	57.35	88.50	46.15	93.78	46.06	91.13
50	51.38	100.00	64.80	100.00	49.21	100.00	50.54	100.00
60	39.56	76.99	47.23	72.88	37.28	75.75	39.15	77.46

***The maximum activity was assumed as the 100%**

The data also, show that the enzyme production were decreased about 10.24, 11.50, 6.22, 8.87, 23.01, 27.12,24.25 and 22.54% for

Bacillus stearothermophilus ATCC7953; *Brevibacillus panacihumi* S-5; *Bacillus aerius*S-8and *Bacillus aerius*S-9 at 40 and 60°C respectively. This indicates that temperatures over or lower 50°C were not suitable for the production of thermo-stable protease by the four tested thermophilic *Bacillus* strains.

These results came to support the findings of **EL- Hawary** and **Ibrahim (1988),** and **Beg** and **Gupta (2003)** who found that 50°C was the optimum incubation temperature for maximum protease production from *Bacills coagulans* strain 135 and *Bacillus mohavensis*. While, **El-Sawah and Zin El-Din (2000)** found that maximum production of alkaline protease by *Bacillus lichiniformiss* was attained when incubated at 60°C**.** They also stated that beyond this temperature , enzyme formation decreased sharply.

c.Effect of fermentation period:

The fermentation period needed for the maximum production of protease is one of the limiting factors for the economic success of the commercial production. **Fogarty** and **Kelly (1980)**and **Kumar *et al.* (2012)** reported that the selected organism must produce a good yield of the enzyme in a relatively short yield ideally in submerged culture.

In our experiment, the effect of incubation period on protease production from the four tested bacterial strains namely *Bacillus stearothermophilus* ATCC7953, *Brevibacillus panacihumi* S-5, *Bacillus aerius*S-8 and *Bacillus aerius*S-9 were grown on modified Luria broth medium. The experiments were incubated for 24, 48, 72, 96 and 120 hours., and the enzyme activity was estimated at 24 hrs.intervals.

Table (4) Effect of fermentation period on the production of protease by tested bacterial strains.

Fermentation period (hrs.)	*Bacillus stearothermophilus* ATCC7953		*Brevibacillus panacihumi* S-5		*Bacillus aerius* S-8		*Bacillus aerius* S-9	
	Protease *Activity µmol/ml	*Activity %	Protease Activity µmol/ml	*Activity %	Protease Activity µmol/ml	*Activity %	Protease Activity µmol/ml	*Activity %
24	49.14	83.95	44.68	68.34	41.55	84.17	38.36	76.35
48	55.43	94.70	57.92	88.60	45.39	91.95	44.91	89.39
72	58.53	100.00	65.37	100.00	49.36	100.00	50.24	100.00
96	56.76	96.97	42.90	65.62	42.24	85.57	45.03	89.62
120	31.20	53.30	34.76	53.17	37.98	76.94	39.49	78.60

***The maximum activity was assumed as the 100 %.**

The results recorded in Table (4) show that, the maximum protease activity for the tested bacterial strains was obtained after 72 hours of incubation. Increasing the incubation period over 72 hours the enzyme

activity decreased by 3.03% , 46.70%; 34.38%, 46.83%; 44.43%, 23.06% and 10.38%, 21.40% for *Bacillus stearothermophilus* ATCC7953, *Brevibacillus panacihumi* S-5; *Bacillus aerius* S-8and *Bacillus aerius* S-9 at 96 and 120 hours respectively. The present results agreed with those found by **El-Bendary *et al*., (2002); Nayl (2004)** and **Lakshmi** and **Prasad (2013**) who stated that maximum protease production was obtained after 3days of incubation.

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عزل وتصنيف وإنتاج إنزيم البروتييز بواسطة البكتريا المحبة للحرارة

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تحتل أنزيمات البروتييز المنتجة بواسطة الميكروبات المحبة لدرجات الحرارة المرتفعة مكانة رفيعة حيث أنها تتحمل درجات الحرارة العالية وهذا لبعض الصناعات وقد دعى ذلك لعزل سلالات مختلفة من البكتريا المتجرثمة المحبة لدرجات الحرارة العالية والتى لها المقدرة على انتاج الانزيم من التربة حيث تم عزل ١٦ سلالة واختبرت القدرة الانزيمية لهذه السلالات لانتخاب أقواها من حيث انتاج الانزيم ووجد أن السلالات رقم ٥ ، رقم ٨ ، رقم ٩ هم أكفأ الميكروبات المنتجة للانزيم وذلك بطريقة المزرعة المغمورة وقد تم دراسة الصفات الموروفولوجية والمزرعية والفسيولوجية والوراثية لهذه العزلات التى أعطت أعلى نشاط للانزيم وتم تصنيفهم على أن العزلات أرقام ٩،٨،٥ هى على التوالـى:

Brevibacillus panacihumi S-5 ،*Bacillus aerius* S-8 ، *Bacillus aerius* S - 9.

ولقد تم دراسة تأثير الظروف البيئية لمعرفة أنسب الظروف الملائمة لانتاج أعلى انتاجية من الانزيم مثل تأثيردرجة pH وحرارة التحضين وكذلك مدة التحضين وقد أوضحت النتائج أن درجة pH ٧ للبيئة التخميرية أعطى أعلى انتاجية للانزيم للسلالة *Bacillus stearothermophilus* ATCC9753 و*Brevibacillus panacihumi* S-5 ،بينما كانت درجة pH ٨ لكلا من السلالتين*Bacillus aerius* S-8،*Bacillus aerius* S-9

واتضح من النتائج أيضا أن لدرجة حرارة التحضين وكذا مدة التحضين أثر واضح على معدل إنتاج الإنزيم للسلالات المختبرة حيث يعتبر الإنتاج أعلى مايمكن عند استخدام درجة حرارة ٥٠ م° لفترة تحضين تصل الى ٧٢ ساعة .