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Isolation and Biochemical Characterization of Extracellular Microbial Proteases

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Abstract: Proteases are a broad family of hydrolytic enzymes with various applications in chemical, cosmetics, and pharmaceutical industries. Owing to their physiological necessity, proteases are found in diverse sources including microorganisms. Our objective study was to search for a high quality and inexpensive source for the production of microbial proteases under different culture and growth conditions. Also, we aimed to characterize microbial proteases. Proteases-producing bacteria were isolated from soil samples collected from a poultry waste site. Soil samples were inoculated in skimmed agar media and 48 h later, colonies producing clear zones were selected as the source of microorganisms producing enzyme. The isolates were used to inoculate liquid media and the clear supernatant was taken as a crude for enzyme preparation. The enzyme was isolated and purified with ammonium sulfate at 60-80% saturation followed by dialysis. Subsequently, characterization of the enzyme fraction with the highest activity was carried out. The results indicated that the isolated enzyme with (60-80%) fractionation of ammonium sulfate exhibited the highest specific activity. In addition, the optimal temperature for enzyme activity was determined at 70ºC at pH values of 0.05M of acetate buffer 3.6 and 0.05M of glycine-NaOH buffer 10.0. Finally, the kinetic parameters (Michaelis–Menten constant, Km and maximal reaction velocity, Vmax) were calculated as 0.11 µmole/ml and $0.5x10^4$ nmole of tyrosine/ml/hour, respectively. In conclusion, our findings provide evidence that the isolated bacteria represent a rich source of thermostable proteases. Indeed, more studies are still required to obtain such proteases in a purified form suitable for studying their applications.

Keywords: *Proteases; Microorganisms; Poultry waste; Skimmed agar media.*

Introduction:

Proteases are a group of enzymes, whose catalytic function is to hydrolyze peptide bonds of proteins and break them down into polypeptides or free amino acids. They constituted 60% of the global market of industrial enzymes. They have a wide range of a commercial usage in detergent, leather, food and pharmaceutical industries **(Bhaskar *et al*., 2007; Jellouli *et al*., 2009; Deng *et al*., 2010).** Proteases are involved in controlling of a large number of key physiological processes such as homeostasis and inflammation. Their involvement in the life cycle of diseases – causing organisms has led them to become a potential target for developing therapeutic agents against fatal diseases such as cancer and AIDS **(Rao *et al*., 1998).** Sources of proteases include all forms of life, that is, plants, animals and microorganisms. Based on their acid – base behavior, proteases are classified into three groups acid, neutral and alkaline proteases **(Gupta *et al*., 2002).**

Microbial proteases have important roles in physiological processes, broad biochemical diversity, their analytical and industrial applications, and feasibility of mass production and ease of genetic manipulation to generate new enzymes with altered properties that are described for their various applications **(Kumar *et al*., 1999; Olivera *et al*., 2007).** Microbial proteases can be either extracellular

or intracellular and their production is greatly influenced by strains, nutritional and physicochemical factors, such as temperature, pH, nitrogen, carbon sources and inorganic salt **(Huston *et al*., 2000; Secades *et al*., 2001; Wang *et al*., 2008; Kuddus *et al*., 2008).**

Several recent publications have shown that bacillus produces a wide variety of extracellular enzymes, including proteases, several bacillus species involved in proteases production are *B.cereus, B.sterothermophilus, B.mojavensis, B.megaterium and B.subtilis.* They are widely distributed in soil and water and certain strains tolerate extreme environmental conditions including highly alkaline conditions **(Shumi *et al*., 2004).** Moreover, the operation of proteases in organic solvents and ionic liquids is an interesting developing area of biochemistry and biotechnology **(Ogino *et al*., 2001).**

The present study aimed to search for high quality and inexpensive source for isolation of microbial proteases from poultry soil under certain conditions and characterization of these proteases was also carried out.

Material and methods:

1. Sample collection

The soil samples were collected from a number of feather processing areas in Kotor city, Gharbia governorate, Egypt in January 2014. The collected samples were transferred, in sterile plastic bags, to the Biochemical and Microbiology Research Lab, Faculty of Science, Tanta University, Egypt for further processing.

2.Isolation of protease-producing bacteria

Bacteria were isolated using the serial dilution method described by **(Sjodahl *et al*., 2002)**. One gram of soil samples was transferred into 10 ml of sterilized distilled water and properly mixed. This initial dilution was activated by heat shock at 70ºC for 15 minutes. Subsequently, each heat treated sample was serially diluted to reduce the initial number of microorganisms (serial dilution was made up to 10^{-9}). Out of each diluted sample, an aliquot of 0.1 ml was inoculated on a 0.8% skimmed milk agar plate containing 0.1% peptone, 0.1% glucose, and 0.5% beef extract medium. The plates were rotated clockwise and anticlockwise to spread the sample uniformly and incubated at 30 ± 2°C for two days **(Uyar *et al*., 2011)**. Bacterial isolates were primarily purified on nutrient agar medium. Pure isolates were maintained in Nutrient agar medium (NAM) slants at 4ºC for further studies.

3. Screening of proteolytic bacteria

The bacterial isolates were inoculated in the basal medium enriched with skimmed milk. The pH was adjusted at 7.0. The medium was incubated in a rotary shaker at a speed of 180 rpm at 30°C for 48 h. After incubation, the cells were removed by centrifugation at 3,000 rpm for 10 min and the supernatant was collected and examined for enzyme activity.

4. Measurement of enzyme activity

Protease activity in the culture supernatant was determined according to the modified method of Lowry **(Suh *et al*., 2001)** using bovine serum albumin (BSA) as a substrate. A mixture of 100 µl of 5mg/ml (w/v) of BSA in 50 mM acetate buffer, pH 5.6 and 400 µl crude enzyme extract, and 500 µl 50 mM acetate buffer, pH 5.6 were incubated in a water bath at 37°C for 30 min. After 30 min, the enzyme reaction was terminated by the addition of 200 µl of 10% (w/v) trichloroacetic acid (TCA) and was kept at room temperature for 15 min. Then, the reaction mixture was centrifuged to separate the unreacted BSA at 3,000 rpm for 10 min. The supernatant was mixed with 2.5 ml of 500 mM alkaline copper reagent and kept at room temperature for 15 min. 0.25 ml of 3-fold diluted Follin Ciocalteu's phenol reagent was added. The resulting solution was incubated at room temperature for 30 min and the absorbance of the blue color developed was measured at 750 nm against a reagent blank using a tyrosine standard. One protease unit is defined as the amount of enzyme that releases 1 nmole of tyrosine per ml per hour under the above mentioned assay conditions. The specific activity of the enzyme is expressed as the number of units per milligram of protein.

5. Protein concentration

Protein concentration was determined according to the method of **(Lowry *et al*., 1951)**, with bovine serum albumin as a standard.

6. Partial purification

6.1 Ammonium sulfate fractionation

The crude extract was precipitated by using (0-80%), (0-60%), (60-80%) saturated ammonium sulfate. The solution was centrifuged at 3,000 rpm for 15 min at 4ºC and the supernatant was discarded. The formed precipitate was re-suspended in a small volume of 50 mM sodium acetate buffer, pH 5.6.The excess salt bound to the protein after the ammonium sulfate precipitation was removed by dialysis of the protein against buffer solution (pH 5.6).

7. Enzyme Characterization

A. Effect of temperature

To study the effect of temperature on enzyme activity, the enzyme reaction mixture was incubated at different temperatures (20–90ºC) in acetate buffer 0.05M (pH 5.6) using BSA as substrate.

B. Effect of pH

The effect of pH on protease's activity, with BSA as substrate, was performed in acetate buffer 0.05M (pH

3.6, 4.4, and 5.6) and glycine-NaOH buffer 0.05M (pH 9, 10, and 10.6).

c. Effect of enzyme concentration

Different enzyme concentrations were studied, where protein was used to investigate enzyme activity.

D. Effect of incubation time

The effect of incubation time on protease activity was determined by incubating the assay mixture under standard conditions at different time points 0, 15, 30, 60, 90,120 min. The percent of relative activity was plotted against time (min).

E. Determination of the K_m & V_{max}

The K_m and V_{max} values for the protease were determined using varying concentrations of BSA (1-5mg) by keeping the enzyme concentration constant. The reaction velocities corresponding to different substrate concentrations were plotted to get a hyperbolic curve. K_m and V_{max} were calculated from the Lineweaver-Burk plot.

Results and Discussion:

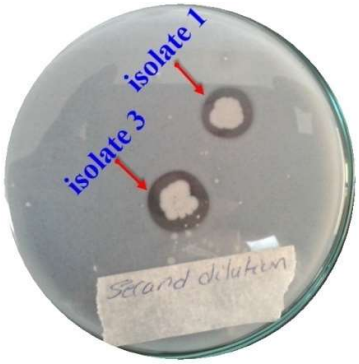
Eight bacterial isolates were isolated from different soil samples. For all the 8 isolates, both qualitative (zone of inhibition) and quantitative (proteases assay U/ml) were done.

Screening and isolation of proteolytic bacteria

Isolated bacterial strains were screened for proteases producing ability on skimmed milk agar plates. The clear zone formation around the bacterial colony indicated that the proteases produce strain due to hydrolysis of casein. The images of clear zone (zone of inhibition) forming isolates are shown below **(Fig. 1a and b).**

The objective of the present investigation was to select the bacterial strains with the highest proteases-producing ability. In order to achieve our aim, we have selected a total of 8 different bacterial isolates from the initial screening. The isolates were checked quantitatively for their ability to produce extracellular proteases in liquid medium. As shown in **Table (1)**, all of the selected bacterial isolates under investigation secreted proteases at various levels. The maximum protease activity (2303 U/ml) was obtained after 48 h by isolate (# 2). The other three isolates # 4, 5, and 8, showed a high level of extracellular protease activity of 1162.8, 1574.2, and 1860 U/ml, respectively. The isolates 1, 3, and 6 exhibited moderate activity of 591.4, 597, 857 U/ml, respectively. On the other hand, the lowest extracellular protease activity was observed by isolate (# 7) with an enzyme activity of 68.5 U/ml. Previous studies carried out on six isolates of bacillus species reported a maximum protease activity of 243 U/ml that was obtained after 72 h. Other three isolates showed high production of extracellular protease of 155, 175 and 149 U/ml, respectively. The lowest extracellular enzyme activity was observed by two isolates with enzyme activity of 55 and 75 U/ml, respectively **(Johnvesly *et al*., 2012).**

Moreover, the maximum protease production noticed among 14 isolates from each of *Endhatia Parasitica and Miehei*, was 5.1 and 369 U/ml, respectively **(Brown *et al*., 1991).** Therefore, the isolate # 2 has been selected for partial purification and characterization.



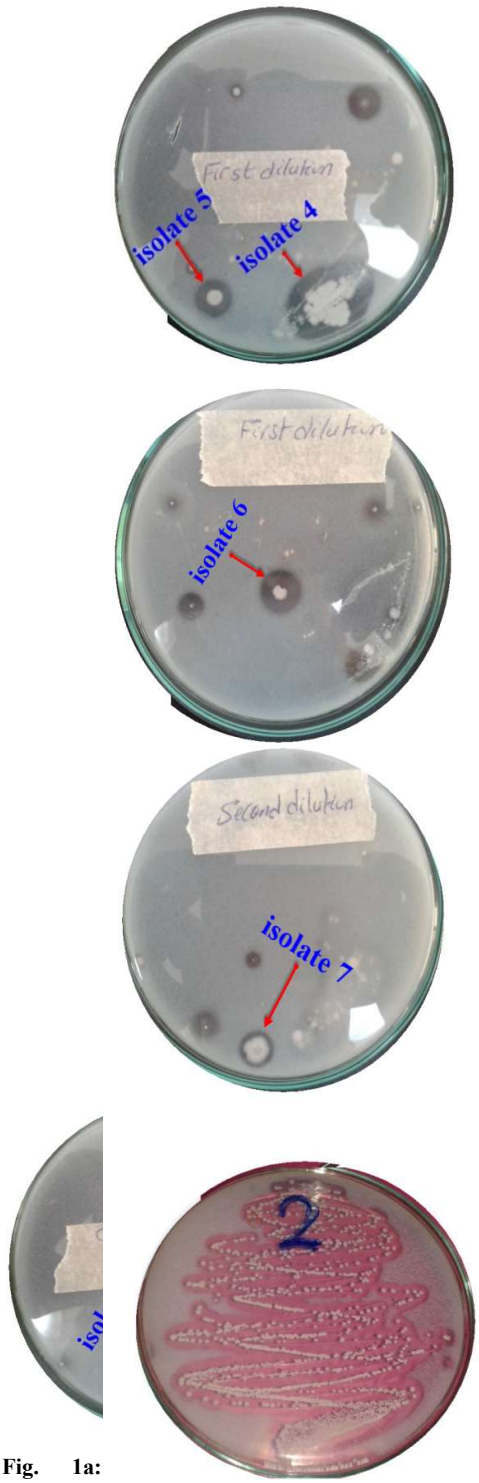


Fig. 1a: Zones of inhibition of 8 isolates of the protease- producing bacteria

Fig. 1b: The sub-culture of the excellent isolate # 2 on skimmed agar medium (SAM)

Table (1) Protease activity from different isolates

Isolate number	Enzyme activity (U/ml)
Isolate 1	591.4
Isolate 2	2303
Isolate 3	597
Isolate 4	1162.8
Isolate 5	1574.2
Isolate 6	857
Isolate 7	68.5
Isolate 8	1860

Ammonium sulphate fractionations. The concentrated cell free-supernatant was subjected to ammonium sulphate precipitation (0-60%), (60-80%), and (0-80%). The main fractions with high activity were pooled, while other precipitations with low specific activity were discarded. This method led to a high fold of purification as shown in Table 2. The present bacterial protease yield is 60%. **(Sanatan *et al.*, 2013)** reported that the yield of the partially purified protease by ammonium sulphate from *Streptomyces* sp. M30 was 15.5% and from *Periplaneta americana* was 4.2%. In the present case, the specific activity of the extracellular protease is 7800 U/mg , while other authors have demonstrated that the specific activity of the protease isolated from *Streptomyces* megaspores strain SDP4 was (95.4 U/mg) and from *Streptomyces* sp. MAB18 was 2,398.36 U/mg **(Moreira *et al.*, 2003;Manivasagan *et al.*, 2013)**.

Characterization of the partially purified protease

The characterization of the protease produced by thermophilic bacteria is relevant not only for their participation in nature cycles in the environment but

also for their possible applications in industry and clinical. .

Effect of enzyme concentration

As shown in **(Fig. 2)**, the relative activity of the partially purified enzyme increases as the concentration of enzyme increases. .

Effect of incubation time

The results represented in **(Fig. 3)** shows that protease activity increases by increasing the incubation time till a maximum is reached at 90 minutes. However, by increasing the incubation time beyond 90 min., a drop in the enzyme activity was observed due to the feedback inhibition exerted by the accumulation of product.

Effect of temperature

At pH 5.6, the activity of the partially purified protease was observed over a range of temperature from 20 to 90ºC, with a maximum activity (2724 U/ml) reported at 70ºC. As shown in **(Fig. 4)**, the enzyme activity increased rapidly above 30ºC followed by thermal inactivation above 70ºC due to enzyme denaturation. The protease showed 53.2 and 35.9 % reduction in relative activity at 80ºC and 90ºC, respectively. Other studies of **(James *et al*., 1991)** reported that the actinomyces proteases including *S. megaspores* (65ºC). Furthermore, the current study shows that the partially purified protease produced by the bacterial strain in isolate # 2 is thermostable compared to other proteases produced by other microorganisms such as the extracellular aspartyl protease *Eap1* from the phytopathogen fungus *Sporisorium reilianum* (45ºC) **(Mandujano *et al*., 2013)** and an extracellular keratinolytic protease from *Asperigullus parasiticus* (50ºC) **(Anitha *et al*., 2013).** .

Effect of pH

It is well known that the pH of culture medium affects the availability of certain metabolic ions and the permeability of bacterial cell membranes, which in turns supports cell growth and enzyme production. The partially purified protease was active in a broad range of pH (3.6 - 10.6) with an optimum pH at (3.6) using 50mM acetate buffer and (10.0) in case of 50mM glycine –NaOH buffer. As shown in **(Fig. 5)**, the results indicate that this bacterial protease has both alkaline and acidic properties. The optimum pH at 10.0 was similar to that of proteases obtained from *bacillus* sp. **(Subba *et al*., 2009; Benkiar *et al*., 2013; Anbu 2013; Annamalai *et al*., 2013; Joshi *et al*., 2013).** .

Determination of K_m and V_{max}

The partially purified bacterial protease was characterized for its kinetic parameters using BSA (bovine serum albumin) as a substrate. The affinity with which the protease binds BSA and on which the rate of reaction depends was evaluated. As shown in **(Fig. 6)**, the K_m and V_{max} of the protease are 0.11 µmole/ml and $0.5x10^4$ nmole of tyrosine/h, respectively. Several earlier reports indicated that the protease from *B. circulans* MTCC 7942, characterized for its kinetic parameters using casein as a substrate, had a K_m and V_{max} values of 3.1 mg/ml and 1.8 µmol/min, respectively **(Bordusa 2002).** Other protease produced by *Bacillus circulans* had a K_m of 0.597 mg/ml and V_{max} of 13825 µmol/min using casein as substrate **(Subba *et al*., 2009).** On the other hand, the protease produced by *Bacillus pseudofirmus* showed higher activity with casein with a V_{max} and K_m values of 6.346 µmole/min and 0.08 mg/ml, respectively **(Raval *et al*., 2014).** Moreover, the *Bacillus clausii* GMBAE42 protease has revealed a K_m of 1.8 mg/ml and a V_{max} of 11.5 µmole/min **(Kazan *et al*., 2005).**

Table (2) Partial purification of protease from isolate # 2 by different ammonium sulphate precipitations

Purification step	Total volume (ml)	Total activity (nmole/ml/h)	Total protein (mg/ml)	Specific activity (nmole/h/mg)	Yield (%)	fold
Crude (extracellular proteases)	10	23140	62	373	100	1
Amm. sulphate 0-60%	1	2082.6	1.0	2082	9	5
Amm. sulphate 60-80%	1	13884	1.78	7800	60	20.9
Amm. sulphate 0-80%	1	22214	10.29	2158	96	5.78

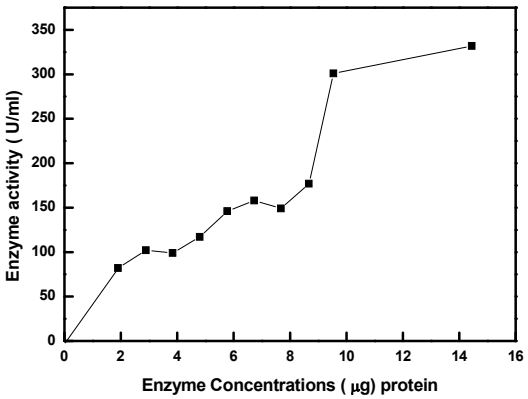


Fig. 2: Effect of different enzyme concentrations on the partially purified protease

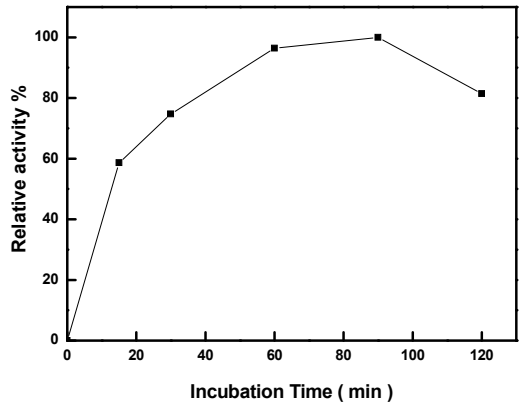


Fig. 3: Effect of incubation time on the partially purified protease

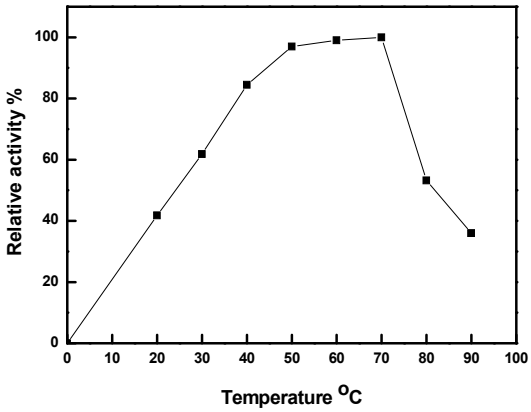


Fig. 4: Effect of temperature on the partially purified protease

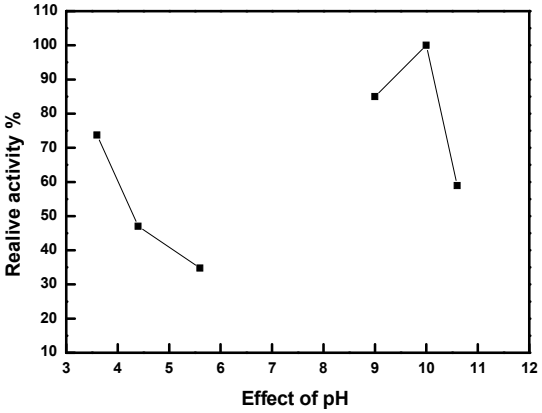


Fig. 5: Effect of pH on partially purified protease

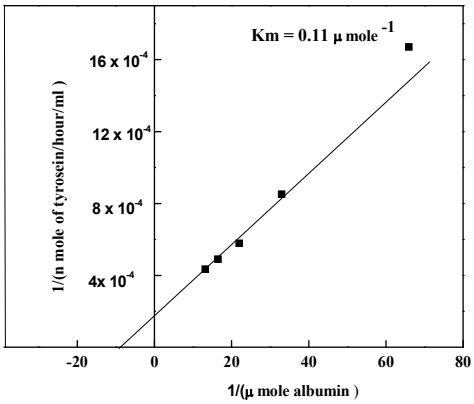


Fig. 6: The Lineweaver- Burk plot of the partially purified protease

Conclusion:

Our findings provide evidence that the bacteria isolated from poultry soil represent a rich source of thermostable protease. Indeed, more studies are still required to obtain such protease in a complete purified form suitable for studying its applications.

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العزل والتوصيف البيوكيميائي لانزيمات البروتيز الميكروبى من خارج الخلية

مها محمد سالم

تهدف دراستنا الي البحث عن انزيم البروتيز بجودة عالية و تكلفة اقل تحت ظروف مختلفة من الثقافة والنمو. تم عزل انزيم البروتيز من خلايا بكتيرية تم الحصول عليها من تربة مخلفات الدواجن حيث تم تلقيح العينات علي وسط يحتوي علي لبن خالي الدسم لمدة48 ساعة. تم اختيار المستعمرات التي تعطي اعلي مناطق واضحة كمصدر للبكتريا التي تنتج انزيم البروتيز. استخدمت هذة العزلات للحصول علي الانزيم في صورتة الخام بواسطة تطعيم وسط من اللبن الخالي الدسم السائل بهذة البكتريا التي تنتج انزيم البروتيز. بعد ذلك تم تنقية هذا الانزيم لتحويلة من الصورة الخام للصورة النقية باستخدام كبريتات الامونيوم. تم اجراء العديد من التجارب علي هذا البروتيز النقي لمعرفة بعض خواصة و لمعرفة الظروف المناسبة لانتاج اعلي نشاط . دلت النتائج علي ان الانزيم الذي تم عزلة و تنقيتة بواسطة كبريتات الامونيوم بترسيب(60%-80) اعطي اعلي نشاط للانزيم. و قد وجد ان الانزيم نشط و مستقر علي نطاق واسع من درجات الحرارة المختلفة مع اقصي قدر من النشاط عند درجة 70°C و في قيم الرقم الهيدروجيني 3.6 و 10.0 باستخدام BSA . تم دراسة الخواص الحركية للانزيم و تم حساب ثابت مايكل (K_m) و (V_{max}) وجدوا ان قيمتهم 0.11 µmole/ml , $5x10^4$nmole/ml/h علي التوالي . توفر هذة النتائج دليلا علي ان البكتريا التي تم عزلها تمثل مصدرا غنيا لانزيم البروتيز الحراري.