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PRODUCTION AND CHARACTERIZATION OF AN EXTRACELLULAR HALO-ALKALINE PROTEASE PRODUCED BY *Bacillus* ISOLATES FROM FESEEKH (*Mujil auratus*) PRODUCTS

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Samah H. Abuhussien[1]; Fatma R. Nassar[1]; Sohair A. Nasr[1] and A.A.M. Abdelhafez[1]

1- Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams Univ., Shobra El-Kheima Cairo, Egypt

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ABSTRACT

Ninety five isolates were collected from commercially and laboratory manufactured feseekh products and tested for protease production on standard skim milk agar and gelatin media supplemented with 15% NaCl salt. They were identified to be *Bacillus* isolates. Out of the 95 isolates, three *Bacillus* isolates (11pb, 14pb, 13pb) were selected based on their highest protease production. Five media were tested for select the most suitable medium for production by the three above isolates. Fish by-product medium gave the highest protease production by the three isolates, reaching 126.5, 136.5, 147.5 u/ml by 11pb, 14pb, 13pb, respectively. These levels were obtained between late logarithmic growth phase and beginning of stationary phase for the isolates. The highest Protease production was obtained at pH 8, temperature 30°C and NaCl concentration 5%, the enzyme was stable at temperature ranged between 60 and 65°C during the period tested (1h) and the protease was optimally active at pH 10 and 60°C. The enzyme was stable for 10 days at 30 °C during the period tested (1 month) where it lost 10 % of its activity after 20 days while lost 85% of its activity after 30 days of storage at 30°C. These properties make the enzyme suitable for detergent industry.

1. INTRODUCTION

Fish and fish products play an important role in the diets of West African countries. In Egypt, "feseekh" is the Arabic name for salted fermented Bouri fish, where whole non-eviscerated fish are washed by tap water, and left to decompose for one day to three days, depending on the weather, before salting. The salting process involves stuffing of the gills and covering the entire fish with approximately 15-25 % salt by weight, followed by aging at room temperature for suitable period of time, depending on salt concentration, and is characterized by its specific flavor and its sharp penetrating odor. This odor permeates the air in and around selling shops and gives the area a characteristic smell (**FAO, 1992**).

Enzymes especially bacterial proteases and lipases, are expected to be greatly responsible for the feseekh ripening process and flavor development. Amino acids and volatile compounds producing during maturation as example are mostly responsible for producing flavor. Therefore, the quantitative determinations of such chemical compounds during ripening may contribute to flavor quality and grade (**Essuman, 1992**). Moreover, enzymes have been used for centuries as processing aids in the manufacture of food products to improve their qualities, solubility, and stability (**Joo *et al* 2003**).

About 50 % of the enzymes used as industrial processing aids are proteases, which have been used in a number of industrial applications such as laundry detergents, feed and leather treatment (**Ming, *et al* 1992**), as well as silk de-gumming, cheese making, chill proofing, meat tenderizing, fermented sauces and the production of pharmaceuticals (**Rao, *et al* 1998**). These enzymes have also been used for waste management and silver recovery (**Gupta, *et al* 2002**). Therefore many types of proteases (acid, alkaline and neutral) have been studied. Moreover, the production of halophilic enzymes using halophilic bacteria may be

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applied in purification of polymer containing wastes using these halophilic microorganisms. Also, these proteases can improve fish sauce production **(Kim and Kim, 2005)**.

Highly active proteases have been purified from soy sauce halophiles, such as *Bacillus subtilis* CCKS-118 **(Choi *et al* 1996)**, *B. subtilis* CCKS-111 **(Choi and Kim, 1997)**, *Halobacterium* sp. **(An *et al* 1990)** and *Aspergillus sp.* FC-10 **(Su and Lee, 2001)**, from fish sauce *B. subtilis* FS-2 **(Nagao and To, 2000)**, soil *B.subtilis* Y-108 **(Yang *et al* 2000)** and CF80 **(Watanabe, 2003)** and poultry waste *B. subtilis* KS-1 **(Su and Lee, 2001)**.

The objective of this study was to isolate and characterize salt tolerant protease from fermented feseekh as a starter for fish sauce with high salt concentration. Hence, strong proteolytic bacteria were isolated from feseekh and sardine samples with 15% NaCl salt during maturation and spoilage stages, and its proteases were studied.

2. MATERIALS AND METHODS

Materials

Sixety feseekh samples were collected from local markets of five regions of cairo, and transported in sanitized plastic containers filled with product brine solution and transferred to lab in the same day. For laboratory manufactured feseekh, samples were manufactured due to **(Rashad, 1986)**.

Methods

2.1. Production of protease by fish isolated bacteria

2.1.1. Screening, Isolation and purification of proteolytic Bacillus Isolates

For isolation of proteolytic isolates, the ninety five isolates were collected from feseekh products using laboratory and commercially manufactured samples, skim milk agar with 10 % NaCl was used **(Shumi, *et al* 2004)**. The isolates were purified by sub-culturing on skim milk agar medium. The purified bacterial isolates were transferred onto trypton glucose yeast extract agar (TGYA) slants and then preserved as stock cultures. The primary screening was done by liquefying of gelatin then hydrolysis of skimmed milk casein with 0 %, 5% and 10% NaCl. Plates were incubated at 37°C for 24 hrs. Clear zones of skim milk hydrolysis were used as an indication of protease production **(Shumi, *et al* 2004)**. Depending on the zone clearness and NaCl concentrations, ninety five isolates were selected for further experimental studies.

2.1.2. Determination of the specific growth rates

Inoculum was prepared by transferring a loop-full from each isolate from 24 old cultures on nutrient agar slants, into 25 ml of TGY broth medium and incubated on a rotary shaker (100 rpm) at 37 °C for 24h. During this period, samples were taken for the determination of total count, in order to estimate the specific growth rates. The inoculum concentration for the actual fermentation of each isolate was based on the optical density at the end of the exponential phase. The aim was to standardize the inoculum amount for each experiment based on the growth rate of the first sub-culture.

2.1.3. Media used for protease production

Five broth media were used to select the most suitable media for protease production with the three isolates. Media used were (1): Casein broth, **(Shumi, *et al* 2004)** (casein, 3%; $NaNO_3$, 0.5%; K_2HPO_4, 0.55; $MgSO_4.7H_2O$, 0.02%; Na_2CO_3,1%), (2): Gelatine broth, **(Shumi, *et al* 2004)** (gelatine,3%; K_2HPO_4,0.2%; glucose 0.1%;peptone 0.5%), (3): Tryptone Glucose Yeast extract broth (TGY), **(Shumi, *et al* 2004)**, medium (4): 1% peptone medium, **(Shumi, *et al* 2004)** containing 1% peptone, 2.9mM K_2HPO_4 and 5mM $MgCl_2$ and medium (5): fish by-product medium was composed only from fish by-products powder (10 g/l), **(Ellouz, *et al* 2001)**. The selected isolates were grown in 250 mL Erlenmeyer flasks containing 50 ml of each of the above production media at pH 7.0. Media were inoculated with 1 ml inoculum (1.8 x 10^8) and aliquot samples were taken at 2h intervals and centrifuged at 15000 rpm for 15 min at 4°C and the supernatants were examined for enzyme activity **(Amozeegar, *et al* 2007)**.

2.2. Protease assay

2.2.1. Protease assay for halotolerant isolates

The protease activity was determined by casienolytic modified method of **Kembhavi, *et al* 1993**. using casein as a substrate, one ml aliquot of the culture supernatant was mixed with 3 ml of 1%

casein (pH 7 adjusted with Tris-HCl buffer) and incubated for 15 min at 60°C. The reaction was stopped by adding 3 ml of Trichloroacetic acid (7.5 %). The mixture was allowed to stand at room temperature for 15 min, filtrated using filter paper Whatman no. 1 and the absorption was measured at 280 nm. Blank was prepared in which 3 ml TCA was added before incubation **(Alsheheri *and* Mostafa, 2004)**. All samples were measured in triplicate. A standard curve was generated using solutions of 0- 50 mg/l tyrosine. One unit of protease activity was defined as the amount of enzyme required to liberate one microgram tyrosine per milliliter in 1 min under the experimental conditions used **(Shumi, *et al* 2004).**

2.2.2. Protease assay for halophilic isolates

The proteolytic activity was assayed using casein as substrate. Casein solution was prepared by dissolving 480 µl of 1% (w/v) casein in 20mM tris HCl buffer (pH 8.5), 50 mM NaCl, and 0.5 mM $CaCl_2$. Reaction was carried out by mixing 3ml of casein solution with 1ml of crude enzyme and incubated at 55°C for 5 min. The reaction was stopped by adding 3ml of 7.5% TCA, kept at room temperature for 15 min. and then filtrated using filter paper whatman no. 1, the absorbance was measured against a blank at 280 nm. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µmol of tyrosine in 1 min at 60°C **(Shumi, *et al* 2004).**

2.3. Effect of growth conditions on protease production

For maximizing protease production, 3 isolates were selected due to their enzymatic activity. TGY medium was selected as the best medium for protease production.

2.3.1. Effect of pH on microbial and protease production

In order to investigate the influence of pH on the growth and protease production, 50 ml of TGY medium in 250 ml Erlenmeyer flasks were prepared at different pH values ranged from 7 to 11, and inoculated with 1 ml (1.5×10^8) culture. Flasks were incubated at 30°C for 48 hrs with shaking at 100 rpm. Ten milliliter samples were taken at 2h intervals and centrifuged at 15000 rpm for 15 min at 4°C and supernatants were examined for enzyme activity as previously described.

2.3.2. Effect of temperature on microbial growth and protease production

To test the effect of temperature on the bacterial growth and alkaline protease production, 50 ml of TGY medium in 250 ml Erlenmeyer flasks were prepared. After inoculation with 1 ml (1.5×10^8) culture, the incubation was carried out at 25, 28, 30, 32, 34, 36 and 40°C, using rotary shaker (100 rpm) for 24 h. Ten milliliter samples were taken at 2h intervals and centrifuged at 15000 rpm for 15 min at 4°C and supernatants were examined for enzyme activity as previously described.

2.3.3. Effect of NaCl concentrations on bacterial growth and protease production

The effect of salt concentrations on growth and protease production was studied by using TGY medium containing 0 – 20% NaCl concentration at pH 8. The growth and enzyme activity were quantified during incubation on rotary shaker at 100 rpm at 37°C for 72 h.

2.4. Kinetics of the protease enzyme activity

2.4.1. Effect of pH on protease activity

Effect of pH on protease activity was studied using pH levels ranged from 3 – 12 in glycine- HCl buffer (pH 3), sodium acetate buffer (pH 4 and 5), Tris- HCL buffer (from 6 to 9) glycine-NaOH buffer (11 and 12). Crude enzyme extract and substrate at a ratio of 1: 3 were mixed in each pH buffer, and then incubated at 60 °C for 15 min. then protease activity was measured.

2.4.2. Effect of Incubation temperature on protease activity

Crude enzyme extract and substrate with pH 10 were incubated at different temperatures ranged from 25 to 75°C for 15 min, then protease activity was measured at 280 nm (Shimadzu model UV 160 A).

2.4.3. Effect of Incubation period on protease activity

pH 10 was the best pH for protease activity so, crude enzyme extract and casein substrate with pH 10 were incubated at 60 °C for different incubation periods ranged from 5 to 95 min, then protease activity was measured at 280 nm as previously described.

2.4.4. Effect of shelf time on protease production

The crude protease was incubated at room temperature for 30 days. The percentage of the remaining activity was measured every 10 days. The reaction was stopped in ice-cold water and the remaining activity was measured at 55°C in the following buffer systems 0.1 M glycine- HCl buffer (pH 3), 0.1 M sodium acetate buffer (pH 4 and 5), 0.1 M Tris HCL buffer (6-9), 0.1 M glycine - NaOH buffer (11 and 12), respectively, then protease activity was measured at 280 nm. as previously described.

3. RESULTS AND DISCUSSION

Ninety five isolates were collected from commercial and laboratory manufactured feseekh products during processing and storage till spoilage occurred and then purified, preserved and tested for their proteolytic ability. The isolates, which showed proteolytic ability in liquid culture media, were finally selected for further studies. Three *Bacillus* isolates, 11pb, 14pb and 13pb showed the highest proteolytic activity and were selected for detail studies.

3.1. Culture conditions affecting protease production

3.1.1. Effect of different media on protease production

The results in **Fig. (1)** showed that the enzyme production determined in TGY increased up to 2 fold compared to gelatin medium, casein medium, and 1% peptone medium. This may be due to the fact that TGY medium contained less easily metabolized able carbohydrates, as reported by **Schaffer, 1969, Moon and Paruleker, (1991), Beg *et al* (2002)**, also mentioned that the depression of protease synthesis by excess of glucose led to 52 % decline in protease production.

3.1.2. Effect of using fish by-products on protease production

Data showed that combined heads and viscera preparations were the best substrate for protease synthesis where the three *Bacillus* isolates reached the maximum production being 147, 136.5 and 126.3 u/ml by 11pb, 14pb and 13 pb, respectively **Fig. (2)** compared with artificial media which reached the highest activity using TGY medium to produce 108, 79.8, 70.6 u/ml by 11pb, 14 pb and 13 pb, after 22, 24, 36 h, respectively. Optimum pH, NaCl, and temperature of protease production were 8, 5% and 30°C, respectively. The highest level of protease production was obtained in the presence of head and viscera instead of peptone, **(Genckel and Tari, 2006)**.

Ellouz *et al* 2001 reported that protease production was significantly low (108 u/ml), in the presence of meat flour, although this substrate contains more protein than other preparations as shown in **Fig. (2)**, protease synthesis was strongly enhanced when cells were grown on combined heads and viscera powders and there was 100 % increase in the production of protease compared to the original medium. Combined heads and viscera flour contains more lipids and less protein than meat sardinelle, and due to higher bone content, there are minerals. Two hypotheses may be considered to explain the enhancement of protease synthesis when cells were cultivated in the presence of a combined heads and viscera flour. Hypotheses (1), the enzyme might be induced by an excess of salts. This hypotheses seems improbable since the *Bacillus subtilis* strain produced the same level of protease whether ash, obtained after heating combined heads and viscera at 600 °C for 24h, was added to meat fish medium or not. Hypotheses (2), there existed in lipid or protein fractions bioactive molecules that stimulated protease synthesis. In order to study such hypotheses, defatted combined heads and viscera flour was prepared and then tested for protease production. Results presented in **Fig. (2)**, shows that protease activity decreased when lipid content was considerably reduced. A second experiment was realized in order to confirm that combined head and viscera flour contains inducers. Since, lower activity was obtained with meat substrate, culture were conducted in fish media containing both meat fish and combined heads and viscera preparations.

3.1.3. Effect of temperature, pH and NaCl on protease production

3.1.3.1. Effect of temperature on protease production

Temperature is one of the most important factors affecting enzyme production .The results illustrated in **Fig. (3)** referred that highest enzyme activity was obtained when *Bacillus* isolates incubated at 30°C for 15 min. to reach 108, 108 and 89.28 u/ml by 11 pb, 14 pb and 13 pb, respectively. These findings agree with **Alsheheri and Mostafa, 2004** where their *Bacillus* isolate was able to produce maximum production at 30°C.

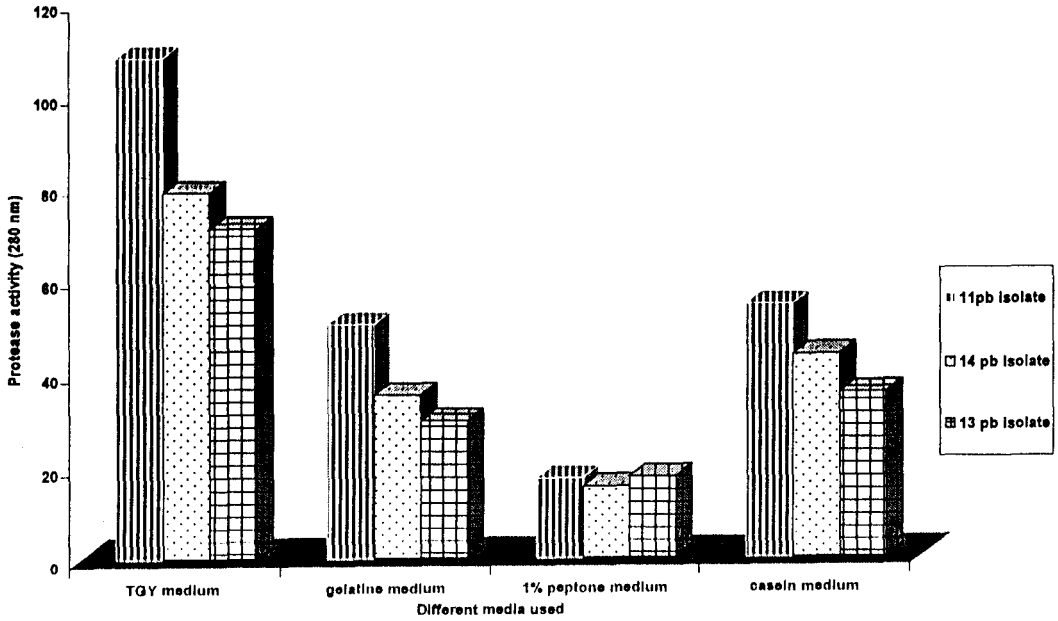

Fig. 1. Effect of different media used for protease production by selected *Bacillus* spp. Isolates

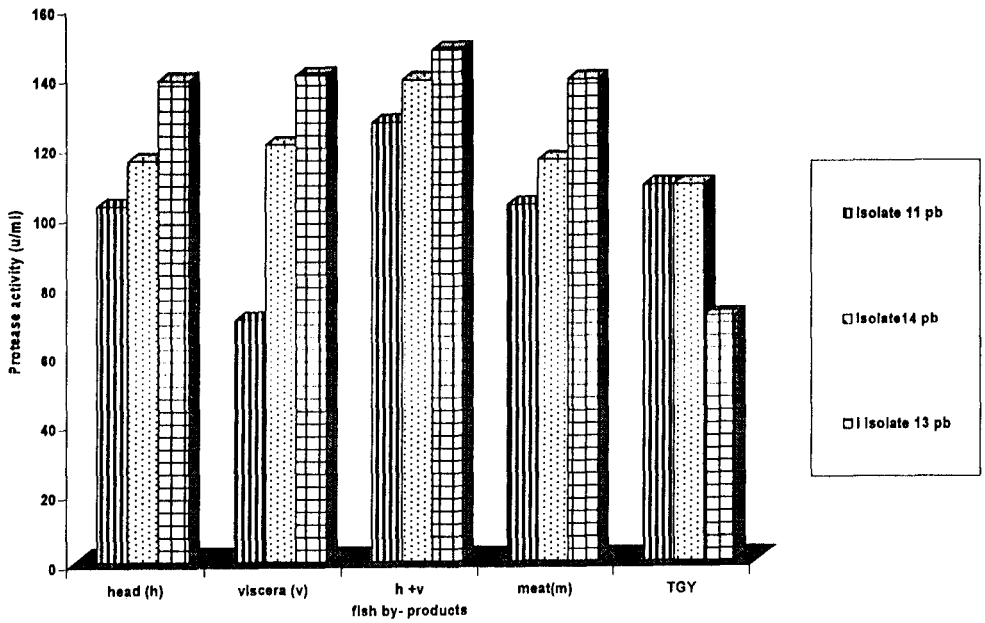

Fig. 2. Effect of fish by-products on protease production by selected *Bacillus* spp. isolates

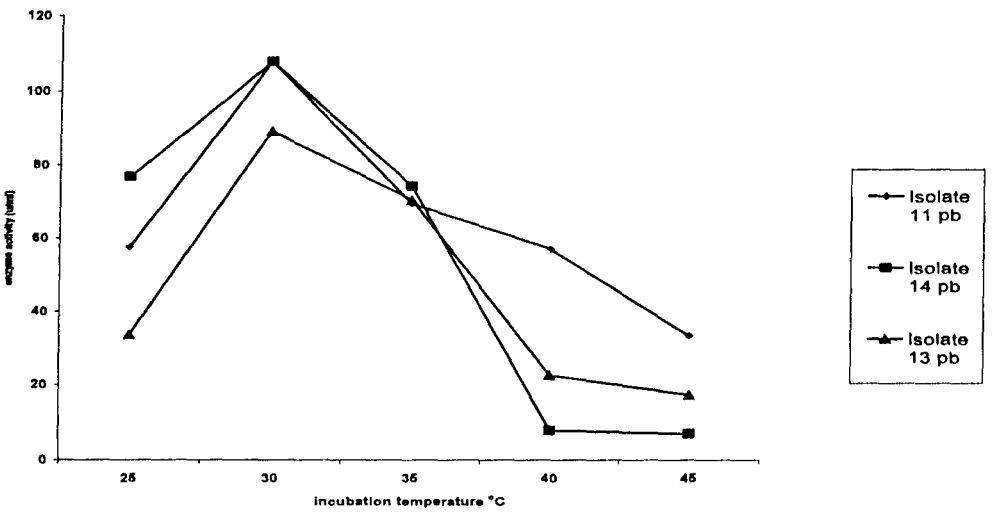

Fig. 3. Effect of temperature on protease production by the selected *Bacillus* spp. Isolates

3.1.3.2. Effect of initial pH on protease production

Results given in **Fig. (4)** revealed that the protease production was sensitive to acidic pH. Maximum protease production was obtained at pH 8 and temperature of 30 °C of reaching 125, 112, 90 u/ml by 11 pb, 14 pb, 13 pb, respectively. The enzyme production was gradually decreased by 37.96, 44.44, and 49.14 % for 11 pb, 14 pb, 13 pb at pH 11, respectively. These observations were in agreement with the results of **Alsheheri, and Mostafa, 2004, & Joo *et al* 2002**. Their *Bacillus* isolate was able to produce protease in a broad pH range from 5.0 to 11.0 and maximum production was observed at pH 7.5 – 9.0 and temperature of 30°C.

3.1.4. Effect of NaCl on protease production

The isolate 13 pb grew in the range of 0 – 20 % (w/v) NaCl, with optimum concentration of 10 % (w/v). At 0 and 20 % (w/v) NaCl, reduction in protease production was observed. The results clearly indicated the halophilic nature of the protease as in **Fig. (5)**. These results are in agreement with **Patel, *et al* (2006), & Zhang, *et al* (2007)**, who indicated that the best concentration of NaCl for protease production was 5 % (w/v). The growth of the *Bacillus* isolate 13 pb was reduced extensively in the absence of salt with no protease production. Similar results have also been reflected by the halophilic archeon, *Natrococcus occultus* in which protease secretion was optimum at 1-2 M NaCl (%) **(Studdert, *et al* 1997)**.

3.1.5. Time course of protease production using five different media

Little synthesis of protease was detected during the earlier part of logarithmic growth phase. Protease activity being 88.36 u/ml at 12 h. A rapid increase in protease production was recorded in the last stage of the logarithmic growth phase. It increased gradually to maximum value of 126.5, 136.5, 147.5 u/ml by 11pb, 14 pb and 13 pb *Bacillus* sp. isolates, respectively. Thereafter, protease production decreased gradually with cell autolysis. From the profile of protease production and cell growth using TGY medium **(Fig. 6)**. It can be observed that cell growth increases parallel to enzyme production. The present results were supporting the findings of **Amozeegar, *et al* (2007)**, where they reported that the maximum protease production usually occurs at the late of logarithmic to the beginning of the stationary phase of growth and continued in the stationary phase of the growth. Also, **Patel *et al* 2006** reported that the production thereafter remained nearly static at optimal level along with the stationary phase up to 36h. Maximum protease production in the basal medium with 10 % (w/v) NaCl was determined in stationary phase (30h), **(Karbalaei-Heidari, *et al* 2007)**.

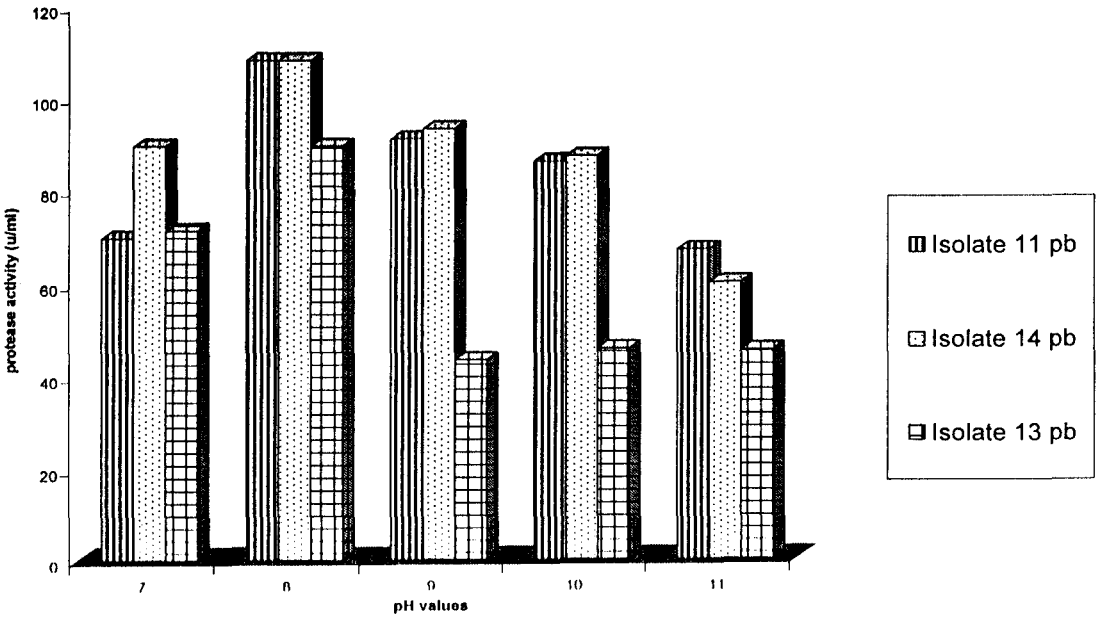

Fig. 4. Effect of pH values on protease production by the selected *Bacillus* spp. isolates

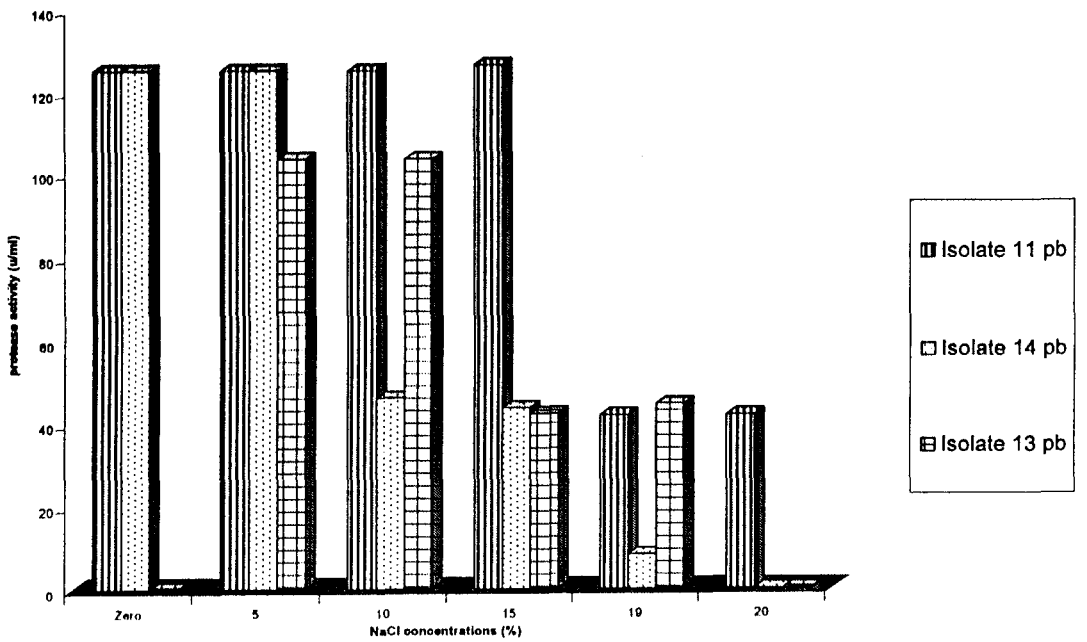

Fig. 5. Effect of NaCl concentration on protease production by selected *Bacillus* spp. isolates

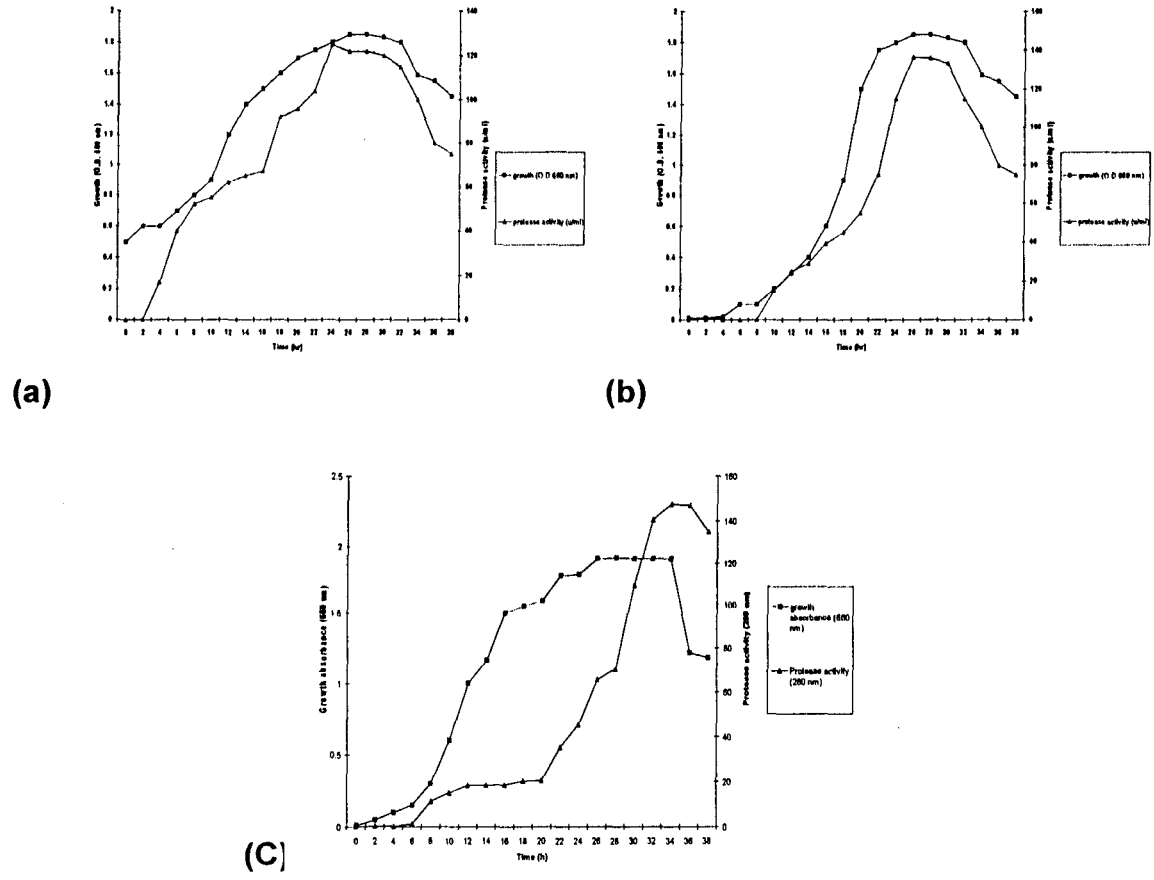

Fig. 6. Kinetics of protease production corresponding to (a) 11pb, (b) 14 pb and (c) 13 pb *Bacillus* isolates growth rate

3.4. Effect of temperature, pH, incubation time and shelf life on protease activity and stability

3.4.1. Effect of temperature on protease activity

The effect of temperature on the protease activity using casein as substrate was examined at various temperatures for 15 min at pH 10. As shown in **Fig. (7)**, the maximum enzyme activity was obtained at 65°C. Then, protease activity decreased. The relative activity at 75 was 46%. The optimum temperature of the crude preparation in the presence of 2 mM of $CaCl_2$ was increased to 70°C and the enzyme activity was 10 % obtained without $CaCl_2$ (65-70C). The thermo activity was increased only at temperature values above 65 °C. A similar result was obtained by **(Hadj-Ali, *et al* 2007), and (Ghorbel, *et al* 2003)**.

4.2. Effect of incubation periods on protease activity

The effects of incubation periods on the protease activity using casein as substrate were examined at different incubation time in min. at pH 10 and 60°C. Protease activity was the highest when casein was incubated with the crude enzyme for 15 min by 11pb and 13 pb *Bacillus* isolates while it was required to be incubated for 30 min to obtain the highest activity by 14pb *Bacillus* isolate as illustrated in **Fig. (8)**.

4.3. Effect of pH on protease activity

The pH profile of the three *Bacillus* isolates alkaline protease was determined at 60°C. The enzyme was active in the range of pH 8.5 - 12 with an optimum between pH 10 and 11. There was a sharp decrease in the enzyme activity at pH lower

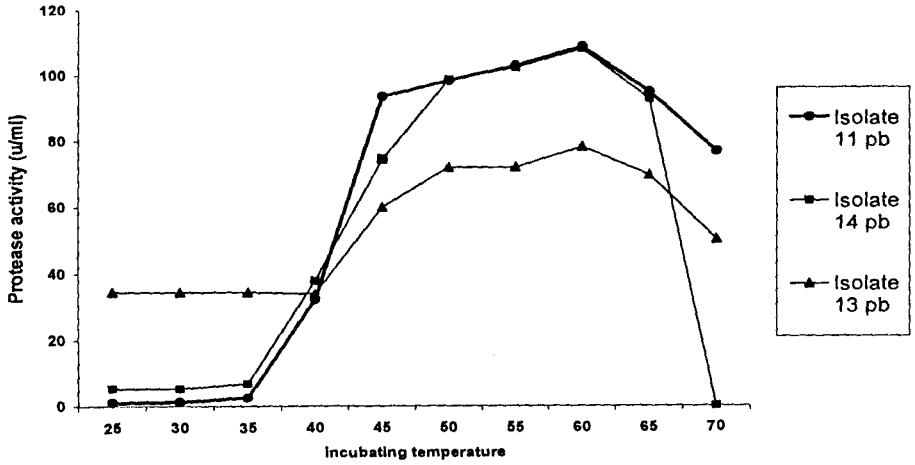

Fig. 7. Effect of incubation temperature on protease activity by selected *Bacillus* sp. Isolates

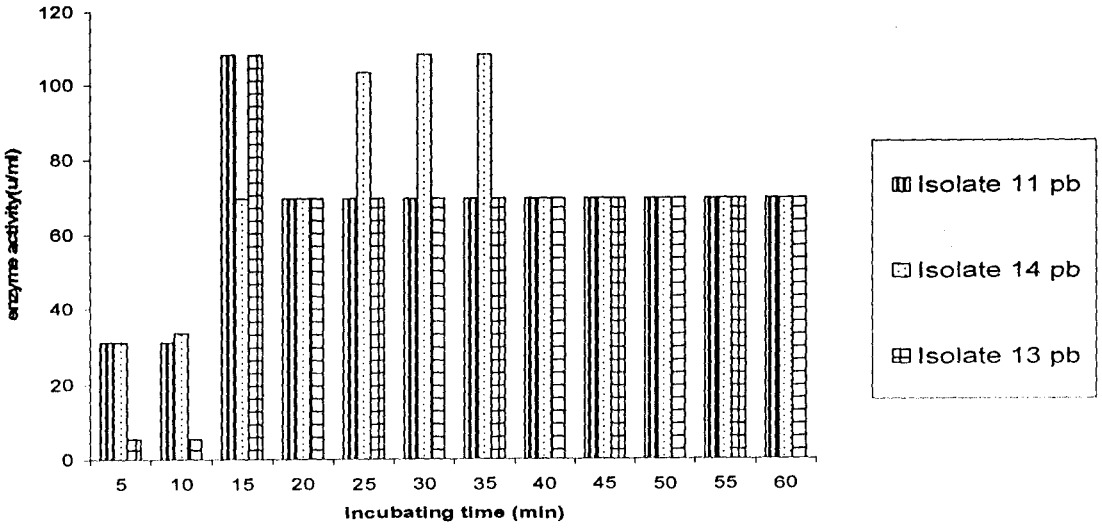

Fig. 8. Effect of incubating period on protease activity and by selected *Bacillus* sp. isolates

than 9, suggesting the alkaline nature of the enzyme **Fig. (9)**. This range of optimum catalysis is relatively narrow proteases where the optimum catalysis was in the range of pH 9-12. The protease was optimally active at pH 10 and 60 °C. The enzyme was most active at temperature 60°C during the period tested (1h). These properties make the enzyme suitable for detergent industry. The effect of pH on enzyme stability was followed by incubating the enzyme in different buffers 5.0 and 12 for 1h at 60°C, followed by activity estimation at pH 10 and 60 °C. As shown in **Fig. (9)**, the enzyme was stable between pH 9 and 12, and highest stability was observed at pH 10.

4.4. Effect of storage time on protease activity and stability at room temperature

Shelf life experiment in **Fig. (10)** showed that the enzyme retained 95.5 % of its activity for 10 days at room temperature, then lost 10.4, 71.55 % of its activity after 20, 30 days, respectively The effect of storage of alkaline protease on its activity was determined as it is an important parameter for commercial utilization of enzyme. These results are on line with **Singh *et al* (1999)**, who found that the enzyme is 91% active for 20 days and the activity is reduced to 85% in 30 days of storage at room temperature suggesting that enzyme may be stored for 20 days at room temperature without much loss in its activity.

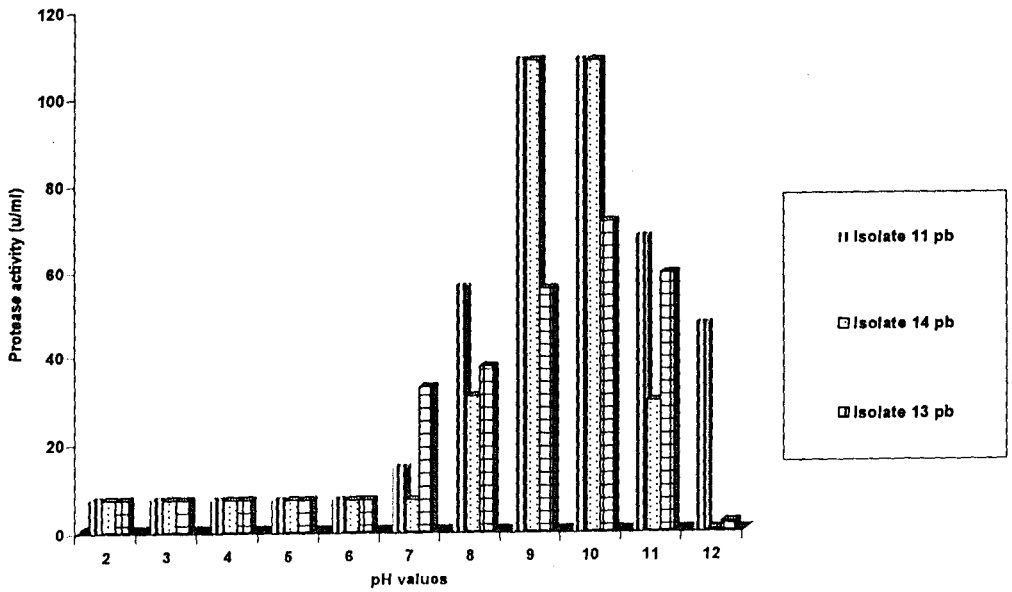

Fig. 9. Effect of pH on protease activity by selected *Bacillus* sp. Isolates

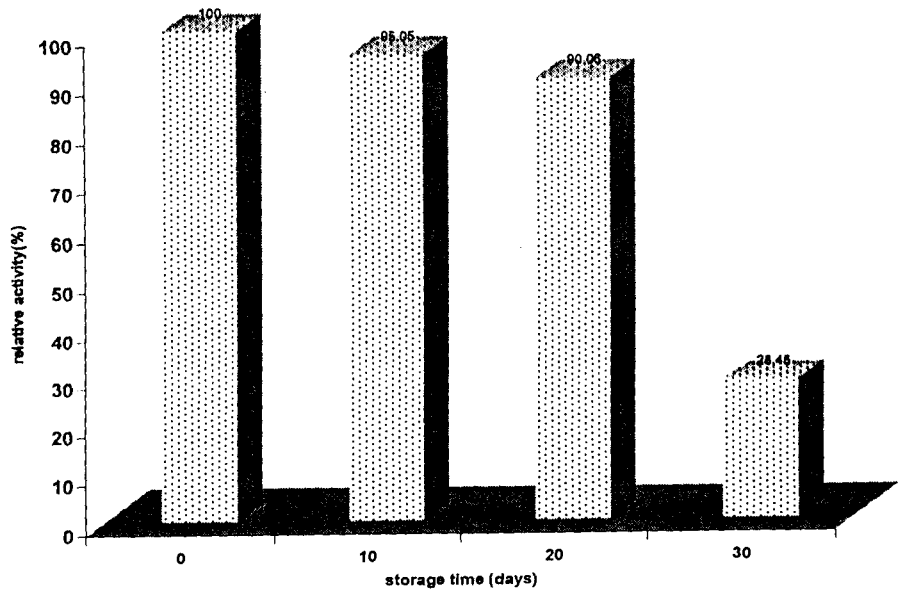

Fig. 10. Effect of storage time on protease stability at 30°C for a month by *Bacillus* sp. isolate 14 pb

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مجلة اتحاد الجامعات العربية
للعلـــــوم الزراعيـــــة
جامعة عين شمس ، القاهـرة
مجلد(١٦)، عدد (٢)، ٣٣٥-٣٤٧، ٢٠٠٨

إنتاج وتوصيف لأحد إنزيمات المحللة للبروتين المتحملة للملوحة باستخدام بكتيريا الباسيلس المعزولة من منتجات الفسيخ

[٢٦]

سماح هاشم السيد محمد[١]- فاطمة رفعت نصار[١] - سهير أحمد إبراهيم نصر[١] -
أحمد عبد الوهاب عبد الحافظ[١]

١- قسم الميكروبيولوجي - كلية الزراعة - جامعة عين شمس - شبرا الخيمة - القاهرة - مصر

الملخص

تم الحصول علـي ٩٥ عزلـة بكتيريـة محللـة للبروتين متحملة للملوحة من عينات الفسيخ المملحـة معمليا وتجاريا وذلك باجراء حصر مبدئى بإسـتخدام بيئتي اجار اللبن الفرز والجيلاتين المـضاف لهمـا ١٥% ملح كلوريد الصوديوم. و قد تم اختيـار أكفـأ العزلات المحللة للبروتين و كانوا ثلاث عزلات هـم 13 pb, 14 pb , 11 pb والذى تم تعـريفهم ليتبعـوا جنس *Bacillus* . ولدراسة أهم العوامل المؤثرة علي انتاج الإنزيم تم دراسة تأثير البيئه المـستخدمة مـن خلال إستخدام ٥ بيئـات مختلفـة ، تـأثير درجـة الحرارة، تأثير درجة الحموضة، تأثير تركيـز ملـح كلوريد الصوديوم. و كانت النتائج كمـا يلـى : تـم التوصل الي اقصي انتاج انزيمى من خلال اسـتخدام بيئة منتجات الاسماك الثانوية ذات رقم حموضـة ٨ وتركيز ٥% من ملح كلوريد الصوديوم عند التحضين علي ٣٠ م° ليصل الانتـاج الـي ١٢٦,٥ و ١٣٦,٥ و١٤٧,٥ باسـتخدام 13 pb, 14 pb , 11 pb علـي التوالي وذلك في نهايه طور النمو اللوغاريتمى وبداية الطور الثابت أما عن العوامل المؤثرة علي النـشاط الإنزيمى فقد تم دراسة تأثير درجة حرارة التحضين ، فترة التحضين، درجة الحموضة ودراسـة تأثير فترة التخزين علي الثبات الإنزيمي وكانت النتائج كما يلى: أعطي الإنزيم أقصي نشاط عند التحضين علي ٦٠م° باستخدام مادة الكازين ذات درجة حموضة ١٠ لمـدة ١٥ ق لكل من 11 pb , 14 pb بينما احتـاج لفتـرة تحضين ٣٠ ق للعزلة ,13 pb. و قد وجد ان للانزيم قدرة ثبات عالية تـسمح باسـتخدامه فـي مجـالات التنظيف الصناعي حيث ظل قادرا علي الإحتفـاظ ب ٩٠ % من نشاطه بعد ٢٠ يوم من التحـضين علـي ٣٠ م° ولكنه فقد ٨٥% من نشاطه بعد ٣٠ يوم مـن التحضين علي ٣٠ م° .

تحكيم: أ.د الشحات محمد رمضان
أ.د محمد إبراهيم شحاته