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PROTEASE-PRODUCING MICROORGANISMS INHABITING SALTED FISH

(With 4 Tables & 5 Fig.)

By

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الميكروبات المحلله للبروتين المصاحبه للأسماك المملحه / الملوحه) مع أشارة خاصة لنشاط أنزيم البروتيز بواسطة بكتريا باسيلوس سيبتلس

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تم فى هذا البحث عزل ٢٥ عتره من البكتريا والفطريات المنتجه لانزيم البروتيز من الاسماك المملحه (الامايه). كما تم أيضاً أختبار قدرة هذه الكائنات على تحليل البروتين بعد ٤ أيام من الزراعه عند ٣٧ م . من بين هذه العترات ثمانية عترات من البكتريا كانت تنتمى لنوع باسيلوس سيبتلس وعزلتين اخريتين لنوع باسيلوس ليكينوفورمس أما الفطريات المعزوله كانت عباره عن ١٥ عتره تشتمل على ٥ أنواع لجنسين من الفطريات هما الاسبرجلس والبنسيلوم .

أظهرت واحده من عترات باسيلوس سيبتلس نشاطاً كبيراً لتحليل البروتين مقارنة بالعترات الاخرى للكائنات المعزوله ولذا أختيرت لاجراء دراسة تفصيليه لدراسة أنتاج أنزيم البروتيز بواسطتها. كانت الظروف المثلى لانتاج الانزيم المحلل للبروتين بواسطة هذه العتره هى ٣٧ م فى وسط أسه الهيدروجينى ٨ فى وجود مصدر كربونى نشا (٤٪) وكذلك مصدر نيتروجينى كازين (٢٪) تميز الانزيم المنتج بهذه العزله بتحمله للحراره العاليه حتى ٨٠ م لمدة ٤٠ دقيقه وكذلك الملوحه المرتفعه حتى ٢٥ ٪.

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SUMMARY

Twenty-five isolates of protease-producing bacteria and fungi were isolated from salted fish and their proteolytic activity was determined after 4 days cultivation at 37°C on media contained 10% NaCl. Eight isolates were belonging to *Bacillus subtilis and two isolates as Bacillus licheniformis.* 15 isolates of fungi including two different genera with five species were identified as three isolates of *Penicillium chrysogenum,* two isolates of *P.nigricans,* five isolates of *P.brevicompactum,* two isolates of *P.citrinum* and three isolates of *Aspergillus terreus.* One isolate of *Bacillus subtilis* was chosen as the most promising protease producer. Optimal conditions for enzyme production by *B.subtilis* AO5 was pH 8.0, 37°C, 4% (W/V) starch as carbon source and 2% (W/V) casein as nitrogen source. Ca^{2+} and Mg^{2+} stimulated enzyme synthesis. Maximal activity of the crude enzyme was at pH 8 and the optimal temperature was 60°C. The protease retained 63% of its original activity after 40 min at 80°C. Protease was stable in NaCl with 62% of the original activity being retained in 25% (W/V) NaCl.

INTRODUCTION

In Arab Republic of Egypt, salted fish is a popular food mostly prepared, at Upper Egypt, distributed all over the country.

There have been three basic methods used in salting of fish. In most countries these methods are in distinguished as dry, wet and mixed methods.

SHEWAN (1961) pointed that in relation to salt, bacteria can be divided into three main groups: The halophobic or salt sensitive group, which includes most of pathogens and most of the putrefactive types such as *Pseudomonas* and *Achromobacter species.* These fail to grow in salt concentrations more than 6%. The halotolerant group, which includes most spore forming baceria; micrococci; some anaerobes can grow in salt concentration greater than 6% and even up to saturation, although the growth and multiplication is more slowly with increasing salt concentration. The halophilic bacteria or salt-living groups grows best in the presence of salt, requiring concentration usually greater than 2%. Microorganisms causing pink and dun

colouration (mould affection) in salted fish are important member of the halophilic group. Commercially salted fish at upper Egypt contained sodium chloride and sodium chloride in water phase (C%) with averages of 14.97% and 21% respectively *(YOUSSEF, 1976)*. The fish product is hydrolysed by joint action of fish and microbial proteases *(ZUBERI et al., 1988)*.

Eleven species of bacteria, one of yeast and three of filamentous fungi were found to occur in salted fish *(CRISAN and SANDS, 1975; HO et al., 1984 and ZUBERI et al., 1988)*. These microbes responsible for considerable deteriorative changes in fishes including loss in essential chemical constituents and proteins.

The present investigation was designed to isolate and identify the proteolytic microorganisms present in salted fish.

MATERIAL and METHODS

Isolation and identification of protease-producing microbes:

Five gram samples of *Alestes nurse* was blended with 45 ml 10% (W/V) NaCl and after thorough mixing, was serially diluted in 10% NaCl. Samples were spread onto nutrient agar containing (g/l bidistilled water): Tryptone, 10; Yeast extract, 5; NaCl, 100, Casein, 5; Agar, 15. The initial pH of the medium was adjusted to 7.3. After 4 days at 37°C, all colonies exhibiting clear halos were isolated. The bacteria and fungi were identified according to Bergey's Manual of Systematic Bacteriology *(SENEATH et al., 1986)* and to the keys of *Penicillium and Aspergillus (PITT, 1985 and RAPER and FENNELL, 1965)*, respectively.

Cultivation for enzyme production: The medium for liquid cultures consisted of (g/l bidistilled water): Tryptone, 10; yeast extract, 5; NaCl, 50; with the pH adjusted to 7.3 with 0.1 M NaOH. Cultivation was in 250 ml ERLENMEYER flasks containing 50 ml of sterile medium which contained various other substrates, as sources of carbon or nitrogen. Various metal ions were also added as indicated and various pH medium were used. Media were inoculated with 1 ml of spore suspension (5×10^6). After 4 days the cells were harvested through glass wool and culture filtrates were assayed for enzyme activity.

Protease assay: The protease activity ws measured by using a reaction mixture of 2 ml containing 10 mg of HAMMARSTEN casein, 7.5 µmol KH_2PO_4/ Na_2HPO_4 buffer, pH 7.2 and an enzyme source. The mixture was incubated for 60 min at 37°C and then the reaction was stopped by adding 3 ml of 5% trichloroacetic acid and the acid soluble material was estimated at 280 nm

after removing the precipitate by filtration through WHATMAN no.1 filter paper. A blank was prepared by adding 3 ml of precipitating agent to 2ml of the casein solution before the addition of the enzyme solution. One protease unit is defined as the amount of enzyme which yields the equivalent of 1 µg of tyrosine per min at 280 nm under control conditions.

EFFECT OF pH and temperture on enzyme activity: The protease activity was measured over pH range of 5.0 to 9.0. The buffer systems used for assay were: 50 mM sodium acetate/acetic acid for pH 5.0, 50 mM sodium potassium phosphate for pH 6.0 to 7.0 and 50 mM Tris/ HCl for pH 8 to 9. The enzyme activity was performed at 35, 40, 50, 55, 60, 65, 70, 75 and 80°C in a thermostatic water bath *(Hagihara et al., 1958).*

pH stability: The stability of the protease preparation was studied in buffer solution of various pH. The mixture was incubated for 24 h at 4°C. The residual activity was measured at optimum conditions *(PANSARE et al., 1986).*

Thermal stability: Crude enzyme was incubated at various temperatures for 40 min, then immediately cooled in ice and its activity was assayed *(UEHARA et al., 1979).*

Salt tolerance test: Enzyme was incubated at 5.0 mM Tris/HCl buffer, pH 8, containing various NaCl concentrations for 24 h and residual activity was determined *(PANSARE et al., 1986).*

RESULTS

The results were recorded in tables 1 to 4 and figures 1 to 5.

DISCUSSION

Isolation and identification: Twenty-five isolates of protease-producing bacteria and fungi were isolated from salted fish. Ten isolates were identified by standard biochemical and bacteriological test as *Bacillus* strains. Eight strains were identified as *Bacillus subtilis* and two strains were identified as *Bacillus licheniformis.* 15 isolates of fungi including two different genera with five different species were identified as *Penicillium nigricans, P.chrysogenum, P.brevicompactum, P.citrinum* and *Aspergillus terreus* (Table 1). Eleven species of bacteria belonging to genus *Bacillus,* three of filamentous fungi and one of yeast were found in salted fish sauce *(CRISAN and SANDS, 1975; ZUBERI et al. 1988).* As previously reported by

YOUSSEF (1976) that commercially salted *Alestes nurse* contained sodium chloride with average 14.97%. The present study agree with the findings of many researchers *(SHEWAN, 1961; SEDIK, 1971, YOUSSEF, 1976; CRISAN and SANDS, 1975; ZUBERI et al., 1988)* that in relation to salt, halotolerant and halophilic bacteria such as *Bacillus sp.* and mould grow in the presence of salted fish contained NaCl more than 6%.

Protease activity in culture filtrates of 25 isolates were determined (Table 1). *Bacillus subtilis* AO5 was the highest producer and its activity (13 U/ml) is greater than those reported previously for *Bacillus subtilis* MT-2 (8 U/ml) *(FUJII et al., 1983).*

Our results indicated that the isolated microbes were capable of producing extracellular proteases. The rate of production varied among the species. Protease production seemed to be related to the loss in protein of salted fish lead to deterioration and spoilage of the products.

Protease production by *Bacillus subtilis* AO5: The cultural conditions were examined to obtain the highest production of proteolytic enzyme and the optimal conditions. The optimum pH and temperature for the maximum production of the enzyme was 8 and 37°C (Figures 1 and 2), in accordance with the results of *LOGINOVA et al. (1980)* and *DISLER (1982).* However, *KANG et al. (1987)* reported that maximum production of proteinase by *saccharomycopsis (Yarrowia) lipolytica* was obtained at pH 9.0.

Effect of carbon source: Utilization of starch at different levels were evaluated and a concentration of 4% found to the best for synthesis of enzyme. Substitution of starch in the basal medium by glucose, fructose or sucrose resulted in a decrease in enzyme production (Table 2). Starch has been found to stimulate the production of proteolytic enzymes from several microbes *(NOVIKOVA et al., 1986 and VALDIMIROVA and KORNIENKO, 1987).*

Effect of nitrogen source: Among the concentrations of the various organic nitrogen sources tested, 2% casein or 4% soybean flour, when added to the basal medium, stimulated enzyme production (Table 3). Hydrolysed proteins, such as Bacto-peptone, Tryptone and casamino acid, did not exhibit any enhancing effect. Thus *Bacillus subtilis* AO5 requires a complex nitrogen soruce with large molecular weight protein fragments for stimulation of enzyme production. Similarly, an exogenously supplied protein was found to increase the production of proteinse by *Neurospora crassa* by five-folds when added to the medium *(DRUCKER, 1972 and COHEN et al., 1975).*

The role of proteins, or their large molcular weight

fragments, in stimulating proteinase production was also demonstrated in *Erwinia chrysanthemi (WANDERSMAN et al. 1986)* and *Aspergillus oryzae (FUKUSHIMA et al. 1989)*.

Effect of metal ions: The effect of addition of metal ions to culture medium was examined. The results obtained were shown in Table 4. Ca^{2+} and Mg^{2+} stimulated enzyme production, Co^{2+}, Zn^{2+}, Cu^{2+} completely stopped the growth. Fe^{2+} and Mn^{2+} had decreased the enzyme activity.

Properties of protease: Optimum temperature for enzyme activity was 60°C (Fig 3). The enzyme was stable at temprature up to 75°C for 40 min but at 80°C only 63% of the original activity was retained after this time. The neutral proteases of *Bacillus stearothermophilus* and *Bacillus subtilis* retained about 80% of the initial activity after treatment at 65°C for 30 min (*FUJII et al. 1983)* and 20min (*UEHARA et al. 1979)*, respectively. In terms of thermal stability, the enzyme is superior to proteolytic enzymes from *Bacillus alvei (NOVIKOVA et al. 1986)* and *Bacillus subtilis (TREITSKII et al. 1987)*.

Optimum pH of the enzyme was 8. The enzyme was stable over the pH range 5 to 9 but at more acidic or alkaline pHs it lost activity rapidly (Figure 4). Most of *Bacillus* proteases were stable between pH 6 to 8 *(FUJII et al. 1983)*.

Salt tolerance of the enzyme was monitored by incubating the enzyme solution in buffer contaning various concentrations of NaCl up to 25% (W/V) for 24h at 30°C and measuring the residual activity. Protease activity was stable in NaCl with 62% of the original activity being retained in 2% NaCl (Figure 5). A literature survey has indicated that this is probably the first report on salt-tolerance proteases from *Bacillus subilis*. The favourable characteristics of the enzyme may be applied extensively in laundry detergents and the tanning industries.

REFERENCES

Cohen, B.L. Morris, J.E. and Drucker, H. (1975): Regulation of two extracellular proteases of *Neurospora crassa* by induction and by carbon-nitrogen and sulfur metabolism repression. Arch. Biochem. Biophy. 169: 324-330.

Crisan, E.V. and Sands, A. (1975): Microflora of four fermented fish sauces. Appl. Microbiol. 92: 106-108.

Disler, E.N. (1982): Suppression of the extracellular proteolytic activity of *Streptomyces reseofungini* by certain environmental factors. Prikladnaya Biokhimiya i Mikobiologiya 18: 391-396.

Drucker, H. (1972): Regulation of exocellular proteases in *Neurospora crassa:* induction and repression of enzyme synthesis. J. Bacteriol. 110: 1041-1049.

Fujii, M.; Takagi, M.; Imanaka, T. and Aiba, S. (1983): Molecular cloning of a thermostable neutral protease gene from *Bacillus stearothermophilus* in a vector plasmid and its expression in *Bacillus stearothermophilus* and *Bacillus subtilis.* J. Bacteriol. 154: 831-837.

Fukushima, Y.; Itoh, H.; Fukase, T. and Motai, H. (1989): Continuous protease production in a carbon limited chemostat culture by salt tolerant *Aspergillus oryzae.* Appl. Microbiol. Biotech. 30: 60-608.

Hagihara, B.; Matsubara, H.; Nakai, M. and Okunuki, K. (1958): Crystalline bacterial proteinase. I. Preparation of crystalline proteinase of *Bacillus subtilis.* J. Biochem. (Tokyo) 54: 185-194.

Ho, C.C.; Toh, S.E.; Ajam, N. and Cheah, K,P. (1984): Isolation and characterization of halophilic yeast and bacteria involved soy sauce fermentation in Malaysia. Food Technol. (Australia) 36: 227-230.

Kang, K.H.; Bae, I.H. and Lee, C.H. (1987): Studies on extracellular protease from *saccharomycopsis lipolytica.* Conditions of enzyme production. Snop Misaengmul Hakahoechi (KOREAN) 15: 279-285.

Loginova, L.G.; Usaite, I.A. and Seregina, L.E. (1980): Isolation of unpigmented producer of proteolytic enzymes. *Thermoactinomyces vulgaris.* Prikladnaya Biokhimiya i Mikrobiologiya 9: 228-233.

Novikova, T.M.; Vybomykh, S.N.; Egorov, N.S. and Lortya, ZH.K. (1986): Extracellular protease of *Bacillus alevi* B-724, Prikladnaya Biokhimiya i Mikrobiologiya 22: 772-779.

Pansare, A.C.; Lewise, N.F and Venngopal, V. (1986): Characterization of extracellular proteases of *Aeromonas hydrophila.* Agric Biol. Chem.

Pitt, J.I. (1985): A laboratory guide to common *Penicillium* species. Commonwealth Scientific and Industral Research Organization, Division of Food Research, Australia.

Raper, K.B. and Fennell, D.J. (1965): The genus *Aspergillus.* Willimas and Wilkins, Baltimore, U.S.A.

Sedik, M.F. (1971): Studies on *Tilapia nilotica* and *Mugil cephalus.* Ph.D. Thesis, Faculty of Vet. Med., Cairo Univ.

Seneath, P.H.A.; Mier, N.S.; Sharpe, N.E. and Holt, J.G. (1986): Bergey's Manual of Systematic Bacteriology, vol. 2 pp. 1104-1139. BALTMORE: Williams and Wikins.

Shewan, J.M. (1961): Microbiology of sea water fish. Fish as food Vol. 1. Edited by G. Borgestrom, Academic Press. New York and London.

Treitskii, A.V.; Bogdanova, L.A.; Gonchar, A.M. and Salganik, R.I. (1987): Properties of bacterial proteases immobilized on a water soluble polymer. Prikladnaya Biokhimya i Mikrobiol. 28: 762-767.

Uehara, H.; Yamana, K. and Maruo, B. (1979): Thermosensitive, extracellular neutral proteinase in *Bacillus subtilis:* Isolation, characterization and genetics. J. Bacteriol. 139: 585-590.

Valdimirova, E.V. and Kornienko, A.E. (1987): Effect of some nitrogen and carbon on the proteolytic activity, growth and development of actinomycetes. Trudy S'ezda Mikrobiologiya Ukrayins 4: 22-33.

Wandersman, C.; Andro, T. and Berthaw, Y. (1986): Extracellular protease in *Erwinia chrysanthemi.* J. Gen. Microbiol. 132: 899-906.

Youssef, H.A. (1976): Studies on the sanitary improvements of locally manufactured salted fish. Ph.D. Thesis, Faculty of Vet. Med., Assiut University.

Zuberi, R.; Shamshad, S.I. and Qadri, R.B. (1988): Bacterial flora of fermented fish sauce prepared from sardines in Pakistan. Tropical science 28: 239-246.

Table 1. Proteolytic enzyme activity in culture filtrates of different microbes recovered from salted fish.

Organism	NaCl%* (average)	C%*	Protease activity (U / ml)
Bacillus subtilis			
AO1			6
AO2			7
AO3			8
AO4	14.97	21	12
AO5			13
AO6			10
AO7			8
AO8			9
Bacillus licheniformis			
AO9			6
AO10	14.97	21	5
Pencillum nigricans			
MS1			7
MS2			9
P. chrysogenum			
MS3			6
MS4	14.97	21	9
MS5			8
P. brevicompactum			
MS6			10
MS7			11
MS8	14.97	21	8
MS9			7
MS10			6
P. citrinum			
MS11			8
MS12			6
Asperigllus terreus			
MS13	14.97	21	4
MS14			5
MS15			6

*As recorded by Youssef 1976

Table 2

Effect of carbon source on 24-h protease production by *Bacillus subtilis* AO5 at 37°C and pH 8.

Carbon source (% , W/V)	Protease activity (U/ml)
control (basal medium)	11.8
Starch	
0.5	12.3
1.0	13.1
2.0	14.2
3.0	15.8
4.0	18.3
5.0	13.7
Glucose	
0.5	6.9
1.0	8.2
2.0	9.8
3.0	5.7
4.0	3.3
5.0	2.8
Fructose	
0.5	6.3
1.0	9.4
2.0	5.5
3.0	3.8
4.0	2.8
5.0	1.8
Sucrose	
0.5	5.3
1.0	7.4
2.0	8.3
3.0	9.8
4.0	6.5
5.0	3.6

Table 3
Effect of nitrogen source on protease production by *Bacillus subtilis* A05 at 37°C and pH 8.

Nitrogen source (%, W/V)	Protease activity of 24-h culture (U/ml)
Control (basal medium)	11.3
Casein	
1.0	11.7
2.0	18.7
3.0	15.3
Bacto-peptone	
1.0	10.4
2.0	9.3
3.0	8.7
Tryptone	
1.0	10.1
2.0	10.8
3.0	9.3
Casamino acid	
1.0	7.7
2.0	7.8
3.0	5.3
Soybean flour	
1.0	8,9
2.0	9.8
3.0	11.3
4.0	17.3

Table 4.

Effect of metal ions on protease production by *Bacillus subtilis* AO5.

Metal ion concentration (0.1% W/V)	Protease activity of 24-h culture (U/ml)
Control (basal medium)	11.3
$MgSO_4.7H_2O$	12.7
$CaCl_2.2H_2O$	15.2
$FeSO_4.7H_2O$	3.5
$MnSO_4.4H_2O$	4.3
$CoCl_2.6H_2O$	0
$ZnSO_4.7H_2O$	0
$CuSO_4.5H_2O$	0

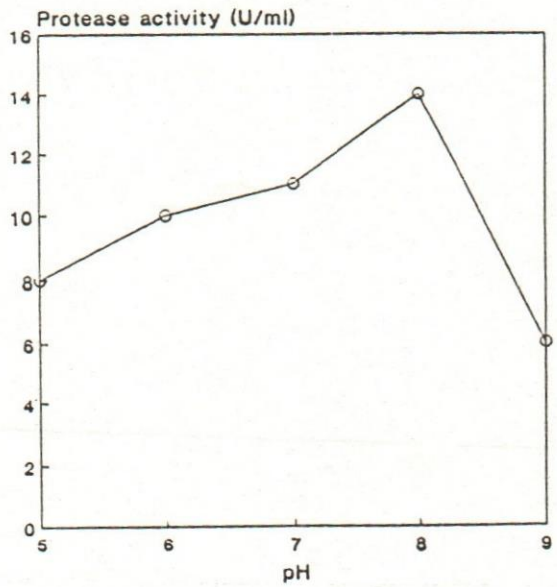

Figure 1. Effect of initial medium pH on protease production by *Bacillus subtilis* AO5.

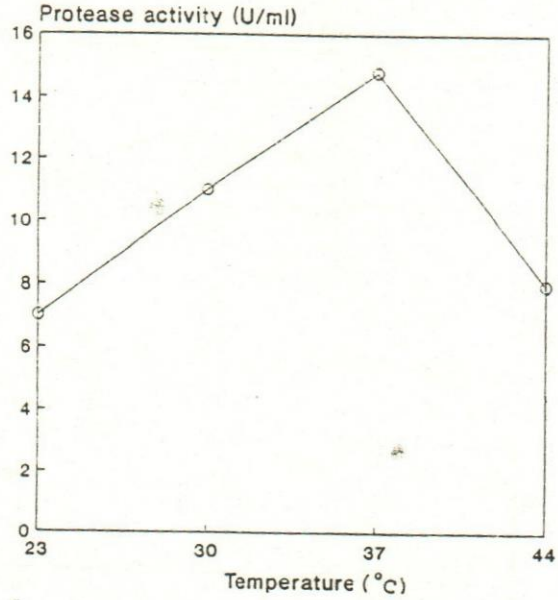

Figure 2. Effect of temperature on protease production by *Bacillus subtilis* AO5.

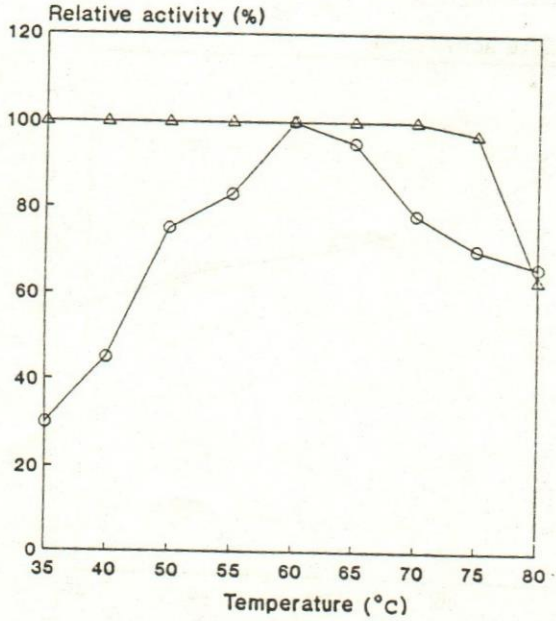

Figure3. Effect of temperature on Protease activity (O)and stability(△).

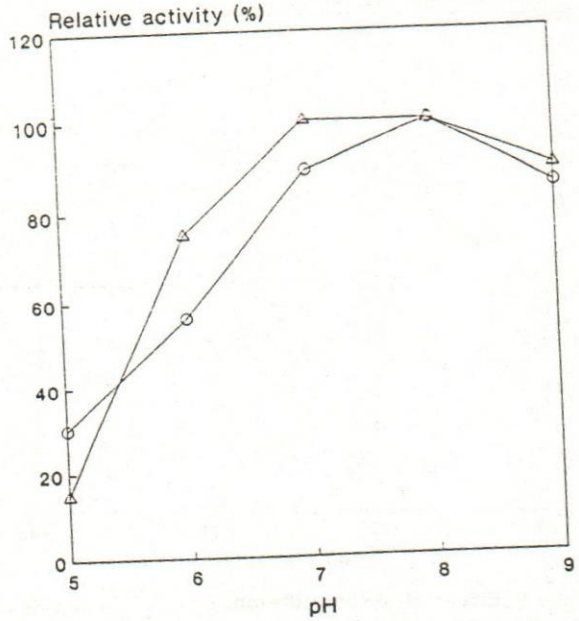

Figure4. Effect of pH on protease activity (O)and stability(Δ).

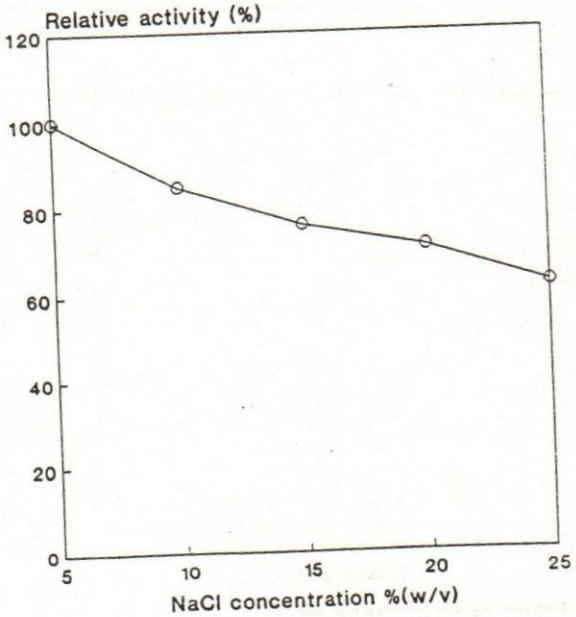

Figure 5. Effect of NaCl on protease activity.