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PROTEOLYTIC MICROFLORA CONTAMINANTS OF ALEASTES NURSE (SALTED FISH)

(With 5 Tables)

By

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البكتريا المحلله للبروتين الملوثة لسمك الأمايه المملح

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يعتبر سمك الأماية المملح من أكثر أنواع الأسماك المملحه. تم جمع ٤٠ عينه من سمك الأماية المملح من مدينة أسيوط لفحصها بكتريولوجيا للتعرف على مدى تلوثها بالميكروبات المحلله للبروتين. كانت هذه البكتريا تنتمي إلى البكتريا المحبه للحرارة العالية، البكتريا المحبه لدرجة المحراره المعتدله، البكتريا المحبه للبرودة والتي تراوحت أعدادها من أقل من ١٠ - ١ x ١٠^٤، ٥ x ١٠^٤، - ٢,١ x ١٠^٨، ١ x ١٠^٤ - ٣,٥ x ١٠^٦، بكتريا/جم على التوالي. تم عزل ٩٩ عترة من هذه الأنواع المختلفه. عزلت وصنفت البكتريا المحبه للحرارة العالية إلى نسب مختلفه من :

Bacillus megaterium, B. cereus, B. licheniformis & B. subtilis.

كما عزلت وصنفت البكتريا المحبه للحرارة المعتدلة إلى نسب مختلفه من :

Virbrio Spp., Staph. aureus, S. epidermidis. B. subtilis, B. licheniformis, M. luteus, M. roseus and P. aeruginosa.

كما عزلت وصنفت البكتريا المحبه للبرودة إلى نسب مختلفه من:

Staph. aureus, S. epidermidis, Flavobacterium, Aeromonas hydrophilia, Strept. faecalis, S. faecium, M. roseus, P. fluorescens & Vibrio Spp.

تم مناقشة الأهمية الصحية للعزلات وكذلك وضع التوصيات اللازمة لمنع تلوث هذه الأسماك بالميكروبات المحلله للبروتين.

SUMMARY

A total of 99 proteolytic bacterial isolates were collected from Aleastes nurse (salted fish). These associated proteolytic bacteria were of thermophilic, mesophilic and psychrotrophic groups. The counts of the aforementioned microorganisms ranged from <10-$1x10^4$, $5x10^4$-$2.1x10^8$ and $1x10^4$-$3.5x10^6$ CFU/g respectively. The identified isolates of thermophilic proteolytic were *Bacillus megaterium, B. cereus, B. firmus, B.licheniformis and B.subtilis* . The mesophilic group includes; *Vibrio spp., Staph. aureus, S. epidermidis, B. subtilis, B.lcheniformis, Micrococcus luteus, M.roseus and Pseudomonas aeruginosa.* The identified psychrotrophic group includes; *Staph. aureus, S. epidermidis, Flavobacterium* and *Aeromans hydrophilia, Strept. faecalis, S.faecium, Micrococcus roseus, P. fluorescens and vibrio spp.* The public health significance of the different proteolytic isolates and the suggestive control measures to prevent contamination of salted fish with proteolytic microorganisms were given.

Key words: *Salted fish - Microflora - Contamination.*

INTRODUCTION

Salting of fish in Egypt is a traditional method of fish preservation since ancient times. The salted fish " Aleastes nurse" are kept for months at room temperature (25^oC-35^oC) or more. Consumption of these fishes has been resulted in a public health hazard (Youssef, 1976, Morshdy et al., 1982, Abdel-Rahman et al., 1988).

Proteolytic microorganisms produce protease enzymes which attack the muscle protein leading to the breakdown of protein resulting in the gradual development of staleness and spoilage of fishes. The temperature of fish holding is of supreme importance. These enzymes are produced by psychrotrophic, mesophilic and/or thermophilic proteolytic bacteria . Some of these enzymes are heat-stable and survive processing temperatures. Even low quantities of enzymes can cause fish quality problems on prolonged storage (Londhall and Nilsson, 1978; Chopra and Marthur, 1983).

In Egypt, data concerning the presence of the proteolytic microorganisms associated with salted fish are not available. Therefore this work was planned to assess the level of thermophilic, mesophilic and psychrotrophic proteolytic microorganisms in these salted fish which may be used as a

criteria for fish quality determination. Besides isolation and identification of these target microorganisms.

MATERIALS and METHODS

Collection of Samples:

A total of 40 samples of ready-to-eat salted fish were obtained from different retailers in Assiut City and transported to the laboratory. These are *Aleastes nurse* type, each of ca 18 cm long and 245 gm weight.

Perperation of samples :

Ten grams of the fish muscle were removed under sterile conditions and blended with 90 ml of 0.1% physiological saline in a blender(8000 rpm) for three min. Serial ten-fold dilutions up to 10^6 were made as described by AOAC (1985).

Enumeration proceedure:

The dilutions were cultivated into skim milk medium (ICMSF,1978) which consisted of nutrient agar with 10% (V/V) skim milk. The components were sterilized seperately at 121 ^{0}C for 15 min., then cooled at 55 ^{0}C and mixed. Another medium of calcium caseinate agar medium (Fraizer and Rupp, 1928) was used. The prepoured plates were inoculated by appropriate dilutions using pour plate method.

1. **Thermophilic proteolytic** . Duplicate plates were incubated at 55^{o}C for three days (ICMSF, 1978).
2. **Mesophilic proteolytic** . Duplicate plates were incubated at 37^{o}C for two days (Harrigan and McCance, 1983).
3. **Psychrotrophic proteolytic** . Duplicate plates were incubated at 4^{o}C for 7 days (APHA, 1985).

After incubation, the colonies having proteolytic activity which are charcterized by clear hallow zone around them were counted.

Confirmation (gelatin liquifaction):

Confirmation of the organisms ability to hydrolyze protein was done by gelatin liquifaction procedure similar to that outlined by Harrigan and McCance, 1983. Tubes of nutrient broth (Difco) were prepared with the addition of 12% (wt/vol) gelatin (Difco). The medium was sterilized by autoclaving for 14 min at 121 ^{o}C. Duplicate tubes were stab inoculated for each test. Representative colonies were picked up from skim milk agar or calcium caseinate agar to nutrient agar slant for further identification.

Identification of the isolates:

The isolates were identified according to Cowan's and Steel, 1974; Mossel, 1977; Harrigan and McCance, 1983 and Krieg and Holt, 1984.

RSULTS

Table 1. Statistical analysis of proteolytic microorganisms associated with Aleastes nurse (salted fish).

Microorganisms	Minimum	Maximum	Mean
Thermophilic	<10	$1x10^4$	$1.3x10^3$
Mesophilic	$5x10^4$	$2.1x10^8$	$1.3x10^7$
Psychrotrophic	$1x10^4$	$3.5x10^6$	$2x10^5$

Table 2. Frequency distribution of proteolytic microorganisms count in Aleastes nurse (salted fish).

Count	Thermophilic		Mesophilic		Psychrotrophic	
	F	%	F	%	F	%
$>10^3$	11	27.5	0	0	0	0
$10^3->10^4$	28	70	0	0	0	0
$10^4->10^5$	1	2.5	2	5	15	37.5
$10^5->10^6$	0	0	2	5	18	45
$10^6->10^7$	0	0	10	25	7	17.5
10^7-10^9	0	0	26	65	0	0
Total	40	100	40	100	40	100

Table 3. Thermophilic proteolytic bacteria isolated from salted fish

Isolates	No	%
Bacillus cereus	9	25.7
B.firmus	5	14.3
B.licheniformis	2	5.7
B.megaterium	13	37.1
B.subtilis	1	2.9
Bacilloid	5	14.3
Total	35	100

Tble 4. Mesophilic proteolytic bacteria isolated from salted fish.

Isolates	F	%
Bacillus licheniformis	3	10
B. subtilis	6	20
Micrococcus luteus	3	10
M.roseus	2	6.7
Pseudomonas aeruginosa	3	10
Staph. aureus	2	6.7
Staph. epidermidis	4	13.3
Vibrio spp.	7	23.3
Total	30	100

Table 5. Psychrotrophic proteolytic bacteria isolated from salted fish.

Isolates	No	%
Aeromonas hydrophilia	6	17.6
Flavobacterium	8	23.5
Micrococus roseus	2	5.9
Pseudomonas fluorescens	2	5.9
Staph. aureus	3	8.8
Staph.epidermidis	7	20.6
Strept. faecalis	2	5.9
Strept. faecium	2	5.9
Vibrio spp.	2	5.9
Total	34	100

DSCUSSION

In Egypt, salting is used not only for fish preservation but also to cater particular taste especially for special fests. The normal Gram-negative spoilage flora of fish is not halotolerant and are replaced by halophilic and halotolerant micrococci, spore formers and moulds. Halophilic microorganisms are of proteolytic action (Hobbs and Hodgkiss, 1982).

Thermophilic proteolytic bacteria recovered from salted fish:

It is evident from Table (1) that; the count of thermophilic proteolytic microorganisms ranged from <10 to $1x10^4$ with a mean of $1.3x10^3$ CFU/g. Table (2) shows that the highest frequency distribution of the examined samples (70%) had 10^3 ->10^4 CFU/g, while 27.5% of the samples had >10^3 and few (2.5%) had count of 10^4 CFU/g. Counts of 10^5 or more were not recorded for thermophilic proteolytic microorganisms in the present study.

The isolated thermophilic proteolytic microorganisms were bacilii. The most dominant isolates were *B.megaterium, B.cereus* and *B.firmus* in percentages of 37.1, 25.7 and 14.3 respectively (Table 3). Nearly similar findings were reported by some authors (Tom and Crisan , 1975) who studied the proteolytic activity of *Bacillus cereus, B.firmus, B.licheniformis, B.megaterium* and *B.subtilis*. Bacilli were isolated from salted fish by Morshody et al., (1982) but no trials were done to study their proteolytic

activity. While the proteolytic activity of *Bacillus subtilis* in fish was attributed to protease enzyme by (Abd-AllA, 1994).

Mesophilic proteolytic bacteria recovered from salted fish:

Mesophilic proteolytic microorganisms associated with the muscle of ready-to-eat salted fish in the current study could be detected in levels ranged from $5x10^4$ to $2.1x10^8$ CFU/g as shown in Table (1). Their frequency distribution as cited in Table (2) pointed that 65% of the samples had 10^7-10^9 while 25% of the samples had 10^6 CFU/g i.e. 90% of the samples had 10^6-10^9 CFU/g. Few samples had 10^4-10^5 CFU/g.

The identified mesophilic proteolytic isolates were *Vibrio spp.*, *Staph.epidermidis*, *Staph.auerus*, *Bacillus substilis*, *Micrococus luteus*, *Micrococus roseus*, *Pseudomonas aeruginosa* and *B.licheniformis* where they recovered in varying percentages from 6.7 to 23.3 as presented in Table (4). These results comply with Karnop (1982b) , where the proteolytic flora associated with German fish were micrococci, staphylococci , vibrio, bacillus, pseudomonas and flavobacterium. In study conducted by Abdel-Rahman et al., 1988; the isolated microorganisms from salted fish were *Staph. aureus*, micrococci and lactobacilli . The proteolytic enzymes produced by Pseudomonas spp. were studied by some investigators (Mckellar, 1982, Murry et al., 1983, Skura et al., 1989 and Myhara and Skura, 1986).While that of micrococci and Staph. aureus were investigated by Tom and Crisan (1975).

Staphylococci and micrococi are halophilic microorganisms, Gram-positive and mesophiles can grow on salted fish at low water activity of 0.75 (Motohiro, 1988). These bacteria originate in salt used for salting of fish and multiply in brine (Sikorski, 1992).

Karnop (1982 b) found that at advanced stage of fish spoilage, the proportion of proteolytic bacteria on muscle of German cod was 30% to the total bacterial count.

Bacillus cereus was isolated in the current study from 25.7% of the thermophilic proteolytic isolates as in Table (2). The presence of *B.cereus* at this low level is insignificant unless the organism is able to grow to $<10^5$ where mild symptoms may occur if ingested (Goepfert et al., 1972). However, no food-poisoning outbreaks caused by B.cereus in seafoods have been reported.

Psychrotrophic proteolytic bacteria recovered from salted fish:

Psychrotrophic bacteria produce proteolytic enzymes during growth (Londhall and Nilsson, 1978). The count of proteolytic psychrotrophic count varied from $1x10^4$ to $3.5x10^6$ CFU/g.

The frequency distribution of their count (Table, 2) showed that 45% of the samples had 10^5 and 37.5% had 10^4 CFU/g . Higher count of 10^6 CFU/g was recorded in 17.5% of the samples. The isolated microorganisms were *Staph.aureus, Staph. epidermidis, flavobacterium* and *Aeromonas hydrophilia* in 8.8%, 20.6%, 23,5% and 17.6% respectively. Others (*Strept. faecalis, Strept. faecium, Pseudomonas fluorescens, Micrococcus roseus* and *vibrio spp.*) could be isolated in varying percentages (Table 5).The attained results agree with Tom and Crisan (1975) who listed Flavobacterium, micrococci and staphylococci among proteolytic microorganisms. Vibrio spp. could be isolated in 23% and 5.9% of the total isolates of mesophilic and psychrotrophic proteolytic respectively. Higher incidence of this microorganism was noted in mesophilic than in psychrotrophic ones in this study and this attributed to the more incidence of such organism in a warmer condition than in cooler ones (Abeyta, 1983).

Gill, 1982 mentioned that the protein breakdown by bacteria can be cleared by an increase in non-protein nitrogen fraction until after prolonged storage. The onset of bacterial proteolytic activity should be predectable on the bases of the conditions known to be necessary for proteolytic enzymes productions. To degrade proteins, bacteria must secret extracellular proteolytic enzymes.

The use of skim milk agar media in the present study gave better results concerning the proteolytic activity and for isolation than using calcium caseinate agar and this indicates that the use of more than one medium seems to be of an absolute necessity for reporting of proteolytic activity (Sikes and Maxy, 1979, Karnop, 1982 a).

The sources of these proteolytic bacteria may be from fish of pollutted water, salt, improper salting, improper hygienic condition during salting in vessels and /or abuse storage temperature. Therefore the following recommendations must be applied to minimize such proteolytic microorganisms:

(1) The use of high quality fish for the purpose of salting, (2) The utilization of high quality salt for salting process, (3) Hygienic and sound salting, and (4) Refrigerated storage of salted fish.

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