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Isolation and Identification of protease producing *Aeromonas hydrophila*

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Abstract

A total of 89 samples were collected from pools of fish farms and gills pieces of fresh fishes from different locations in Baghdad governorate. From these samples, a total of 91 bacterial isolates were obtained and identified as suspected *Aeromonas* spp. according to the cultural and microscopical characteristics. After subjection to the biochemical tests, seven isolates were identified as *Aeromonas hydrophila*. Results of identification were confirmed by using Api 20E system. Ability of these isolates for protease production was screened. Results showed that all the seven isolates were protease producers; among them the *A. hydrophila* A4 obtained from gills pieces of fresh fishes was the most efficient in protease production. Enzyme specific activity of protease of the crude filtrate of this isolate was 31.01U/mg protein.

Introduction

A. hydrophila are Gram-negative, facultative anaerobic bacteria that can be isolated from many sources, such as food, drinking water, sewage, environmental water and human clinical samples with a world-wide distribution, these bacteria can develop in refrigeration temperatures and are responsible for food and water-borne diseases that can cause a range of human diseases that vary in severity from a self-limiting gastroenteritis to potentially fatal septicemia [1]. The virulence of *A. hydrophila* is multifactorial. Surface-associated factors include adhesins (e.g., pili), the S-layer, and lipopolysaccharide. Extracellular factors include an array of exoenzymes and exotoxins, i.e., enterotoxins, hemolysins, lipases, gelatinase, caseinase, elastase, lecithinase, deoxyribonuclease and proteases. Many of the proteins involved in pathogenicity are reliant on the genera secretory pathway for export [2].

One of the most important extracellular enzymes produced by *Aeromonas* spp. Proteases (E.C.3.4.24.4) are highly complex group of enzymes which occupy a central position with respect to their applications in both physiological and commercial fields. Proteolytic enzymes, which are produce intracellularly and extracellularly, play an important role in the metabolic and regularory processes of animal and Plant cells, as well as in those of prokaryotic and eukaryotic microorganisms, extracellular proteases are involved mainly in the hydrolysis of large polypeptide substrates, such as proteins into smaller entities which can subsequently be absorbed by the cell. There are different types of protease include serine, cysteine, aspartic and metallo proteases [3].

According to those mentioned above, this study was aimed to isolate a locally higher protease producer *A. hydropphila* and to improve its ability in protease production and this was achieved by:

1. Isolation of *A. hydropphila* from different fish and water samples.
2. Screening the ability of local isolates in protease production and select the efficient one for enzyme production.

Material and Methods:

Isolation of *A. hydrophila*

For the isolation of *A. hydrophila*, a total of 89 samples of fresh fish samples (55 sample), and water samples (34 sample) from pool of living fish were collected from lake of fish farm in Baghdad governorate. These samples were transferred quickly in sterilized tube containers and nylon bags to the lab for further analysis.

One gram of each fish sample (pieces of gills), and one milliliter of each water sample were transferred aseptically to test tubes containing 9 ml of alkaline peptone water (pH 8.9) and incubated at 30ºC for 4-6 h. under aerobic conditions, then 1 ml aliquots of each sample was added to test tubes containing 9 ml of distilled water and mixed vigorously. Each sample was serially diluted, then 0.1 ml

aliquots from the appropriate dilution were taken and spread on blood and MacConky agar media, and on selective medium TCBS agar for *Aeromanas* ssp. and incubated at 30ºC for 24h., then the suspected colonies were selected and subjected to bacteriological and biochemical assays and comfirmed by using Api 20E system [4].

Screening the ability of local isolates of *A. hydrophila* in production of protease.

Semi quantitative screening

Local isolates were streaked on nutrient agar medium and incubated at 30 °C for 24h. A single colony was then taken and placed on the center of skim milk agar medium plate. Plates were then incubated at 30°C for 24h. Ability of *A. hydrophila* in protease production was measured according to the ratio of hydrolysis based on presence of clear halo around each colony [5].

Quantitative screening

A volume of 100 µl of fresh culture of each bacterial isolate were used to inoculate nutrient broth medium in conical flasks and incubated at 30ºC for 24 hrs. with shaking at 150 rpm. After incubation, cultures were centrifuged, pellets were discarded, while supernatants were taken and assayed for protease activity. Activity of protease was assayed according to Manachini *et al.* [6] by measuring the release of trichloroacetic acid soluble peptides from 1% (w/v) casein solution at 280 nm. One unit (U) of enzyme activity was defined as the amount of enzyme required to produce an increase in absorbance at 280 nm equal to 0.01 in one minute under experimental conditions and the enzyme activity and specific activity [6] were calculated as the following equations:

$$\text{Enzyme activity} \left(\frac{\text{U}}{\text{ml}}\right) = \frac{\text{Absorbance at 280 nm}}{0.01 \text{ x } 30 \text{ x } 0.2}$$

0.01: Constant
30: Reaction time (min)
0.2: enzyme volume (ml)

$$\text{specific activity} \left(\frac{\text{U}}{\text{mg}}\right) = \frac{\text{Activity} \left(\frac{\text{U}}{\text{ml}}\right)}{\text{Protein concentration} \left(\frac{\text{mg}}{\text{ml}}\right)}$$

Results and Discussion

Isolation of *Aeromonas hydrophila*

A total of 89 samples of gills pieces of fresh fish and water samples from pool of fish farms were collected from different locations in Baghdad governorate. From these samples, ninety one isolates were obtained.

Identification of the bacterial isolates

- Morphological charactristcs

The isolates were firstly identified according to their cultural and morphological characteristics. Results showed that ten of these isolates were yellow in color when grown on nutrient agar medium, and have musty odor.

- Microscopical examination

These isolates were rod in shape, non-spore forming and occurs singly when examined under light microscope.

- Biochemical tests

These isolates were subjected to biochemical analyses. Results in Table (1) showed that seven of these isolates were positive for catalase, oxidase, methyl red, indole, voges-proskauer, and citrate utilization tests, and negative for urease, and lactose fermentation.

Table (1)
Biochemical properties of A. hydrophila isolates using biochemical assays.

Isolate symbol Test	*A. hydrophila (standard) strain*	*A1*	*A2*	*A3*	*A4*	*A5*	*A6*	*A7*	*A8*	*A9*	*A10*
Catalase	+	+	+	-	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+
MR	+	+	+	+	+	+	+	+	+	+	+
VP	+	+	+	+	+	+	+	+	+	+	+
Indole	+	+	+	+	+	+	+	+	+	+	+
Citrate utilization	+	+	+	+	+	+	+	+	_	_	+
Urease	-	-	-	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+	+	+	+	+
β-hemolysis blood agar	+	+	+	+	+	+	+	+	+	+	+
Lactose fermentation	-	-	-	-	-	-	-	-	-	-	-

According to these results, these isolates could be to *A. hydrophila*. Moreover, these seven isolates were re-identified by using Api 20E system and the results in Table (2).

Table (2)
Biochemical properties of A. hydrophila isolates using API 20E system.

Test	*isolates*										
	Standard strain	*H1*	*H2*	*H3*	*H4*	*H5*	*H6*	*H7*	*H8*	*H9*	*H10*
ONPG	+	+	+	+	+	+	+	+	+	+	+
ADH	+	+	+	+	+	+	+	+	+	+	+
LDC	+	-	-	-	-	-	-	-	-	-	+
ODC	-	-	-	-	-	-	-	-	-	-	-
CIT	-	-	-	+	-	-	-	-	-	-	-
H_2S	-	-	-	-	-	-	-	-	-	-	-
URE	-	-	-	-	-	-	-	-	-	-	-
TDA	-	-	-	-	-	-	-	-	-	-	-
IND	+	+	+	+	+	+	+	+	+	-	+
VP	+	+	+	+	+	+	+	+	+	+	+
GEL	+	+	+	+	+	+	+	+	+	+	+
GLU	+	+	+	+	+	+	+	+	+	+	+
MAN	+	+	+	+	+	+	+	+	+	+	+
INO	-	-	-	-	-	-	-	-	-	-	-
SOR	-	-	-	+	-	-	-	-	-	+	-
RHA	-	-	-	-	-	-	-	-	-	-	-
SAC	+	+	+	+	+	+	+	+	+	+	+
MEL	-	-	-	-	-	-	-	-	-	-	+
AMY	+	+	+	+	+	+	+	+	+	+	-
ARA	-	-	-	+	-	-	-	-	-	-	-

3. Screening the ability of *A. hydrophila* isolates in protease production

Two methods were used to screen the ability of *A.hydrophila* isolates for production protease. The first one was semi quntitative screening which depends on the formation of halo of hydrolysis on skim milk agar medium, while the second was quantitative screening which depends on the determination of specific activity of protease.

Ability of local isolates *A. hydrophila* in protease production Semi-quantitative screening

Fig.(1) showed that *A. hydrophila* isolates were able to hydrolyze skim milk and forming halo of hydrolysis with variable degrees.

Table (3) the diameter of hydrolysis zone ranged between 6 and 24mm for different isolates, among them the isolate A4 (isolated from fish gills) was the most efficient in protease production because it recorded the highest diameter of hydrolysis (24 mm). While the other isolates had low efficient in protease production due to the lower formation of zones of hydrolysis around their colonies.

Quantitative screening for protease production by local isolates of *A. hydrophila*

Local isolates of *A. hydrophila* were screened quantitatively to examine their ability in protease production. This was achieved by growing each of the seven isolates in production broth medium for 24 hours at 30°C, then they were centrifuged and the specific activity of protease in crude filtrates was determined. Results indicated in Table (4) showed that all of the isolates were protease producers with variable degrees. Specific activity of protease in culture filtrates was ranged between 2.11 and 31.01 U/mg protein. Among them, *A. hydrophila* A4 was the most efficient in protease producion. The specific activity of protease in crude filtrate of this isolate was 31.01 U/mg protein.

Fig.(1) Proteolytic activity of A. hydrophilaA4 on 10% skim milk agar after incubation at 30•C for 24hrs.

Table (3)
Diameter of clear zone around colonies of A. hydrophila isolates grown on skim milk agar medium for 24 hours at 30•C.

Isolate	*Diameter of clear zoon (mm)*
A1	6
A2	16
A3	14
A4	24
A5	19
A6	10
A7	10

The differences in the ability of the isolates to produce protease were due to genetic variations of geneses responsible for the production of protease [7]. In another study [8] it was found that 100% of *A. hydrophila* isolates were able to produce protease but with variable degrees. While [9] confirmed 81% of *streptococcus pyogenes* isolates were able to produce protease, another study [7] found 77% of *S. pyogenes* isolates was able for producing protease.

Table (4)
Specific activity of protease in culture filtrate of locally A. hydrophilaafter incubation at 30°C for 24hr.

Isolate	*Specific activity (U/mg protein)*
A1	2.11
A2	12.03
A3	3.86
A4	31.01
A5	5.05
A6	7.89
A7	4.55

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الخلاصة

جمعت 89 عينة بيئية شملت عينات ماء اخذت من حقول تربية الاسماك و عينات اسماك طازجة، تم الحصول على 91 عزلة بكتيرية شخصت على انها *Aeromonas* spp وفقا لخصائصها المزرعية والمظهرية. اخضعت جميع هذه العزلات للاختبارات الكيموحيوية المختلفة وقد اظهرت النتائج الى ان هناك سبعة عزلات بكترية شخصت على انها *Aeromonas hydrophila* وقد تم تاكيد نتائج التشخيص باستخدام نظام العدة التشخيصية للعائلة المعوية Api-20 E. اختبرت قابلية العزلات البكترية المشخصة على اساس قابليتها في انتاج انزيم البوتييز وقد اظهرت النتائج الى ان جميع هذه العزلات السبعة كانت منتجة للبروتيز ولكن بدرجات متفاوتة، وكانت العزلة البكتيرية A4 هي الاكفا في انتاج الانزيم. حيث بلغت الفعالية النوعية للانزيم في رائق مزرعتها 31.01 وحدة/ ملغم بروتين.