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Susceptibility of the fungus Saprolegnia sp. On the secretion of extracellular enzymes

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Abstract

Some species of aquatic fungi were isolated during a period of August 2008 to July 2009 .from the environment of Shat – Al – Arab (represented by Qurna , Garma , and Ashar) and the aquariums in the Marine Sciences Center and College of Agriculture , some of the environmental factors of thewater sample such as the salinity , temperature , and the pH have been measured . Five species have been isolated and diagnosed as belonging to two genera (Saprolegniaparasitica, S. hypogena, S. ferax , Saprolegnia sp., and Achlya sp.).The enzymic activity of all the isolated fungal species has been examined. The test involves all species of enzymes (Protease, Lipase, Amylase, Phenol oxidase, Keratinase). All the examined species have given positive results concerning discovering all enzyme.

Keywords: *aquatic fungi. Enzymes, Saprolegnia , Achlya, protease.*

Introduction

Aquatic environments are a good habitat for several biological communities, including aquatic fungi (water molds), especially Oomycota. They are of economic and environmental importance, especially those belonging to the water mold order Saprolegniales (Webster & Weber, 2007). These fungi are distinguished by their ability to secrete exoenzymes that help them with nutrition and pathogenesis (Hube et al., 1994).

Microorganisms, especially fungi, play an important role in the decomposition of organic materials found in nature. Fungi exploit these materials to obtain raw materials to build their cells and obtain energy to carry out vital activities. Fungi secrete many types of extracellular enzymes, and therefore they are able to grow on Various food sources and also play an important role in the food chain because they are considered decomposers (Alexopolous et al., 1996).

Aquatic fungi are characterized by the production of swimming spores and have a wide ability to spread in rivers and lakes and are not found in seas (Chauvet, 1992 and Czeczuga, 1994).

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Researchers (Alderman &Polyase, 1986, Czeczuga et al., 1999, Czeczuga&Orolowska, 2000) mentioned that these fungi play a major role in the decomposition of plant, animal, and other remains because they are reparative, in addition to the fact that a number of them are self-sufficient. It is parasitic on fish, their eggs, economic plants, algae, crustaceans, and other living organisms. Therefore, it is considered of economic importance, as it causes economic damage to fish and plant wealth. The process of parasitism includes stages, first attacking the host with motile spores through the process of chemotaxis.

(Rand &Munden, 1993), secondly, its attachment to the host through its secretion of adhesive substances such as glycoproteins (Sing and Bartinikia-Garcia, 1975), and thirdly, the growth of spores and their penetration into the host through its secretion of enzymes (Peterson et al., 1997).

Materials and methods of work:

Water samples collection

Water samples were collected from three sites (Al-Qurna, Al-Karma, and Al-Ashar) in Basra in the middle of each month for three consecutive days and over the course of a full year, starting from August 2008 until July 2009, in addition to collecting water samples from fish farming ponds at the Marine Science Center and the College of Agriculture. University of Basra, using sterilized glass bottles using an autoclaveAt a temperature of 121°C and a pressure of 15 lb/in2 for 20 minutes. The bottle was opened under water, and the water was taken at a depth of 20-30 cm from the surface of the water. Then the bottles were closed, and after arriving at the laboratory, the samples were planted directly.

Isolation and Identification of Aquatic fungi

Immediately after bringing the water samples to the laboratory, the Baiting technique method was used to isolate water fungi (Rattan et al., 1978), using sterile Petri dishes (three replicates for each sample). A specific volume of sample water was placed in it, i.e. approximately half of the dish, then 1. One ml of anti-Chloramphenicol (250 mg/L) was added to the culture medium to avoid bacterial contamination, and then sterilized sesame seeds were added to the incubator, five seeds per plate (Al Rekabi et al., 1996), then the plates were incubatedAt a temperature of 18-20°C, it was then examined every 24 hours for 7 days using a dissecting microscope to see the undivided fungal hyphae, Coenocytic hyphae, and after the appearance of the asexual reproductive organs represented by the zoosporangia, on the basis of which the fungus was identified as a genus, and upon the formation of the sexual reproductive organs represented by the oogonia. And Antheridia, and with the help of other morphological and structural characteristics, the species was accurately identified using Lactophenol cotton blue dye, prepared according to the method (Eills, 1994).

Part of the growing colony was transferred onto glass slides containing a drop of dye, and then the glass slides were examined using a light microscope and the fungal species were diagnosed based on the following references:

(1956) Johnson, (1970) Seymour, and (1977) Muhsin

After identifying the fungal species, these species were preserved by transferring fungal hyphae from a pure colony onto plates containing corn meal agar for later use. The plates were incubated at a temperature of 18-20°C for three days, and after growth appeared, they were transferred to the refrigerator for preservation (Dick, 1965).

Culture media

The following agricultural media were used to purify and preserve the isolated fungi after their diagnosis:

A- Sabouraud's Dextrose Agar medium

This medium was prepared by dissolving 65 grams of ready-made SDA powder, produced by HiMedia.India, in a liter of distilled water, adding 250 mg/liter of the antibiotic Chloramphenicol, produced by ZMC, and sterilizing the medium in an autoclave at a temperature of 121°C and a pressure of 15 pounds/inch2 for 20 minutes.

Corn Meal Agar

This medium was prepared by dissolving 17 grams of ready-made CMA medium powder, produced by HiMedia.India, in a liter of distilled water, adding 250 mg/liter of the antibiotic Chloramphenicol, produced by ZMC, and sterilized as before.

Enzymatic Activity

Test the ability of the isolated fungal species to produce extracellular enzymes, namely lipase, protease, amylase, phenol oxidase, urease, and keratinase, on special solid culture media to detect the ability of the fungal isolates to produce these enzymes, as follows:

Lipase

The culture medium described by Sierra (1975) was used, which was prepared from the following materials

Peptone	**10gr**
Nacl	**5gr**
CaCl2.2H2O	**1.0gr**
Agar	**20gr**
D.W.	**1000ml**

Tween20 was sterilized individually in a glass beaker, then added to the culture medium at an amount of 1 ml/100 ml of the medium, then the pH of the medium was adjusted to 5.5, and the medium was poured into sterile Petri dishes and left to solidify at room temperature. After that, a 3 mm diameter disk of the isolate to be tested was transferred using a cork borer to the culture medium, and two replicates were used for each sample. The dishes were incubated at a temperature of 20°C for 48 hours. After incubation, the dishes were examined, and a halo appeared around the fungal colony indicating the secretion of the enzyme. Based on the diameter of the corona, the enzyme activity of the isolate was measured.

Protease

Two methods were used to test the effectiveness of this enzyme, as follows:

*** The first method:-**

The medium described by Hankin and Anagnostakis (1975) was used, which contains gelatin as a protein substance and is composed of:

Nutrient agar	**20gr**
D.W	**1000ml**

Sterilize the gelatin solution (8%) separately, then add it to the sterile culture medium in the amount of 5 ml/100 ml culture medium and adjust the pH to 7.4. Pour the culture medium into sterile Petri dishes and leave to solidify, then transfer a 3 mm diameter disk of the fungal colony to be tested using a cork piercer

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to the medium. The culture was used and two replicates were used for each isolate. The dishes were incubated at 20°C for 48 hours. After incubation, a transparent halo appeared around the colony indicating the secretion of the enzyme. Then the diameter of the halo was measured to determine the effectiveness of the enzyme.

***The second method:-**

He used a ready-made culture medium called Milk Media Agar, produced by the Oxoid (England) company, according to the method of Cowan (1986). This medium was prepared by dissolving 24 grams of the ready-made medium powder in a liter of distilled water, and after sterilizing it, the medium was poured into sterile Petri dishes, left to solidify, and the isolates were tested in the same manner as before.

Amylase

The culture medium was prepared from the materials mentioned below to detect the enzymatic activity of fungal isolates according to the method of Gessner (1980):

Soluble Starch	**2gr**
Peptone	**1gr**
Yeast extract	**15gr**
Agar	**18gr**

The components were dissolved in distilled water and the volume was brought to 1000 ml, then the medium was sterilized and poured into sterile petri dishes to solidify. Then a 3 mm diameter disk of the fungal colony to be tested was transferred using a cork piercer to the culture medium and two replicates were used for each isolate. The dishes were incubated at 20°C for 48 hours. After incubation, a transparent halo appeared around the colony indicating the secretion of the enzyme. The diameter of the halo was then measured to determine the effectiveness of the enzyme.

Phenol Oxidase

To detect this enzyme, the culture medium described by Gessner (1980) was used, which consists of:

Malt extract	**15gr**
Tannic acid	**20gr**
Agar	**0.8gr**

Dissolve the agar and the malt extract in 900 ml of distilled water and sterilize it alone. As for the sterile tannic acid, dissolve it in 100 ml of sterilized distilled water, then mix it with the agar medium and the sterilized malt extract and pour it into sterile Petri dishes and leave it to solidify. Then, transfer a 3 mm diameter disk from the fungal colony to be tested. To the culture medium, the dishes were incubated at 20°C for 48 hours, after which the back of the colony was colored dark brown, and the color surrounded the colony. This indicates the production of the enzyme that breaks down tannic acid, and the degree of color and its spread indicates the activity of the fungus in producing the enzyme.

Urease

This test was conducted using Urea Base Agar medium prepared according to the specifications of the manufacturer (Difco) by dissolving 2.4 g of the medium in 95 ml of distilled water, sterilizing the medium, and after cooling the medium to 50°C, 5 ml of 20% urea solution was added to it, sterilized by filtration.

Mix the mixture until it is homogeneous, then pour it into sterile test tubes and leave it to solidify into a slanted layer. The tubes were inoculated with the fungal culture by stabbing using a sterile needle. The tubes were incubated at 20°C for 48 hours. The color of the medium turned from yellow to pink, and this indicates that the test result is positive. If the color does not change, it indicates that the test result is negative, according to the method of the two scientists (Prescott). & Harley, 1996).

Keratinase

The method described by Wawrzkiewicz and his group (1991) was adopted with some modifications, and the medium consists of the following materials:

Keratin pow.	**2gr**
$MgSO_4 7H_2O$	**2.5gr**
$KH_2.PO_4$	**0.1gr**
$FeSO_4.7H_2O$	**0.01gr**
$ZnSO_4.7H_2O$	**0.005gr**
Na_2HPO_4	**3.97gr**
NaH2PO4	**3.86gr**
Agar	**15gr**
D.W.	**1000ml**

The components of the medium, except for the keratin powder, were dissolved in a liter of distilled water and the pH was adjusted to 5.6, then the medium was sterilized. After sterilization, the sterilized wool keratin powder was added to the medium, then it was poured into sterile Petri dishes and left to solidify. A 3 mm diameter disk of the fungal colony to be tested was transferred by The cork was pierced into the culture medium and two replicates were used for each isolate. The dishes were incubated at 20°C for 48 hours. A transparent halo appeared around the colony indicating the secretion of the enzyme. Then the diameter of the halo was measured to determine the effectiveness of the enzyme.

Results

Fungal species isolated during the study:

Five species belonging to two genera of aquatic fungi were isolated and identified: Saprolegnia and Achlya, which were isolated from the selected study sites represented by the Shatt al-Arab sites (Al-Ashar, Karma Ali, and Qurna) and two fish pond sites (the ponds of the Marine Sciences Center and the College of Agriculture) at the University of Basra. (Table 1).

Table (1) The total number of isolates of fungal species belonging to the genera Saprolegnia and Achlya isolated from the study sites during 12 months.

Months	Temperature range (°C)	pH range	Salinity range(‰)	*S.parasitica*	*S.ferax*	*S.hypogena*	*Saprolegnia*sp.	*Achlya* sp.
August/ 2008	26-30	7.3-8.2	2.5-2.8	0	0	0	0	0

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September/2008	21-30	7-8.3	2-2.8	4	0	0	0	0
October/ 2008	20-28	7.9-8	2-2.5	7	0	0	0	0
November/2008	16-19	7.4-8	1.7-2	11	0	0	0	2
December/2008	11-12	8.05-8.3	2-2.7	5	2	3	0	1
January/2009	10-11	8-8.3	2-2.5	7	0	1	1	3
February/ 2009	12-13	7.6-8.2	2.4-2.8	2	0	0	0	0
March/2009	18.5-18	8-8.2	2.2-2.7	3	0	0	0	0
April/2009	20-22	7.2-8.2	1.2-2.1	4	1	0	1	0
May/2009	24-26	7.9-8.2	1.2-2.7	0	0	0	0	2
June/2009	28-30	8-8.2	0.9-1.7	0	0	0	0	0
July/2009	26-28	7.9-8.2	0.9-1.8	0	0	0	0	0
total summation				43	3	4	2	8

The results showed that the total number of fungal isolates was 60 isolates, represented by 13 isolates for the Marine Sciences Center pond site, 23 isolates for the College of Agriculture pond site (Table 2), 7 isolates for the Karma Ali River site, 13 isolates for the Qurna site, and 4 isolates for the Al-Ashar site (Table 2). 3).

Table (2) Number of isolates of fungal species isolated from fish farming ponds for the Marine Sciences Center and College of Agriculture sites.

Months	**Marine Science Centre**					**Collage of Agriculture**				
	S.parasitica	**S.ferax**	**S.hypogena**	**Saprolegniasp.**	**Achlyasp.**	**S.parasitica**	**S.ferax**	**S.hypogena**	**Saprolegniasp.**	**Achlyasp.**
August/ 2008	0	0	0	0	0	0	0	0	0	0
September/2008	2	0	0	0	0	2	0	0	0	0
October/ 2008	2	0	0	0	0	3	0	0	0	0
November/2008	3	0	0	0	0	6	0	0	0	1
December/2008	0	1	0	0	1	3	1	3	0	0
January/ 2009	2	0	0	0	0	1	0	0	0	0
February /2009	0	0	0	0	0	0	0	0	0	0
March/2009	0	0	0	0	0	0	0	0	0	0
April/2009	2	0	0	0	0	2	0	0	1	0
May/2009	0	0	0	0	0	0	0	0	0	0
June/2009	0	0	0	0	0	0	0	0	0	0
July/2009	0	0	0	0	0	0	0	0	0	0
total	11	1	0	0	1	17	1	3	1	1

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summation										

Table (3) Number of isolates of fungal species isolated from the sites of Qurna, Karma Ali, and Al-Ashar

Months	Al-Ashar					**Karma Ali**					**Qurna**				
	S. parasitica	*S.ferax*	*S.hypogena*	*Saprolegnias* p.	*Achlyasp.*	*S.parasitica*	*S.ferax*	*S.hypogena*	*Saprolegnias* p.	*Achlyasp.*	*S.parasitica*	*S.ferax*	*S.hypogena*	*Saprolegnias* p.	*Achlyas* p.
August/ 2008	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
September/ 2008	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
October/ 2008	0	0	0	0	0	**1**	0	0	0	0	**1**	0	0	0	0
November/ 2008	0	0	0	0	0	0	0	0	0	**1**	**2**	0	0	0	0
December 2008/	0	0	0	0	0	**2**	0	0	0	0	0	0	0	0	0
January/2009	**1**	0	**1**	0	0	**1**	0	0	**1**	**1**	**2**	0	0	0	**2**
February/ 2009	**2**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
March/2009	0	0	0	0	0	0	0	0	0	0	**3**	0	0	0	0
April /2009	0	0	0	0	0	0	0	0	0	0	0	**1**	0	0	0
May/ 2009	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**2**
June/ 2009	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

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July /2009	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
total summation	**3**	0	**1**	0	0	**4**	0	0	**1**	**2**	**8**	**1**	0	0	**4**

Enzymatic activity of isolated aquatic fungi:

The isolated fungal species were tested to measure their enzymatic activity capacity for the following enzymes (Protease, Lipase, Amylase, Keratinase, Phenol oxidase, and Urease). All of the tested fungi showed "positive" detection for these enzymes. (Tables 4 and 5).

Regarding the protease enzyme, the fungus S. ferax showed a high enzyme activity reaching 25 mm, followed by the fungus S. parasitica (15 mm), and the fungi S. hypogena and Saprolegnia sp. The effectiveness reached 10 mm, while the fungus sp. Achlya showed an effectiveness of 5 mm, which is the least effective of the fungi. (Table 4).

As for lipase enzyme, the fungus Achlya sp. The highest effectiveness reached 25 mm, followed by the fungi S. parasitica and S. ferax, whose effectiveness was 20 mm. As for the fungus Saprolegnia sp. Its effectiveness was 15 mm. As for the fungus S. hypogena, its effectiveness was the least active type, reaching (10 mm). (Table 4).

As for the amylase enzyme, the fungus S. parasitica showed a high activity of 20 mm, followed by the fungus Saprolegnia sp. And Achlya sp., their effectiveness reached 15 mm each. While the fungi S. hypogena and S. ferax showed an enzyme activity of 10 mm. (Table 4).

The fungi S. parasitica and S. ferax showed a high activity of the keratinase enzyme, reaching 25 mm, then the fungus Saprolegnia sp. The effectiveness of which reached 20 mm, and then the fungus Achlya sp. Its effectiveness reached 15 mm, while the fungus S. hypogena was the least active fungus, reaching 10 mm (Table 4).

As for the phenol oxidase enzyme, the S. parasitica fungus showed a high activity of 15 mm. As for the fungi S. ferax and Achlya sp., the activity of each was 10 mm. As for the fungi S. hypogena and Saprolegnia sp. Their effectiveness reached 5 mm each. (Table 4).

All fungi showed the ability to secrete the enzyme urease by changing the color of the culture medium from yellow to pink. (Table 5).

Table (4) Enzymatic activity of aquatic fungi isolated in the study

Species	Keratinase		Protease		Lipase		Amylase		Phenol oxidase	
	Diameter* of colony	Diameter* of the areola	Diameter* of colony	Diameter* of the areola	Diameter* of colony	Diameter* of the areola	Diameter* of colony	Diameter* of the areola	Diameter* of colony	Diameter* of the areola
Saprolegniaparasitica	**20**	**25**	**10**	**15**	**20**	**20**	**25**	**20**	**15**	**15**
S. hypogena	**20**	**10**	**20**	**10**	**15**	**10**	**15**	**10**	**10**	**5**
S.ferax	**25**	**25**	**25**	**25**	**30**	**20**	**30**	**10**	**10**	**10**
Saprolegnia. Sp.	**20**	**20**	**30**	**10**	**25**	**15**	**20**	**15**	**15**	**5**
Achlya Sp.	**15**	**15**	**25**	**5**	**20**	**25**	**30**	**15**	**15**	**10**

Colony diameter and areola diameter measured in (mm)

Table (5): Enzymatic activity of aquatic fungi isolated in the study

Species	Keratinase	Protease	Lipase	Amylase	Phenol oxidase	Urease
S.parasitica	+	+	+	+	+	+
S. hypogena	+	+	+	+	+	+
S.ferax	+	+	+	+	+	+
Saprolegnia. Sp.	+	+	+	+	+	+
Achlya Sp.	+	+	+	+	+	+

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Discussion

Isolated fungal species:

Some aquatic fungi of the Saprolegniaceae family were isolated and identified from the waters of the Shatt al-Arab and fish farming ponds. Five species were identified with both sexual and asexual phases (Saprolegniaparasitica, S. ferax, S. hypogena, and Saprolegnia. sp. and Achlya sp. These species were previously recorded by Muhsin (1977) and some of their species were also recorded by Ismail et al (1979) and some of them were also recorded by (Toma Zia, 1985). As for the species S. hypogena, it was recorded by Muhsin et al. al. (1994).

Enzymatic activity of fungi:

The enzymatic activity of the isolated fungal species was effective for all species and for all enzymes tested. As for the lipase enzyme, it works to maintain the function of the fungal cell membrane and helps the pathogenic fungi in invading the host tissues (Das and Banerjee, 1974), as all species gave a "positive" detection for this. This enzyme is consistent with what Cochrane (1985) stated that the lipase enzyme is produced by most fungi.

As for the protease enzyme, all fungal species showed a positive result in the test. This is due to the high ability of these species to degrade proteins. This enzyme plays an effective role in the pathogenicity of fungi, as the fungus decomposes the protein contents of the membranes of fish or eggs when they are infected.

(St-Leger et al., 1997).

As for the amylase enzyme, all fungal isolates gave a positive test result, and this indicates the high ability of these fungi to break down starch molecules into simple sugar molecules to benefit from them for nutrition. Thus, such fungi are able to grow on plant materials available in the aquatic environment.

As for the enzyme phenol oxidase, all of the fungal isolates tested gave a positive test result, and this indicates the ability of these fungi to degrade lignin, which is a different polymer made up of heteropolymer units of phenylpropanoid, which is found in the tissues of woody plants and gives them hardness and resistance to biological agents (Saparrat et al., 2002).

As for the keratinase enzyme, all of the tested fungal isolates gave a positive test result, and this confirms the ability of these fungi to degrade keratin available in the aquatic environment in fish and crustaceans, which is a protein substance with a complex structure.

As for the urease enzyme, all of the tested fungal isolates gave a positive test result, and this indicates the ability of these fungi to decompose urea into ammonia and carbon dioxide.

The secretion of such enzymes by aquatic fungi helps them grow on nutrient media

There are a variety of sources available in aquatic environments, and some of these enzymes help in the mechanics of injuryFor living organisms such as fish, especially the enzyme protease and lipase.

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