RESEARCH ARTICLE

Morphological and Molecular Identification of Aquatic Fungi in Tigris River for some Areas in Salah Al-Din province and Evaluating their Enzymatic Activity

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ABSTRACT

Thirty-eight fungal isolates were isolated from the water of Tigris river for three districts within Salah Al-Din Governorate, including Tikrit (the university site), Al-Dur, and Samarra, from November 2020 to January 2021. These isolates were identified phenotypically and molecularly into 16 species using nucleotide sequence analysis of the 5.8s rRNA gene, four isolates for each of *Phytopythium palingenes* (J21, J33, J5, and J14), *Fusarium graminearium* (D1, D10, J4, and J31) and *Penicillium expansum* (D8. D37, D38, and J36) with an appearance of 10.53% for each of them, three isolates for each species *Saprolegnia australis* (S20, S18, and D2), *Aspergillus sydowii* (S35, D12, and J11) and *Geotrichum candidum* (S17, J15, J27) with an appearance rate of 7.90 %, two isolates of species *Saporlegnia polymorpha* (S24, S26), *Boeremia exigua* (J6, S25), *Cladosporium allicinum* (J19, J3), *Pencillium spinulosum* (S22, D34), *Cytospora chrysosperma* (J7, J16) and *Pencillium polonicum* (D9, D30) with an appearance rate of 5.26%. Finally, one isolate of the species *Cladosporium cladosporioids* (J13), *Phytopythium oedochilum*(J28)*, Pilidium concavum* (D29), *Phytophthora hydropathica* (J32*)* and isolate S23 with an appearance rate of 2.63%. The fungal isolate S23 was identified phenotypically within sterile hyphae, with no match for this isolate in the NCBI World Genetic Bank, as it is either a new isolate that has not been discovered or that has not been registered at the NCBI site yet. The results showed the ability of these fungal isolates to produce protease, lipase, and laccase. The production of all fungal isolates of these enzymes was recorded with the superiority of isolates of *P. polonium* (D9), *Saprolegnia australis* (S18 and S20) in protease activity reached 1.57, 1.17, and 1.15 units per mg (U/mg), respectively. The fungal isolates *B. exigua* (J6), *S. australis* (S18), and *F. graminearium* (D1) were significantly superior on other isolates, with the highest lipase-specific activity reaching 5.81, 5.65 and 5.22 U/mg, respectively. At the same time*, P. expansum* (D8), *C. allicinum* (J3) and *A. sydowii* (J11) were significantly superior to other isolates, with the highest laccase specific activity reaching 0.42, 0.32 and 0.26 unit/mg protein, respectively.

Keywords: Aquatic fungi, Enzymatic activity, Molecular diagnosis, Tigris river.

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INTRODUCTION

Aquatic fungi are organisms that have adapted themselves to life in the aquatic environment, and their structures have been modified to suit this environment. That characterizes the fungi that inhabit the aquatic environment; they spend their entire life cycle in the water, while some of them spend a part of their life in the water. Such fungi are called amphibious, and fungi that spend their entire life cycle on land, called terrestrial fungi.[1,2] Therefore, it is necessary to differentiate between these fungal species according to the environment in which they are endemic. Despite the possibility of the presence and spread the terrestrial fungi in the water as a result of their spores that spread by wind. Therefore, some of these fungi may spend a temporary period in the water[3,4] named some terms to distinguish between freshwater fungi such as indigenous organisms, which are aquatic fungi that spend all their life cycle in the fresh aquatic environment. In contrast, the term immigrant organisms refer to the fungi that naturally possess habitats outside the water and are sometimes transported to the water at regularly alternating intervals. Finally the versatile

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fungi that are found in the watery by chance and not regularly. The fungi endemic to the water are completely adapted to aquatic life, and the level of their biomass is more stable from year to year, especially when the conditions are appropriate and with the availability of nutrients.[5,6] As well as forming zoospores that can swim in the water.[2]

Aquatic fungi are important microorganisms in the ecological balance, especially in the freshwater environment, as they decompose and break down organic materials by possessing a distinct enzymatic system that enables them to degrade the organic materials,[7] such as photolytic enzymes which are among the important virulence factors produced by fungi that are pathogenic to fish, crustaceans, and even insects found in the aquatic environment.[8] In addition to the great role played by aquatic fungi through their activity of protease, laccase, and lipase in the decomposition of organic matter and many pollutants in the aquatic environment.[9-11] Aquatic fungi are important organisms within the food chains of the ecosystem, as they represent decomposing organisms that can secrete wide ranges of enzymes that help them to degrade the dead organisms. Plant-animal remains, as well as parasitize on aquatic organisms.[12]

Aquatic fungi play major roles in aquatic ecosystems and in the transitional regions, similar to terrestrial fungi in the nutrients cycle and decomposing organic compounds.[13] For the importance and limited studies of aquatic fungi in the Salah El-Din Governorate, the current study aims to isolate aquatic fungi from Tigris river passing through Salah El-Din Governorate, morphological and molecular identification of these fungi, and estimation of their enzymatic activities.

MATERIALS AND METHODS

Sample's Location

Three locations were selected for collecting samples in the Tigris River within Salah al-Din Governorate. The following sites were included :

University of Tikrit site in the Tikrit region, water samples were collected on 22 November 2020, at a temperature of 18°C in the morning. The collection site was determined by the global positioning system (GPS) at the line Longitude 43.663529 and latitude 34.680198.

At the second location in Al-Dour city, water samples were collected on 20 December 2020, at a temperature of 15°C in the morning, at 43.778975 longitudes and 34.474023 latitudes.

The third location is in Samarra city. Samples were collected on 10 January 2021 at 11°C in the morning, at longitude 43.845057 and latitude 34.192938.

Sample Collection Method

Water samples were collected in clean and sterilized plastic bottles of 2 liters capacity. The water temperature was measured at the collection sites, while the total dissolved salts and pH was measured in the laboratory.

The samples for biological examinations and isolation of aquatic fungi were collected in dark- sterile 250 mL glass bottles. The bottle was opened under the surface of the water at a depth of 10 to 30 cm to avoid contamination with non-aquatic fungi from air fungi. The bottles were closed while they were underwater, then transferred to the laboratory by a chilled cork container to preserve the properties of the water. The samples were kept at a temperature of 4°C.[14,15]

Determination of Some Physical and Chemical Properties of Studied Water Site in Tigris River

Water Temperature

The water temperature was measured at the study sites using a digital thermometer.

Water pH

The water pH was measured by pH meter after calibrating the device using standard solutions (pH9,7,4) according to the method of Richards (1954).[16]

Total Dissolved Solids (TDS)

Total dissolved salts were measured by the TDS device, and expressing the results in mg/liter according to the method of Cooper (1977).[17]

Chemical Oxygen Demand (COD)

COD was measured using the volumetric method by oxidation with potassium permanganate, according to Gupta, (2000),[18] the COD was calculated by the flowing equation:

COD ($mg.L^{-1}$) = (V1-V2) C/Vs

C: concentration titrate solution ($m.mole.\ L^{-1}$)

V1: Volume of blank titrate solution

V2: Volume of sample titrate solution

Vs: Volume of water sample

Determination of Microbial Account

The total account of fungi was estimated depending on the standard method of counting using the dilution method,[19] by mixing 10 mL of the water sample for each of the three samples separately, with 90 mL of distilled water to get a dilution of 10^{-1}, the series of dilutions was achieved until 10^{-2}. 1-mL of the last diluted solution was placed in a Petri dish, then the sterile and cooled potato dextrose agar (PDA) containing Chloramphenicol (250 mg/L), was poured before it solidified, mix carefully and the dishes were incubated at 25 ± 2ºC for 3–5 days with the observation of fungi growth. The grown fungi were calculated according to the equation:

Total account of fungi (CFU) = number of colonies x inverted dilution

Then purified by transferring part of the growing colonies into new PDA media separately and incubated as the above method.

For the bacterial account, the same method is followed. One mL of 10^{-3}–10^{-5} dilution was placed in a Petri dish, then the sterile and cooled nutrient agar (NA) was poured before it solidified, mixed carefully and the dishes were incubated at 35±2ºC for 24–48 hours, with observation of bacterial growth. The grown bacteria were calculated according to the equation:

Total account of bacteria (bacterial cell) /mL) = number of colonies x inverted dilution

Isolation and Purification of Aquatic Fungi by Dilution Method

Isolation of aquatic fungi was carried out according to the dilution method as above method. The growing fungal colonies were purified by transferring part of the growing colonies into new PDA media separately and incubated at 25 ± 2ºC for 3-5 days.

Isolation of Aquatic Fungi by Bait Method (Direct Isolation)

The method of Al-Shibli and Zamili (2017)[20] was followed for isolation of aquatic fungi from water samples with three replicates for each studied location. 15 mL of water sample was added to sterile Petri dishes (diameter 9 cm), 10 sterilized seeds including wheat *Triticum aestivum*, millet *Pennisetum glaucum*, sesame *Sesamum indicum*, and rice *Oryza sativa* (sterilization in autoclave at 121°C , pressure of 1.5 kg/cm^2 for 15 minutes) distributed to each Petri dish, incubated at 18–20°C for 7–10 Days, after the appearance of the developing fungal hyphae on the seed bait, transferred by a sterile needle to Petri dishes containing PDA.

Evaluation of Several Media for Isolation of Aquatic Fungi

The commercial media, including PDA, Malt extract agar (MEA), and Peptone yeast dextrose agar (PYDA), were evaluated for the isolation of aquatic fungi.[19] In addition to preparing the modified media from the wheat, millet, sesame, and rice grains, briefly, 50 g of each grains powder were added to 1-L. of DW then 5 g of Agar was added after homogenized in a magnetic stirrer at 80°C, the media were sterilized in autoclave at 121°C, the pressure of 1.5 kg/cm^2 for 15 minutes. These media were inoculated with water samples from the studied sites, as mentioned above.

Morphological Identification of Fungi

The morphological identification of isolated fungi was carried out according to the macroscopic characteristics that included the color, texture, and sizes of fungal colonies, microscopic characteristics (shape, size and number of conidia cells), conidiophore branching, sporangia and fungal hyphae, depending on the taxonomic keys of fungi.[21]

Preparation of Crude Enzyme

The nutrient medium is prepared for the production of protease and laccase according to the method of Khushal *et al*. (2010)[10] by dissolving 10 g of glucose, 0.4 g of HPO4 K2, 0.001 g of ZnSO4, 0.05 g of MnSO4, 0.5 g of MgSO4, 0.6 g of KH_2PO_4, 0.0005 g of FeSO4, 3 g of Peptone in one liter of distilled water, the pH is adjusted to 6. For the lipase medium, the method of Falong *et al*. (2006)[22] is adopted in the preparation of this medium using olive oil (as a substrate) 20 mL, 12 g of NaH_2PO_4, 2 g of KH_2PO_4, 0.3 g of $MgSO_4.7H_2O$ 0.25 g of $CaCl_2$ and 10 g of (NH_4) SO_4 dissolved in one liter of distilled water and adjusted the pH to 6.

Then all media were sterilized in an autoclave at 121°C, pressure of 1.5 kg/cm^2 for 15 minutes. After cooling at room temperature, the media were inoculated with a piece of the fungal colony (0.5 cm in diameter) from each isolates then incubated at 18–20 C for 10 days. After the incubator, the media were filtered using filter paper (Whatman NO.1) and centrifuged at 5000 rpm for 10 minutes to obtain a filtrate free of fungi cells as a crude enzyme.[23]

Determination of Protease Activity

Protease activity was Estimated as follows:

A 0.1 mL of the crude enzyme was mixed with 0.9 mL of casein solution(1%), then incubated in a water bath for 20 minutes. A 2 mL of TCA is added to the mixture to stop the reaction, then centrifuged at 6000 rpm for 5 minutes. Absorbance was measured in a spectrophotometer with a wavelength of 280 nm. One unit of protease activity was expressed as the amount of enzyme required to casein hydrolyses and produced a 0.001 change in absorbance ($\lambda = 280$)

Specific activity (enzyme unit/mg protein) = enzyme activity (unit/mL) ÷ protein concentration (mg/mL).

Determination of Laccase Activity

It was estimated according to Mahadevan and Sridhar (1986). 5 mL of the guaicol solution (1%) at a temperature of 25°C was mixed with 0.1 mL of the crude enzyme. After 5 minutes, the absorbance was measured at a wavelength of 470 nm, then the laccase activity was estimated according to the following equation:

$$\text{One unit} = \Delta A_{470} \text{ of } 0.01/\text{min}$$

Then the specific activity was calculated

Determination of Lipase Activity

Lipase activity was estimated according to the titration method as mentioned by Watanabe *et al*. (2002)[24] through titration of the fatty acids resulting from lipolysis by lipase against NaOH solution. One unit of lipase is defined as the amount of enzyme that releases 1-µmol of fatty acids in one minute. Then the specific activity was calculated.

Protein Estimation

The Biuret method was used to determine the protein in the crude enzymes; 1-mL of the crude enzyme was added to 4 mL of Biuret solution and then incubated for 20 minutes at 37°C. The absorbance is measured at a wavelength of 540 nm. Protein concentration was determined to depend on the standard curve.[17]

Appearance Rate of Aquatic Fungi

Appearance rate of aquatic fungi isolated from the studied sites was calculated according to the equation:

Appearance rate = number of appearance times of isolate / (total samples number) x 100%

Molecular Identification

The fungal isolate was diagnosed to the species level according to the molecular method based on the analysis of nucleotide sequences of the 5.8 S rRNA gene.

Genomic DNA Isolation

A swab (100 mg) of a newly grown colony was used for each

fungal isolate separately. The genomic DNA was extracted using the ready-made ZR Fungal/Bacterial/Yeast DNA Mini-Prep TM kit supplied by ZR American company. The genomic DNA was extracted according to the manufacturer's instructions.

Polymerase Chain Reaction (PCR)

Maxime PCR PreMix Extraction Kit (i-Taq) 20µLrxn (Cat. No. 25025) was used for PCR. The 5.8S rRNA gene was amplified using PCR technology with the use of the primer pair, Forward 5′- TCCGTAGGTGAACCTGCGG -3′ Reverse 5′ TCCTCCGCTTATTGATATGC-3′ [25] this universal primer supplied from (Integrated DNA Technologies company, Canada).

Procedure and Preparation of the PCR Reaction

PCR technique was used to amplify the ITS region in DNA by the following components depending on the leaflet attached to the Premix reaction mix (i-Taq DNA Polymerase at 5U/µl, DNTPS at 2.5Mm, Reaction buffer (10X) at 1X, Gel Loading buffer at 1X. The components of the reaction mixture with their concentrations were a final volume of 25 µL as follows: Taq PCR PreMix at a concentration of 5 µL, Forward Primer at a concentration of 10 picomol/mL, 1 µL Reverse primer at a concentration of 10 picomol/mL(1 µL), DNA at a concentration of 1.5 µL, Distill water (16.5) µL)

Agarose Gel Electrophoresis

Applied biosystem gene-amp PCR System 9700 thermal polymerization device (TPD) was used to amplify the mentioned gene. Table 1 shows the standard reaction conditions. The PCR product was separated using electrophoresis on agarose gel (1.5%), then the genomic DNA bands were shown using UV rays with a length of wavelength (302 nm) after treatment with the dye (Intron Korea red stain).

Table 1: Polymerase chain reaction conditions

No.	*Phase*	*Tm(C)*	*Time*	*No.of cycle*
1	Initial denaturation	95 ˚C	5min	1 cycle
2	Denaturation	95 ˚C	45sec	
3	Annealing	58 ˚C	45sec	35 cycle
4	Extension -1	72 ˚C	45sec	
5	Extension -2	72 ˚C	7min	1 Cycle

Nucleotide Sequencing Analysis

The nucleotide sequences of the PCR amplified gene were determined after obtaining the 5.8S rRNA amplification directly by sending a volume of 25 µL of PCR product and a volume of 10 µL (10 picomole concentration) of each primer to the Korean company Biotechnology Lab (Applied Biosystem 3730XL, DNA Sequencer device used). The results were compared using a web-based computer program (the basic in-situ nucleotide sequence search tool named basic local alignment search tool (BLAST) with the database at the National Center for Biotechnology Information (NCBI), which matches the nucleotide sequences of the studied genes with respect to the fungal isolates placed in the search and knows their species according to the match in the database mentioned above.

Statistical Analysis

The study experiments were implemented using the completely randomized design (CRD), the analysis of variance was conducted using the SPSS program, and the means were compared according to the least significant difference (LSD) test at the probability level of 0.05.[26]

RESULTS AND DISCUSSION

Water Analysis

Table 2 shows the water temperatures varied in the studied locations, ranging between 11 to 18°C. Tigris River water in Samarra siete recorded the lowest temperature because samples were collected in winter. In contrast, the Tikrit site recorded the highest water temperature of 18°C when water samples were collected in autumn. Temperature is an important factor in aquatic fungi's growth and spread. The temperature range of 15 to 25°C is optimum for the growth and flourishing of fungi in aquatic environments. This is consistent with the study of Al-Shindah (2008).[27] The Tigris River was characterized by moderate pH in all studied areas, pH values were 7.3, 7.4, and 7.3 for Tikrit, Al-Dur and Samarra sites, respectively, the highest pH was 7.4 in December at Al-Dur site, while the lowest pH was 7.3 in January and November in Tikrit and Samarra sites, this agreed with Issa *et al*. (2011)[12] and Mahmoud *et al*. (2018).[28]

Tigris River is one of the fresh rivers, and this is evident by the low total dissolved salts (TDS) of the three sites, which were

Table 2: Average some physical, chemical, and biological properties in three sites of Tigris River.

Water parameters	*Tikrit sites*	*Al-Dur sites*	*Samara sites*	*Standards*
Water temperature (°C)	18	15	11	35 fewer than
PH	7.3	7.4	7.3	*6.5–8.5 **5–9
COD(mg.L^{-1})	1.53	1.06	1.5	*150 **90
Total dissolved salts (TDS (mg/L	228	225.6	207	*100 **45
Total bacteria account of cell/mL)	3×10^5	4.1×10^3	1.94×10^4	
Total fungi account of (CFU/mL)	0.15×10^2	0.1×10^2	0.1×10^2	

* Iraqi Standard ** International Standard according to FAO

228, 225.6, and 207 mg/liter in Tikrit, Al-Dur, and Samarra, respectively. The chemical oxygen requirement COD values in the three collection sites reached 1.53, 1.06, and 1.5 mg/L in Tikrit (Table 2), Al-Dur, and Samarra, respectively. According to these results, TSS and COD were within the environmentally permissible limits of the Iraqi and international specifications for drinking and irrigation purposes, which ranged between 45-100 mg/L. for TDS and 1.06 - 1.53 mg/L. for COD, The results agreed with Mohammed (2018)[28] in his study in some Tigris river sites. The results showed that the total account of bacteria in the waters of Tigris river sites were 3×10^5, 4.1×10^3, and 1.94×10^4 bacterial cells/mL, while the total account of fungi were 0.15×10^2 and 0.1 $10^2\times$ and 0.1×10^2 CFU /ml in Tikrit, Al-Dur and Samarra sites, respectively, (Table 2). These results indicate that the water of the Tigris river for all collection sites was free of pollutants and were within the permissible limits for drinking and irrigation purposes as well as for industrial purposes according to the Iraqi specification for 1991.[29] and International specifications for the year 2011.[15]

Appearance Rate of Fungal Isolates by Baits Method

Eleven fungal isolates were isolated using the bait method; the results in Table 3 showed that the bait of sesame, *Sesamum indicum*, was significantly superior to other baits, as it recorded the highest appearance rate reached 45.46%, followed by 36.36% in the bait of rice *Oryza sativa*. In contrast, the lowest appearance rate was 9.09% for both baits, millet *Pennisetum glaucum*, and wheat *Triticum aestivum*.

Appearance Rate of Fungal Isolates Cultivated in Laboratory Media

Table 4 showed that the total number of fungal isolates reached 27 isolates, with a significant superiority of the PYDA medium specialized for aquatic fungi, with an appearance rate of 29.63 %, followed by MEA and sesame Agar, which recorded an appearance rate of 18.52% for both media. In contrast, no growth of fungi was recorded in the wheat agar medium. The Tikrit site showed the highest number of fungal isolates, reaching 11 isolates compared to 8 isolates in Al-Dur and Samarra regions.

Identification of Fungal Isolates

A total of 38 fungal isolates were isolated from the water of the Tigris River passing in Salah al-Din Governorate at three sites (Tables 2 and 3). Depending on the morphological characteristics, the fungal isolates were classified to the genus level, such as fungal colony shape, color, colony diameter, and height, as well as microscopic features such as the presence or absence of septa in the fungal hyphae, shape, presence or absence of conidia, size, and color of conidia and presence or absence of sexual and sexual structures, according to Available taxonomic sources.[21,24,30-35] While the fungal isolates were classified to the species level using the molecular method.

Molecular Identification of Fungal Isolates

Figure 1 shows the bands resulting from the electrophoresis of genomic DNA. A single band for each fungal isolate indicates the accuracy of the genomic DNA extraction. Figure 2 shows the electrophoresis of the PCR product using the specialized primer for 5.8S rRNA gene amplification. The results show the presence of bands with a size of 650 base pairs, indicating the accuracy of this test and all the bands related to fungal isolates.[25] Table 5 shows the molecular diagnosis of these isolates to the species level. The results listed in this table show the percentage of similarity between the fungal isolates and the extent of their conformity with the world registered fungal species in the country from which they are isolated. The similarity ratio reached 83.49–100% with the world registered strains, confirming the diagnosis's accuracy. Although there is a similarity to the aquatic fungi

Table 3: Appearance rate of fungal isolates by baits method.

	Water sites				
Type of bait	*Tikrit*	*Al-Dur*	*Samarra*	*Total number of isolates*	*Appearance rate (%)*
Season	2	1	2	5	45.46
Rice	2	1	1	4	36.36
millet	1	0	0	1	9.09
wheat	1	0	0	1	9.09
Total number of isolates	6	2	3	11	

Table 4: Appearance rate of fungal isolates cultivated in laboratory media

	Locations				
Media	*Tikrit*	*Al-Dur*	*Samarra'*	*Total number of fungal isolates*	*Appearance rate of fungal isolates (%)*
MENU	3	1	1	5	18.52
PDA	2	1	1	4	14.81
PYDA	3	3	2	8	29.63
Rice Agar	1	1	1	3	11.11
Millet Agar	1	0	1	2	7.41
Wheat Agar	0	0	0	0	0
Total number of isolates	11	8	8	27	

Table 5: Molecular identification of the aquatic fungi, according to the percentage of matches of the 5.8S rRNA gene sequences with the strains of fungal strains in the World Genetic Bank.

Code of fungi isolate	*fungal species that match*	*Accession number*	*Country*	*Similarity (%)*
J6	*Boeremia exigua* isolate H3	MT154624.1	china	99.80
13J	*Cladosporium cladosporioides* strain MCCC3A00182	MT258647.1	china	99.40
20S	*Saprolegnia australis* isolate SC1	MK046073.1	Portugal	99.57
35S	*Aspergillus sydowii* isolate M4-C3	MN968365.1		99.61
21J	*Phytopythium palingenesis* isolate VN429	MN872742.1	Czech republic	83.49
7J	*Cytospora chrysosperma* isolate HMBF_CNHrm1	KC880156.1	china	99.14
23S	Doesn't match	-	-	-
24S	*Saprolegnia polymorpha genes*	AB219394.1	Japan	98.68
22S	*Penicillium spinulosum* clone mcs11.k	MK131675.1	Canada	99.62
19J	*Cladosporium allicinum*	MF472917.1	Holland	99.63
1D	*Fusarium graminearum* isolate Fg-W18	MT636316.1	India	99.44
17S	*Geotrichum candidum* strain JYC554	MN244396.1	Taiwan	99.71
29D	*Pilidium concavum* isolate OP83	MF776040.1	America	100
9D	*Penicillium polonium strain TLN1*	KX421790.1	Pakistan	96.36
8D	*Penicillium expansum strain QZ1*	MK660357.1	Spain	99.64
32J	*Phytophthora hydropathic isolate HV177*	KC734446.1	Switzerland	99.86
28J	*Phytopythium oedochilum isolate 77*	KJ865237.1	America	100

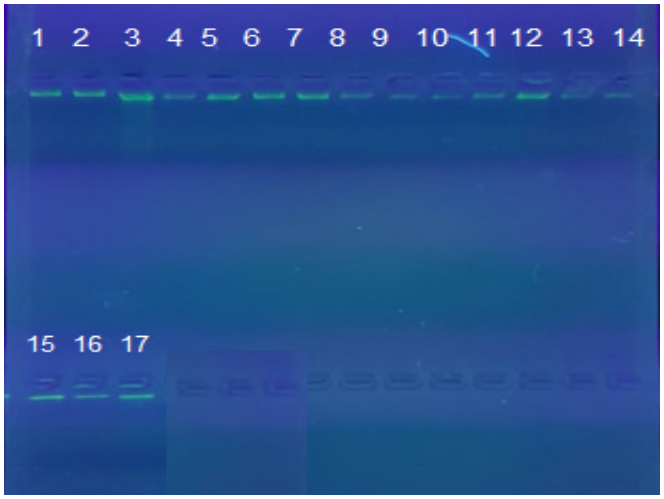

Figure 1: Electrophoreses of genomic DNA of aquatic fungi **1-S20, 2-S24, 3-J35, 4-J28, 5-J21, 6-S17, 7-D9, 8-J6, 9-S32, 10-J19, 11-J32, 12-D29, 13 -J7, 14-S22, 15-D1, 16-D8, 17-S23.**

isolated in this study in matching the nucleotide sequences, they are genetically different isolates, so the match was not 100%. This may be due to the recombination from sexual reproduction or mutations that occur in the fungi as a result of their presence in different environments, which may contain chemical compounds such as pesticides, hydroxyl radicals, and phenols formaldehyde and purine analogs.[36] Other studies have confirmed that the excessive use of chemical and agricultural pesticides of various kinds leads to genetic changes in the community of aquatic fungi.[37-40] The variation in the genetic and identification of fungal strains consistent with Hassan *et al.* (2022)[41] and Hassan and Ajaj (2022)[42] in their studies of fungal classification depend on nucleotide sequences of the ITS1-ITS4 region.

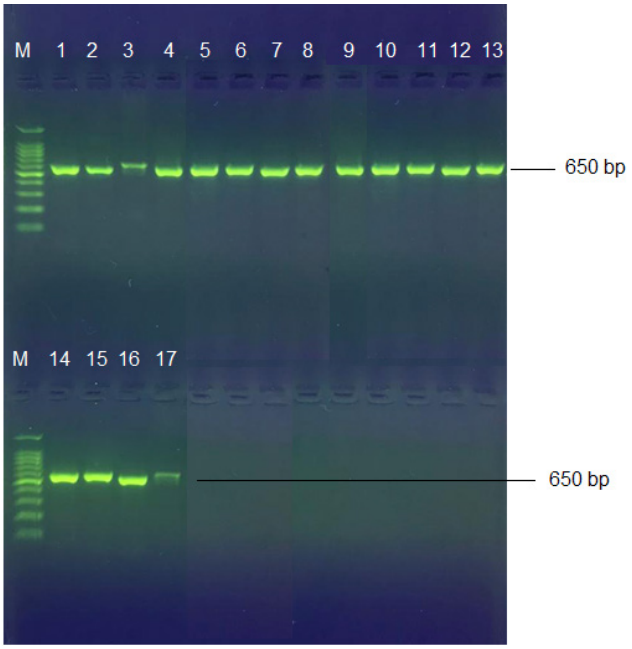

Figure 2: Electrophoresis of PCR products of aquatic fungi **1-S20, 2-S24, 3-J35, 4-J28, 5-J21, 6-S17, 7-D9, 8-J6, 9-S32, 10-J19, 11-J32, 12-D29, 13 -J7, 14-S22, 15-D1, 16-D8, 17-S23.**

The results included in Table 4 showed that the taxonomic ranks of the isolated aquatic fungi according to the NCBI website were distributed in 2 phylum; Oomycota related to Chromista and Ascomycota related to Mycota (Fungi). The species related to Oomycota including; *Saprolegnia australis, Saporlegnia polymorpha Phytopythium, palingenesis, Phytopythium oedochilum Phytophthora hydropathical,* while Ascomycota included the fungal species*: Boeremia exigua* and *Cladosporium, cladosporioids, Cladosporium*

allicinum, Aspergillus sydowii Penicillium, spinulosum, Penicillium polonium, Penicillium expansum and *pilidium, concavum, Fusarium graminearium, Geotrichum candidum* and *Cytospora chrysosperma.* Although the isolate S23 was phenotypically diagnosed as a fungal isolate via its response to the universal fungi primer, we did not get a match with any fungal strains.

The diversity of these fungal isolates is attributed to their presence in different geographical areas along the Tigris river within Salah El-Din Governorate. The difference between this geographical diversity and its impact on the water of the Tigris river, especially with organic materials content that provides the nutrients such as amino acids, carbohydrates, and others that are necessary for the growth of these fungi as well as the remnants of dead plants from stems and other parts of plants that fall into the river water. Filtration of water from crops irrigation is another important source of these nutrients, as well as the presence of aquatic organisms, including fish and others, which encourages the presence and flourishing of many types of aquatic fungi, some of which are important pathogens of fish, as they cause significant economic losses to the thick wealth[2] In addition to the availability of suitable environmental conditions for the growth and flowering of water fungi in the Tigris river, such as the purity of the water in the studied sites, where the average of TDS reached 220.2 mg/L., as well as the moderate pH which reached 7.37 with the low temperature during autumn and winter seasons, which are among the best seasons for the reproduction and spread of aquatic fungi. This is consistent with the study of Al-Shibli and Al-Zamili, (2017) on aquatic fungi in southern Iraq.[20]

Appearance Rate of the Molecularly Identified Fungal Species

Table 6 shows the appearance rate of the molecularly identified aquatic fungi under species level. According to molecular identification, 16 fungal isolates were identified to the species level. The highest appearance rate was recorded for the fungal species *Phytopythium palingenes*is, *Fusarium graminearium, Penicillium expansum*, resulting in 10.53%, followed by *Saprolegnia australis*, *Aspergillus sydowi, Geotrichum candidum*, which reached 7.90%, while the species *Cladosporium, cladosporioids*, *Phytopychilum hydrothium*, *Pilopathium conidium* recorded the lowest appearance rate of 2.63%.

The seventeenth fungal isolate (S23), although it gave a positive result for the reaction with specialized primer to amplify the fungi gene 5.8 SrRNA, this isolate did not match any of the fungi recorded in NCBI World Genetic Bank, as it is either a new isolate that has not been discovered or that it has not been registered at the NCBI site yet.

Enzymatic Activities of the Aquatic Fungi

Protease Activity

The results in Figure 3 showed that all isolated aquatic fungi have a specific activity of the protease with a significant superiority of the fungal isolates *Penicillium polonium* (D9). The isolates of *Saprolegnia australis* (S18 and S20) where the highest specific activity of the protease was 1.57, 1.17, and 1.15 unit/mg protein, respectively, while the fungal isolates *Aspergillus sydowii* (D12), *Geotrichum candidum* (J14) and *Cladosporium cladosporioids* (J13) recorded the lowest specific

Table 6: Appearance rate of the molecularly identified fungal species

Code of fungal isolate	*Fungal species*	*Sites*			*Total number of isolates*	*The appearance of the fungal isolate (%)*
		Tikrit	*Al-Dur*	*Samarra*		
S20,S18,D2	*Saprolegnia australis*	0	1	2	3	7.90
S24, S26	*Saprolegnia polymorpha*	0	0	2	2	5.26
J6, S25	*Bohemia exigua*	1	0	1	2	5.26
J13	*Cladosporium cladosporioids*	1	0	0	1	2.63
J19,J3	*Cladosporium allicinum*	2	0	0	2	5.26
S35,D12,J11	*Aspergillus sydowii*	1	1	1	3	7.90
S22, D34	*Penicillium spinulosum*	0	0	2	2	5.26
J7, J16	*Cytospora chrysosperma*	2	0	0	2	5.26
J21,J33,J5,J14	*Phytopythium palingenesis*	2	0	2	4	10.53
J28	*Phytopythium oedochilum*	1	0	0	1	2.63
J32	Phytophthora hydropathical	1	0	0	1	2.63
S17,J15, J27	*Geotrichum candidum*	2	0	1	3	7.90
D1,D10,J4,J31	*Fusarium graminearium*	2	2	0	4	10.53
D29	*Pilidium concavum*	0	1	0	1	2.63
D9,D30	*Penicillium polonicum*	0	2	0	2	5.26
D8, D37, D38, J36	*Penicillium expansum*	2	2	0	4	10.53
S23	Fungal isolate SA23	0	0	1	1	2.63
Total number of isolates		17	9	12	38	
Total number of fungal species		11	6	8		

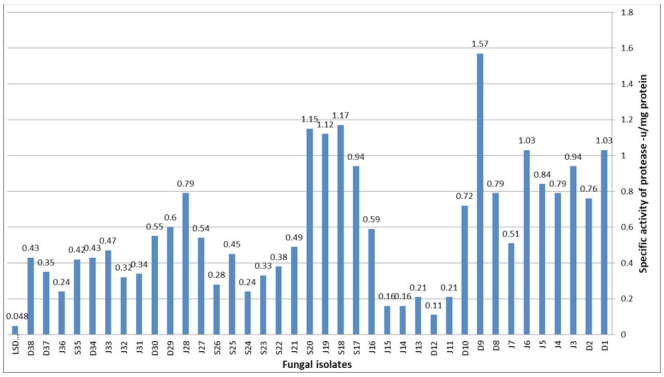

Figure 3: Specific activity of protease (unit/mg protein) of the aquatic fungal isolates.

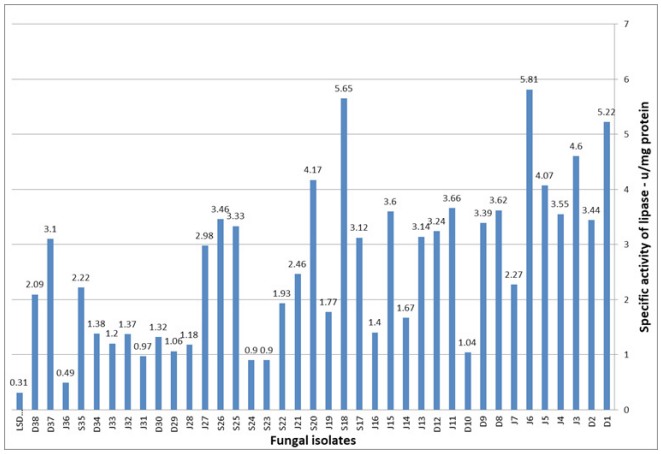

Figure 4: Specific activity of lipase (unit/mg protein) of the aquatic fungal isolates.

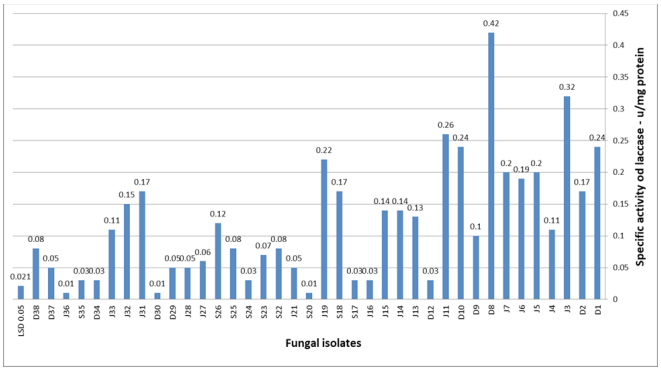

Figure 5: Specific activity of laccase (unit/mg protein) of the aquatic fungal isolates.

protease activity reached to 0.11,0.16, 0.21 unit/mg protein, respectively.

Lipase Activity

Figure 4 shows that all aquatic fungal isolates have the lipase activity. The isolates *Boeremia axigua* (J6), *Saprolegnia australis* (S18) and *Fusarium graminearium* (D1) significantly superior on other fungal isolates, as they recorded the highest specific activity of the lipase, which were 5.81, 5.65 5.22 unit/mg protein, respectively, while the isolates *S.polymorpha* (S24), S23 and *P.expansum* (J36) recorded the lowest specific activity of lipase reached 0.9, 0.9, 0.49 unit/mg protein, respectively.

Laccase Activity

Figure 5 showed a significant superiority for isolates *P.expansum* (D8), *Cladosporium allicinum* (J3) and *Aspergillus sydowii* (J11) which recorded higher specific activity of laccase reached 0.42, 0.32 and 0.26 unit/mg protein, respectively, while the isolates *Saprolegnia australis* (S20) and *Penicillium polonium* (D30) showed the lowest specific activity of this enzyme which was 0.01 unit/mg protein.

The results of our study agreed with other studies[43,20] as the results showed that aquatic fungal isolates have an efficient enzymatic system. This is evidence of the fact that fungi are decomposers of organic matter in the aquatic environment, as well as the role of these enzymes in infecting fish and aquatic animals. Protein and lipid-degrading enzymes are among the important virulence factors produced by fungi that are pathogenic to fish and crustaceans. The production of these enzymes contributes to also infecting fish eggs with zoospores because the fish eggs contain high protein and lipid contents, which are the substrates for the protease and lipase activities, respectively. Our results are also consistent with the study of Wade *et al*. (2008) on the several fungal isolates, especially the fungus *Geotrichum* spp., which has a high activity for some enzymes, the most important of which are lipase and protease enzymes that degrade the cell wall of plants.[44]

CONCLUSION

There are a diversity of aquatic fungi in the aquatic environment of the Tigris River passing through Salah al-Din Governorate, which was among the phylum Ascomycota and the Oomycota phylum represented by the three families Saprolegniaceae, Peronosporaceae, and Pythiaceae. All fungal isolates isolated from the Tigris river showed a high ability to produce the enzymes protease, laccase, and lipase, with significant superiority for isolate *P.polonicum* (D9) and isolates S18 and S20 of *S.australis*, which recorded the highest specific activity of the protease enzyme.

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