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Enzymatic estimation of some fungi isolated from manuscripts preserved at the Al-Hussein holy shrine

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***Abstract*---**The study was carried out to diagnose the fungi that attack the historical manuscripts in the holy shrine of the city of Karbala, as well as its enzymatic activity. (375) samples were taken using sterile cotton swabs for three locations of the manuscript, represented by the beginning, middle and end of the manuscript. The total number of isolates and isolated fungal species, the percentage of frequency (Frequency %), percentage of presence (Occurrence %), and the Distribution Intensity Index (DII) were calculated for each of the fungi isolated from the manuscripts. The fungi decomposing cellulose and protein and the enzyme amylase and lipid were also detected at the time of the manuscripts. For the purpose of knowing the effects of fungi and their damage to the manuscripts. Five genera of fungi were recorded in addition to yeasts and sterile fungal hyphae. The highest percentage of the total numbers of fungi isolated at the beginning of the manuscript was 40.54%, followed by the end of the manuscript with a rate of 34.66%, while the lowest percentage of the total numbers was for the middle of the manuscript, which was 24.32%. The results showed that the fungus Aspergillus niger had the highest incidence of 61% and the frequency of 29.33. It also had the highest density coefficient of distribution with a rate of 330.3. Then the fungus Aspergillus flavus with a percentage of 54%, a frequency of 26.13, and a distribution density coefficient of 276.2 , The fungus Penicillium verrucosum had a percentage of 52%, a frequency of 25.06, and a density coefficient of .260.3, while Mucor got a presence of 11%, a frequency of 5.61 and a density coefficient of 26.3. And each

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of Rhizopus, Yeast, and Mycelia sterilia fungi had the lowest incidence of 10%,8%, and 6%, respectively, while the frequency was 5.06,4.26,3.20, respectively, while the density coefficient was 22.5,16.5,10.7, respectively. The results also showed that the enzymatic activity of producing cellulose enzyme for both sexes Aspergillus niger and A. flavus was of high intensity, while Penicillium verrucosum was moderately active, while the rest of the species were ineffective in producing cellulose enzyme. The protein analysis test also showed Penicillium verrucosum and A.flavus highly effective, while Aspergillus niger, the activity was moderate, and the results of the amylase degradation test showed, Penicillium verrucosum was highly effective, while A. flavus, Mucor species were m oderately effective. As for the results of the test of the ability to secrete the enzyme lipase, most of the isolated fungal species have the ability to secrete the enzyme lipase, which is Aspergillus niger, A. flavus, Penicillium verrucosum, Mucor , Mycelia .sterilia fungi.

***Keywords*---**opportunistic fungi, manuscripts fungi, fungal enzymes.

Introduction

Cystic fungi are widespread fungi and live in different environments, differing in ways of living ranging from parasitic, saprotrophic and symbiotic, or opportunistic and many of them are pathogenic to humans, as they are found on organic materials, grains, vegetables, bread, wood and leather . It causes a large number of diseases to plants, humans and animals, some of which are known for their industrial, medical, agricultural and industrial importance and the most important compounds produced by enzymes, organic acids and antibiotics (Carrie *et al.*, 1994).

The type of medium and environmental conditions are an important factor in the enzymatic activity, and there is more than one type of these enzymes that contribute to the damage of manuscripts. There are many studies that dealt with the enzymatic activity of cellulose enzyme, protease, amylase and lipase produced by fungi (Hussein and Muhammad, 2014; Romanie *et al.*, 2006). Where it was studied for the first time by Al-Waeli, (2018) where he referred to the enzymatic activity of the fungi isolated from the manuscripts, and he referred to the role that fungi play in this damage to the enzymes and studied four of them. The fungi, in the way they infect the leaves and skins of manuscripts, depend on the secretion of specialized enzymes, which can break down the cellulose of leaves and the protein of leather and parchment. Cellulose-degrading enzymes are known as cellulose enzymes, while the enzyme that breaks down leather and wafers known as lipase enzymes, and these enzymes are complex organic substances that are very sensitive to changes in temperature, pH and alkalinity (PH) (Linko, 1997).

It was proved that there is an enzymatic activity and a high enzymatic capacity of the cellulose enzyme for some fungi isolated from the scriptures of the Al Hussein holy shrine. Among the fungi that analyze are *Trichoderma harzianum, T.viride, T.longibrachiatum, Alternaria alternate, Fusarium solan, Aspergillus niger,*

Rhizoctonia solani and Fusarium oxysporum (Mushrif *et al.*, 2017). The presence of cellulose material in the middle of development or production, stimulates microorganisms to produce cellulose enzyme, and the products of the decomposition are quickly exploited by those organisms, (Singh, *et al.*, 2009). Starch is one of the fastest degrading carbohydrates, as it follows simple sugars in the rate of decomposition (Robyt and Mukerjea, 2013). Therefore, the numbers of starch-degrading microbes are much greater than those capable of degrading other carbohydrates (Zeeman, *et al.,* 2010). In view of the lack of studies on the ability of fungi to damage manuscripts, the study aimed to estimate the enzymatic activity of cellulose enzymes, proteases, and amylase lipases of the fungi isolated from them.

Material and Methods

The culture media used in the study

The culture media used in the study was prepared according to the instructions of the supplying company and sterilized in a sterilizer at a temperature of 121 °C and a pressure of 15 bar / inch ^{2} for 15 minutes. Sabouraud's Dextrose Agar Medium , Czapex Agar (CA), Potato Dextrose Agar (PDA) , Malt Extract Agar Medium , Cellulose Agar, Starch Agar, Tween 80 Agar.

The solutions and reagents used

Iodine solution , Casein solution (0.5%). , HCL .Iodine Solution , pure cellulose solution , Sodium hydroxide solution NaOH at a concentration (0.5M), Hydrochloric acid (HCl) solution (5) N , potassium iodide solution (KI).

Collection, isolation and identification of fungi

Samples were collected from 11/12/2019 to 15/2/2020 and some fungi were isolated from manuscripts . Old and preserved in the Manuscripts Conservation and Restoration Center, using sterile cotton swabs (Swab) The cultures were in Petri dishes containing Sabouraud's Dextrose Agar (S.D.A) nutrient medium, supplemented with the antibiotic Amoxicillin at a concentration of 100 mg L-1. The dishes were incubated at 2 ± 26 °C for a period of 5-7 days, and the plates were examined preliminary for the purpose of counting the colonies growing on the culture media. The fungal species were purified by transferring parts of isolated developing colonies initially using a sterile needle into dishes containing Sabouraud's Dextrose Agar (SDA), and incubated at a temperature of 2±26°C for 5-7 days for the purpose of obtaining colonies pure Glass slides were prepared from pure cultures to study the microscopic characteristics of fungi under a light microscope. The fungi were also re-cultivated on Czapex Agar and Potato Dextrose Agar medium and the fungi were diagnosed according to their morphological shapes and colony colors in addition to the diagnostic characteristics of each fungus. A fungus. The following sources were used in the diagnosis: - (Moubasher, 1993 ; 1997, Hocking&Pitt ; 1972, Bary&Barnett ; Ellis, 1971).

Quantitative and qualitative estimation of fungi isolated from manuscripts

The following has been calculated: According to the method of Booth *et al* ., (1988)

- Total number of isolates and fungal species isolated for each manuscript
- The percentage of frequency was calculated from the following law:
  The number of isolates of the same sex
  Frequency % = ---------- x 100 .
  The total number of all isolates
- The percentage of appearance was calculated from the following law:
  The number in which the sex or gender appeared
  Occurrence % = ---------- x 100
  The total number of samples during the study
- DII (Distribution Intensity Index)
  The density distribution index for all isolated fungi is calculated from the following law: DII = % occurrence × $\sqrt{\%\text{Frequency}}$

Detection of decomposing fungi

To find out the effects of fungi and their damage to the manuscripts, the fungi that analyze the materials of the manuscripts were discovered. Three replicates were used for each type of fungi and for each enzyme, as well as for the control treatment. The medium of the plates for all treatments was inoculated with a 5 mm diameter disc from a pure fungal culture grown on PDA medium, three days old, at a temperature of 26 ± 2 °C.

Cellulosenzyme

Inoculated the cellulose medium. The hydrolysis of cellulose was detected using the HCl–Iodine reagent. Add the reagent to the dish and leave for 5 minutes, then pour the solution and leave the dish for 10 minutes. The appearance of a transparent halo with a high diameter around the fungal colonies was observed, indicating the transformation of cellulose into simple sugars by the action of the cellulose enzyme (Yoeh *et al.*, 1985).

Proteinenzym

Used agar medium - Skimmed - milk Agar. Hydrolyzed protein (casein) was detected in milk. The appearance of a transparent halo around the fungal colonies to indicate the presence of enzymatic activity, where the greater the diameter of the halo, the greater the enzymatic activity of the fungus (Hankin and Anagnostakis 1975).

Starch

Inoculated the starch medium. The decomposition of starch is detected using an iodine reagent. The reagent was added to the dish and left for 5 minutes, then the solution was poured and the dish was left for 5 minutes. A transparent halo

appeared around the fungal colonies, indicating the production of amylase enzyme. The diameter of the halo is a function of the fungus' activity in the production of the enzyme (Pandey *et al.,* 2000).

Lipidenzyme

Dishes containing the medium containing the substrate (peptone supported with Tween80) were inoculated. Observations are recorded through white precipitates around the colonies or through the appearance of a transparent halo around the colony of mushrooms to indicate the enzymatic activity of the Lipidenzyme. (Takó *et al.,* 2012).

Table 1
The mechanism for calculating the diameter of the transparent corona around the fungal colonies of cellulose, protease and amylase media to reveal the decomposition activity

decomposition efficacy	Decomposition area diameter (mm)	Symbol
unanalyzed	Zero	-
weakly effectiveness	< 10 mm	+
Moderate effectiveness	10 – 15 mm	++
Highly effectiveness	> 15 mm	+++

Table 2
Names of fungi isolated from some historical manuscripts at a temperature of 2±26 °C and on PDA

No	Name of fungi
1	*Aspergillus niger*
2	*Aspergillus flavus*
3	*Penicillium verrucosum*
4	*Mucor*
5	*Rhizopus*
6	Yeast
7	Mycelia sterilia fungi

Results and Discussion

Results
Isolation and identification of fungi from manuscripts

Fungi were collected and isolated from historical manuscripts by 375 isolates that were purified from the beginning, middle and end of the manuscript. The highest percentage of the total numbers of isolated fungi at the beginning of the manuscript was 40.54%, followed by the end of the manuscript with 34.66%, while the lowest percentage of the total numbers was for the middle of the manuscript, where it was 24.32%. Table (3).

Table 3
The number of fungal isolates and their percentages from the beginning, middle and end of the manuscript on PDA medium at a temperature of 26 ± 2 °C for 5-7 days

No	The location where the sample was taken	number of isolates	% of whole numbers
1	Beginning	150	40.54
2	Center	90	24.32
3	End	130	34.66
Total	------	375	99.52

The reason for this difference is attributed to the nature of the covers of the manuscript, which consists of the skin, and the protein is the main component of it, while the middle of the manuscript, whose basic structure is cellulose, of which the paper is composed, While cellulose makes up the bulk of the manuscript, fungi find it difficult to analyze the cellulose components. As well as other materials such as adhesives, inks, etc., and their effect on changing the humidity and the acidic medium pH, as it is one of the determining factors for the enzymatic activity with the appropriate temperatures (Silva *et al.*, 2001).

Diagnosis of fungi by the traditional method

375 fungal isolates were obtained from the historical manuscripts, represented by three sites, the beginning, middle and end of the manuscript, represented by three replicates for each site table (4) . The fungi isolated from the manuscripts were diagnosed by the traditional method and it was found that they are represented by five fungal species in addition to yeasts and sterile fungal hyphae: *,Aspergillus niger,A. flavus, Penicillium verrucosum, Rhizopus, Mucor,* Yeast, Mycelia sterilia fungi .

Table 4
The number of isolates and fungal species isolated from the beginning, middle and end of the manuscript during the period from 15/11/2019 to 15/1/2020

fungal species	Beginning	Center	End	Total number of isolates
Aspergillus niger	45	40	25	110
A. flavus	40	30	28	98
Penicillium verrucosum	37	25	32	94
Mucor	9	6	7	21
Rhizopus	7	6	6	19
Yeast	4	7	5	16
Mycelia sterilia fungi	4	4	4	12
Total				375

Occurrence % , **Frequency % and Distribution Density Coefficient**

The results showed that there were differences in the incidence of fungal species during the study (Table 5) where *Aspergillus spp.* recorded The highest

percentage of presence is represented by both genders *A. niger*, and *A. flavus* . Where the fungus *A. niger* recorded the highest incidence of 61%, followed by *A. niger. A. flavus* with 54%, followed by *Penicillium verrucosum* 52% . The percentage of appearance of the genus *Mucor* was 11%, and the genus *Rizopus* was 10%, while the yeasts came by 8%, and the white sterile fungal hyphae were 6%. While the percentage of frequency , the result showed *Aspergillus niger* recorded the highest frequency value with 29.33%, followed by *A. flavus* with 26.13, followed by *Penicillium verrucosum* with a frequency of 25.06%, *Mucor* 5.61%, followed by *Rhizopus* with a frequency of 5.06%, while the lowest frequency was for the two yeasts Yeast and sterile white hyphae with 4.26 %, 3.20%, respectively. The results of determining the distribution density coefficient of the fungi isolated from the manuscripts showed that the species *A. flavus*. *A. niger, , Penicillium verrucosu* . Were the most densely distributed. While the least dense species were *Mucor, Rhizopus*, and Yeast, in addition to Mycelia sterilia fungi, the sterile white hyphae.

Table 5
Total appearance percentage, frequency percentage and distribution density coefficient

Fungus	Occurrence %	Frequency %	Distribution %
Aspergillus niger	%61	29.33	330.3
A. flavus	%54	26.13	276.2
Penicillium verrucosum	%52	25.06	260.3
Mucor	%11	5.61	26.3
Rhizopus	%10	5.06	22.5
Yeast	%8	4.26	16.5
Mycelia sterilia fungi	%6	3.20	10.7

Detection of analyzing fungi
Cellulose

The results of the cellulose activity test of the fungi isolated from the parenchyma on agar-cellulose medium showed, as shown in Table (6). There are two types of fungi belonging to the fungus *Aspergillus spp*. isolated, had the ability to produce cellulose enzyme with different efficiency, where the species *A. niger, A flavus*. highly effective, The diameter of the transparent areola was greater than (15 mm) during 3, 6, 9 consecutive days, while the fungus *Penicillium verrucosum* was moderately effective during 3, 6, 9 days, as the diameter of the transparent areola was between (10-15) mm. As for the rest of the fungal species were unanalyzed. This result is consistent with what was stated that most of the true fungi have the ability to decompose natural cellulose for the purpose of growth and perpetuation of life through the production of cellulose-degrading enzymes (1989, Armstrong). A discrepancy was also observed between the isolates, from weak to good. The weak ability of some isolates to secrete cellulose can be explained by several reasons, including the insufficient incubation period to stimulate its secretion and its difference in the ability to exploit the culture medium or the inappropriateness of the pH of the medium for these isolates (Abdul-Hadi; 2011, Luiza, 2000) . And through the results of the study, we find that the reason for the presence of these fungi on the manuscripts may be due to their ability to secrete the cellulose

enzyme on the manuscript paper, of which cellulose fibers are the main component.

Table 6
Decomposition of cellulose by fungi on a cellulose agar medium at a temperature of 26±2°C. For a period of incubation 3 , 6 , 9 days

Fungal species	Decomposition diameter (mm)	decomposition efficacy
Aspergillus. niger	>15	+++
Aspergillus flavus	>15	+++
Penicillium verrucosum	10 - 15	++
Mucor	Zero	-
Rhizopus	Zero	-
Yeast	Zero	-
Mycelia sterilia fungi	Zero	-

+++ = Highly Effective ++ = Medium Effective

Protein

The results of the proteolytic efficacy test of fungi that were isolated from the parenchyma were recorded on medium Skimmed milk- agar media. as shown in the table (7). The species, *A. flavus, Penicillium verrucosum*, were highly effective, as the diameter of the transparent areola was greater than (15 mm), while the species, *A. niger* had medium activity, the diameter of the lytic recorded between (10-15) mm, while the rest of the fungal species were It was unresolved , Also, the fungi *A.niger, A. flavus*, has the ability to produce protease enzyme. These results were in agreement with what was stated by researcher Nakagowa, (1970) about the production of protease enzyme by fungal species *A. flavus, A. niger* when grown on two different media.

Table 7
Protein degradation by fungi on skimmed milk agar medium at ±262°C for an incubation period of 3,6,9 days

fungal species	Decomposition area diameter (mm)	decomposition efficacy
Penicillium verrucosum	>15	+++
A.flavus	>15	+++
A. niger	10 – 15	++

+++ = Highly Effective ++ = Medium Effective

Whereas, protease enzymes were produced on a large scale in industry using molds of the genus *Aspergilius,* where it was found that the majority of fungi specialized in analyzing leather and parchment are attributed to the genera *Aspergilluse, Penicillium, Alternaria and Helmintho sprium.* It is noted that these genera have an important role in the analysis of protein materials (Baldrian and Valášková, 2008). And (Nieves, 2003) showed that fungi attack the skins because they are an organic substance that includes proteins, carbohydrates and fats in

their composition, and this confirms what we have found about the presence of fungi on the covers of manuscripts. The reason for using leather in the packaging of printed books and manuscripts is that the chemical composition of leather is the same as that of parchment and parchment, as parchment and parchment are protein materials extracted from leather. Therefore, most of the fungi specialized in analyzing leather and parchment are attributed to the genera Aspergilluse, Alternaria Penicillium and Helminthosprium. It is noted that these genera play a role in the analysis of protein substances (1971, Ellis).

Starch

The results of the test of the amylase activity of the fungi that were isolated from the parenchyma on the agar-starch medium, and as shown in table 8, showed that the fungus, *Penicillium verrucosum*, was highly effective as the diameter of the transparent areola was greater than 15 mm, While the species *A.niger A. flavus* and *Mucor* were weakly active, the corona diameter was less than 10 mm, the rest of the fungal species were unanalyzed d The results also showed that the presence of these fungi on the manuscripts may be due to the presence of starch, which is one of the materials that enter into the composition of manuscripts as an adhesive to bindings, papers and the heels of manuscript books. As starch is a complex compound of glucose, there are some fungi specialized in analyzing it and feeding on its components through the secretion of their enzymes (Sakaki *et al.,* 1986 ; Dijkhuizen , 2002) .

Table 8
Degradation of amylase by fungi isolated on starch medium at 26±2°C for an incubation period of 3,6,9 days

fungal species	Decomposition area diameter (mm)	Casein hydrolysis efficacy
Penicillium verrucosum	>15	+++
A.niger	<10	+
A. flavus	<10	+
Mucor	<10	+

+++ = Highly Effective + = Weakly Effective

Lipase

Table 9 indicated that the results of this test show that most of the isolated fungi have the ability to secrete lipase enzyme, including *A. flavus., A.niger, Mucor, Penicillium verrucosum, Rhizopus,* and Mycelia sterilia fungi .(Sumathy et al ., 2012) indicated the ability of some fungi, namely *Aspergillus, Fusarium, Geotrichum*, and *Penicillium*, to produce the enzyme lipase. These results are agree with the findings of (Bramono *et al* .,2006; Yenişehirli *et al* ., 2010), about the ability of some studied fungi to secrete lipase enzyme, while Yeast was not analyzed.

Table 9
Degradation of lipase by fungi on lipase medium at ±262°C for an incubation period of 3.6,9 days

fungal species	Inference on the ability of fungus to produce the enzyme lipase
Aspergillus flavus	A visible white precipitate is formed around the colony
A.niger	A visible white precipitate is formed around the colony
Penicillium verrucosum	A visible white precipitate is formed around the colony
Mucor.	A visible white precipitate is formed around the colony
Rhizopus.	A visible white precipitate is formed around the colony
Mycelia sterilia fungi	A visible white precipitate is formed around the colony

As indicated (Park and Jung, 2013) to the importance of lipases and phospholipases in the pathogenesis, as it is one of the virulence factors of lipophilic fungi, and they have an effect on the growth, shape and spread of fungal cells across the host.

Conclusion

- The presence of the genus *Aspergillus niger*, which formed the highest incidence, frequency, distribution coefficient and speed, in addition to *Aspergillu flavus, Penicilliu.verrucosum, Mucor, Rhizopus,* Yeast and Mycelia sterilia fungi in the studied manuscripts.
- Most of the fungi isolated from the manuscripts have the ability to secrete protease enzymes, cellulose, amylase, and lipase, which confirms their ability to damage these parenchyma when the appropriate environmental conditions are available.
- The results showed that the presence of the incubation period of nine days gave high results in the rate of production of enzymes by the fungi and the behavior of some fungi in a second direction in the event of food depletion for permanence of survival and adaptation.

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