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Enzymatic activity of some fungi isolated from submerged plant parts in aquatic habitats southern Iraq

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Abstract

Twenty-six fungal species were isolated from different dead plant substrates submerged in aquatic habitats southern Iraq. These isolates were screened for their ability to produce five extracellular enzymes on solid media. Exoenzymes detected included (cellulase, laccase, lipase, ligninase and amylase). Two species, *Clavatospora bulbosa* and *Lentithecium* sp. 3 were positive for all enzymes tested. *Acremonium sclerotigenum, Cladosporium xanthochromaticum,* and *Lentithecium varaginesporum* were positive for all enzymes except laccase and *Exserohilum rostratum* was positive for all enzymes except cellulase. Lignase enzyme was produced by 22 species, while laccase was screeted only by 7 species. The study observed significant differences between the different fungal species in their enzymatic activities.

Keywords: Fungi, aquatic habitat, Enzymatic activity.

Introduction

Aquatic ecosystems receive large quantities of fixed carbon inputs in wood residues and plant herbs. These materials are sources of energy and carbon in the environment (Anuja *et al*., 2017). In general, Fungi produces a wide range of extracellular enzymes such as (cellulase, laccase, lipase, ligninase, and amylase). The microbes need to produce an extracellular enzyme to convert polymeric compounds– such as carbohydrates, cellulose, lignin, starch, pectin, and other plant components into smaller molecules that can be assimilated (Sunitha *et al*.,2013). Many researchers have tended to exploit these sources to produce simple saccharides of industrial importance or biofuels using the lytic enzymes produced by fungi. Fungal enzymes are also natural products used in the food, beverage, confectionery, textile, and leather industries to simplify raw materials processing. They are also used in the biological treatment of pollutants and organic and inorganic wastes and are often more stable than enzymes derived from other sources. Therefore, enzymes produced from fungi make up nearly 40% of enzymes' global production (Kubicek and Kubicek 2016). Despite many studies in Iraq on fungi associated with plant parts submerged in water, such as Al-Saadoon and Abdullah (2001), Muhsin and Khalaf (2002), Al-Saadoon, and Al-Dossary (2010), However, there is no study on the extracellular enzymatic activity of fungi which isolated from these environments. Therefore, the present study aims to test some fungi accompanying plant residues immersed in water to produce extracellular enzymes on solid media.

Materials and Methods

1. Fungal Isolation

Fungal species were isolated from different dead plant substrates (stems, wood, twigs, and herbaceous plants) submerged in aquatic habitats in Basra governorate by using a moist chamber; isolated species were grown on two different types of media potato dextrose agar (PDA) and malt extract agar (MEA). The isolation media preparation was done according to the direction of the manufacturing company (Hi media). The cultures were incubated at 25 °c until tested. The isolated fungi were identified according to the following references Hyde *et al*. (1999); Wanasinghe *et al.* (2017) and Jayasiri *et al*. (2019).

2. Study the enzymatic activity of isolated fungi

Special media were used to study fungi' enzymatic activity by inoculating each medium with discs taken from the edges of the fungal colony. The dishes were incubated at 25 ° C for 5-10 days. After the incubation time, special reagents were added to each dish to determine each species' ability to produce a different enzyme; three triplicates were used for each test.

2.1: Cellulase enzyme

The medium used in this test contained 7.0g KH_2PO_4, 2.0g K_2HPO_4, 0.1g $MgSO_4.7H_20$, 1.0g $(NH_4)_2SO_4$, 0.6g yeast extract, 10g carboxymethylcellulose, and 15g agar per liter (Ireri *et al*., 2015). After 5-7days of incubation, the plates were flooded with 0.2% aqueous congo red solution and distained with 1M NaCl for 15minutes. The appearance of yellow areas around the fungal colony in an otherwise red medium indicated cellulase activity.

2. 2: Lipase enzyme

The fungi were grown on peptone agar medium (peptone 10g, NaCl 5g, CaCl. $2H_2O$ 0.1g, agar- 16g, distilled water-1L, pH 6.0) supplemented with 1% Tween 20 separately sterilized and added to the medium. At the end of the incubation period, a visible precipitate around the colony due to the formation of calcium salts of the lauric acid liberated by the enzyme indicated positive lipase activity (Sunitha *et al*., 2013).

2.3: Laccase enzyme

Glucose yeast extract peptone agar medium with 0.05g α-naphthol per 1L, pH 6.0 was used. As the fungus grows, the colorless medium turns blue due to the oxidation of α-naphthol by laccase enzyme, indicating a positive reaction (Sunitha *et al*., 2013).

2. 4: Ligninase enzyme

The culture was inoculated onto tannic acid agar plates containing 0.2% tannic acid. A positive reaction is indicated by forming a yellow to the light brown zone around the colony (Sharma *et al*., 2017).

2. 5: Amylase enzyme

The medium used to study the ability to degrade starch contained malt extract plus 0.2% soluble starch, pH9. After 5-7 days of incubation, the plates were flooded with an iodine solution. A yellow zone around a colony appeared, which indicates a positive reaction, in an otherwise blue medium indicated negative activity (Ireri *et al*., 2015).

2.3 Statistical Analysis

ANOVA analysis was used by applying Minitab ver.16 to analyze the results statistically. The mean was tested using the least significant difference RLSD test under the probability level 0.05.

3. Results and Discussion

The enzymatic efficacy of 26 fungi species was examined to test their ability to produce cellulose, liginase, lipase, laccase and amylase enzymes. Significant differences were observed between the different fungal species in their enzymatic activity. The results showed qualitative differences, whether between the species belonging to one genus or between species of different genera, and this may be due to the enzymatic capacity that differs from one species to another according to its adaptation to the environment in which it lives and the enzyme secretion ability it possesses (Sunitha *et al*., 2013; Patil *et al*., 2015). In general, the results obtained in our study may not be consistent with other studies due to the different isolates, as Trigiano & Fergus (1979) stated that their fungi give different results. The fungus *Clavatospora bulbosa* and *Lentithecium* sp.3 showed the capacity to secret all studied enzymes. The ability of the rest fungi ranged from their ability to secrete one to four

enzymes. Some enzymes' negative results may refer to either the enzyme does not produce, or it produces but does not break free from the hyphae, or it produces and liberates. Still, the medium limits its secretion, and therefore the negative results do not represent an absolute confirmation of the species' inability to produce the specific enzyme (Abdel- Raheen & Shearer, 2002).

The results showed that most of the tested fungi were able to produce ligninase enzyme, as 22 fungi were able to give a positive reaction by forming a yellow-brown colored halo around the fungal colony. The secretion rates ranged from low as in *Zopfiella* to high secretion as in *Phomopisis bougainvilleicol* (Table 1, Fig.1).

The statistical analysis showed significant differences (P <0.05) between the tested fungi in their ability to secrete the ligninase enzyme. The ability of fungal isolates to produce this enzyme with high efficiency in this study indicates their ability to lyse this compound and other organic and plant compounds present in the environment and use them as sources of carbon and energy (Saini *et al*., 2015). This study's results were consistent with Sharma et al. (2017) and Bi et al. (2012), that isolated 72 fungal species and indicated their ability to secret ligninase and use it to degrade the organic material.

Most fungal species achieved good efficiency in the production of lipase enzyme. The results of the statistical analysis showed significant differences (P <0.05) between the tested fungi in their ability to produce lipase enzyme (Table 2, Fig.2). 13 fungal species including *Diplodia mutila*, *Acremonium sclerotigenum*, managed to give a positive reaction and secretion at a very high rate, through the formation of a transparent halo due to the formation of a white precipitate or white crystals around the fungal colony. In comparison, only seven species gave an adverse reaction, such as *Aniptodera chesapeakensis*, *Lentithecium* sp.2.

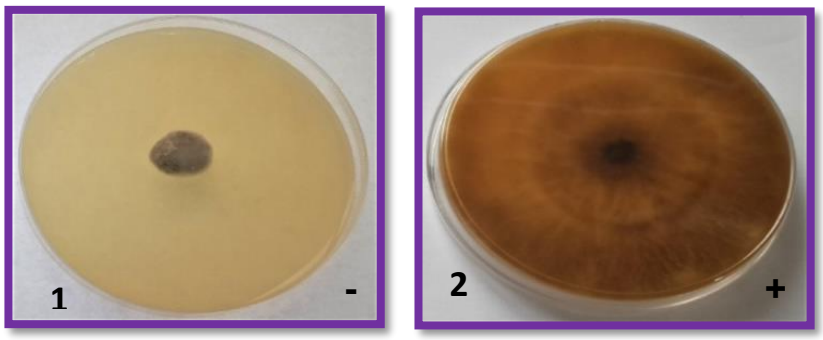

Fig. 1: Ligninase enzyme test: (1) *Zopfiella marina* (2) *Phomopisis bougainvilleicola*

Table 1: Ligninase activity of fungal isolates on solid medium

No.	Fungal isolates	Activity
1	*Phomopisis bougainvilleicola*	+++

No.	Fungal isolates	Activity
2	*Diplodia mutila*	+++
3	*Amorosia littoralis*	+++
4	*Leptosphaeria* sp.	+++
5	*Lentithecium* sp.2	+++
6	*Lentithecium voraginesporum*	+++
7	*Trematosphaeria pertusa*	+++
8	*Lentithecium* sp.3	+++
9	*Halosapheia* sp.	+++
10	*Gilmaniella humicola*	+++
11	*Aniptodera chesapeakensis*	+++
12	*Chaetomium globosum*	+++
13	*Lentithecium* sp.4	+++
14	*Clavatospora bulbosa*	+++
15	*Alternaria mauchaccae*	++
16	*Lentithecium* sp.1	++
17	*Alternaria molesta*	++
18	*Acremonium sclerotigenum*	+
19	*Coprinus* sp.	+
20	*Alternaria infectoria*	+
21	*Cladosporium xanthochromaticum*	+
22	*Exserohilum rostratum*	+
23	*Zopfiella latipes*	+
24	*Z. marina*	–
25	*Preussia terricola*	–
26	*Paraconiothyrium cyclothyrioides*	–
	RLSD = 15.5	

- Negative no secretion ; + medium (3-5)mm.

++ good (5-8) mm. ; +++ strong (8-11)mm.

Giving an adverse reaction by these fungi is not necessarily an indicator that they cannot lyse lipids. Abdel- Raheen & Shearer (2002) stated that lipase is present in most fungi but not appearing in the test, and this may be due to the short duration of the test. They mentioned that some microorganisms require up to ten days to give visual lysis of fats. Still, most do not require more than five days for this purpose. The other reason may be that the enzyme excreted by the fungus is unable to hydrolyze the ester bond present in the Tween 80 substance. Meanwhile, the tested fungi's higher ability to produce this enzyme may be attributed to the isolated fungi type (Savitha *et al*., 2007; Kempka *et al* ., 2008; Gopinath *et al* ., 2013).

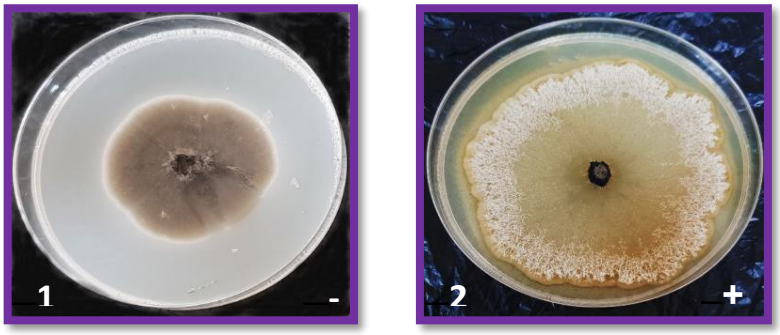

Fig. 2: Lipase enzyme test: (1) *Lentithecium* sp.2 (2) *Diplodia mutila*

Table 2: Lipases activity of fungal isolates on solid medium.

No.	Fungal isolates	Activity
1	*Diplodia mutila*	+++
2	*Acremonium sclerotigenum*	+++
3	*Alternaria infectoria*	+++
4	*Lentithecium* sp.3	+++
5	*Alternaria molesta*	+++
6	*Alternaria mauchaccae*	+++
7	*Paraconiothyrium cyclothyrioides*	+++
8	*Gilmaniella humicola*	+++
9	*Lentithecium* sp.1	+++
10	*Coprinus* sp.	+++
11	*Phomopisis bougainvilleicola*	+++
12	*Zopfiella marina*	+++
13	*Halosparpheia* sp.	+++
14	*Preussia terricola*	++
15	*Clavatospora bulbosa*	+
16	*Exserohilum rostratum*	+
17	*Cladosporium xanthochromaticum*	+
18	*Lentithecium voraginesporum*	+
19	*Zopfiella latipes*	+
20	*Trematosphaeria pertusa*	–
21	*Chaetomium globosum*	–
22	*Leptosphaeria* sp.	–
23	*Amorosia littoralis*	–
24	*Lentithecium* sp.2	–
25	*Lentithecium* sp.4	–
26	*Aniptodera chesapeakensis*	–
RLSD = 15.5		

- Negative no secretion ; + medium (3-5)mm.

++ good (5-8) mm. ; +++ strong (8-11)mm.

The cellulase enzyme came in third place. Eighteen fungal species gave a positive reaction by forming a yellow halo around the fungal colony due to the complex carbohydrates turning into simple sugars. The widest the halo, the more influential the fungi in producing this enzyme, with different capabilities of secretion rates from low as in *Aniptodera chesapeakensis* to high secretion as in *Lentithecium voraginesporum* (Table 3, Fig.3). The statistical analysis results showed significant differences ($P < 0.05$) between the tested fungi in their susceptibility to the production of the cellulase enzyme.

Many microorganisms participate in the process of cellulose decomposition. Still, fungi played the main role in this process. It possesses an extracellular enzymatic system for cellulose degradation into simple saccharide units in the form of glucose that can dissolve in water to be easy to use as a source of energy. Therefore the cellulase enzyme plays an important role in the biological activity of fungi due to its environmental importance in the decomposition of plant wastes in which cellulose forms approximately 94% of the composition of the plant cell wall and about 45% of the composition of the secondary wall, and this explains its spread over the plant parts(Payne *et al*., 2015). This result is consistent with the study of Khalid *et al*. (2006) that showed 42 species of fungi to grow on a medium containing cellulose.

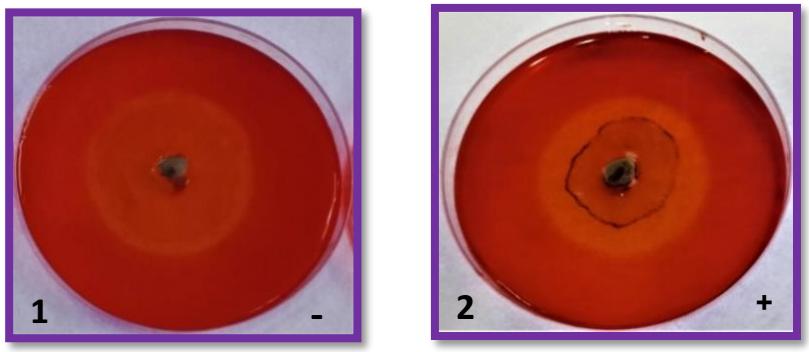

Fig.3: Cellulase enzyme test:(1) *Aniptodera chesapeakensis* (2) *Lentithecium voraginesporum*

Table 3: Cellulases activity of fungal isolates on solid medium.

No	Fungal isolates	Activity
1	*Trematosphaeria pertusa*	+++
2	*Clavatospora bulbosa*	+++
3	*Amorosia littoralis*	+++
4	*Acremonium sclerotigenum*	+++
5	*Coprinus* sp.	+++
6	*Cladosporium xanthochromaticum*	+++
7	*Lentithecium voraginesporum*	+++
8	*Lentithecium* sp.4	+++
9	*Lentithecium* sp.2	++
10	*Halosarpheia* sp.	++
11	*Lentithecium* sp.1	+
12	*Lentithecium* sp.3	+
13	*Gilmaniella humicola*	+
14	*Zopfiella latipes*	+
15	*Paraconiothyrium cyclothyrioides*	+
16	*Preussia terricola*	+
17	*Leptosphaeria* sp.	+
18	*Zopfiella marina*	+
19	*Aniptodera chesapeakensis*	–
20	*Exserohilum rostratum*	–
21	*Alternaria molesta*	–
22	*Phomopisis bougainvilleicola*	–
23	*Alternaria infectoria*	–
24	*Alternaria mauchaccae*	–
25	*Diplodia mutila*	–
26	*Chaetomium globosum*	–
RLSD = 15.5		

.- Negative no secretion ; + medium (3-5)mm.

++ good (5-8) mm ; +++ strong (8-11)mm.

Moreover, 11 species of the tested fungi were able to give a positive reaction for amylase enzyme by forming a yellow halo around the colony, indicating the breakdown of starch into simple sugars while staining the rest of the medium with purple color as a result of the interaction of starch with iodine. The greater the areola's width, the more influential the fungus in the production of the enzyme. At the same time, 15 fungal species gave an adverse reaction as *Zopfiella marina.* The results of the statistical analysis showed the presence of significant differences ($P < 0.05$) between the tested fungi in their ability to secrete the amylase enzyme (Table 4, Fig.4).

Many microorganisms, including fungi, secrete the amylase enzyme and lyse the starch due to its abundance in the plant residues that store starch, such as wheat and corn; therefore, it is easy to decompose use by fungi and exploit it in its growth and survival in the environment. (Aiyer, 2005; Sunitha *et al*., 2013.; Desire *et al*., 2014).

This explains the reduced secretion of this enzyme compared to the other types. The base material from which the fungi were isolated is a dead plant that cellulose and lignin constitute the bulk of their formation.

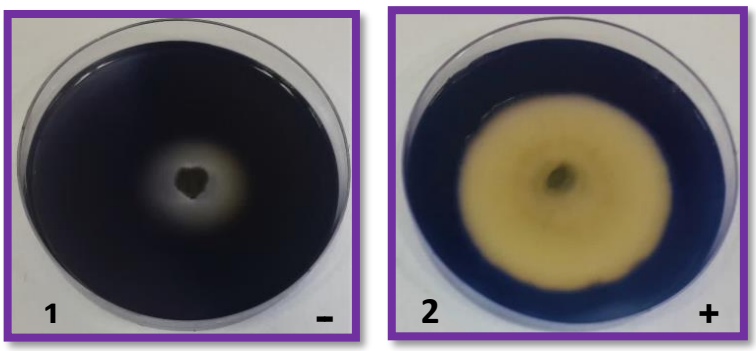

Fig.4: Amylase enzyme test:(1) *Zopfiella marina* (2) *Clavatospora bulbosa*

Table 4: Amylases activity of fungal isolates on solid medium

No.	Fungal isolates	Activity
1	*Clavatospora bulbosa*	+++
2	*Acremonium sclerotigenum*	+++
3	*Trematosphaeria pertusa*	++
4	*Alternaria molesta*	+
5	*Exserohilum rostratum*	+
6	*Lentithecium* sp.3	+
7	*Lentithecium voraginesporum*	+
8	*Zopfiella latipes*	+
9	*Lentithecium* sp.2	+
10	*Cladosporium xanthochromaticum*	+
11	*Lentithecium* sp.4	+
12	*Lentithecium* sp.1	-
13	*Aniptodera chesapeakensis*	-
14	*Phomopisis bougainvilleicola*	-
15	*Preussia terricola*	-
16	*Alternaria infectoria*	-
17	*Zopfiella marina*	-
18	*Alternaria mauchacca*	-
19	*Paraconiothyrium cyclothyrioides*	-
20	*Coprinus* sp.	-
21	*Diplodia mutila*	-
22	*Gilmaneilla humicola*	-
23	*Leptosphaeria* sp.	-
24	*Amorosia littoralis*	-
25	*Halosarpheia* sp.	-
26	*Chaetomium globosum*	-
RLSD = 15.5		

- Negative no secretion ; + medium (3-5)mm.

++ good (5-8) mm ; +++ strong (8-11)mm.

On the other hand, only seven fungi were able to give a positive reaction to laccase enzyme by forming a blue halo around the colony due to the oxidation of α-nephthol. The results of the statistical analysis showed the presence of significant differences ($P < 0.05$) between the tested fungi for their ability to produce laccase enzyme (Table 4, Fig.4).

The fungus *Clavatospora bulbosa* achieved a distinct activity in laccase production, while the remaining six fungi production ranged from a medium such in *Paraconiothyrium cyclothyrioide* to weak *Cladosporium xanthochromaticum*. Fungi that can secrete laccase enzymes usually have copper atoms in their composition responsible for their distinctive blue color. the fluctuation of production ratio among fungi may depend on the type of fungus and its latent secretion capacity, which leads to a difference in its enzymatic activity. (Brijwani *et al*., 2010). The reason for the small number of fungi that can secrete this enzyme in the current study may be attributed to that laccase is one of the enzymes usually produced by the bazidomycetes. In contrast, ascomycetes and asexual fungi excrete at a lower level, furthermore, the way to detect the enzyme using the medium of glucose yeast extract peptone agar medium may be suitable for fungi that contain copper atoms responsible for their distinctive blue color, and not suitable for the other fungi that lack it which produce different types of laccase enzyme known as yellow and white that requires the use of other media to detect it (Claudia *et al*., 2013; Abeer *et al*., 2015; Brijwani *et al*., 2010). These results were consistent with the results of the study by Abeer *et al*. (2015) that was able to test and examine twenty-six fungal isolates to ensure the presence of laccase enzyme activity; only eight fungal isolates were able to give a positive reaction, while 17 fungal isolates gave a negative response. This finding also agreed with the study of Pragathi *et al*. (2013) that showed a few fungi could secrete the laccase enzyme depending on the type of the fungal isolates and the groups to which they belong.

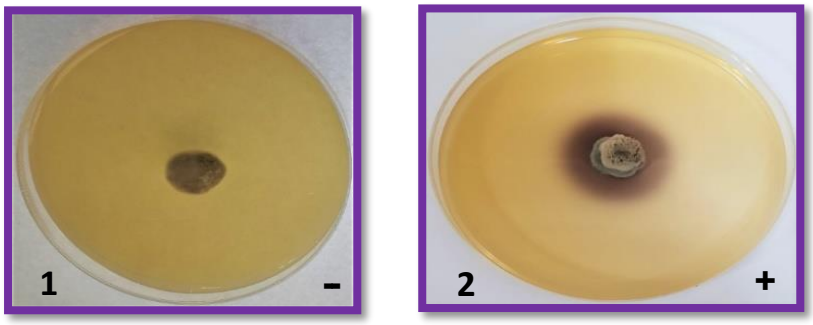

Fig.5: Laccase enzyme test:(1) *Zopfiella marina* (2) *Clavatospora bulbosa*

Table 5: Laccases activity of fungal isolates on solid medium.

No	Fungal isolates	Activity
1	*Clavatospora bulbosa*	**+++**
2	*Paraconiothyrium cyclothyrioides*	**+**
3	*Leptosphaeria* sp.	**+**
4	*Exserohilum.* Sp	**+**
5	*Lentithecium* sp.3	**+**
6	*Alternaria infectoria*	**+**
7	*Amorosia littoralis*	**+**
8	*Cladosporium xanthochromaticum*	**-**
9	*Aniptodera chesapeakensis*	**-**
10	*Alternaria molesta*	**-**
11	*phomopisis bougainvilleicola*	**-**
12	*Preussia terricola*	**-**
13	*Lentithecium voraginesporum*	**-**
14	*Zopfiella latipes*	**-**
15	*Acremonium sclerotigenum*	**-**
16	*Termatosphaeria pertusa*	**-**
17	*Zopfiella marina*	**-**
18	*Alternaria mauchaccae*	**-**
19	*Coprinus sp.*	**-**
20	*Diplodia mutila*	**-**
21	*Chaetomium globosum*	**-**
22	*Halosphaeria* sp.	**-**
23	*Gilmanilla humicola*	**-**
24	*Lentithecium* sp.2	**-**
25	*Lentithecium* sp.4	**-**
	RLSD = 15.5	

- Negative no secretion ; + medium (3-5)mm.

++ good (5-8) mm ; +++ strong (8-11)mm.

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النشاط الأنزيمي لبعض الفطريات المعزولة من الاجزاء النباتية شبه المغمورة في البيئات المائية جنوب العراق

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المستخلص

في هذا البحث عزل 26 نوعًا من الفطريات من بقايا نباتية مختلفة مغمورة في بيئات مائية متنوعة جنوب العراق، واختبرت قابليتها على إنتاج خمسة إنزيمات خارج خلوية على اوساط صلبة مختلفة. شملت الانزيمات الخارج خلوية المختبرة كلا من انزيم السليليز، اللاكيز ، اللاييز ، اللاجنينيز والأميليز. اظهر النوعان *Clavatospora bulbosa* و *Lentithecium* sp. كشفا ايجابيا لجميع الإنزيمات التي تم اختبارها. أما الفطريات *Acremonium sclerotigenum* ، *Cladosporium xanthochromaticum* و *Lentithecium varaginesporum* فقد اظهرت قابلية إيجابية لانتاج جميع الإنزيمات باستثناء اللاكيز ، اما الفطر *Exserohilum rostratum* فقد اعطى كشفا ايجابيًا لجميع الإنزيمات باستثناء السليليز. تم إنتاج اللجنينيز بواسطة 22 نوعًا، بينما تم افراز انزيم اللاكيز من قبل7 أنواع فقط. لاحظت الدراسة وجود فروق معنوية بين الأنواع الفطرية المختلفة في أنشطتها الإنزيمية .