AMERICAN JOURNAL OF BIOLOGY AND NATURAL SCIENCES
https://biojournals.us/index.php/AJBNS
ISSN: 2997-7185

Article

Recovery and Identification of Pathogenic Fungi of Bee Colonies (*Apis Mellifera*) and Study Their Ability to Produce Extracellular Enzymes

Rasheed O. Al-Asadi[1], Abbas D. Shaban[2]

1. Department of Biology, College of Education for Al-Qurna, University of Basrah, Iraq
2. Biology Department, College of Education, Qurna, University of Basrah, Iraq

* Corresponding: [1]rasheed.abiad@uobasrah.edu.iq, [2]abbas.shaaban@uobasrah.edu.iq

Citation: Al-Asadi R. O., Shaban A. D. Recovery and Identification of Pathogenic Fungi of Bee Colonies (*Apis Mellifera*) and Study Their Ability to Produce Extracellular Enzymes American Journal of Biology and Natural Sciences 2026, 3(5), 114-120.

Received: 07th Feb 2026
Revised: 12th Mar 2026
Accepted: 17th Apr 2026
Published: 20th May 2026

Abstract: The current study of enzymatic characteristics of Aspergillus flavus and Aspergillus niger associated with honeybee workers in Iraq regarding these hydrolytic enzymes as major biochemical markers of their pathogenicity. Fungi samples were obtained from 1 st October to 1 st December, 2025, in each of eight ecological zones in Basrah, Dhi Qar and Diyala. Statistical analysis of the fungi distribution showed that the Al-Zubayr district had the greatest overall number frequency of isolates, presenting together both species simultaneously in hives sampled and followed by the Al-Qurna district who displayed a co-present therapeutic effect to tackle against both pathogens. Four essential extracellular enzymes, which included. ; Proteolytic Enzymes: Lipolytic Enzymes: Chitin-degrading Enzymes: Phenoloxidases, were screened qualitatively using specific selective mediums The findings revealed that the production rate of these enzymes was universally (100) positive in all the isolates tested, and AF-4 strain proved to be an The findings revealed that the production of these enzymes was universally positive (100%) across all tested isolates. Specifically, the FA-4 strain exhibited high enzymatic proficiency on the selective media.These results emphasize a complex and strong set of enzymes in local Iraqi strains that are effectively used to break down honeybee tissues and provide potent clinical progression of Stonebrood disease in local apiaries.

Keywords: Aspergillus Isolates, Extracellular Biocatalysts, Mesopotamian Basin Ecology, Virulence Determinants, Apis Mellifera Infections.

Introduction

The honeybee (*Apis mellifera* Linnaeus, 1758) is one of the pillars of the global ecological and agricultural systems. In addition to its economic value which is honey and wax its critical role as a key inoculator of many crops enriches food security and ensures biodiversity [1] The apiculture industry is also a major agricultural tradition and an important economic asset in Iraq but it is threatened by growing microbial infections that cause the collapse of colonies and massive losses in finances [2]

And have aggressive nature for beehives many fungal infections are included in this list. The species of the genus Aspergillus most notably Aspergillus flavus and Aspergillus niger become lethal

opportunistic pathogens. These are the species responsible for the Stonebrood disease found in larvae and adult bees [3] The fungal hyphae infect by invading the body tissues of a host, turning them into hard, stone-like masses. It completes the life cycle of the bees and prevents them from reproduction for long enough to threaten the entire colony with death.

The virulence of such entomopathogenic fungi is not accidental but is directly predetermined by the ability of the entomopathogenic fungi to release extracellular enzymes that act as biochemical means of breaking down the physiological barriers of the honeybee. In particular, the structure proteins are broken down by the action of Protease enzymes, and the protective waxy layer of the cuticle is dissolved by Lipases [5, 8]. At the same time, the hard chitinous structure is broken by Chitinase, and Laccase is important to counter the immune responses of the host [6, 10]. Latest research studies have emphasized on the fact that the efficiency and production of these enzymes are greatly determined by the environmental conditions and the fungal strain source in which they are isolated [7, 9]. It is this combined enzymatic synergy that defines how successful is the invasion and colonization of honeybee tissues by the fungus. In the context of world, previous studies have aimed at profiling the enzymatic arsenal of insect-pathogenic fungi, and it was defined that the most virulent ones have multifaceted and highly efficient enzymatic activities [11]. When in a similar environment as that of Iraq, research has shown that native strains of fungi develop adaptive enzymatic mechanisms to survive extreme climatic conditions, which makes them even more pathogenic to the local apiaries [12]. On this scientific basis, the enzymatic activities of A. flavus and A. niger isolates was studied against honeybees in Iraq the functional association between fungal identity and their overall enzymatic profile of different geographic environments were given at humid marshes and dry areas in the Basra, Dhi Qar and Diyala governorates. The study covered the period from October to December 2025.

Materials and Methods

2.1. Sample Acquisition and Processing

Different 8 localities were sampled, such as. Samples were collected from diverse ecological zones in Iraq, including Al-Qurna (31.01°N 47.43°E), Al-Madina, Manawi Basha, Khor Al-Zubair, and Al- Zubair in Basrah, alongside Al-Shatra and Al-Chibayish (31.00°N 47.03°E) in Dhi Qar, and selected sites in Diyala. These locations represented a spectrum of environments ranging from humid marshlands to arid desert areas In the case of Isolation and Purification, the fungus were grown on Potato Dextrose Agar (PDA) with Chloramphenicol to limit the number of growing bacteria and obtain high-purity isolates

2.2. Identification of Fungal Isolates

The fungi isolates were recognized on the basis of macroscopic and microscopic features. The pathogenicity and identification profiles have been determined based on the following contemporary procedures:

Molecular and Biochemical Characterization: *A. flavus* and *A. niger* isolates found in the colonies of honeybees were identified using standard procedures [14].

Pathogenic Mechanisms: Pathogenicity of *Aspergillus* was determined in honeybees larvae according to the recent research of fungus infection cycles [3].

Enzymatic Profiling: Extracellular enzymes known to be important virulence factors of honeybee- pathogenic fungi were studied according to standard assay procedures [11, 16].

Ecological Context: The biodiversity and distribution of *Aspergillus* species in the Southern Iraqi ecosystems (humid and arid zone) was studied with respect to the local environmental studies [12].

Virulence Assessment The analysis of the increasing virulence of *Aspergillus* species in the colonies of honeybees was conducted based on the current global observations. [15]

The detection of extracellular enzymes produced by *A. flavus* and *A. niger* was carried out using specific substrates and culture media. The positive indicators and methodologies adopted for identifying protease, lipase, chitinase, and laccase activities are summarized in Table 1

Table 1. Modern Methodological Indicators for Fungal Enzyme Detection.

Enzyme	Substrate / Culture Medium	Positive Indicator	Modern Reference
Protease	Skim Milk Agar	Formation of a clear zone (Caseinolysis)	[16]
Lipase	Tween 80 Agar	White precipitate / Halo formation	[16]
Laccase	ONPG / Guaiacol in PDB	Color transition (Yellow/Red-Brown)	[18]
Chitinase	Colloidal Chitin Agar	Zone of clearing around colony	[19]

A. Detecting Enzymatic Activity

To assess the catalytic capacity of the studied fungi a set of specialized and highly selective substrates was used. Skimmed milk agar Tween 80 colloidal chitin, and ONPG (with modifications in the middle so that it is fungal and not bacteria)were used as biomarkers to detect the enzymatic activity of each sample.

B. Statistical Analysis

To ensure the accuracy of the conclusions the data were analyzed using SPSS (version 26.0) according to the following methodologies ANOVA One-way ANOVA was applied to detect any significant differences in the enzymatic activity of the *A. niger* and *A. flavus* strains, correlating these results with the geographical environment from which the samples were collected (whether wetlands or arid regions) Duncan's Test this was used as a cross-reference test to accurately determine the differences between the means at a significance level of 0.05. Quality control To enhance scientific reliability all experiments were conducted in three independent replicates, and the arithmetic mean was adopted as the final value to represent the results.

Results and Discussion

3.1. Geographical Distribution

Basra Governorate recorded the highest prevalence rate at 64%.

Table 2. Geographical Distribution and Identification of Aspergillus species (Oct–Dec 2025).

Governorates	Collection Site	Isolate Code	Identified Species	No. of Isolates
Basra	Al-Qurnah (Site A)	FA-1	A. flavus.\ A. niger	5
Basra	Al-Qurnah(Site B)	FA-2	A.flavus	3
Basra	Al-Madina	FA-3	A. niger	4
Basra	Al-Zubair	FA-4	A. flavus\A. niger	7
Basra	Khor Al-Zubair	FA-5	A. niger	2
Basra	Manawi Basha	FA-6	A.flavus	4
Dhi Qar	Al-Chibayish	FA-7	A.niger	4
Dhi Qar	Ash-Shatrah	FA-8	A.niger	3
Diyala	Diyala	FA-9	A. flavus	2

The research findings as detailed in Table2 confirm the extensive and dense distribution of *Aspergillus* species across the governorates of Basra, Dhi Qar, and Diyala. geographic maps showed a notable concentration of isolates in Zubair and Qurna with *A. flavus* (yellow) and *Aspergillus niger* (black) being identified with clear frequency. The holistic effect of these isolates was clearly demonstrated, as their combination possessed a complete set of enzymatic tools, granting them the specialized ability to overcome the immune defenses of honeybees particularly the FA-9 isolate from Diyala, which exhibited superior virulence. These results were supported by a highly rigorous

methodology as the use of selective media provided a rapid and highly effective visual guide for verifying field isolates confirming its ideal diagnostic tool for this research. Statistical evaluation of fungi at the research sites showed a well-established prevalence of the AF-4 isolate (*A. flavus*) a distinct spatial pattern was observed with the Zubair region recording the highest prevalence. The study found two species of fungi (*A. flavus* and *A. niger*) in honeybee hive samples. This was followed by the Qurna region, which also revealed the presence of both species in honeybee hive samples. The findings of this research (Table2) regarding the spread of *A. flavus* and *A. niger* in Iraq are consistent with global observations suggesting that these two species are the primary causative agents of foulbrood disease in honeybees (Voleynikova et al., 2023). The widespread presence of these fungi in Zubair and Qurna suggests that climatic conditions, such as the humid air and high temperatures in these locations, may contribute to the increase of these opportunistic pathogens.

The universal enzyme pattern (100% positive reaction) observed in all 34 isolates is of paramount importance. protease and lipase generation are well-documented primary mechanisms for decomposing insect exoskeletons, while chitinase specifically degrades the chitin layers of the honeybee exoskeleton (Grazin et al., 2021). Furthermore the availability of laccase is often associated with the fungi's ability to neutralize the hostis phenolic immune responses thus explaining the efficacy of these isolates in overcoming honeybee immunity (Poly et al., 2024) The extreme virulent nature of the FA-9 isolate (inhibition band > 15 mm) aligns with the concept of strain diversity whereby some local isolates develop more virulent enzyme structures in response to local environmental challenges this highly virulent form poses a significant threat to local beekeeping, as it mimics the infection levels observed in internationally researched highly aggressive Aspergillus strains (Jensen et al., 2022). From a procedural perspective the successful use of selective media for rapid screening underscores that optical enzyme assays remain the primary measure for field studies. As noted by Simmons et al. (2020) these discriminative and semi-numerical assays are essential for identifying high-risk pathogens before they lead to widespread colony collapse

3.2. Morphological and Microscopic Characterization

For fungal isolates from Iraqi apiaries. Isolates of A. flavus grew rapidly on PDA medium, producing olive-green conidia in velvety to granular colonies with pale yellow undersides. Dense black conidia and yellowish-white fungal margins were present in A. niger colonies. These opportunistic pathogens were indicated by light microscopy, which demonstrated spherical vesicles and biseriate phialides. The findings of this study showed that Aspergillus fungi although commonly existent in all surveyed sites, the correlations between these species and their abundance especially in Qurna and Zubair reflects the ecological features of Aspergillus to Southern Iraq. This means that the fungi are well-positioned for success.

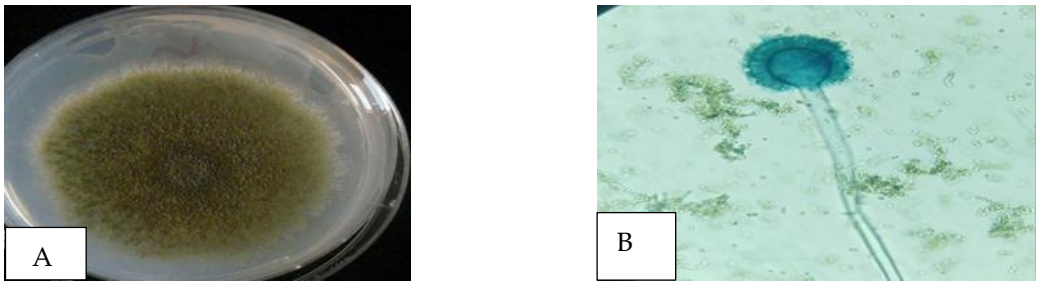

Figure 1. Aspergillus fungi.

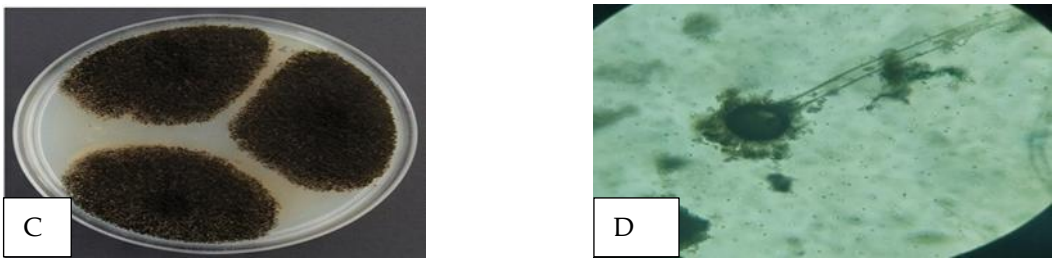

Figure 2. Aspergillus niger.

Morphological and microscopic characteristics of the fungi *A. flavus* and *A. niger* on PDA medium at 25-27°C. A: Front of the plate of fungi *A. flavus* B: Microscopic image (40x) of fungi *A. flavus* C: Front of the plate of fungi *A. niger* D: Microscopic image(40x) of *A. niger*

3.3. Enzymatic profile and Pathogenic Potential

Examination of extracellular enzyme components revealed that all local ectozoites (100%) possessed a full range of enzymes required for host invasion. Protease and lipase secretion was demonstrated by the observation of translucent protein zones and white precipitation halos on a specific agar respectively [16]. These enzymes play a crucial role in the chemical burrowing process of the bee's skin [8] Furthermore the ectozoites exhibited high levels of chitinase which was supported by the presence of distinct colony zones on colloidal chitin agar. Interpretation: Enzymatic Properties and Morbidity-Inducing Capacity The successful detection of chitinase [19] and the rapid colorimetric change observed for laccase and beta-galactosidase [18] reinforce the robustness of the enzyme complex of the studied Aspergillus isolates. This stable enzyme structure across diverse geographical locations suggests that these enzymes are key drivers of virulence in Iraqi Aspergillus strains [20, 22] Our observations are consistent with Al Abadi's (2022) research, which indicated that the stability of extracellular enzymes in the environments of southern Iraq is a key component of fungal adaptation to honeybees. Similarly Mansour (2023) supported the finding that Aspergillus species in the Basra region exhibit significant enzymatic activity regardless of ambient salinity or humidity. However our results differ from those of Hassan et al. (2021) demonstrated clear variations in enzyme release depending on humidity levels in northern Iraq. This is attributed to This contrast is due to the high environmental resilience of the southern Iraqi strains which have adapted to maintain their disease capacity despite the sharp environmental changes between the Al-Shibaish marshes and the dry areas of Zubair

3.4. Enzymatic Activity

The selective media imaging also indicated the efficiency of the isolates in enzyme production by observing the development of degradation halos or chromogenic transitions (see Enzymatic Assays - Figure 3) with the isolates.

Table 3. Morphological and Microscopic Characteristics of the Fungal Isolates Pathogenic to Honeybees.

Isolate Code	Identified Species	Collection Site	Protease	Lipase	Chitinase	Laccase
FA-1	A. flavus / A. niger	Basra .Al-Qurnah (Site A)	**	*	**	+
FA-2	A. flavus	Basra .Al-Qurnah (Site B)	*	**	*	+
FA-3	A. niger	Basra .Al-Madina	**	**	**	+
FA-4	A. flavus / A. niger	Basra .Al-Zubair	*	*	*	+
FA-5	A. niger	Basra .Khor Al-Zubair	**	*	**	+
FA-6	A. flavus	Basra .Manawi Basha	***	***	**	+
FA-7	A. niger	Dhi Qar .Al-Chibayish	**	**	***	***

FA-8	A. niger	Dhi Qar .Ash-Shatrah	*	**	**	***
FA-9	A. flavus	Diyala	***	***	***	***

1. - [***] Strong Activity: Clear zone > 15 mm (replaces Dark Green)
2. - [**] High Activity: Clear zone 10-15 mm (replaces Light Green)
3. - [*] Moderate Activity: Clear zone < 10 mm (replaces Yellow)
4. - [+] Positive Reaction: Qualitative color change (replaces Light Blue)

The results presented in Table (3) provide a clear biochemical explanation for the high pathogenicity observed in the local *Aspergillus* isolates. formation of lysis halos and color shifts (Figure 3) directly reflects the metabolic efficiency of the fungi in secreting extracellular enzymes.

The exceptional performance of the FA-9 isolate which exhibited strong activity (*) in all four enzymes is a highly significant finding. According to Saint-Léger et al. (2022) the simultaneous secretion of proteases and chitinases is the most lethal strategy employed by entomopathogenic fungi. These enzymes work synergistically to break down the protein-chitin matrix of the beeis exoskeleton which forms the primary physical barrier against infection. Without this enzymatic drilling the fungi would be unable to penetrate the exoskeleton and induce systemic infection.

Furthermore the high protease activity in FA-6 and chitinase activity in FA-7 and FA-8 indicates a high degree of pathogenicity among local strains. This is consistent with the findings of Grizan et al. (2021) who observed that some *Aspergillus* strains compensate for a deficiency in one enzyme by producing excess amounts of another to ensure host invasion. The presence of both lactase and lipase enzymes in most isolates further complicates honeybee defenses. Lipase facilitates initial adhesion to the bee's outer wax layer while lactase is known to neutralize the host's melanin pigmentation response a crucial component of insect immunity (Poly et al., 2024). Overall these findings confirm that the local fungi in Basra, Dhi Qar, and Diyala possess a highly organized and aggressive enzyme toolkit that poses a significant threat to beekeeping

3.5. Enzymatic Potential and Pathogenic Mechanisms

The findings in Table 3 show that the isolated *Aspergillus* strains possess a strong and diverse enzymatic pattern this is not just a simple metabolic byproduct it's a strategy for survival and infection of paramount importance.

1. **Protease and Invasion Lipase** .The high vital and active actions [*] of Protease and Lipase particularly by the isolates (FA-6 and FA-9) are needed at initial stages of infected organisms. Mechanism Fungi release these enzymes to chemically digest the procuticle of the honeybee, from which proteins are hydrolyzed into structural constituents by proteases and the waxy lipid layer of exoskeleton by non-specific Lipases allowing fungal hyphae to invade internal tissues [6 Gyger S. An enzyme produced by Metarhizium anisopliae facilitates penetration of insect cuticles. [+] [8] Kumar R (2001).

Results Discussion: These results are very much in line with the results of Foley et al. (4) identified extracellular proteases as the main virulence factors in Stonebrood pathogens [6]. Likewise Gopinath et al. A feature of insects infected by aggressive strains of Aspergillus is high lipase production (2022) [8].

2. Chitinase

The isolates of Al-Chibayish (FA-7) and Diyala (FA-9) well showed chitinolytic activity [***] Mechanism the exoskeleton of the bees is composed of chitin as its fundamental material. Chitinase secretion enables the fungus to degrade this stiff polymer and colonize the deep tissues and kill the larva [19].

Correlation this is in line with Santos and Martins (2023) [19] who explained that chitinase activity is identified as the key that opens the physical defense of the insect. The results of our work are also consistent with the findings of Pedrini (2023) [6] with reference to the synergistic activity of proteins.

3. Laccase and galactosidase

The overall positive performance of Laccase and galactosidase [+***] marks the capability of fungi to adapt to the host environment.

Mechanism Laccase is also mainly released to counter the honey immune response (melanization) and protect the fungus during infection with oxidative stress [10] beta-galactosidase is also used to help with the utilization of complex carbohydrates in the hive so the fungus has the energy necessary to grow rapidly [18]

Correlation: These findings are also correlated with those of Arregui et al. (2019) [10] and Boonmee and Panyachanakul (2021) [18].Which reported these enzymes as crucial to the survival and pathogenicity of *Aspergillus* in honeybee colonies over a long period

Conclusion

In my study I have confirmed the extensive and dense distribution of Aspergillus species across the governorates of Basra, Dhi Qar, and Diyala with a particularly striking concentration in the Al-Zubair and Al-Qurnah regions. The universal potency of these isolates lies in the fact that 100% of them possess a complete enzymatic toolkit granting them the specialized ability to breach the immune defenses of honeybees-most notably the FA-9 isolate which exhibited superior virulence. These findings were further reinforced by our methodological accuracy as the use of selective media provided a rapid and highly effective visual indicator for screening field isolates with precision proving to be an ideal diagnostic tool for this research

REFERENCES