ORIGINAL ARTICLE

Soil Fungi from Extreme Environments with Enzymatic Activity of some Isolated Taxa

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Key words:
Soil fungi, Extreme environments, Fungal activity, New records

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ABSTRACT

Background: *Fungi growing in extreme environments are a potency massive reservoir of novel bioactive natural products. Extremophilic fungi can survive in extreme habitats, they can be divided into thermophilic, psychrophilic, acidophilic, alkaliphilic, halophilic, and barophilic fungi.* ***Objective:*** *To employ the fungi isolated from soils and collected from various extreme sites to study their enzymatic activity.* ***Methodology:*** *A total of 161 fungal isolates were isolated from different sites. Morphological identification, molecular identification and enzymatic activity of selected fungal isolates was done.* ***Results:*** *The highest occurrence was for genus Aspergillus and their species starting from A. niger (87.5%) and followed by both A.flavus and A. terreus 62.5%, while A. flavus gave the highest frequency were (27%), followed by A. niger (23.8%) and A. terreus (21.9%) respectively. Six isolates were selected for molecular identification and three of them (Aspergillus cejpii, Penicillium camembertii, Trichoderma asperellum) where selected for enzymatic activity studies.Aspergillus cejpii and Trichoderma asperellum represent new record for Iraqi mycobiota.* ***Conclusion:*** *The study showed that the diversity and high dominance of Aspergillus in different environments. When testing one of its species, Aspergillus cejpii, which was recorded for the first time in Iraq, it did not show any activity to the tested enzymes, while Trichoderma asperellum gave a positive detection for all tested enzymes, perhaps the second species is an extremophilic fungus, while A. cejpii is an immigrant species.*

INTRODUCTION

Fungi are a highly various group ofheterotrophic eukaryotes microorganisms. They are characterized by several features like the presence of a chitinous cell wall, they can be observed unicellular or multicellular, and which have a great ability to produce a high metabolic diversity, including organic acids, antibiotics, and enzyme[1]. Fungi growing in extreme environments are a potency massive reservoir of novel bioactive natural products. The opportunity of isolating new fungal species is greater if the samples come from non-mesophilic environments, such as those characterized by high salinity, high radiation, restricted nutrients, extreme temperatures, pressures and changing acidity. Extremophilic fungi can survive in extreme habitats, they can be divided into thermophilic, psychrophilic, acidophilic, alkaliphilic, halophilic, and barophilic fungi[2]. Organisms from these extreme habitats have advanced duration strategies for growing and reproduction[3], osmotically active compounds such as polyols in xerotolerant fungi[4], and fungal melanins for protection against freezing and UV radiation[5]. Extreme environment conditions stimulate fungi to synthesize several specific compounds for adaptation, in addition, fungi from extreme environments seem therefore, promising candidates to isolate new bioactive compounds. Fungi represent the most extreme-tolerant organisms with a very multilateral lifestyles and amazing ecological and morphological pliability[6]. Relevant relationship between fungal metabolites production and the growth conditions[7] and therefore, the evaluation of fungal growth environment is key to conception the fungal role in the nutrient rotation and leads to a better understanding of the type of fungus collected. In recent years, molecular identification has been performed as the main method to proceed the identification of fungi. Particularly, the ITS region has been used to recognize a large number of fungi with a high success rate[8]. This region also shows issues for highly specious genera such as *Aspergillus sp., Cladosporium sp., Fusarium sp., Penicillium sp*., and *Trichoderma sp.* since their taxa have strict or invalid gaps in the bar code of their ITS regions[9].

Enzymes play an essential role in basic biological reactions in the nutrition of life. They are widely distributed in all forms of living organisms. Fungi represent an interesting source of industrial enzymes[10]. Fungal enzymes are characterized by high production power, especially for filamentous fungi and effective

catalysis with the required stability against extreme conditions[11]. Enzymes play serious function in the challenges which facing humanity to use biological systems for a wide range of uses. Presently, fungal enzymes accounted for more than 50% of the total enzyme market[11], and these are attributed to a small number of species of the genera *Aspergillus, Trichoderma, Rhizopus* and *Penicillium* that meet the commercial requirements to produce enzymes[12]. The aim of this work is employ the fungi isolated from soils and collected from various extreme sites to study their enzymatic activity.

METHODOLOGY

Collection and isolation of fungi

The soil samples were collected from different extreme environments in Iraq which included: 1-contaminated soil with hydrocarbons (Basrah city Center) 2-soil from Qurna oil Area 3-soil from Mount Sanam 4-sediments from the Faw area 5-soil from the Nasiriyah desert 6-soil from the Ramadi desert 7-soil from the Hammam al-Alil (hot water springs) in Mosul 8-soil from the sulfuric springs area in Kubeesa. The 13 soil samples were cultured on potato dextrose agar (PDA) medium with chloramphenicol (0.05 mg/ml), then the petri dishes were incubated at 27 C for two weeks. The developing fungal colonies were examined and identified according to phenotypic traits.

Morphological identification of fungal isolates[13-15]

Morphological analysis was made for the growing colonies on PDA such as colour (obverse and reverse), shape, appearance, surface, margin, growth rate and the features of the hyphae, sexual and asexual reproductive organs. We Identified the isolated colonies of filamentous fungi by using compound microscope. The morphological identification was then confirmed by the molecular method and sequencing.

Molecular identification of fungal isolates

Genomic DNA extraction of six fungal isolates (P1, E2, T3, N4, R5, F6), primers synthesis, PCR amplification and sequencing were achieved at Macrogen Company Inc., South Korea. The universal primers ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used to amplify the ITS gene encoding the 5.8S rRNA (ITS1-5.8SrRNA-ITS2 fragment)[16]. The sequence results were analysed by using web-based blasting program, Basic Local Alignment Search Tool (BLAST) and data were compared with the Genbank database at the National Center for Biotechnology Information (NCBI) Nucliotide Sequence database to find the closest sequence similarities[17,18]. The sequence was deposited to Bankit for accession Number. Alignments for each data set were made in MEGA11 and refined with MUSCLE. Sequence phylogeny was constructed employing Maximum Liklihood (ML) and the most suitable nucleotide substitution model determined by MEGA11. Models with the lowest Bayesian Information Criterion (BIC) scores are regarded as the most accurate descriptors of the substitution pattern. Bootstrap analysis of 1000 replicates assessed the reliability of the constructed phylogenies. *Candida albicans* (NR-1253321.1) was used as an outgroup isolate.

Enzymatic activity test of selected fungal isolates

An enzymatic efficacy of the selected fungal colonies was tested to evaluate the production of amylase, lipase and protease on solid media, with three replicates for each fungal isolate. Negative controls were made to own a reference point. A specific substrate of each enzyme was added to the culture medium as a carbon source. After inoculation cultures were incubated for 2-5 days according to growth rate of each fungal isolate, appearance of a clear zone or precipitation around each cultured fungus refers to enzyme production.

Amylase activity was estimated on nutrient agar medium supplemented with 0.2% of soluble starch. After incubation period (2-5 days) at 25 °C, cultures were flooded with a 1% solution of iodine. The clear zone around the colony refers to the existence of amylase[19].

Lipase activity was specified on culture medium including tween 80 as a lipid substrate (10 g/L peptone, 5 g/L NaCl, 0.1 g/L $CaCl_2$ $2H_2O$, 17 g/L agar and 10 mL/L tween 80). Tween 80 was sterilized separately then added to the sterile medium. After incubation of cultured petri dishes at 25 °C, an opaque precipitation had appeared around the fungal colony[19] . Protease activity was revealed on nutrient agar containing 0.4% gelatin. gelatin solution was sterilized separately then added to the sterile medium. After incubation at 25 °C, cultures were flooded with a saturated solution of ammonium sulfate degradation of gelatin was detected by displaying a clear zone around the colony[19].

RESULTS

Overall, 161 fungal isolates were isolated from all selected sites (Table 1). 157 fungal isolates were subjected to microscopical identification to genera level. Four isolates were not identified (sterile mycelia). 149 fungal isolates were belonging to hyphomycetes, 11 fungal isolates were belonging to teleomorphic ascomycetes and 1 isolate of Mucorales. The isolated fungi showed a variation in their occurrence and frequency, the highest occurrence was for genus *Aspergillus* and their species starting from *A. niger* 87.5% and followed by both *A.flavus* and *A. terreus* (62.5%), while *A. flavus* gave the highest frequency percentage 27%, followed by *A. niger* 23.8% and *A. terreus* 21.9%. respectively.

Table 1: List of fungal isolates that were obtained from the search sites with their occurrence

Fungal isolates	Sample number	Number of isolates	Occurrence %	Frequency %
Aspergillus flavus	2, 3, 5, 7, 8	42	62.5	27
A. fumigatus	2, 5, 8	4	37.5	2.58
A. niger	1, 2, 3, 4, 6, 7, 8	37	87.5	23.8
A. cejpii	7	6	12.5	3.87
A. terreus	1, 2, 3, 5, 6	34	62.5	21.9
Aspergillus sp.	8	2	12.5	1.29
Bipolaris sp.	8	1	12.5	0.64
Cladosporium sp.	8	3	12.5	1.93
Curvularia lunata	5	1	12.5	0.64
Emericella sp.	1	1	12.5	0.64
Eurotium sp.	3, 5, 8	10	50	18.18
Penicillium camemberti	8	1	12.5	0.64
Penicillium sp.	2, 6, 7	10	50	18.18
Rhizopus sp.	6	1	12.5	0.64
Trichoderma asperellum	1	1	12.5	0.64
Ulocladium chartarum	4, 5	3	25	1.93
Sterile mycelium	4	4	12.5	2.58
Total of isolates		161		

Phylogeny

The PCR amplification of the ITS region of the six fungal isolates produced PCR products measuring between 500 to 650 base pairs (bp). Molecular identification of fungal isolates showed 6 fungal species, grouped in two clades, where *Candida albicans* was used as out group (Figure 1). Drawing the phylogenetic tree, the result shown the six isolates were related to Ascomycota. The most dominant genus (66.66%) was *Aspergillus*, 4 fungal isolates, followed by 16.67% *Penicillium* and *Trichoderma* one species for each (Figure 2). For identification at species level, the sequences generated in this study were aligned against all sequences of well-documented species in GenBank. The outcome of blasting searching, indicating that Aspergillus species include *Aspergillus fumigatiaffinis* (F6), *Aspergillus niger* (N4), *Aspergillus terreus* (R5), *Aspergillus cejpii* (E2), while (T3) isolate identified as *Trichoderma asperellum* and (P1) as *Penicillium camemberti*. DNA sequences of these isolates were submitted to the databases of GenBank. The gene bank accession numbers were issued and confirmed as shown in (Table 2).

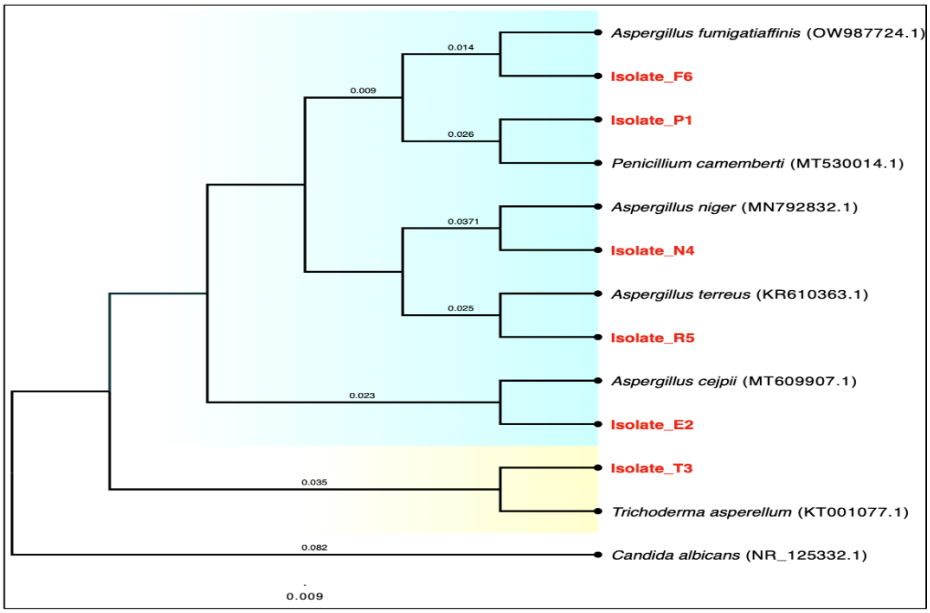

Fig. 1: Phylogenetic tree represents Neighbor-joining analysis of ITS domain sequences depicting the relationships of 6 isolated fungi (isolate_No. **P1, E2, T3, N4, R5** and **F6**, red colour) with closely related reference sequences of species retrieved from NCBI. Each numerical value represents the percentage of bootstrap samples, a total of 1000 samples, that support the internal branches with a confidence level of 50% or higher. The scale bar represents number of nucleotide substitutions per site. *Candida albicans* (NR_1253321.1) represented an out group.

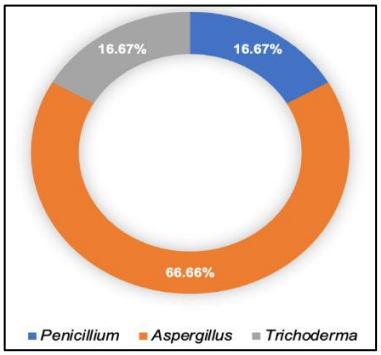

Fig. 2: Frequency of isolated fungi by genus level

Table 2: Identification of 6 fungal isolates from desert sand contaminated with oil based on sequencing of rDNA - ITS1-ITS4 region during this study

No.	Isolate Code	NCBI-Blast Identification	Accession No. (NCBI)	ID %
1	**P1**	*Penicillium camemberti*	PP345600	100
2	**E2**	*Aspergillus cejpii*	PP345599	100
3	**T3**	*Trichoderma asperellum*	PP345598	98.02
4	**N4**	*Aspergillus niger*	PP345596	100
5	**R5**	*Aspergillus terreus*	PP345597	100
6	**F6**	*Aspergillus fumigatiaffinis*	PP345595	100

New Record isolates

Morphological and molecular identification

Trichoderma asperellum Samuels, Lieckf and Nirenberg, Sydowia 51:81(1999) (Figure 3). Colonies growing rapidly on PDA reaching 45mm in 7 days at 25 °C, initially hyaline turn yellow, and soon becoming green or remained creamy yellow without concentric rings but scattered throughout the colony due to the dense production of conidia: reverse cream coloured. Conidiophores branched, paired, grow within aggregates. Phialides form at the tips of branches in whorls, ampuliform, straight, slightly wider in the middle than the base. Conidia hyaline, green in mass, fine ornamented, ovoidal or globose to subglobose 3.5-5 x 2.5-3.5 µm. Chlamydospores present.

Material examined: The strain *Trichoderma asperellum* (**T3**) has been isolated from soil contaminated with hydrocarbons (Basrah city Center), Iraq, 11January 2022. Living culture deposited at Mycology Laboratory, Department of Biology, Collage of Science, University of Basrah. NCBI (PP345598).

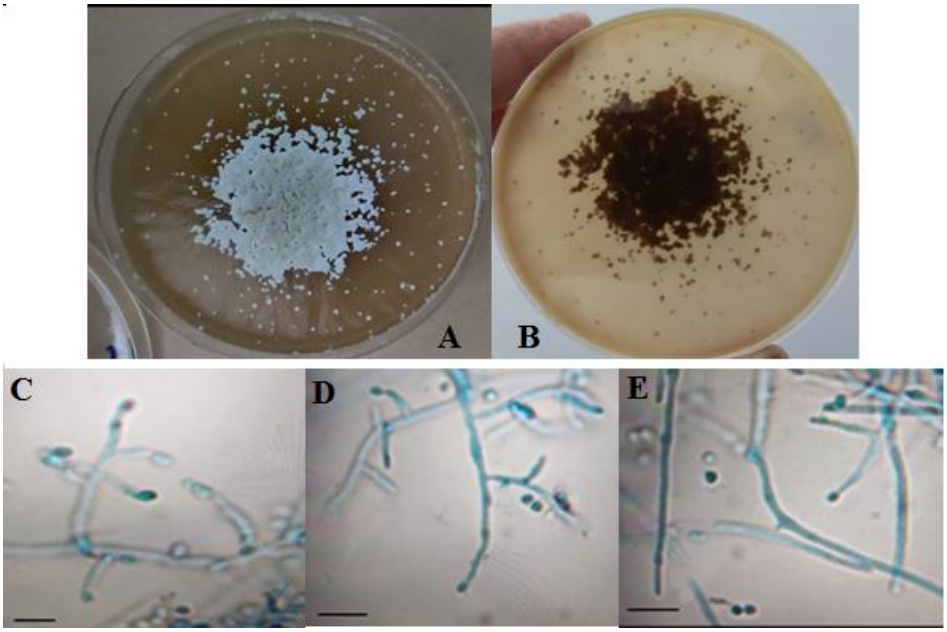

Fig. 3: Morphological identification of *T. asperellum.* (A) The cultural view of *T. asperellum* grown on PDA at 25 °C for 7 days, (B) the reverse of the colony, (C, D, E) Microscopic view of conidia and conidiophores. Scale bar = 10 µm *Aspergillus cejpii* (Milko) Samson, Varga, Visagie and Houbraken, Stud. Mycol., 78: 155 (2014). Basionym: *Talaromyces cejpii* Milko, Novosti. Sist. Nizsh. Rast. 1: 208 (1964) =*Dichotomomyces cejpii* (Milko) D. B. Scott, Trans. Brit. Mycol. Soc. 47: 428 (1970). (Figure 4). Colonies growing rapidly on PDA attaining 57mm in 7 days at 25 ºC, light yellow, floccose, with white cleistothesia sscattered on the colony surface. Ascomata spherical, 500-1000 µm white to cream coloured. Asci spherical, 8-spored, (10-15) µm. Ascospores hyaline to light yellow at maturity, lenticular with two thin equatorial crests, (3-4.5 x 3.5-5 µm), convex wall smooth. Anamorph is not determined.

Material examined: The strain *Aspergillus cejpii (***E2)** has been isolated from soil of the Hammam al-Alil area, Mosul, Iraq, 12 January 2022. Living culture deposited at Mycology Laboratory, Department of Biology, Collage of Science, University of Basrah. NCBI (PP345599).

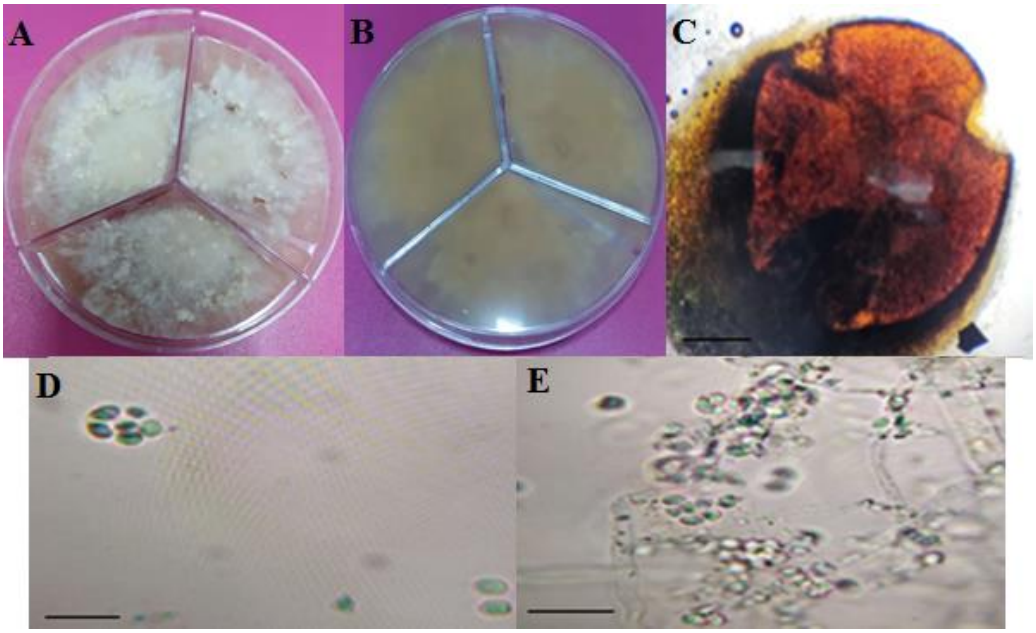

Fig. 4: Morphological identification of *Aspergillus cejpii*, (A) The cultural view of *Aspergillus cejpii* grown on PDA at 25°C for 7 days, (B) the reverse of the colony, microscopic view of (C) cleistothecia, ascospores (D, E). Scale bars: C= 250 µm, D, E = 10 µm

Enzymatic activity of selected fungi that isolated from soil of extreme environments

The results have obtained from the assessment of the enzymatic capabilities of isolated fungi are shown in (Table 3) and (Figure 5).

Table 3: Enzymatic activity of some fungal isolates

Fungal isolate	Amylase	Lipase	Protease
Aspergillus cejpii	-	-	-
Penicillium camemberti	-	+	+
Trichoderma asperellum	+	+	+

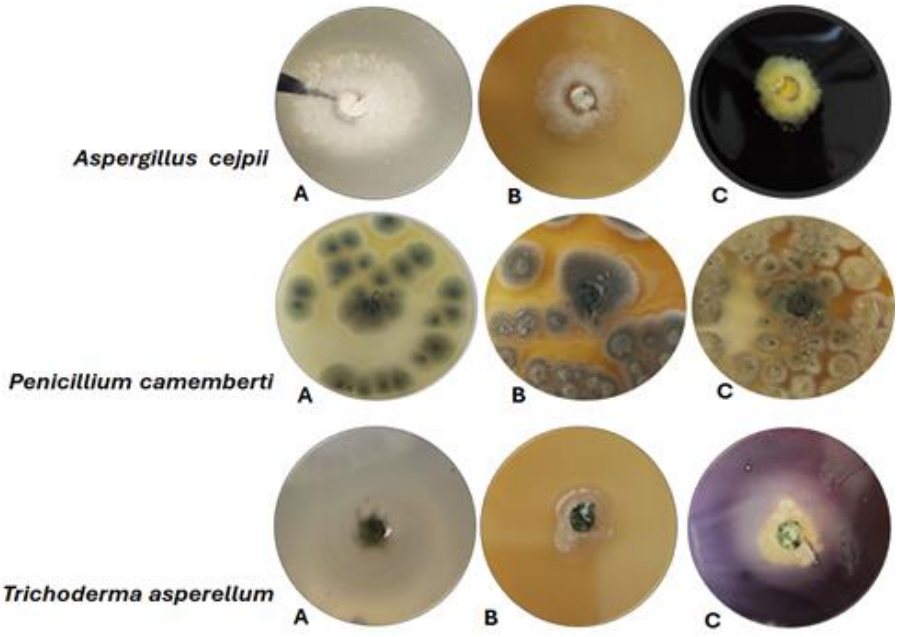

Figure 5: Enzymic activity of selected fungi which isolated from soil on solid medium. A= Lipase enzyme test, B=Protease enzyme test, C= Amylase enzyme test

The enzymic test revealed that the fungal isolate *T. asperellum* produced the three types of tested enzymes (Amylase, lipase, and protease), while the fungal isolate *P. camemberti* produced two enzymes. However, the fungal isolate *A. cejpii* did lose its ability to produce any kind of studied enzymes as shown in (Table 3) and (Figure 5).

DISCUSSION

Recent study has the occurrence pattern of isolated fungi in soil of extreme environments and, the method of fungal isolation is like the study was performed by (Hamim, and Hassan,2017)[20]. They isolated and identified fungi from 40 samples, which were taken from six extreme sites in Nasiriyah city, Iraq. Also, they reported that *Aspergillus* was the most common genus (28.14%). Generally, current environmental studies reveal that clime change is impact significantly the occurrence and distribution pattern of fungi in soil of extreme environment. According to the United Nations reports about clime change crisis, Iraq has been recognised as one of the five countries most susceptible to the effects of clime change, the negative consequences include increasing temperature, insufficient rainfall and, repeated dust and sand storms[21], possibly resulting in structural and functional changes in microbial community[22]. Six fungal isolates were selected that gave good growth on the culture medium and were molecularly diagnosed, three of which were studied for their enzymatic efficacy.

Microbial community plays a critical role in ecosystems due to their bioactive products like enzymes, antibiotics, and vitamins[23]. Fungi are one of key players in extreme environment[6].

The study of Elsababty *et al.*[24] showed that *T. asperellum* and *A. cejpii* could make various pectinase activities and caused disintegration of the pectin medium. Gliotoxins have been isolated from different fungi, including *A. cejpii* [25]. *T. asperellum* is a filamentous fungus that can produce and secrete a wide range of extracellular hydrolytic enzymes used for plant cell wall degradation[26]. Gueye *et al.*[27] study the factors affecting the chitinase activity of *T. asperellum*, while Berini *et al.*[28] study the production and characterization of *T. asperellum* chitinases and their use in synergy with *Bacillus thuringiensis* for lepidopteran control.

Recently, it was found that the production of microbial enzymes retains an advanced subject of innovation. The use of enzymes is still preferable to the use of chemical processes because they can carry out many important types of reaction more efficiently under moderate conditions[29]. Among microbial enzymes, fungal ones are in the best positions, due to their ability to be produced in large quantities, more quickly and at reasonable costs. The production of fungal enzymes has had an incredible impact not only on industrial sectors but also on the processes of biochemical synthesis and healthcare services[29]. The advancement of Biotechnology in these fields offers various ways to economically reduce the cost of producing enzymes. Moreover, the use of microbial enzymes, especially fungal ones, has proved to be extremely important for processing and quality improvement. Rapid progress in the commercialization of enzymes has occurred during recent periods, due to the development of industrial and biotechnological uses.

CONCLUSION

Fungi are known to produce a wide range and high levels of interesting enzymes that are important industrially and medically. The study showed that the diversity and high dominance of Aspergillus in different environments. Interestingly, when testing one of its species, *Aspergillus cejpii*, which was recorded for the first time in Iraq, it did not give activity to the tested enzymes, while *Trichoderma asperellum* gave a positive detection for all tested enzymes, perhaps the second species is an extremophilic fungus, while *A. cejpii* is an immigrant species. Continuous isolation from extreme environments may lead to the addition of new taxa to the Iraqi mycobiota. Focusing on such environments plays an important role in discovering fungi that may contribute to the production of various secondary metabolites useful in medicine, pharmaceutical industries, and biotechnology.

Conflict of interest

The authors have no conflict of interest.

Acknowledgments

We would like to thank Dr. Alyaa Albadr for reading this manuscript and Ms. Haneen M. Abdulhafedh for help with collecting.

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