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Extraction of biologically active substances of fungi isolated from various ecosystems and evaluation of their effect

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ABSTRACT

Fungi represent a vast and largely unexplored source of biologically active metabolites. This study aimed to isolate fungi from ecologically distinct regions of the Republic of Azerbaijan and evaluate their antioxidant and antimicrobial potential. During 2022–2025, 105 pure cultures were obtained from soil, water, and plant samples collected across the Greater Caucasus, Lesser Caucasus, Kura–Araz lowland, and the southern region (Lankaran–Astara), including 79 micromycetes and 26 macromycetes. Initial screening of exogenous metabolites revealed variable activity profiles: among micromycetes, antioxidant activity was classified as weak (32 strains), medium (27), and strong (5), while antimicrobial activity was weak (26), medium (37), and strong (4). Comparable variability was observed in macromycetes. Four highly active strains were selected for detailed study. Based on classical and ITS rDNA analysis, the most promising micromycetes were identified as *Trichoderma citrinoviride* AEF-2024 and *T. harzianum* AEF-2024 (99.83% and 99.66% sequence similarity, respectively). Optimization experiments demonstrated that liquid Čapek-Doks medium yielded maximum biomass (12.5 and 10.7 g/L for *T. citrinoviride* and *T. harzianum*, respectively). Under optimized conditions, culture solution (CS) exhibited pronounced antimicrobial activity (34–35 mm inhibition zones), whereas biomass extracts (BM) showed higher antioxidant capacity (29.6–31.2% DPPH radical scavenging). The findings highlight significant strain-level variability and confirm that fungal isolates from diverse Azerbaijani ecosystems constitute promising sources of bioactive metabolites with potential biotechnological and pharmaceutical applications.

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INTRODUCTION

Fungi isolated from ecologically different areas of the Republic of Azerbaijan were evaluated for biological activity. It was determined that the isolated cultures have both antioxidant and antimicrobial activity, among which species such as *Trichoderma citrinoviride* AEF-2024 and *T. harzianum* AEF-2024 are characterized by relatively high activities for both indicators.

Although both fungal species, which were selected as active producers with antioxidant and antimicrobial activity, were present in both the culture solution and biomass, the culture solution was characterized by relatively high antimicrobial activity, and the biomass by relatively high antioxidant activity. The fact that the activity level of a particular metabolite varies even at the strain level allows us to note that research aimed at searching for highly active natural strains of the same species is still relevant and that it is possible to isolate natural strains with higher activity under specific conditions. The isolation of such natural strains can also be considered a positive factor, as they are more promising material for obtaining more active strains through genetic means.

Fungi, which are one of the most diverse groups of eukaryotic organisms and are characterized by a wide variety of species, include yeasts, molds, micromycetes, and macromycetes. It has also conditioned the ability of fungi, among which cosmopolitans are the majority, to spread in any part of the world where organic matter is present, even in extreme conditions (in Antarctic glaciers, thermal waters, areas with high salinity, etc.). According to the modern systematics of fungi, which currently have between 150,000 and 170,000 species known to science, and estimates of the actual number of species in nature to be up to 5.5 million, they are classified into 1 kingdom, 8 divisions, and 46 classes as of 2017 (Spatafora *et al.*, 2017). Such diverse fungi perform a wide and diverse range of functions in nature. Thus, from a practical point of view, there are both useful and harmful (Bakhshaliyeva *et al.*, 2024; Hyde *et al.*, 2024) ones among them, which is the basis of the practical interest in them. For this reason, mushrooms have been a direct food source for humans since ancient times (cap mushrooms), but they have also been a source of a number of products (sour milk products, bakery products, alcoholic beverages, etc.), and this process continues in modern times. For this reason, microorganisms, including fungi, are considered a great source of biodiversity and an inexhaustible source of secondary metabolites with biological activity that differ in structure and effect (Besharati *et al.*, 2025). The global food problem caused by the increasing population in developing countries, the decreasing availability of usable land, climate change, natural disasters, biodiversity loss, and other problems are driving the growing practical interest in fungi (Berry, 2020; Petrescu *et al.*, 2020). Thus, the use of mushrooms for one or other purposes makes it possible to note that they are more effective in solving the above-mentioned problems, both from economic, technological, and ecological considerations. Thus, the composition of mushrooms is rich in carbohydrates, including cellulase, which also provides them with sources of other organic substances (Dimopoulou *et al.*, 2022; Gopal *et al.*, 2022). The amino acid composition of mushroom proteins is comparable to that of animal-derived foods (meat, eggs, and milk). On the other hand, the biomass produced by fungi also contains other bioactive components, the use of which in enriching nutrients has also been shown to be effective in a number of studies (Anusiya *et al.*, 2021; Venturella *et al.*, 2021).

It should be noted that the practical interest in fungi is also due to their active synthesis of metabolites with pharmacological activity, and currently, remedies obtained from them are used in both folk and clinical medicine (Bolesławska *et al.*, 2024). Interestingly, among fungi that synthesize metabolites with biological, including pharmacological, activity, there are species that belong to both the edible and poisonous mushroom categories. For this reason, obtaining various products from mushrooms, primarily for food and

medical purposes, is characterized as a complex issue. The reason for this may be the toxicological properties of some mushrooms, insufficiently studied mushroom categories, incorrect identification of mushrooms, the content of heavy metals and pesticides, pathogenicity and the ability to cause allergies, as well as other problems. Therefore, the expansion of the use of fungi requires an expansion of the search for safer fungi and the metabolites they synthesize.

The Republic of Azerbaijan has a vast and rich nature, which has led to the spread of various taxonomic groups of fungi in its various territories. Thus, the conducted studies determined the distribution of fungi in the soil, water and plants, and also revealed the presence of species that are simultaneously pathogens, allergens and of practical interest (Bakshaliyeva *et al.*, 2020).

However, the number of fungal species obtained from these results cannot be considered sufficient in terms of exceeding the potential of fungi in either case, as it covers a small part of the species known to science in Azerbaijan, as in the world.

Therefore, the purpose of the presented work is to isolate fungi from ecologically different areas of the Republic of Azerbaijan and to initially evaluate them in pure culture for the biologically active substances they synthesize, and to clarify the purposes for which they may be useful in the future..

MATERIALS AND METHODS

Areas of research

The studies were conducted in the Greater Caucasus, Lesser Caucasus, Kur-Araz Lowland, and southern region (Lankaran-Astara economic region) of the Republic of Azerbaijan during 2022-2025. The mentioned territories differ from each other in terms of area, natural soil and climatic conditions, flora and fauna, anthropogenic impact load, etc. For example, 49% of the forests in the territory of the Republic of Azerbaijan are located in the Greater Caucasus, 34% in the Lesser Caucasus, 2% in the Kur-Araz lowland, and 15% in the southern region [https://eco.gov.az/az/fealiyyet-istiqametleri/mesheler]. More than half of the irrigated lands in the Republic of Azerbaijan are located in the Kur-Araz lowland (Orujova, 2022). The differences between areas according to other indicators can also be characterized as ecosystems with specific indicators in a certain sense.

Taking samples to isolate fungi and obtain pure culture

Samples were taken from soils, waters, and plants located in the above-mentioned areas, using known methods and approaches used in similar studies (Jangid and Ojha, 2025; Maheshwari, 2016). Agarized Čapek-Doks medium (Yusifova *et al.*, 2021) was used to isolate pure cultures of fungi from the samples, to which an antibiotic such as chloramphenicol (100 mg/l) was added to prevent bacterial contamination. The purity of the separated cultures was monitored using a microscope with a magnification of up to 2500 times (OMAX 40X-2500X LED Digital Lab Trinocular Compound Microscope). The separated cultures were sequentially numbered.

Extraction of exogenous and endogenous metabolites from fungi

During the initial screening, the culture medium (CM) of the fungi grown in pure culture after cultivation in a liquid Čapek-Doks medium for 5 days under deep culture conditions was used as a source of exogenous metabolites.

Initial assessment of the biological activity of exogenous metabolites

Antioxidant and antimicrobial activities were used to assess the biological potential of exogenous metabolites.

Antioxidant activity was determined by a method based on the absorption of 2,2-diphenyl-1-picrylhydrazylin (DPPH) free radicals (Gerasimova *et al.*, 2022). The culture solution and the solution obtained from the biomass are dissolved in methanol and 50 µl of the resulting mixture is mixed with 550 µl of DPPH solution(A_N). After 30 minutes of

incubation at room temperature, the absorbance is measured at a wavelength of 517 nm (Spectrophotometer SF 2000, Russia) was used as control (A_K). Free radical neutralization activity (FRNA) was calculated according to the following formula:

$SRNA = (A_K - A_N/A_K) \times 100$

The determination of antimicrobial activity was carried out according to the disk-diffusion method (Prodip *et al.*, 2024), in which bacteria such as *Bacillus megaterium, Staphylococcus aureus, Escherichia coli, Pseudomonas aureus*, and fungi such as *Aspergillus flavus, A. niger*, and *Candida alpicans* were used. Standard discs were used for comparison: a disc with kanamycin as an antibacterial agent and a disc with ketoconazole as an antifungal agent.

During the optimization of cultivation conditions of fungi selected as active producers from the initial screening and the study of their synthesis of metabolites with biological activity, liquid Čapek-Doks, Saburo media, and malt juice (2°B) were used. Cultivation was carried out under deep cultivation conditions (10 days, 200 cycles/min). After the expiration of the period, the resulting biomass and CS were separated from each other by filtration, and both substances were used to obtain active fractions. In the case of direct use of CS, the biomass was used in the following sequence: After washing the biomass (BM) separated from CS 3 times with phosphate buffer with neutral pH, 50 ml of sterile distilled water was added and the cell structure was disrupted in a tissue grinder 3 times with intervals of 3 minutes each. After the expiration of the period, the extract obtained is centrifuged and the supernatant obtained is used. For the precipitation of active ingredients in both CS and BM was used acetone. At this time, the ratio is 1:2 and the dissolved substances in the mixture are precipitated by centrifugation (5000 rpm, 10 min) and the precipitated part is dehydrated in a lyophilizer and analyzed according to the purpose of the work. The results are calculated based on the amount of protein in both sources, where the amount of protein is determined spectrophotometrically (Anisimovich *et al.*, 2017).

Statistical analysis

All experiments conducted during the research were performed in 4 replicates and statistically analyzed (Pollard *et al.*, 2019). The difference was considered statistically significant at a confidence level of at least 0.95.

RESULTS AND DISCUSSION

As a result of analyzing water, soil, and plant samples taken from various ecosystems for fungal biota, 105 pure cultures were obtained, which differed in their morphological appearance, of which 79 belonged to micromycetes and 26 to macromycetes. The distribution of the obtained pure cultures according to the geomorphological units from which they were isolated is summarized in Table 1. As can be seen, although the majority of strains isolated in general are from the Kura-Araz lowland and the least from the Greater Caucasus, this differs slightly between micromycetes and macromycetes. Thus, most of the isolated macromycetes belong to subtropical forests located in the Southern zone.

Table 1. General characteristics of the distribution of cultures identified in the studies across geomorphological units

Geomorphological units	Micromycetes	Macromycetes
Greater Caucasus	17	8
Lesser Caucasus	12	5
Kura-Araz lowland	29	3
Southern zone	21	10
Total	79	26

As is known, the metabolites produced by fungi as a result of their vital activities are of endogenous and exogenous nature, and exogenous metabolites are considered more valuable in terms of practical use (Muradova and Jabrailzade, 2023). This is due to the fact that their separation and purification are cheaper than endogenous metabolites, i.e. more profitable. For this reason, during the research, the isolated cultures were initially evaluated for the biological activity of exogenous metabolites. The results showed that the isolated cultures differed in their biological

activity, and this difference was evident in both antioxidant and antimicrobial activities. Based on the results obtained, it was considered appropriate to divide the fungi into 3 groups and characterize them as having strong, medium and weak activities (Table 2). As can be seen, differences in biological activity indicators are observed at the level of both micromycetes and macromycetes.

Table 2. Evaluation of exogenous metabolites of strains isolated in studies for biological activity

Activity	Activity level	Micromycetes	Micromycetes
Antioxidant	Weak	32	8
	Medium	27	10
	Strong	5	2
Antimicrobial	Weak	26	10
	Medium	37	7
	Strong	4	2
Total		67	20

More precisely, some of the isolated strains did not possess the mentioned activities at all. Thus, antioxidant activity was not observed in 12 strains of micromycetes, and antimicrobial activity was not observed in 10 strains. Analogous indicators were 6 and 7 in macromycetes, respectively. As a result of the studies conducted at this stage, 4 strains with the highest activity indicators were selected, 2 of which belonged to micromycetes and 2 to macromycetes. While both cultures selected from micromycetes had higher antioxidant and antimicrobial activity than the others, this issue was not unequivocally confirmed in macromycetes. Thus, a strain with antimicrobial activity is relatively lower than others in terms of antioxidant activity. For this reason, it was considered appropriate to use micromycetes in further research.

It would be appropriate to touch on one point here, which is related to the fact that strains belonging to the same species, determined by classical mycological methods, may not have different levels of activity among the isolated strains. Thus, the difference between the antioxidant activities of strains belonging to species such as *Trichoderma harzianum* and *Penicillium cyclopium* is 1.2-1.6 times, and the difference between the antimicrobial activities is 1.3-1.5 times. It should be noted that these types of differences at the species and strain levels have also been confirmed in other studies (Muradova and Jabrailzade, 2023; Ulusu *et al.*, 2025).

This fact is due to the ecological plasticity of fungi, their high ecophysiological capabilities, the universality of their metabolism (Bahram and Netherway, 2022), and other characteristics.

The species composition of the cultures selected as active producers based on their biological activity indicators was determined using both classical and molecular genetic methods. It was clear that, according to classical mycological methods, these cultures have characteristics typical of species such as *Trichoderma citrinoviride* and *T. harzianum*. These results have been confirmed by molecular genetic methods (Goncharova *et al.*, 2016; Xu, 2016), as the nucleotide sequence of the ITS gene in GenBank (Benson *et al.*, 2010) corresponds to *Trichoderma citrinoviride* accession number KY764860.1 and *T. harzianum* accession number MH865865.1 by 99.83% and 99.66%, respectively. These strains were named *T. citrinoviride* AEF-2024 and *T. harzianum* AEF-2024 (Muradov *et al.*, 2025).

Studies have also been conducted on optimizing the environment for selected cultures, or rather, finding an environment that allows for maximum biomass yield, and the results obtained have shown that the use of the liquid Čapek-Doks medium for this purpose is appropriate (Table 3) since the quantitative indicators of the optimal parameters of the Čapek medium are characterized by either the same (for example, cultivation temperature and initial pH of the medium) or different (carbon and nitrogen sources, preparation time of the planting material, etc.) indicators for both fungi. Despite this the material obtained from the sedimentation of both biomass and culture solution obtained during the cultivation of both fungi in an optimized environment for 10 days was characterized by higher activity, and it was determined that CS has high antimicrobial activity and BM has antioxidant activity in both fungi (Table 4).

Table 3. Selection of a liquid nutrient medium that provides the maximum biomass yield (g/l) for fungi selected as active producers.

Nutrient medium	*T. citrinoviride* AEF-2024	*T. harzianum* AEF-2024
Čapek	12,5	10,7
Saburo	10,1	9,1
2% malt juice	9,9	8,2

Table 4. Antimicrobial and antioxidant activity of CS and BM produced of fungi selected as active producers during cultivation under optimal conditions

	T. citrinoviride AEF-2024		*T. harzianum* AEF-2024	
	CS	BM	CS	BM
Antimicrobial activity (mm)	34	10-15	35	9-16
Antioxidant activity (%)	21,7	29,6	23,7	31,2

It should be noted that fungi of the genus Trichoderma, which are widespread in various parts of the world, including *T. citrinoviride* and *T. harzianum*, are widely studied as producers of metabolites with various effects (Guo *et al.*, 2022). When analyzing the results of studies conducted in this regard, it becomes clear that the activity level of a particular metabolite can vary even at the strain level, and from this point of view, studies aimed at searching for highly active natural strains of the same species remain relevant.

CONCLUSION

Fungi are an integral and valuable component of any ecosystem where organic matter is present. As a result of the conducted studies, it was also determined that fungi isolated from various sources contain rich sources of metabolites with a wide spectrum of biological activity, which are distinguished as active producers. In this regard, fungi such as *T. citrinoviride* AEF-2024 and *T. harzianum* AEF-2024 have attracted more attention. Thus, exogenous and endogenous metabolites synthesized by both fungi have both antioxidant and antimicrobial activity. This allows them to have high potential for targeted antibacterial applications and to create a more sustainable platform for biotechnological processes aimed at producing bioactive metabolites from them. Research aimed at obtaining biologically active metabolites from one or another living organism, more precisely from fungi, is considered a priority research direction even today. This is due to the fact that fungi are characterized by a wide diversity in various aspects, the fact that abiotic and biotic factors of the environment in which they are distributed also play a certain role in the level of biological activity of this or that metabolite. If we add to the above that the species studied in this direction today do not even constitute 1% of the fungi known to science, then there is no doubt that the scientific and practical basis for obtaining specific metabolites with biological activity will expand the technologies for their preparation solely at the expense of unstudied fungi.

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