Biologically active metabolites of *Penicillium* fungi

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**Abstract**

The present overview highlights potential biomolecules produced by microscopic fungi *Penicillium*. These fungi are a large part of microbial diversity and open up exciting prospects in the search of various biologically active natural scaffolds. In connection with the observed signs of global warming (a decrease of the Arctic Ocean ice cover), the most topical are the results obtained for fungi isolated from permafrost. The strategy for the search of secondary metabolites was successfully implemented as evidenced from the isolation of novel compounds and important metabolites. Studies of the toxigenic potential of the fungi grown on artificial media and natural substrates, the observed correlation between them, confirm the importance of the obtained data and are of ecotoxicological significance.

**Keywords**

Fungi *Penicillium*, Secondary metabolites, Mycotoxins, Ergot alkaloids, Global warming, Permafrost

1. Introduction

Penicillia are some of the most widespread hyphomycetes among microscopic fungi. Their natural habitats are soils of northern latitudes. They are known as producers of various types of biologically active compounds such as ergot alkaloids, diketopiperazines, quinolines, quinazolines and polyketides [1].

*Penicillium* fungi synthesize clavine ergot alkaloids whose structural specificity is that they share the tetracyclic ergoline nucleus. Clavine alkaloids can be divided into three groups. The first includes 6-N-methylergoline derivatives such as festuclavine, epicostaclavine, fumigaclavines and iso-fumigaclavines A and B with a completely saturated ring D. Ergolenes having a double bond in position 8,9, such as agroclavine, agroclavine-1, epoxyagroclavine-1 are assigned to the second group. The third group
consists of clavine alkaloids with the modified rings C and D; rugulovasines A and B, aurantioclavine, and \(\alpha\)-cyclopiazonic acid (CPA) are some of the members of this group of compounds.

Ergot alkaloids possess various kinds of biological activity. They can act peripherally leading to uterine vasoconstriction and contraction; neurohormonally, by blocking the action of adrenaline and serotonin; and can also affect the central nervous system, by reducing the activity of the vasomotor centre and stimulating the sympathetic structures in the midbrain, thereby causing a hallucinogenic effect, and also inhibit the secretion of prolactin, hyperthermia and vomiting [2,3].

The pharmacology of ergot alkaloids finds its origins in numerous Middle-Age accounts of ergot toxicity in man and animals. This toxicity called ergotism was due to the ingestion of (a product from) rye grain infected with the ergot fungus *Claviceps purpurea* [4,5]. Ergotism had been well known from the Middle Ages to the 19th century as “Holy Fire” or “St. Antony’s Fire”. Convulsive ergotism is connected with poisoning with clavine alkaloids, and gangrenous ergotism is caused by peptide alkaloids produced by *Claviceps* [3]. Earlier research into the pharmacological activity of epoxyagroclavine-1 has shown this compound to possess a neurotropic activity, to have a mild hypotensive effect, to slow down the heart rate, to lower the reactivity of the vascular system to noradrenaline [6]. Some ergot alkaloids were found to have antibiotic and cytostatic activities as well [7]. Thus, agroclavine, festuclavine, and their alkyl derivatives exhibit bacteriostatic effects with respect to *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and other bacterial strains [8,9]. Festuclavine derivatives, by suppressing nucleoside transport in some tumor cells, inhibit DNA and RNA syntheses [10].

Another group of biologically active compounds synthesized by penicillia are cyclic peptides diketopiperazines consisting of residues of two amino acids and mevalonic acid. The precursors of roquefortine and related alkaloids such as 3,12-dihydroroquefortine, glandicolines A and B, meleagrine, oxalin are tryptophan, histidine and mevalonic acid [11]. Tryptophan and mevalonic acid are also the precursors of diketopiperazine alkaloids fellutanines and isofellutanines [12]. Brevianamides A and B and the new alkaloids piscarines A and B are formed from tryptophan, proline and from one or more mevalonic acid molecules via the same pathway [13]. Diketopiperazine alkaloids whose precursors are tryptophan and leucine include leucyl tryptophanyl diketopiperazine and verrucosine [11]. Compounds formed from the residues of tryptophan and phenylalanine are represented by such alkaloids as rugulosuvine, isorugulosuvine, puberulin (= rugulosuvine A), puberulin A (= rugulosuvine B) [14].

Roquefortine is the most widespread and well studied mycotoxin from the point of view of biological activity. It is known as a neurotoxic substance [1]. Under certain conditions, it inhibits enzymes of the digestive tract [15] and activities of cytochrome P 450 [16]. Some diketopiperazines, e.g. rugulosuvines and brevianamides, were shown to have antibiotic and antitumor activities [14,17].

If anthranilic acid begins the biosynthetic chain, the result of the metabolic processes are benzodiazepine compounds (cyclopeptin, cyclopenin and cyclopenol), quinoline compounds (viridicatin, viridicatol, quinocitrinines A and B) and quinazoline compounds (fumiquinazolines F and G). Quinocitrinines A and B exhibit various types of biological activity [18]. They are efficient against a range of Gram-positive and Gram-negative bacteria, yeasts and fungi, and were found to possess a moderate antiproliferative activity with respect to L-929 cells and HeLa cells. Thus, the inhibitory concentrations (LC\(_{50}\), \(\mu\)g/mL) of quinocitrinines A and B for L-929 murine fibroblast cells are 33.1 and 18.6, respectively; for K-562 human leukemic cells, 19.5 and 7.8; and HeLa >50, >50, respectively. In turn, fumiquinazolines F and G exhibit moderate cytotoxicities in the P388 lymphocytic leukaemia test system in cell culture [19].

Interest in these metabolites increases owing to their valuable pharmacological and therapeutic properties (Table-1). The most
numerous group of mycotoxins produced by penicillia are polyketides. The most well investigated mycotoxins among them are patulin, citrinin, as well as ochratoxins A and B [4].

Ochratoxin A is a nephrotoxin and has been entered into the list of five main mycotoxins the levels of which in food and feed are strictly controlled [20].

**Table 1. Biological activity of secondary metabolites from *Penicillium* fungi**

<table>
<thead>
<tr>
<th>Kind of biological activity</th>
<th>Metabolites</th>
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<tbody>
<tr>
<td>Effects on the central nervous system, neurohumoral, peripheral</td>
<td>Agroclavine-I</td>
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<td></td>
<td>Aurantioclavine</td>
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<tr>
<td></td>
<td>Isofumigaeclavines A and B</td>
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<td>Isochanoelavine-I</td>
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<td>Costaclavine</td>
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<td>Rugulosavins A and B</td>
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<td>Cyclopiazonic acid</td>
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<td>Chanoclavine-I</td>
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<td>Festuclavine</td>
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<td>Fumigaclavines A and B</td>
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<td>Chanoelavines-III</td>
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<td>Epicostaclavine</td>
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<td>Epoxyagroclavine-I</td>
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<td>Dimer epoxyagroclavine-I</td>
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<td></td>
<td>Dimer agroclavine-I</td>
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<td>Antibiotic, antitumor activity</td>
<td>Agroclavine-I [2]</td>
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<td></td>
<td>Festuclavine [9, 10]</td>
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<td>Piscarinines A and B [13]</td>
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<td></td>
<td>Roquefortin [1]</td>
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<td>Meleagrin [24]</td>
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<td></td>
<td>Rugulosuvines A (puberulin A) and B (puberulin) [14]</td>
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<td></td>
<td>Fellulantine A–E, Isofellutamine B and C [23]</td>
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<tr>
<td></td>
<td>Viridicatin, viridicatol [1]</td>
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<td></td>
<td>Quinocitrinins A and B [18]</td>
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<td></td>
<td>Questiomyicine, Xantocilllin [36]</td>
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<td></td>
<td>Griseofulvin [1]</td>
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<td></td>
<td>Citrinin, Patulin [20]</td>
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<tr>
<td>Nephrotoxin</td>
<td>Ochratoxins A and B, Citrinin, Cyclopiazonic acid [20]</td>
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2. Strategy of the search for new mycotoxin producers and strains synthesizing earlier unknown biologically active compounds

Various approaches have been used to search for new biologically active molecules that pose a hazard as mycotoxins. This study used strains from microbial culture collections of a certain official status, which are maintained under controlled conditions. Strains were cultured under conditions optimal for the synthesis of secondary metabolites. Ordinarily, we used a simple synthetic modified Abe’s medium, containing mannitol and succinic acid as a carbon source and mineral salts, which is favourable and optimal for secondary metabolite biosynthesis. The strains were cultured under optimal conditions on a rotary shaker for submerged cultivation at 24°C and 220-240 rpm. The fungi were grown in 750 mL flasks with 150 ml of the media. The secondary metabolites were isolated from the culture liquid by extraction. Chloroform was used to recover substances from culture liquid filtrate and a mixture of chloroform and methanol (3:1) for extraction from mycelium. Extracts were analyzed by thin-layer chromatography using Ehrlich’s, Dragendorf’s and similar reagents for functional groups and groups of compounds. These data enabled a preliminary identification of the metabolites by using the mycotoxin samples available at the laboratory. At this stage, we also selected promising strains taking into account the synthesis of new, earlier unknown metabolites. The selected strains were cultured to produce the required amounts of secondary metabolites. The produced samples of the secondary metabolites were used for further isolation and purification of required individual substances. The last stage was to elucidate the structures of the isolates. Biological tests are an integral part of work.

The strains belonging to species creative with respect to their ability to produce new biomolecules were screened. Metabolome components in the producers isolated from the regions subjected to anthropogenic stress and from other unusual habitats were studied. Considering the specific features of the habitats, it was important to assess the toxigenic potential of fungal microflora in sites, e.g. such as in the Mir orbital space station. In view of the observed climate changes and global warming predictions, it is of undoubted interest to study the toxigenic potential of the fungi isolated from permafrost.

2.1. Search for biologically active compound producers among representatives of species creative with respect to secondary metabolite biosynthesis

An earlier study established an ability of the fungus \textit{P. fellutanum} (=\textit{P. sizovae}) to produce ergot alkaloids of unusual stereochemistry in carbon atoms 5 and 10 of the ergoline nucleus. These are agroclavine-1 and epoxiagroclavine-1, as well as ergot alkaloids of a dimeric structure with the N-N bond of the indolic ring — dimer of agroclavine-1, dimer of epoxyagroclavine-1 and mixed dimers of agroclavine-1 and epoxyagroclavine-1 (Figure 1) [21, 22]. Screening of five \textit{P. fellutanum} strains found one of them – VKM-3020 – to be able to produce a group of evidently biogenetically related diketopiperazine alkaloids in which tryptophan was the precursor [12]. These metabolites were named fellutanine A, fellutanine B, isofellutanine B, fellutanine C, isofellutanine C, fellutanine D and fellutanine E. Fellutanine D was cytotoxic for L-969, K-562 and HeLa cells (Figure 2) [23].

\textit{P. piscarium} fungi are well known as producers of indole-containing tremorgenic mycotoxins – jantitrem B, fumitremorgin B, verruculogen and penitrems [24]. Information on \textit{P. piscarium} synthesis of alkaloids not belonging to the tremorgenic group is scarce. The metabolome studies of three \textit{P. piscarium} strains have found that they can produce various diketopiperazine alkaloids [21]. Verrucosine, prolyl tryptophanyl diketopiperazine, puberulin A, isorugulosuvine and fellutanine A were found in the VKM F-325 strain. The fungus F-1823 produced only isorugulosuvine and fellutanine A. The strain VKM F-691 synthesized prolyl tryptophanyl diketopiperazine. Two new metabolites were isolated and purified from the culture liquid of this strain, their structures were elucidated [13]. These compounds belong to the
Figure 1. Examples of few ergot alkaloids and quinocitrins

Figure 2. Fellutanines A-D
diketopiperazine family of unusual structure of the dihydropyranyl-substituted indole nucleus. The metabolites were named piscarinine A and piscarinine B (Figure 3). The substances exhibited an average antimicrobial activity against bacteria and fungi and an average cytotoxicity. The metabolites are active against the prostate cancer cell line LNCAP with IC_{50} values of 2.195 μg/mL for piscarinine A and 1.914 μg/mL for piscarinine B [13].

2.2 Strains of fungi isolated from habitats submerged to anthropogenic stress

It is known that anthropogenic factors cause changes in soil micromycete communities and fungal successions [26]. It has also been found that the composition and amount of mycotoxins synthesized by a producing strain depend on various external factors [27]. However, information on the toxigenic potential of the strains isolated from habitats subjected to anthropogenic stress is scarce [28].

This work investigated mycotoxins in 19 strains of 12 species of the Penicillium genus [29]. The examined species were isolated from areas with various environmental conditions at various times. Major part of the strains were isolated from plots exposed to high levels of anthropogenic impact – urban soils, soils polluted by lead, acid rain, oil fuel and pesticide fundazole. Some strains were isolated from extreme natural conditions – primitive arctic soils (Kola Peninsula), primitive alpine soils (Pamir) and light serozems (Gray Desert Soils) (Turkmenistan). Besides, fungal isolates from the snow of urban areas and domestic dust in blocks of flats (Moscow) were investigated. All species were chosen based on the preliminary analysis of the general set of the filamentous fungi in a location and were typical of them.

The culture conditions have a significant effect on the quantity of fungal secondary metabolites produced, and the relationship between them [30]. The qualitative composition of the metabolites is, however, constant as a rule [30,31]. Therefore, in most cases submerged cultivation of a producer in Abe’s medium with mannitol and succinate as carbon and energy sources, favourable for production of secondary metabolites, makes it possible to reveal major part of the mycotoxins produced by strains examined.

The highest productivity was observed in fungi of those species whose strains are known in the literature to have a high toxin-forming capability: *P. chrysogenum*, *P. granulatum*, *P. spinulosum*, *P. vulpinum*. It should be noted that several strains including *P. aurantiogriseum* № 3, *P. chrysogenum* № 4, *P. spinulosum* № 15 and *P. vulpinum* № 113 isolated from urban soils and those polluted with nitrogen fertilizers were able to produce biologically active substances earlier unknown for these species.

Thus, *P. chrysogenum* is known as a producer of penicillin, rugulovasine and diketopiperazine alkaloids of the roquefortine group [32]. One of the strains of this species – № 105 – isolated...
from primitive alpine Pamir soils also synthesized alkaloids of the roquefortine group. Another – strain № 4 – isolated from domestic dust produced a considerable quantity (up to 10 mg/L) of clavine alkaloids (fumigaclavine A, fumigaclavine B and pyroclavine) unknown before for representatives of this species.

*P. vulpinum* № 113 (*P. claviforme*) isolated from an urban environment showed a high production of α-CPA and its imine. Roquefortine, oxaline, viridicatin, cyclopenin and patulin described for this species [32] were not detected in this strain. On the other hand, strain № 16 isolated from Moscow downtown snow produced roquefortine, which is characteristic of the typical strains of this species. *P. granulatum* is known as a producer of such mycotoxins as patulin, viridicatin and roquefortine [32]. Strain № 128 was characterized by a high production (up to 10 mg/L of culture broth) of alkaloids of the roquefortine group – meleagrine, roquefortine, 3,12-dihydroroquefortine. New producers of tryptophanyl tryptophanyl diketopiperazine (fellutanine A) – *P. spinulosum* № 15 and *P. aurantiogriseum* № 3 – were found. Both strains were isolated from an urban environment. Fungi of the species *P. janczewskii* are widely known as producers of griseofulvin [32]. The strain *P. janczewskii* № 2 isolated from soddy-podzolic soils polluted by acid rains also synthesized this metabolite.

In strains of the fungi assigned to such species as *P. glabrum*, *P. dangeardii*, *P. sp.*, *P. funiculatum* the ability to synthesize noticeable amounts of secondary metabolites was not found. It should be noted that strains of these species do not belong to pronounced producers of mycotoxins.

The examined strains of *P. aurantiogriseum*, *P. chrysogenum*, *P. granulatum*, *P. griseofulvum*, *P. spinulosum* and *P. vulpinum* were found to have other secondary metabolite profiles than those described in the literature. Among them are such mycotoxins as α-CPA and clavine alkaloids extremely hazardous to man and animals. The highest toxigenic potential was characteristic of the isolates of *P. vulpinum* N113 (content of α-CPA, up to 100 mg/l) and *P. chrysogenum* N4 (content of clavine alkaloids, up to 10 mg/L).

These results are of ecotoxicological significance inasmuch as the fungal producers of mycotoxins are becoming typical of and dominant in microfungal communities in ecosystems experiencing human impact [33].

### 2.3 Search for new producers among fungal strains isolated in unusual, little investigated habitats

#### 2.3.1 Mir orbital space station as a habitat for mycotoxin-producing fungi

Such an ecological system as Mir orbital space station, which for many years underwent a persistent influence of various factors, is considered as a unique material for in-depth studies [22]. Space flights, both manned and unmanned, are accompanied with setting out a great variety of microorganisms on onboard equipment outside the limits of their natural conditions. Investigations carried out onboard Mir space station have shown that its microflora includes more than 100 species of filamentous fungi [34]. Some of them were capable of resident long-term population of structural materials of the interior, equipment and devices of the station. The strains were reliably diagnosed in the microflora tests in 1997-1998 and assigned to the species *P. expansum* and *P. chrysogenum* – remote descendants of the cultures isolated in the living compartments of the station more than eight years ago. Strains of *P. expansum* IMBP 2-7, which had become dominant by the end of the long-term flight, and *P. chrysogenum* IMBP strains 1-5 and 1-6 were able to produce biologically active compounds such as
xanthocillin X and questiomycin A — the antibiotics of a broad range of action [24].

2.3.2 Fungal strains isolated from permafrost as new producers of biologically active compounds

The profiles of the secondary metabolites (mycotoxins) were investigated in 91 fungal strains isolated from various permafrost regions – North America, North-East Asia and Kamchatka [37-43]. Cultures were isolated from soils, cryopegs and volcanic ash of various geological ages [44]. Different secondary metabolites were identified in half of the cultures. By their ability to produce secondary metabolites, the strains can be divided into several groups.

The most numerous group includes strains, which can produce ergot alkaloids. Fungi of *P. commune* (10 strains) and *P. palitans* (2 strains) synthesize a well known mycotoxin, α-CPA [42,43]. Seven strains of *P. palitans* can also produce clavine alkaloids such as fumigaclavine A, fumigaclavine B and festuclavine. Five strains of *P. variabile* were first found to produce rugulovasines A and B. Two strains belonging to *P. waksmanii* and *P. citrinum* were found to synthesize clavine alkaloids of unusual stereochemistry such as agroclavine-1 and epoxyagroclavine-1 [38, 42]. These strains are also producers of new, previously unknown quinoline compounds quinocitrinine A and quinocitrinine B [39].

Well known mycotoxins ochratoxin A and ochratoxin B were produced by six strains of *P. verrucosum* [41]. Biosynthesis of the roquefortine-group diketopiperazine compounds was observed in *P. chrysogenum* (5 strains) and *P. aurantiogriseum* (3 strains) [37, 40]. Tryptophanyldehydrohistidinyl-diketopiperazine identified in *P. chrysogenum* (2 strains) was the first intermediate of the biosynthetic pathway leading to the formation of roquefortine. It is assumed that 3,12-dihydroroquefortine found in all strains of *P. aurantiogriseum* and in one strain of *P. chrysogenum* is the next link in the pathway of roquefortine biosynthesis. In several strains, roquefortine was the final metabolite of the biosynthetic pathway, for others, it undergoes a number of transformations. Thus, meleagrine produced by *P. chrysogenum* (3 strains) was the result of roquefortine modification. Glandicoline A and B isolated from a strain of *P. chrysogenum* were intermediates in biosynthesis from roquefortine to meleagrine.

*P. solitum* species (6 strains) produced benzodiazepine compounds cyclopenin and cyclopenol [43]. Two of them were found to synthesize viridicatgin. All of these metabolites are linked by a common biosynthetic chain. The first metabolite in this pathway was cyclopeptin, from which cyclopenin and cyclopenol are formed, which are further transformed into quinoline alkaloids viridicatin and viridicatol. Quinazoline compounds fumiquinazolines F and G and polyketide metabolite PC-2 were identified in two strains of *P. thymicola*. Fumiquinazolines exhibited an activity against tumor cells [19].

*P. rugulosum* (3 strains) and *P. brevicompactum* (2 strains) produced mycophenolic acid. This mycotoxin was first found in *P. rugulosum*. Mycophenolic acid possesses antibiotic and immunodepressive activities.

*P. griseofulvum* synthesized griseofulvin. This metabolite is known as an antibiotic of fungicide action little used at present due to its high toxicity.

Thus, it has investigated the secondary metabolites, including mycotoxins, in penicillia, which are components of ancient microbial communities in perennially frozen permafrost deposits of various geneses and ages. It has been shown that the strains that have existed under long-time cryopreservation conditions possess a high biosynthetic potential. The fungi produce various biologically active compounds - secondary metabolites – ergot alkaloids, diketopiperazines, benzodiazepines, quinolines, quinazolines and polyketides.

3. Studies of fungal toxigenic potential using artificial media and natural substrates

As is known, production of secondary metabolites depends on many factors, first and foremost, on the composition of culture liquid,
temperature and pH [45]. For this reason, it was important to establish a correlation between the data on the biosynthetic potential of fungi grown on artificial media and on natural substrates.

The validity of the approaches used to assess the fungal toxigenic potential was studied in [47]. *Penicillium* fungi of the species *P. palitans*, *P. expansum* and *P. farinosum* species were grown by means of surface and submerged cultivation in simple and complex media, such as summer wheat and apple juice. These species are well known as contaminants of food and producers of mycotoxins – ergot alkaloids including α-CPA, benzodiazipines, diketopiperazines [24]. It was established that biologically active compounds detected in submerged cultivation in Abe’s medium were synthesized under surface growth conditions, including on dense media. The spectra of alkaloids were the same as in submerged culture on a simple Abe’s medium, but in the case of natural substrates the relation between particular components could vary. Thus, the ability of fungal species to synthesize biologically active metabolites under laboratory conditions correlated with that in nature.

These data are in conformity with current views that secondary metabolism enzymes are in many cases constitutive and that genes controlling their synthesis arranged in corresponding clusters [24].

4. Conclusions

*Penicillium* fungi are a large part of microbial diversity and offer exciting prospects in the search of biomolecules with various biological and pharmacological potentials and other related properties. In connection with the observed signs of global warming (a decrease of the Arctic Ocean ice cover), the most topical are the results obtained for fungi isolated from permafrost. The strategy of the search for secondary metabolites was successfully implemented, which is confirmed by earlier unknown novel compounds and important metabolites. Studies of the toxigenic potential of the fungi grown on artificial media and natural substrates, the observed correlation between them, confirm the importance of the obtained data and are of ecotoxicological significance.

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