Metal Nanomaterials and Hydrolytic Enzyme-Based Formulations

Subjects: Microbiology

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Combination of metals and enzymes as effective antifungal agents is currently being conducted due to the growing antifungal resistance problem. Metals are attracting special attention due to the wide variety of ligands that can be used for them, including chemically synthesized and naturally obtained variants as a result of the so-called “green synthesis”. The main mechanism of the antifungal action of metals is the triggering of the generation and accumulation of reactive oxygen species (ROS). Further action of ROS on various biomolecules is nonspecific. Various hydrolytic enzymes exhibit antifungal properties by affecting the structural elements of fungal cells (cell walls, membranes), fungal quorum sensing molecules, fungal own protective agents (mycotoxins and antibiotics), and proteins responsible for the adhesion and formation of stable, highly concentrated populations in the form of biofilms.

green synthesis MOFs amyloid proteins prionase mycotoxins growth inhibition

1. Introduction

The accumulation of information about the role that microscopic fungi can play in the development of a number of negative processes affecting human health [1][2][3] has led to increasing interest in antifungals that can control and reduce the growth, as well as the metabolic activity, of these biological objects, especially those associated with pathogens [4]. The seriousness of these tasks is increasing due to the fact that in some cases, fungal cells may develop resistance to the chemical formulations used against them [5][6][7].

A number of current scientific studies are related to the development of effective antifungals [8]. Among the new trends in the development of effective antifungals, the prospects of a possible combination of various chemical compounds [7] with different mechanisms of action on fungal cells are being considered. This approach can enable researchers to overcome the development of adaptive processes in fungi and, possibly, reduce the doses of the substances used, increasing the effectiveness of their action in such combinations. When implementing such a combined approach to suppressing the growth and metabolic activity of fungi, the main question arises about what is better to combine with what, and what may be unpromising. One of the possible answers to this question is based on the use of metal nanomaterials such as metal-nanoparticles, metal-organic frameworks, etc., to which no resistance is formed by most microorganisms since the mechanism of suppression of biological processes is primarily associated with the generation of reactive oxygen species (ROS) in the cells. Metals such as Ag, Cu, Fe, Zn, Se, Ni, Au, Zr, Ce, Ti, and Pd have been studied in regard to compounds possessing antifungal activity [9][10][11]

[12]. At the same time, current scientific research on the antifungal properties of metals is mainly focused on the study of Ag and Au nanoparticles (NPs) [10][11][12][13][14][15] since the antimicrobial effectiveness of their action has been known for a long time.

Among the various organic synthetic ligands for the metals used in research in this direction, the so-called “green synthesized” metal-containing NPs should be noted. These “green synthesized” metal-containing NPs are formed inside the cells of microorganisms (bacteria, fungus, yeast) in vivo or using plant extracts, polysaccharides of phototrophic microorganisms, and extracellular enzymes of mycelial fungi [10][14][15][16][17]. “Green synthesis” is an environmentally friendly synthesis technique that avoids the formation of undesired by-products and costs less. Moreover, it was found that “green synthesis” makes it possible to obtain NPs with identical antifungal properties compared to similar chemically synthesized metal-containing compounds that are, in some cases, superior to them [17].

It is known that the combination of metal NPs with known chemical fungicides makes it possible to reduce the minimum inhibitory concentration (MIC) of the latter by more than eight times [17]. However, despite this researchers decided to consider the possibility of combining metal-containing compounds with biological molecules having catalytic properties, in particular, with various enzymes exhibiting antifungal activity instead of chemically synthesized fungicides. It has been previously shown that the efficiency of the use of metal NPs can be increased by combining them with cyclic peptides that exhibit antifungal properties [18]. Unlike peptides that exhibit antimicrobial activity, the enzymes have catalytic activity [19], which allows them not just to trigger destructive processes against fungi but to repeatedly participate in these acts of biocatalysis, deepening antifungal processes. In addition, a wide substrate range of action of the enzymes themselves allows researchers to consider the possibility of not only their destructive activity against fungal cells but also against the most important fungal molecules involved in the formation of their quorum sensing (QS) and adhesion [20] and molecules that ensure their own safety (antibiotics [21] and mycotoxins [22]).

2. Antifungal Agents Based on Metal Nanoparticles, Metal–Organic Frameworks and Their Composites

Multiple antifungal agents have been developed to date on the basis of metal nanoparticles (NPs) and/or metal–organic frameworks (MOFs) ([11][12][23][24][25][26][27][28][29][30][31][32][33][34][35][36][37], **Figure 1**).

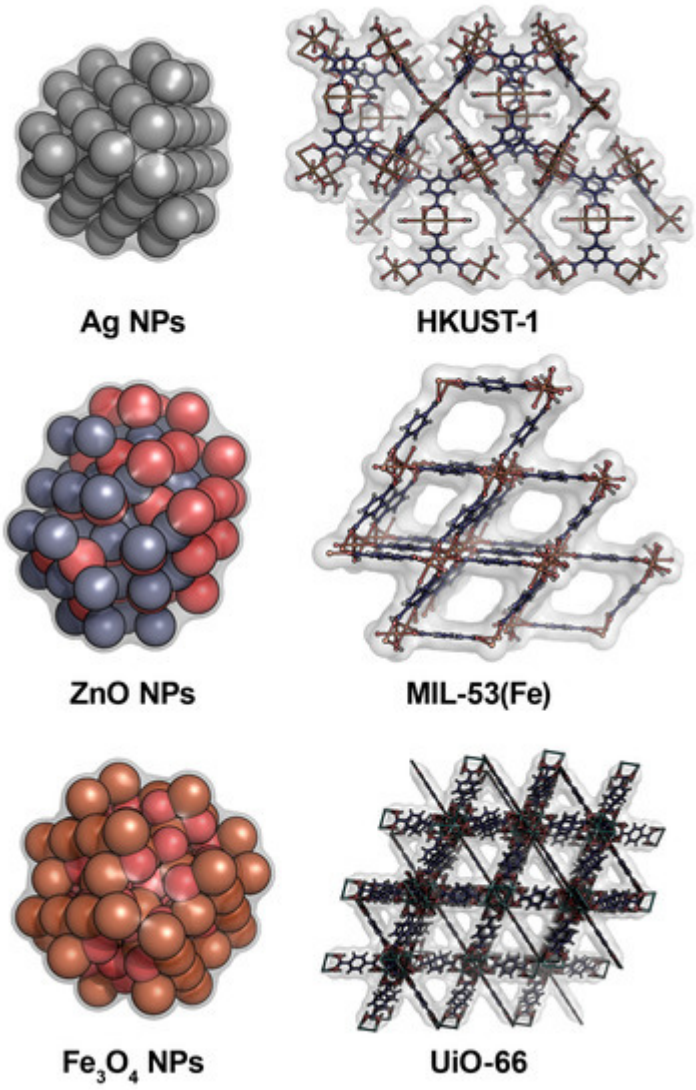

Figure 1. Some representative metal NPs and MOFs with antifungal activities. Crystal structures of Ag (1741252), ZnO (13950), Fe_3O_4 (1612598), HKUST-1 (2091261), MIL-53-Fe (2088536), and UiO-66 (2054314) were obtained from CCDC, then expanded in Mercury (v.4.2.0, CCDC, Cambridge, UK) and visualized in PyMOL (v.1.7.6, Schrödinger Inc., New York, NY, USA). Water-accessible molecular surface is indicated by light grey while atoms are colored by element: Ag–grey, Zn–slate, O–red, Fe–orange, C–deep blue, H–white, Zr–cyan.

Table 1. Antifungals based on metal nanoparticles (NPs), metal–organic frameworks (MOFs), and their composites *.

Antifungal Agent [Reference]	Target of Action	Antifungal Activity	Efficiency of Antifungal Action
ZrO_2-Ag_2O (14–42 nm) [23]	*Candida albicans*, *C. dubliniensis*, *C. glabrata*, *C. tropicalis*	The growth rate inhibition	89–97% inhibition

Antifungal Agent [Reference]	Target of Action	Antifungal Activity	Efficiency of Antifungal Action
WS_2/ZnO nano-hybrids [24]	*C. albicans*	Inhibition of biofilm formation	91% inhibition
CuO@C (36–123 nm) [25]	*Alternaria alternata*, *Fusarium oxysporum*, *Penicillium digitatum*, *Rhizopus oryzae*	Inhibition of the hydrolytic activity of fungal enzymes used by them for their own metabolism	Inhibition (100 µg/mL) of cellulases and amylases secreted by fungi: 38% and 42% for *A. alternata*, 39% and 45% for *F. oxysporum*, 24% and 67% for *P. digitatum*, and 20% and 24%for *R. oryzae*, respectively
ZnO NPs [26]	*C. albicans*, *Aspergillus niger*	Inhibition of growth	Large enough zone of growth absence (8-9 mm)
ZnO NPs (20–45 nm) [27]	*Erythricium salmonicolor*	Notable thinning of the hyphae and cell walls, liquefaction of the cytoplasmic content with decrease in presence of a number of vacuoles	Significant inhibition (9–12 mmol/L) of cell growth
ZnO–TiO_2 NPs (8–33 nm) [28]	*A. flavus*	High level of ROS production and oxidative stress induction. Treated objects have a lower count of spores and damaged tubular filaments and noticeably thinner hyphae compared to the untreated fungi	Fungicidal inhibition (150 µg/mL) zone is 100 %
ZnO NPs (40–50 nm) [29]	*C. albicans*	High level of ROS production	MIC = 32–64 µg/mL MFC = 128–512 mg/mL
Fe_2O_3 NPs (10–30 nm) [30]	*Trichothecium roseum*, *Cladosporium herbarum*, *P. chrysogenum*, *A. alternata*, *A. niger*	Inhibition of spore germination	MIC = 0.063–0.016 mg/mL
Fe_3O_4 NPs (70 nm) [31]	*C. albicans*	Inhibition of cell growth and biofilm formation	MIC = 100 ppm MFC = 200 ppm
Cu-BTC (10–20 µm) [32]	*C. albicans*, *A. niger*,	ROS producing, the damage of the cell membrane	Inhibition of *C. albicans* colonies is 96% by 300 ppm and up to 100% by 500 ppm.

Antifungal Agent [Reference]	Target of Action	Antifungal Activity	Efficiency of Antifungal Action
	A. oryzae, *F. oxysporum*		Inhibition growth of *F. oxysporum* and *A. oryzae* is 30% with 500 ppm. No significant effect on the *A. niger* growth.
HKUST-1 or HKUST-1 NPs (doped with NPs of Cu(I)) (49–51 nm) [33]	*A. niger*, *F. solani*, *P. chrysogenum*	Appearance of Cu^{+2} inhibiting of cell growth	100% growth inhibition of *F. solani* by 750–1000 ppm and *P. chrysogenum* by 1000 ppm; for *A. niger*—no inhibition
$[Cu_2(Glt)_2(LIGAND)](H_2O)$ [34]	*C. albicans*, *A. niger* spores	The apoptosis-like fungal cell death, ROS production	50–70% death of *C. albicans* and 50–80% germination inhibition of *A. niger* at 2 mg/mL of the MOFs
MIL-53(Fe) and Ag@MIL-53(Fe) composite [35]	*A. flavus*	Inhibition of cell growth	MIC = 40 µg/mL for the MIL-53(Fe); MIC = 15 µg/mL for the Ag@MIL-53(Fe)
MOF on the basis of Ce and 4,4′,4″-nitrilotribenzoic acid [11]	*A. flavus*, *A. niger*, *Aspergillus terreus*, *C. albicans*, *Rhodotorula glutinis*	Enzyme-like activity: catalase, superoxide dismutase, and peroxidase	Inhibition efficiency of 93.3–99.3% based on the colony-forming unit method
TiO_2 co-doped with nitrogen and fluorine (200–300 nm) [12]	*F. oxysporum*	Peroxidase-like activity, production of ROS under light irradiation	100% inhibition of fungal growth
Fe_3O_4@MoS_2-Ag (~428.9 nm) [36]	*C. albicans*	Peroxidase-like activity	80% damage of cell membranes
CoZnO/MoS_2 nanocomposite [37]	*A. flavus*	Peroxidase-like activity under light irradiation	MIC = 1.8 mg/mL

* BTC—1,3,5-benzenetricarboxylate; Glt—glutarate; HKUST-1—type of MOFs composed of $[Cu_3(BTC)_2(H_2O)_3]_n$; MFC—minimum fungicidal concentrations; MIC—minimum inhibitory concentration; MIL-53(Fe)—type of MOFs composed of $[Fe_4(OH)(1,4\text{-benzenedicarboxylate})_4]$; LIGAND—1,2-bis(4-pyridyl)ethane, 1,2-bis(4-pyridyl)ethylene, or 1,3-bis(4-pyridyl) propane.

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3. Enzymes as Antifungal Agents

3.1. Antifungal Enzymes Using Cell Structural Components of Fungi as Substrates

Discussing the possibilities and prospects for the use of various enzymes in the composition of antifungals, it should be noted that their diversity is determined by the spectrum of targets on which these enzymes can have notable effect, leading to a halt in the fungal growth, disruption of metabolism and death of fungi. Such targets for enzymatic action may include: structural elements of fungal cells (cell walls, membranes) [38], nucleic acids [39], fungal Quorum Sensing molecules (QSMs) regulating fungal resistance to various negative factors and protect them (mycotoxins, antibiotics) [40]), peptides and proteins involved in the formation of stable forms such as biofilms (adhesives, hydrophobins) (**Figure 2**).

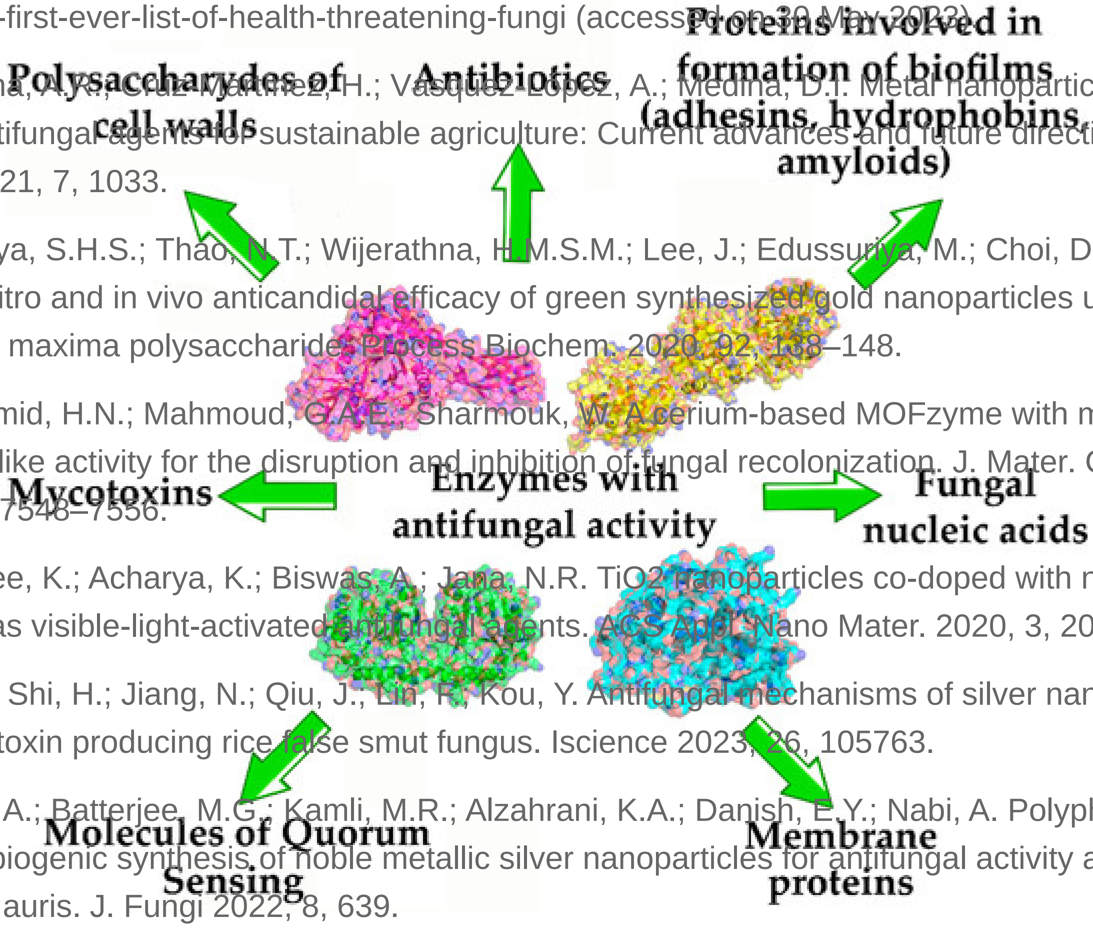

Figure 2. Enzymes (pink—chitinase (PDB ID: 1edq), yellow—keratinase (PDB ID: 5wsl), green—lactonase AidC (PDB ID: 4zo2), blue—peroxidase (PDB ID: 1mnp) with antifungal activities due to their catalytic action on different targets as substrates.

Enzymes are interesting as antifungal agents because they are proteins present in various natural sources (plants, microorganisms, animal tissues), which are designed to protect living objects from the effects of fungi. The recombinant forms of necessary enzymes can be produced in various host cells.

The analysis of enzymes exhibiting a variety of antifungal activities indicated that most of them were hydrolases acting on polysaccharides present in the structure of the fungal cell wall or involved in the formation of biofilms. The greatest effect was observed in the case of chitinases [41][42][43][44][45][46][47][48][49][50][51][52], among which there were

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associated with a lower efficiency of their action in comparison with hydrolases and the need to introduce additional H_2O_2 into the medium with fungi.

3.2. Enzymes Hydrolyzing Fungal Proteins with Amyloid Characteristics

Special attention should be paid to the fact that yeast and mycelial fungi are able to form amyloids, which are unbranched fibrils consisting of monomers stacked on top of each other and stabilized by intermolecular β-layers. For example, monomers of hydrophobins of class I, small surface-active proteins produced by fungi, form amyloid fibrils that perform many functions [68]. It is known that the specific functions of hydrophobins synthesized by fungi can enhance their pathogenicity. Thus, *A. fumigatus* can cause invasive aspergillosis in patients with weakened immunity due to the amyloid-forming ability of hydrophobin RodA [69][70]. The formation of amyloid by hydrophobin MPG1 in *M. oryzae* contributes to rice pyriculariosis [71]. One of the most well-described examples of amyloid proteins in yeast cells is the Cdc19 protein from *S. cerevisiae*, which, in the absence of glucose, self-aggregates into an amyloid-like aggregate to avoid degradation under stressful conditions [72].

It is known that the yeast cells of *C. albicans*, often used in studies of antifungals, also contain proteins with amyloid characteristics. Thus, the proteins As1, As3, and As5 from the ALS-type adhesion family have the ability to self-aggregation. The presence of an amyloid sequence in the monomers of these proteins leads to the formation of hydrophobic nanodomains that promote the cell adhesion of *C. albicans* on biotic or abiotic surfaces and improve their ability to form biofilms [73][74]. It is assumed that Sap6, Rbt1, Pga59, and Pga62 proteins, as well as adhesins, play a significant role in the appearance of *C. albicans* biofilms due to the presence of an amyloid-forming sequence in their structures [75][76][77][78].

Today, due to their ability to be transmitted from “mother” cells to “daughter” cells, yeast prions are classified as infectious, for example, [URE3] and [PSI+], HET, and HELLP in *S. cerevisiae*, *Podospora anserina*, and *Chaetomium globosum* cells, correspondently [79]. The presence of similar conditions for the formation of yeast prions and common molecular properties with pathogenic human amyloids has now led to the creation of models of neurodegenerative diseases based on yeast prions. The methods of their regulation are being investigated in order to develop new effective therapeutic agents and approaches to the treatment of diseases associated with prion proteins [80]. In this regard, the interest in enzymes capable of hydrolyzing amyloid aggregates formed by fungi is huge. This is due to the possible development of antifungals that reduce the level of biofilm formation and the potential use of enzyme-containing formulations for the treatment of neurodegenerative diseases in humans. Information about such proteases hydrolyzing amyloid proteins is presented in [81][82][83][84][85][86][87][88][89][90][91][92][93][94][95][96].

Discussing the prospects for the possible use of enzymes hydrolyzing fungal amyloid proteins, it should be noted that so far there are a few such studies. The ability of several proteolytic enzymes, such as subtilisin, keratinases, and proteinase K, to degrade yeast prion aggregates of protein Sup35NM under various conditions was investigated [91][92][93][94]. It has been shown that hexameric AAA^+-ATPase (Hsp104), which is a yeast chaperone, is involved in the fragmentation of large fungal amyloid fibrils. It is believed that the direct binding of Hsp104 to

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amyloid fibrils prevents the reproduction of yeast prions. Since Hsp104 is absent in the cells of multicellular animals, including mammals, the possibility of constructing variants of Hsp104 with the potential for use for the degradation of abnormal human proteins is being investigated [80].

Despite the limited number of studies in the field of enzymatic degradation of yeast prions, a number of proteolytic enzymes are known today that can degrade prion proteins and amyloids associated with human diseases, including subtilisin-like serine proteases TK-SP from hyperthermophilic archaeon *T. kodakarensis* [81], nattokinase from *Bacillus subtilis natto* [82], subtilisin 309 and protease from *B. lentus* [83][85], two proteases from *B. subtilis* and *B. lentus* [84][86], subtilisin-like protease MSK103 from *B. licheniformis* [87], enzyme E77 from *Streptomyces* sp. [88], subtilisin-homolog from the extremophile archaea *Aeropyrum pernix* [89], and serine protease from lichens [90].

Multiple metalloenzymes have been reported to have an important role in the degradation of Aβ [95][96], including two metal-activated keratinases, Ker1 and Ker2, from an actinomycete *Amycolatopsis* sp. MBRL 40; NEP—a zinc-dependent metalloprotease, cleaving various vasoactive peptides; and ECE—another zinc-dependent metallopeptidase, which could degrade and cleave amyloid Aβ. The ability to cleave amyloid precursor proteins has been confirmed in Zn-containing transmembrane metalloproteases [96]. At the same time, the influence of redox-active metals such as Cu and Fe (affecting the pathogenesis of Alzheimer's disease) was established, which consists in increasing the biosynthesis of the metalloproteases under discussion. The influence of the same metals on the activity of these enzymes has not yet been investigated but is of great scientific and practical interest.

Despite the fact that there is still no effective enzymatic formulation for the cleavage of prion proteins, new proteolytic enzymes continue to be discovered and studied, the prionase activity of which still needs to be investigated [97][98][99]. Researchers focus readers' attention on such enzymes as a potential basis for the development of new antifungals, probably with some anti-neurodegenerative effect.

3.3. Enzymes Hydrolyzing Mycotoxins, Antibiotics, and QS Molecules (QSMs) of Fungi

To date, a significant amount of information has been accumulated about QS in the cells of various fungi and molecules that are produced by the fungi themselves in response to an increase in their concentration per unit volume. These QSMs are produced in order to trigger the processes of fungal cell transition to a state of stable intercellular communication, synchronization of the functions of multicellular populations, and biochemical changes in the cells themselves [100][101][102][103][104]. The ability of individual enzymes to catalyze the hydrolysis of fungal QSMs allows them to be attributed to the so-called Quorum Quenching enzymes (QQE). Gluconolactonase- [105]) and hexahistidine-containing organophosphorus hydrolase (His$_6$-OPH) [106][107], esterases [108][109]) have been identified as such enzymes acting against fungi today.

Discussing the potential of these enzymes as candidates for inclusion in combined antifungals with metal-containing compounds, it can be noted that for His$_6$-OPH, such possibilities have already been demonstrated and

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proved promising, while ZnO NPs [110][111] appeared to be the most effective option for such a combination. However, so far, such combined antimicrobials have been investigated only against bacterial cells [112], and their effectiveness against fungal cells has yet to be confirmed.

Interesting use cases for combining with metal-containing compounds are enzymes that carry out the destruction of mycotoxins synthesized by fungi in the OFR state. At the same time, it should be noted that, as in the case of QSM hydrolysis, among the enzymes that carry out the destruction of various mycotoxins (zearalenone, patulin, deoxynivalenol, ochratoxin), there are all the same enzymes, namely lactonases, esterases, lipases [22], and His$_6$-OPH [39][113]. In this regard, with their involvement in combined antifungal formulations, a very interesting option may turn out to provide a multi-targeted action due to the promiscuous activities of these enzymes.

Continuing to analyze possible variants of enzymes that can be considered as candidates for creating combined variants with metal NPs, it is worth noting the enzymes that are able to catalyze the hydrolysis of antibiotics synthesized by fungi among other secondary metabolites in their QS state. Here, the undisputed leaders are β-lactamases, known to everyone due to studies of bacterial antibiotic resistance to natural and semi-synthetic penicillins and cephalosporins [114].

It is interesting to note that QQE including His$_6$-OPH are close “relatives” for metallo-β-lactamases [115]. Moreover, the structural analogy revealed between phosphotriesterase (of the same His$_6$-OPH) and some nucleases indicate that these enzymes can catalyze to one degree or another similar reactions with a certain preference for individual substrates. Since these enzymes have been mentioned here more than once in connection with their various targets of action in fungal cells, their use in research on the development of new antifungals may be not only new but also promising. Surprisingly, an active search for data on the use of metallo-β-lactamases in the content of any antifungals to give them a number of catalytic activities, as discussed above, did not reveal any.

It should be noted here that many of mentioned enzymes contain different transition metals, particularly Zn(II), Mn(II), and Fe(II)/Fe(III) in their active sites [115], which can be positively taken into account when creating combinations with metal-containing compounds against the fungi sensitive to these metals. In addition, the combination of these enzymes with metal-containing compounds that are not embedded in the active site of enzymes but can exhibit significant antimicrobial activity at low MIC values [110][111] looks interesting and promising.

4. Combination of Antifungal Enzymes and Metal-Nanoparticles

It is known currently that many sources and types of enzymes can be used to prepare antifungal formulations with metal NPs: bacterial keratinase [116] and chitinase [117], archaeal protease and lipase [118], fungal β-1,3-glucanase, N-acetylglucosaminidase, chitinase, and acid protease [119][120], etc. Such formulations can possess secondary antioxidant [116][117] and/or specific inhibitory activity [116]. The additional antibacterial action mode of these combinations is widely present [116][118][120][121].

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Green synthesized metal NPs are of great interest for the production of enzyme formulations [116][118][119][120]. β-1,3-glucanase(s) and, to a lesser extent, N-acetylglucosaminidase(s) are predominantly adsorbed on Ag NPs as compared to chitinase(s) and acid protease(s) [119]. Altogether, these enzymes on Ag NPs not only inhibit mycelium growth but also prevent the formation of sclerotia thereby leading to lifecycle arrest.

Interestingly, the "un-capping" of Ag NPs (i.e., desorption of enzymes) leads to a detectable increase of their size and is likely to be a result of their aggregation [120]. At the same time, the negative net charge of "uncapped" Ag NPs argues for the substitution of enzymes for [illegible] used as a solubilizer. This can contribute to the increased toxicity of such "un-capped" NPs towards [illegible] organisms and cells [120]. Surprisingly, "un-capped" Ag NPs are ineffective in mycelium growth test and only decrease the number of sclerotia by twofold as compared to the control experiment without any effector.

Similar to germination, the formation of sclerotia is known to be regulated by multiple genes though there are a lot of gaps about this process [122]. As a result, the biochemical composition of the cell wall changes dramatically; for example, the most abundant components of *Sclerotium rolfsii* hyphae—polysaccharides and proteins—shift by 1.5–2 times [illegible] while [illegible] compounds (so-called melanin-like pigments) increase numerously and become the second prevalent subclass (after polysaccharides). The last ones have been shown to propagate resistance of sclerotia towards environmental factors and, for example, to slaughter via the hydrolytic action of extracellular glucanases and chitinases [123]. Moreover, the leakless thick rind can be formed from such melanized cells on the sclerotia surface [124], further limiting enzymatic hydrolysis and antifungal penetration. Thus, polyphenol-degrading activity may be useful in addition to antifungal formulation. Another rational functionality in such formulation(s) to treat sclerotia appears to be the antioxidant activity discussed previously since ROS also affects sclerotial development somehow [122].

During field trials of chitinase-based formulation against filamentous fungi [117], it was found to be slightly less effective than the same formulation with a live biocontrol agent (*Streptomyces cellulosae*). This may be a consequence of differing profiles of protective gene modulation in the plant by these formulations.

As was determined for peptide melittin, slow release of active compound from the Zn-MOF matrix occurs and the maximal amount (60%) is released at pH 6 during 24 h [125]. The antifungal activity of melittin is [illegible] decreased threefold during its encapsulation within Zn-MOF at 30% loading. However, [illegible] formulation improves almost twofold. Altogether, yeast adhesion to the surface during biofilm formation and (pseudo)hyphal transformation are inhibited.

Melittin is known to disturb membranes of different (micro)organisms, activate several transmembrane receptors, depolarize membranes, etc. Some of these effects are also manifested in the composite formulation [125]. Moreover, lactoferrin being a transporter of iron ions and having other possible activities [126] greatly improves the antifungal activity of melittin, especially towards pre-formed biofilms [125]. The synergic action of lactoferrin and melittin can also be [illegible]. Lactoferrin can bind to the fungal cell surface itself and affect biofilm formation and yeast-to-hyphal transition in combination with conventional drugs [127]. Thus,

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