Original Article
Food Science and Technology
ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)
DOI: https://doi.org/10.5327/fst.00271

Inhibitory action of mycocins from *Wickerhamomyces anomalus* on filamentous fungi present in cornmeal

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Abstract

Some species of filamentous fungi present in grain crops can be mycotoxigenic. Mycotoxins are secondary metabolites that are potentially carcinogenic, hepatotoxic, and nephrotoxic to humans and animals. In addition, they are mostly thermostable, i.e., they resist the processing and refining of grains. *Wickerhamomyces anomalus* is a yeast found widely in nature and was the first reported yeast capable of producing mycocins that act on both eukaryotic and prokaryotic microorganisms. Mycocins are glycoproteins that act on sensitive cells of other microorganisms without direct cell-to-cell contact. This study aimed to identify the filamentous fungi present in cornmeal and verify their inhibition against the mycocins of *W. anomalus* WA92, all the cornmeal samples analyzed (eight) presented colony-forming units (CFU) of filamentous fungi including some known as potential mycotoxin producers, and 10 fungal genera were identified—*Acremonium* sp., *Alternaria* sp., *Aspergillus* sp., *Chrysosporium* sp., *Cladosporium* sp., *Fusarium* sp., *Mucor* sp., *Penicillium* sp., *Rhizopus* sp., and *Scopulariopsis* sp. Tests were carried out on a solid medium containing the supernatant of mycocins from *W. anomalus* and showed total inhibition of the growth of these fungi. Mycocins from *W. anomalus* are a promising agent in the biocontrol of grain fungal populations.

Keywords: cornmeal; filamentous fungi; mycocins; mycotoxins; *Wickerhamomyces anomalus*.

1 INTRODUCTION

Brazil is a country that excels in the cultivation of corn and other grains, and one of the reasons is the subtropical climate that predominates in our country and favors cultivation throughout the year. However, due to the high temperatures and humidity resulting from the climate, fungus development also occurs, which usually begins in the field during the grain ripening phase and continues in the following stages: harvesting, drying, storage, processing, and refining. The fungi that affect grains can be classified into two groups: field fungi that affect grains while they are still in the field, in which the main genera are *Alternaria* spp., *Cladosporium* spp., *Cephalosporium* spp., *Fusarium* spp., *Giberella* spp., and *Nigrospora* spp., and storage fungi that affect the grains during the period in which they are stored, in which the main genera are *Aspergillus* spp., *Mucor* spp., *Penicillium* spp., and *Rhizopus* spp. (Márcia & Lázzari, 1998).

Mycotoxins are secondary metabolites of low molecular weight produced by some species of filamentous fungi, including some of the main ones present in the corn crop (Bondy et al., 2023). Among the necessary factors for the production of mycotoxins by fungi are favorable conditions of humidity, pH, temperature, and chemical composition of the food where the fungi are installed. Mycotoxins affect not only the food *in natura* but also its by-products because they are thermostable, and it is possible for the fungus that produced it to be destroyed and for it to remain (Pereira et al., 2002). In addition to the financial losses that these mycotoxins cause in the field, there is also concern for the health of the individuals who consume these foods because these mycotoxins are considered potentially carcinogenic, hepatotoxic, and nephrotoxic (Anderson et al., 2010).

Wickerhamomyces anomalus is a mycocin-producing yeast found in nature in widely diverse habitats such as marine environments, plants, and fruit peels, and its isolation in insect intestines has also been described. It has no complex nutritional requirements and is able to withstand hostile environments for long periods, being highly competitive. It is considered a yeast that is adaptable to extreme environmental stress conditions, which allows it to grow on different carbon, phosphorus, and nitrogen sources in pH variations, low water and oxygen activity, and high osmotic pressure (Hua et al., 2015; Walker, 2011). The yeast in question can produce groups of mycocins with different molecular masses, and antimicrobial activity tests confirm the ability of mycocins to act on cell wall glucans (Rosseto et al, 2022).

Mycocins are proteins or glycoproteins that act on sensitive cells of other microorganisms without direct cell–cell contact, but with a mechanism that includes receptors in the cell wall and cell membrane of sensitive cells (Fuentefria, 2007), mycocins are capable of exerting a lethal action on sensitive microorganisms and in nature are used as a mechanism in the competition for nutrients (Vieira et al., 2021). They are used in various applications as biocontrol agents. The antimicrobial action of mycocins from *W. anomalus* against strains of *Staphylococcus aureus* coagulase

Received 31 Jan., 2024.
Accepted 10 Mar., 2024.
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Practical application: Mycocin activity in fungi isolated from cornmeal.

positive isolated from meat was proven (Calazans et al., 2021). In the food industry in wine fermentation, mycocin-producing yeasts have been used as starter cultures to prevent the growth of spoilage yeasts in the fermentation process (Yap et al., 2000). They are also used in biological control in agricultural applications and in the inhibition of wood-degrading and phytopathogenic basidiomycete fungi (Walker et al., 1995).

The aim of this research was to identify the presence of potentially mycotoxin-producing filamentous fungi in cornmeal samples and verify their inhibition by mycocins produced by *W. anomalus*.

2 MATERIALS AND METHODS

2.1 Cornmeal samples

Samples of cornmeal from eight different brands were purchased in sealed 250- and 500-g packages from commercial establishments in Cascavel, PR.

2.2 Isolation and identification of filamentous fungi present in cornmeal

To isolate and identify the fungi, the cornmeal samples were handled in a laminar flow hood to avoid any contamination by fungi present in the environment, and the tests were carried out in duplicate. For each sample of cornmeal, 50 g of it was removed and 200 mL of a solution of peptone water prepared with 1.6% peptone and 0.85% sodium chloride was added, and the mixture was stirred for 5 min. Then, a 2 mL aliquot was transferred to a tube containing 2 mL of peptone water. The mixture was then homogenized again and 100 µL was deposited in a Petri dish containing agar composed of 1.2% agar, 1% glucose, 1% peptone, and 0.1% chloramphenicol, using a Drigalski spatula to spread the liquid over the entire dish. The plates were incubated in an oven at 25°C for 7 days. After this period, a macroscopic analysis was carried out and the colonies were placed in tubes containing Sabourad Dextrose agar, where they were found for another 7 days. To identify the colonies, in addition to the macroscopic appearance, the microculture technique was used for microscopic identification.

2.3 Microcultivation to identify filamentous fungi

For microscopic identification of the genera of filamentous fungi present in cornmeal, the microcultivation technique was used. On a sterilized slide positioned on top of a small curved glass rod inside a Petri dish, also previously sterilized, a cube of Sabourad Dextrose agar was placed, the previously isolated filamentous fungus was sown on all four sides of the cube, and a coverslip was placed on top of the agar cube. Next to the slide, sterile cotton wool was soaked with approximately 1–2 mL of sterile water to form a moist chamber, and the Petri dishes were closed and allowed to remain for 7 days. Then, the coverslip and the agar cube were removed, one drop of lactophenol was added to the slide, the coverslip was placed back on top, and the fungi were examined under the microscope to identify them to genus level.

2.4 W. anomalus mycocins production

For the production of mycocins, the molecularly identified *W. anomalus* (WA92) yeast was used (deposited in GenBank access number: KT580792—available at: www.ncbi.nlm.nih.gov/BLAST). The yeast strains were previously reactivated on modified Sabouraud agar (2% agar agar, 1% peptone, 2% glucose, 1. 92% citric acid, and 3.48% dibasic potassium phosphate) at 32°C for 48 h, after which they were inoculated into Roux flasks with 200 mL of modified Sabouraud broth (1% peptone, 2% glucose, 1.92% citric acid, 3.48% dibasic potassium phosphate, pH 4.7) and incubated at 25°C for 5 days. The broth was centrifuged at 6,000 rpm for 10 min, and the supernatant was obtained, which was then sterilized using a 0.22-µm filter membrane and stored at 4°C until the *in vitro* tests were carried out.

2.5 Determination of β-glucanase activity

The β-glucanase activity present in the supernatant containing the mycocins of *W. anomalus* WA92 was determined according to the methodology described by Miller (1959), with adaptations, using 1% laminarin (*Laminaria digitata*), 50 mM acetate buffer, and pH 5.0. A solution contained 62.5 µL of the supernatant with the mycocins of *W. anomalus* WA92 and 125 µL of 1% laminarin, and then the solution was incubated at 37°C for 10 min. A volume of 100 µL of the solution was removed, and 100 µL of 3,5-dinitrosalicylic acid (DNS) was added. The solutions were incubated in boiling water for 5 min, after which 500 µL of sterile distilled water was added to stop the reaction, and the reaction product (reduced sugar) was read at 550 nm. For the blank reading, the same test solution was used, but without the addition of laminarin. An enzyme unit was defined as the amount of protein required to produce 1 µmol of reducing sugar per minute (U/min/mL). This test was carried out in duplicate. Protein quantification in the WA92 supernatant containing mycocins was carried out using the Bradford methodology (1976), using bovine albumin as a standard curve, and the equation of the line was applied to calculate the total protein concentration in mg/mL. The specific activity of β-glucanases was calculated using the ratio of the concentration of enzyme activity to the concentration of protein.

2.6 Inhibition test of filamentous fungi present in cornmeal on solid media

To carry out the inhibition test in a solid medium, 50 g of cornmeal was removed and 200 mL of a solution of peptone water prepared with 1.6% peptone and 0.85% sodium chloride was added. The mixture was stirred for 5 min. An aliquot of 500 µL of this solution was removed and placed in two tubes: the tube called control, containing 1 mL of the broth used for the production of mycocins (1% peptone, 2% glucose, 1.92% citric acid, 3.48% dibasic potassium phosphate, pH 4.7), and the test tube, containing 1 mL of supernatant with mycocins at a concentration of 3.8 U/mg of β-glucanases. The tubes were incubated at 25°C for 24 h, after which an aliquot of 50 µL was taken from each tube and placed on plates containing Sabourad Dextrose agar. The tests were carried out in duplicate.

3 RESULTS AND DISCUSSION

Corn kernels are susceptible to several fungal contaminations. The main fungi that affect the corn crop are *Aspergillus* sp., *Fusarium* sp., and *Penicillium* sp. Among the factors that influence the involvement of corn kernels by toxigenic fungi, the climatic conditions, such as the predominance of rainy periods from planting to harvest and post-harvest storage, during the cultivation period stand out. Fungi reproduce through spores, which can be spread by wind, water, and other environmental factors. In addition, they are resistant to temperature fluctuations and can remain dormant in the soil for years, making it difficult to control these microorganisms in the field and making them present in cultivated grains (Márcia & Lázzari, 1998). The presence of fungi in corn kernels can lead to a variety of impacts on the crop, such as economic losses due to reduced yields and altered product quality, as well as contamination by mycotoxins that are harmful to human and animal health (Mudili et al., 2014; Weaver et al., 2021).

Approximately 300–400 mycotoxins are known, and climatic conditions are determining factors for the development of the fungi that produce these metabolites. In Brazil, there are favorable conditions for the growth of all genera of mycotoxin-producing fungi, among which we can highlight five genera that are considered the main ones: *Alternaria*, *Aspergillus*, *Claviceps*, *Fusarium,* and *Penicillium*. The main mycotoxins found in food are aflatoxins (B_1, B_2, G_1, G_2, and M_1), fusaric acid, fumonisins (B_1 and B_2), ochratoxins (A, B, and C), citrinin, trichothecenes, patulin, and zearalenone (Maziero & Bersot, 2010). In addition to infection by a single mycotoxin, humans are also susceptible to infection by multitoxins. This is due to the heterogeneity of mycotoxin-producing fungi in the environment and the ability of these microorganisms to produce multiple mycotoxins that can affect the individual simultaneously (Nleya et al., 2018).

Cornmeal is a by-product of corn obtained from grinding the grains. It is a significant part of the Brazilian diet, and like all corn-based products, it is susceptible to contamination by fungi and molds that can potentially be mycotoxigenic, i.e., produce toxins that are harmful to health (Araújo Alhadas et al., 2004). In this study, eight samples of cornmeal were analyzed, a total of 1,850 CFU/mL were found, and 10 fungal genera were identified—*Acremonium* sp., *Alternaria* sp., *Aspergillus* sp., *Chrysosporium* sp., *Cladosporium* sp., *Fusarium* sp., *Mucor* sp., *Penicillium* sp., *Rhizopus* sp., and *Scopulariopsis* sp. In addition to the fungal diversity between the samples, diversity was also observed in specific samples of cornmeal, such as samples A and B, which showed six and seven different genera of fungi, respectively. The presence of potentially mycotoxigenic filamentous fungi isolated in cornmeal was also demonstrated in another study, five samples of cornmeal were analyzed, and seven different fungal genera were found, namely, *Aspergillus* sp., *Penicillium* sp., *Cladosporium* sp., *Rhizopus* sp., *Acremonium* sp., *Paecilomyces* sp., and *Cunninghamela* sp., where the first three showed a higher incidence of mycotoxigenic fungi (Araújo Alhadas et al., 2004). The occurrence of the same fungal genera in the studies is justified by the fact that they are known fungi in grain crops such as maize.

Table 1 shows the incidence of fungal genera in each brand of cornmeal analyzed and also the number of CFU/g of corn found:

Figure 1 shows the fungal diversity present in one of the cornmeal samples analyzed.

Table 1. Incidence of filamentous fungi isolated from cornmeal.

BRAND	CFU/g*	GENERA IDENTIFIED
A	360 CFU/g	*Alternaria* sp., *Aspergillus* sp., *Cladosporium* sp., *Mucor* sp., *Penicillium* sp., *Scopulariopsis* sp.
B	200 CFU/g	*Alternaria* sp., *Aspergillus* sp., *Chrysosporium* sp., *Cladosporium* sp., *Mucor* sp., *Penicillium* sp., *Scopulariopsis* sp.
C	380 CFU/g	*Acremonium* sp., *Aspergillus* sp., *Cladosporium* sp., *Penicillium* sp., *Scopulariopsis* sp.
D	400 CFU/g	*Aspergillus* sp., *Fusarium* sp.
E	100 CFU/g	*Aspergillus* sp., *Chrysosporium* sp., *Scopulariopsis* sp.
F	70 CFU/g	*Acremonium* sp., *Aspergillus* sp., *Cladosporium* sp., *Penicillium* sp., *Rhizopus* sp.
G	220 CFU/g	*Acremonium* sp., *Alternaria* sp., *Aspergillus* sp., *Chrysosporium* sp., *Cladosporium* sp., *Scopulariopsis* sp.
H	120 CFU/g	*Alternaria* sp., *Aspergillus* sp., *Cladosporium* sp.

CFU/g*: Colony-forming units per gram of cornmeal.

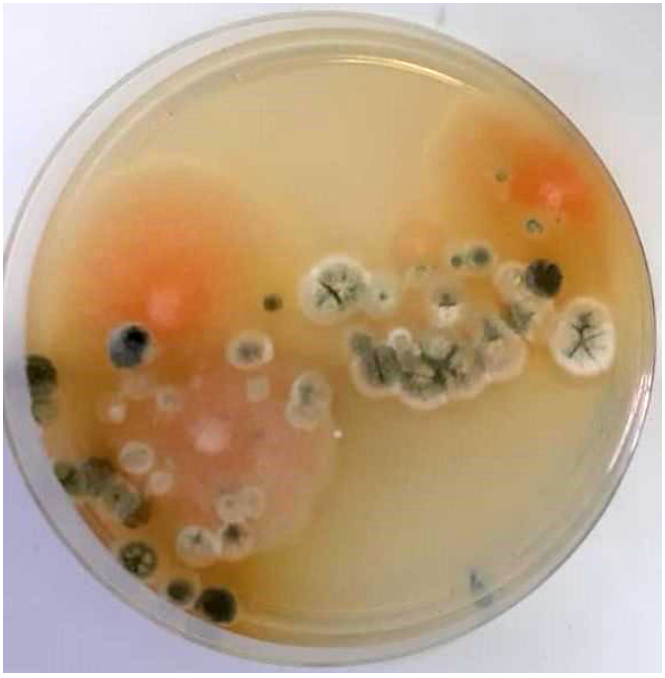

Figure 1. Fungal diversity present in cornmeal samples analyzed.

In this study, the three predominant fungal genera were *Aspergillus* sp. representing 38.9% of the total genera identified, followed by *Cladosporium* sp. with 16.7% and *Penicillium* with 13.0%. The occurrence of the fungal genera identified is shown in Figure 2.

The three predominant fungal genera in this study are widely distributed in nature. *Aspergillus* sp. is considered an industrially important group. However, some species have a negative impact on agricultural crops and the food safety of humans and animals. The higher incidence of *Aspergillus* sp. in the cornmeal samples analyzed in this study shows a possible presence of mycotoxins, especially aflatoxins commonly produced by species such as *Aspergillus flavus* and *Aspergillus parasiticus*, which are considered carcinogenic and mutagenic and also the causative agent of aflatoxicosis. *Cladosporium* sp. has wide ecological adaptability and has already been reported in other studies as being a common presence in the corn crop, often affecting the quality of the grain (Salvatore et al., 2021). *Penicillium* sp. affects grains mainly in the storage and drying phases through inadequate practices, some species such as *Penicillium verrucosum* are producers of ochratoxin A, and species of *Aspergillus* are also capable of producing this mycotoxin. Ochratoxin A is a chemically stable compound, which makes it difficult to eliminate from food because conventional processing procedures fail to reduce the presence of this metabolite (Tong, 2017). Early identification of fungal invasion in the cultivation or storage stages is a way of preventing the production of these metabolites which, when present in food, cannot be destroyed by normal cooking processes (Kumar et al., 2017; Shabeer et al., 2022).

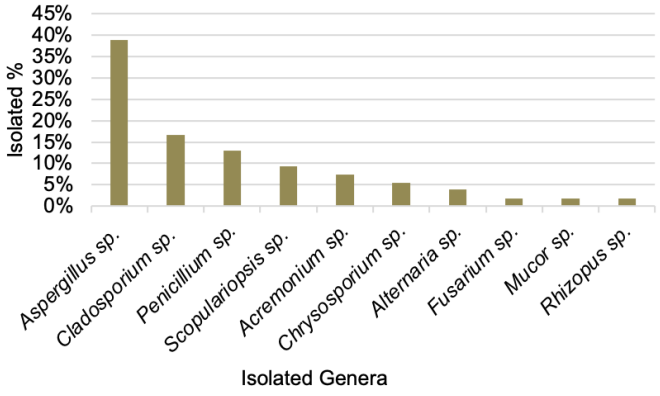

Figure 2. Occurrence of fungal genera in cornmeal samples.

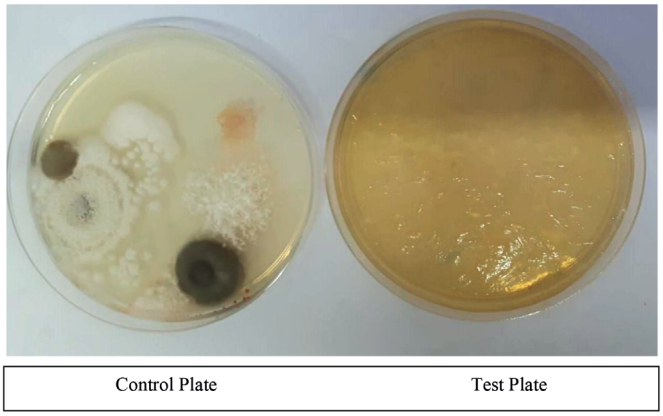

Figure 3. Control plate: growth of fungal colony-forming units. Test plate: Inhibition of colony-forming units by mycocins from *W. anomalus* WA92.

3.1 Determination of β-glucanase activity

Beta glucans are molecules classified as glucose polysaccharides, which have different molecular weights, branching, and bonding patterns (Zhu et al., 2015). They are widely present in the cell wall of yeasts, fungi, and some bacteria. β-Glucanases can activate a cell lysis system through the hydrolysis and degradation of β-1,3;1,6-glucan, which is present in the cell wall of some microorganisms (Nascimento et al., 2020). As mammalian cells do not have this constituent in the membrane, this cell lysis mechanism becomes selective for microorganisms.

In this study, the specific activity of 3.8 U/mg of β-glucanases was found in the culture supernatant of *W. anomalus*. Calazans et al. (2021) reported 0.40 U/mg of specific activity in mycocins produced by *W. anomalus*. With the same study, Lima et al. (2013) obtained an amount of 0.071 U/mg. The difference in results may be related to the strain and optimization of the reactivation, as well as some variations in the culture medium, pH, and temperature.

3.2 Inhibition test of filamentous fungi present in cornmeal on solid media

In the eight samples of cornmeal analyzed, all showed inhibition of filamentous fungi that could potentially produce mycotoxins, as shown in Figure 3.

The antimicrobial activity test on solid media is a very effective method, as it offers a visual reading of the activity of the mycocins on the microorganism being tested (Junges et al., 2020).

4 CONCLUSION

We found the presence of a wide range of fungi in the cornmeal samples. The identification of these fungi is important because it is an important indicator of the presence of mycotoxins in food and also helps ensure safer grain handling in order to avoid harm to human and animal health. The mycocins present in the supernatant of *W. anomalus* WA92 showed inhibitory action against these fungi. Mycocins are, therefore, a promising agent for biocontrol of the fungal population in the process of growing or storing grains.

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