Microbiology Insights

ORIGINAL RESEARCH

Microbiological Control of Flour-Manufacture: Dissemination of Mycotoxins Producing Fungi in Cereal Products

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Abstract: Wheat grain and its products are widely consumed as fodder and basic daily food stuffs in Kyrgyzstan. Mycobiota is known to produce hazardous effects to a consumer since it produces mycotoxins. Henceforth, mycobiota starting from the field stage to flour, grain and flour samples were selected for mycological analysis from eight sites of flour manufacture: grain stored in storehouses before milling, mechanically cleaned grain, washed grain, grain dried and prepared for mill, roughly-milled flour, first grade flour and high grade flour. The samples were analyzed using classical mycological and immunoassay methods in order to detect mycotoxins producing fungi species. We isolated overall 27 species belonging to 7 genera. Mycotoxins producing species like *Aspergillus flavus*, *Aspergillus ochraceus* and *Penicillium cyclopium* were detected in the stored grains and in mechanically-cleaned grains. The species of *Penicillium*, *Alternaria* and *Fusarium* genera dominated in roughly-milled flour samples, so this site of flour manufacture still has a risk and danger of contamination with mycotoxins producing fungus. Only the final product i.e. the high grade flour lacked any fungal contamination. We recommend to scrutinize flour samples at the last stages of processing, particularly in the mills like B1, C1 and C4.

Keywords: flour milling, microbiological control, mycotoxins producing fungi, mycotoxins, safety of cereal products

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Introduction

Certain species of fungi growing in food are able to produce metabolites toxic for animals, birds and human. Such metabolites are incorporated into a group of mycotoxins. Physicochemically, mycotoxins are thermostable and in most cases aromatic and nonantigenic low-molecular metabolites. Mycotoxins exert a diverse range of toxic evicts because their chemical structures are very heterogeneous. They develop a cancerogenic, mutagenic and teratogenic effect on human beings, animals and birds.

Mycotoxicosis is a disease that results from the ingestion of food contaminated with mycotoxins. For many centuries in some developed European countries Mycotoxicosis was not considered as a dangerous disease. Since 1970 cases of Mycotoxicosis caused by mycotoxins have intensively increased in many countries of the world.[1] In particular, cases of Mycotoxicosis diagnoses have increased in developing countries lately. Thus, the analysis conducted in Kyrgyzstan in 2004 shows that 44 samples out of 1123 samples of raw food taken from Osh, Chu and Issyk-Kyl regions were infected with mycotoxins.[2]

Representatives of *Aspergillus, Penicillium, Fusarium* and *Alternaria* genera are more responsible for producing mycotoxins. Microscopic fungi can produce these poisonous substances not only during the vegetation on grain crops, but also during storage time.[3–6] High frequency of *Alternaria* species in grains of wheat, barley, oats, rye and other cereals has been discovered in many countries of the world.[7,8] Scientists from Russia have marked a wide spread of fusarioz (*Fusarium*) of cereal heads in the European part of Russia, in the Urals and West—Siberian regions.[4]

Aflatoxin producing strains of *A. flavus* and *A. parasiticus* has been isolated from the fields of California. In California, on a fig plantation scientists have found isolates of *A. ochraceus*, *A. melleus* and *A. sclerotiorum*.[3,9] Regular pollution of grapes with *Aspergillus sp.* has been marked in tropical and subtropical countries. For example, *A. niger var. niger, A. flavus, A. niger var. awamori, A. foetidus,* and *A. candidus* strains have been detected.[10,11] *A. niger, A. flavus, A. terreus* and *A. ustus* strains have been found in grapes from Argentina and Brazil.[12]

Penicillium species are different on their needs for climatic conditions from *Aspergillus*. Representatives of *Penicillium sp.* can grow at the temperature of 4–31 °C, whereas *Aspergillus* species can grow at the temperature of 12–39 °C.[9,13] *Penicillium* fungi are spread out in all regions of the globe. *Penicillium chrysogenum* was isolated in France from grape samples and this strain has a potential to produce the *Ochratoxin A*.[14]

The first step in the diagnostic process of the pathogen or disease associated with the production of mycotoxins is to know and understand the biology of organisms responsible for mycotoxicosis. The biology, ecology and dissemination of mycotoxins producing fungi found in agricultural crops and food is still unknown in Kyrgyzstan.

This research describes the results of microbiological analysis of grain and its products after harvesting and during processing in order to detect contamination with mycotoxins produced by fungi and risk points at the manufacture.

Material and Methods

Grain and flour samples, the points of their collection for the analysis

Wheat grains were stored at the temperature of 27–28 °C (in summer months) and at the temperature of 15 °C (in winter months), air humidity was 16.5%–17%, so these parameters do not meet standard requirements for the storage of grains in optimum conditions. Samples were collected once in two months from the sites:

1. Grain stored in storehouses before processing
2. Grain after mechanical cleaning
3. Grain after washing
4. Grain after drying and preparation for grinding
5. Flour after rough mill
6. Flour after middle mill
7. First-grade flour
8. High-grade flour

Samples were taken by 3 kg from each point and were subjected to a triple microbiological analysis.

Nutrient mediums used for fungi cultivation

Czapek-Dox Agar (CDA) medium (by adding antibacterial antibiotics 200 mg cycloheximide/litre) was used in order to isolate microscopic fungi and their pure culture; also a peptone—dextrose agar and a potato agar were used to study their culture, morphological and physiological characteristics.

Mycobiota analysis

From each collection, 20–25 grains per plate, usually in triplicate, were superficially sterilized for 5 minutes in 70% spirit to enumerate the storage fungi, then washed thoroughly in sterile distilled water for 2 minutes and plated onto Czapek-Dox Agar (CDA). The plates were incubated at the temperature of 22–27 °C for 10 days. Colonies of fungi growing around the grains were isolated to a pure culture by a standard technique. Individual species of fungi were counted separately and their numbers were expressed as Colony Forming Units per gram (CFU/g).

The percentage of abundance, density, frequency, occurrence and contribution was calculated for each species.[15] Common fungi species were identified using the determinants.[6,11,16–21,32,36,37] The *Aspergillus* and *Penicillium* genera were identified after.[22,23] Nomenclature of *Aspergillus* was accomplished.[24]

The strains were deposited in the Microbiology Laboratory of Kyrgyz-Turkish International University. The Shenon index[25] was used to estimate biodiversity of fungi species in wheat grain. The Simpson's index[26] was used to determine a domination rate.

The frequency criteria were used to determine the importance of fungi species.

Frequency of occurrence in space (%):

$$= \frac{\text{Number of samples in which the species were found}}{\text{Common number of samples}}$$

Test on the antagonistic activity of micromycetes cultures to bacteria

To test the activity against bacteria species, the fungus was spotted at the center of the plate containing MRS.02 agar. After 48 hours of incubation of fungus culture at 25 °C, the bacteria culture of *Bacillus thuringiensis*, *Staphylococcus sp.,* and *Erwinia sp.* were spotted to the fungus mycelium. Bacterial growth inhibition was estimated using measurements of the colony density towards the edge in the direction of the grown fungal colonies. Controls of bacterial growth in the selected media were performed at the absence of fungus culture.

All tests were conducted in duplicates.

Results and Discussion

Level of fungal contamination of grain at flour-manufacture

Wheat grain stored in storehouse before processing

Grains covered and not covered with mycelium were recorded to calculate the level of fungal contamination. Grains in the storehouse before processing showed quite a high level of fungal contamination –15 to 30.5% (Fig. 1). Apparently, this mycobiota contains various species of fungi like representatives of ephytic microflora in plant grains.

Wheat grain after mechanical cleaning

Grain from the granary goes to a cleaning machine where the grains are exposed to 70% of

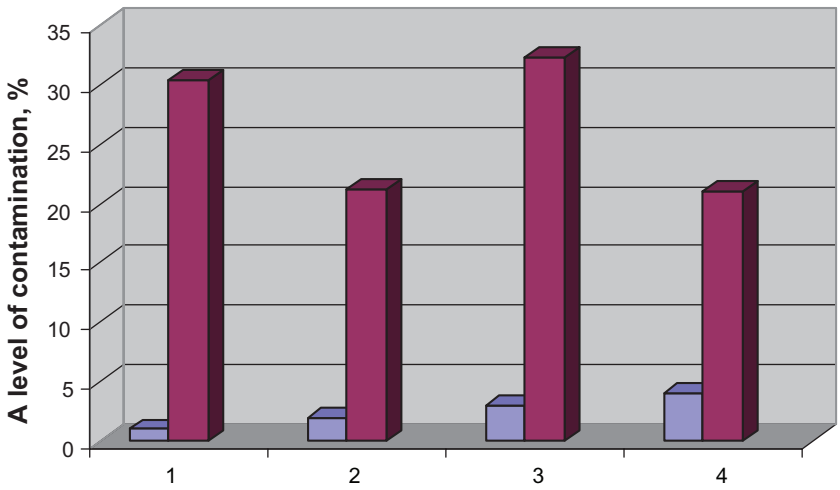

Figure 1. Fungal contamination of grains at the flour manufacture.
1. The grain stored in storehouse before processing 2. The grain after mechanical cleaning 3. The grain after washing 4. After drying and prepared for mill.

mechanical cleaning. The number of fungal spores on the cleaned grains reduced almost twice in comparison with the first point (Fig. 1). Obviously, during the mechanical cleaning fungal spores are eliminated with dirt and dust stuck on the grain coat.

Wheat grain after washing with room-temperature water

Wheat grain after mechanical cleaning goes to a washing machine to be washed up with water without disinfectants. The temperature of water during the winter time is 14°–16 °C and 20 °C in summer. Grain moisture was 14.8%–15.1% before washing in the machine, but after washing moisture rose up to 20%. The level of fungal contamination of the washed grain increased to 32.3% (Fig. 1). This might be caused by the increase of moisture contents in grains that leads to intensive growth and reproduction of fungi spores.

Wheat grain after drying and preparation for mill

At this point of flour-manufacture the grain gets dried. Grain moisture reduces to 16%–17%, and then the grain is sent to a mill. The level of fungal contamination of the dried grain decreased to 21%. It occurs because moisture contents decrease in grains before going to a mill (Fig. 1).

Mycobiota of grain and flour

Mycobiota of grain stored in storehouse before processing

Fifteen species classified under seven genera were isolated from the grains which included *Penicillium, Alternaria, Helmintosporium, Cladosporium, Aspergillus* and *Mucor*. Representatives of *Penicillium* genus showed maximum contribution (40,0%) and appeared to be the most predominant species in the grain stored before processing. *Cladosporium* species took the second position with 31.0%. *Alternaria* species took the third position with 13%. The percentage contribution of other fungi (*Fusarium, Helmintosporium, Aspergillus* and *Mucor* was equal (4.3%) (Fig. 2).

Mycobiota of grain after mechanical cleaning

Twelve species belonging to six genera were isolated from the cleaned grains. They included *Cladosporium, Penicillium*, *Alternaria, Fusarium, Helmintosporium* and *Aspergillus* species. *Cladosporium* species were abundant in the grain and constituted maximum percentage (65,0%) of the total. Although *Penicillium* species took the second position, its percentage contribution was much lower (11.0%) compared to that of stored grain. *Alternaria, Fusarium*, *Helmintosporium* and *Aspergillus* species were recorded more frequently than in stored grains (6,0%) (Fig. 2).

Mycobiota of grain after washing with water of room temperature

Ten species classified under five genera were isolated from the grains washed up with room-temperature water. They included *Helmintosporium, Fusarium, Penicillium, Cladosporium* and *Alternaria* species. *Helmintosporium* representatives showed maximum contribution (50.0%) and appeared to be the most predominant species. *Fusarium* species occupied the second position with 19.0%. It was much higher than in the stored and washed grains. *Penicillium* species occupied the same position with 19.0%. Percentage contribution of remaining genera (*Cladosporium* and *Alternaria) was* notable too (6.0%). *Aspergillus* species were not detected (Fig. 2).

Mycobiota of grain after drying and preparation for mill

At this phase grains are dried. Grain moisture reduces to 16%–17%. After drying the grains went to a mill. Ten species classified under five genera were isolated from the grains dried and prepared for mill. They included *Helmintosporium, Penicillium, Fusarium, Cladosporium* and *Alternaria* species (Fig. 3). *Helmintosporium* representatives showed maximum contribution (46. 6%), and appeared to be the most predominant species. *Penicillium* and *Fusarium* species showed higher and more notable contribution (20.0%) compared to that of the washed grains. *Aspergillus* species were not detected (Fig. 2).

Mycobiota of flour samples subjected to different degree of fineness and milling

Mills at flour-manufacture line up in the following sequence: B1, B2, B3, B4, C1, C2, C3, and C4. Each mill fulfills 40% of grinding. For example, grains which were not grinded in B1 mill do not go

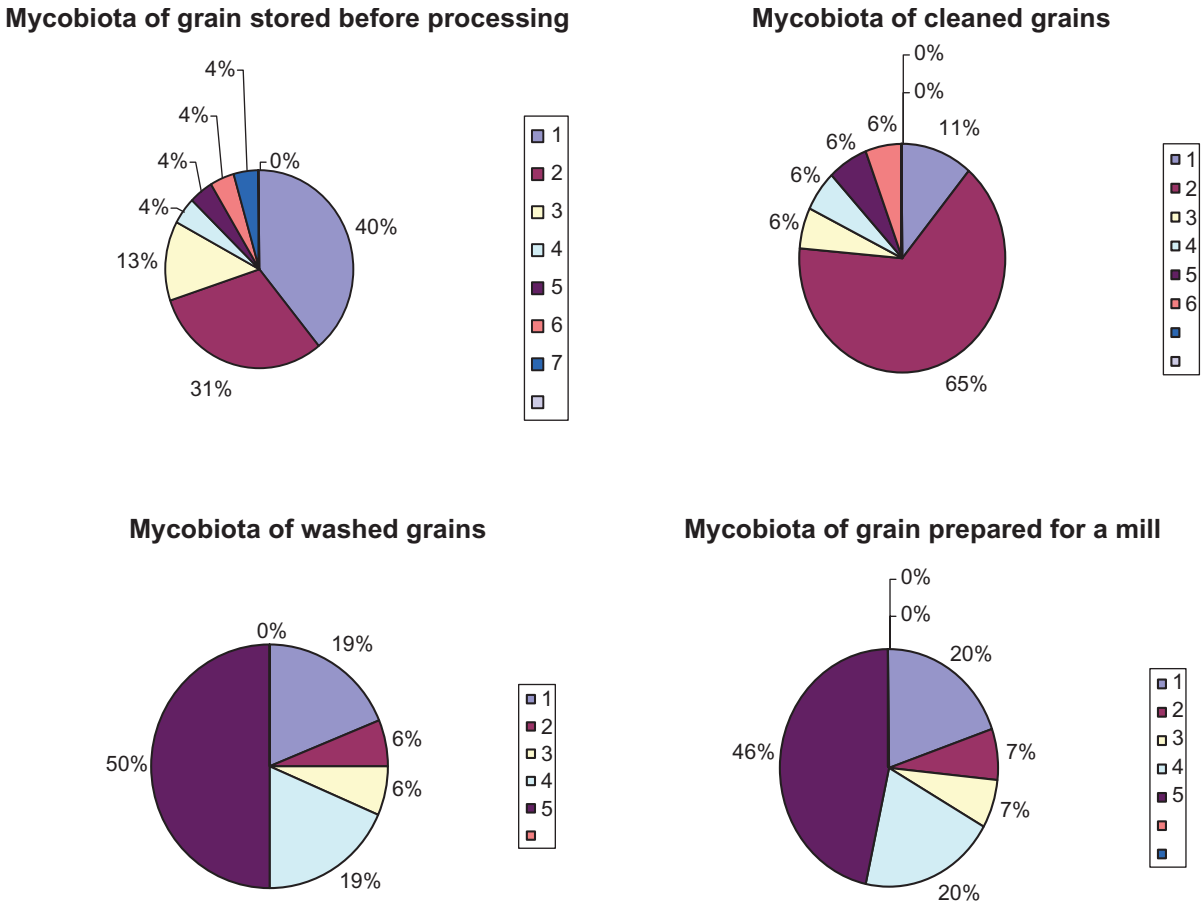

Figure 2. Species of fungi frequently isolated from different grain samples and their percentage contribution: 1. *Penicillium* 2. *Cladosporium* 3. *Alternaria* 4. *Fusarium* 5. *Helmintosporium* 6. *Aspergillus* 7. *Mucor.*

to the next B2 mill. Grains which were not grinded in "B" mills are not accepted in "C" mills. Flour after complete processing in mills "B" and "C" and going through a sieve is separated into supreme-grade flour, first-grade flour, second-grade flour, and then packed in bags on electronic scale and transported in tanks. We selected samples from mills B1, B4, C1 and C4 for mycological analysis.

Mycobiota of flour after processing in B1 mill

Ten species belonging to six genera were isolated from a rough fraction of flour. They included *Cladosporium, Mucor*, *Penicillium*, *Alternaria, Fusarium* and *Helmintosporium* species. *Cladosporium* and *Mucor* species dominated and constituted maximum percentage (29,0%) of the total. *Helmintosporium* and *Alternaria* species formed 14.0%; *Fusarium* and *Penicillium* formed 7.0% (Fig. 4).

Mycobiota of flour after processing in B4 mill

Eight species belonging to three genera were isolated from a rough fraction of flour. They included *Cladosporium, Mucor* and *Penicillium* species. *Cladosporium* and *Mucor* species were recorded frequently and made a maximum contribution (43.7% each) of the total. *Penicillium* species reached 12.5% (Fig. 5).

Mycobiota of flour after processing in C1 mill

Again the representatives of *Cladosporium* and *Mucor* genera were abundant in flour fractions (43,0% and 50.0%). However, representatives of *Alternaria* were also detected (7.0%.) (Fig. 6).

Mycobiota of flour after processing in C4 mill

Only 2 species were isolated from a fraction of flour after processing at the last mill. They included *Cladosporium and Mucor* species. They demonstrated equal percentage—50.0%. (Fig. 7).

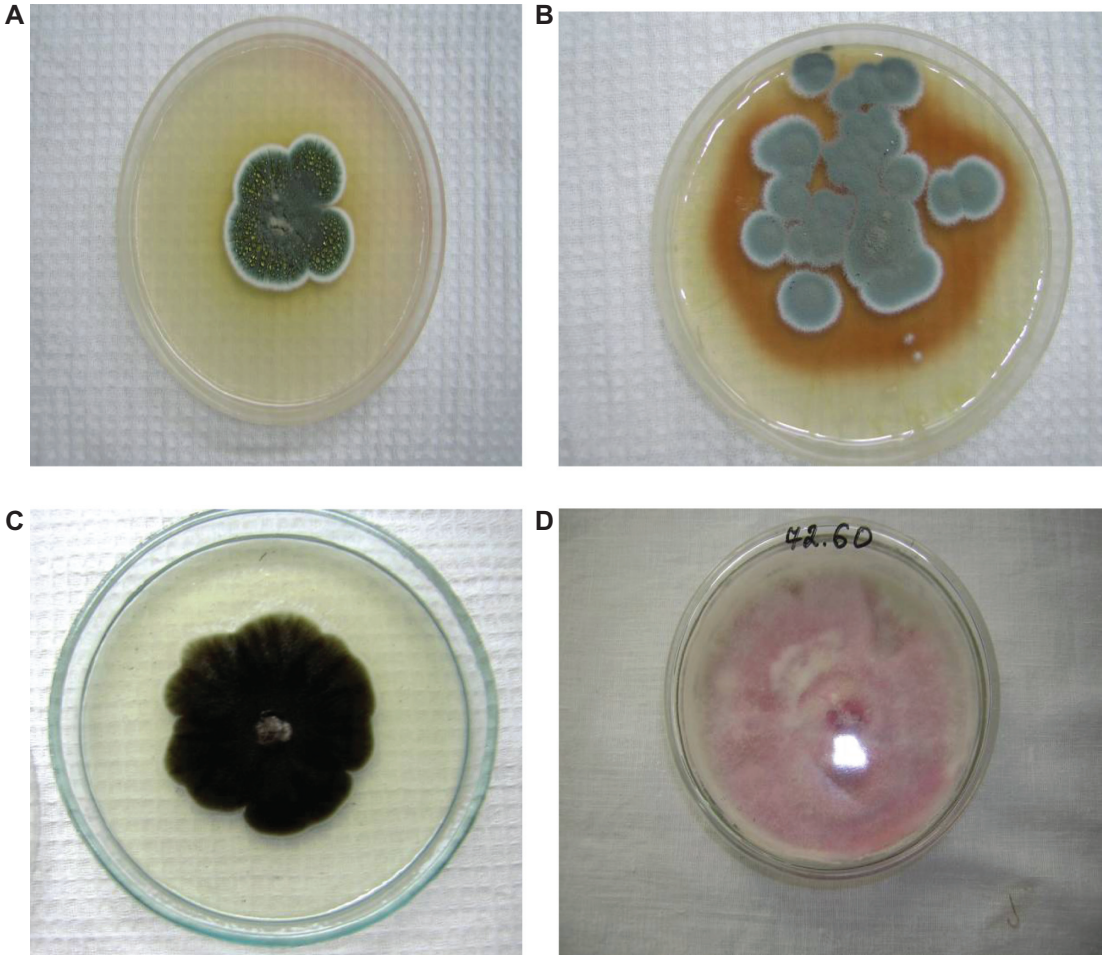

Figure 3. The colonies of fungi. **A,B**) *Penicillium species* **C**) *Alternaria sp.* **D**) *Fusarium sp*. found in grain before processing in mill.

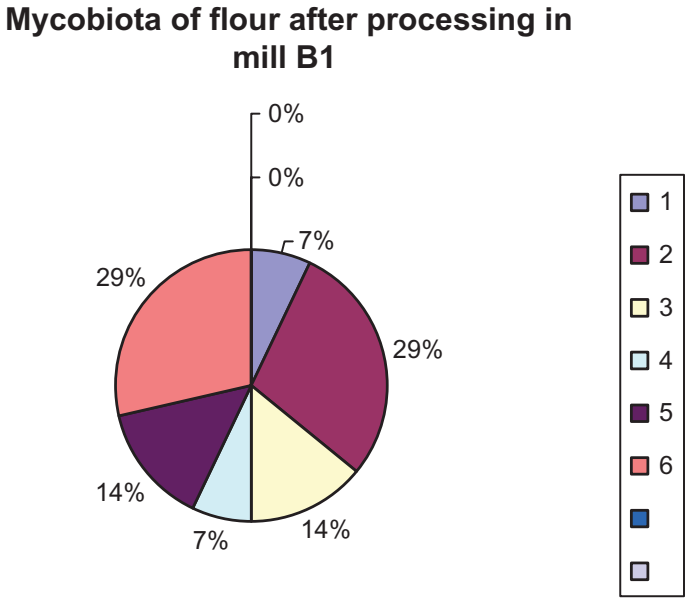

Figure 4. Species of fungi frequently isolated from flour samples and their percentage contribution. 1. *Penicillium* 2. *Cladosporium* 3. *Alternaria* 4. *Fusarium* 5. *Helmintosporium* 6. *Mucor*.

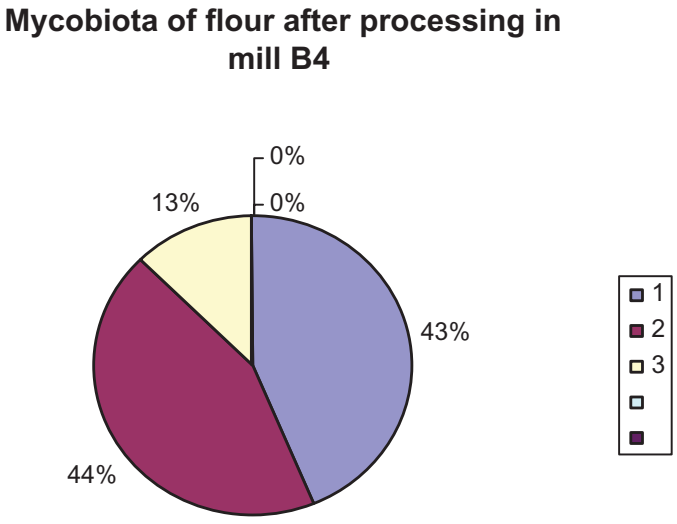

Figure 5. Species of fungi isolated from flour samples and their percentage contribution. 1. *Mucor* 2. *Cladosporium* 3. *Penicillium*.

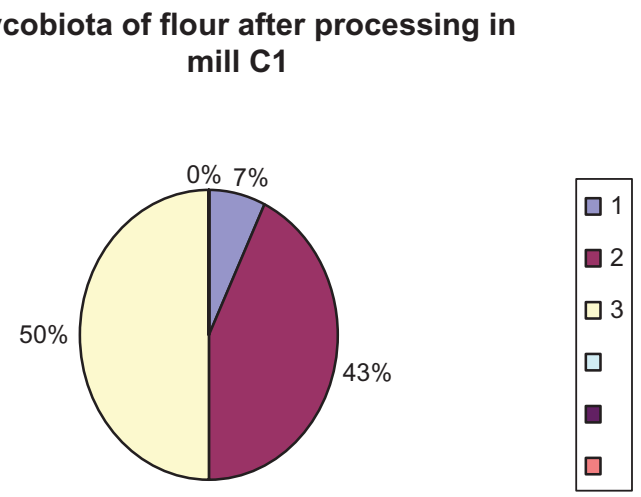

Figure 6. Species of fungi isolated from flour samples and their percentage contribution. 1. *Alternaria* 2. *Cladosporium* 3. *Mucor*.

Differentiation of Mycotoxins Producing Micromycetes from Saprophytic Species

Species belonging to 7 fungi genera (*Aspergillus, Alternaria, Cladosporium, Helmintosporium, Fusarium, Penicillium, and Mucor)* were isolated from grain and flour samples taken at different stages of flour-manufacture.

In further studies we paid more attention to the isolates of *Aspergillus* and *Penicillium* genera as producers of the most important mycotoxins responsible for mycotoxicosis of animals and human.

As it is known from some researches,[8,10] mycotoxins producing micromycetes have specific physiological properties. They can grow at the temperature of 37 °C.

To specify fungi properties and to grow at the temperature of 37°, their pure cultures were cultivated on the Czapek-Dox Agar at the temperature of 4°, 25° and 37 °C.

We checked growth rate, the sizes of radial circles of growing colonies within 20–25 days. Only one isolate—*Aspergillus sp*. *Asp-4* formed colonies at 37 °C on the 5th day (Fig. 8). At the temperature of 25 °C growth of this culture was more intensive, the diameter of a colony reached 4–4. 5 cm. Other *Aspergillus* isolates were not able to grow at 37 °C; however, at 25 °C their colony growth was normal. At 4 °C growth of *Aspergillus strains* was not noted within 20 days.

Penicillium cultures displayed a more distinctive need for temperatur*e* than *Aspergillus* isolates. Strains such as *Pen-4, Penicillium sp.,* and Pen-3, *Penicillium* sp., were able to grow at 37 °C. In particular, *Pen-3, Penicillium s.* showed intensive growth, the diameter of a colony reached 2.8 cm in 5 days (Fig. 8). *Penicillium* cultures did not show intensive growth and showed a small size of colonies (1.6 cm) at 25 °C. All analyzed *Penicillium* isolates were able to grow at 4 °C (Fig. 9).

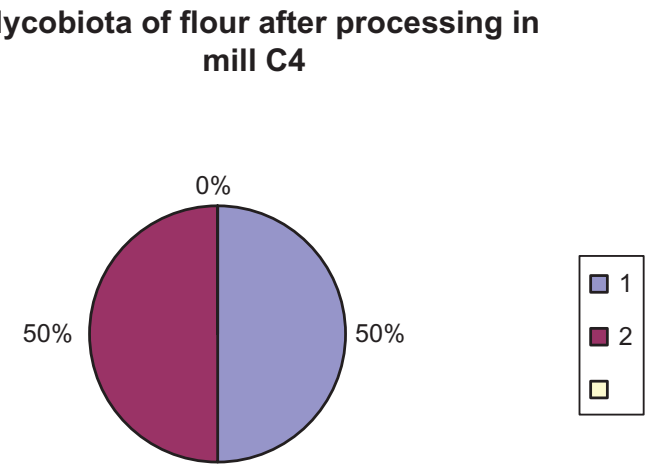

Figure 7. Species of fungi isolated from flour samples and their percentage contribution. 1. *Mucor* 2. *Cladosporium*.

Antagonistic Activity of Micromycetes Cultures to Other Microorganism Groups

Antagonistic activity of *Aspergillus* and *Penicillium* species isolated from grain and flour was assayed against the *Bacillus thuringiensis,* toxins forming bacteria for insects, *Staphylococcus*—toxin forming bacteria in food that causes human intoxication; *Erwinia sp* a phytopathogen.

One strain of *Aspergillus,* Asp-4 showed a high inhibition activity towards all test-cultures. Totally this strain inhibited the growth of two strains of *Bacillus thuringiensis* (55-P2 and 12-06), which produce crystal toxins—intestinal poisons of *Lepidoptera* insects. This strain not only inhibited the growth of *Staphylococcus aureus*, but also showed a hyper parasitic effect on this strain growing in its colonies. Also it inhibited the growth of *Erwinia* bacteria (strain *Erwinia* sp.15-ó), that is a phytopathogen of plants. The isolate *Asp-3* showed a hyper parasitic effect on the two strains of *Bacillus*

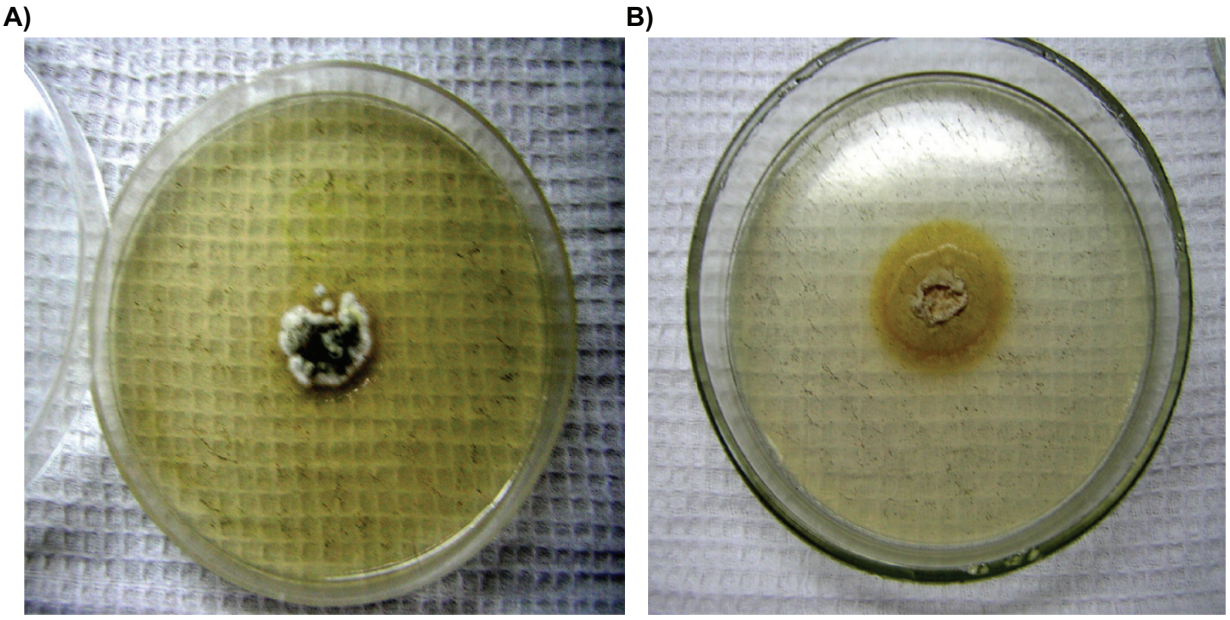

Figure 8. The growth of Pen-3, Penicillium sp and Asp-4, Aspergillus sp colonies at 37 °C temperature.

thuringiensis. However, it did not affect any other test-cultures. The isolate *Asp-1* had a hyper parasitic effect on *Erwinia* bacteria, it inhibited the growth of *Bacillus thuringiensis* 55-P2 strain, but it had no antagonistic effect on the other strain—*Bacillus thuringiensis* 12-06. The isolate *Asp-2* was not active towards all test-cultures.

The isolate *Penicillium,* Pen-3 had the best antagonistic capacity against all test-cultures. Mycelium of this isolate fully covered the surface of the test-culture and used it as a source of supply. Other two isolates of *Penicillium*—Pen-2 and Pen-1 also showed the highest inhibition capacity when tested against *Bacillus thuringiensis, Staphylococcus aureus* and *Erwinia* sp. (Table 1). Three isolates of *Penicillium* genus—Pen-3, Pen-2 and Pen-1 can be preliminarily ranked to mycotoxins producing fungi. Two isolates of *Aspergillus* genus—*Asp-4 and Asp-1* can be ranked to mycotoxins producing fungi. These cultures showed a high antagonistic activity towards all tested bacteria species. Among them only one Asp-4 was able to grow at the temperature of 37 °C.

Table 1

Strain of micromycetes	The test—culture			
	55-П2, Bacillus thuringiensis	***12-06, Bacillus thuringiensis***	***7-а, Staphylococcus sp.***	***15-в, Erwinia sp.***
Asp-4, *Aspergillus sp.*	Inhibited the growth	Inhibited the growth	Hyper parasitic effect	Inhibited the growth
Asp-3, *Aspergillus sp.*	Hyper parasitic effect	Hyper parasitic effect	No effect	No effect
Asp-1, *Aspergillus sp.*	Inhibited the growth	No effect	No effect	Hyper parasitic effect
Asp-2 *Aspergillus* sp.	No effect	No effect	No effect	No effect
Pen-2, *Penicillium sp.*	Inhibited the growth	Inhibited the growth	Hyper parasitic effect	Hyper parasitic effect
Pen-1, *Penicillium sp.*	Inhibited the growth	Inhibited the growth	Inhibited the growth	Hyper parasitic effect
Pen-3, *Penicillium* sp.	Hyper parasitic effect	Hyper parasitic effect	Hyper parasitic effect	Hyper parasitic effect

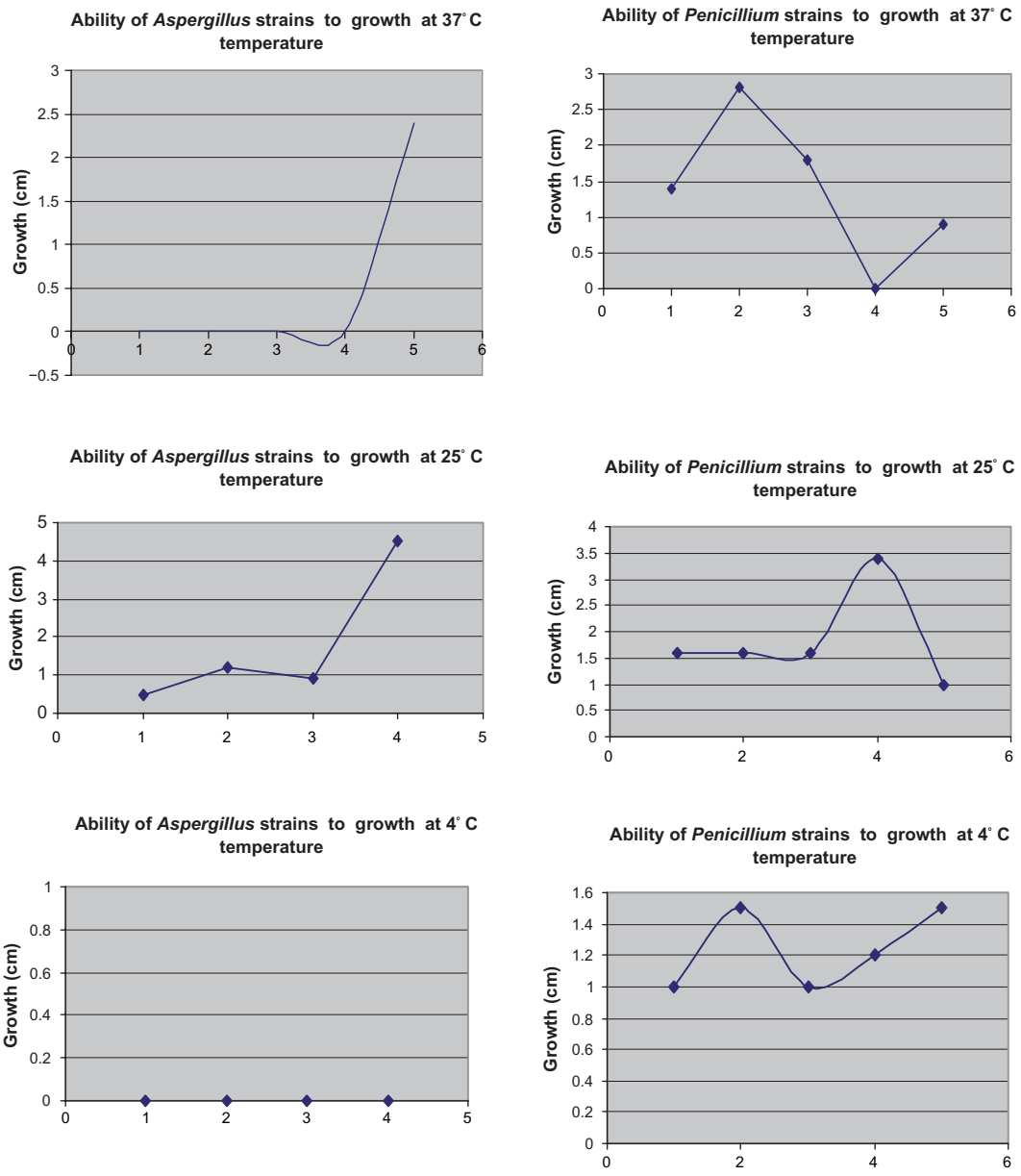

Figure 9. Growth ability of *Aspergillus* and *Penicillium* strains at different temperature.

Identification and Characterization of Selected *Aspergillus and Penicillium* Strains which were Considered to be Producers of Mycotoxins

Strain Asp—4

For 10–14 days this strain produces colonies 3–4 cm in the diameter at the temperature of 24–26° on Czapek-Dox agar. Colonies are usually flat or slightly furrow.

Air mycelium is of ochre—light yellow color. The reverse part of colonies has red—purple shades. Exudation is of amber color and produces a drop on the colony surfaces. A vesicle is spherical, colorless and 35–50 μ in diameter. Mature vesicle departs into 2–3 wide complete columns. The phialides are in two tiers and closely covers the vesicle. Chains of conidia are 1–1.5 mm in length, 10–14 μ in thickness; they have a pale—brown color and a thick (1–2 μ) rough coat. Conidia are spherical 2.5–3 μ. Sclerotia are 1 mm in diameter, purple and cylindrical.

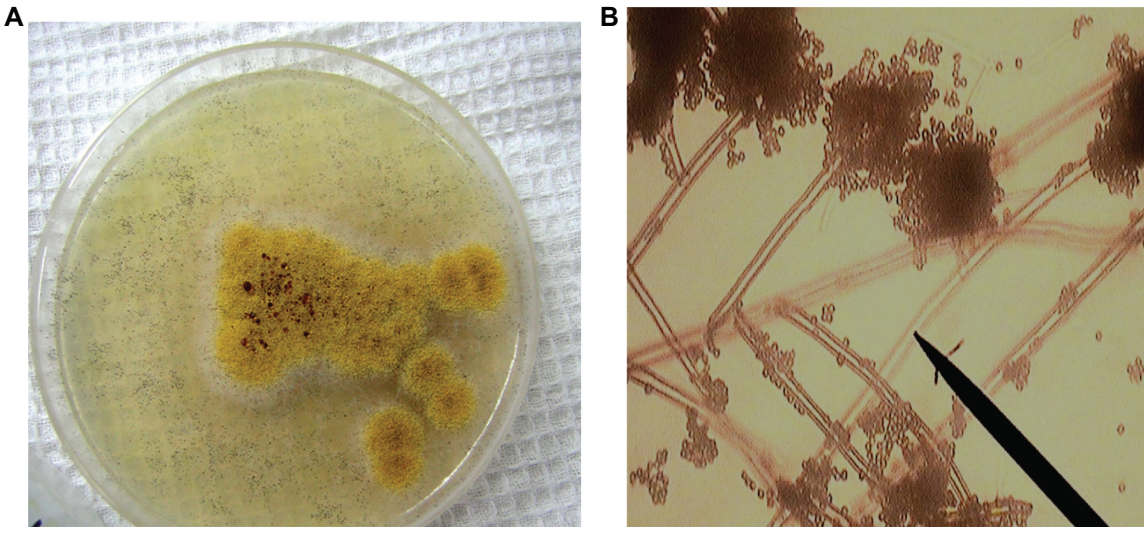

Figure 10. A) *Aspergillus ochraceus* colony on Czapek-Dox agar **B)** *Aspergillus ochraceus* conidiophores and conidia, × 900.

(Fig. 10). According to its cultural and morphological characteristics, this strain was determined as ***Aspergillus ochraceus*** (Wilhelm, Raper, Fennell).

Aspergillus ochracues was isolated from the second point where wheat grains were cleaned mechanically. *Aspergillus ochracues* is known as a species producing the following **mycotoxins**—Ochratoxin A, Ochratoxin B, Ochratoxin C, Penicillic acid, Viomellein.

Strain Asp—1

For 10 days, at the temperature of 24–26 °C on Czapek-Dox agar this strain produces colonies 6–7 cm in diameter. Colonies are usually radially folded; air mycelium is of olive-yellowish-green color. Exudation is plentiful of yellow red color with an unpleasant smell. Immature vesicle is oblong, later more spherical, 10–65 μ in diameter. Mature vesicle is fine radial; they split into badly expressed columns. Chains of conidia are colorless, 3 mm in length. The phialides are in one tier. Conidia is spherical and thorn, 3.5–4.5 μ in diameter (Fig. 11). According to its cultural and morphological characteristics this strain was determined as ***Aspergillus flavus*** **(**Link; Raper, Fennel).

Aspergillus flavus was isolated from the first point where grain was stored in storehouses before processing. *Aspergillus flavus* is considered to be a

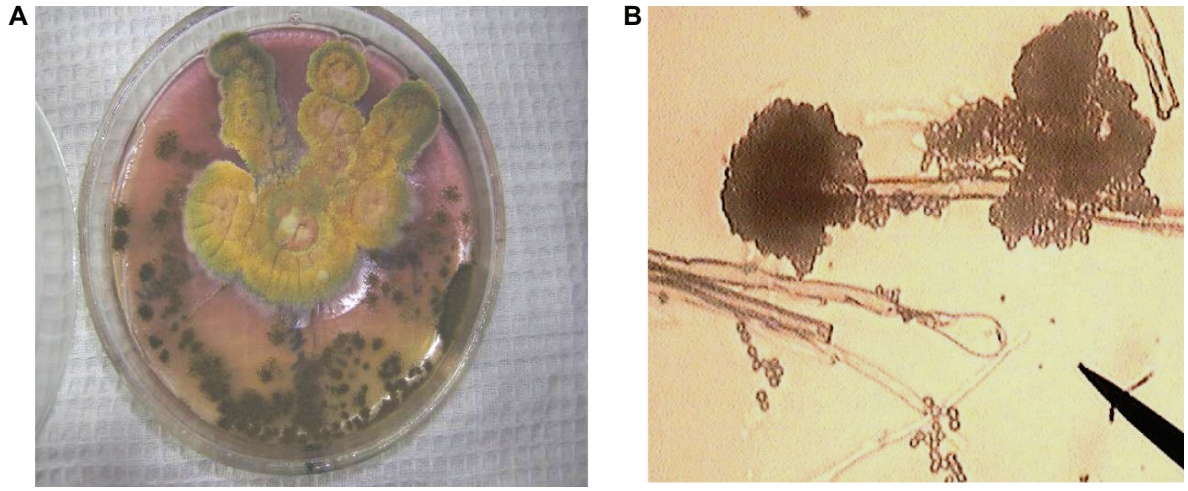

Figure 11. A) *Aspergillus flavus* colonies on Czapek-Dox agar **B)** *Aspergillus flavus* conidiophores and conidia, × 900.

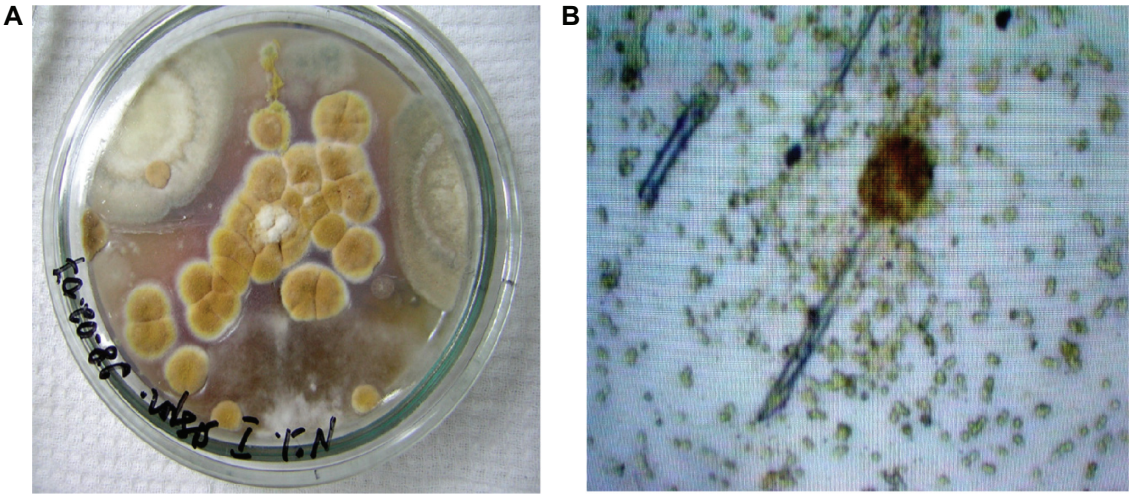

Figure 12. A) *Aspergillus sulphures* colonies on Czapek-Dox agar **B)** *Aspergillus sulphures* conidiophores and conidia, × 900.

producer of the following toxic metabolites: Aflatoxin B1, Aflatoxin B2, Aflatoxin M1, Aflatrem (alkaloid), Aflatrem (indol alkaloid) and other.

Strain Asp—3

For 10–14 days, at the temperature of 24–26 °C on Czapek-Dox agar this strain produces colonies 3.5–4.5 cm in diameter. Colonies are of a furrow form with radial traces. Air mycelium is from white to the cream or pale yellow color forming a dense layer. The reverse part of colonies is pale yellow and has pink shades. Exudation is light and imperceptible. The vesicle is spherical and colorless, sometimes oblong. The phialides are in two tiers, of brightly yellow color. They are friable radial. Chains of conidia reach 1 mm in length with a smooth and colorless coat. Conidia are spindle—shaped and slightly rough, 2–2.5 μ in diameter. Sclerotia are of a cubical form of dark blue color (Fig. 12). According to its cultural and morphological characteristics this strain was determined as ***Aspergillus sulphureus*** **(Fres Thom et Church; Raper, Fennell**). *Aspergillus sulphureus* was isolated from the first point where grain stored in storehouses before processing. *Aspergillus sulphureus* is known as Ochratoxin A producing species.

Strains Pen-2 and Pen-5

These strains produce fast-growing colonies on Czapek-Dox agar. Colonies are of a radially furrow form with a granular surface. Air mycelium of immature colonies is bluish-gray. Air mycelium of mature colonies is dim grey. The reverse part of immature colonies is orange brown, of mature ones is in purple tones. Exudation is plentiful and light and has a strong and mouldy smell. Metula depart from a substratum, 200–400 × 3–3.5 μ, slightly rough, with 2 branches. The phialides of 4–8 in a bunch, 7–9 × 2.5 μ. Conidia are from elliptic to spherical, 3.3–4 × 3–3.3 μ., chains form irregular mass (Fig. 13). According to its cultural and morphological characteristics these strains were determined as ***Penicillium martensii*** (Biourge; Raper, Thom).

Penicillium martensii was isolated from the first and second point of manufacture when grains were in storehouse after mechanical cleaning. There are no data about the mycotoxins produced by *Penicillium martensii.*

Strain Pen-1

This strain on Czapek-Dox produces fast-growing colonies. Colonies are of a less radial furrow form with abundant spore forming. Air mycelium of immature colonies is light blue, of mature ones is dark green and mealy. The reverse part of immature colonies is yellowish, of mature ones is orange—brown. Colonies have a strong mouldy smell. Metula is slightly rough and depart from a substratum, 200–400 × 3–3.5 μ, with several branches. The phialides are 7–10 × 2.2–2.8 μ, of 4–8 in a bunch, with the truncated top. Conidia are more or less spherical, 3.5–4 μ in diameter (Fig. 14). According to its cultural and morphological characteristics this strain was determined as ***Penicillium cyclopium*** **(Westl; Raper, Thom)**. *Penicillium cyclopium* was isolated from the second

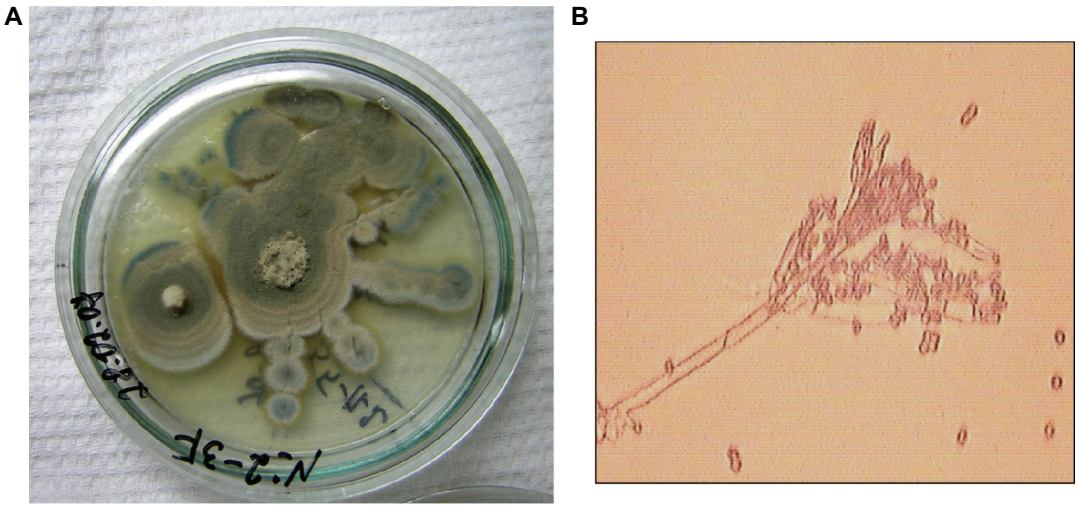

Figure 13. A) *Penicillium martensii* colonies on Czapek-Dox agar **B)** *Penicillium martensii* conidiophores and conidia, × 900.

point where wheat grains have a mechanical cleaning. *Penicillium cyclopium* is known as Patulin producing species.

Strain Pen-3

On Czapek-Dox agar this strain produces radially folded and velvety colonies. Air mycelium is abundantly spore forming from bright—yellow green to bluish dark green shades. The reverse part is of yellow—brown color. Exudation is white like brilliant drops with a mouldy smell. Metula reach 100–200 µ in length, 3–3.5 µ in thickness. Phialides are of 8–10 in a bunch (Fig. 15). According to its cultural and morphological characteristics this strain was determined as ***Penicillium frequentans (Westl; Raper, Thom, 172; Atlas, 69).***

Penicillium frequentans was isolated from the second point of manufacture where wheat grains had a mechanical cleaning. There are no data about the mycotoxins produced by this fungi species.

Immuno-Enzyme Assay of Fungi Isolates Able to Produce Mycotoxins

The study of morphological, cultural, physiological, biochemical properties of fungi strains can give basic information on their ability to produce toxins; however, only specific researches, particularly an immuno-enzyme analysis method can detect the

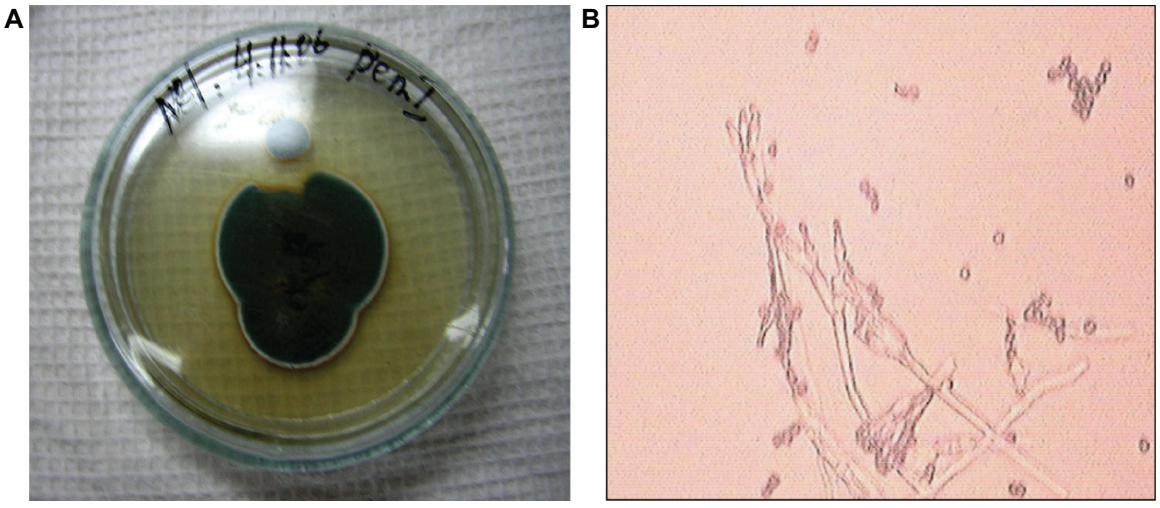

Figure 14. A) *Penicillium cyclopium* colonies on Czapek-Dox agar **B)** *Penicillium cyclopium* conidiophores and conidia, × 900

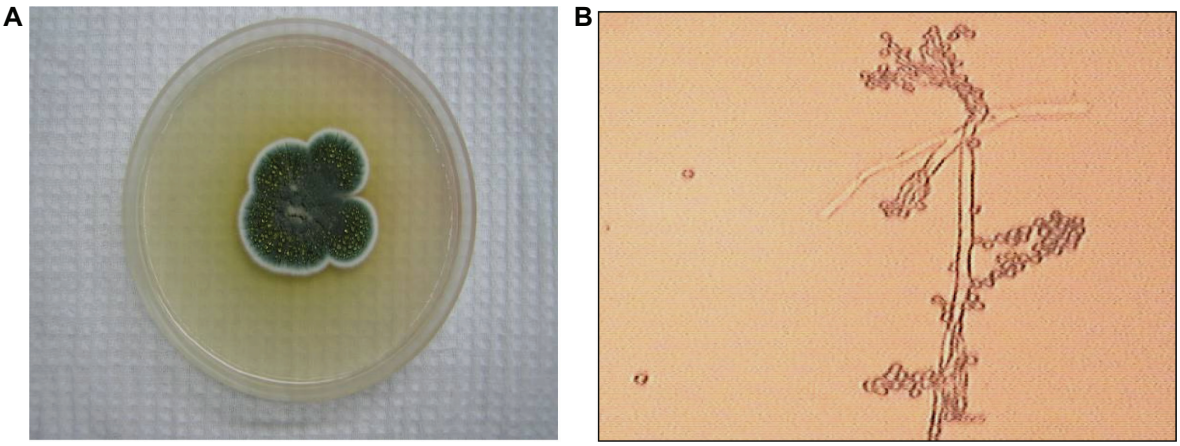

Figure 15. A) *Penicillium frequentans* colonies on Capek's agar **B)** *Penicillium frequentans* conidiophores and conidia, × 900.

toxic level.[27,28] The evaluation of toxin production was conducted in laboratory conditions according to a general scheme, which includes preparation of a fungi subculture, plating on a sterile grain substratum (on rice) with humidity of 30%, stationary incubation in darkness at the temperature of 25–26 °C for 14 days. Subculture inoculum was represented by homogeneous suspension of fungi cells in 0.01%-s' water solution of Tween 80 with approximate concentration of 20 thousand spores per ml. Mycelium—grain biomass was homogenized in the mix of acetonitrol—water (6:1), kept for 14 hours at room temperature, intensively mixed and filtered through a paper filter. The received extract was analyzed by immune enzyme method to detect concentration of Ochratoxin A, aflatoxin B1 and Patulin. Special antibodies were used to each species of mycotoxins. The results showed that *Aspergillus ochraceus i*solated from wheat grain had ability of Ochratoxin biosynthesis in a number of 1.2–4. 8 mg/kg. *Aspergillus flavus* also was able to synthesize aflatoxin B1; however, this strain was poor toxigenic. The number of toxins in a mycelium—grain biomass was only 0.04–1.0 mg/kg. Toxin producing *Penicillium cyclopium* constituted 0.08 to 2.4 mg/kg.

Discussion

In Kyrgyzstan wheat grain, cereal products and flour are used for food preparation daily. Under favorable environmental conditions, some toxigenic molds can produce mycotoxins on agricultural commodities during plant growth and storage. Being overspread in the environment, toxigenic molds can be found in food products and forages without visible mycelium growth. Therefore, the absence of microscopic fungi mycelium does not indicate that a product does not contain mycotoxins. On the contrary, the presence of toxin producing fungi in food products is not a ground to assume that they contain mycotoxins. Generally, there are three types of food contamination with mycotoxins. Primary pollution of agricultural products occurs in the field during vegetation, after harvesting and during storage time. Secondary pollution can occur during processing in poor sanitary conditions. Finally, the last pollution can occur as mycotoxins residual effect as a result of feeding animals by fodder containing mycotoxins.[29]

This study with the wheat grain and flour samples asserts that fungal infection occurs during harvest as well as storage and further continues until the last processing stages of flour though their incidence vary with the different samples. This might be caused by the differences in their moisture contents and cleaning degrees. Grain samples showed high levels of fungal contamination due to their moisture content. As for grains washed with water, contamination with fungi increased abruptly (32.3%) corresponding to the increase of moisture content (20%). Fungal contamination decreased to 21% in dried grain samples with the decreasing moisture content (to 16%). In cleaned grains due to the elimination of dirt and dust stuck on the grain coat, fungal contamination decreased almost twice (7.0%–15.0%) in comparison with grains in the storehouse before processing.

High levels of mycotoxins producing fungi were found in grains during storage time and at the mechanical cleaning point at flour-manufacture. Here *Penicillium, Aspergillus, Alternaria* and *Fusarium s*pecies abounded in grain samples. At the fourth point, where the grains were prepared for mill after drying, there were no *Aspergillus s*pecies but there was a significant number of *Penicillium* and *Fusarium* species (20.0%). Thus, grain before going to mill was free from *Aspergillus species*; however, *Penicillium* and *Fusarium* species were still detected. They cause a risk as they are mycotoxin-producing agents.

Three different species of *Aspergillus,* mycotoxin producers, were detected from grain samples. *Aspergillus flavus* was detected from the grains stored in storehouses before processing. *Aspergillus flavus* is the main source of aflatoxin, the most important mycotoxin in the world's food supplies.[30] These toxins badly affect the liver of warm-blooded animals. Aflatoxin B1 is the most dangerous in this group (LD50 = 6.5–16.5 mg/kg). However, for chickens and ducklings this rate amounts for 1 mg/kg. In a doze of 0.7 mg/kg aflatoxin B1 depresses DNA, concentration of vitamin A in the liver reduces, and concentration of fat rises.[10,14,31,32]

Aspergillus ochraceus was detected from the second point where wheat grains have mechanical cleaning. *Aspergillus ochracues* is known to be the source of the following mycotoxins—Ochratoxin A, Ochratoxin B, Ochratoxin C, Penicillic acid, Viomellein. Ochratoxin production occurs over a wide temperature range and, apparently, below the freezing point too. Ochratoxin—contaminated fodder has its major economic impact on the poultry industry.[3,12,14,33] *Aspergillus sulphureus* was isolated from the first point where grain was stored in storehouses before processing. *Aspergillus sulphureus* is known as Ochratoxin A producing species.

During this study 3 different species of *Penicillium* were isolated from the grain samples. *Penicillium martensii* and *Penicillium frequentans* were recorded from the first and second point of research where the grains were in the storehouse after mechanical cleaning. They are not pathogen. *Penicillium cyclopium* was isolated from the second point where wheat grains have mechanical cleaning. *Penicillium cyclopium* is known as Patulin producing species.

Penicillium species produce toxins—*Patulin* and *Citrinin*. Citrinin is basically related to cereals and rice grain. It is produced by cells of P. *citreonigrum, P. citrinum*, *P. expansum* and *P. verrucosum*. Patulin is produced by cells of *P. expansum, P. claviforme, P. cyclopium, P. equinum, P. glandicola, P. commune, P. lapidosum, P. melinii, P. novaezeelandiae,* and *P. griseofulvum*.[5,9,11,33]

The researches show that Patulin occurs on bananas, pineapples, grapes, peaches, apricots, plums, and tomatoes.[34,35] Inoculation experiments showed that Patulin may be produced by *Penicillium* species on a variety of food and especially fruit, but natural occurrence of Patulin is limited to fruit and is mainly associated with apples and apple products.

Demirici et al[36] detected Patulin in cherries, mulberry, raspberry, and strawberry. Cherries were contaminated with Patulin most frequently, 9 of 10 samples contained Patulin with concentration of 37 mg/kg.

Since the present study detects mycotoxin-producing species like *Aspergillus flavus, Aspergillus ochraceus* and *Penicillium cyclopium* in the grain samples, it is apparent that wheat bran as a waste of flour grinding manufacture contains these toxins; thus it is not suitable fodder for chicken or other animals. Even flour ready for sale contains a significant number of saprophytic fungi.

Only the final product, i.e. high-grade flour was free from *Aspergillus, Penicillium* and *Fusarium* contamination. We assert that the grinding process under several-sequence mills and through sieves eliminates the fungal spores with bran from grain covers. But rough grain fractions still contain fungal spores which are used for preparation of porridge and other food for daily need. Wheat bran, which is proven to have a high level of mycotoxins produced by their fungal component, is given to animals as fodder. Therefore, it is suggested that a severe scrutiny of the samples to be given to animals be done. Also, it is found that supreme-grade flour is safe for consumption since there is no possibility of mycotoxins production.

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Disclosure

This manuscript has been read and approved by the author. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The author reports no conflicts of interest.

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