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EXPERIMENTAL ARTICLES

Species Composition of Food-Spoiling Mycelial Fungi

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Abstract—We investigated the composition of the microflora that spoils foodstuffs (the surface of hard cheeses and sausages) at agribusiness factories. Mycelial fungi, mostly ascomycetes of the order *Eurotiales* belonging to the genus *Penicillium* play the main role in spoiling food. Most representatives of these fungi are mesophiles and possess the capacity for utilizing nutrient substrates in surface and submerged cultures.

Key words: mycelial fungi, *Penicillium*, foodstuffs, surface and submerged cultivation.

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The international community considers the 21st century the Age of Biotechnology. Mycelial fungi are regarded as major biotechnological producers. In the recent decades, these organisms have been employed for obtaining a number of biologically active substances that are used in agriculture, food industry, and especially in medicine. However, negative effects produced by fungi are also receiving increasing attention. These effects result in (i) the destruction of paper (including wallpaper), granite and marble monuments, concrete, sewage pipes, wires, devices, textiles, leather, optical glass, and photographic and movie films [1, 2]; (ii) a drastic increase in the incidence of mycoses; and (iii) active spoilage of foodstuffs that is often accompanied by formation of mycotoxins with strong hepatotoxic and hepatocarcinogenic effects [3]. Mycelial fungi destroy approximately 25% of globally produced food [4, 5].

Recent data have demonstrated that food spoilage largely depends on the novel enzyme activities of mycelial fungi that result from adaptive mutations. In contrast to other microorganisms, fungi possess a powerful mechanism enabling quick invasion of food substrates. They are capable of polarized apical growth resulting in intense fungal biomass accumulation, hypha spreading to new medium zones, and rapid territory colonization.

Food conservation is more often based on chemical antimicrobial agents than on heating or UV irradiation. In recent years, antimicrobial, particularly antifungal agents used for food protection have received much attention, because fungi tend to dominate among food-spoiling microorganisms.

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Despite the diversity of preservative agents used in the food industry, inefficient fungicide preparations are still available in the present-day market. Nevertheless, it is fungi that currently pose the main threat, as far as food spoilage is concerned. The choice of the substances used to inhibit fungal growth at agribusiness factories is made on empirical grounds, without using scientific criteria. To some extent, this is due to a lack of precise information concerning the species composition for the fungal microflora involved.

This work dealt with hard cheeses and sausages whose surface is particularly susceptible to fungal invasion. Developing a scientific concept concerning food surface and raw material protection against the destructive influence of mycelial fungi requires (i) long-term research on the composition of the microflora that invades foodstuffs (hard cheese and hard smoked sausages), (ii) identification of the main contaminating organisms, and (iii) elucidation of the differences among these organisms in terms of food substrate utilization. These issues were the goals of the present work.

MATERIALS AND METHODS

Microscopic fungi were isolated from the surface of hard cheeses (Posad, Shveitsarskii, Rossiiskii, Staryi Gollandets, Sovetskii, Kostromskoi, and others) and raw-smoked (Servelat, Braunshveigskaya, and Pikantnaya), boiled and smoked sausages (Moskovskaya, Servelat, and others) in the course of their production and storage at cheese-producing and meat-processing factories in Moscow and the Moscow, Smolensk, Yaroslavl', and Vladimir oblasts in the Russian Federation.

Methods for isolating and identifying fungi from the samples. Cheese and sausage samples were examined to locate fungi-spoiled parts. A microbiological

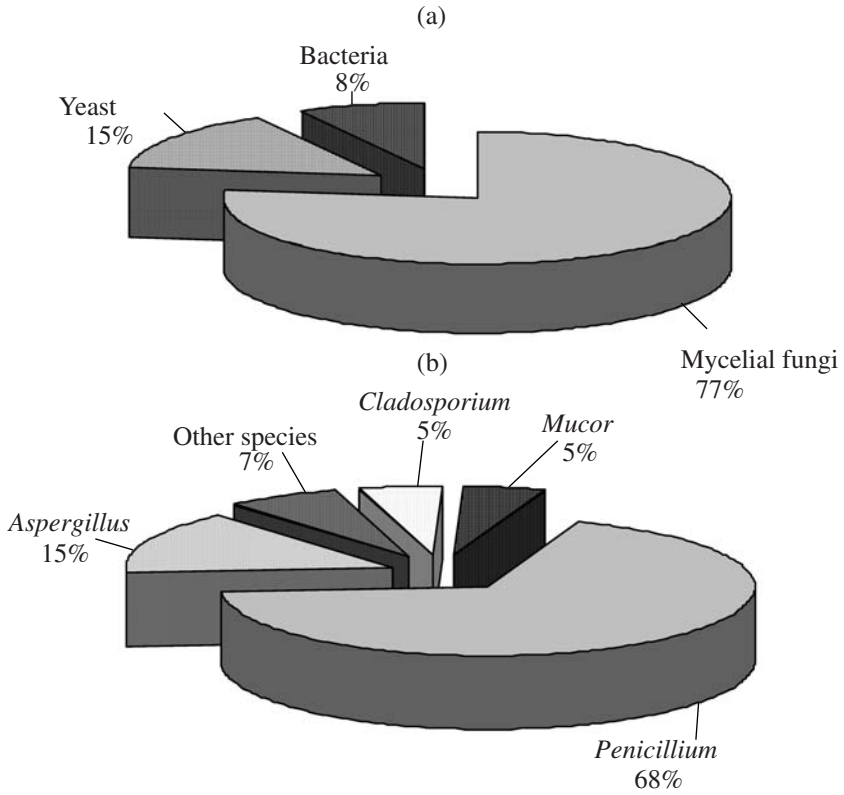

Fig. 1. Cheese-spoiling microflora: a, composition of microorganisms; b, composition of predominant mycelial fungi.

hook was used to prepare scrapes from the sites where mycelium growth was visible. They were inoculated to obtain stab cultures on sterile petri dishes with the Czapek–Dox medium. Fungi belonging to all taxa except zygomycetes develop on this medium. Malt agar was used to isolate zygomycetes. After inoculation, the petri dishes were incubated for 10 days at 25–28°C.

For species identification pure cultures of the fungi involved were obtained. For this purpose, all visually distinct fungi were inoculated on separate dishes with the Czapek–Dox medium and malt agar. Identifying the species of the isolates belonging to the genera *Aspergillus* and *Penicilluim* was performed using the following media: (i) Czapek agar with yeast extract; (ii) the same medium with 20% sucrose; (iii) 25% glycerol–nitrate agar; and (iv) concentrated Czapek medium and malt extract agar plus the above media. The cultivation time was 7 days. Owing to the high hydrophobicity of *Penicillium* and *Aspergillus* conidia, we used water containing 0.2% agar and 0.5% detergent (e. g., Twin-80) for preparing the spore inoculum. Manuals [6–9] were consulted to identify the fungal isolates.

Fungi were cultivated at 27°C. Surface cultivation was carried out by the stab culture method. Submerged cultivation was carried out on a shaker (220 rpm) in 250-ml flasks (50 ml of medium). The morphology of the invading microflora was determined by light microscopy and by scanning electron microscopy using a T-300 scanning microsope (JEOL, Japan).

RESULTS AND DISCUSION

In 2002–2003, we started our research on surface microbial contamination of sausage and cheese obtained from agribusiness factories. It was established that the main microflora for all samples consisted of ascomycete fungi of the order *Eurotiales*. Among the latter, mycelial fungi of the genus *Penicillium* predominated (Figs. 1 and 2). These mycelial fungi account for about 68% and up to 50–53% of the food-spoiling fungi on the surface of hard cheeses and sausages, respectively. Among other mycelial fungi, the role of the genus *Aspergillus* is important (17–20% and 15% on the cheese and sausage surface, respectively). Representatives of other genera of mycelial fungi, *Mucor, Thamnidium*, and *Cladosporium*, are less widely spread. Apart from mycelial fungi, we also detected yeast and anaerobic bacteria (Figs. 1a, 1b, Figs. 2a, 2b).

Similar results concerning cheese and sausage surface-spoiling microflora were obtained by us in 2006–2007 at the same agribusiness factories. However, the *Penicillium* content on the cheese and sausage surfaces increased to almost 80%. For the first time, the composition of the predominant mycelial fungal genus, *Penicillium*, was studied in detail. The list of the representa-

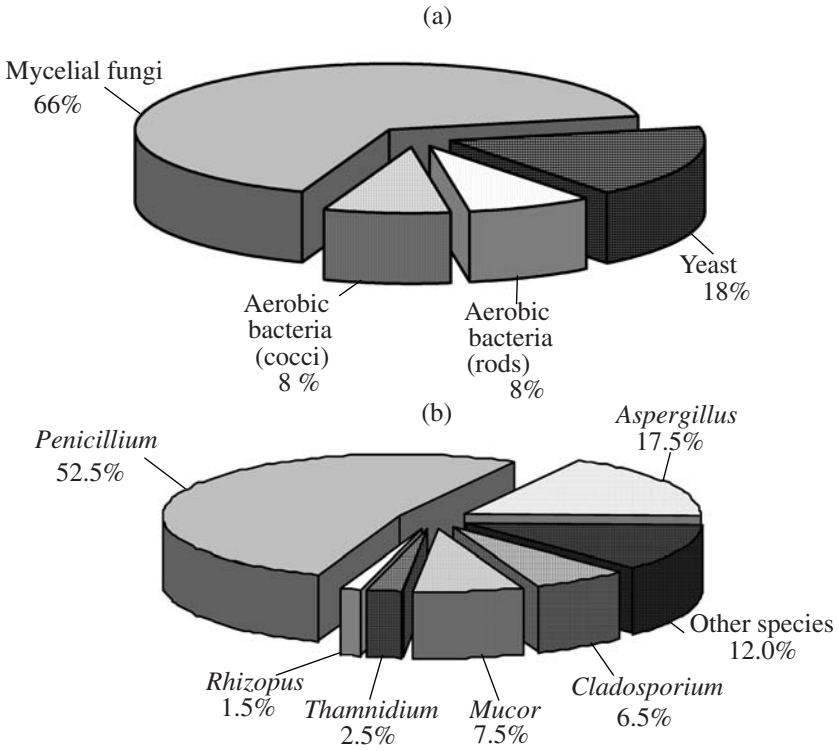

Fig. 2. Sausage- and meat specialties-spoiling microflora: a, composition of microorganisms; b, composition of predominant mycelial fungi.

tives of the genus *Penicillium* isolated and identified by us includes the following species: *Penicillium aurantiogriseum, P. roquefortii, P. verrucosum, P. brevicompactum, P. expansum, P. citrinum, P. commune, P. rugulosum*, and *P. chrysogenum.* As for representatives of other classes of fungi, *Mucor plumbens, Eurotium (Aspergillus) amstelodami, Aspergillus niger*, and *Geotrichium candidum* were detected. Presently, these microorganisms are stored in the All-Russian Collection of Microorganisms of the Skryabin Institute of Physiology and Biochemistry of Microorganisms.

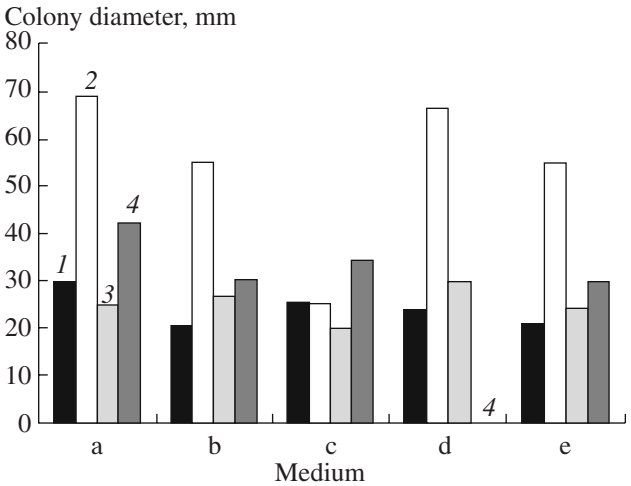

Fig. 3. Colony diameter (mm) of *Penicillium* specieson the 7th day of cultivation at 27°C on the following media: a, malt agar; b, potato–carrot medium; c, nutrient agar; d, whey; e, maize–soybean medium. *P. commune* (*1*); *P. roquefortii* (*2*); *P. aurantiogriseum* (*3*); *P. chrysogenum* (*4*).

We selected a number of strains of mycelial fungi that are particularly widespread on the surface of food substrates and highly active in destroying foodstuffs. The list of the tested fungi included *P. roquefortii, P. aurantiogriseum, P. commune*, and *P. chrysogenum.* The data in Fig. 3 indicate that the fungi isolated by us differ in their requirements with respect to growth substrates. Some of them demonstrate a correlation between the capacity for growing on specific media and the ability to colonize foodstuffs. All tested *Penicillium* strains grow on natural media but differ in their substrate colonization rate. *P. roquefortii* is characterized by the most active growth. Its colony size on milk whey is up to 13.2 mm on the second day of cultivation. *P. chrysogenum* displays a similar growth rate. *P. roquefortii* is a typical representative of cheese-spoiling fungi. This organism requires special media. Its growth is optimum on whey and malt agar and much worse on the meat–peptone medium. *P. chrysogenum* that was isolated from the surface of meat foodstuffs failed to grow on solid whey-containing media but quickly spread over the surface of nutrient agar. The same pattern also occurred when cheeses or sausages

Biomass Accumulation by Submerged *Penicillium* Cultures on Various Media

Culture	Malt, 7 °B		Whey		Nutrient broth	
	27 h	4 days	27 h	4 days	27 h	4 days
P. chrysogenum	3.04	17.06	Traces	17.28	Traces	1.51
P. roquefortii	2.61	9.91	1.99	9.45	Traces	1.13
P. aurantiogriseum	1.98	14.24	Traces	7.30	Traces	1.87
P. commune	Traces	Traces	Traces	7.47	Traces	0.69

were used as substrates. Inoculating the surface of a Rossiiskii cheese sample with mature *P. roquefortii* conidia resulted in the growth of its mycelium after 48 h. The cheese surface was completely overgrown on the fourth day of cultivation. *P. chrysogenum* failed to grow on the cheeses' surface. However, it grew on meat products' surface. Other strains isolated by us from the cheese/sausage surface were not characterized by such manifest differences in terms of substrate utilization.

These species-specific patterns in terms of substrate utilization drastically change upon the transition to submerged cultures (table). Interestingly, *P. chrysogenum* grows better than other species on the tested substrates under these conditions. It is characterized by the highest biomass accumulation rate on whey. Its growth in nutrient broth is extremely scarce, i.e. its enzyme activities differ from those on solid media.

The tested strains are to be considered mesophiles according to their temperature requirements. From the data in Fig. 4 it is evident that growth of the species *P. commune, P. roquefortii*, and *P. aurantiogriseum* is optimal at 27°C. It is worse at 9°C and completely absent at 33–34°C. An exception is *P. chrysogenum* that was detectable at high temperatures on day 5 of growth on malt agar, potato–carrot medium, and nutrient agar. The fact that most tested strains grow at low temperatures is of particular importance because mycelial fungi grow even in refrigerator compartments under industrial conditions.

The new data obtained by us are primarily related to the issue of food colonization by ascomycete fungi. Of special interest is the fact that the surface of such foodstuffs as hard cheeses and sausages is predominantly infected by *Penicillium*, not *Aspergillum*, whose quantity does not exceed 10%. The *Penicillium* percentage in food-contaminating microflora clearly tends to increase. *Pennicillium* species accounted for 68–71% of the fungi detected in 1985 [10] and 2003 and for over 80% in 2007. The content of another ascomycete genus, *Aspergillus*, decreased; only two species, *E. amstelodami* and *A. niger*, were revealed. The results of these studies suggest that these two ascomycete groups, which were earlier regarded as closely related taxa, differ in terms of their metabolic activity and adaptability. Of particular interest in this context are the data that while over 30 *Aspergillus* species were detected in the late 20th century among pathogenic fungi, a pathogenic *Penicillium* species, *P. marneffei* that is responsible for endemic penicilliosis, was discovered only recently [11]. This finding can be considered in terms of the habitat enlargement strategy and the development of new enzyme activities in the fungi from the genus *Penicillium*. This *Penicillium* species is characterized by dimorphism (lacking in *Aspergillus*), i.e. it exists in yeast-like and mycelial forms. It is presently believed that dimorphism significantly increases the adaptive potential of a species. In addition, *Penicillium* species are more resistant to fungicides than *Aspergillus* species, according to the data obtained by mycologists that work at milk-processing factories [12].

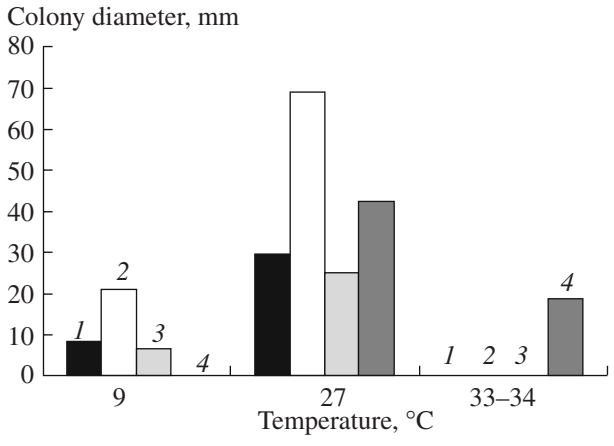

Fig. 4. Colony diameter (mm) of *Penicillium* species on the 7th day of cultivation at various temperatures. For designations see Fig. 3.

Of special interest are the data on the requirements of various fungi in terms of nutrient substrates. *P. roquefortii* and *P. chrysogenum* are characterized by a relationship between their preferences on solid nutrient media and food items. Substrate utilization patterns vary depending on whether solid-phase or submerged cultures are used. Presumably, it is the oxygen requirement that influences the enzyme activities of the fungi involved. This fact was established in studies with ascomycete superproducers of citric acid that are industrially grown either in submerged or surface cultures [13].

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