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EXPERIMENTAL ARTICLES

# Strategies of Food Substrate Colonization by Mycelial Fungi

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**Abstract**—This article deals with a new research area related to mycology and the food industry. It is concerned with the strategies used by ascomycete mycelial fungi to utilize and colonize food items. The data of scanning and transmission electron and light microscopy revealed that fungi growing on such food items as cheese and sausage are characterized by the following colonization strategies: (i) spreading growth; (ii) burrowing growth; (iii) growth with the formation of up to 4–5 new branches on the apexes of the old hyphae; (iv) growth with the formation of long strands, and (v) growth with biofilm formation.

*Keywords*: mycelial fungi, colonization strategies, contamination, food substrates, *Penicillium*

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The activity of food-spoiling mycelial fungi considerably increased over the course of the last decades. It has been estimated that up to 25% of the food produced on the planet is subject to the destructive action of ascomycete fungi. Therefore, developing a scientific strategy aimed at conserving foodstuffs has become one of the main goals of mycology [1].

In our earlier work, we listed the main mycelial fungal species that affect such widely used, expensive food items as hard cheese and sausage varieties [2]. It was emphasized that some representatives of the genus *Penicillium*, e.g., *Penicillium roqueforti*, colonize food substrates at a sufficiently high rate.

The strategies enabling mycelial fungi to be among the first microorganisms to colonize nutrient-rich substrates have not yet received sufficient attention in mycology. However, this subject, defined as "natural spatial expansion", is presently considered one of the central issues of biology and biopolitics [3].

Several strategies of colonizing food substrates are widespread among microorganisms. A particularly well-understood strategy involves the formation of motile flagellated cells (swarmers) in *Proteus mirabilis, Pr. vulgaris*, and *Serratia marcescens*. They migrate towards new food substrate areas in front of other bacterial cells [4]. Once a new substrate area or a new nutrient source has been colonized, the swarmers lose their flagella and convert into ordinary vegetative cells. Of still greater interest is the motility type used by myxobacteria on the nutrient substrate. The most recent studies indicate that these microorganisms do not use their mucilage as "lubrication" facilitating their movement over the substrate. Instead, the mucilage serves as a "jet fuel", i.e., the microorganism uses a "jet engine" [5] allowing them to colonize nutrient sources very quickly. Myxomycetes, which are presently not considered true fungi, are characterized by another motility type used by them to seek nutrient substrates. At a certain stage of their development, the cells move like amoebas: they form pseudopodia [6].

Unlike other microorganisms, such representatives of the kingdom *Fungi* as mycelial fungi possess an extremely powerful tool enabling them to quickly colonize nutrient sources. They are characterized by a polarized apical growth pattern of their hyphae, which results in rapid accumulation of the fungal biomass. The growth of hyphae is accompanied by cell wall formation. As a result, a high turgor pressure is maintained in the hypha, and substrate-degrading enzymes are released at the growing hypha apex. A hypha with a diameter of 10–15 μm elongates at a rate of 100 μm per minute, and this process is controlled by specialized aggregated microvesicles referred to as "vesicle aggregation centers" (*Spitzenkorper*) [7]. Actinomycetes, a different group of mycelial microorganisms, lack such microvesicles, and these bacteria are characterized by a significantly lower growth rate (0.5 μ per minute).

As noted above, mycelial fungi, due to their adaptive mutations, have recently become increasingly active in utilizing food substrates and infecting the objects of the agricultural sector producing such food items as cheeses and sausages. These substrates are colonized at a sufficiently high rate, even in refrigerators. In order to eradicate the contamination of food and production premises, it is necessary to know how fungal hyphae spread over food items, i.e. how they colonize new nutrient substrates.

The goal of this work was to investigate the strategies used by the fungi of the genus *Penicillium* for colonizing cheese and sausage surfaces. Light, transmis-

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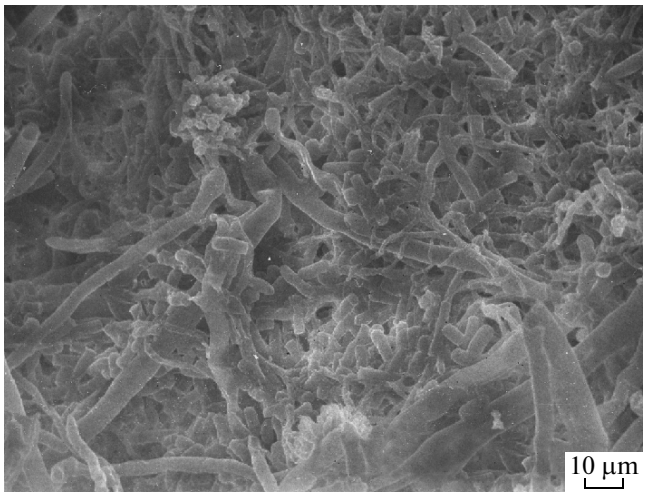


**Fig. 1.** *P. roqueforti* hyphae formed on the Posad cheese surface (sample 1). Scanning microscopy. Scale bar, 10 μm.

sion electron, and scanning electron microscopy were used.

## MATERIALS AND METHODS

Two cheese samples were tested. Sample 1 was taken from the Posad cheese, whose dense structure lacks pores, and sample 2 from the Rossiyskiy cheese characterized by a looser structure with small pores. The cheese-contaminating organism used by us was the *Penicillium roqueforti* VKM FW-3057 strain isolated from the Posad cheese (Yaroslavskie Syry Company). It grows well on whey in solid-phase culture [1].

We also tested uncooked smoked Servelat, Braunschweigskaya, and Pikantnaya sausages. In these systems, *Penicillium* species (*P. aurantiogriseus* and *P. commune*) were also the predominant contaminants. Cheese and smoked sausage samples were obtained from cheese and meat-processing plants located in Moscow and in the Moscow, Smolensk, Yaroslavl', and Vladimir Regions of the Russian Federation. Isolation and identification of the fungi from the above food items were performed using the method described in an earlier publication [2]. The samples were infected with the suspension of 7- to 8-day conidia. They were placed in glass containers with a volume of 3–5 L that were covered with gauze layers. The containers were stored at room temperature or at 27–28°C. The samples were examined daily under a microscope.

Fungal contamination was investigated using (i) light microscopy; (ii) scanning electron microscopy (examining gold-coated samples under a JEOL ISM-T300 microscope, Japan), and (iii) transmission electron microscopy under a JEM-100CXII microscope (Japan) at an accelerating voltage of 80 kV and a magnification of 8.000–20.000×. Ultrathin sections were obtained with an LKB-NOVA ultramicrotome (Sweden). Scanning microscopy makes it possible to evaluate the growth pattern of the surface mycelium and, therefore, to focus the research on a well-defined goal. Transmission microscopy makes it possible to conduct detailed studies on the pattern of hypha spreading and substrate invasion and assess the physiological state of the hyphae of the substrate and aerial mycelium.

Fungal mycelium and food sections were prefixed with 1.5% $KMnO_4$ solution in phosphate buffer (pH 7) for 1 h and thereupon in 1% $OsO_4$ solution in acetate–veronal buffer (pH 7) for 12–14 h at a low temperature. Thereafter, the samples were dehydrated by treating them consecutively with solutions with increasing ethanol concentrations [8], up to absolute acetone. The samples were thereupon embedded in EPON-812. They were additionally contrasted with 3% aqueous uranyl acetate solution at room temperature for 40 min and then with lead citrate according to Reynolds [9]. Cell morphology was assessed with an Axio Imager.DI light microscope (Carl Zeiss, Germany) at a 400×.

## RESULTS AND DISCUSSION

Well-developed aerial mycelium was formed on the Posad cheese after inoculation with *P. roqueforti* conidia. The substrate part of the mycelium consisted of thickened submerged hyphae. On *P. roqueforti*-inoculated Rossiiskiy cheese, a visually analogous aerial mycelium formed. However, the substrate mycelium, which appeared to be looser and less homogeneous, was of special interest.

Using scanning and transmission electron microscopy, we revealed that cheese surface colonization by the fungus could be based on two different strategies that were arbitrarily denoted by us as the spreading and the burrowing growth strategy. The first strategy involved filling all macro- and microscopic fissures and cavities on the cheese surface by the fungus. This growth pattern was characteristic of sample 1 and developed relatively quickly. On the third day of growth at 27°C, virtually the whole cheese surface was covered with aerial mycelium (Fig. 1). On the fourth day, active conidia formation set in. No further cheese destruction or penetration of the hyphae into the cheese occurred.

The burrowing growth strategy implicating the penetration of the fungus into cheese (Fig. 2) was a more complex process including several stages. This pattern was typical of sample 2. It can arbitrarily be broken down into four stages. The first stage involved colonization of the substrate by spreading over it. The second stage was characterized by the formation of a penetrating substrate mycelium and was probably accompanied by the synthesis of cheese surface-degrading enzymes. The third stage was characterized by the death of the primary penetrating hyphae and by

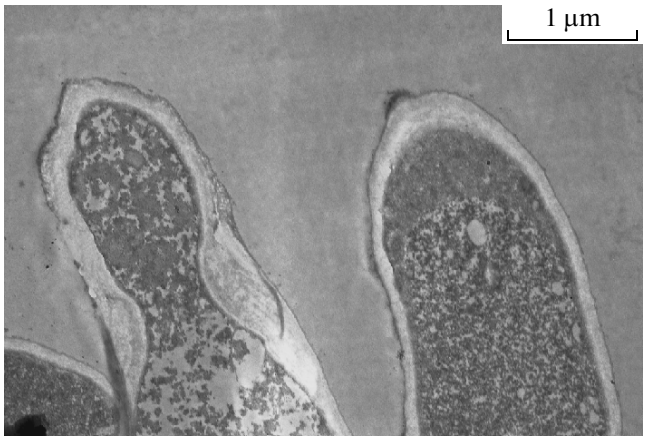


**Fig. 2.** *P. roqueforti* hyphae penetrating into the interior of Posad cheese (sample 2) from its surface. Electron microscopy. Scale bar, 1 μm.

the penetration of the new hyphae. The fourth stage actually repeated the third stage but was performed in the deeper layers of the cheese. Interestingly, we did not detect any hyphae at a depth of more than 1–2 cm, probably due to the changed aeration and humidity levels in the deeper layers.

Currently, it is difficult to account for the above cheese variety-dependent differences in cheese colonization patterns of the fungal hyphae (they may be related to the structure of the tested cheese varieties). However, this fact is of indisputable importance for practical cheese production in terms of food conservation.

If *P. roqueforti* grew on the cheese surface in conformity with the spreading strategy, an unusual type of hypha branching occasionally occurred. Older hyphae growing on the surface formed new branching points at the apex. They consisted of three to five hyphae that subsequently spread rapidly over the substrate surface. Such growth may be considered a new type of rapid substrate colonization strategy.

In mycelial fungi, one more strategy of growth on the surface of food substrates was observed. In model studies, stab inoculation of *P. commune* conidia on agarized wort was performed. The petri dishes were kept in the dark for 7 days at 27°C and then incubated in the light at room temperature. During the observation period, we noted a growth pattern change that resulted in the formation of concentric circles. The density of fungal biomass was significantly less in the areas between the circles. In some cases, mycelium-free zones were detected on the substrate surface (Fig. 3). This was probably due to the onset of the pulsed growth mechanism [10]. Scanning microscopy revealed that long strands of thickened surface mycelium characterized by mass conidia formation were arranged between the circles of the colony. The central zone of each concentric circle was made up of concentrated conidia. We suggest that these strands are involved in transferring conidia to more distant mycelium-free areas, facilitating further utilization of the nutrient substrate. Possibly, these processes are subject to regulation by circadian rhythms that promote circular colony growth.

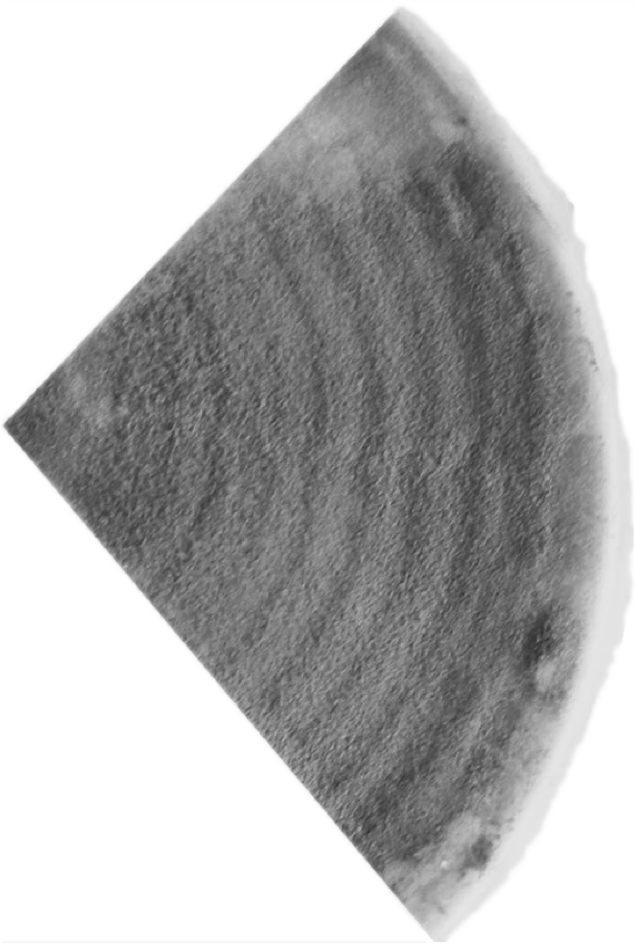

**Fig. 3.** *P. commune* colony sector on a petri dish (wort agar). Light microscopy.

Our studies on the spreading of mycelial fungi on the sausage surface enabled us to reveal an additional strategy of colonizing the food substrates. The surface of hard smoked sausages is coated by a special cover made of natural materials. This cover is exposed to the destructive action of microorganisms during storage. This process includes three stages. Initially, the cover surface is colonized by yeasts. The yeasts are thereafter replaced by bacteria. The last stage is accomplished by rapidly growing fungal mycelium. The sausage cover is quickly destroyed, and fungal hyphae penetrate into the interior of the sausage (Fig. 4). This is accompanied by a change in the sausage meat color under the surface; it becomes dark brown. This process predominantly involves representatives of the genus *Penicillium,* such as *P. commune* and *P. aurantiogriseum*. Hence an additional pattern of colonizing new nutri-

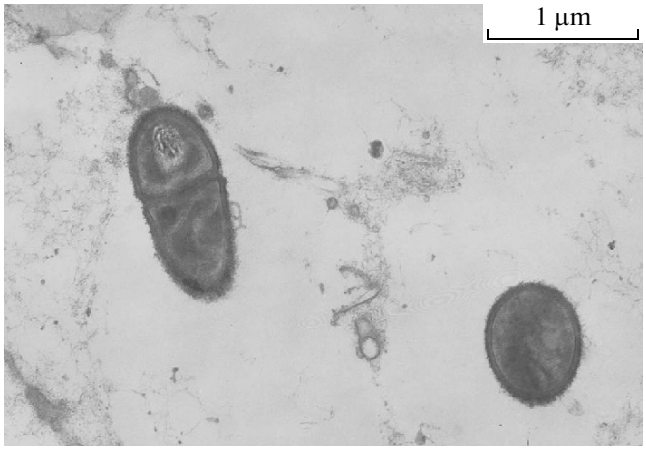


**Fig. 4.** Fungal hyphae formed in the interior of a sausage. Electron microscopy. Scale bar, 1 μm.

ent substrates by fungi was established in this system. It implicates the role of the fungi in forming a biofilm on the sausage cover and gradual replacement of the preceding bacterial microflora by fungi.

The data presented in this work are the first results of our research on the strategies used by fungi to colonize food items. They hold much theoretical value in terms of biopolitics because the motility of microorganisms and food substrate colonization by them are regarded as adaptive traits [11]. The research results are also of practical importance. The colonization strategies used by fungi on food substrates are to be taken into account while making decisions concerning the quantity and composition of preservatives that can be recommended in terms of food conservation. Our preliminary studies demonstrated that the conservation of cheese varieties contaminated by fungi preferring the second colonization strategy required a significantly larger amount of preservative E-200. The data obtained can be of interest to agro-industrial businesses that use fungicides to protect food from colonization by fungal mycelium.

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## REFERENCES

1. Lyuk, E. and Yager, M., *Konservanty v pishchevoi promyshlennosti* (Preservative Agents in Food Industry), St. Petersburg: GIORD, 1998.
2. Feofilova, E.P., Kuznetsova, L.S., Sergeeva, Ya.E., and Galanina, L.A., Species Composition of Food-Spoiling Mycelial Fungi, *Microbiology*, 2009, vol. 78, no. 1, pp. 112–116.
3. Oleskin, A.V., Ecologically Important Properties of Microbial Populations, *Soros. Obrazovat. Zh.*, 2001, vol. 7, no. 8, pp. 7–12.
4. Alison, C., Lai, H.C., and Hughes, C., Co-Ordinate Expression of Virulence Genes During Swarm-Cell Differentiation and Population Migration of *Proteus mirabilis, Mol. Microbiol.*, 1992, vol. 6, no. 12, pp. 1583–1591.
5. Fiegna, F., Yuen-Tsi, N., Kadam, S.V., and Velier, G.Y., Evolution of an Obligate Social Cheater to a Superior Cooperation, *Nature*, 2006, vol. 441, pp. 310–314.
6. Ermilova, E.V., Zalutskaya, Zh.M., and Lapina, T.V., *Podvizhnost' i povedenie mikroorganizmov, t. 2. Eukarioty* (Motility and Behavior of Microorganisms. Vol. 2. Eukaryotes), St. Petersburg: Izd-vo St.-Petersburg Gos. Univ., 2010.
7. Potapova, T.V., Intercellular Interactions in *Neurospora crassa* Hyphae—20 Years Later, *Biol. Membr.*, 2004, vol. 21, no. 3, pp. 163–191.
8. Biryuzova, V.I., *Membrannye struktury mikroorganizmov* (Membrane Structures of Microorganisms), Moscow: Nauka, 1973.
9. Reynolds, E.S., The Use of Lead Citrate at High pH as an Electron-Opaque Stain in Electron Microscopy, *J. Cell Biol.*, 1963, vol. 17, no. 1, pp. 205–212.
10. Lopez-Franco, R., Bartnicki-Garcia, S., and Bracker, C., Pulsed Growth of Fungal Hyphal Tips, *Proc. Natl. Acad. Sci. U. S. A.*, 1994, vol. 91, pp. 1228–1232.
11. Oleskin, A.V., *Biopolitika v kontekste istorii biologii i filosofii* (Biopolitics in the Context of the History of Biology and Phylosophy), Moscow: Gumanitarii, 2011.