UDC 582.28:579.66:678.02

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MICROMYCETES – POTENTIAL BIODESTRUCTORS OF RUBBER TECHNICAL MATERIALS WASTES IN THE CLAMATE OF UKRAINE

Aim *of the presented paper was the preliminary screening of microscopic fungi promising for development of the biotechnology of destruction of the wastes of rubber technical materials (RTM).* ***Methods.*** *The cultures of micromycetes were isolated from RTM reposited on agarized mash (4.5° sensu Balling) on 4 °C. An ability of investigated micromycetes to grow on RTM was estimated on the model caoutchouc-based medium (CBM) imitated rubber substrate. The presence of carboxylesterase and oxidases was educed by the qualitative methods and the enzymatic index was calculated. The compatibility of the selected cultures of micromycetes has been estimated by the method of agarinic blocks.* ***Results.*** *It has been shown that 77 micromycetes from 85 studied strains were able to form clear colonies on CBM. Among them only five strains – Aspergillus fumigatus F-41484, Mucor racemosus F-41411, M. racemosus F-41412, Rhizopus cohnii F-2 and R. cohnii F-3 have high enzymatic indexes by the carboxylesterase activity and five strains – Alternaria alternata F-41431, Aspergillus flavus F-41432, A. sydowii F-41426, A. ustus F-41437 and A. versicolor F-41469 – have high enzymatic indexes by the oxidase activity. It has been ascertained an ability of using of the strains that have high enzymatic indexes in the eight mixed cultures.* ***Conclusion.*** *Ten fungal strains that have been characterized by high enzymatic indexes for carboxylesterase or complex of oxidative enzymes and were able to combined cultivation, were selected for development of technology of biodestruction of RTM wastes.*

Key words: rubber technical materials, micromycetes, biodestruction, enzymes, enzymatic index, mixed culture.

Until today the problem of concentration of RTM wastes is current not only for Ukraine but for the whole world. On an annual basis approximately 150 000 tons of rubber wastes get into a place on Ukrainian dumps, while in the world this number achieve 7–10 million tons [14].

Traditional technology of RTM utilization by the pyrolysis is dangerous for the environment due to its secondary products: oxides of carbon, sulfur, nitrogen, aromatic hydrocarbons etc [11]. The subsequent employment of RTM for producing of technical materials is not appropriate release of their utilization as well because manufactured goods have low funginertness [4].

Biodestruction is an alternative way of the conversion of the RTM wastes. The first step of the development of the technology of biodestruction is searching of the

strains of microorganisms able to degrade RTM. According to literature data and the results of our previous investigations it has been established that microscopic fungi are able to colonize the surface and inner layers of rubber substrates and destruct the main components of RTM – plasticizers and caoutchouc [1, 5, 13, 17]. We associated these processes with the action of enzymes of microscopic fungi, in particular – carboxylesterase that breaks up the ethereous group of plasticizers and complex of oxidative enzymes that are accountable for caoutchouc consuming. For this reason it is completely well to use microscopic fungi – the potential producers of above-mentioned enzymes as biodestructors of rubber wastes. The aim of present work is to detect microscopic fungi promising for developing of the biotechnology of destruction of the wastes of rubber technical materials (RTM).

Materials and methods

The objects of investigation were 80 strains t belonged to 31 species of 16 genera of micromycetes of phyla *Zygomycota* and *Ascomycota* that had been isolated earlier from biodeteriorated rubber technical materials (RTM) – fully-molded and pneumatic rubber tires and their main components (granulated rubber, rubber mix and plasticizers) [3]. As a control we have used five strains-producers of lipases and oxidative enzymes and destructors of different technical materials – *Penicillium sclerotiorum* J.F. H. Beyma F-1, *Rhizopus cohnii* Berl. & De Toni F-2, *R. microsporum* F-3, *P. chrysogenum* Thom F-16719 and *Trichoderma viride* Pers. F-16173. These cultures were gained from the collection of Department of Physiology and Taxonomy of D.K. Zabolotny Institute of Microbiology of NASU.

Studied strains of microscopic fungi were stored on agarized mash (4.5° sensu Baling) on 4 °C. For the support of enzymatic activity all the cultures were subcultivated once per year on CBM media that was similar by its composition to Czapek-Doks media, but it contained natural caoutchouc in concentration 5 g/l as a source of carbon [15]. This media after our modifications was used on the first stadium of the screening for detection the ability of investigated micromycetes to grow on rubber substrates [6]. Microscopic fungi that formed distinct colonies on CBM in diameter at least 5 mm on the 14-th day of our experiment we considered capable to grow on RTM.

The presence of carboxylesterase and oxidase activities was educed by the qualitative methods on the third day of fungal growth on 29±2 °C [12]. Signification of carboxylesterase guided on agarized medium contained Tween-80 (ester of oleinic acid and sorbitol) as substrate and had forthcoming composition (g/l): Tween-80 – 10,0; peptone – 10,0; NaCl – 5,0; $CaCl_2 \cdot H_2O$ – 0,1; agar – 20,0. Conclusion of carboxylesterase presence was given in case of appearance of zone of crystallinic precipitation of calcium oleate (halo) around fungal colonies. The ability to produce oxidases was estimated by the oxidase test. Fungal colonies on Czapek-Doks' medium were treated by 1 ml of 1 % fresh solution of tetramethyl-*p*-phenylendiamine that changed its colour from pink to dark-blue in case of presence of oxidative enzymes. Accounting of the results was carried out in 10 minutes after treatment of the colonies by tetramethyl-*p*-phenylendiamine.

Enzymatic index estimated as ratio of diameter of halo to diameter of fungal colony [8]:

$$EI = \frac{d_h}{d_к}, \text{ where:}$$

EI – enzymatic index; d_h – diameter of halo, mm; $d_к$ – diameter of fungal colony, mm.

The strain that has EI over 1,5 is considered a potential producer of enzymes [7, 8]. The investigated strains were conventionally divided by the values of their enzymatic indexes into four groups: enzymatic index was absent (0), low (1.0–1.24), moderate (1.25–1.49) and high (1.5 and over).

The characterization of compatibility of the selected cultures was estimated by the method of agaric blocks [20]. As a criteria of compatibility we considered absence of suppression of one culture by another. Fungicidal and fungistatic cooperation, hyperparasitism (growth of mycelia of one culture on the surface of another) and contest for nutrient solution were considered as criteria of suppression. All the experiments were carried out in three-time replication. The statistical processing of gained results was carried out by computer program Microsoft Excel 2007.

The hazard of selected fungi for human health was evaluated by the data of literature [18, 19].

Results and their discussion

It has been shown that 77 strains from 85 (74 were distinguished from RTM and 3 control) formed on CBM clearly expressed colonies in diameter from 5 to 30 mm (Fig. 1), that testifies to their potential ability to colonize rubber substrates and cause their biodamage.

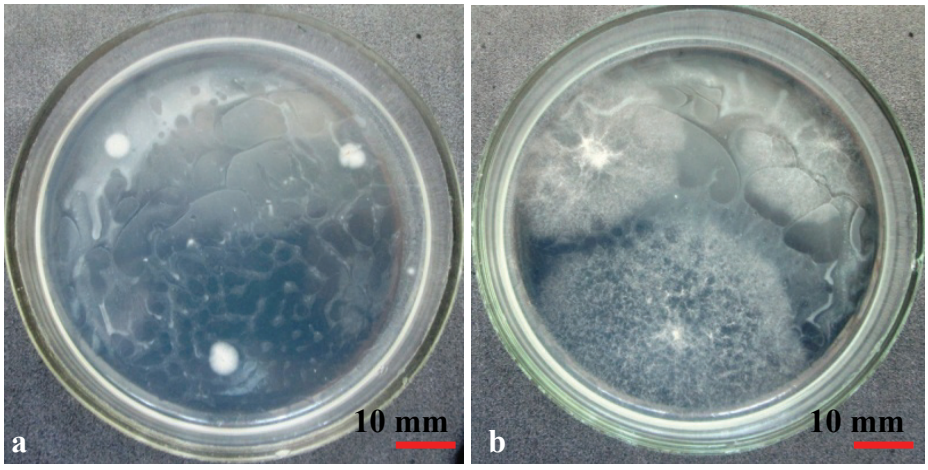

Fig. 1. Growth of microscopic fungi on the CBM (14-th day):
a) *Penicillium sclerotiorum* F-1; b) *Rhizopus cohnii* F-2

10 mm

It has been established the ability to form extracellular carboxylesterases in 74 % of the investigated cultures and complex of oxidative enzymes – in 91 % of strains (Fig. 2).

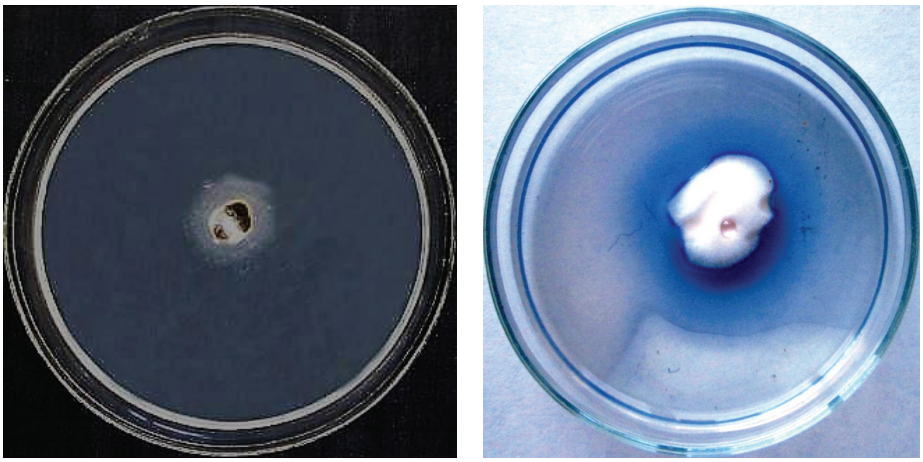

Fig. 2. Revelation of extracellular enzymes of the studied fungi (3-th day): a) carboxylesterase; b) complex of oxidative enzymes

Studied micromycetes were divided into three groups:

- group 1 (EI ≥ 1,5 by carboxylesterase);
- group 2 (EI ≥ 1,5 by complex of oxidative enzymes);
- group 3 (EI < 1,5 by carboxylesterase and complex of oxidative enzymes).

Five strains – *Aspergillus fumigatus* Fresen. F-41484, *Mucor racemosus* Bull. F-41411, *M. racemosus* F-41412, *Rhizopus cohnii* F-2 and *R. cohnii* F-3 obtained the enzymatic indexes by carboxylesterase in limits 1.67±0.1 – 2.42±0.03 were belonged to the first group; five strains – *Alternaria alternata* (Fr.) Keissl. F-41431, *A. flavus* Link F-41432, *A. sydowii* (Bainier & Sartory) Thom & Church F-41426, *A. ustus* F-41437 (Bainier) Thom & Church and *A. versicolor* (Vuill.) Tirab. F-41469 obtained the enzymatic indexes by complex of oxidative enzymes in limits 1.52±0.07 – 2.7±0.04 were belonged to the second group (Fig. 3). Other 75 investigated micromycetes were belonged to the third group.

It has been established that the strains which have high EI by carboxylesterase had low values of EI by complex of oxidative enzymes and vice versa. We suspect that it may be due to different functions of investigated micromycetes in consortium, which was formed during storage RTM *in vivo*.

We confirmed that the strains of the first group prioritily inhabited rubber substrate and caused destruction of its most affordable components – plasticizers, which decomposition products (organic acids and alcohols) could be a source of power for micromycetes of the second group, which in turn oxidized inaccessible component of RTM – caoutchouc to form oligoaldehydes and oligoketones [2, 16, 17]. We assume that the formation of a number of low molecular weight, readily

available compounds on the surface of affected RTM during their destruction by micromycetes of the first and second groups promotes colonization of RTM by large third group of micromycetes, representatives of which are characterized by low values of EI by carboxylesterase and complex of oxidases activity and the lack of growth on the CBM.

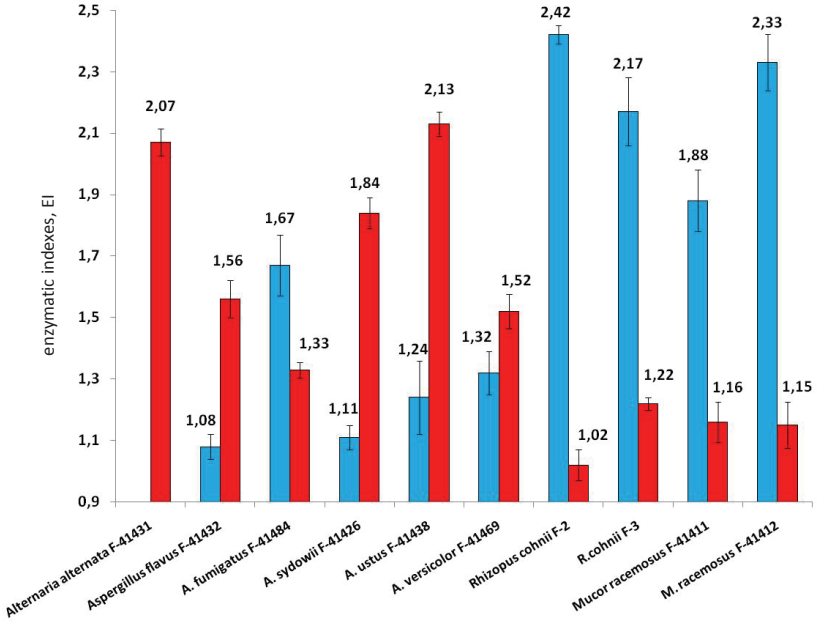

Fig. 3. Micromycetes that have enzymatic indexes over 1.5

In our opinion, for biodegradation technology of RTM it is optimal to use not only single cultures of micromycetes but their complexes. The cultures of microscopic fungi considered compatible in case of absence of signs of reciprocal inhibition (Fig. 4).

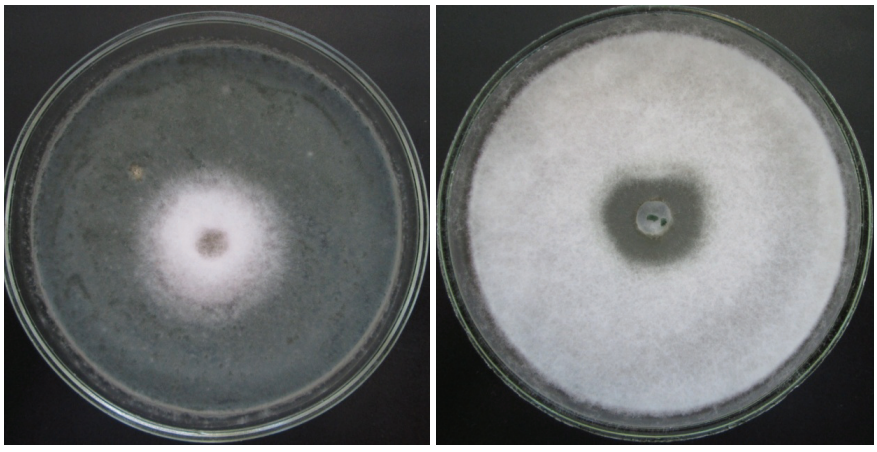

Fig. 4. Interaction of studied microscopic fungi:
a) Cultures are compatible; b) Cultures show reciprocal inhibition

We consider that this approach will not only ensure the simultaneous formation of both classes of enzymes, but also increase the level of their activity.

Micromycetes that were Compatible:

1). *A. alternata* F-41431, *A. sydowii* F-41426, *A. ustus* F-41437 and *A. versicolor* F-41469;

2). *M. racemosus* F-41411, *M. racemosus* F-41412, *R. cohnii* F-2 and *R. cohnii* F-3;

3). *A. alternata* F-41431, *R. cohnii* F-2 and *R. cohnii* F-3;

4). *A. alternata* F-41431, *A. flavus* F-41432;

5). *A. flavus* F-41432, *A. fumigatus* F-41484;

6). *A. flavus* F-41432, *Rhizopus cohnii* F-2 and *R. cohnii* F-3;

7). *A. fumigatus* F-41484, *A. ustus* F-41437;

8). *A. fumigatus* F-41484, *R. cohnii* F-2 and *R. cohnii* F-3;

The most selected strains, and particularly *A. flavus* F- 41432 and *A. fumigatus* F-41484, are referred to opportunistic micromycetes because according to the literature, they can cause a variety of human diseases and produce mycotoxins (Table).

Table

Characteristics of selected micromycetes by negative impact on human health [18, 19]

№	Fungal specie	Group of patogenicity	Diseases	Mycotoxins
1.	*A. alternata*	IV	Sinusitis, keratomycosis, onychomycosis	Alternariols
2.	*A. flavus*	III	Pulmonitis, infarcts and necrosis	Aflatoxins
3.	*A. fumigatus*	III	Infections of viscera	
4.	*A. sydowii*	IV	Keratomycosis, onychomycosis sinusitis, otitis	
5.	*A. ustus*	IV	Middle otitis, infections at stings	Sterigmatocystine
6.	*A. versicolor*	IV	Onychomycosis	
7.	*M. racemosus*	IV	Zygomycosis	No data
8.	*R. cohnii*	IV		

Thus, from the 85 investigated strains, 10 cultures that grew on CBM (*Alternaria alternata* F-41431, *Aspergillus flavus* F-41432, *A. fumigatus* F-41484, *A. sydowii* F-41426, *A. ustus* F-41437, *A. versicolor* F-41469, *Mucor racemosus* F-41411, *M. racemosus* F-41412, *Rhizopus cohnii* F-2 and *R. cohnii* F-3) were considered as the potential biodestructors of RTM. They are characterized by high enzymatic indexes by carboxylesterases and complex of oxidative enzymes and able to co-cultivation. The final conclusion on the suitability of use of the above-mentioned

strains for creation of biodegradation technology of RTM can be given only after the additory studies of their nonpathogenicity and nontoxity.

Author express sincerely thanks to Nakonechna L.T., the senior engineer of Department of Physiology and Taxonomy of D.K. Zabolotny Institute of Microbiology of NASU, for kindly given control strains of microscopic fungi.

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МІКРОМІЦЕТИ – ПОТЕНЦІЙНІ БІОДЕСТРУКТОРИ ВІДХОДІВ ГУМОТЕХНІЧНИХ МАТЕРІАЛІВ В УМОВАХ УКРАЇНИ

Реферат

Метою *роботи був попередній скринінг мікроскопічних грибів, перспективних для створення біотехнології деструкції відходів гумотехнічних матеріалів (ГТМ).* ***Методи.*** *Культури мікроміцетів, виділені з ГТМ, зберігали на агаризованому суслі (4,5° за Балінгом) при 4 °С. Здатність досліджених мікроміцетів до росту на ГТМ оцінювали на 14-у добу на модельному каучуквмісному середовищі (КВС), що імітувало гумовий субстрат. Наявність карбоксилестеразної та оксидазної активності виявляли якісними методами, розраховували ензиматичний індекс. Сумісність відібраних культур мікроміцетів оцінювали за методом агарових блоків.* ***Результати.*** *Показано, що з 85 досліджених штамів мікроскопічних грибів 77 здатні утворювати чітко виражені колонії на середовищі КВС діаметром від 5 до 30 мм. З них лише 5 штамів – Aspergillus fumigatus F-41484, Mucor racemosus F-41411, M. racemosus F-41412, Rhizopus cohnii F-2 та R. cohnii F-3 мали високі ензиматичні індекси за карбоксилестеразною активністю та 5 штамів – Alternaria alternata F-41431, Aspergillus flavus F-41432, A. sydowii F-41426, A. ustus F-41437 і A. versicolor F-41469 – за оксидазною. Встановлено можливість використання штамів з високими ензиматичними індексами у восьми змішаних культурах.* ***Висновок.*** *Для створення технології біодеструкції відходів ГТМ відібрано 10 штамів мікроміцетів, що характеризуються високими ензиматичними індексами за карбоксилестеразною або оксидазною активністю та здатні до сумісного культивування.*

Ключові слова: гумотехнічні матеріали, мікроміцети, біодеструкція, ферменти, ензиматичний індекс, змішана культура.

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МИКРОМИЦЕТЫ – ПОТЕНЦИАЛЬНЫЕ БИОДЕСТРУКТОРЫ ОТХОДОВ РЕЗИНОТЕХНИЧЕСКИХ МАТЕРИАЛОВ В УСЛОВИЯХ УКРАИНЫ

Реферат

Целью *работы был предварительный скрининг микроскопических грибов, перспективных для создания биотехнологии деструкции отходов резинотехнических материалов (РТМ).* ***Методы.*** *Культуры микромицетов, выделенные из РТМ, хранили на агаризованном сусле (4,5° по Баллингу) при 4 °С. Способность исследованных микромицетов расти на РТМ оценивали на 14-е сутки на модельной каучуксодержащей среде (КСС), имитирующей резиновый субстрат. Наличие карбоксилэстеразной и оксидазной активности выявляли качественными методами, рассчитывали энзиматический индекс. Совместимость отобранных культур микромицетов оценивали методом агаровых блоков.* ***Результаты.*** *Показано, что из 85 исследованных штаммов микроскопических грибов 77 способны образовывать четко выраженные колонии на среде КСС диаметром от 5 до 30 мм. Из них лишь 5 штаммов – Aspergillus fumigatus F-41484, Mucor racemosus F-41411, M. racemosus F-41412, Rhizopus cohnii F-2 та R. cohnii F-3 имели высокие энзиматические индексы по карбоксилэстеразной активности и 5 штаммов – Alternaria alternata F-41431, Aspergillus flavus F-41432, A. sydowii F-41426, A. ustus F-41437 і A. versicolor F-41469 – по оксидазной. Установлена возможность использования штаммов с высокими энзиматическими индексами в восьми смешанных культурах.* ***Вывод.*** *Для создания технологии биодеструкции отходов РТМ отобрано 10 штаммов микромицетов, характеризующихся высокими энзиматическими индексами по карбоксилэстеразной или оксидазной активности и способны к совместному культивированию.*

Ключевые слова: резинотехнические материалы, микромицеты, биодеструкция, ферменты, энзиматический индекс, смешанная культура.

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Стаття надійшла до редакції 10.07.2015 р.