SZENT ISTVÁN UNIVERSITY

PH.D. THESIS

MYCOLOGICAL ANALYSIS OF COMPOSTS OF BIOMASS ORIGIN

FLÓRA SEBŐK

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Ph.D. School

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1. BACKGROUND AND AIMES

Minimalization of energy demand and replacement of soil organic matter are two main considerations for magaging the wastes of industrial, agricultural and communal origin. As the mixed wastes contain organic matterial of actual or processed biomass origin, composting technologies having been spreding all over the word. Their compost end-product, due to its high humus content, is excellently suitable for soil amendment in agricultural and horticultural practice. A lot of side-products in food processing, textile and pharmaceutical industries, as well as the fresh manure from animal farms and miscellaneous green wastes also are considered of biomass origin. All of them can be optimally managed by this was. Composting is declared in the Thematic Strategy for Soil Protection (COM(2006)231) as an advisable mean of waste treatment. As a result of this decision, in 2011, 15 % of the total solid municipal waste produced in the European Union was composted, accounting for about 200 million tonnes (Eurostat 2013).

The process of decomposing and transforming organic matter which perform during the compostation are related to microbial activities. Therfore the knowledge of presence and role of different microorganism groups is necessary for the success of composting technologies.

In cosequence of the exotherm character of the process, temperature of the composting mass markedly increases, for some days it reaches 65-70 ºC. So degrading processes are due to the activities of thermophilic microorganisms, by bacteria and fungi in similar rate. In that temperature latitude, where higienization also performs, only some bacteria, endospore-forming and actynobacteria can grow. Maybe because of this situation, the scientific results dealin with compostation refer in majority to the activity of bacteria. The big difference between the fungal and bacterial metabolisms

and ecological features accounts an intensive research of the fungal colonization and the diversity of fungal communities in composting process.

Concerning the composition and taxonomical diversity of thermophilic fungal communities in composting processes, the following aims are assigned during my PhD activity:

- Investigation of quantitative relations and taxonomic diversity of thermophilic fungal mycobiota in composted municipal waste from an urban and a village environment, i. e. what the kinetics of mesophilic and thermophilic mycota like, and which thermophilic species are dominant in composting processes.
- To reveal the thermophilic fungal propagula in the air of composting plants. Besides to reveal the sesquiterpene emission from these fungi. Contribution to the knowledge of the environmental effect of composting technologies.
- To answer the question of the distribution of thermophilic fungi in natural ecosystems I intended to study their presence in the soil and the litter of forest reservation.
- To contribute to the knowledge of the tolerance of thermophilic fungi of the effect fo toxic heavy metals I intended to investigate their sensitivity to Cu^{2+}, Cd^{2+}, Ni^{2+} and Pb^{2+} ions.
- Furthermore, among the objectives, I planned to compile a collection from the strains obtained during my work. This local culture collection can serve as a source of strains for scientific and technological researcees in the future.

2. MATERIALS AND METHODS

2.1. Sampling

Samples were taken at a composting plant where the municipal mixed solid waste from a town was treated, and at an other composting plant where mixed solid waste from a village was treated. Compost and air were sampled at both places. The waste from the town consisted of cellulose-containing haushold materials, in majority, while the waste from the village consisted, beside the household waste, lignocellulose-containing greenwaste and gardening waste as well. As a result of a final riddking compost became a mass of crumbling consistance, with a high humus content.

The samples of 5-5 kg compost were taken according to the standard method and were stored for (less than 24 hours) at 4 ºC until the culturing process.

Air samples were collected with a three-stage Andersen air sampler at 1.5 m a.g.l. Results were expressed as colony-forming units per cubic meter of air (CFU/m^3) calculated using the positive hole correction method. The sampler was loaded with 90 mm Petri-dishes containing Martin's medium (Martin 1950) and potato-dextrose agar (PDA). Repetations were done on three calm and rain-free days of three consequtive weeks. Five plates were exposed successively per sampling day. The operating time of the sampler was based on calibrating test measurements at a flow rate of 28.3 liters/min performed before each sampling.

Soil samples were taken at three sites in different forest types in the Forest Reservation of Vár-hegy in Hungary. The samples of forest litter were collected at the asme places.

2.2. Microbial methods

2.2.1. Cultivation method and isolation

The quantity of thermophilic fungi in the compost and soil samples was determined with a serial delution combined with microbiological cultivation. Five-five g of compost and soil samples were mixed in 45 ml steril destilled water, the mixtures were intensively vortexed and finally, in order to dissolve microbial aggregates, they were sonicated for 5 minutes. Aliquots of 100 µl of each step of the dilluted suspension were spread on PDA supplemented with streptomycin of 10 mg/l.

2.2.2. Maintance of the strains

The spores or any other propagula of the strains harvested from colony plates were suspended in 20 % glycerol solution and homogenized in a tissue homogenizer tube of Potter- Elvehjem type. Suspensions were stored at -80 ºC.

2.2.3. Taxonomic identification

Taxonomic identification of the strains was carried out on the base of their morphological characterictics and the analysis of the nucleotide sequence of their internal transcribed spacer (ITS) region. As for the morphological characteristics, ability of forming fruiting bodies, as a result of sexual process, the way of conidium ontogeny, and also the conidium forming organs and structure were examined for identification. In the coures of the analysis of the ITS-region sequence, DNA was extracted form the mycelia using the MasterPure™ Yeast DNA Purification (Epicentre, USA) according to the instructions of the manufacturer. The ITS region was amplified by PCR using the primer pairs ITS1 and ITS4 according to White et al (1990). PCR product were purified with DNA, RNA and protein purification Kit

(Macherey-Nagel). The purified PCR products were used in sequencing reactions using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Sequencing was performed on an ABI 3130 genetic analyzer. Sequences were compared with those of all known fungal species available from the NCBI GenBank Sequence Database (http://www.ncbi.nlm.nih.gov/BLAST/) used the current name of the fungi based on the online CABI databases, Index Fungorum (http://www.indexfungorum.org/names/names.asp).

2.3. Calculation of diversity indices

The diversity of propagule-communities was measured and expressed with the Shannon's index (H) and the Simpson's index (D) as well.

Shannon diversity which quantifies the uncertainty (entropy) associated with this prediction was calculated with the following formula:

$$H = -\sum_{i=1}^{S} p_i \, ln \, p_i ,$$

where S is the number of the species, p_i is the the proportion of individuals belonging to the i^{th} species in the dataset of interest.

Simpson's index which quantifies the probability of interspecific encountering was calculated with the following formula:

$$D = 1 - \frac{\sum_{i=1}^{S} n_i (n_i - 1)}{N(N-1)},$$

where S is the number of the species, n_i is the abundance of the ith species and N is the number of individuals.

2.4. Examination od sesquiterpene emission

The examination of sesquiterpene emission was carried out at the departure of Eart and Environmental Sciences, University of Pannonia, Veszprém,

Hungary with the analysis of the extracted volatiles by gas-chromatograph. The samples were taken in a sterile flow-through apparatus (Fig. 1) designed in the laboratory based on the principle of the experimental setup developed by Nilson et al. (1996). The volatila metabolites emitted by solid phase microextraction (SPME) based on a multiphase equilabrum process. The analysis of the extracted volatiles was performed with an Agilent G890 gas chromatograph coupled to a quadropole mass spectrometer 5973 MSD. For quantitative analysis of the headspace extracts the peak areas of the sesquiterpenes in the ion chromatograms were used to calculate the amount of the extracts adsorbed on the filter during the sampling period.

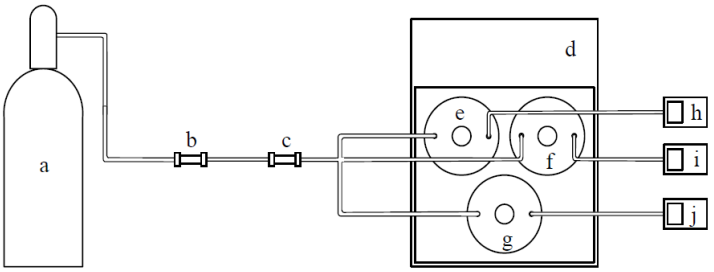

Fig. 1. Scheme of the developed flow-through apparatus: (a) compressed air, (b) activated charcoal filter, (c) HEPA filter, (d) thermostat, (e, f, g) sampling vials, and (h, i, j) portable carbon dioxide monitor

2.5. Methods of the investigation of the sensitivity to heavy metals

As all thermophilic fingi produce mycelial on solid media, I could choose the method of poisoned agar growth for studying their sensitivity to heavy metals. The compounds $Cu(NO_3)_2 \cdot 2{,}5H_2O$, $Cd(NO_3)_2 \cdot 4H_2O$), $Ni(NO_3)_2 \cdot 6H_2O$ and $Pb(NO_3)_2$ were used in the experiments. All the four strains, *Rasamsonia emersonii* TK07, *Thermomyces lanuginosus* TK11, *Thermothelomyces*

thermophila TK11 and *Mycothermus thermophilus* TK09 assigned for the test developed well formed and pigmented colonies on Sabouraud-glucose agar. I measured the diameter of the colonies at a milimeter scale two times a day until they reached the plate margin (92 mm), and calculated the effective concentrations (EC_{50}) from the actual colony territories.

2.6. Statistical methods

Average values and standard deviation were calculated from the results obtained form the 3 replications of every microbiological culturing processes. The one-way analysis of variance was performed in Microsoft Excel 2007 to determine whether there are any statistically significant differences between the means of the different treatments.

3. RESULTS AND DISCUSSION

3.1. Diversity of thermophilic mycota

The quantity and taxonomic diversity of the thermophilic mycota in the composting solid organic municipal wastes from a town and a village, in the air composting environment, and in forest ecosystems showed ecological regularities.

3.1.1. Diversity during composting process of urban solid waste

The mixed solid urban waste appropriately prepared for composting process contained 5.6×10^5 CFU/g mesophilic fungal propagula and only the amount 2.8×10^2 CFU/g of thermophilic ones. Up to the 12^{th} day of composting process, the mesophilic mycota decreased to 8.5×10^2 CFU/g but the thermophilic one increased to the level of magnitude 10^6, and then also at the 16^{th} day in remained at the level of 2.0×10^6 CFU/g (Fig. 2). This result suggested that the latter group had the most important role in composting process. Regarding that the thermophilic fungi belong to different taxonomic groups, I intended to follow also the species composition and taxonomic diversity of the thermophilic mycobiota during the composting process.

From the mixed solid urban waste, I obtained 128 isolates which belonged to 6 species. Among them, the *Thermomyces lanuginosus* and the *Rasamsonia emersonii* proved to be dominant, and even the least frequent species, *Chaetomium thermophilum* showed a proportion 8.6 %. Half of the species obtained from the wastes occurred above 20 %, and other species also appeared between 8.6 and 11.7 %, so the Shannon's index (H=1.70) calculated from their occurrence seemed to be relatively low, as well as the

Simpson's diversity index expressing the probability of the sameness of two random isolates scaled 0.81 (Table 1).

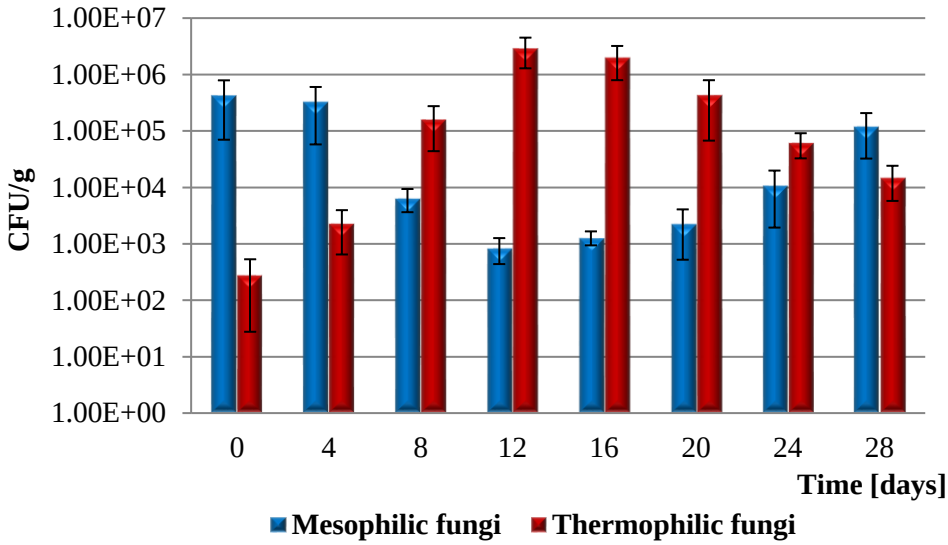

Fig. 2. Number of mesophilic and thermophilic fungal propagula during the composting process of municipal waste from an urban environment

At the 8th day of composting process, in the logarithmic phase (10^5 CFU/g), the 139 obtained isolates distributed among not more than 5 species. The 3 species, dominant at the beginning, showed a fast growth also in the first stage of the process, furthermore, their common starting proportion (70.3 %) went on increasing (78.5 %) in that time. While at this period the thermophilic mycota acheaved a three-magnitude increase by the growth of that 3 species, the diversity form the starting state, in convenience of ecological rules, surprisingly went narrow (H = 1.52, D = 0.77) (Table 1).

On the 16th day, at the end of their 5-7 days long maximum-period (2.0×10^6 CFU/g), the 146 random isolates distributed among 9 species. *Thermomyces lanuginosus*, with the broad spectrum of its catabolic activity, proved to be the most dominant species (23.3 %), in spite of its frequency

had slightly decreased. The quantity and frequency of the other 3 species (*Rhizomucor pusillus*, *Rasamsonia emersonii*, *Thermomyces thermophilus*) was similarly formed. The latter 2 species have got a property of strong lignocellulose-degradation. The diversity of the thermophilic mycobiota (H = 2.10, D = 0.88) composed by 9 species essentially exceeded the diversity of the starting phase.

The last sampling of the urban solid waste composting was doneat the end of the intensive phase, on the 28th day. Basing on the identification of 149 random isolates the reduced thermophilic mycota (4.4×10^4 CFU/g) distributed among 11 species, with the outstanding abundance of *Thermomyces lanuginosus* (20.8 %) and *Rasamsonia emersonii* (19.5 %). Regarding the big proportion of these 2 species in each phase of composting and regarding their well known broad catabolic activity (Rosenberg 1978, McHale and Coughlan 1981, Puchart et al. 1999, Singh et al. 2003, Waters et al. 2010) their important role in composting can be supposed. Further 4 of the 6 species being present with frequencies of 5-12 %, the *Malbranchea cinnamomea*, the *Thermomyces thermophila*, the *Rasamsonia composticola* and the *Mycothermus thermophilus* appeared, i. e. become culturable only in the metabolic and in the maturing phase of composting. Therefore their role should be concluded as an essential factor of compost maturation.

3.1.2. Diversity during composting process of village solid waste

The kinetics of mesophilic and thermophilic mycota in composting village soil wastes, in spite of the technological differences, showed some similarities to those of composting urban wastes. In particular, very similar changes performed in mesophilic mycota after the magnitude-larger starting value. Nevertheless, some smaller divergences were found between the ecological processes of the urban and the village waste composting. I could

demonstrate the presence of 7 thermophilic fungal species with the dominance of *Thermomyces lanuginosus* in the starting mixture, *Acremonium alabamense* also appeared in this mixture. Thge thermophilic fungal community found in the village waste mixture probably originates directly from the colonized waste surfaces, therfore the other 4 species of lower frequency, namely the *Acremonium thermophilum* (6.6 %), the *Chaetomium thermophilum* (8.1 %), the *Rhizomucor pusillus* (7.3 %) and *Thermoascus aurantiacus* (9.5 %) can be considered species of ubiquitous occurrence. The diversity of the community originated from the village waste also containing mixed green cuttings was broader (H = 1.79, D = 0.81) than that of the urban mixed solid waste (Table 1).

Table 1. Diversity of thermophilic fungal community during the composting process of municipal waste from an urban environment

	Time of composting process			
	Day 0	**Day 8**	**Day 16**	**Day 28**
Shannon diversity index (H)	1.70	1.52	2.10	2.21
Simpson diversity index (D)	0.81	0.77	0.88	0.91
Number of species	6	5	9	11

NEW SCIENTIFIC RESULT (based on results of 3.1.1. and 3.1.2. chapters): Thesis I. I proved, by culturing the samples taken from the composting mixtures at the consecutive phases, that the dynamics of fungal communities at the urban and the village plants showed big similarities. The thermophilic mycobiota in composting municipal solid waste mixtures developed in concinience of ecological rules. Its maximum values reached at the end of the thermophilic phase of composting process. The taxonomic diversity of the thermophilic mycobiota with 11-12 species grew even during the maturation phase.

3.1.3. Diversity in the air spora communities

I found the thermophilic funhal concentration 320 CFU/m3 in January, 510 in April, 870 in July and 980 in October in the environment of the piles of stored urban waste compost. Although increases were experienced from January to October , a marked change could be measured only in the period between April and July. In the air surrounding the stored village waste compost, the following air propagule concentrations were found: 292 CFU/m3 in January, 960 in April, 3150 in July and 2700 in October. At this site the quantity measured in spring was really higher then that in winter, and the maximum value was found in summer (Fig. 3).

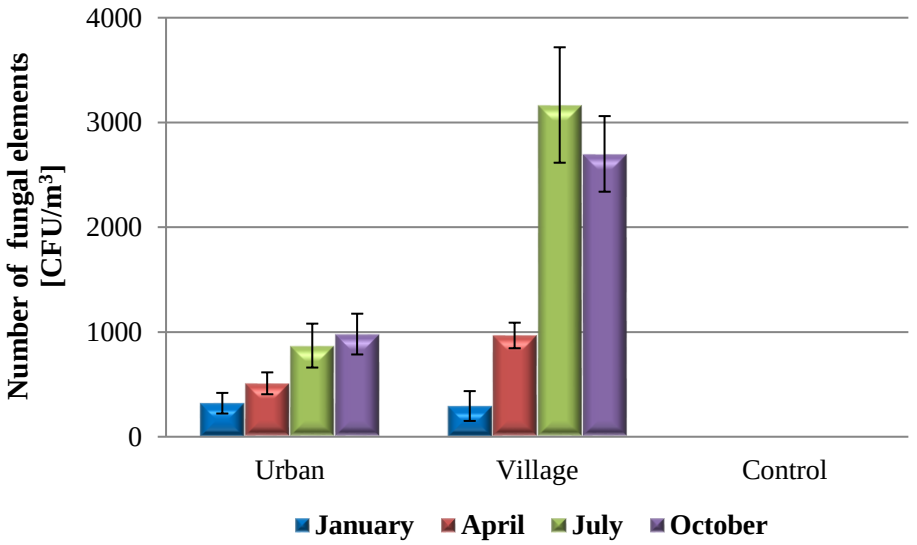

Fig. 3. Aeral concentration of thermophilic fungal propagula in the surrounding of compost plies in composting facilities treating urban and village waste.

Table 2. Relative frequency of thermophilic fungal species in the compost and the in air samples in surroundings of compost (October 2011)

Species	Relative frequency (%)			
	Urban composting facility		Village composting facility	
	Compost	Air	Compost	Air
Acremonium alabamense	2.1	9.9	2.7	6.5
Chaetomium thermophilum	8.9	6.2	6.5	9.8
Malbranchea cinnamomea	6.8	2.5	5.9	0.0
Melanocarpus albomyces	0.0	0.0	2.7	0.0
Mycothermus thermophilus	7.9	0.0	8.6	0.0
Rasamsonia emersonii	14.7	43.2	10.3	43.5
Rhizomucor pusillus	7.9	8.6	6.5	9.8
Thermoascus aurantiacus	5.3	12.3	7.6	13.0
Thermomyces lanuginosus	26.8	8.6	26.5	10.9
Thermomyces thermophilus	11.1	3.7	10.3	3.3
Thermothelomyces thermophila	8.4	4.9	12.4	3.3

NEW SCIENTIFIC RESULT (based on results of 3.1.3. chapter): Thesis II. I proved, by Andersen's air sampling method and culturing the samples, that the concentraition of thermophilic air propagula in the environment of stored compost reached 300 CFU/m^3 even in the coldest month January than in increased to tentimes higher level during the year. The compost origin of air thermophilic fungal communities is proven by their decrease with horisontal distance. As well as I revealed some differene between the diversitiy of compost thermophilic mycobiota and that of the thermopilic mycobiota of air propagula composition.

3.2. Strain collection of thermophilic fungi

Total of 87 thermophilic fungal strains is maintained in our culture collection. Strains were isolated form composts of different origin, air samples in surroundings of composts, soil and litter of of forestal ecosystems. Based on their fenotipic charasceritics and the results of analysis of their ITS sequence, the strains belong to 12 species. The strain collection consists of 7 *Acremonium alabamense*, 8 *Chaetomium thermophilum*, 5 *Malbranchea cinnamomea*, 3 *Melanocarpus albomyces*, 6 *Mycothermus thermophilus*, 2 *Rasamsonia composticola*, 10 *Rasamsonia emersonii*, 11 *Rhizomucor pusillus*, 8 *Thermoascus aurantiacus*, 12 *Thermomyces lanuginosus*, 7 *Thermomyces thermophilus* and 8 *Thermothelomyces thermophila* strains.

3.3. Sesquiterpene emission of composts and thermophilic fungi

The 5 days old culture of the tested *Thermothelomyces thermophila* Tb085 strain emitted 8 different sesquiterpene in a quantity of 943.4 pg/h (Fig 5). All of the other testes strains – except a *Thermomyces lanuginosus* – also emitted sesquiterpenes in a well detectable amount. Based on the retention time, it could be state that some fungi were able to synthetise the same sesquiterpenes as others

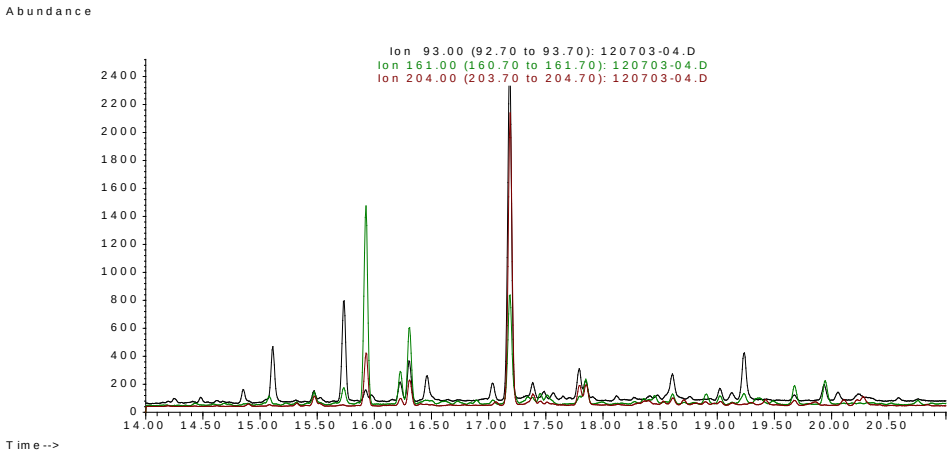

Fig 5. Ion-chromatogram proving the sesquterpene-production of *Thermothelomyces thermophila* strain Tb085. (Pannon University, Air Chemistry Research Group, 2012)

Compost processed from solid urban waste emitted 16 different sesqiterpenes in the quantity of 378.1 pg/h, while compost processed from solid village waste emitted only 14 different sesqiterpenes in the quantity of 55.8 pg/h.

In some cases, the retention time of sesquiterpenes emitted form composts were the same as that of emitted by the thermophilic fungi. Consequently, partly thermophilic fungi are responsible for the sesquiterpene emission of composts.

NEW SCIENTIFIC RESULT (based on results of 3.3. chapter): Thesis IV. The sesquiterpen emission of composts processed from solid municipal waste and that of 7 compost-inhabiting thermophilic fungi was proved. Some sesquiterpes can be synthetised by different thermophilic fungi. Partly thermophilic fungi are responsible for the sesquiterpene emission of composts.

3.4. Tolerance of thermophilic fungi to heavy metals

Tolerance of the 4 tested thermophilic fungal strains to copper(II), a cadmium(II), a nickel(II), and lead(II) ions differed from each other. The calculated EC_{50} value of copper, lead, nickel and cadmium against *Mycothermus thermophilus* strain TK09 were 34.4 ppm, 11.6 ppm, 10.4 ppm and 1.7 ppm, respectively. The order of tolerance in the case of *Rasamsonia emersonii* starin TK07 and *Thermothelomyces thermophila* strain TK04 was the same as in the case of *Mycothermus thermophilus* strain TK09. However, *Thermomyces lanuginosus strain* TK11 could tolerate nickel better than lead (Table 5).

Table 5. EC_{50} values of heavy metals against thermophilic fungal strains

	EC_{50} (ppm)			
Strains	**Copper**	**Cadmium**	**Nickel**	**Lead**
Mycothermus thermophilum TK09	34,4	1,7	10,4	11,6
Rasamsonia emersonii TK07	105,2	4,7	15,3	20,2
Thermomyces lanuginosus TK11	70,4	1,8	30,4	16,3
Thermothelomyces thermophila TK04	90,6	5,5	17,3	26,5

NEW SCIENTIFIC RESULT (based on results of 3.4. chapter): Thesis V. Among the tested strains *Rasamsonia emersonii* TK 07 and *Thermothelomyces thermophila* TK04 proved to be the most tolerant ones, while *Mycothermus thermophilus* TK09 the least tolerant one. Based on the sensitivity of *Mycothermus thermophilus* TK09, it seems to be suitable to detect toxicity of heavy metals.

5. CONCLUSIONS AND SUGGESTIONS

Numerous scientific and practical conclusions can be drawn from the results of the mycological analysis of composts of biomass origin, mainly solid urban and village wastes.

The low but well detectable quantity of thermophilic fungal contant of urban and village solid wastes suggest that some members of the group have ubiquiter occurrence. Organic contant of municipal wastes are favourable nutrients for a dozen of thermophilic fungi based on their high presence in the thermophilic phase. Since the majority of these organic materials are biopolimers of biomass origin, the species must have borad spectral and high activity lignocellulotic enzyme system. Based on the minimal quantity of thermophilic fungi in the initial mixture it can be supposed that certain species are present under the quantity of the detection treshold and can grow as the environmental conditions favoure them. The increasing diversity can be a consequence of the spontaneous inoculation of the new prism with the residual spores in the old one at the composting facilities. It can be deduced from the broad diversity and low quantity of thermophilic mycota in the maturing phase that the majority of the species has dormant propagula which can persist for a long time even in the matured compost. I suggest that inoculation of wastes containing biomass components with thermophilic fungi is not necessery for a succesful composting process, onyl the optimal condition for their growth is needed to provide.

Quantity and diversity of thermophilic fungi in the air samples in the surroundings of composts can be well determined, since these are mainly effected by two factors, namely emission and deposition. However, these two factors are influenced by several other factors.

The occurrence of thermophilic fungal community in soils in the quantity of 10^3 CFU/g proves that propagula, able to form colonies are actually present. The seasonal changes in the size of thermophilic mycota suggest that their mycelia can even grow in the upper layer of the soil which can be warmed by insolation. The occurrence of some thermophilic fungi in the soil of different forestrial ecosystems confirm the opinion which is widespread in the literature (Cooney és Emerson 1964, Johri et al. 1999, Salar és Aneja 2007) that thermophilic fungi are cosmopolitan and can occure in natural habitats.

In taxonomy mycologyist try to enforce the new „one fungus = one name" principle. In spite of the fact that some genera containing thermophilic fungi was revised and taxonomically arranged as the effect of changes in regulation, still there are species in the group of thermophilic fungi whose taxonomical status is not determined. Because of the ecological and technological importance of thermophilic fungi I think that studying the phenotypic and genotypic charatceristics of strains from the natural ecosystems. Moreover the ITS-sequence of every described thermophilic fungal species sholud be upload to the mycologial databases.

The ability of thermophilic fungi to produce sesquiterpenes confirm the hypothesis that the big taxonomical group of fungi produce sesquiterpene as a secondary matabolite (Kramer és Abraham 2012). Sesquiterpene emission from composts proved that solid colloids does not absorbe the total amount of the produced sesquiterpene molecules.

Tolerance of thermophilic fungi to heavy metal ions was studied with the method of artificially contaminated media and *Mycothermus thermophilus* was proved to be the most sensitive species. Since its EC_{50} values are under the „B" soil and water contaminanting treshold (6/2009. (IV. 14.) KvVM-EüM-FVM) this species could be used for the detection of heavy metal contamination in soils or in other environmental elements.

6. PUBLICATIONS

Scientific papers:

Sebők, F., Dobolyi, C., Bobvos, J., Szoboszlay, S., Kriszt, B., Magyar D. (2016): Thermophilic fungi in air samples in surroundings of compost piles of municipal, agricultural and horticultural origin. *Aerobiologia* 32:255–263. **IF: 1.452**

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Kósa-Kovács M., **Sebők F.**, Szoboszlay S., Kriszt B., Dobolyi Cs. (2012): Termofil gombaközösségek a Vár-hegy erdőrezervátum talajaiban és avarjában. *Tájökológiai Lapok* 10(1): 163-175.

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Communication published in congress publications (concerning ISBN, ISSN or other, authenticated publications)

Sebők, F., György Kérész, Csaba Dobolyi, Sándor Szoboszlay, Balázs Kriszt (2015): Sensitivity of members of thermophilic compost mycobiota to toxic compounds. *Acta Microbiologica et Immunologica Hungarica* 62 (Suppl.): 94-95.

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Horváth, E., **F. Sebők**, A. Hoffer, Cs. Dobolyi, S. Szoboszlay, B. Kriszt, A. Gelencsér (2011): Sesquiterpene emission of fungi based on pure culture experiments. *Acta Microbiologica et Immunologica Hungarica* 58 (Suppl.): 158.