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Studies of Keratinolytic and Keratinophilic Fungi in Sewage Sludge by Means of a Multi-Temperature Hair Baiting Method

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Abstract

Sewage sludge was examined for keratinolytic and keratinophilic fungi by means of a multi-temperature (23, 26, 29, 33, 37 and 45°C) hair baiting method. Results indicate that this method can be a useful tool for determination of the influence of temperature and other factors on fungal composition in sludge and evaluation of health risk posed by fungal pathogens. The use of the method facilitated recognition of fungal flora much more than a traditional room-temperature method. The method was adapted to quantitative purposes. The effect of Petri dish sludge weight on fungal composition was determined. A 10 g weight should be used in qualitative studies while larger weights are more suitable for determination of the influence of environmental factors on fungal growth and composition.

Keywords: keratinolytic and keratinophilic fungi, sewage sludge, multi-temperature hair-baiting method, environmental factors

Introduction

Due to high micro- and macro-element contents sewage sludge is often used for reclamation of agricultural, forest and devastated soils. However, sludges also contain many harmful chemicals, e.g., heavy metals and PAHs and pathogenic organisms. Therefore, each time sludge land use must be preceded with sanitary analyses and health risk evaluation. The occurrence and survival of bacteria, viruses, protozoa and helminths in sewage sludge have been relatively well recognized [1, 2]. Still little is known on pathogenic fungi in the sludge environment [1, 3, 4]. Due to high quantities of keratinous debris of human and animal origin (mostly hair), this environment should especially favor the growth of keratinolytic and keratinophilic fungi.

A modified Majchrowicz & Dominik's [5] definition of keratinolytic and keratinophilic fungi is used in the present paper. In this definition, keratinolytic fungi are able to attack and destroy keratin, whereas keratinophilic fungi accompany them utilizing only non-keratinous components of keratinous substrata or the products of keratin decomposition. Fungal keratinolytic activity on hair *in vitro* is expressed by superficial erosion and producing pocket-like structures or penetrating hair with radial hyphae [6, 7]. The examined fungi are mostly geophilic (soil) microorganisms, with resistance to actidione and pathogenic or potential pathogenic properties to animals, including humans [7, 8]. Korniłłowicz [9] and Czeczuga & Muszyńska [10] have recently studied keratinolytic and keratinophilic fungi in Polish superficial waters.

Vanbreuseghem's [11] hair baiting method has been mainly used for examination of keratinolytic and keratinophilic fungi in sewage sludge and other organic waste and waste-contaminated habitats [4]. In this method, the so-called room temperature is usually employed (incubation temperature usually does not exceed 30°C). Hav-

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Table 1. The quantitative and qualitative compositions of keratinolytic and keratinophilic fungi in sewage sludge at six temperatures.

Fungal species and indices	Number of strains and fungal growth indices at temperature:						Total	Frequency (%)
	23°C	26°C	29°C	33°C	37°C	45°C		
Keratinolytic fungi								
Chrysosporium anamorph *Aphanoascus clathratus Cano* & Guarro	0	0	3	2	14	0	19	14.0
Chrysosporium keratinophilum D.Frey ex Carmichael	0	0	3	1	1	0	5	3.7
Chrysosporium queenslandicum Apinis & Rees	0	0	0	0	2	0	2	1.5
Microsporum gypseum (Bodin) Guiart & Grigorakis	23	27	30	30	0	0	110	80.9
Sum of strains (SS)	23	27	36	33	17	0	136	-
Isolation frequency (IF; %)	76.7	90	100	100	56.7	0	70.5	-
Number of species (NS)	1	1	3	3	3	0	4	-
Keratinophilic fungi								
Aspergillus fumigatus Fresenius	0	0	1	5	25	30	61	17.8
Aspergillus versicolor (Vuill.) Tiraboschi	20	17	0	0	0	0	37	10.8
Fusarium oxysporum Schlecht.	11	10	3	12	0	0	36	10.5
Fusarium solani (Mart.) Saccardo	22	20	5	13	0	0	60	17.5
Paecilomyces lilacinus (Thom) Samson	5	9	18	0	0	0	32	9.3
Phialophora melinii (Nannf.) Conant	24	17	0	0	0	0	41	12.0
Plectosphaerella cucumerina (Lindf.) W.Gams	2	3	1	2	0	0	8	2.3
Pseudallescheria boydii (Shear) McGinnis *et al*.	1	2	2	11	19	0	35	10.2
Verticillium lecani (Zimm.) Viegas	9	13	11	0	0	0	33	9.6
Sum of strains (SS)	94	91	41	43	44	30	343	-
Isolation frequency (IF; %)	100	100	96.7	80	90	100	94.4	-
Number of species (NS)	8	8	7	5	2	1	9	-

ing considered results obtained under different climate conditions [12 and other papers], however, it is thought that more than one temperature should be used for better recognition of fungal flora in examined habitats. The goal of this study was to evaluate a multi-temperature hair baiting method for examination of keratinolytic and keratinophilic fungi in sewage sludge.

Two experiments were performed. The first experiment was to determine the influence of temperature and other factors on the qualitative and quantitative composition of keratinolytic and keratinophilic fungi in sludge. Subsequently, the second experiment was to demonstrate the effect of Petri dish sludge weight on fungal composition.

Material and Methods

Sewage sludge from the Bytom-Miechowice wastewater treatment plant was used for experimentation. It was the activated sludge after prolonged aeration (without primary settling tank) and C, N and P removal in the Biomix system, dewatered by centrifugation, and piled with plant remains for at least 12 months. 20 kg samples were taken from four places on the slopes and one place in the middle of the sludge pile and mixed together. The mixed sample was first cleaned from stones and plant remains, crumbled, then dried at open air, sieved through a 3 mm diam net and thoroughly mixed again. Physico-chemical characteristics of the sludge were presented elsewhere [13].

In the first experiment, sterilized redistilled water was added to dry sludge to obtain 40-50% of moisture. The moist sludge was distributed into sterilized Petri dishes. Forty grams of the sludge were put into each dish and the dishes were covered with 400 mg of defatted, cut into 1cm long pieces and autoclaved children's hair. The dishes were incubated for 4 months in the dark at 23, 26, 29, 33, 37 and 45°C. Thirty dishes (repetitions) were set up for each temperature. The moisture was kept at the same level during the whole incubation period. At 1-month intervals, macro- and microscopic observations of hair were performed. Single hair strands inhabited by fungi were inoculated on Sabouraud 1:10 medium supplemented with mineral

salts, chloramphenicol (100 mg/L) and actidione (500-mg/L) [14]. The medium is often called TK. Inoculations were performed in 2 repetitions and the inoculated dishes were incubated at 23 and 37°C. The appearance of a given species on hair confirmed by its growth on the medium meant the presence of this species in a given Petri dish (one strain). Pure fungal cultures were identified to species or genera based on macro- and micromorphological characteristics and using selected taxonomic monographs [15-20]. The test for keratinolytic activity was that of Ulfig *et al.* [21]. The fungal growth indices were as follows: SS – sum of strains; IF – isolation frequency (number of dishes with fungal growth *100/total number of dishes set up); and NS – number of species. Results also demonstrated the effect of an increasing sample (4-Petri dish/temperature sets) quantity on fungal growth indices.

In the second experiment, Petri dishes were incubated at 23, 29, 33 and 37°C. The moist sludge weights were 10, 20, 30 and 40 g per dish. Ten dishes (repetitions) were set up for each temperature and weight.

Results

The compositions of keratinolytic and keratinophilic fungi at six temperatures are presented in Table 1. In contrast to keratinophilic fungi, keratinolytic species were rather poorly represented in the sludge. Altogether, 136 keratinolytic strains from four species were isolated from the sludge. Total isolation frequency was 70.5%. *Microsporum gypseum* was the predominating species. The fungus occurred frequently at 23-33°C; being observed in all dishes at 29 and 33°C. Its best growth was noticed at 29°C. Subsequently, *Chrysosporium* anamorph *Aphanoascus clathratus* occurred with the highest frequency at 37°C. The other two species, i.e., *Chrysosporium keratinophilum* and *Chrysosporium queenslandicum* were seldom isolated from the sludge. The best growth of keratinolytic species was observed at 29 and 33°C. No keratinolytic fungi occurred at 45°C.

Altogether, 343 keratinophilic strains from nine species were isolated from the sludge. The total isolation frequency was 94.4%. *Fusarium solani*, *Phialophora melinii*, *Aspergillus versicolor* and *Fusarium oxysporum* predominated at 23 and 26°C. However, the last species was also recorded frequently at 33°C. Subsequently, the temperatures 26 and 29°C favored the growth of *Verticillium lecani*. *Paecilomyces lilacinus* prevailed at 29°C. The best growth of *Pseudallescheria boydii* was observed at 33 and 37°C. *Aspergillus fumigatus* prevailed at 37 and 45°C. At 45°C, it was the only species identified. The growth of keratinophilic fungi on hair was good at all temperatures. However, their best growth was observed at 23 and 26°C.

It was acknowledged that temperatures 23, 29, 33 and 37°C differentiated best the fungal flora examined. Results obtained at these temperatures were taken for further studies. In general, the main process of the

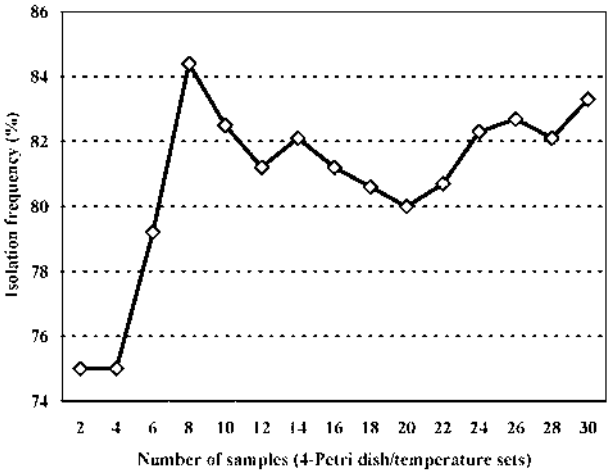

Fig. 1. The effect of sample (4-Petri dish/temperature sets) quantity on the isolation frequency (%) of keratinolytic fungi from sewage sludge.

growth indices "stabilization" for both keratinolytic and keratinophilic fungi took place up to ten samples (4-Petri dish/temperature sets). An example of such stabilization is illustrated in Figure 1. However, "new" low-frequency keratinolytic species appear when the number of samples (sets) exceeds 10.

The effect of Petri dish sludge weight on fungal composition is presented in Table 2. The SS and NS values were the highest in the dishes with a 10 g sludge weight. No distinct differences were observed in IF values. The 10 g weight favored the ascomata production by *Aphanoascus clathratus* and the growth of *Trichophyton terrestre* with its teleomorph *Arthroderma quadrifidum*. In contrast to keratinolytic fungi, the sum of keratinophilic strains was the lowest in 10 g sludge weight dishes. However, no significant differences in fungal qualitative compositions were observed in dishes with different sludge weights.

Discussion

Microsporum gypseum favors soils with high organic carbon (Tiurin) content [22, 23]. In the sludge examined, the organic carbon content was very high (25.9% d.w.). The prevailing of this geophilic dermatophyte in the sludge was, therefore, not surprising. Ulfig [13] also found that the incidence of *Microsporum gypseum* was associated with total sulfur content in sludge. A considerable part of the total sulfur is the organic sulfur "accumulated" in keratinous debris of human and animal origin (mainly in sulfur amino acids, i.e., cystine, cysteine and methionine). It is thought that the abundance of keratinous debris favors the growth of keratinolytic and keratinophilic fungi in sludge, *Microsporum gypseum* in particular.

In pure culture and artificial media, *Microsporum gypseum* grows between 4-40°C, with optimal and maximal ranges of 28-30°C and 38-40°C, respectively [23, 24]. On hair laid on sludge, the dermatophyte also grew at a

Table 2. The effect of Petri dish sludge weight on fungal composition in sewage sludge.

Fungal species and indices	Number of strains and fungal growth indices for Petri dishes with sludge weights [g moist mass]:			
	10	20	30	40
Keratinolytic fungi				
Chrysosporium anamorph *Aphanoascus clathratus* Cano & Guarro	13	4	8	7
Teleomorf *Aphanoascus clathratus* Cano & Guarro	6	2	0	0
Chrysosporium keratinophilum D.Frey ex Carmichael	3	0	0	1
Chrysosporium queenslandicum Apinis & Rees	3	2	2	0
Microsporum gypseum (Bodin) Guiart & Grigorakis	22	30	30	28
Trichophyton terrestre Durie & Frey	6	0	0	0
Teleomorph *Arthroderma quadrifidum* Dawson & Gentles	6	0	0	0
Sum of strains (SS)	59	38	40	36
Isolation frequency (IF; %)	85	80	85	80
Number of species (NS)	5	3	3	3
Keratinophilic fungi				
Aspergillus fumigatus Fresenius	9	10	12	10
Aspergillus versicolor (Vuill.) Tiraboschi	4	6	5	6
Fusarium oxysporum Schlecht.	7	7	9	8
Fusarium solani (Mart.) Saccardo	9	10	12	13
Paecilomyces lilacinus (Thom) Samson	5	9	9	8
Phialophora melinii (Nannf.) Conant	6	7	5	8
Plectosphaerella cucumerina (Lindf.) W.Gams	2	1	1	1
Pseudallescheria boydii (Shear) McGinnis *et al*.	4	8	10	12
Verticillium lecani (Zimm.) Viegas	6	7	5	6
Sum of strains (SS)	52	65	68	72
Isolation frequency (IF; %)	82.5	87.5	92.5	90
Number of species (NS)	9	8	8	8

wide temperature range (23-33°C), with the best growth observed at 29°C. The fungus did not inhabit hair at 37°C. Cano & Guarro [20] reported that *Aphanoascus clathratus* did not grow at 37°C. In this study, however, the fungus grew well at this temperature on both TK and hair. Having considered fungal temperature requirements, an agreement between the growth on hair and temperature optima in pure culture/artificial media can be generally acknowledged for the sludge examined. It should be emphasized, however, that such agreement could be considerably modified by the influence of environmental factors [25].

Aphanoascus clathratus grew best in dishes with a 10 g sludge weight. In these dishes, the fungus produced ascomata, and *Trichophyton terrestre* with its teleomorph *Arthroderma quadrifidum* also occurred. It is believed that the higher sludge weight, the more restrictive conditions for growth of keratinolytic fungi, which is attributed to the impact of environmental factors associated with the weight. One of these factors can be the activity of sludge microflora, certainly lower in a smaller sludge weight. However, this thesis requires elucidation in a separate study. The results have an important practical implication. Small weights (≤10 g) should be used in qualitative studies. Larger weights are more suitable for ecological studies, in which the influence of sludge environmental factors on fungal growth and composition is to be determined.

In the sludge examined, the diversity of keratinophilic fungi was higher than the diversity of keratinolytic species. Antagonistic activity of keratinolytic fungi against

keratinophilic species on hair also was demonstrated. These observations were possible with the multi-temperature and -weight method.

A health risk evaluation system for pathogenic fungi was proposed by de Hoog [8]. The system included fungal pathogenic properties, phylogenetic position, transmission vectors, immunological system factors, therapeutic methods and others. In the system, keratinolytic *Microsporum gypseum, Trichophyton terrestre* and *Chrysosporium keratinophilum,* isolated from the sludge, represent biosafety level BSL-2. This level includes saprophytic species capable of surviving in vertebrate tissues and attacking them. These fungi cause opportunistic mycoses in immunocompromised patients. Keratinophilic *Aspergillus fumigatus, Pseudallescheria boydii, Fusarium solani* and *Fusarium oxysporum* also represent BSL-2. Subsequently, *Paecilomyces lilacinus* and *Aspergillus versicolor* are listed in BSL-1. This biosafety level includes saprophytic species or plant pathogens, rarely causing mycoses, which are superficial and mild in character.

Microsporum gypseum is a geophilic dermatophyte relatively frequently isolated from skin lesions. Therefore, this species is of special epidemiological importance. The sludge favored the growth of *Microsporum gypseum* on keratinous substrata in a wide temperature range. It can be expected, therefore, that the sludge on a wastewater treatment plant area or applied to land poses an elevated health risk to immunocompromised individuals. Other species of special epidemiological importance are *Pseudallescheria boydii* and *Aspergillus fumigatus,* well-known opportunists with temperature optima over 30°C [26].

More databases must be established to thoroughly evaluate the applicability of a multi-temperature hair baiting method to sewage sludge and other habitats. These results, however, indicate that the method is potentially useful for determining the influence of temperature and other factors on fungal composition in sludge and evaluation of health risk posed by fungal pathogens. The use of the method facilitates the recognition of fungal flora much better than a traditional room-temperature method. The results also indicate that temperatures 26 and 45°C can be neglected in further studies, unless the studies are focused on thermophilic and thermotolerant species. The method was adapted to quantitative purposes. However, when the number of Petri dishes is ten per temperature, some low-frequency keratinolytic species may be lost. The conclusions generally agree with those drawn for bottom sediments [25].

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