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SEWAGE SLUDGE OPEN-AIR DRYING AFFECTS ON KERATINOLYTIC, KERATINOPHILIC AND ACTIDIONE-RESISTANT FUNGI

WPŁYW POWIETRZNEGO SUSZENIA OSADU ŚCIEKOWEGO NA GRZYBY KERATYNOLITYCZNE, KERATYNOFILNE I AKTIDIONO-OPORNE

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The goal of the study was to demonstrate the effect of sewage sludge open-air drying on the quantitative and qualitative composition of keratinolytic, keratinophilic and actidione-resistant fungi. The open-air drying altered the composition of keratinolytic fungi and considerably enriched the population of keratinophilic and actidione-resistant fungi in the sludge examined. Sludge drying in sludge drying beds at wastewater treatment plants and during land use could increase the quantities of opportunistic fungi in the environment and the subsequent risk to public health posed by these organisms.

Key words: keratinolytic and keratinophilic fungi, actidione-resistant fungi, sewage sludge, open-air drying
Słowa kluczowe: grzyby keratynolityczne i keratynofilne, grzyby aktidiono-oporne, osady ściekowe, powietrzne suszenie

INTRODUCTION

According to *Majchrowicz* and *Dominik's* definition [8], keratinolytic fungi are able to decompose keratin in keratinous substrates (hair, nails, keratinized cells of the epidermis, fur, horn, etc.), whereas keratinophilic fungi accompany keratinolytic fungi on these substrates; utilizing their non-protein components or the products of keratin decomposition.

Keratinophilic fungi are also known as non-keratinolytic fungi inhabiting keratinous substrates. Many of these fungi are resistant to the antibiotic, actidione (cycloheximide), and potentially pathogenic to animals, including humans [6]. Due to these potential pathogenic properties, recognition of the factors affecting keratinolytic/keratinophilic and actidione-resistant fungi in the environment is of epidemiological significance.

Sewage sludge is more and more frequently being used for fertilization or reclamation of soils. It has been demonstrated that keratinolytic and keratinophilic fungi occur abundantly in sludges and sludge-amended soils [11, 20, 23]. Temperature, pH, ammonium nitrogen, proteolytic activity, organic carbon, total nitrogen, C:N ratio, total sulfur, C:S ratio, available phosphorus and particle size distribution have been found to be the most important factors affecting fungal populations in sewage sludge [19, 20]. Additionally, liming considerably alters fungal composition in the sludge environment [21]. The goal of the present study was to demonstrate the effect of sewage sludge open-air drying on keratinolytic/keratinophilic and actidione-resistant fungi.

MATERIAL AND METHODS

Sewage sludge from the Bytom-Miechowice municipal wastewater treatment plant (Upper Silesia, Poland) was used in the experiment. It was the excess sludge, after extended aeration (without primary settling tank) and the integrated biological C and N removal process, dewatered by centrifuging, mixed with plant residues (hay and straw) and piled for 1-2 years. Stabilized organic matter evidenced by low ammonium nitrogen concentration (14.9 mg $N\text{-}NH_4$/kg d.w.), low proteolytic activity (534 $N\text{-}NH_3$ mg/100 g d.w.), and C/N ratio = 11.2 characterized this sludge [19]. The sludge was taken from a pile at different depths (from the surface to the bottom of the pile), collected in plastic barrels disinfected with 60% ethyl alcohol and delivered to the laboratory within 2-5 hours. The sludge was cleaned from stones, plant remains and large particles, crumbled and mixed thoroughly. The sludge was sampled in the summer season in three ca. 200-kg portions. One part of each sludge portion remained wet; these were the fresh sludge samples. Another part was being dried in open air in the laboratory at 25-30°C. The open-air drying of sludge portions took from seven to thirty days (on average fourteen days); depending on the sample and ambient conditions (air humidity and temperature). The sludge was continuously crumbled and mixed while drying. The dried sludge samples were mixed and sieved through a 3-mm diameter net and mixed again each. The sludge handling was performed under aseptic conditions. Fresh and air-dried sludge samples were kept at 4°C for no longer than two days before physico-chemical and microbiological examination.

Selected physico-chemical parameters, i.e. moisture, pH in H_2O, and sulfate, ammonium nitrogen, nitrite ammonium and nitrate ammonium concentrations were measured in fresh and dry sludge samples. Other physico-chemical characteristics of the sludge examined along with the methods used were presented elsewhere [19].

Keratinolytic and keratinophilic fungi were examined in fresh and dried sludge samples using a modified hair baiting method [18]. Petri dishes were filled with 40 g of sludge and covered with 0.4 g of detergent-defatted, fine cut, and autoclaved children hair each, and incubated in the dark at 23, 29, 33 and 37°C for four months. Ten dishes responded to each incubation temperature. Forty dishes responded to each sludge sample. During incubation, stable moisture conditions (ca. 40%) by adding autoclaved redistilled water were maintained in the dishes. The fungal growth indices used were as follows: number of occurrences; isolation frequency (number of Petri dishes positive for fungal growth*100/total number of Petri dishes set up); number of species; and the frequency of a species (number of occurrences of a given species*100/total number of fungal occurrences).

Serial dilutions in physiological saline and the Wiegand medium [14] supplemented with chlo-

ramphenicol (100 mg/L) and actidione (500 mg/L), which was incubated in the dark at 23 and 37°C, were used for determination of qualitative and quantitative compositions of actidione-resistant fungi in sludge samples. From among fungal propagule numbers obtained at two temperatures, higher values were presented for each species.

Pure fungal strains were identified to the species level using selected taxonomic monographs [2, 5, 13, 15, 16]. The *in vitro* hair degradation test was that of *Ulfig* et al. [22]. The test relies on the incubation of fungal strains for one month on Sabouraud 1:10 + mineral salts medium with sterilized child hair laid over its surface. Strains with strong and moderate keratinolytic activity, forming penetrating bodies, pockets or radial hyphae in hair were recognized as keratinolytic. Fungi with no or weak keratinolytic properties, colonizing hair only superficially, were ranked as keratinophilic. Based on the test results, fungal species were included in keratinolytic and keratinophilic groups.

The mycological and physico-chemical results presented in this study are the means for three samples examined.

RESULTS

The incidence of keratinolytic and keratinophilic fungi in fresh and dried sludge samples is presented in tables I and II, respectively. Altogether, 136 keratinolytic fungi occurrences belonging to at least ten species were observed in sludge samples. The *Chrysosporium* anamorph of *Aphanoascus clathratus* (25.6%), *Microsporum gypseum* (23.9%), *Trichophyton terrestre* with its teleomorph *Arthroderma quadrifidum* (16.2%), and *Trichophyton ajelloi* (10.3%) prevailed in the samples. The other species were observed with lower numbers of occurrences (frequency <10%).

The sludge open-air drying did not considerably affect the keratinolytic fungi growth indices. However, the number of *Microsporum gypseum* and, except for *Chrysosporium indicum*, the number of *Chrysosporium occurrences* was higher in the dried sludge than in the fresh sludge. The highest number of *Microsporum gypseum* occurrences was observed at 29°C. Contradictorily, the sludge open-air drying eliminated *Chrysosporium indicum* from the sludge (no occurrences observed on hair) and considerably decreased the number of *Trichophyton ajelloi* occurrences. In the fresh sludge, the highest numbers of *Chrysosporium indicum* and *Trichophyton ajelloi* occurrences were noticed at 33 and 23°C, respectively.

As far as keratinophilic fungi are concerned 81 their occurrences belonging to twelve species were observed. *Verticillium lecani* (29.6%), *Aspergillus fumigatus* (27,2%) and *Pseudallescheria boydii* (14.8%) prevailed in sludge samples. The other species were noticed with lower numbers of occurrences (frequency <10%).

Broadly, the sludge open-air drying considerably increased the numbers of keratinophilic fungi occurrences and species. This process decreased the number of *Aspergillus fumigatus* occurrences, whereas the number of Pseudallescheria boydii occurrences increased. The highest number of *Aspergillus fumigatus* occurrences was observed at 37°C. In the fresh sludge, *Pseudallescheria boydii* occurred at 23 and 29°C, whereas in the dried sludge this species was observed with higher number of occurrences at 33 and 37°C. *Paecilomyces lilacinus* and some other species only occurred in the dried sludge. The temperature range for *Paecilomyces lilacinus* was 23-33°C.

The results on the effect of sludge open-air drying on the composition of actidione-resistant fungi are presented in Table III. In the fresh sludge, the numbers of actidione-resistant

Table I. The incidence of keratinolytic and keratinophilic fungi in fresh sludge
Występowanie grzybów keratynolitycznych i keratynofilnych w osadzie świeżym

Fungal species and growth indices	Number of fungal occurrences at temperature				Total
	23°C	29°C	33°C	37°C	
Keratinolytic fungi					
Chrysosporium anamorph of *Aphanoascus clathratus* Cano & Guarro	0	4	8	1	13
Trichophyton ajelloi (Vanbreuseghem) Ajello	10	1	0	0	11
Trichophyton terrestre Durie & Frey	10	1	0	0	11
Teleomorph *Arthroderma quadrifidum* Dawson & Gentles	9	0	0	0	9
Microsporum gypseum (Bodin) Guiart & Grigorakis	1	7	2	0	10
Chrysosporium indicum (Randhawa & Sandhu) Garg	0	0	9	0	9
Chrysosporium keratinophilum D.Frey ex Carmichael	0	2	1	0	3
Malbranchea fulva Sigler & Carmichael	0	0	0	1	1
Number of occurrences	30	15	20	2	67
Isolation frequency (%)	100	100	100	20	80
Number of species	3	5	4	2	7
Keratinophilic fungi					
Aspergillus fumigatus Fres.	0	3	6	10	19
Verticillium lecani (Zimm.) Viegas	7	3	0	0	10
Pseudallescheria boydii (Shear) McGinnis *et al.*	2	1	0	0	3
Aspergillus terreus Thom	2	1	0	0	3
Penicillium nigricans Bain. ex Thom	0	1	0	0	1
Number of occurrences	11	9	6	10	36
Isolation frequency (%)	80	70	60	100	78
Number of species	3	4	3	1	5

fungi were 2.6×10^0 and 2.6×10^1 CFU (Colony Forming Units)/100 g d.w. (sludge dry weight) at 23 and 37°C, respectively. The sludge open-air drying increased these numbers to 3.1×10^4 and 2.3×10^3 CFU/100 g d.w., respectively. The numbers of species were two and ten in fresh and dried sludges, respectively. *Arthrographis alba* (57.8%) and *Trichophyton terrestre* (22.3%) prevailed in the dried sludge. The other fungi occurred in the sludge with frequencies <10%.

The open-air drying decreased the sludge moisture from 60.9 to 7.4%, while the pH in H_2O increased from 4.6 to 6.5. Subsequently, the sulfate, ammonium nitrogen, nitrite nitrogen and nitrate nitrogen concentrations increased from 0.08 to 0.21% d.w., from 15 to 42 mg $N\text{-}NH_4$/kg d.w., from 2.1 to 5.3 mg $N\text{-}NO_2$/kg d.w., and from 1.1 to 10.3 mg $N\text{-}NO_3$/kg d.w., respectively.

Table II. The incidence of keratinolytic and keratinophilic fungi in open-air dried sludge
Występowanie grzybów keratynolitycznych i keratynofilnych w osadzie powietrznie suchym

Fungal species and growth indices	Number of fungal occurrences at temperature				Total
	23°C	29°C	33°C	37°C	
Keratinolytic fungi					
Microsporum gypseum (Bodin) Guiart & Grigorakis	3	10	5	0	18
Chrysosporium anamorph of *Aphanoascus clathratus* Cano & Guarro	0	6	7	4	17
Trichophyton terrestre Durie & Frey	8	0	0	0	8
Teleomorph *Arthroderma quadrifidum* Dawson & Gentles	8	0	0	0	8
Chrysosporium keratinophilum D.Frey ex Carmichael	0	1	2	3	6
Tel. *Aphanoascus keratinophilus* Punsola & Cano	0	0	1	1	2
Chrysosporium zonatum Al-Musallam & Tan	0	0	0	4	4
Chrysosporium an. *Aphanoascus reticulisporus/fulvescens*	0	0	2	1	3
Amauroascus mutatus (Quelet) Rammeloo	2	0	0	0	2
Trichophyton ajelloi (Vanbreuseghem) Ajello	1	0	0	0	1
Number of occurrences	22	17	17	13	69
Isolation frequency (%)	100	100	100	50	88
Number of species	4	3	4	4	8
Keratinophilic fungi					
Verticillium lecani (Zimm.) Viegas	7	7	0	0	14
Pseudallescheria boydii (Shear) McGinnis *et al.*	0	0	4	5	9
Paecilomyces lilacinus (Thom) Samson	3	4	1	0	8
Aspergillus fumigatus Fres.	0	0	0	3	3
Penicillium janthinellum Biourge	2	1	0	0	3
Plectosphaerella cucumerina (Lindf.) W.Gams	1	0	1	0	2
Fusarium oxysporum Schlecht.	2	0	0	0	2
Fusarium solani (Mart.) Saccardo	2	0	0	0	2
Phialophora cinerescens (Wollenw.) van Beyma	1	0	0	0	1
Aspergillus versicolor (Vuill.) Tiraboschi	1	0	0	0	1
Number of occurrences	19	12	6	8	45
Isolation frequency (%)	100	100	50	60	78
Number of species	8	3	3	2	10

Table III. The effect of sewage sludge open-air drying on the composition of actidione-resistant fungi
Wpływ powietrznego suszenia osadu na skład grzybów aktidiono-opornych

Fungal species	Number of fungal propagules (CFU/g d.w.)	
	Fresh sludge	Air-dried sludge
Arthrographis alba Gené, Ulfig & Guarro	-	$2{,}2 \times 10^3$
Trichophyton terrestre Durie & Frey*	-	$8{,}5 \times 10^2$
Verticillium lecani (Zimm.) Viegas	-	$2{,}8 \times 10^2$
Paecilomyces lilacinus (Thom) Samson	-	$1{,}7 \times 10^2$
Penicillium nigricans Bain. ex Thom	-	$1{,}2 \times 10^2$
Phialemonium dimorphosporum W.Gams & W.B.Cooke	-	$1{,}0 \times 10^2$
Scopulariopsis candida (Guéguen) Vuill.	-	$5{,}8 \times 10^1$
Aspergillus fumigatus Fresenius	$2{,}6 \times 10^1$	$2{,}0 \times 10^0$
Mycelia sterilia (white)	-	$6{,}0 \times 10^0$
Pseudallescheria boydii (Shear) McGinnis *et al.*	-	$1{,}0 \times 10^0$
Aphanoascus reticulisporus (Routien) Hubálek*	$3{,}0 \times 10^0$	-

* – keratinolytic species
CFU – Colony Forming Units
d.w. – dry weight of sludge

DISCUSSION

The open-air drying decreased the number of *Trichophyton ajelloi* occurrences and eliminated Chrysosporium indicum from the sludge (no occurrences observed on hair). *Trichophyton ajelloi* is hygrophilic [3] and this characteristic can explain the decrease of the fungus occurrences resulted from sludge drying. Subsequently, *Chrysosporium indicum* is ubiquitous in hot climate countries [1, 4]. *McAleer* [9] demonstrated its high tolerance to temperature, moisture and other environmental factors. Based on available data, however, it is difficult to indicate a factor responsible for the elimination of *Chrysosporium indicum* from the sludge during drying. It is possible that microbiological competition could have eliminated the fungus from this sludge.

The incubation temperature considerably affected the composition of keratinolytic and keratinophilic fungi isolated from sewage sludge. The fungal temperature spectra found in this investigation generally agree with spectra presented in a previous paper [19].

The open-air drying changed the composition of keratinolytic fungi and considerably enriched the population of keratinophilic (non-keratinolytic) and actidione-resistant fungi in the sludge. This can be explained with that the drying process took up to thirty days (fourteen days on average) and was associated with slow sludge moisture decrease, sludge laceration due to crumbling, and with the subsequent improvement of sludge aeration and organic matter biodegradation conditions. The sludge pH increase could have resulted from ammonium nitrogen concentration increase. Both ammonium and sulfate are among the

final products of keratin decomposition [12]. Therefore, the increase of ammonium nitrogen along with the increase of sulfate concentration could testify to the activity of keratinolytic microorganisms (including fungi) on keratinous substrata in the sludge. Finally, the increase of nitrite and nitrate ammonium concentrations could have resulted from the nitrification process in the sludge.

The findings appear to be important from the sanitary point of view. When sludges are being dried in sludge drying beds at wastewater treatment plants and also when applied to land, the abundant growth of keratinolytic and keratinophilic along with actidione-resistant fungi can be expected. This conclusion agrees with our previous findings [19, 23] and with findings of *Kacprzak* and *Stańczyk-Mazanek* [10]. From among the fungi occurring in sludge, *Microsporum gypseum* and *Pseudallescheria boydii* are of special epidemiological concern [7]. Microsporum gypseum is the agent responsible for skin mycoses, while *Pseudallescheria boydii* (one of its anamorphs: *Scedosporium apiospermum*) is the emerging opportunistic pathogen causing various infections in humans. These pathogenic fungi could increase the risk of mycoses to workers of municipal wastewater treatment plants and to farmers and other individuals when contacted with sludge-amended soil. This suggestion agrees with that of *Śpiewak* [17]. According to this author, mycoses are the most prevalent diseases in farmers and soil is one of the fungal infection sources.

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Summary

The study was to demonstrate the effect of sewage sludge open-air drying on the quantitative and qualitative composition of keratinolytic/keratinophilic and actidione-resistant fungi. The sludge was being dried for up to thirty days (on average fourteen days) at 25-30°C. The composition of these fungi was determined with the hair baiting method along with the dilution method, using the Wiegand medium supplemented with chloramphenicol (100 mg/L) and actidione (500 mg/L). The open-air drying altered the composition of keratinolytic fungi and considerably increased the population of keratinophilic and actidione-resistant fungi in the sludge. This phenomenon can be explained with that the drying process was associated with slow sludge moisture decrease, sludge laceration due to crumbling and the subsequent improvement of sludge aeration and organic matter biodegradation conditions. A considerable increase of fungal populations can be expected in sludges being dried in drying beds at wastewater treatment plants and in sludge-amended soils. Two sludge opportunistic fungi, i.e. *Microsporum gypseum* and *Pseudallescheria boydii*, require special attention from the epidemiological point of view. Sludge land applications may increase the number of these fungi in the environment and the subsequent risk to public health posed by them.

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Streszczenie

Celem badań było określenie wpływu powietrznego suszenia osadu ściekowego na skład ilościowy i jakościowy grzybów keratynolitycznych, keratynofilnych i aktidiono-opornych. Osad suszono średnio przez okres od siedmiu do 30 dni (średnio 14 dni) w temperaturze 25-30°C. W oznaczaniu składu grzybów wykorzystano metodę przynęty włosowej oraz metodę rozcieńczeń, z wysiewami na pożywkę *Wieganda* z dodatkiem aktidionu (500 mg/L) i chloramfenikolu (100 mg/L). Powietrzne suszenie osadu ściekowego zmieniło skład grzybów keratynolitycznych i znacząco wzbogaciło populację grzybów keratynofilnych i aktidiono-opornych. Zjawisko to można wytłumaczyć wolnym obniżeniem wilgotności osadu, jego rozdrobnieniem i w konsekwencji poprawą warunków napowietrzania i biodegradacji materii organicznej. Silnego wzrostu populacji omawianych grzybów można się spodziewać podczas powietrznego suszenia osadów, np. na poletkach osadowych, jak również w glebach rekultywowanych osadami ściekowymi. Wśród badanych grzybów na szczególną uwagę, z epidemiologicznego punktu widzenia, zasługują dwa gatunki oportunistyczne, tj. *Microsporum gypseum* i *Pseudallescheria boydii*. Stosowanie osadów ściekowych do użyźniania i rekultywacji gleb może przyczynić się do wzrostu ich liczebności, a tym samym do wzrostu zagrożenia epidemiologicznego ze strony tych grzybów w środowisku.

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