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Original Research

Examination of Keratinolytic and Associated Non-Keratinolytic Fungi in Sewage Sludge

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Abstract

Sewage sludge is frequently applied to land. It has been shown, however, that sludges contain many toxic components and pathogenic organisms. Keratinolytic and associated non-keratinolytic fungi are considered potentially pathogenic. Our knowledge on the incidence of the fungi in the sludge environment is still insufficient. Sludge from the Bytom-Miechowice wastewater treatment plant in Upper Silesia, Poland, was examined. The objective of the study was to compare fungal compositions in the sludge using three methods:

(1) dilution pour plating method
(2) hair-baiting method, and
(3) most probable number method.

The MPN method combined sludge dilution in sterile clay or sand and hair-baiting techniques. Actidione-resistant fungi were examined in the sludge. The dilution pour plating method provided poor data on the incidence of keratinolytic fungi, while using the method more information was obtained on non-keratinolytic fungi in the sludge. Subsequently, the conventional hair-baiting method provided extensive data on the growth of fungi in the hair spread over the sludge, but the method did not allow determining fungal quantities. Such quantities were obtained by means of the MPN method. The method complemented results obtained with two other methods. The ecological and epidemiological significance of MPN values was discussed.

Keywords: keratinolytic and non-keratinolytic fungi, quantitative and qualitative composition, sewage sludge, dilution pour plating method, hair-baiting method, MPN method

Introduction

Sewage sludge is being more and more frequently applied to land. However, sludges contain many toxic components and pathogenic organisms. The incidence of pathogenic bacteria, viruses, and zooparasites in sewage sludge has been relatively well recognized, while the knowledge of pathogenic fungi in the sludge environment is still insufficient [1-4].

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Keratinolytic fungi specialize in biodegradation of keratin, being the main component of keratinous substrates. Non-keratinolytic fungi (earlier named keratinophilic) accompany keratinolytic fungi, utilizing non-protein components of the substrates and/or the products of keratin biodegradation [5-7]. Sewage sludge contains high amounts of keratinous substrata of human and animal origin, mainly hair and keratinized epidermal cells. It is not surprising, therefore, that the fungi occur abundantly in the sludge environment. However, keratinolytic and associated non-keratinolytic fungi have recorded the agents responsible for

mycoses [8] and mycotoxin producers [9]. Consequently, studies of the fungi in sludges are of epidemiological significance.

The composition of the sludge keratinolytic and non-keratinolytic mycobiota is dependent on sewage and sludge treatment technologies and of the influence of a variety of biological and physico-chemical factors [3, 10-19]. The dilution pour-plating method, using solid media supplemented with actidione, and the hair-baiting method were mostly used in the quoted studies. In order to complement knowledge of the mycobiota in sewage sludge, a series of experiments was performed to compare fungal compositions using the above-mentioned methods and the Most Probable Number method. This paper presents results of the first experiment.

Material and Methods

Sewage sludge from the Bytom-Miechowice wastewater treatment plant, Upper Silesia, Poland, was used in the experiment. The methods of sludge sampling and physico-chemical analyses were described in previous papers [3, 6]. The sludge was dried in open air for 7 days before being used in the experiment.

The methods for examination of keratinolytic and associated non-keratinolytic fungi in the sludge were as follows:
(1) dilution pour-plating method;
(2) hair-baiting method [20] modified according to Ulfig [14]; and
(3) MPN method combining sludge dilution in clay or sand, and hair-baiting techniques.

In the dilution pour-plating method, sludge dilutions (1:10 - 1:100,000) were prepared in sterile physiological saline. The Wiegand medium containing bacteriological peptone (Difco) – 10 g, glucose – 40 g, bacteriological agar (Difco) – 20 g, chloramphenicol – 100 mg, actidione – 500 mg, 0.5-% alkaline phenol red solution – 40 mL, and redistilled water – 960 mL, was used for examination of actidione-resistant fungi. Among them, keratinolytic and non-keratinolytic species were identified. The final pH of the medium was 5.6 [21]. Five plates were set up for each dilution and temperature. The plates were incubated for 10-14 days at 23 and 37ºC. Strains with proteolytic properties changed the color of the medium from yellowish to red, due to pH increase caused by peptone ammonification. The detection limit of the method was 200 CFU/100 g d.w. (dry weight).

In the hair-baiting method, 30-g sludge portions were placed in sterile standard (9-cm diam) glass plates and then sterile redistilled water was added to each plate to obtain ca. 40-% moisture. A 0.4-g portion of fine cut, detergent-defatted and autoclaved children's hair was spread over the sludge in each plate. The plates were incubated at 23, 29, 33 and 37ºC for 4 months. Ten plates were set up for each temperature. During incubation of the plates the moisture was kept stable by adding sterile redistilled water.

In the MPN method, open-air dried sand and clay were first autoclaved (30 minutes at 121ºC) three times at 24-hour intervals. Sludge dilutions (1:10 - 1:1,000,000) were then prepared. The first dilution contained 100 g of sludge and 900 g of sterile clay or sand (900 g), and the components were mixing for 30 minutes in a laboratory ball mill under aseptic conditions. The following dilutions were performed by taking a 100-g portion of the previous dilution, introducing it into 900 g of sterile clay or sand, and mixing the material in the above-mentioned manner. Subsequently, 10-g sludge dilution portions were placed in sterile standard glass plates. A 0.4-g portion of fine cut, detergent-defatted, autoclaved children's hair was spread over the sludge dilution in each plate. Ten plates were set up for each dilution, substrate (clay or sand) and incubation temperature. Autoclaved redistilled water was added to each plate to obtain ca. 20- and 30-% moisture for sludge dilutions with sand and clay, respectively. The plates were incubated for 4 months at 23, 29, 33 and 37ºC. During incubation of the plates, the moisture was kept stable by adding sterile redistilled water.

At monthly intervals, the hair was examined by microscopy and also spread over plates of Sabouraud 1:10/mineral salts agar (TK medium) [22], supplemented with chloramphenicol (100 mg/L) and actidione (500 mg/L). After incubation at 23 and 37ºC for 10 days, growth of a particular species was taken as confirmation of the previous observation by microscopy of that species occurring in the hair.

Purified fungal strains were identified to species level using selected taxonomic monographs [23-29]. The *in vitro* hair degradation test was that of Ulfig et al. [30]. All isolates were actidione-resistant.

In an MPN combination a single positive or negative result in a repetition derived from observations of four plates incubated at 23, 29, 33 and 37ºC. If a given species appeared at least once in any of the four plates, it was taken as a positive result for the repetition in the MPN combination. Ten repetitions were performed to increase the precision of the method. MPN values were calculated following standard rules and a formula by Geldreich [31, 32]. The MPN method detection limit was 2 MPN/100 g d.w.

Results

Ten actidione-resistant species were isolated from the sludge (Table 1). One species was ranked as keratinolytic and nine as non-keratinolytic. Keratinolytic *T. terrestre* complex occurred in the sludge with a high quantity (22.4% in relation to the total number of actidione-resistant strains). *A. alba* was found to be the most numerous non-keratinolytic fungus in the sludge (58.1%). The other fungi occurred in the sludge with lower quantities.

Nine keratinolytic and eleven non-keratinolytic species were isolated from the sludge by means of the hair-baiting method (Table 2). Among the keratinolytic fungi, the *Chrysosporium* anamorph of *A. clathratus* with the teleomorph *A. clathratus* occurred in the sludge with the highest number of strains (34.1% in relation to the total number of strains), followed by *C. keratinophilum* with its teleomorph

Table 1. The composition of actidione-resistant fungi in the sludge from the Bytom-Miechowice wastewater treatment plant. Data obtained by means of the dilution pour-plating method on Wiegand medium.

Fungal species	Fungal number [CFU/100 g d.w.]
Arthrographis alba Gené, Ulfig et Guarro	2.2×10^5
Trichophyton terrestre complex Durie & Frey*	8.5×10^4
Verticillium lecani (Zimm.) Viegas	2.8×10^4
Paecilomyces lilacinus (Thom) Samson	1.7×10^4
Penicillium nigricans Bain. ex Thom	1.2×10^4
Phialemonium dimorphosporum W. Gams & W.B. Cooke	1.0×10^4
Scopulariopsis candida (Guéguen) Vuill.	5.8×10^3
Mycelia sterilia (white)	6×10^2
Aspergillus fumigatus Fresenius	2×10^2
Pseudallescheria boydii complex (Shear) McGinnis et al.	1×10^2

* - keratinolytic species,
d.w. – dry weight,
CFU – Colony Forming Units.

A. keratinophilus (17%), *C. zonatum* (15.9%), *M. gypseum* (12.5%) and *T. terrestre* complex with its teleomorph *A. quadrifidum* (11.4%). *T. terrestre* with its teleomorph *A. quadrifidum* predominated in the hair at 23ºC, *M. gypseum* at 29ºC, while *Chrysosporium* species occurred most numerously at 33 and 37ºC. The simplified (without some teleomorphs) temperature spectrum of keratinolytic fungi in the sludge was illustrated in Fig. 1.

Among the non-keratinolytic fungi, *V. lecani* occurred in the sludge with the highest number of strains (22.4%), followed by *P. nigricans* (13.4%), *P. cinerescens* (11.9%), *A. fumigatus* (10.4%) and *P. cucumerina* (10.4%). Except for *A. fumigatus*, the other non-keratinolytic fungi grew in the hair at 23 and 29ºC. *A. fumigatus* prevailed at 37ºC.

Twelve keratinolytic and sixteen non-keratinolytic species were isolated from the sludge by means of the MPN method (Table 3). Among the keratinolytic species, the highest MPN values were noted for the *Chrysosporium* anamorph of *A. clathratus* with its teleomorph *A. clathratus*, and *T. terrestre* complex with its teleomorph *A. quadrifidum* (Table 3). The *Chrysosporium* anamorph of *A. clathratus* had high MPN values on both clay and sand, though its teleomorph was found to be more numerous on clay. Subsequently, *T. terrestre* complex had a higher MPN on clay, though its teleomorph was observed more frequently on sand. The *Chrysosporium* anamorph of *C. keratinophilum* along with *Microsporum gypseum* complex had higher MPN values on clay, while the MPN of *C. zonatum* was higher on sand. The other fungi had low MPN values.

Among the non-keratinolytic fungi, *P. lilacinus* showed the highest MPN values, followed by *P. cinerescens*, *A. fumigatus*, *V. lecani* and *P. cucumerina*. *P. lilacinus* and *A. fumigatus* along with *F. oxysporum* and *P. boydii* complex had higher MPN values on sand than on clay, while *V. lecani* and *P. cucumerina* showed similar MPN values on both substrates. The other fungi occurred in the sludge with low MPN values.

The sludge pH in H_2O was 6.5. The organic carbon and total nitrogen contents were 17.4 and 1.46% d.w., respectively, with the C:N ratio 9.6. The ammonium, nitrite and nitrate nitrogen concentrations were 42 mg $N\text{-}NH_4$/kg d.w., 5.3 mg $N\text{-}NO_2$/kg d.w. and 10.3 $N\text{-}NO_3$/kg d.w., respectively. The total sulfur content was 0.34% d.w., and the C:S ratio was 51.2.

Discussion

The dilution pour-plating method provided poor data of the incidence of keratinolytic fungi, while using the method more information was obtained on non-keratinolytic fungi in the sludge. Subsequently, the conventional hair baiting method provided extensive data on the growth of the fungi in the hair spread over the sludge. In fact, the hair baiting method has frequently been used for semiquantitative purposes [3, 33], but does not allow determining fungal quantities. Such quantities were determined by means of the MPN method. The method significantly complemented results obtained with two other methods.

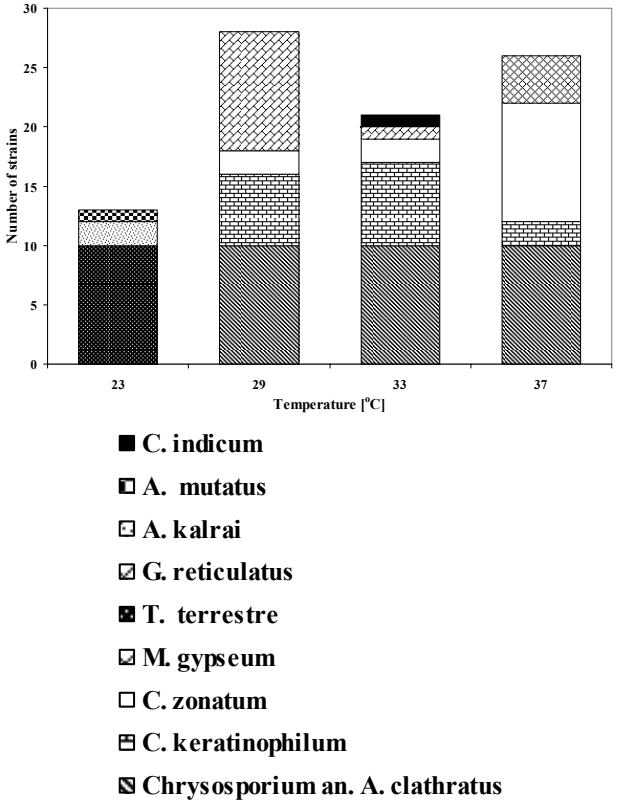

Fig. 1. The temperature spectrum of keratinolytic fungi in sludge.

Table 2. The composition of keratinolytic and associated non-keratinolytic fungi in sludge from the Bytom-Miechowice wastewater treatment plant. Data obtained by means of the hair-baiting method.

Fungal species	Number of strains at temperature:				Total
	23ºC	29ºC	33ºC	37ºC	
Keratinolytic fungi					
Chrysosporium anamorph Aphanoascus clathratus Cano & Guarro	-	10	10	10	30
Teleomorph *Aphanoascus clathratus* Cano & Guarro	-	2	10	4	16
Chrysosporium keratinophilum D.Frey ex Carmichael	-	6	7	2	15
Tel. *Aphanoascus keratinophilus* Punsola & Cano	-	-	1	-	1
Chrysosporium zonatum Al-Musallam & Tan	-	2	2	10	14
Microsporum gypseum complex (Bodin) Guiart & Grigorakis	-	10	1	-	11
Trichophyton terrestre complex Durie & Frey	10	-	-	-	10
Tel. *Arthroderma quadrifidum* Dawson & Gentles	10	-	-	-	10
Gymnoascus reticulatus Zukal	-	-	-	4	4
Arthrographis kalrai (Tewari & Macpher.) Sigler & Carmichael	2	-	-	-	2
Amauroascus mutatus (Quelet) Rammeloo	1	-	-	-	1
Chrysosporium indicum (Randhawa & Sandhu) Garg	-	-	1	-	1
Non-keratinolytic fungi					
Verticillium lecani (Zimm.) Viegas	7	8	-	-	15
Penicillium nigricans Bain. ex Thom	5	4	-	-	9
Phialophora cinerescens (Wollenw.) van Beyma	4	4	-	-	8
Aspergillus fumigatus Fres.	-	-	-	7	7
Plectosphaerella cucumerina (Lindf.) W.Gams	6	-	1	-	7
Fusarium oxysporum Schlecht.	6	-	-	-	6
Paecilomyces lilacinus (Thom) Samson	4	1	1	-	6
Verticillium psalliotae Treschow	4	-	-	-	4
Mycelia sterilia (brown)	2	-	-	-	2
Verticillium chlamydosporium Goddard	-	2	-	-	2
Penicillium janthinellum Biourge	1	-	-	-	1

All keratinolytic and non-keratinolytic fungi isolated from the sludge with the hair-baiting method were quantified using the MPN method. However, keratinolytic species such as Chrysosporium anamorphs of *A. reticulisporus/fulvescens*, *Chrysosporium* anamorph of *A. curreyi* and *T. ajelloi*, and non-keratinolytic species such as *Candida* sp., *F. solani* complex, *P. cyclopium*, *P. boydii* complex and *T. koningii,* were "additionally" quantified by means of the method. Except for *P. boydii* complex, the above-mentioned fungi occurred in the sludge with low MPN values. The results indicate that the selectiveness of the MPN method was even higher than that of the hair baiting method. It is believed that environmental factors associated with the undiluted sludge inhibited the growth of some fungi, but the sludge dilution in sterile clay or sand stimulated the organisms to grow in the hair. Interestingly, a similar phenomenon was observed for *Uncinocarpus reesii*, while measuring MPN values of keratinolytic and non-keratinolytic fungi in municipal landfill soils [34].

In some cases, MPN values were dependent on the specified substrate. For instance, *C. keratinophilum* and *M. gypseum* complex obviously favored clay for growth, while *F. oxysporum*, *P. cinerescens* and *P. boydii* complex had much higher MPN values on sand. On the one hand, the preference of *M. gypseum* complex to grow better on clay agrees with the finding of Ulfig et al. [35]. On the other hand, however, this dermatophyte was found to occur in sludges with total sulfur content >1% d.w. and C:S ratio <30 [3]. In the sludge examined, due to the total sulfur content 0.34% d.w. and C:S ratio 51.2, the conditions should not, but did, favor the growth of the fungus. The phenomenon requires explanation in further studies.

Table 3. MPN values of keratinolytic and associated non-keratinolytic fungi in sludge from the Bytom-Miechowice wastewater treatment plant.

Fungal species	Fungal numbers [MPN/100 g d.w.] on substrate:	
	Clay	Sand
Keratinolytic fungi		
Amauroascus mutatus (Quelet) Rammeloo	<2	4
Arthrographis kalrai (Tewari & Macpher.) Sigler & Carmichael	18	728
Chrysosporium anamorph of *Aphanoascus clathratus* Cano & Guarro	>3.8 x 10^6	>3.8 x 10^6
Teleomorph *A. clathratus* Cano & Guarro	>3.8 x 10^6	1 x 10^4
Chrysosporium an. *Aphanoascus reticulisporus/fulvescens*	55	<2
Chrysosporium an. *Arthroderma curreyi* Berkeley	36	<2
Chrysosporium keratinophilum D.Frey ex Carmichael	3.9 x 10^4	183
Chrysosporium tropicum Carmichael	4	<2
Chrysosporium zonatum Al.-Musallam & Tan	4.9 x 10^4	2.8 x 10^5
Gymnoascus reticulatus Zukal	<2	9
Microsporum gypseum complex (Bodin) Guiart & Grigorakis	1.8 x 10^4	227
Trichophyton ajelloi (Vanbreuseghem) Ajello	4	4
Trichophyton terrestre complex Durie & Frey	>3.8 x 10^6	1.3 x 10^6
Tel. *Arthroderma quadrifidum* Dawson & Gentles	7.9 x 10^3	2.9 x 10^5
Non-keratinolytic fungi		
Aspergillus fumigatus Fres.	1.7 x 10^5	2.7 x 10^5
Candida sp.	<2	4
Fusarium oxysporum Schlecht.	9	1.6 x 10^4
Fusarium solani complex (Mart.) Saccardo	121	<2
Mycelia sterilia (brown)	16	73
Paecilomyces lilacinus (Thom) Samson	2.7 x 10^5	4.3 x 10^5
Penicillium janthinellum Biourge	80	<2
Penicillium nigricans Bain. ex Thom	<2	16
Penicillium cyclopium (West.) Samson, Stolk & Hadlok	4	<2
Phialophora cinerescens (Wollenw.) van Beyma	<2	5.3 x 10^5
Plectosphaerella cucumerina (Lindf.) W.Gams	2.7 x 10^3	2.8 x 10^5
Pseudallescheria boydii complex (Shear) McGinnis et al.	15	3.8 x 10^3
Trichoderma koningii Oudemans	<2	797
Verticillium chlamydosporium Goddard	4	<2
Verticillium lecani (Zimm.) Viegas	1.6 x 10^5	2.0 x 10^5
Verticillium psalliotae Treschow	9	<2

It has been demonstrated that the mycobiota can be used to indicate the sludge organic matter stabilization process [3, 6, 18]. The concept was based on the composition of keratinolytic fungi at 23ºC. At this temperature, *T. terrestre* complex with its teleomorph A. quadrifidum favors sludges with organic carbon content <20% d.w. and C:N ratio between 10-15, while most Chrysosporium species, especially *C. keratinophilum*, occur more frequently in sludges with higher C and N contents and C:N ratio <10. In addition, the *Chrysosporium* species are alkalophilic in nature

and resistant to ammonium released in high amounts by the activity of proteolytic microorganisms. In the sludge examined, the prevalence of *T. terrestre* complex and its teleomorph *A. quadrifidum* at 23ºC (evidenced by both hair baiting and MPN methods) along with the organic carbon content 17.4% d.w., C:N ratio 9.6 and low ammonium concentration (42 mg N-NH4/kg d.w.) testify that the sludge organic matter stabilization process was highly advanced.

Some of the fungi such as *M. gypseum* and *P. boydii* complexes together with *A. fumigatus* have higher pathogenic potential compared to others [36, 37]. Attention should also be paid to *C. zonatum*, which occurred in the sludge with high MPN values. According to Sigler et al. [29] and Sigler [38], the fungus can colonize lungs and cause deep mycoses in immunocompromised patients. Hayashi et al. [39] also described a case of lung mycosis caused by the fungus in a patient with healthy immunological system. The pathogenicity of *C. zonatum* has been recently confirmed in experimentally infected mice [40]. Although the number of mycoses caused by *C. zonatum* is low, the appearance of the fungus on the list of pathogenic fungi reflects the general increase in the number of opportunistic mycoses. This increase may be associated with increasing contamination of the environment with organic waste, including keratinous remnants and xenobiotics, and with more and more favorable conditions for growth and expansion of opportunistic fungi.

The fungal pathogenic potential is dependent of a variety of factors, including spore type and their quantities required to initiate infection. However, the so-called minimal infection doses have only been determined for the dermatophytes and some other fungal pathogens [40, 41]. For instance, the MIDs for saprophytic spores of the dermatophytes, including *M. gypseum* complex, vary from 1×10^4 to 15×10^6. If the conventional hair-baiting data obtained at 23ºC are taken for consideration, one can conclude that the sludge was free of *M. gypseum* complex. However, the dermatophyte grew abundantly in the hair at 29ºC (Fig. 1). Thus, the results obtained at 23ºC can be confusing. As mentioned earlier, the conventional hair baiting method provides extensive data on fungal growth in the hair spread over the sludge. In the context of the qualitative character and, paradoxically, high selectiveness of the method, however, the data appear to be of moderate epidemiological significance. In fact the MPN method is not a precise tool, but a high number of repetitions (10) with incubation at four temperatures considerably improves the precision of the measurement [31]. In the examined sludge, the MPN of *M. gypseum* was 1.8×10^4/100 g d.w. (on clay). Such a quantity of the dermatophyte (at the lowest MID level) along with high quantities of other potentially pathogenic fungi cannot be ignored from the epidemiological point of view.

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