Polish J. Environ. Stud. Vol. 15, No. 2 (2006), 341-346

Original Research

Sludge Liming Decreases the Growth of Keratinolytic and Keratinophilic Fungi

K. Ulfig

Institute for Ecology of Industrial Areas, 40-832 Katowice, Kossutha St. 6, Poland

Received: March 5, 2005
Accepted: July 19, 2005

Abstract

Due to the content of pathogenic organisms, including fungi, sewage sludge land application poses risks to both human health and the environment. One of the ways for reducing pathogens in sludge is liming. This study aims to determine the effect of sludge liming on the composition of keratinolytic and keratinophilic fungi in model experiments. The fungi were examined using the hair baiting method and the dilution method with incubation on a Wiegand medium supplemented with chloramphenicol (100 mg/L) and actidione (500 mg/L). The sludge liming considerably decreased the number of actidione-resistant fungi propagules and eliminated many fungal species, including *Pseudallescheria boydii*. The influence of this process on hair-baited fungi was that the liming eliminated keratinolytic and keratinophilic fungi at 37°C. In the range of 23-33°C, the liming considerably restricted the growth of keratinolytic fungi, including *Microsporum gypseum*, but only slightly affected keratinophilic fungi, including *Pseudallescheria boydii*. The sludge liming decreases the risk posed by geophilic dermatophytes and other keratinolytic fungi, as well as by keratinophilic fungi to humans and the environment. The process affected more keratinolytic fungi than keratinophilic ones.

Keywords: keratinolytic and keratinophilic fungi, sewage sludge, liming, health and environmental risks

Introduction

Keratinolytic fungi specialize in the decomposition of keratin, being the main component of keratinous substrata. Keratinophilic fungi associate keratinolytic fungi, utilizing non-protein components of the substrata or the products of keratin decomposition [1]. Since many keratinolytic and keratinophilic fungi have been found to be the agents responsible for human mycoses [2], and since the fungi occur in abundance in sewage sludge and sludge-amended soils, studies on fungal incidence in these environments are of hygienic, epidemiological and ecological significance. A recent report [3] demonstrated that many environmental factors affect keratinolytic and keratinophilic fungi in sewage sludge. It was also found that under model conditions, sludge alkalization (to pH 9) and acidification (to pH 4) considerably altered the composition of these fungi [4]. However, liming is often used for sludge hygienization [5] but no data have been found on the effectiveness of this process in reducing fungal pathogens in the sludge. Therefore, the study was to determine the effect of sewage sludge liming on the composition of keratinolytic and keratinophilic fungi in model experiments. An attempt to assess health and environmental risks posed by the fungi also was performed.

Material and Methods

Sewage sludge from the Bytom-Miechowice wastewater treatment plant was used in the experiments. It was excess sludge, after extended aeration (without primary

*e-mail: ulf@ietu.katowice.pl

Table 1. The composition of keratinophilic and keratinophilic fungi in the limed sludge and the control sludge.

Fungal species	Number of fungal appearances	
	Limed sludge	Control sludge
Keratinolytic fungi		
Chrysosporium anamorph of *Aphanoascus clathratus* Cano & Guarro	11	24
Microsporum gypseum (Bodin) Guiart & Grigorakis	3	27
Arthrographis kalrai (Tewari & Macpher.) Sigler& Carmichael	1	14
Trichophyton terrestre Durie & Frey	-	10
Teleomorph *Arthroderma quadrifidum* Dawson & Gentles	-	10
Chrysosporium keratinophilum D.Frey ex Carmichael	5	4
Chrysosporium zonatum Al-Musallam & Tan	1	5
Chrysosporium an. *Aphanoascus reticulisporus/fulvescens*	3	2
Gymnoascus petalosporus (Orr *et al*.) v. Arx	-	2
Keratinophilic fungi		
Pseudallescheria boydii (Shear) McGinnis *et al.*	25	24
Fusarium solani (Mart.) Saccardo	-	11
Phialophora melinii (Nannf.) Conant	5	-
Paecilomyces lilacinus (Thom) Samson	-	3
Aspergillus fumigatus Fresenius	-	3
Narasimhella hyalinospora (Kuehn *et al*.) v. Arx	2	1
Mycelia sterilia (white)	1	1
Paecilomyces marquandii (Massee) Hughes	-	1
Trichoderma koningii Oudem.	-	1

settling tank) and the integrated biological C and N removal process in the Biomix system, dewatered by centrifuging, and piled with plant residues for 1-2 years. These processes provided the sludge with stabilized organic matter and kept it free of *Salmonella* and helminth ova. Methods for sludge sampling and preparation for model experiments, as well as sludge physico-chemical characteristics, were presented elsewhere [3, 4].

Prior to the final experiment, several preliminary experiments were performed to determine the quantity of burnt lime (CaO) to maintain sludge pH of over 12 for at least two hours and over 11.5 for the following 22 hours. The quantity was 63 g CaO per 1 kg of dry sludge. The limed sludge moisture was ca. 40%. After the lime quantity determination, two autoclaved (at 121°C for 20 minutes) glass beakers were prepared. Dry sludge, autoclaved redistilled water, and burnt lime in a quantity to reach the appropriate pH and 40% of moisture were put into the first beaker. Dry sludge and autoclaved redistilled water in a quantity to reach 40% of moisture were put into the second beaker. This sludge served as control. The sludges in beakers were thoroughly mixed. The pH values were 12.5 and 6.4 after two hours and 12.3 and 6.3 after 24 hours for the limed sludge and the control sludge, respectively.

Keratinolytic and keratinophilic fungi were examined in sludges using the hair-baiting method [6]. Petri dishes were filled with 40 g of sludge and covered with 0.4 g of detergent-defatted, fine cut, and autoclaved children's hair each, and incubated in the dark at 23, 29, 33 and 37°C for four months. Ten dishes responded to each temperature and sludge sample. During incubation, stable moisture conditions (ca. 40%) were maintained in the dishes. The fungal growth indices were as follows: number of appearances; isolation frequency (number of Petri dishes positive for fungal growth*100/total number of Petri dishes set up); and the number of species.

Qualitative and quantitative compositions of actidione-resistant fungi were also examined in the limed sludge and the control sludge. Dilutions in physiological saline and the Wiegand medium supplemented with chloramphenicol (100 mg/L) and actidione (500 mg/L), with incubation in the dark at 23 and 37°C were used. The number of actidione-resistant fungi (CFU/g d.w.) was determined for each incubation temperature. From among fungal propagule numbers obtained at two temperatures, higher values

Table 2. The growth indices of keratinophilic and keratinophilic fungi in the limed sludge and the control sludge.

Fungal growth indices	Limed sludge	Control sludge
Keratinolytic fungi		
Number of appearances	24	98
Isolation frequency (%)	32.5	100
Number of species	6	8
Keratinophilic fungi		
Number of appearances	33	45
Isolation frequency (%)	67.5	85
Number of species	4	8

were presented for each species. Pure fungal strains were identified to the species level using selected taxonomic monographs [7-11]. The in vitro hair degradation test was that of Ulfig et al. [12].

After the experiment, sludge pH was measured in three Petri dishes for each sample and incubation temperature. Before the pH measurement, sludge from each Petri dish was thoroughly mixed in a plastic container.

Results

The data on the influence of sewage sludge liming on keratinolytic and keratinophilic fungi are presented in Tables 1 and 2. A total of 98 keratinolytic fungi appearances belonging to at least eight species were observed in the control sludge. Keratinolytic fungi were found in all Petri dishes (isolation frequency 100%). The sludge liming considerably decreased the number of keratinolytic fungi appearances, isolation frequency and the number of species. The process eliminated *Trichophyton terrestre* with its teleomorph *Arthroderma quadrifidum* and decreased the number of *Microsporum gypseum, Chrysosporium* anamorph of *Aphanoascus clathratus*, *Arthrographis kalrai* and *Chrysosporium zonatum* appearances.

A total of 45 keratinophilic fungi appearances were observed in the control sludge. The effect of sludge liming on hair-baited keratinophilic fungi was small. The sludge liming had no effect on *Pseudallescheria boydii*. However, the process eliminated *Fusarium solani*, *Paecilomyces lilacinus*, *Aspergillus fumigatus* and some other species from the sludge. *Phialophora melinii* was isolated exclusively from the limed sludge.

The influence of sludge liming on keratinolytic fungi growth indices was observed at four incubation temperatures (Table 3). The sludge liming decreased the number of fungal appearances, isolation frequency and the number of species at 23, 29 and 33°C, and eliminated keratinolytic fungi at 37°C.

The results on the influence of sludge liming on the quantitative and qualitative composition of actidione-resistant fungi are presented in Table 4. In the control sludge, the numbers of actidione-resistant fungi were 9.2E+05 and 7.5E+04 CFU/100 g d.w. (dry weight) at 23 and 37°C, respectively. The sludge liming decreased these numbers to 8.8E+02 and 8.2E+03 CFU/100 g d.w. at 23 and 37°C, respectively. The number of species in the limed sludge and in the control sludge was six and eight, respectively. The process considerably decreased the number of *Trichophyton terrestre*, which predominated in the control sludge, and eliminated four keratinolytic species, i.e., *Arthrographis kalrai*, *Chrysosporium* anamorph of *Aphanoascus reticulisporus*/*fulvescens* and *Gymnoascus reticulatus,* and three keratinophilic species, i.e., *Penicillium nigricans*, *Paecilomyces lilacinus* and *Pseudallescheria boydii*. Keratinolytic *Chrysosporium* anamorph of *Aphanoascus clathratus*, *Chrysosporium zonatum* and *Gymnoascus petalosporus* and keratinophilic *Beauveria bassiana* were exclusively isolated from the limed sludge. The sludge liming slightly increased the number of *Phialophora melinii*.

The control sludge pH in H_2O decreased on average from 6.4 to 5.2-5.6 during four months of the experiment. Subsequently, the limed sludge pH decreased from 12.2 to 8.5-8.8 during the same time.

Discussion

The majority of keratinolytic and keratinophilic fungi is included in two biosafety level categories: BSL-1 and BSL-2 [13]. The BSL-2 fungi are opportunists; belonging to the dermatophytes and other fungal groups and posing a higher risk to man than the BSL-1 fungi does. In earlier studies [3, 14-18] and in the present one, neither zoophilic nor anthropophilic dermatophytes have been recorded in sludges. It was found, however, that the zoophilic dermatophytes did not survive in sludge longer than one month and the dermatophytes were strongly affected by environmental factors [19-20]. Since the ability of anthropophilic dermatophytes to survive outside the host was found not to exceed eight days, the survival of these fungi was not

Table 3. The influence of sewage sludge liming on keratinolytic fungi growth indices at four temperatures.

Sample	Fungal growth indices	Temperature			
		23°C	29°C	33°C	37°C
Limed sludge	Number of appearances	11	10	3	-
	Isolation frequency (%)	40	70	20	-
	Number of species	4	4	2	-
Control sludge	Number of appearances	44	23	15	16
	Isolation frequency (%)	100	100	100	100
	Number of species	6	5	3	3

Table 4. The influence of sewage sludge liming on the composition of actidione-resistant fungi (on Wiegand medium supplemented with chloramphenicol 100 mg/l and actidione 500 mg/l).

Fungal species	Incubation temperature (°C)	Fungal number (CFU/100 g d.w.)	
		Limed sludge	Control sludge
Trichophyton terrestre Durie & Frey*	23	7.8E+02	5.0E+05
Penicillium nigricans Bain. Ex Thom	23	-	4.0E+05
Arthrographis kalrai (Tewari & Macpher.) Sigler & Carmichael*	37	-	6.3E+04
Paecilomyces lilacinus (Thom) Samson	23	-	2.0E+04
Chrysosporium anamorph of *Aphanoascus clathratus* Cano & Guarro*	37	5.0E+03	-
Pseudallescheria boydii (Shear) McGinnis *et al*.	37	-	5.0E+03
Chrysosporium an. *Aphanoascus reticulisporus*/*fulvescens**	37	-	4.5E+03
Phialophora melinii (Nannf.) Conant	37	2.2E+03	2.0E+03
Chrysosporium zonatum Al.-Musallam & Tan*	37	1.0E+03	-
Gymnoascus reticulatus Zukal*	23	-	1.0E+02
Beauveria bassiana (Bals.) Vuillemin	23	5.0E+01	-
Gymnoascus petalosporus (Orr *et al*.) v. Arx*	23	5.0E+01	-

* - keratinolytic species; CFU – colony forming units; d.w. – dry weight

examined in sewage sludge [21]. The data testify to that, generally, sewage sludge does not favor the survival of zoophilic and anthropophilic dermatophytes. Thus, the health risk posed by these dermatophytes to man and animals, by direct contact with sludge or sludge-amended soil, appears to be minimal.

Except for the geophilic dermatophyte, *Microsporum gypseum*, the other keratinolytic fungi isolated from sewage sludge have been rarely recorded as agents responsible for mycoses [2]. From the epidemiological point of view, therefore, the above-mentioned dermatophyte should not be considered equally with the other fungi. Sewage sludge contains high amounts of keratinous remnants of human and animal origin, which provide favorable conditions for the growth of keratinolytic fungi, including *Microsporum gypseum*. It is also possible that apart from keratinous remnants, temperature and other physical and chemical factors, the production of gypsetin and other secondary metabolites favor the prevalence of the fungus in sewage sludge [22]. It can be assumed that in sludge and sludge-amended soils the inocula of this fungus are sufficient to initiate infection and disease.

Pseudallescheria boydii (anamorph: *Scedosporium apiospermum*) is a fungus of increasing epidemiological significance; occurring in sewage, soil, manure, sewage-polluted water and sewage sludge [2, 23-25]. In a previous study [3] and in the present one, *Pseudallescheria boydii* was found to be the most common keratinophilic species in sludges examined, with the incidence associated with the incidence of Microsporum gypseum. This association may be of epidemiological significance.

Aspergillus fumigatus is another keratinophilic spe-

cies of increasing epidemiological significance [26]. The species occurs in composts and other environments, in which temperatures reach 40-60°C or higher [5]. In the present study, the number of *Aspergillus fumigatus* was low. However, the fungus was found to prevail on hair at 45°C [6]. It can be assumed that the abundance of keratin remnants and high temperatures favor the incidence of the fungus in sewage sludge.

Keratinolytic and keratinophilic fungi are widely distributed but, except for soils receiving organic waste containing keratinous remnants, do not occur abundantly in the soil environment [27]. As mentioned earlier, sludge-amended soils receive high amounts of keratinous remnants. Therefore, a separate ecological problem is to explain the impact of sludge land application, including high keratinolytic and keratinophilic fungi quantities and activity, e.g., mycotoxin production, on soil microbial consortia, including their composition, homeostasis and biodiversity.

Liming using burnt lime is considered the most efficient method for eliminating many pathogens, mainly bacteria and some viruses, from sewage sludge [28, 29]. In the available literature, however, no data on the influence of liming on fungal pathogens in sewage sludge was found. In the present study, two aspects of the influence of the sludge liming process on fungal composition were demonstrated. On the one hand, liming considerably decreased the number of actidione-resistant fungi propagules and eliminated many fungal species, including *Pseudallescheria boydii*. On the other hand, the influence of liming on hair-baited fungi was different. In fact, the process eliminated keratinolytic and keratinophilic fungi at 37°C. In the range of 23-33°C, the liming considerably restricted the growth of keratinolytic fungi, mainly *Microsporum gypseum*. However, the process affected keratinophilic fungi, including *Pseudallescheria boydii*, to a much smaller degree. The different selectivity of the methods along with pH decrease after sludge liming, which enabled partial recolonization of the sludge by fungi, could cause the differences. The general conclusion is that sludge liming decreases the risk posed by geophilic dermatophytes and other keratinolytic fungi, as well as by keratinophilic fungi to humans and the environment. The process affected more keratinolytic fungi than keratinophilic ones.

Acknowledgements

The study was performed within a KBN grant (6P04G0812). The author wishes to thank Mrs. I. Biedroń for her excellent technical assistance.

References

1. MAJCHROWICZ I., DOMINIK T. Further contribution to the knowledge of keratinolytic and keratinophilic soil fungi of the region of Szczecin – keratinolytic and keratinophilic fungi in the immediate surroundings of cattle. Ekol. Pol. (Seria A) **17**, 87, **1969**.
2. DE HOOG G.S., GUARRO J., GENE J. AND FIGUERAS M.J. Atlas of Clinical Fungi; Centraalbureau voor Schimmelcultures & Universitat Rovira i Virgili: Utrecht, Reus, **2000**.
3. ULFIG K. Factors influencing the occurrence of keratinolytic and keratinophilic fungi in sewage sludge. Mycotechnology: Current Research and Future Prospectives, S. Deshmukh Ed., Oxford IBH (in press), **2005**.
4. ULFIG K. The effect of sewage sludge alkalization and acidification on keratinolytic and keratinophilic fungi. Pol. J. Environ. Studies (in press), **2005**.
5. DUMONTET S., DINEL H., BALODA S.B. Pathogen reduction in sewage sludge by composting and other biological treatments: a review. Biol. Agricul. Horticul. **16**, 409, **1999**.
6. ULFIG K. Studies of keratinolytic and keratinophilic fungi in sewage sludge by means of a multi-temperature hair baiting method. Polish J. Environ. Studies **12**, 461, **2003**.
7. SIGLER L., CARMICHAEL J.W. Taxonomy of Malbranchea and some other Hyphomycetes with arthroconidia. Mycotaxon **4**, 349, 1976.
8. RAPER K.B., FENNEL K.D. The genus Aspergillus. R.E. Krieger Publishing Company: Huntington, New York, **1977**.
9. VAN OORSCHOT C.A.N. A revision of Chrysosporium and allied genera. Studies in Mycology **20**, 1, **1980**.
10. DOMSCH K.H., GAMS W., TRAUTE-HEIDI A. Compendium of Soil Fungi. Academic Press: London-San Francisco, **1980**.
11. CANO J.F., GUARRO J. The genus Aphanoascus. Mycol. Res. **94**, 355, **1990**.
12. ULFIG, K., GUARRO, J., CANO, J., GENÉ, J., VIDAL, P., FIGUERAS, M.J., ŁUKASIK, W. A preliminary study of the occurrence of actidione-resistant fungi in sediments of Catalonian river mouths (Spain). I. Keratinolytic fungi and related Onygenales. Mycopathologia **141**, 143, **1998**.
13. DE HOOG G.S. Risk assessment of fungi reported from humans and animals. Mycoses **39**, 407, **1996**.
14. ULFIG K., KORCZ M. Isolation of keratinophilic fungi from sewage sludge. Sabouraudia **21**, 247, **1983**.
15. ULFIG K. Keratinophilic fungi in sewage and waters (in Polish). Ochrona Środowiska (Wydawnictwo PZITS, Wrocław) **488/3**, 43, **1986**.
16. ULFIG K. Keratinophilic fungi in sewage sludge (in Polish). Roczn. PZH **42**, 309, **1991**.
17. ULFIG K., KORCZ M. Keratinolytic fungi in sewage sludge applied to devastated urban soil. A preliminary experiment. Inter. J. Environm. Health Res. **4**, 244, **1994**.
18. ULFIG K., TERAKOWSKI M., PŁAZA G., KOSAREWICZ O. Keratinolytic fungi in sewage sludge. Mycopathologia **136**, 41, **1996**.
19. ULFIG K. A preliminary study on the growth and survival of selected pathogenic dermatophytes on bottom sediments and sewage sludge (in Polish). Roczn PZH **37**, 336, **1986**.
20. ULFIG K. The growth and survival of selected pathogenic dermatophytes in bottom sediments and sewage sludge (in Polish). Roczn. PZH **39**, 151, **1988**.

21. GRIN E.I., OŽEGOVIĆ I. Influence of the soil on certain dermatophytes and their evolutionary trend. Mycopathol. Mycol. Appl. **21**, 23, **1963**.
22. DESHMUKH S.K., AGRAWAL S.C. Biology of keratinophilic fungi and related dermatophytes. Microbes: For Health, Wealth and Sustainable Environment, A. Varma Ed.; Malhotra Publishing House: New Delhi, pp 253-272, **1998**.
23. COOKE W.B. The effect of sludge additives on soil fungus populations. Mycopathol. Mycol. Appl. **44**, 205, **1971**.
24. ABDEL-HAFEZ A.I.I., EL-SHAROUNY H.M.M. The occurrence of keratinophilic fungi in sewage sludge from Egypt. J. Basic Microbiol. **30**, 73, **1990**.
25. ALI-SHTAYEH M.S., JAMOUS R.M.F., ABU-GHDEIB S.I. Ecology of cycloheximide-resistant fungi in field soils receiving raw city wastewater or normal irrigation water. Mycopathologia **144**, 39, **1999**.
26. STEVENS D.C., KAN V.L., JUDSON M.A., MORRISON V.A., DUMMER S., DENNING D.W., BENNETT J.E., WALSH T.J., PATTERSON T.F., PANKEY G.A. Practice guidelines for diseases caused by Aspergillus. Clinical Infections Diseases **30**, 696, **2000**.
27. ULFIG K. The occurrence of keratinolytic fungi in waste and waste contaminated habitats. Biology of Dermatophytes and other Keratinophilic Fungi, R.K.S. Kushwaha and J. Guarro Eds.; Revista Iberoamericana de Micología, pp 44-50, **2000**.
28. STRAUB T.M., PEPPER I.L., GERBA C.P. Hazards from pathogenic microorganisms in land-disposed sewage sludge. Rev. Environm. Contam. Toxicol. **132**, 55, **1993**.
29. DUMONTET S., SCOPA A., KERJE S., KROVACEK K. The importance of pathogenic organisms in sewage and sewage sludge. J. Air & Waste Management Assoc. **51**, 840, **2001**.