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KRZYSZTOF ULFIG[1], GRAŻYNA PŁAZA[2], KATARZYNA JANDA-ULFIG[3]

THE GROWTH OF PROTEOLYTIC MICROORGANISMS AFFECTS KERATINOLYTIC FUNGI IN SEWAGE SLUDGE

WPŁYW MIKROORGANIZMÓW PROTEOLITYCZNYCH NA GRZYBY KERATYNOLITYCZNE W OSADACH ŚCIEKOWYCH

[1] Polymer Institute, Technical University of Szczecin,
Pułaskiego St. 10, 70-322 Szczecin
Head: prof. dr hab. inż. *T. Spychaj*
[2] Institute for Ecology of Industrial Areas, Kossutha St. 6, 40-832 Katowice
Head: dr hab. inż. *J. Skowronek*
[3] Department of Microbiology and Environmental Biotechnology
Agriculture University of Szczecin, Słowackiego St. 17, 71-434 Szczecin
Head: prof. dr hab. *A. Nowak*

The study was to demonstrate the effect of proteolytic microorganisms on growth and composition of keratinolytic fungi in sewage sludge. In the sludge solidified with agar medium, high peptone concentrations and the associated growth of proteolytic microorganisms considerably restricted the growth of keratinolytic fungi. The antibiotics (chloramphenicol and actidione) added to the medium inhibited, to a high degree, the growth of proteolytic microorganisms and enabled many keratinolytic fungi to grow.

Key words: keratinolytic fungi, sewage sludge, proteolytic microbiota, antibiotics
Słowa kluczowe: grzyby keratynolityczne, osady ściekowe, proteolityczne mikrobiota, antybiotyki

INTRODUCTION

Keratinolytic fungi are specialized in decomposition of keratin, being the main component of keratinous substrata, while keratinophilic fungi (also known as non-keratinolytic fungi) accompany keratinolytic fungi, utilizing non-protein components of these substrata or the products of keratin decomposition [5]. Since many keratinolytic and keratinophilic fungi have been recorded the agents responsible for human and animal mycoses [3], and since these fungi occur abundantly in sewage sludge and sludge-amended soils, studies on fungal incidence in the above-mentioned environments are of epidemiological and ecological significance.

Temperature, pH, moisture, ammonium nitrogen, organic carbon and total nitrogen, C:N ratio, total sulfur, C:S ratio, available phosphorus, particle size distribution and oxygen supply have all been found to affect keratinolytic fungi and associated keratinophilic fungi in sewage

sludge [10, 12-14]. In addition, liming considerably alters fungal composition in the sludge environment [11]. The quoted studies have also indicated that sludge proteolytic microbiota affect the fungi. The present study was to demonstrate the effect of proteolytic microbiota on growth and composition of keratinolytic fungi in sewage sludge.

MATERIAL AND METHODS

In the present study, sewage sludge from the Bytom-Miechowice municipal wastewater treatment plant (Upper Silesia, Poland) was used. 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. The sludge was crumbled, dried in open air for ca. 14 days and thoroughly mixed. Stabilized organic matter characterized the sludge [10, 12]. Two sludge samples were used in two experiments.

The three-layer hair baiting method (1st layer - agar medium-solidified sludge; 2nd layer - agar medium-sludge cover, and 3rd layer – child hair) was originally applied in this study. Glass crystallizers (300-mL of volume, 5.5-cm high and with a 9-cm diameter) were used in this method. Prior to the main experiment, a preliminary experiment was performed. 15-g dry sludge portions were carefully placed in sterile crystallizers. The crystallizers were divided into three parts. In the first part, sludge portions were first watered with autoclaved redistilled water to reach the moisture of ca. 40% and then covered by a 0.4-g of detergent-defatted, fine cut and autoclaved (121°C for 30 minutes) child hair each. In second and third parts, sludge portions were covered by water agar (15 g of Bacteriological agar in 1 L of redistilled water) and peptone agar (1 g of Pancreatic Digest of Casein + 15 g of Bacteriological agar in 1 L of redistilled water), respectively, and cooled down to room temperature (ca. 23°C). After solidification of the first agar medium layer, the second layer with the same medium was formed. The second layer was to completely cover sludge particles by the medium. The thickness of both medium layers was ca. 2-cm. Difco Lab. (Michigan) provided the microbiological components of the media. In each crystallizer, a 0.4-g portion of sterilized child hair was spread over the surface of the medium. The experiment was set up with and without addition of antibiotics. The antibiotics were chloramphenicol and actidione in concentrations of 100 and 500 mg/L, respectively.

In the main experiment, 15-g sludge portions in crystallizers were solidified and covered by agar media containing increasing peptone concentrations (0, 0.5, 1, 2, 5 and 10 g/L of Pancreatic Digest of Casein, Difco), with or without addition of the above-mentioned antibiotics. The crystallizers with the medium without peptone served as control. Ten crystallizers were set up for each variant of the experiments.

The incubation was conducted in the dark for two months at 23°C. After one and two months of incubation, microscopic observations of hair and inoculations of hair attacked by fungi on Sabouraud 1:10 + mineral salts (TK medium) [9], supplemented with chloramphenicol (100 mg/L) and actidione (500 mg/L) were performed. The inoculated TK dishes were incubated at 23 and 37°C for 10 days. The rule was accepted that the growth of a given species on hair, confirmed by its growth on TK medium with antibiotics meant one occurrence of the species in a given *Petri dish*. Pure fungal strains were identified to the species level using selected taxonomic monographs [1, 2, 6-8]. The fungal growth indices were as follows: number of occurrences; isolation frequency (number of *Petri* dishes positive for fungal growth*100/total number of *Petri* dishes set up); and the number of species.

RESULTS

122 fungal occurrences belonging to at least eight species were observed (tables I and II) in the preliminary experiment. *Trichophyton terrestre*, with its teleomorph *Arthroderma*

quadrifidum (62.2%), and *Microsporum gypseum* (19.5%) prevailed in the sludge. The other species were noticed with frequencies <10%.

Table I. The effect of sewage sludge supplementation with antibiotics (chloramphenicol and actidione in concentrations of 100 and 500 mg/L) and/or solidification with water or peptone (1 g/L) agar on qualitative and quantitative compositions of keratinolytic fungi. Results of the preliminary experiment
Wpływ dodatku antybiotyków (chloramfenikol i aktidion w stężeniach 100 i 500 mg/L) i/lub zestalenia osadu ściekowego agarem wodnym lub agarem zawierającym pepton (1 g/L) na składy jakościowe i ilościowe grzybów keratynolitycznych. Wyniki doświadczenia wstępnego

Fungal species	Number of fungal occurrences					
	Sludge	Sludge + antibiotics	Sludge + water agar	Sludge + water agar + antibiotics	Sludge + peptone agar	Sludge + peptone agar + antibiotics
Trichophyton terrestre Durie & Frey	9	7	10	10	7	8
Teleomorph *Arthroderma quadrifidum* Dawson & Gentles	4	7	6	10	7	6
Microsporum gypseum (Bodin) Guiart & Grigorakis	6	1	-	1	1	7
Chrysosporium keratinophilum D. Frey ex Carmichael	-	-	-	-	4	3
Chrysosporium anamorph of *Aphanoascus reticulisporus/fulvescens*	-	-	-	-	-	2
Microsporum fulvum Uriburu	-	-	-	-	2	-
Gymnoascus petalosporus (Orr et al.) v. Arx	-	-	-	-	-	2
Chrysosporium anamorph of Aphanoascus clathratus Cano & Guarro	-	-	1	-	-	-
Trichophyton ajelloi (Vanbreuseghem) Ajello	-	-	1	-	-	-

The addition of chloramphenicol and actidione to the sludge slightly decreased the number of fungal occurrences and isolation frequency. No significant changes in the number of *Trichophyton terrestre* (tel. *Arthroderma quadrifidum*) occurrences were observed. However, the number of *Microsporum gypseum* occurrences was found to be lower after the addition of the antibiotics. In the sludge solidified with water agar, both with and without antibiotics, no significant changes in the number of *Trichophyton terrestre* occurrences were noticed compared to the sludge non-solidified with agar media. Single occurrences of the *Chrysosporium anamorph* of *Aphanoascus clathratus*, *Trichophyton ajelloi* and *Microsporum gypseum* were observed in sludges solidified with water agar non-supplemented and supplemented with antibiotics, respectively. The addition of pepton (1 g/L) to the medium used for sludge solidification caused the growth of proteolytic microorganisms (bacteria and fungi) and increased the number of keratinolytic fungi species. Among the "new" species isolated were *Chrysosporium*

Table II. The effect of sewage sludge supplementation with antibiotics (chloramphenicol and actidione in concentrations of 100 and 500mg/L) and/or solidification with water and peptone (1-g/L) agars on keratinolytic fungi growth indices. Results of the preliminary experiment
Wpływ dodatku antybiotyków (chloramfenikol i aktidion w stężeniach 100 i 500 mg/L) i/lub zestalenia osadu ściekowego agarem wodnym lub agarem zawierającym pepton (1 g/L) na wskaźniki wzrostu grzybów keratynolitycznych. Wyniki doświadczenia wstępnego

Fungal growth indices	Sludge	Sludge + antibiotics	Sludge + water agar	Sludge + water agar + antibiotics	Sludge + peptone agar	Sludge + peptone agar + antibiotics
Number of occurrences	19	15	18	21	21	28
Isolation frequency (%)	100	70	100	100	100	100
Number of species	2	2	3	2	4	5

keratinophilum (four occurrences) and *Microsporum fulvum* (two occurrences). In the sludge solidified with the peptone agar supplemented with antibiotics, the growth of proteolytic microorganisms was weaker, whereas the number of keratinolytic fungi occurrences and species was the highest. *Microsporum gypseum* was observed in this sludge with a relatively high number of occurrences. Among the "new" species isolated were *Chrysosporium anamorphs* of *Aphanoascus reticulisporus/fulvescens* and *Gymnoascus petalosporus*.

In the main experiment, 106 and 201 fungal occurrences belonging to at least five and ten species were observed in sludges solidified with agar media non-supplemented and supplemented with antibiotics, respectively (tables III and IV). *Trichophyton terrestre* with its teleomorph *Arthroderma quadrifidum* (62.1 and 40.6%) and *Scopulariopsis brevicaulis* (12.1 and 12.6%) were found to be the predominating species in both sludges. *Gymnoascus petalosporus* prevailed in the sludge solidified with agar media supplemented with antibiotics. The other species were noticed with frequencies <10%.

In the sludge solidified with agar medium without peptone and in the sludge solidified with agar medium containing 0.5 g of peptone, both non-supplemented with antibiotics, only *Trichophyton terrestre* with its teleomorph *Arthroderma quadrifidum* was observed. The number of fungal occurrences increased up to the peptone concentration of 2 g/L and then decreased upon higher peptone concentrations. The number of species was four in the sludge solidified with agar media containing 2-10 g of peptone. Upon the highest peptone concentration (10 g/L), the number of fungal occurrences was the lowest (11), *Trichophyton terrestre* was not observed, and *Scopulariopsis brevicaulis* was the predominating species.

In the sludge solidified with agar medium without peptone, supplemented with antibiotics, only *Trichophyton terrestre* with its teleomorph *Arthroderma quadrifidum* was observed. A two-step increase of fungal occurrences was noticed with increasing peptone concentration. The first step concerned the sludge solidified with agar media containing peptone in concentrations of 0.5 and 2 g/L (27 and 31 occurrences) and the second step included the sludge solidified with media containing peptone in concentrations of 5 and 10 g/L (49 and 45 occurrences). The number of species also increased with increasing peptone concentrations in such a two-step way.

Table III. The influence of the proteolytic microbiota growth in the agar medium-solidified sludge on the composition of keratinolytic fungi. The medium contained increasing peptone concentration (1-10 g/L)
Wpływ wzrostu mikroflory proteolitycznej w pożywce użytej do zestalenia osadu ściekowego i zawierającej wzrastające stężenia peptonu (1-10 g/L) na skład grzybów keratynolitycznych

Fungal species	Number of occurrences					
	Sludge	Sludge + 0.5-g peptone agar	Sludge + 1-g peptone agar	Sludge + 2-g peptone agar	Sludge + 5-g peptone agar	Sludge + 10-g peptone agar
Media non-supplemented with antibiotics						
Trichophyton terrestre Durie & Frey	6	10	10	8	7	-
Teleomorph *Arthroderma quadrifidum* Dawson & Gentles	6	8	9	6	7	-
Scopulariopsis brevicaulis (Sacc.) Bain.	-	-	-	-	-	8
Chrysosporium anamorph of *Aphanoascus clathratus* Cano & Guarro	-	-	-	4	1	1
Microsporum gypseum (Bodin) Guiart & Grigorakis	-	-	1	2	2	1
Chrysosporium anamorph of *Aphanoascus reticulisporus/fulvescens*	-	-	-	2	2	1
Aphanoascus reticulisporus (Routien) Hubálek	-	-	-	2	2	-
Media supplemented with chloramphenicol (100 mg/L) and actidione (500 mg/L)						
Trichophyton terrestre Durie & Frey	10	10	10	10	10	8
Teleomorph *Arthroderma quadrifidum* Dawson & Gentles	10	10	10	10	10	8
Gymnoascus petalosporus (Orr et al.) v.Arx	-	2	8	7	8	8
Scopulariopsis brevicaulis (Sacc.) Bain.	-	-	-	-	8	10
Chrysosporium anamorph of *Aphanoascus clathratus* Cano & Guarro	-	-	-	-	6	4
Malbranchea fulva Sigler & Carmichael	-	1	2	1	4	-
Chrysosporium zonatum Al-Musallam & Tan	-	3	1	1	1	2
Chrysosporium keratinophilum D.Frey ex Carmichael	-	-	-	-	2	2
Chrysosporium anamorph of *Aphanoascus reticulisporus/fulvescens*	-	-	-	-	-	2
Amauroascus mutatus (Quelet) Rammebo	-	-	-	-		
Myceliophthora sp.	-	1	-	-		

Trichophyton terrestre, with its teleomorph *Arthroderma quadrifidum*, prevailed in all sludge samples solidified with media supplemented with antibiotics. However, two occurrences of *Gymnoascus petalosporus* were observed in the sludge solidified with agar medium

Table IV. The influence of the proteolytic microbiota growth in the agar medium-solidified sludge on growth indices of keratinolytic fungi. The medium contained increasing peptone concentration (1-10 mg/L)
Wpływ wzrostu mikroflory proteolitycznej w pożywce użytej do zestalenia osadu ściekowego i zawierającej wzrastające stężenia peptonu (1-10 g/L) na wskaźniki wzrostu grzybów keratynolitycznych

Fungal growth indices	Sludge	Sludge + 0.5-g peptone agar	Sludge + 1-g peptone agar	Sludge + 2-g peptone agar	Sludge + 5-g peptone agar	Sludge + 10-g peptone agar
Media non-supplemented with antibiotics						
Number of occurrences	12	18	20	24	21	11
Isolation frequency	60	100	100	100	100	100
Number of species	1	1	2	4	4	4
Media supplemented with chloramphenicol (100 mg/L) and actidione (500 mg/L)						
Number of occurrences	20	27	31	29	49	45
Isolation frequency	100	100	100	100	100	100
Number of species	1	5	4	4	7	8

containing peptone in the concentration of 0.5 g/L. Upon higher peptone concentrations, this species prevailed in the sludge. Upon peptone concentrations of 5 and 10 g/L, *Scopulariopsis brevicaulis* also prevailed. Upon high peptone concentrations (5 and 10 g/L), the fungi from the genus Chrysosporium occurred relatively frequently the fungal diversity was the highest in the sludge.

DISCUSSION

Since the three-layer hair baiting method has been used here for the first time, the results obtained are difficult to compare with literature data. In our method, the peptone addition to the agar medium was to stimulate the growth of sludge proteolytic microbiota. The microbiota also include keratinolytic fungi, which have been demonstrated to have proteolytic properties [4]. Subsequently, the addition of chloramphenicol and actidione to the medium was to inhibit the growth of proteolytic bacteria and fungi affecting keratinolytic fungi. Finally, the use of agar medium had two purposes: (1) improvement of transport (diffusion) and action conditions for nutrients and antibiotics; and (2) identification of the fungi, which under rigorous competition conditions are able to penetrate the agar medium and then attack hair.

In our preliminary experiment, chloramphenicol and actidione added to the sludge inhibited the growth of *Microsporum gypseum*. This fungus was not able to penetrate both the water agar, with or without antibiotics, and the peptone agar without antibiotics, and to attack hair. However, the antibiotics added to the peptone agar inhibited the growth of proteolytic microbiota and stimulated *Microsporum gypseum* to grow. Thus, this dermatophyte was found to be sensitive to the activity of sludge microbiota but, interestingly, was also resistant to high

ammonium concentrations [10]. The addition of peptone to the medium stimulated the growth of *Chrysosporium keratinophilum*.

In the main experiment, another sludge sample and higher peptone concentrations were used. The number of occurrences along with other fungal growth indices increased up to the peptone concentration of 2 g/L. Upon the highest peptone concentration (10 g/L), however, the abundant growth of proteolytic bacteria and fungi eliminated most of keratinolytic fungi, including the predominating fungus, *Trichophyton terrestre* with its teleomorph *Arthroderma quadrifidum*. Under these conditions, only *Scopulariopsis brevicaulis* was able to penetrate the medium and attack hair. The antibiotics added to the peptone agar inhibited, to a high degree, the growth of sludge proteolytic microorganisms and enabled many keratinolytic fungi to grow.

It is clear from the presented data that the growth of sludge proteolytic microorganisms considerably affects the growth and composition of keratinolytic fungi, including species of epidemiological concern. The results have generally confirmed findings from a screening study [10] and from a study on the influence of different organic nitrogen sources added to sewage sludge and of ammonium content on these fungi [13].

However, the effect of direct peptone addition to the sludge (data obtained with the conventional hair baiting method) was the abundant growth of fungi from the genus *Chrysosporium*, chiefly *Chrysosporium keratinophilum*, *Chrysosporium* anamorph of *Aphanoascus clathratus* and *Chrysosporium* anamorphs of *Aphanoascus reticulisporus*/*fulvescens* [13]. In the present study, the peptone addition to the medium used for sludge solidification gave rather different fungal compositions. Although *Chrysosporium* occurrences were relatively frequent, *Scopulariopsis brevicaulis* and *Gymnoascus petalosporus* prevailed in the pepton agar-solidified sludge. These differences possibly resulted from different conditions provided by the two methods used. In the conventional hair-baiting method, the natural gas exchange between the sludge and air takes place and keratinolytic fungi grow on hair laid directly on sludge. In the three-layer hair baiting method, the gas exchange undergoes through the medium covering the sludge, what provides anoxic conditions and decreases fungal growth. The effect of anoxic conditions on keratinolytic and keratinophilic fungi was recently demonstrated by *Ulfig* et al. [14]. Moreover, in the three-layer hair baiting method keratinolytic fungi form colonies in the agar medium before they attack hair. Thus, ambient conditions, including microbial competition, are more rigorous in this method compared to the conventional hair-baiting method.

Since *Microsporum gypseum* and *Scopulariopsis brevicaulis* are both the fungi of epidemiological concern [3], the results have public health implications. The first species prevails in sludges with high total sulfur content, while the second occurs in sludges with high proteolytic activity (low degree of organic matter stabilization).

K. Ulfig, G. Płaza, K. Janda-Ulfig

WPLYW MIKROORGANIZMÓW PROTEOLITYCZNYCH NA GRZYBY KERATYNOLITYCZNE W OSADACH ŚCIEKOWYCH

Streszczenie

Badania miały na celu określenie wpływu mikroflory proteolitycznej na wzrost i skład grzybów keratynolitycznych w osadach ściekowych. W doświadczeniach modelowych zastosowano 3-warstwową metodę przynęty włosowej (warstwa I – osad zestalony pożywką agarową; warstwa II – pożywka agarowa pokrywająca warstwę I; oraz warstwa III – włosy). Pożywka agarowa zawierała wzrastające stężenia peptonu (0, 0,5, 1, 2, 5 oraz 10 g/L). Liczba szczepów i wskaźniki wzrostu grzybów rosły aż do stężenia 2 g/L peptonu w pożywce. Najwyższe stężenie peptonu w pożywce (10 g/L) spowodowało intensywny wzrost drobnoustrojów proteolitycznych i zahamowało wzrost grzybów keratynolitycznych. W tych warunkach jedynie szczepy *Scopulariopsis brevicaulis* (gatunek o znaczeniu epidemiologicznym) były zdolne do penetracji pożywki i atakowania włosów. Dodatek antybiotyków (chloramfenikolu i aktidionu w stężeniu odpowiednio 100 i 500 mg/L) w znaczącym stopniu zahamował wzrost drobnoustrojów proteolitycznych, co umożliwiło wzrost wielu grzybów keratynolitycznych.

K. Ulfig, G. Płaza, K. Janda-Ulfig

THE GROWTH OF PROTEOLYTIC MICROORGANISMS AFFECTS KERATINOLYTIC FUNGI IN SEWAGE SLUDGE

Summary

The present study was to demonstrate the effect of proteolytic microorganisms on the growth and composition of keratinolytic fungi in sewage sludge. In model experiments, the 3-layer hair baiting method (layer I – sludge solidified with agar medium; layer II – agar medium layer covering layer I; and layer III – hair) was used. The agar medium contained increasing peptone concentrations (0, 0.5, 1, 2, 5 and 10 g/L). The number of occurrences along with other fungal growth indices increased up to the peptone concentration of 2 g/L. Upon the highest peptone concentration (10 g/L), the abundant growth of sludge proteolytic bacteria and fungi inhibited the growth of most keratinolytic fungi. Under these conditions, only *Scopulariopsis brevicaulis*, the fungus of epidemiological concern, was able to penetrate the medium and to attack hair. The antibiotics (chloramphenicol and actidione in concentrations of 100 and 500 mg/L) added to the peptone agar inhibited, to a high degree, the growth of sludge proteolytic microbiota and enabled many keratinolytic fungi to grow.

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