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Keratinolytic Fungi as Indicators of Hydrocarbon Contamination and Bioremediation Progress in a Petroleum Refinery

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Abstract

Keratinolytic fungi were examined in soil heavily contaminated with petroleum waste during long-term bioremediation in a biopile at the Czechowice Oil Refinery located in southern Poland. Soil chemistry and associated microbiological parameters were monitored for 39 months to assess the bioremediation process at this site. These fungi were found to occur relatively frequently in the biopile, with the geophilic dermatophyte, *Trichophyton ajelloi* (teleomorph *Arthroderma uncinatum*) as the predominant fungal species. Fungal growth was found to depend on the concentration of petroleum hydrocarbons and their polar derivatives in soil. Results demonstrate that the keratinolytic fungi are a potential tool for assessment of soil petroleum hydrocarbon contamination and associated bioremediation progress.

Keywords: keratinolytic fungi, petroleum contamination, bioremediation, indicators

Introduction

Keratinolytic fungi are specialized in degradation of keratin, which is the main component of keratinous substrata (cornified epidermis, hair, feather, wool, horn, hoof, nails, etc.) [1]. These fungi also display lipolytic activity and remove petroleum hydrocarbons from the medium during degradation of proteins [2-4].

It was demonstrated in a previous study [5] that inhibition of growth and biomass production of keratinolytic fungi could be used as indicators of leachate toxicity during bioremediation. The incidence of these fungi also was examined in an acidic petroleum waste lagoon before bioremediation [6]. The goal of this study was to determine whether keratinolytic fungi could be used as indicators of soil petroleum hydrocarbon contamination and bioremediation progress in a biopile at the Czechowice refinery site.

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Material and Methods

The focus of this study was the bioremediation of soil heavily contaminated with petroleum waste at the Czechowice Oil Refinery site. The biopile technology designed for environmental restoration of this site was previously described [7]. In 1997-1999, the aeration and addition of mineral NPK fertilizers and the surfactant, Rokafenol N8, to the biopile considerably accelerated the biodegradation of petroleum contaminants [8]. Activities in 2000-2001 were conducted with natural aeration and application of NPK fertilizers to passively stimulate the removal of recalcitrant hydrocarbons from the biopile.

Eight sampling campaigns were conducted to monitor the bioremediation process in the biopile over a 39-month period. The first and last campaigns were performed after the 4th and 39th months of the process. During each campaign, soil was sampled at 23 locations from shallow (ca. 20 cm of depth) and deep (ca. 80 cm of depth) layers of the biopile. Samples were analyzed for petroleum hydrocarbons (TPH = Total Petroleum Hydrocarbons; measured by infrared spectrometry FT-IR with extraction in CCl_4; PB-07: 1999, PB-10: 1999, PN-82/C-04565.01, EPA Method 3620: 1992; EPA Method 8440: 1995; PN-V-04007: 1997PB-07: 1999), petroleum hydrocarbons + their polar derivatives (TPOC = Total Petroleum Organic Carbon; also measured by infrared spectrometry FT-IR with extraction in CCl_4), polyaromatic hydrocarbons (PAHs = fluoranthene + pyrene + benzo(b)fluoranthene + benzo(k)fluoranthene, benzo(a)pyrene, benzo(g,h,i)-perylene, dibenzo(a,h)anthracene, and indeno(1,2,3-cd)-pyrene; by HPLC with fluorescence detection; PB-06: 1999; PB-09: 1999; ISO/FDIS 13877: 1998), pH in H_2O (by electrometric method; PN ISO 10390: 1997), moisture (HUM; by gravimetric method; PN-75/C-04616.01), dehydrogenase activity (by TTC method; Russel [9]), microbial densities and other parameters. This paper presents selected mycologically important results.

Soil samples were examined for keratinolytic fungi using the hair-baiting method [10]. For each sample one hair-supplemented Petri dish was prepared. The weight of soil was 30 g per dish. If necessary, autoclaved redistilled water was added to the dishes to obtain ca. 20% of moisture. Each dish was then covered with 300-mg of defatted, cut and autoclaved children's hair. Dishes were incubated in the dark for 4 months at ca. 23°C. Moisture was kept stable during the 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) [11]. 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 [12-16].

Relationships between indices of fungal growth and physico-chemical parameters were determined with simple linear correlation analysis, analysis of variance (ANOVA), and cumulative frequency analysis. The Statistica 5.1 for Windows program was used for statistical analysis of data.

Results

Data on the incidence of keratinolytic fungi in the biopile are summarized in Table 1. Altogether, 346 fungal strains, belonging to 13 species, were isolated and identified. Among these species, the geophilic dermatophyte, *Trichophyton ajelloi* (teleomorph *Arthroderma uncinatum*) predominated in soil (78.3%). Other species were sporadically isolated from the soil (frequencies less than 5.5%). No statistically

Table 1. The occurrence of keratinolytic fungi in soil in the biopile during bioremediation (1997-2001).

Fungal species and indices	Number of strains or species	Frequency (%)
Trichophyton ajelloi (Vanbreuseghem) Ajello	271	78.3
Teleomorph *Arthroderma uncinatum* Dawson & Gentles	6	1.7
Chrysosporium keratinophilum D.Frey ex Carmichael	19	5.5
Chrysosporium anamorph of *Aphanoascus reticulisporus*/ *fulvescens*	8	2.3
Teleomorph *Aphanoascus reticulisporus* (Routien) Hubálek	6	1.7
Chrysosporium an. *Arthroderma curreyi* Berkeley	8	2.3
Myceliophthora vellerea (Sacc. & Speg) van Oorschot	6	1.7
Chrysosporium europae Sigler, Guarro & Punsola	6	1.7
Malbranchea fulva Sigler & Carmichael	5	1.4
Amauroascus mutatus (Quelet) Rammeloo	3	0.9
Microsporum sp.	2	0.6
Chrysosporium pannicola (Corda) van Oorschot & Stalpers	2	0.6
Scopulariopsis brevicaulis (Sacc.) Bain.	2	0.6
Microsporum gypseum (Bodin) Guiart & Grigorakis	1	0.3
Trichophyton terrestre Durie & Frey	1	0.3
Total number of strains	346	-
Total number of species	13	-

Table 2. Changes in the means of fungal indices and physico-chemical parameters during bioremediation in the biopile.

Month of bioremediation	*T. ajelloi* frequency (%)	Number of strains	Number of species	pH in H_2O	Moisture (%)	TPH (g/kg d.w.)	TPOC (g/kg d.w.)	PAHs (mg/kg d.w.)	Dehydrogenase activity (mg TF/g d.w.)
4	65.2	41	6	6.6	20.9	22.6	36.6	4.16	31.7
7	82.6	53	7	7.2	19.0	14.2	24.0	5.60	30.3
9	63.0	40	7	7.2	16.8	14.3	23.5	5.89	5.3
13	63.0	31	3	7.2	na	na	na	na	na
18	78.3	41	5	6.4	16.2	16.8	24.3	2.14	23.5
21	73.9	38	4	6.7	18.9	18.3	27.5	2.56	16.8
24	89.1	46	5	6.6	9.4	7.4	15.3	2.18	19.8
39	73.9	56	7	7.3	18.7	14.9	17.0	3.44	20.4

na – data not available
d.w. – dry weight

significant differences in fungal incidence between shallow and deep layers of the biopile were observed.

During the study, soil moisture and pH in H_2O ranged between 1.9-35.6% (mean 17.4%) and 2.64-9.94 (mean 6.95), respectively. Subsequently, TPH, TPOC and PAHs ranged between 0-116-g/kg d.w. (mean 16.1 g/kg d.w.), 0-168.6 g/kg d.w. (mean 23.8 g/kg d.w.) and 0.05-47.53 mg/kg d.w. (mean 4.12 mg/kg d.w.), respectively. The dehydrogenase activity (TTC) range was 0-287.6 mg TF/g d.w. (mean 25.8 mg TF/g d.w.).

The changes in the means of fungal indices and other parameters during bioremediation in the biopile are presented in Table 2. The bioremediation process can be divided into four major stages. The first stage occurred through the 7th month and was characterized by a considerable decrease of TPH/TPOC and a corresponding increase in fungal indices (number of strains and the frequency of *Trichophyton ajelloi*) in soil. However, an increase of contaminants was noticed between the 7th and 21st months of bioremediation (second stage). The fungal indices decreased between the 7th and 13th months to increase in the 18th and 21st months of bioremediation. The second considerable decrease of contaminants was observed between the 21st and 24th months of bioremediation (third stage of the process). An increase of fungal indices accompanied this decrease. The fourth bioremediation stage was between the 24th and 39th months of bioremediation and characterized by an increase of contaminants and the number of fungal strains, and by the decrease of the *Trichophyton ajelloi* frequency. The number of fungal species ranged between 3-7 during bioremediation. The PAH changes were less considerable than the TPH/TPOC (mostly aliphatic compounds) changes in the biopile. Factors influencing the TPH/TPOC changes in the biopile will be explained in a separate paper.

The number of strains and species and the frequency of *Trichophyton ajelloi* were negatively correlated with TPOC ($r = -0.44, -0.44, -0.47$ at $p \leq 0.05$, $n = 322$), TPH (-0.42, -0.43, -0.46), and PAHs (-0.26, -0.27, -0.33) and positively correlated with pH in H_2O (0.18, 0.18, 0.15).

The mean frequencies of *Trichophyton ajelloi* gradually decreased with increasing TPOC (Figure 1). The highest frequencies were observed at TPOC lower than 15 g/kg d.w., while no fungal strains were isolated from samples with TPOC higher than 100 g/kg d.w.. Relationships also were obtained for the number of strains and species vs. TPH and PAHs. However, a statistical difference (general ANOVA effect at $p \leq 0.05$) was the most significant for the frequency of *Trichophyton ajelloi* vs. TPOC.

Figure 2 shows relationships between cumulative frequencies for the number of *Trichophyton ajelloi* strains vs. selected parameters.

The number of strains and species increased with increasing pH. No fungi were observed at $pH \leq 3$. At pH between 3-5, the relative numbers of isolates and species were much lower than isolates and species obtained at $pH \geq 5$. *Trichophyton ajelloi* did not occur in samples with $pH \leq 4.5$.

Discussion

Keratinolytic fungi occurred relatively frequently in the biopile at the refinery, with the geophilic dermatophyte, *Trichophyton ajelloi* (teleomorph *Arthroderma uncinatum*), as the predominant species. The occurrence of the above-mentioned fungi in the biopile environment can be possibly explained by the vicinity of a human population (the refinery is centrally located in the city of Czechowice-Dziedzice), and/or activity of the refinery workers and animals inhabiting or visiting the refinery site (birds, roe-deers, and hares). The accepted hypothesis is that such a population together with workers and animals is the main supplier of keratinous debris to the soil. Subsequently, the keratinous debris is the main substratum for growth of soil keratinolytic fungi [1].

Environmental factors influencing keratinolytic fungi in the clayey soil from the refinery site and its surround-

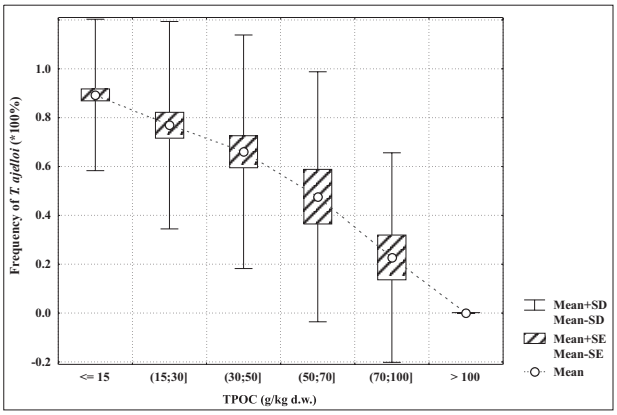

Fig.1. Relationship between the frequency of *Trichophyton ajelloi* and TPOC concentrations in soil in the biopile.

ings were explained in a previous paper [6]. However, some more attention should be paid to pH and moisture. Böhme & Ziegler [17] showed that fungal enzymatic activity is optimal at pH 6-9. Considerable inhibition of this activity was observed at pH below 4 and above 10. Our data confirmed that fungal growth is inhibited at pH < 4 and that *Trichophyton ajelloi* occurs in a wide pH range (4.5-8.5). However, the cumulative frequency plots (Figure 2) suggested no influence of soil pH and moisture on this fungus. Since most of the pH values ranged between 6-8, the *Trichophyton ajelloi* vs. pH cumulative frequency plot is quite understandable. Since most of the soil moisture values ranged between 15-20%, and since the moisture of soil in hair-supplemented Petri dishes was ca. 20%, the *Trichophyton ajelloi* vs. moisture cumulative frequency plot is also explainable.

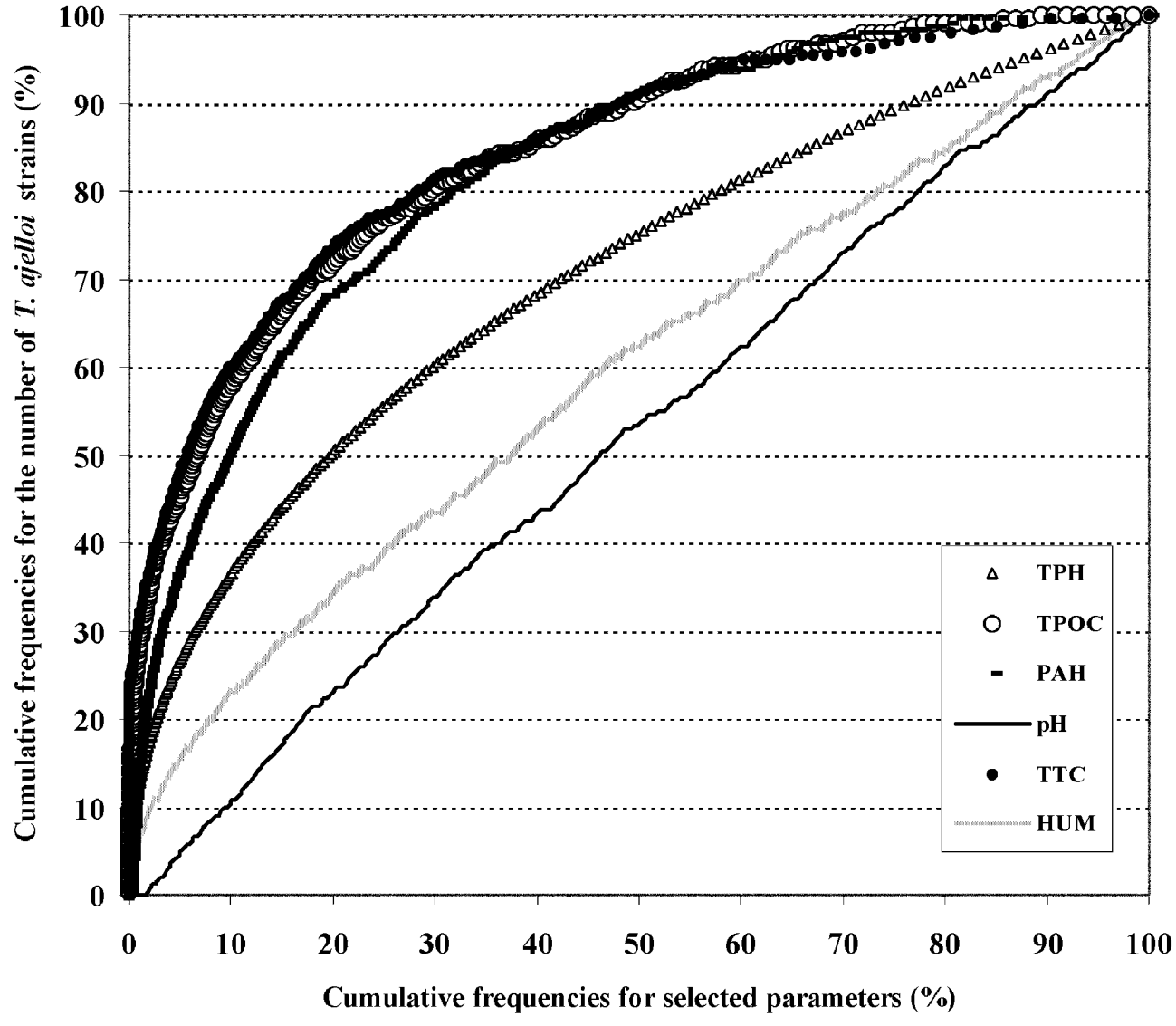

Fig. 2. Relationships between the cumulative frequencies for the total number of *Trichophyton ajelloi* strains and the cumulative frequencies for selected parameters.

The changes in the means of selected parameters during bioremediation already indicated that fungal growth depended on the concentration of petroleum contaminants in the biopile. Determining negative correlations between fungal indices and TPH, TPOC and PAHs confirmed this relationship. Subsequently, the cumulative frequency plots showed those polar products of petroleum hydrocarbon microbiological degradation (component of TPOC), PAHs and dehydrogenase activity (TTC) chiefly inhibited the growth of *Trichophyton ajelloi* in soil. Non-polar compounds (TPH) appeared to play a minor role in this inhibition. The results agree with data on the inhibitory effect of fatty acids, fats and oils against dermatophytes [18] and on the incidence of keratinolytic fungi in an acidic petroleum waste lagoon before bioremediation [6]. No information on the influence of PAHs on keratinolytic fungi was found in available literature. Generally, the keratinolytic fungi, *Trichophyton ajelloi* in particular, are a useful tool for rough assessment of hydrocarbon contamination and associated bioremediation progress of soils heavily contaminated with petroleum waste. This tool has already been used, successfully, for assessment of hydrocarbon contamination in another biopile at the same refinery site (unpublished data).

Similarly to the data obtained in leachate studies [5], however, *Trichophyton ajelloi* displayed rather moderate sensitivity to petroleum hydrocarbons and their derivatives. Still, high TPOCs (below 15-g/kg d.w.) did not affect, or affected to a minimal degree, the incidence of the fungus in soil. This moderate sensitivity may be connected with the ability of *Trichophyton ajelloi* isolates from petroleum-contaminated habitats to remove petroleum hydrocarbons from the medium during degradation of proteins [3, 4]. There is a need for finding other fungal species with higher sensitivity to petroleum hydrocarbons and their derivatives in soil.

Geophilic dermatophytes and other keratinolytic fungi are considered to be pathogens or potential pathogens to animals, including humans. Among the identified species, only *Microsporum gypseum* is more frequently found in medical laboratories. However, the biopile soil does not favor the growth of this dermatophyte; only one *Microsporum gypseum* strain was isolated from this environment. The predominating species, *Trichophyton ajelloi*, is a typical soil fungus. It causes epidermal mycoses but such cases are very rare [16]. Therefore, the biopile bioremediation process can be regarded as safe from the fungal epidemiological point of view.

Extensive testing is required to determine the applicability of the results obtained. The necessary biological databases must be established before keratinolytic fungi could be applied for environmental monitoring. These findings and potential applications could lead to more cost-effective and safe technologies to naturally monitor remediation of petroleum-contaminated soils.

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