BIODEGRADATION OF PHENOL ADSORBED ON SOIL IN THE PRESENCE OF POLYCYCLIC AROMATIC HYDROCARBONS

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ABSTRACT

Degradation of phenol and polycyclic aromatics hydrocarbons (PAHs) presented in artificially contaminated and/or real soil collected from a contaminated site of former coke plant by free cells of *Candida tropicalis* was studied in batch cultures and at the ratio of liquid culture media : soil = 2 : 1 with the initial concentration of phenol 560 mg kg^{-1} and 152 mg kg^{-1} (real soil), respectively. The results confirmed that aerobic biodegradation of phenolic compounds is a viable method for their destruction both linked on solid matrix of soil and in aqueous liquids extracted from soil. Nevertheless, in the case of mixed contamination where phenols with PAHs are presented, PAHs are mostly resistant to biodegradation. Based on that, mixed strains of *Candida tropicalis* and fungus *Phanerochaete chrysosporium* were simultaneously applied on biodegradation of both phenol and PAHs contained in aqueous slurry of contaminated soil with high efficiency. A common mixture of yeasts and white rot fungus proved to be a potent tool for effective biotreatment of contaminated site of former coke plants.

Keywords: Polycyclic artomatic hydrocarbons, Phenol, Bioremediation, *Candida tropicalis*, *Phanerochaete chrysosporium.*

INTRODUCTION

Phenols are common pollutants found in wastewaters from oil refineries, chemical plants, coke manufactures, coal gasification (Matejkova et al., 2015), and textile industries (Polat et al., 2006). Literature on phenol biodegradation introduces many microorganisms which metabolize phenolic compounds. Aerobes are more efficient at degrading because they grow faster than anaerobes and transform phenol into CO_2 and H_2O completely while the process is easy to follow. Mainly wastewaters containing phenol in the range of 5 - 1500 mg L^{-1} are considered as highly suitable for biodegradation (Rosenkratz et al., 2013). Phenol biodegradation was widely studied in the past (Levén and Schnürer, 2010; Banerjee and Ghoshal, 2011; Alemzadeh et al., 2013). Phenol has generally been considered as a model pollutant for the study of biodegradation of recalcitrant compounds. It was demonstrated that biological treatment of phenol is very effective (Wang and Loh, 1999; Zhou et al., 2016). The most commonly used microorganisms within such research were aerobes including *Pseudomonas* sp. (Kostal et al., 1998; Kim et al., 2002; Shourian et al., 2009), *Alcaligenes* sp.

(Jiang et al., 2007), *Arthrobacter* sp. (Li et al., 2016), *Comamonas* sp. (Liu et al., 2016) and *Azotobacter* sp. (Juárez et al., 2008), respectively. Namely, free or immobilized cells of *Pseudomonas putida* were used to degrade phenol (Monteiro et al., 2000; Loh et al., 2000; Reardon et al., 2000) and phenol biodegradation by freely suspended or immobilized cells of *Rhodococcus* sp. (Oh and Han, 1997; Prieto et al., 2002), *Acinetobacter* sp. (Prieto et al., 2002; Beshay et al., 2002) was reported too.

Some yeast like *Rhodotorula glutinis* and *Candida tropicalis* also efficiently utilize phenol as a sole carbon source (Chen et al., 2002; Basak et al., 2004; Varma and Gaikwad, 2009). It is *Candida tropicalis*, which shows a particular capability of effective degradation even at high concentration of phenol ranging from 1000 mg L^{-1} to 5000 mg L^{-1} (Chen et al., 2002).

However, in spite of the considerable amount of data devoted to biodegradation of phenol in simulated phenol-water mixtures, studies focusing on complex mixtures of pollutants, such as phenol/PAHs, which are typical for industrial wastewater and soils from coke manufactures, are rare. Globally, severe problems are caused by coking wastes from the coke industry. Microbial degradation of organic compounds in a mixture can be influenced by other substituents usually in a negative manner (Reardon et al., 2000). Reasons for decreased biodegradation rates include competitive inhibition and/or formation of toxic intermediates (Kryst and Karamanev, 2001). On the other side, in some cases of complex mixtures of contaminants, phenol can play a positive role as a co-substrate and its presence can enhance the rate of biodegradation of more recalcitrant compounds.

Phenol is partially soluble in water (83 g L^{-1} at standard temperature) and its water- solubility is high enough to warrant its continuous extraction from contaminated soils and sediments to the aqueous environment. However, mutual presence of BTEX (benzene, toluene, ethylbenzene and xylenes) and PAH (polycyclic aromatic hydrocarbons), which is typical for soils in coke manufactures sites, negatively influences its release from the soil. Unlike studies engaged in bioremediation of phenol in water, papers dealing with biodegradation of phenol sorbed to a solid matrix are rarely issued.

In contrast to polar organic compounds such as phenol and/or its homologues, one of the main reasons for prolonged persistence of hydrophobic hydrocarbons (particularly PAHs) in contaminated environment is their low water solubility, which increases their sorption to solid particles and limits their availability to biodegrading microorganisms (Barkay et al., 1999; Rodrigues et al., 2013).

Hence, bioremediation strategies for sites impacted by complex contamination of mixed low-molecular weight phenolic compounds and structurally more complex PAHs are needed to be developed.

Similarly to some yeast, many bacteria are capable of degrading phenol. However, biodegradation of PAHs by soil microorganisms such as *Alcaligenes*, *Mycobacterium* and various strains of *Pseudomonas* is rather low (Leblond et al., 2001; Chung and Kim, 2001). Moreover, the biodegradation potential of Candida strains regarding PAHs has not been mentioned yet (see e.g. (Juhasz and Naidu, 2000)). Therefore, investigation of biodegradation of phenol in the sites at the presence of other organic coal-tar components (BTX and PAHs) as well as the study of simultaneous biodegradation of phenol and PAHs are indeed needed.
The impact of organic coal-tar compounds on the degradation rate of phenols in aquifer is rather limited because of low concentrations of PAHs. However, high concentration of

phenols should still be present at the sites associated with coal-tar compounds in unsaturated zone (Broholm and Arvin, 2000). Probably, the mixture of PAHs and low molecular weight organics compounds (like phenol) behaves in such a way that when exposed to air and water, semi-rigid films at tar-water interfaces are formed (Luthy et al., 1993; Liu and Haderlein, 2013). These films are composed of complex high molecular weight water insoluble compounds and can reduce mass transfer rates even of all low molecular compounds with high water solubility. The sorption of PAHs to soil over time and the formation of interfacial films can significantly decrease their bioavailability and simultaneously suppress the extraction of low molecular and more water soluble compounds from soil particles to the aqueous environment (Riding et al., 2013).

Obviously, when considering phenol as a sole contaminant of soil or sediment, the rate of its leaching into the environment is remarkably high. Most attention devoted to biodegradation of phenol is therefore focused on the fate of phenol in water phase. In the case of mixed contamination of both water insoluble organic compounds and more water soluble compounds, such as PAHs and phenols in a soil, a significant portion of both phenol and PAHs can still be retained in the solid matrix for a long time. Thus, the attempt to enhance biodegradation should be primarily focused on the increase in the apparent solubility of organic compounds retained in interfacial films.

Phanerochaete chrysosporium, a well-known white rot fungus, has a strong ability to degrade various refractory and toxic organic compounds such as polycyclic aromatic hydrocarbons (Zheng and Obbard, 2002). Enzymes involved in the degradation of PAHs are oxygenases, dehydrogenases and lignolytic enzymes. They are extracellular and catalyze a radical formation by oxidation to destabilize bonds in the molecule. The addition of biosurfactant-producing bacteria and light oils can increase the bioavailability of PAHs and metabolic potential of bacterial community (Haritash and Kaushik, 2009).

Recently, white rot fungus has also been employed for biodegradation of phenolic compounds in coking wastewater. It shows rather limited efficiency towards phenolic compounds: removal of only 72.09 % in 6 days (Lu et al., 2009). García et al. (2000) also discovered limited efficiency of degradation of phenol from olive mill wastewater using *P. chrysosporium*. In their case, a considerable amount of phenol, i.e. 96 mg L^{-1}, remained in the wastewater from the original content of 1200 mg L^{-1}. It also was documented that *P. chrysosporium* depleted less than 90 % of phenol from the water (Singh, 2006).

Apparently, some bacteria and/or yeast prove to be highly efficient in degradation of phenols but less effective in destroying PAHs; on the other hand, white rot fungus prove to be very good disruptors of PAHs but their efficiency of biodegradation of more simple compounds such as phenol is mediocre. Surprisingly, utilizing bioactive synergy of mixed yeast and fungus population might possibly be efficient in biodegradation of both phenol and PAHs in contaminated soils.

The aim of the presented study was to evaluate the biodegradation potential of a comprehensively contaminated soil by both phenol(s) and PAHs under aerobic conditions with selected population of microorganisms of yeast and fungus. Particularly, the experiments were focused on the comparison of biodegradation efficiency of two different microbial populations (*Candida tropicalis* and *Candida tropicalis* with *Phanerochaete chrysosporium*).

In our experiments, an artificial soil contaminated with pure phenol and two types of real contaminated soils taken from unsaturated zone in a former coke producing plant (with PAHs only and PAHs with phenols) in soil-aqueous medium slurries in batch laboratory conditions were investigated.

MATERIALS AND METHODS

Preparation of Artificially Contaminated Soil with Phenol

3 kg of dry samples of clean soil were intensively mixed with 3 kg of aqueous solution of phenol (p.a., Lachema, Czech Republic) of 10 g L^{-1} concentration. The suspension was stored for 14 days at the temperature of 5 ^{o}C and then mechanically mixed. In another 14 days, the superfluous water evaporated at the temperature of 40 ^{o}C to the ultimate 12 % humidity. The concentration of phenols was determined at 560 mg kg^{-1} of dry material. Soil characteristics such as pH, Cox and particle size can be seen in Table 1.

Soil Used

For these experiments, a soil material from the contaminated area at the field-site of former coke and gas plants in Ostrava region (North Moravia, Czech Republic) was chosen. Two types of soil samples were collected from excavation trenches with different contents of phenolic and polyaromatic compounds, A and B, respectively. Both samples of soil were similar regarding the proportion of individual mineralogical phases and were declared as silt-loam soil with a typical higher content of clay phase. Soil characteristics are also shown in Table 1, where Cox stands for organic carbon oxidation.

Table 1 Characteristics of the artificial soil before contamination and real soil

Parameter		Unit	Artificial soil	Real soil
pH		-	7.17	6.8
Cox		%	1.01	1.41
Particle size:	0.25-2.0 mm	%	47.9	39.4
	0.05-0.25 mm	%	16.8	21.0
	0.01-0.05 mm	%	17.2	15.3
	< 0.01 mm	%	11.0	24.3
Clay phase:	< 0.002 mm	%	not determined	17.6
	< 0.001 mm	%	9.6	15.0

Candida Tropicalis Cultivation

Candida tropicalis was obtained from the Culture Collection of the Biochemical Engineering Department, University of Chemistry and Technology, Prague, Czech Republic. Yeasts were maintained on agar slants at 4^{o}C. Liquid growth media type YBN contained phenol was supplemented with (per 1 L of media): $(NH_4)_2SO_4$ 5 g, KH_2PO_4 1 g, $MgSO_4.7\,H_2O$ 0.5 g, NaCl 0.1 g (all p.a., Lach-Ner, a.s.), Yeast nitrogen base (Difco) 1.7 g, solution of amino acids (L-histidine, DL-methionine, DL-tryphtophan; all p.a., Sigma-Aldrich Co.) 1 mL, glucose 4 g, phenol 0.2 g. Culture media were sterilized at 121^{o}C for 20 min. Phenol was added after sterilization. The cultivation time was 72 hours under constant shaking of 50 rpm and at 28^{o}C. 1000 mL Erlenmayer flasks containing 200 mL of culture media were used. Microorganisms were determined by optical density at a wavelength of 600 nm and dry cell measurement (by filtering culture samples through 1.2 µm filter (Whatman) and dried at 100^{o}C subsequently).

Phanerocheate Chrysosporium Cultivation

In this study, a white rot fungus, *P.chrysosporium* from the collection of the Department of microbiology and biochemistry, University of Chemistry and Technology, Prague, Czech Republic, was used. To obtain the inoculum, fungus was cultivated in the above mentioned medium and under the same conditions (0.04 g L^{-1} ammonium tartarate was added). Cultures were established by inoculating the medium with spores as described (Bumpus, 1989). Respecting the fact that naphthalene is the most abundant PAH presented in soil, this PAH was selected to be added to the growth media. Thus, naphtalene in acetone was added to cultures of *P.chrysosporium*. Owing to that, the amount (final concentration of naphthalene was 50 mg L^{-1}) of naphthalene could be lethal to *P.chrysosporium* when added on day 0; therefore, cultures were allowed to grow before the addition of naphthalene at the atmosphere of air for 72 hours. In 3 days, the culture was flushed with oxygen, sealed, and let to incubate for another 30 days (approx. mycelium dry density was 0.015 g L^{-1}). Culture conditions were as described above (5 mL of inoculum of *P. chrysosporium* was added additionally).

Experiment Conditions

A comparative study of phenol degradation sorbed on solid matrix of soil by free cells of above mentioned microorganisms was carried out in batch cultures. Biodegradation was performed in a series of 500 mL Erlenmayer flasks, containing 200 mL of distilled water, 0.3 g Yeast nitrogen base, 0.5 g of glucose, and 100 g of prepared samples of artificially contaminated soil or samples of real soil. This mixture was inoculated with 5 mL of the inoculum (approx. cell density in culture media was 0.025 g of dry cells L^{-1}) and was incubated in a shaker at 50 rpm at 37 °C. After the course of time-intervals (12 hours), the solid content of individual flasks was separated by filtration, dried under mild conditions at 40 °C and analyzed. During the time of evaporation the vapors were collected in the solution of 1M NaOH. Alkaline solution was analyzed for a possible content of phenol. Simultaneously, the content of phenol in aqueous phase (in the filtrate) was analyzed.

Analytical Procedure

The residual phenol concentration in aqueous solutions was determined quantitatively by a modified colorimetric method using 4-aminoantipyrine as a color reagent. Phenol in artificially contaminated soil samples after their partial biodegradation was determined by its stripping with water vapor to 0.5 M NaOH and by an absorbance measurement (UV-VIS spectrophotometer; Varian, Canada). Phenols in samples of soil containing PAHs were determined by the extraction with di-isopropylether and analyzed using GC - EI.
The loss of PAHs was measured by gas chromatography-mass selective detection after organic solvent extraction. Slurries were acidified to pH of 2 with 5 M HCl and were mixed with n-hexane. The organic phase was separated by centrifugation. Samples were assayed with GC-EI.

RESULTS
Biodegradation of Phenol in Artificially Contaminated Soil

At the beginning of the contact of solid and liquid phases, the concentration of phenols in the cultivation solution increased rapidly and attained the maximal value of its concentration as a consequence of deliberation of phenols from a solid phase. This phenomenon is clearly seen in Figure 1 indicating the time dependence of phenol concentration in water phase. The

successive decrease of phenol concentration in solutions was obviously caused by the biodegradation activity of a microorganism. Correspondingly, the concentration of phenols in the solid phase first decreased significantly, followed by a slower drop, which could be associated with biodegradation of the remaining amount of non- extracted phenols from particles of soil (Figure 2). The rate of phenol biodegradation in the aqueous phase in the presence of *C. tropicalis* was rapid. Phenols removal efficiency reached the value of over 98% and the average phenol degradation rate achieved 12.2 mg L^{-1} h^{-1}. Nevertheless, it must be mentioned that under the applied conditions biodegradation of phenols in soil was not fully completed within 144 hours and some residual amount of phenols (approx. 13 mg kg^{-1} dry) always persisted.

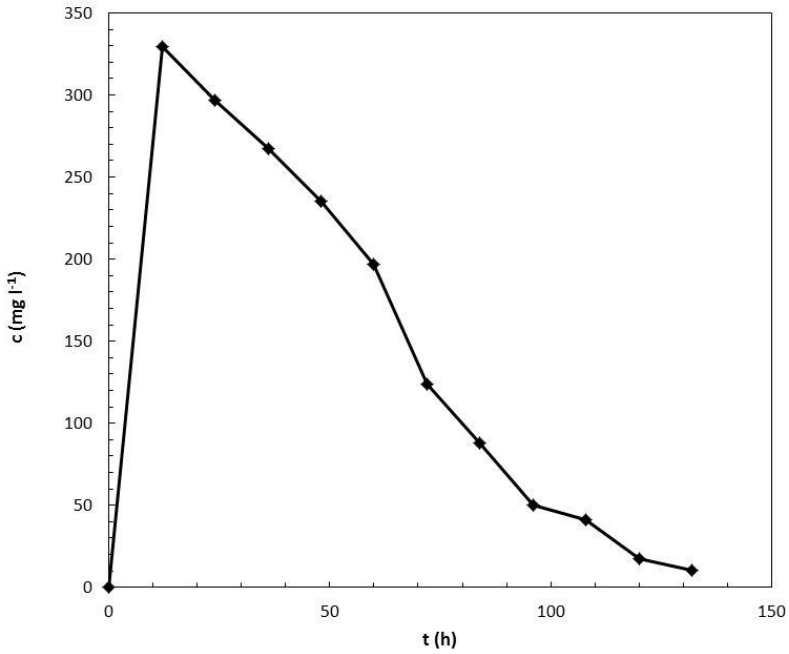

Figure 1 Time dependence of residual phenol concentration in aqueous phase contacting artificially contaminated soil in the course of biodegradation using *Candida tropicalis*

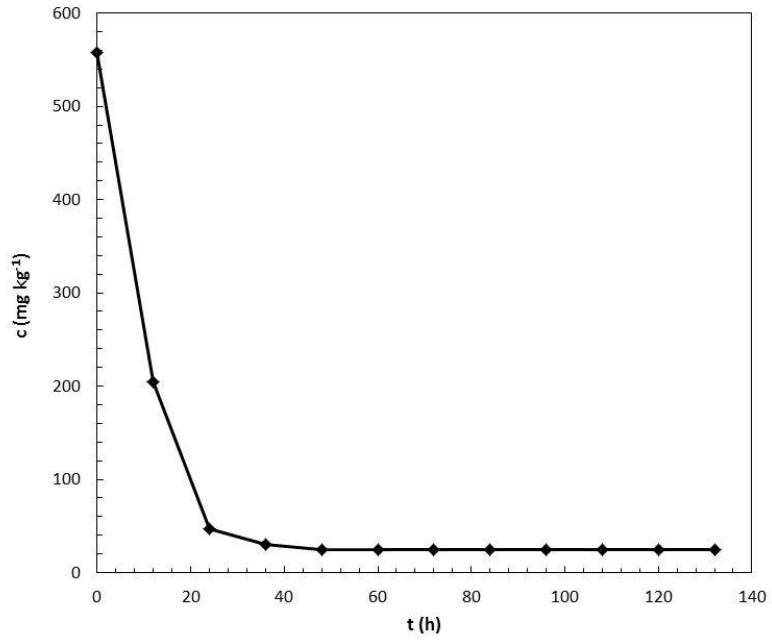

Figure 2 Residual phenol concentration in dependence on biodegradation time with *Candida tropicalis* in artificially contaminated soil

Biodegradation of Real Soils by C. Tropicalis and Phanerochaete Chrysosporium

To obtain information of biodegradation efficiency in a real environment, two types of soil materials with different contents of phenolic and polyaromatic compounds from the contaminated area at the field-site of former coke and gas plants in Ostrava region were collected. The contents of PAHs and phenols in these samples were analyzed, and obtained results are summarized in Table 2. It is obvious that sample A contains both PAHs and phenols contaminants, whereas sample B only PAHs.

Table 2 Content of PAHs and phenols in the real soils

Compound	Soil A (mg kg^{-1} dry)	Soil B (mg kg^{-1} dry)
Naphthalene	160	82
2-methylnaphtalene	62	51
Acenaphtylene	80	40
Acenaphtene	16	9
Fluorene	60	48
Phenentrene	212	190
Anthracene	12	12
Fluoranthene	408	380
Pyrene	320	310
Benzo (b and k) fluoranthene	290	200
Benzo(a)pyrene	120	111
Benzo(g,h,i) perylene	80	58
Total PAHs	**1820**	**1491**
Phenol	107	0
o-cresol	21	0
m-,*p*-cresol	20	0
Xylenols	4	0
Total phenols	**152**	**0**

It must be stressed that this study was not primarily focused on biodegradation of PAHs. Therefore, biodegradation of two contaminated real soils (Type A containing phenols and PAHs, and Type B only PAHs) under comparable conditions was investigated. After the period of 144 hours since contacting soils with the cultivation solution containing a mixed culture of *C.tropicalis* and *Phanerochaete chrysosporium* population, the original concentration of total PAHs in soil Type A decreased to the residual concentration of PAHs equivalent to 200 mg kg^{-1}. The treatment time with biodegrading media was relatively short (Figure 3). Similar results were obtained for biodegradation experiments with soil Type B, using the same population (see Figure 4).

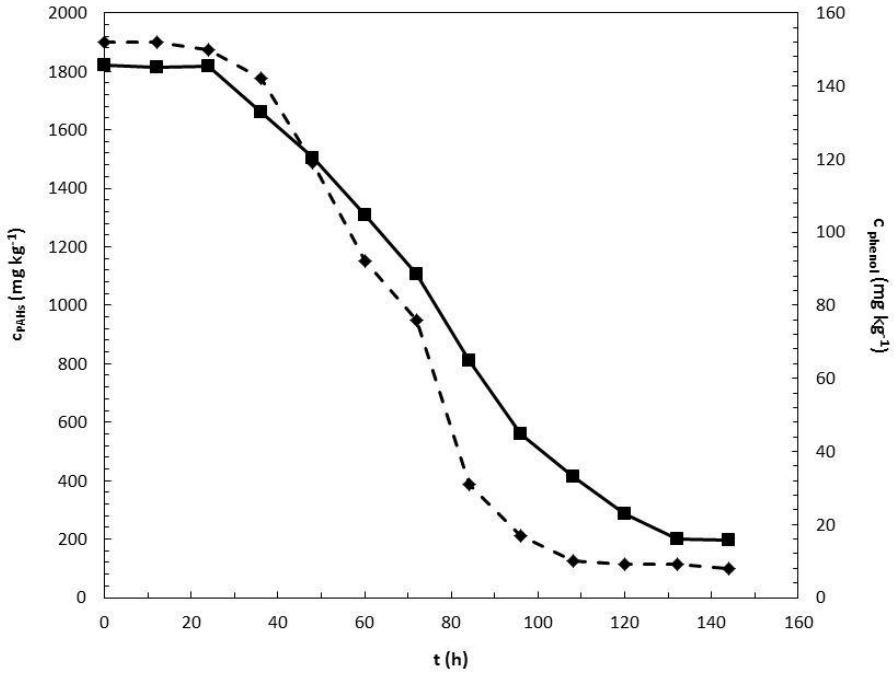

Figure 3 Time course of decontamination of soil Type A with mixed culture of C.tropicalis and Phanerochaete chrysosporium—■— PAHs, , - - ♦- - phenol

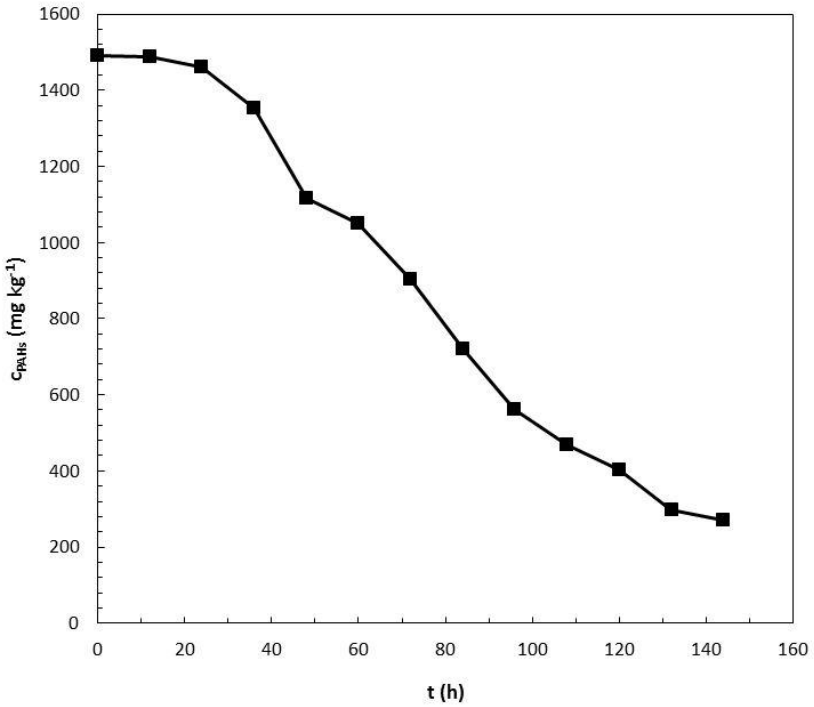

Figure 4 Time course of decontamination of soil Type B with mixed culture of C.tropicalis and Phanerochaete chrysosporium

Fast and complete degradation of phenols was tested under aerobic conditions at the presence of non-soluble hydrocarbons. The conditions were identical with those mentioned above for PAHs degradation. Figure 3 clearly shows that the degradation curve of phenol corresponds to PAHs degradation curve.

DISCUSSION

With respect to obtained results concerning biodegradation of phenol in artificially contaminated soil (see Figures 1 and 2) it is obvious that the continuous decrease of phenol concentration is caused by the biodegradation activity of *C. tropicalis*. Despite of rather high efficiency of biodegradation of phenol, the average phenol degradation rate (12.2 mg L^{-1} h^{-1}) was somewhat lower than published for *C. tropicalis* strains (Juárez-Ramírez et al., 2001). It is well known that the rate of biodegradation strongly depends on cell density (Beshay et al., 2002) which is considered to be the major factor for the time required for the total phenol biodegradation (density of free cells for maximum degradation rate at 0.2 g L^{-1}). Considering the fact that in this study relatively low free cell densities were applied in the experiments, the biodegrading activity of used *C. tropicalis* strain could be regarded as satisfactory and slightly more efficient than other microbial strains (Beshay, 2002).

Owing to the fact that this study was not primarily focused on biodegradation of PAHs, two types of the real contaminated soil were studied with an emphasis to biodegradation of phenol in the presence of PAHs by the mixed culture of *C.tropicalis* and *Phanerochaete chrysosporium*. It was successfully proved that the used mixture is able to effectively degrade phenols persisted at low concentrations (about 150 mg kg^{-1}) in real contaminated soil even in the presence of PAHs. The rate of biodegradation of PAHs was even slower in case of decontamination of soil B, perhaps as a result of missing molecules of phenol, which could contribute to some synergy during enzymatic elimination of PAHs.

Nevertheless, more investigation focused on elucidation of mutual relation between low and high molecular organic compounds presented in mixed contaminants submitted to aerobic biodegradation are needed. This co-metabolic breakdown may become an important mechanism for removing PAHs from soils.

At the presence of phenolic compounds, PAHs were also successfully biodegraded by this mixture in a relatively short time. Although the phenol concentration was at a relatively low level, it was evidently proved that biodegradation of phenolic compounds is possible even at the presence of PAHs. This result should be considered crucial for the future treatment of old coking plants or other brownfields.

CONCLUSIONS

Aerobic biodegradation of phenol in an artificially contaminated soil and in real soils collected from the site with a long history of contamination and containing mixed contamination of phenols and polyaromatic hydrocarbons was investigated. Two types of different microorganisms, namely yeasts *Candida tropicalis* and *Phanerochaete chrysosporium,* were applied. The results prove that biodegradation is a viable method for the destruction of phenol dissolved in liquid effluents and/or in solid matrix of soils. It was found that degradation of phenol in soils, even by phenol accommodated *C. tropicalis*, was not sufficiently completed. The maximum average value of phenol biodegradation rate in aqueous solution obtained at the initial concentration of phenol of about 300 mg L^{-1} was 12.2 mg L^{-1} h^{-1} at cell density of 0.025 g L^{-1}. It was proved that the mixed culture of *C.tropicalis* and *Phanerochaete chrysosporium* population is able to effectively degrade phenols persisted at low concentrations (about 150 mg kg^{-1}) in real contaminated soil even in the presence of PAHs. At the presence of phenolic compounds, PAHs were also successfully biodegraded by this mixture in a relatively short time.

It can be concluded that the mixture composed from yeast and fungus could be worthwhile for biodegradation of complex organic contamination of industrial soils, particularly those contaminated by phenolic and polyaromatic compounds.

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