Biodegradation **14:** 1–8, 2003.

Application of microscopic fungi isolated from polluted industrial areas for polycyclic aromatic hydrocarbons and pentachlorophenol reduction

Rafał Szewczyk, Przemysław Bernat, Krystyna Milczarek & Jerzy Długoński*
*Department of Industrial Microbiology and Biotechnology, University of Łódź, Banacha 12/16, PL 90-327 Łódź, Poland (*author for correspondence: e-mail: jdlugo@biol.uni.lodz.pl)*

Accepted 13 January 2003

Key words: anthracene, biodegradation, *Mucor ramosissimus*, pentachlorophenol, phenanthrene

Abstract

The growth abilities of fifteen fungal strains isolated from contaminated areas, in the presence of xenobiotics compounds mixture (overworked cutting fluid, crude and waste oil) were examined. Strains with the richest growth were chosen for anthracene, phenanthrene and pentachlorophenol biodegradation in Sabouraud medium (with initial xenobiotic concentration 250 mg/l in cultures with polycyclic aromatic hydrocarbons and 10 mg/l for the chlorinated substrate). Strains IM 1063 and IM 6325 were able to attack phenanthrene forming its derivative 9-phenanthrenol with the yields 5.22 mg/l and 2.82 mg/l, respectively. Strain IM 1063 and IM 6325 transformed pentachlorophenol to an intermediate compound – pentachloromethoxybenzene. Final content of pentachloromethoxybenzene reached 3.46 mg/l and 3.2 mg/l, respectively. Strain IM 6203 (contrary to other strains) released an intermediate product of pentachlorophenol metabolism – 2,3,5,6-tetrachlorohydroquinone (8.73 mg/l substrate remaining and 1.2 mg/l 2,3,5,6-tetrachlorohydroquinone forming). The IM 6203 strain was identified as *Mucor ramosissimus*. The chlorinated pesticide degradation by *M. ramossimus* was improved significantly on a medium with overworked oil. Only 8.3% of pentachlorophenol and 4.3% of 2,3,5,6-tetrachlorohydroquinone in relation to the introduced substrate (10 mg/l) were found, after 7 days of incubation. The growth of *M. ramosissimus* on medium with overworked oil in pentachlorophenol presence was associated with oil emulgation, which enhanced fungal growth and the pesticide degradation.

Abbreviations: CO – crude oil; CS – contaminated soil; HCB – hexachlorobenzene; OCF – overworked cutting fluid; OCS – oil contaminated soil; OWO – overworked oil; PCMB – pentachloromethoxybenzene; PCP – pentachlorophenol; TeCH – 2,3,5,6-tetrachlorohydroquinone

Introduction

The ultimate degradation of organic chemicals in polluted agricultural and industrial areas is mainly a result of microbial activity. Contrary to naturally occurring compounds, xenobiotics with structures seldom present in natural products are slowly mineralised. However, some microorganisms are able to attack degradation resistant and toxic pollutants prosperously, and even evolved biochemical pathways to utilise this kind of compounds as a sole source of carbon and energy (Commandeur & Parsons 1990; Singh et al. 1999). It was shown by us that microbial strains isolated from industrial areas in Silesia region (Poland) are very active in biodegradation of polycyclic aromatic hydrocarbons (PAHs) as well as heavy metals (lead and zinc) accumulation (Fijałkowska et al. 1998; Słaba & Długoński 2000).

Biocides extensively applied for plant protection or wood preservation also possess a complex chemical formula not present in the nature and are recalcitrant for microbial degradation. The study into breakdown of agricultural pollutants is focused in the main on soil and water bacteria, lignolitic fungi and actinomycetes (Singh et al. 1999). It was shown that bacterial strains originating from crude oil contaminated

soils or metal works dumps can be also successfully applied for chlorinated pesticide (pentachlorophenol) elimination (Fijałkowska et al. 1998). The wastes of metalworking industry, e.g., overworked cutting fluids and oils also contain high amount of cyclic hydrocarbons, chlorinated paraffins, sulphur compounds and heavy metals. Additionally, at a high temperature of machining, great amounts of complex organic compounds highly toxic and resistant to microbial attack are formed (Apostoli et al. 1993; Scerri & Dalziel 1996; Dąbrowski et al. 1988). Nevertheless, some microorganisms have an ability of the industrial wastes degradation and can be used for contaminated soil and water bioremediation (Guenther et al. 1994; Jaros-Kamińska & Długoński 1996; Rudd et al. 1996).

The aim of the presented work was to check usefulness of microscopic fungi isolated from overworked stored cutting fluids and oil contaminated soils for PAHs and pentachlorophenol (PCP) degradation.

Materials and methods

Chemicals

Crude oil "Lotos" was produced by CPN Poland, cutting fluids were supplied by MAHLE Krotoszyn. Xenobiotic substrate: anthracene, phenanthrene and pentachlorophenol (PCP) came from Sigma and Supelco. Other chemicals were from J. T. Baker or POCH (Poland). All the chemicals were high purity grade reagents. The chemical formulas of the studied xenobiotics are presented in Figure 1.

Stock solutions

Stock solutions of anthracene and phenanthrene were prepared in dimethylformamide (DMF) at concentrations of 12.5 mg/ml. PCP was dissolved in ethanol to give the final solvent concentration less than 0.1% v/v in the medium culture.

Microorganisms

The filamentous fungi were isolated from a mixture of overworked cutting fluid stored for a period of over 6 months (strains: IM 6202, IM 6203, IM 6204, IM 6205, IM 6207), from oil contaminated soils collected in the industrial areas of Silesia (strains: IM 6002, IM 6006, IM 6009, IM 6015, IM 6107, IM 6113, IM 6114), and Łódź (strains: IM 1063, IM 6324, IM 6325) regions (Poland). The IM 6203 strain was identified as *Mucor ramosissimus* (Samutsevitsch) by Department of Plant Taxonomy and Geography, University of Warsaw, using standard diagnostic methods.

Waste media

Undissolved: overworked cutting fluid (OCF), crude (CO) and overworked (OWO) oils (2 ml of appropriate oil with 16 ml of deionised water) were used as media with waste components. The media were sterilised at 121 °C for 30 min.

Preculturing of fungal strains and cultivation on waste media

Preculturing on Sabouraud medium and cultivation on media with waste components were carried out at 28 °C on rotary shaker at 200 rpm. Fungal cultures (10 day-old) on ZT (Wilmańska et al. 1992) agar slants were used as inoculum for 20 ml Sabouraud liquid medium (10 g of neopeptone [Difco Laboratories, Detroit MI. USA] and 40 g of glucose per litre of deionised water). The incubation was performed in 100 ml Erlenmayer flasks for 24 h. The preculture was transformed to fresh medium (in the ratio 1 : 9) and cultivated for the next 24 h (Lisowska & Długoński 1999). Two ml of the obtained homogenous precultures of the fungi was introduced into 100 ml flask containing 18 ml of appropriate waste medium and incubated for 10 days. A control culture was prepared by adding 2 ml of fungal homogenous precultures into 100 ml flask with 18 ml Sabouraud liquid medium.

Reduction of xenobiotics on Sabouraud medium

Two ml of preculture (obtained as described above) was introduced into 18 ml of Sabouraud medium with PAHs (anthracene or phenanthrene, 250 mg/l) or pesticide (PCP, 10 mg/l). The flasks were incubated for 7 days, at 28 °C on rotary shaker (200 rpm). Additionally, two controls were performed, the first one without fungal inoculum and the second one with killed biomass prepared by autoclaving the culture at 121 °C for 40 min, before adding the xenobiotic substrate.

Xenobiotics extraction

Xenobiotics were extracted according to the conditions described by Cerniglia & Yang (1984) with modifications presented in our previous paper (Lisowska & Długoński 1999). The samples were filtered. The mycelium was suspended in water and

disintegrated using sonicator (MISONIX, England). Then fungal homogenates and culture filtrate were extracted three times separately with ethyl acetate. The extracts were dried over anhydrous sodium sulphate and the solvents were evaporated under reduced pressure at 40 °C. Extraction efficiency depended on kind of xenobiotic and mycelium and was 84.0–91.0%, 80.6–88.4% and 94.1–99.1% for anthracene, phenanthrene and PCP, respectively.

PCP degradation on overworked oil medium

The fungal preculture (2 ml) was transformed into OWO medium (18 ml) with PCP (10 mg/l) in 100 ml flask. The inoculated OWO medium without PCP and Sabouraud medium with and without PCP were applied as control cultures. The cultivation was carried out at 200 rpm, 28 °C for 7 days. After the time indicated in the text macroscopic observations of the cultures were made.

Chromatography methods

Gas chromatographic analyses of ethyl acetate extracts were performed on a Hewlett-Packard HP 6890 series gas chromatograph equipped with mass selective detector HP 5973, using a HP-5MS capillary column (30 m × 0.25 mm). Injector temperature was maintained at 250 °C and helium constant flow was 1 ml/min. Hexachlorobenzene (HCB) was applied as an internal standard. Other parameters depended on a kind of estimated chemical (Table 1). The intermediate and oxidation products of xenobiotic metabolism (not present in abiotic controls) were identified on the basis of mass spectrum compared to NIST GC-MS spectra database. All library matched compounds exhibited the degree of match better than 90%. Typical results of quantitative analysis are shown from one of two or three experiments.

Table 1. GC-MS parameters

Oven program	Set of Gc-MS parameters*			
	A	B	C	D
Initial temp (°C/hold time, min)	110/2	80/1	110/2	80/2
Ramp rate 1 (°C/min)	20	20	20	30
Tem1 (°C/hold time, min)		220/0	250/6	230/0
Ramp rate 2 (°C/min)		5	2	10
Temp2 (°C/hold time, min)		230/0	290/15	260/5
Ramp rate 3 (°C/min)		20	2	2
Temp3 (°C/hold time, min)				290/15
Ramp rate 3 (°C/min)				2
Final temp (°C/hold time, min)	250/6	290/3	300/5	300/10

* A, C: anthracene and phenanthrene; B, D: PCP and TeCH; extracted from cultures on Sabouraud medium (A, B) or OWO medium (C, D).

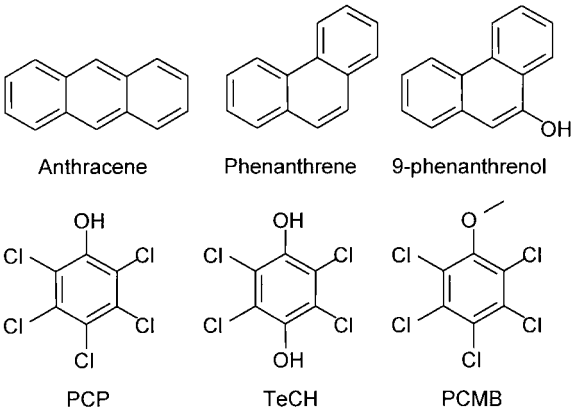

Figure 1. Chemical patterns of the tested xenobiotics.

Results and discussion

Growth of applied strains on media with waste components

In the preliminary experiments of the presented work the growth ability of applied strains in the presence of toxic and very complex industrial wastes, used at the fungi isolation, was checked. It was expected that the study would show not only the strain resistance towards xenobiotics present in the wastes but also their potential utilisation ability of hardly degraded pollutants. The results are presented in Table 2.

All of the fifteen strains were able to grow in the presence of at least one of the wastes used. Six strains demonstrated the most abundant growth in xenobiotics presence which was comparable with the growth intensity on Sabouraud medium (or even higher, IM 6107) after 6 days of culturing. The growth of strain IM 6015 on OCF, CO media was also better than on the control Sabouraud medium. However, waste components present in OCF and OWO media significantly retarded the growth of this fungus.

Anthracene and phenanthrene removal

The strains showing the most vigorous growth in the presence of industrial wastes (IM 1063, IM 6009, IM 6107, IM 6113, IM 6203, IM 6325) were checked in the respect of transformation ability of PAHs (anthracene and phenanthrene) which belong to the main

Table 2. Fungal strains growth ability on OCF, CO, OWO and Sabouraud medium

Strain		Growth intensity* on medium											
		Sabouraud			OCF			CO			OWO		
Number	Originality**	Day of incubation											
		3	6	10	3	6	10	3	6	10	3	6	10
IM 1063	CS	+++	+++	+++	+	+	+	+	+	+	+++	+++	+++
IM 6002	OCS	++	++	++	+	++	++	+	+++	+++	−	+	+
IM 6006	OS	++	+++	+++	−	+	+	+	+++	+++	−	+	+
IM 6009	OCS	+++	+++	+++	+	+++	+++	+	++	++	++	+++	+++
IM 6015	OCS	+	+	+	+	++	++	+	++	+++	−	+	+
IM 6107	OCS	++	++	++	+++	+++	+++	+++	++	++	+++	++	+++
IM 6113	OCS	+++	+++	+++	+	+++	+++	+	++	++	+	+++	+++
IM 6114	OCS	+	+++	++	+	++	++	++	++	++	+	+	+
IM 6202	OCF	+++	+++	+++	−	+	+	±	+	++	+	++	+++
IM 6203	OCF	+++	+++	+++	+	+	+	+	++	++	++	+++	+++
IM 6204	OCF	++	+++	+++	−	+	+	−	++	+++	−	+	+
IM 6205	OCF	++	+++	+++	−	−	±	+	−	−	+++	+++	++
IM 6207	OCF	++	+++	+++	−	−	−	+++	+++		−	−	−
IM 6324	CS	++	++	++	−	−	−	−	−	−	−	+	−
IM 6325	CS	+++	+++	+++	+	+	+	−	+	+	++	++	+++

* The relative scale was used of turbidity growth estimation: (−), lack of growth; (±), very weak growth; (+), moderate growth; (++), abundant growth; (+++), most abundant growth.
** Originality of the samples: OCS, oil contaminated soil; OCF, overworked cutting fluid; CS, contaminated soil.

components of applied wastes (Apostoli et al. 1993; Wilson & Jones 1993).

The chromatographic analysis of ethyl acetate extracts revealed a lower content of anthracene in all fungal cultures after seven days of incubation (Figure 2). However, no derivatives of this xenobiotic were identified on the basis of mass spectrum of applied GC-MS database. On the other hand, the GC-MS analysis disclosed that fungal strains IM 1063 and IM 6325 were able to attack phenanthrene giving its derivative 9-phenethrenol at concentrations 5.22 mg/l and 2.82 mg/l, respectively (Figure 3).

The obtained results correspond with metabolising activity of *Penicillium janthinellum* VUO 10,201 which was isolated form creosote conatminated soil and partially degraded PAHs if cultured in nutrient broth (Boonchan et al. 2000). It was also documented that *Cunninghamella elegans* (growing in rich fungal media) had an ability to transform a wide variety of PAHs to metabolites which are less toxic and mutagenic than parental compounds (Cerniglia & Yang 1984; Cerniglia 1992; Lisowska & Długoński 1999). The significant mutagenicity reduction was noticed during *C. elegans* and *P. zonatum* growth on Pennsylvania crude oil as a sole carbon source, too (Rudd et al. 1996). The ability of strains IM 1063 and IM 6325 to grow abundantly on overworked oil and to transform phenanthrene in Sabouraud medium indicates that both strains can be used as interesting research models for the study on mechanisms of oil components metabolisation in fungi.

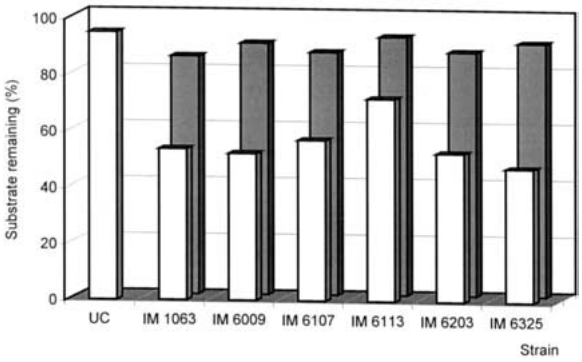

Figure 2. Recovery of anthracene (□) from cultures of fungal strains and uninoculated control (UC) after 7 days of incubation on Sabouraud medium. The grey bars in the graph show the recovery of anthracene from autoclave-killed fungus controls (■).

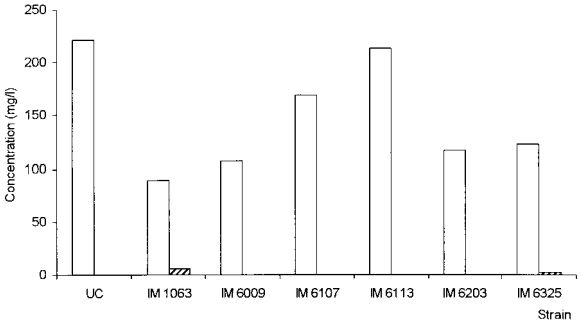

Figure 3. Phenanthrene (□), 9-phenanthrenol (▨) amount in fungal cultures after 7 days of culturing on Sabouraud medium with initial PAH concentration 250 mg/l; UC, uninoculated control.

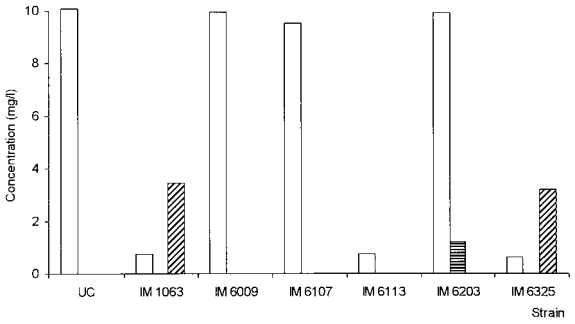

Figure 4. PCP (□), TeCH (▤) and PCMB (▨) content in cultures of fungal strains after 7 days of incubation on Sabouraud medium with initial PCP content 10 mg/l; UC, uninoculated control.

Pentachlorophenol transformation

The following experiments showed that polyaromatic xenobiotics reducing fungi were also able to attack aromatic pesticide – pentachlorophenol (PCP).

The pesticide was transformed by strains IM 1063 and IM 6325 to an intermediate compound – pentachloromethoxybenzene (PCMB) (Figure 4) with retention time 8.29 identified on the basis of mass spectrum compared to NIST GC-MS library (Figure 5). Final concentrations of this derivative reached 3.46 and 3.2 mg/l in IM 1063 and in IM 6325 cultures, respectively. The presented data are comparable with the activity of five bacterial strains isolated earlier by us from the same soils (polluted by crude oil) and industrial dumps. The isolates were able to eliminate PCP at a rate exceeding 50% substrate content (10 mg/l) (Fijalkowska et al. 1998). The xenobiotic utilisation by the most active strain *Rhodococcus equii* IM 6KB3 was concurrent with the culture growth. The varying degrees of pesticide degradation were also noticed by Shelton et al. (1996) who studied metabolism of twelve structurally different herbicides in cultures of *Streptomyces* (strain PS1/5). It was suggested that the differences in rates of utilisation can be a consequence of selective induction of certain metabolic enzymes (transformation consistent with growth kinetics) or activity of enzymes associated with secondary metabolism (conversion not associated with culture growth).

The gas chromatography analyses carried out in this study, revealed additionally that the strain IM 6203 (contrary to other strains) released an intermediate product of PCP metabolism – 2,3,5,6-tetrachlorohydroquinone (TeCH) (1.2 mg/l derivative forming) with the retention time 8.54 identified on the basis of mass spectrum compared to NIST GC-MS library and chemical standard (Figure 5). Lange et al. (1996) documented that *Flavobacterium* sp. ATCC 39723 catalyses the oxygenolytic removal of the first chlorine from PCP by intracellular 4-monoxygenase giving TeCH. On the other hand, the lignin-degrading basidiomycete *Pchanerochaete chrysosporium* is able to degrade PCP via combination of intracellular reductive and extracellular oxidative dechlorination reactions (Reddy & Gold 2000). Both PCP and its derivative TeCH appeared only in the culture filtrate of IM 6203 strain which may suggest that enzymes involved in PCP metabolism of the fungus are part of exterior cell wall enzymatic complexes or they are extracellurar enzymes secreted to the growth medium. It is also possible that PCP and TeCH are metabolised by intracellular enzymes and transported via cell wall to the growth medium. Further experiments are being made in our laboratory to clarify this question.

Pentachlorophenol degradation on OWO medium by the fungal strain IM 6203

Taking into account the rich growth of the strain IM 6203 on overworked oil medium, PCP degradation ability of the fungus on OWO medium was checked. The macroscopic observation of the culture (Table 3) revealed that the fungal growth on OWO medium with PCP was similar to the growth on Sabouraud medium without PCP. Additionally, significant oil emulgation was observed on OWO medium with PCP in contrast to the OWO medium without the xenobiotic. No oil emulgation was noticed on control sterile OWO media with or without PCP.

Some microbial strains are able to synthesise biosurfactants, which, as excellent and dispersing agents, may enhance lipophilic compounds degrad-

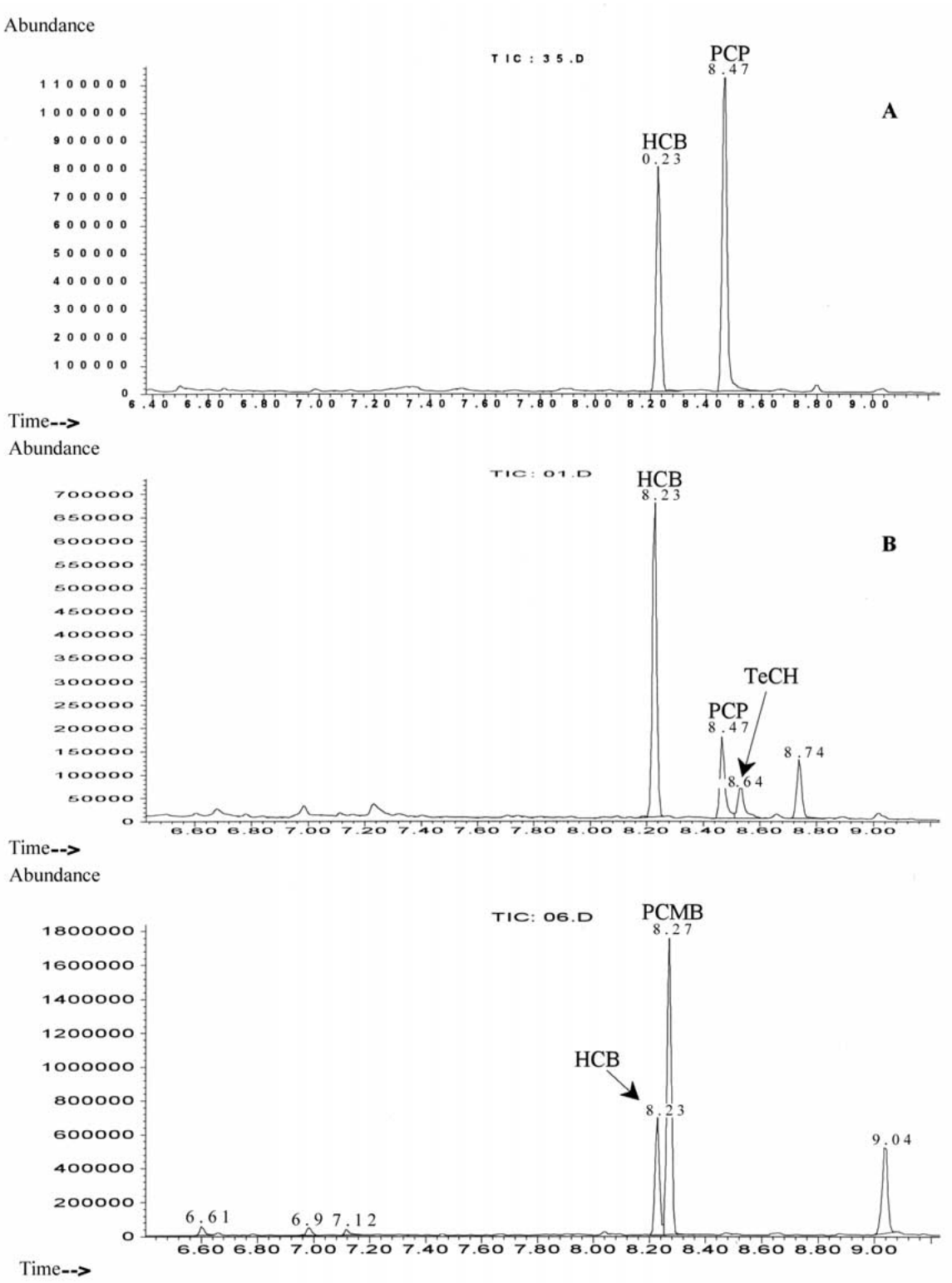

Figure 5. GC-MS chromatograms of sterile control medium with PCP (A), IM 6203 culture filtrate (B), and mycelium (C) of IM 6325 strain, after 7 days of incubation with PCP.

Table 3. Effect of PCP on growth of fungus IM 6203 in Sabouraud and OWO medium

Day incubation	Growth intensity* on medium:			
	Sabouraud	Sabouraud with PCP	OWO	OWO with PCP
2	++	+	++	++ e
5	+++	++	++	+++ e
7	+++	++	+++	+++ ie

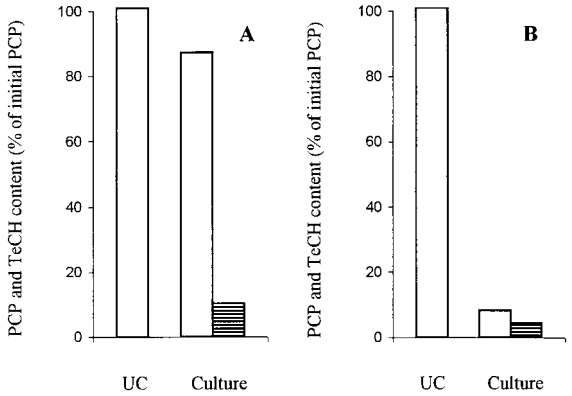

Figure 6. Accumulation of PCP (□) and TeCH (▤) in *Mucor ramosissimus* IM 6203 cultures during 7 days of PCP transformation on Sabouraud (A) and OWO (B) medium. UC, uninoculated control.

ation and/or detoxification (Angelova & Schmauder 1999; Banat et al. 2000; Paraszkiewicz et al. 2002). The chromatography analyses performed by us showed that only 8.4% of PCP and 4.3% of TeCH were present in the culture on OWO medium after 7 days of incubation (Figure 6). The obtained results suggest that overworked oil stimulates PCP utilisation. On the other hand, the pesticide induces oil emulgation by the fungus and, in consequence, accelerates the fungal growth on OWO medium.

The data presented above indicate that the strain IM 6203, identified as *Mucor ramosissimus* (Samusevitsch), is a valuable research tool for the study on chlorinated pesticide (PCP) transformation and industrial waste degradation by fungi. The mechanisms of PCP degradation by *M. ramosissimus* are currently under investigation in our laboratory.

Acknowledgement

This work was supported by a grant from European Commission, DG XII, INCO-Copernicus (No. IC 15-CT98-0114).

References

Angelova B & Shmauder HP (1999) Lipophilic compounds in biotechnology – interactions with cells and technological problems. J. Biotechnol 8: 13–32

Apostoli P, Crippa M, Fracasso ME, Cottica D & Alessio L (1993) Increases in polycyclic aromatic hydrocarbon content and mutagenicity in a cutting fluid as a consequence of its use. Int. Arch. Occup. Environ. Health 64: 473–477

Banat L, Makkar RS & Cameotra SS (2000) Potential commercial applications of microbial surfactants. Appl. Microbiol. Biotechnol. 53: 495–508

Boonchan S, Britz ML & Grant SA (2000) Degradation and mineralisation of high-molecular-weight polycyclic aromatic hydrocarbons by defined fungal-bacterial cocultures. Appl. Environ. Microbiol. 66: 1007–1019

Cerniglia CE (1992) Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation 3: 351–368

Cerniglia CE & Yang SK (1984) Stereoselective metabolism of anthracene and phenanthrene by the fungus *Cunninghamella elegans*. Appl. Environ. Microbiol. 47: 119–124

Commandeur LCM & Parsons JR (1990) Degradation of halogenated aromatic compounds. Biodegradation 1: 207–220

Dąbrowski JR, Firkowski A & Gierzyńska-Dolna M (1988) Cutting fluids. In: Janus K (Ed) Copyright by Wydawnictwa Naukowo-Techniczne, Warsaw, Poland (in Polish)

Fijałkowska S, Lisowska K & Długoński J (1998) Bacterial elimination of polycyclic aromatic hydrocarbons and heavy metals. J. Basic Microbiol. 38: 5–6, 361–369

Guenther K, Schlosser D, Shmauder HP & Rausch U (1994) Bioremediation of contaminated groundwater. Appl. Biochem. Biotechnol. 48: 11–14

Jaros-Kamińska B & Długoński J (1996) Microbiological degradation and remediation of organic and inorganic wastes. In: Chuto J (Ed) 4th International Symposium Integrated Management and Processes for Waste from Industry and Human Settlements (pp 271–278). Environmental Engineering Company "Ekochem", Szczecin, Poland

Lange CC, Schneider BJ & Orser CS (1996) Verification of the role of PCP 4-monooxygenase in chlorine elimination from pentachlorophenol by *Flavobacterium* sp. strain ATCC 39723. Biochem. Biophys. Res. Commun. 219: 146–149

Lisowska K & Długoński J (1999) Removal of anthracene and phenethrene by filamentous fungi capable of cortexolone 11-hydroxylation. J. Basic Microbiol. 39: 117–125

Paraszkiewicz K, Kanwal A & Długoński J (2002) Emulsifier production by steroid transforming filamentous fungus *Curvularia lunata*. Growth and product characterization. J. Biotechnol. 92: 287–294

Reddy GVB & Gold MH (2000) Degradation of pentachlorophenol by *Phanerochaete chrysosporium*: intermediates and reactions involved. Microbiology 146: 405–413

Rudd LE, Perry JJ, Houk VS, Williams RW & Claxton LD (1996) Changes in mutagenicity during crude oil degradation by fungi. Biodegradation 7: 335–343

Scerri & Dalziel KL (1996) Occupational contact sensitisation to the stabilised chlorinated paraffin fraction in neat cutting oil. Am. J. Contact Dermat. 7: 35–37

Shelton DR, Khader S, Karns JS & Pogell BM (1996) Metabolism of twelve herbicides by *Streptomyces*. Biodegradation 7: 129–136

Singh BK, Kuhad RC, Singh A, Lal R & Tripathi KK (1999) Biochemical and molecular basis of pesticide degradation by microorganisms. Crit. Rev. Biotechnol. 19: 197–225

Słaba M & Długoński J (2000) Selective recovery of Zn^{2+} from slag a metal-processing plant by the microscopic fungus *Verticillium marquandii*. Biotechnol. Lett. 22: 1699–1704

Wilmanska D, Milczarek K, Rumijowska A, Bartnicka K & Sedlaczek L (1992) Elimination of by-products in 11β-hydroxylation of Substance S using *Curvularia lunata* clones regenerated from NTG-treated protoplasts. Appl. Microbiol. Biotechnol. 37: 626–630

Wilson S.C. & Jones KC (1993) Bioremediation of soil contaminated with polynuclear aromatic hydrocarbons (PAHs): A review. Environ. Pollut. 81: 229–249