ISOLATION AND CHARACTERIZATION OF *PHANEROCHAETE* STRAINS SUITABLE FOR BIOREMEDIATION PURPOSES

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ABSTRACT
The most unsettled problem of organic farmlands are related to the following two groups of pollutants: POP (Persistent Organic Pollutant) and PAH (Polycyclic Aromatic Hydrocarbon) compounds. These are recognized as being directly toxic to biota even at low concentration; furthermore, they accumulate in organisms. The white rot fungus *Phanerochaete chrysosporium* has a potential for bioremediation of soils polluted by xenobiotic compounds. Our aim was to isolate of *P. chrysosporium* strains, which can efficiently degrade POP and PAH compounds by their enzyme systems. A new selective medium, containing rose bengal, dichloran and carbendazim was used to isolate *P. chrysosporium* from soil. We isolated one *P. sordida* and five *P. chrysosporium* strains from Hungarian environmental samples. In soil microcosm experiments the investigated *Phanerochaete* strains were able to degrade distinct herbicides and parabens in soils of different origin (agricultural field soil, garden soil and sandy soil).

Keywords: bioremediation, PAH, *Phanerochaete chrysosporium*, POP, soil pollution

INTRODUCTION

The air, rain water, spraying water and pesticide treatments, continuously contaminate agricultural areas with polluting compounds. Some of them remain in the soil for a long time, e.g. the persistent POPs compounds (Persistent Organic Pollutants) and polycyclic aromatic hydrocarbons, or PAHs (Polycyclic Aromatic Hydrocarbons). These xenobiotics are classified as dangerous because they could be directly toxic to the organisms and also accumulate in the food chain. Some microorganisms are capable for the degradation of various PAH and POP compounds: *Phanerochaete chrysosporium*, a basidiomycetous filamentous fungus, is belonging to this group of microbes where this phenomenon is confirmed.
P. chrysosporium is a white rot fungus which has a highly efficient lignin degrading enzyme system. With these enzyme systems the fungus can also break down different xenobiotic pollutants. In these types of degradation processes, the lignin peroxidase and the manganese peroxidase have great significance. There are data in the literature, that when these enzymes are used alone or in combinations they target and could degrade pesticides, polycyclic aromatic compounds, chlorinated aromatic compounds, paints and many other xenobiotics (CAMERON et al, 2000).
The aim of our present study was to isolate strains of P. *chrysosporium* capable for efficient degradation of a variety of aromatic pollutants like PAH compounds (e.g. naphthalene, anthracene), pesticides (e.g. diuron, linuron) and various other xenobiotics (e.g. phenol, aniline, methylparaben).

MATERIAL AND METHODS

Isolation of strains

A new type of selective medium was used for the isolation of *Phanerochaete* strains. This contained peptone (0.5%), KH_2PO_4 (0.1%), glucose (1%), $MgSO_4$ (0.05%) and agar (2%) supplemented with 500 µl 5% rose bengal, 1 ml 0,2% dichloran, 100 mg streptomycin and 15 mg carbendazim after autoclaving. The isolates collected from this selective medium and one *P. chrysosporium* strain (DSM 9620) purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ) were examined.

Molecular identification of strains

Molecular identification of the isolates was carried out with the involvement of two targets. The presence of ligninase H8 sequence was examined (JOHNSTON AND AUST, 1994). Furthermore, the ITS regions of the strains were also amplified by ITS4 and ITS5 primers (WHITE ET AL., 1990) and sequenced.

Investigation the in vitro xenobiotic degrading abilities

The degradation of xenobiotics was examined in artificially polluted field soil, garden soil and sandy soil. 20-20 g of soils were measured and spiked with 1-1 ml xenobiotic mix. This contained PAH compounds (naphthalene, anthracene, phenanthrene, pyrene and benzo(A)pyrene), herbicides (diuron, linuron, chlortoluron and isoproturon) and fungicides (captan and carbendazim) 500 ng ml^{-1}, 5 µg ml^{-1} and 5 µg ml^{-1} concentrations, respectively. This mixture also contained certain degradation products of pesticides and other xenobiotics (phenol, aniline, bisphenol A, 2,4-dichloro-phenol, 3,4-dichloro-aniline, 4-chloro-2-methylphenol, 2-amino-benzimidazole, 1,2-diamino-benzene, methylparaben, ethylparaben, propylparaben and butylparaben) in 2.5-5 µg ml^{-1} concentrations. The different polluted soils were inoculated with 5 ml concentrated fungal spore-mycelium suspension. Degradation abilities of *P. chrysosporium* DSM 9620 and three isolated strains (SZMC 20595, SZMC 20960 and SZMC 20961) were examined. The polluted soil samples were incubated at 25 ^{0}C for 40 days. Two controls were used: a non-sterile control, which contained xenobiotics without fungal inoculation and a sterile one, which contains sterilized soil with xenobiotics. The non-sterile control shows the fate of xenobiotics without *P. chrysosporium* and the sterile one shows their behavior in the absence of soil microbes.

The degradation of herbicides and parabens were measured in a HPLC-MS-MS (Agilent) with Zorbax Eclipse C18 column. Mobil phase flow: 0.5 ml min^{-1} and composition: acetonitrile: water=95:5. For soil extraction the QUECHERS method were used: to 10 g of soil 10 ml acetonitrile was added. After mixing for 1 min, 4 g $MgSO_4$ and 1 g NaCl were added and mixed for 1 min again. The samples were centrifuged (5000 rpm, 5 min). To the supernatant 25 mg PSA and 150 mg $MgSO_4$ were added, mixed for 30 sec and centrifuged (6000 rpm, 1 min). The clear supernatant was evaporated to 0.5 ml in inert atmosphere, filled to 1 ml with acetonitrile and acidified with formic acid prior to HPLC analysis (ANASTASSIADES ET AL., 2003).

RESULTS

DIETRICH AND LAMAR (1990) reported the isolation of *P. chrysosporium* strains from soil by application of a selective media containing benomyl. Our efforts to isolate *P. chrysosporium* in this medium were unsuccessful. However, a new modified medium which contained

dichloran, rose bengal and carbendazim was efficiently used for the isolation of *P. chrysosporium* from different Hungarian environmental samples.
Besides a micromorphological investigation, there is a possibility for rapid identification via the detection of the lignin peroxidase H8 gene of *P. chrysosporium* (JOHNSTON AND AUSTEN, 1994). The special primer pair for this gene was used to identify our isolates. Fragments of the proper size (600 bp) were detected in 6 strains after the specific PCR reactions. Besides this sequencing of the ITS region were used for the exact identification of the strains. The sequences were analysed with the NCBI BLAST service. These results proved that 5 of our strains were *P. chrysosporium* and one was *P. sordida*.
Degradation of xenobiotics was examined with 4 strains (DSM 9620, SZMC 20959, SZMC 20960 and SZMC 20961) in soil microcosm experiments. Until now the degradation of herbicides (chlortoluron, diuron, isoproturon and linuron) and parabens were measured. The rates of decomposition of inoculated samples were compared to the controls. The degradation of herbicides appeared also in the non-sterile control samples, at the same time, the presence of the inoculated fungus highly improved this process in certain cases. The most intensive of herbicide degradation took place in field soil by SZMC 20959: the decomposition was 62% compared to the sterile control (0%). The best degradation of herbicides was accomplished by the strain SZMC 20961, the decomposition was 65% *(Table 1.)*. The degradation of parabens (methylparaben, ethylparaben, propylparaben and butylparaben) was also investigated. Their degradations were also enhanced the presence of *P. chrysosporium (Table 2.)*.

Table 1. Treatment of herbicide contaminated soil samples with *Phanerochaete* strains

	field soil		**garden soil**		**sandy soil**	
	µg/20 g	Degradation %	µg/20 g	Degradation %	µg/20 g	Degradation %
Sterile control	8.7	0	7.2	0	2.4	0
Non-sterile c.	6.7	23.0	3.7	49	2.0	17
DSM 9620	5.1	41.4	5.4	25	2.4	0
SZMC 20959	3.3	62.1	5.6	22	2.4	0
SZMC 20960	6.1	29.9	3.5	51	2.4	0
SZMC 20961	6.5	25.3	2.5	65	1.6	33

Table 2. Treatment of paraben contaminated soil samples with *Phanerochaete* strains

	field soil		**garden soil**		**sandy soil**	
	µg/20 g	Degradation %	µg/20 g	Degradation %	µg/20 g	Degradation %
Sterile control	7.1	0	8.5	0	2.8	18
Non-sterile c.	5.6	21.1	4.1	52	2.3	0
DSM 9620	4.4	38.0	5.6	34	2.8	0
SZMC 20959	2.7	62.0	4.7	45	2.8	0
SZMC 20960	4.0	43.7	3.8	55	2.8	50

SZMC 20961	3.7	47.9	1.5	82	1.4	18

Statistics

The data were analyzed with the OpenStat software. The correlation coefficients of fungal remediation effectiveness to herbicides were 0.461, -0.201 and -0,264, in field soil, in garden soil and in sandy soil, respectively (0.95 of confidence). The correlation coefficients of the remediation effectiveness to parabens were 0.807, 0.050, and -0,113, in field soil, in garden soil and in sandy soil, respectively (considering all the four fungal treatments). The correlation coefficient values of our best strain (SZMC 20961) were 0.340 in the case of herbicides and 0.692 for parabens if we consider the data from all soil types.

CONCLUSIONS

The application of a new selective medium proved very useful to isolate *P. chrysosporium* from soil samples. The isolated *P. chrysosporium* strains efficiently degraded herbicides and parabens in soil microcosm experiments. The intensity of xenobiotics degradation was highly different in distinct soil types. One of the isolated *P. chrysosporium* strains was able for significant degradation activities even in sandy soil.

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