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ORIGINAL PAPER

Cresols utilization by *Trametes versicolor* and substrate interactions in the mixture with phenol

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Abstract The ability of the white rot fungus *Trametes versicolor* strain 1 to degrade and utilize methylated phenols (cresols) was established for the first time in a medium not containing any other carbon components. The data obtained demonstrated the better potential of the strain to assimilate *p*-cresol instead of *o*- or *m*- cresol. The 0.5 g/l *p*-cresol provided was degraded in full after 96 h. The effect of a dual substrate mixture (0.3 g/l phenol + 0.2 g/l *p*-cresol) on the growth behavior and degradation capacity of the investigated strain was examined. The cell-free supernatants were analyzed by HPLC. It was established that the presence of *p*-cresol had not prevented complete phenol degradation but had a significant delaying effect on the phenol degradation dynamics. Phenol hydroxylase, catechol 1.2-dioxygenase and *cis,cis*-muconate cyclase activities were obtained in conditions of single and mixed substrates cultivation. The influence of different phenolic substrates on phenol hydroxylase activity in *Trametes versicolor* 1 was established. The mathematical models describing the dynamics of single substrates' utilization as well as the mutual influence of phenol and *p*-cresol in the mixture were developed on the bases of Haldane kinetics. The estimated interaction coefficients ($I_{ph/cr} = 4.72$, $I_{cr/ph} = 7.46$) demonstrated the significant inhibition of *p*-cresol on phenol biodegradation and comparatively low level of influence of phenol presence on the *p*-cresol degradation. Molecular 18S RNA gene taxonomy of the investigated strain was performed.

Keywords *Trametes versicolor* · Cresol utilization · Enzyme activities · Substrate interaction kinetics · 18S rDNA

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Introduction

The selection and investigation of strains with potential to be used as biocatalysts in the detoxification of toxic pollutants is an important step for improving and developing of new environmental technologies. Phenol and its various derivatives, as well as many other aromatic compounds, are known as hazardous pollutants. They can be detected in effluents from oil refineries, coal and chemical industries (Busca et al. 2008; Dhaouadi and Marrot 2008; Uğurlu et al. 2008). The methylated phenol derivates are one of the most toxic for the environment compounds (Huang et al. 2007; Hussain et al. 2008; Lin and Juang

2009). *p*-Cresol has been classified as a pollutant of group C (possible human carcinogens) by US Environmental Protection Agency (EPA).

A number of investigations with bacterial and yeast strains capable of aerobic phenol degradation have been reported (Jiang et al. 2006; Bai et al. 2007; Alexieva et al. 2008; Saravanan et al. 2008a). Some studies have shown that mycelial fungi can exert an important role in aromatic compounds aerobic recycling as well. Mycelial fungi of the genera *Fusarium*, *Graphium*, *Penicillium*, *Aspergillus* have been cited for their potential to degrade phenol (Santos and Linardi 2004; Stoilova et al. 2006). In all of mentioned strains, the phenol catabolism could go through the ketoadipate pathway, i.e. through the *ortho*-fission of catechol.

The first step in aerobic metabolism is phenol hydroxylation to catechol by NADPH-dependant phenol hydroxylase. Catechol, the product of the reaction catalyzed by phenol hydroxylase, is a central intermediate in the degradation pathways of various aromatic compounds. It is metabolized by different strains via either the *ortho*- or the *meta*-fission pathway. Catechol 1.2-dioxigenase catalyzes the second step of phenol *ortho*- pathway of phenolic compounds catabolism. The third key enzyme participating in phenol biodegradation is cis,cis-muconate cyclase, the inducible enzyme catalyzing the conversion of *cis,cis*-muconate to (+)-mucono-lactone (Alexieva et al. 2004).

The ability of white rot fungi to remove a variety of toxic organic pollutants from wastewaters has recently attracted the attention of scientists. This group of microorganisms is capable of degrading polymers of phenolic origin, including lignin. The biotransformation activity towards some wide spread phenolic pollutants is studied together with expression of specific enzymes (Buswell 2001; Stoilova and Krastanov 2008).

The successful bioremediation of phenolic wastewater by white rot fungus *Trametes versicolor* was found to be dependent on fungal growth, enzyme production (laccase) and some inductors (Ryan et al. 2007). There are numerous investigations on ability of *T. versicolor* to produce laccase and by this way to implement aromatics oxidation i.e. transformation of such compounds (Han et al. 2004; Stoilova and Krastanov 2008). The cultivation media has always been complemented with readily assimilated carbohydrates. The first data about phenol based growth of *T. versicolor* as well as phenol utilization as a single carbon substrate by this fungal strain were reported in our recent publication (Yemendzhiev et al. 2008).

The simultaneous presences of different organic mixtures in most of the industrial wastes have made the investigations on the microbial destruction of composite substrates of great importance. The removal or degradation of one or all components could be slowed down and/or stopped depending on the composition of the studied mixture. The influence of other compounds in a mixture of toxic carbon substrates on the biodegradation of a certain chemical could be positive or negative depending on the rate of competitive inhibition toxicity and the formation of toxic intermediates by non-specific enzymes (Bielefeldt and Stensel 1999; Yotova et al. 2009).

In the conditions of co-metabolism or induction of required degrading enzymes the sum of the mutual interactions could lead to a positive effect (Kar et al. 1997; Reardon et al. 2002; Juang and Tsai 2006a, b; Saravanan et al. 2008b). The effect of mixtures of phenol- and methyl-substituted phenols (*o*-, *m*-, *p*-cresol) on the growth behavior and degradation capacity of yeast strain *Trichosporon cutaneum* R57 has been investigated (Alexieva et al. 2008). Systems that exhibit co-metabolic behavior with chlorinated aromatics have been reported (Field and Sierra-Alvarez 2008).

Simultaneous metabolism of phenol and cresol were reported by Hutchinson and Robinson, who have studied the degradation kinetics of both, phenol and cresol by *Pseudomonas putida* in mixture where the concentrations were kept well below the inhibitory level of the toxic substrate (Hutchinson and Robinson 1988). The patterns of multiple substrate utilization and substrate interactions in the biodegradation of paired substrates (phenol/*p*-cresol, phenol/*o*-cresol) by *Arthrobacter* sp. MTCC1553 have been quantified and categorized (Kar et al. 1997). Similar experiments were reported for yeast *Candida tropicalis* where the authors examined the co-utilization of phenol and *m*-cresol (Jiang et al. 2006).

Quantitative estimation of interaction parameters is essential to facilitate the application of single or mixed cultures to the bio-treatment of hazardous compounds. The most mathematical models reported in the literature have been developed to describe only one substrate biodegradation. A few mathematical models of mixed homologous substrate consumption

and microbial growth have been proposed. It has been found that a competitive inhibition models could be used to predict the co-metabolic growth rate of *Pseudomonas* species on mixed toxic substrates (Chen et al. 2008). Completely empirical, exponential inhibition model has given the best fit for a *Flavobacterium's* species co-metabolizing pentachlorophenol, dichlorophenol and trichlorophenol (Gu and Korus 1995). The experimental results with *Pseudomonas putida* strain growing individually and together on 2-chlorophenol, 4-chlorophenol and phenol have been presented and mathematical models are created to describe different inhibition and toxicity effects on the cells and their degradation capacities (Loh and Wu 2006). They have demonstrated that the simple models do not accurately predict the outcome of these biodegradation experiments, and described the development of a new model for substrate mixtures, the sum kinetics with interaction parameters (SKIP) model. Recently, the biodegradation behavior of *Candida tropicalis* in dual-substrate system was described by kinetic equations adapted to fit the investigated process (Juang and Tsai 2006a, b).

In this study the degradation ability of *T. versicolor* 1 to degrade and utilize methylated phenols (cresols) as single carbon and energy sources was investigated for the first time. The binary mixture effect of phenol and *p*-cresol on the growth behavior and degradation capacity of the strain was studied, as well. Kinetic models describing the dynamics of *p*-cresol degradation and the mutual influence of both mixed compounds were developed. The three key enzyme activities and its variability depend on the substrate used in the degradation experiments were established. Molecular taxonomy of the investigated strain based on 18S rDNA sequence was performed.

Materials and methods

Microorganisms and growth conditions

The investigated strain of *T. versicolor* was grown on slants on a medium of the following composition: malt extract 3.0 g/l, yeast extract 3.0 g/l, peptone 5.0 g/l, glucose 10 g/l and agar 20 g/l. The organism on the slants was allowed to grow for 72 h at 28°C and then stored at 4°C.

Biodegradation was conducted on the carbon free Chapek-Dox medium (in %), as follows: $NaNO_3$—0.2, KH_2PO_4—0.1, KCI—0.05, $MgSO_4.7H_2O$—0.05, $FeSO_4.7H_2O$—0.001. The culture media pH was 6.5. The medium was supplemented with appropriate concentrations of cresol and phenol, as single or mixed carbon and energy sources. The tested concentrations of *o*-, *m*- and *p*-cresol ranged between 0.2 g/l and 1.0 g/l in single substrate experiments. The mixture of substrates contained 0.3 g/l phenol + 0.2 g/l *p*-cresol.

The flasks containing 50 ml inoculated culture medium were agitated on a New Brunswick rotary shaker (240 rpm) at temperature of 28°C. Samples were taken at every 12 h interval and centrifuged at 5,000 rpm for 20 min to settle down the cells. The dry weight of the cells was determined by ULTRA X apparatus for drying (Yemendzhiev et al. 2008).

Analytical methods

The content of phenols in the supernatant was determined by the HPLC analyses performed in C_{18} 10 μm Bondapac Column (3.9 mm × 300 mm) and waters 484UV detector (260 nm). The mobile phase was methanol–water (70:30), flow rate 0.2 ml/min and 22°C (Stoilova et al. 2006).

The availability and activity of enzymes, involved in the catabolism of aromatic hydrocarbons were determined by the following methods: Phenol hydroxylase (EC 1.14.13.7)—spectrophotometrically at 340 nm, by measuring NADH oxidation in the presence of phenol; Catechol 1, 2-dioxygenase (EC 1.13.11.1)—spectrophotometrically at 260 nm, by the accumulation of *cis, cis*-muconic acid in the medium leading to increasing the light absorption at this wavelength; *Cis, cis*-muconic acid cyclase (EC 5.5.1.5)—by the decreasing of light absorption at 260 nm due to the decomposing of *cis, cis*-muconic acid in the reaction mixture (Gaal and Neujahr 1979). The presence of laccase (EC 1.10.3.2) was searched for spectrophotometrically at 420 nm by measuring the oxidation of ABTS (2, 2′-azinobis-3-ethylbenzothiazoline-6-sulphonic acid; Bourbonnais and Paice 1990). One unit of enzyme activity is defined as the amount of enzyme transforming 1 μmol of substrate in 1 min under the assay conditions. Specific activities were expressed as units (U) per mg total cell protein.

Protein concentration in cell-free extracts was determined by the method of Lowry with bovine serum albumin as the protein standard (Lowry et al. 1951).

DNA isolation procedure

The cells were frozen at −20°C and then grinded mechanically and collected in a plastic tube with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH = 7.5). The obtained mixture was treated in liquid nitrogen followed by instant incubation at 90°C water bath. The following step presents a treatment with lysis buffer (50 µl 1 M Tris, 50 µl 0.5 M EDTA, 100 µl 5 M NaCl, 10% SDS, pH = 7.5). DNA extraction was accomplished by phenol–chloroform extraction. The DNA samples were purified with GFX columns (GE Healthcare, UK).

Agarose gel electrophoresis

DNA was resolved on a 0.7 or 1.5% agarose gel with TBE buffer by electrophoresis, stained with ethidium bromide, and visualized by UV irradiation. The DNA fragment sizes were estimated by comparison with 50 bp ladder (Fermentas).

PCR conditions and sequencing

The primers used for PCR amplification are: PFf 5′AGGGATGTATTTATTAGATAAAAAATCAA 3′ and PFr 5′ CGCAGTAGTTAGTCTTCAGTAAATC 3′ (Jaeger et al. 2000). The amplification was performed on thermocycler Eppendorf Mastercycler personal (Eppendorf AG, Hamburg, Germany) by using PuReTaq™ Ready-To-Go™ PCR Beads (Amersham Biosciences, Piscataway, NJ, USA). The PCR reactions were carried out in a 25 µl volume containing 10 pmol each primer and 50 ng genomic DNA. PCR procedure for 18S RNA gene amplification was carried out using an initial 5 min 95°C step followed by a 30 s 95°C denaturizing step, a 30 s 58°C annealing step, and then a 45 s 72°C extension steps. Steps 2–4 were repeated 35 additional times, with a final 7 min 72°C extension step before storage of the PCR products at 4°C.

The obtained PCR product was purified by GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Piscataway, NJ, USA). The sequencing of amplified fragment was performed on ABI Prism 310 Genetic Analyzer by using BigDye® Terminator Kit version 3.1. The row data from Genetic Analyzer were editing by Sequence scanner version 1.0 software (Applied Biosystems, Foster City, CA, USA).

Chemicals

All chemicals were of the highest purity grade available (Fluka AG, Sigma–Aldrich and Merck).

Statistics

The experiments for determination of biodegradation capacity of the investigated strain were performed in triple and the data shown in corresponding figures and tables in the text were the average values. Mean and standard deviation values of three replicates are shown on figures demonstrated the model inputs.

Results and discussion

18S rDNA PCR amplification and sequencing

The investigated *T. versicolor* strain has not been taxonomically determined by molecular methods previously. The panfungal primer pair was used in PCR procedure with the purpose to obtain products from 18S rDNA genes of the strain *T. versicolor* 1. The partial sequences of the PCR products were determined. The size of the nucleotide sequence obtained was 664 bases. The comparison of our data with NCBI Gene Bank Data Base reference sequences was conducted by Blast program. The phylogenetic analysis of 18S RNA gene sequence was performed by using ClastalW2. This molecular taxonomic analysis established the strain belonging to *T. versicolor* species. The nucleotide sequence obtained in this investigation has been deposited in the NCBI nucleotide sequence databases under *Accession Number*: GQ472774.

Degradation of *o*-, *m*- and *p*-cresol by *T. versicolor* strain 1

Microorganisms capable of degrading one aromatic compound are often able to degrade other similar compounds (Alexieva et al. 2008; Varma and Gaikwad 2008). Previously the ability of *T. versicolor* 1 to

utilize phenol was demonstrated. It has been demonstrated that the strain had mineralized phenol (0.5 g/l) as a sole carbon source (Yemendzhiev et al. 2008). These results gave us a reason to investigate the strain capability to degrade other toxic industrial pollutants such methyl-phenols: *o*-, *m*- and *p*- cresols.

The degradation ability of the investigated strain was examined in a mineral medium without carbon components except for the investigated compounds. Firstly, the experiments were carried out in a medium comprising 0.3 g/l *o*-, *m*- or *p*-cresol. It was observed that *T. versicolor* 1 had a different capability to degrade the isomeric forms of cresol. For example, the complete degradation and assimilation of *p*-cresol as a sole carbon source was performed for about 72 h. The other two compounds (*o*- and *m*- cresol) were degraded much slower and the observed decrease of its concentrations for the same period of time was 30 and 23%, respectively (Fig. 1a).

The test with 0.5 g/l *o*-, *m*- and *p*-cresol as a sole carbon source in the medium demonstrated a similar tendency of growth and degradation. The time for complete *p*-cresol degradation was 96 h. At the same time, both *o*- and *m*- cresol were partly degraded (34 and 26% respectively) and the cells' growth stopped (Fig. 1b). Despite of some cell growth observed, in view of the small concentrations degraded there could as well be an assumption for ad- or absorption of *o*- and *m*- cresol.

Some kinetic studies were performed on the basis of the data obtained. The values of both specific growth (μ) and degradation (q) rates are presented in Table 1. The comparison between the estimated values of these process characteristics for all three cresol isomers degradation proved the best *p*-cresol degradability by the investigated *T. versicolor* strain.

In the experiments carried out with higher concentrations of cresol substrates the rates of growth and degradation showed significant decrease i.e. a significant substrate inhibitory effect was observed. For example, there was no growth at a concentration of *p*-cresol equal to 1 g/l during 120 h of cultivation.

The effects of phenol on *p*-cresol biodegradation

Since *p*-cresol was the best degradable investigated compound, we used a p-cresol/phenol mixture.

The next set of experiments was carried out with 0.3 g/l phenol and/or 0.2 g/l *p*-cresol. The substrate concentrations were kept below the inhibitory level with the purpose to avoid the well-known high toxicity of both compounds and their negative effects on the entire cells' development and that way to optimize the strain cultivation (Hutchinson and Robinson 1988; Alexieva et al. 2008; Saravanan et al. 2009).

The complete assimilation of 0.3 g/l phenol was observed in 48 h. The experiments involving the degradation of 0.2 g/l p-cresol as a sole carbon source

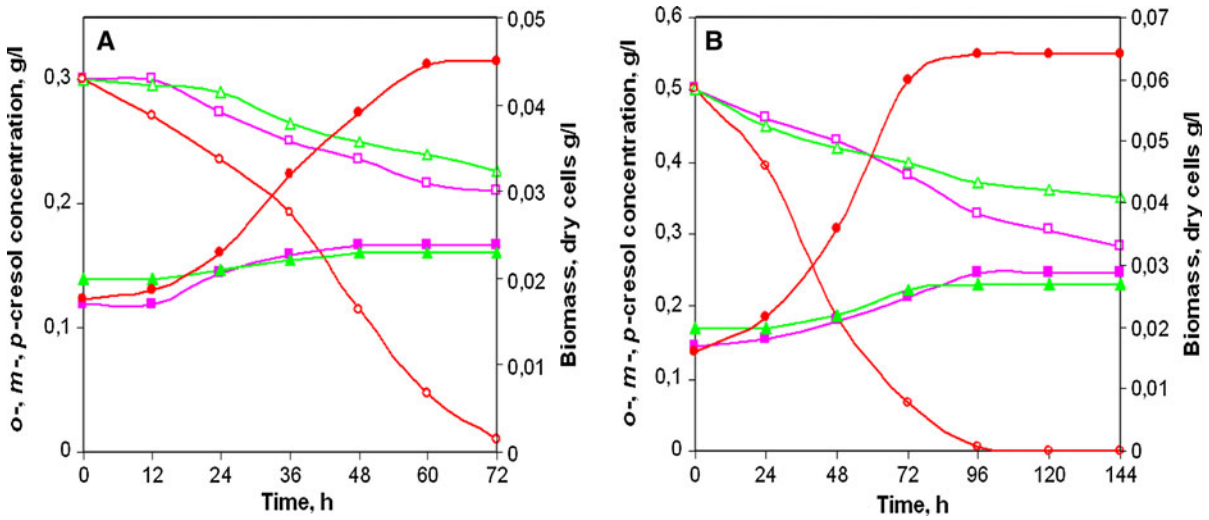

Fig. 1 Growth and degradation of strain *T. versicolor* 1 in a synthetic medium complemented with 0.3 g/l (**a**) and 0.5 g/l (**b**) *o*-cresol (■), *m*-cresol (▲), *p*-cresol (●). *Filled symbols* indicate microbial growth, unfilled symbols indicate biodegradation. Mean values of three replicates are shown

Table 1 Specific growth and degradation rate* of *Trametes versicolor* 1 cultivated in a medium supplemented with 0.3 g/l or 0.5 g/l cresols as a sole carbon source

Substrate	μ (h^{-1})		q (h^{-1})	
	0.3 g/l	0.5 g/l	0.3 g/l	0.5 g/l
o-Cresol	0.008	0.0052	0.053	0.052
m-Cresol	0.004	0.0036	0.045	0.039
p-Cresol	0.012	0.009	0.09	0.08

* Mean values of three replicates are shown

demonstrated its complete utilization in 60 h. The growth kinetics of these single substrate experiments was studied and described by Haldane kinetics:

$$\mu(S_{\mathrm{ph}}) = \frac{\mu_{\max(\mathrm{ph})} S_{\mathrm{ph}}}{k_{s(\mathrm{ph})} + S_{\mathrm{ph}} + S_{\mathrm{ph}}^2 / k_{i(\mathrm{ph})}}$$

$$\mu(S_{\mathrm{cr}}) = \frac{\mu_{\max(\mathrm{cr})} S_{\mathrm{cr}}}{k_{s(\mathrm{cr})} + S_{\mathrm{cr}} + S_{\mathrm{cr}}^2 / k_{i(\mathrm{cr})}}$$

where S_{ph} was the phenol concentrations; $S_{\mathrm{cr}}$was the *p*-cresol concentrations; $k_{s(\mathrm{ph})}$ was the saturation constant of the phenol; $k_{s(\mathrm{cr})}$ was the saturation constant of the *p*-cresol; $\mu_{\max(\mathrm{ph})}$ was the maximum specific growth rate for phenol; $\mu_{\max(\mathrm{cr})}$ was the maximum specific growth rate for *p*-cresol; $k_{i(\mathrm{ph})}$ was the inhibition constant of the phenol; $k_{i(\mathrm{cr})}$ was the inhibition constant of the *p*-cresol. The experimental data and model curves are presented on Fig. 2.

The degradation of binary mixture containing 0.3 g/l phenol and 0.2 g/l *p*-cresol by *T. versicolor* 1 was investigated. The data obtained showed a simultaneous start of degradation of phenol and *p*-cresol. We observed a significant influence of phenol on the *p*-cresol degradation. In the studied mixture *p*-cresol was utilized faster than in the media containing it as a sole carbon source. In contrast the effect exerted by *p*-cresol on the phenol degradation was rather negative. Although complete phenol degradation was observed the degradation rate was significantly slowed down. The results are illustrated on Fig. 3.

There are results from similar degradation experiments with some bacterial, yeast and fungal strains capable to remove phenol/cresol mixture published by other authors. A growth kinetic model was used to characterize the substrates interactions in the dual substrates system experiments with *A. faecalis* cells cultivated in a medium with a mixture of phenol and

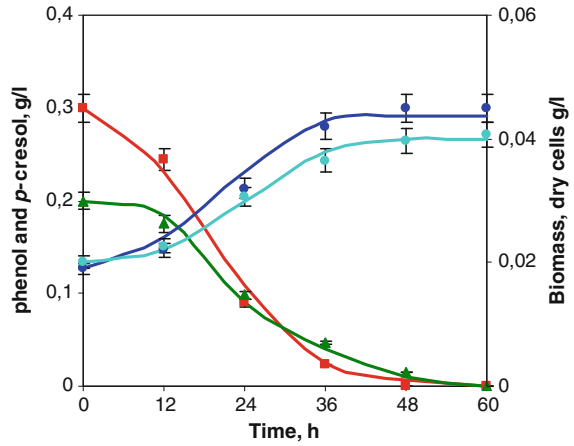

Fig. 2 Experimental data (*symbols*) and model output (*curves*) for biodegradation of single substrates by *T. versicolor* 1: 0.3 g/l phenol [biomass (●), phenol (■)]; 0.2 g/l *p*-cresol [biomass (○), *p*-cresol (▲)]. Mean and standard deviation values of three replicates are shown

m-cresol, where both compounds strongly inhibited the biodegradation of each other (Bai et al. 2007). On the other hand the results obtained in experiments with *Candida tropicalis* strain grown on phenol and *m*-cresol as single and mixed substrates in batch cultures showed that the presence of *m*-cresol intensely inhibited phenol biodegradation. On the contrary, the phenol of low concentration supplied a sole carbon and energy source for *C. tropicalis* in the initial phase of biodegradation and accelerated the assimilation of

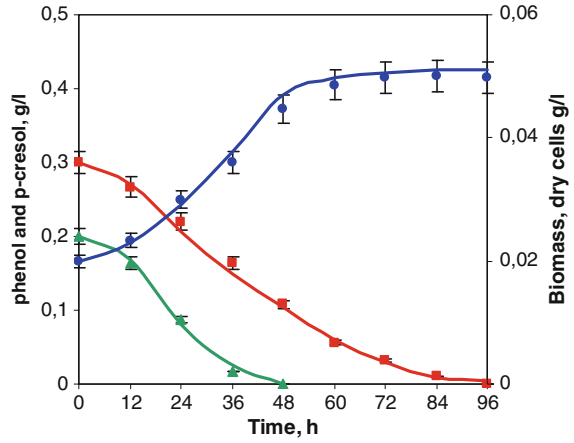

Fig. 3 Experimental data (*symbols*) and model output (*curves*) for biodegradation of a phenol/*p*-cresol mixture by *Trametes versicolor* 1: biomass (●), phenol (■) and *p*-cresol (▲) concentrations. Mean and standard deviation values of three replicates are shown

m-cresol (Jiang et al. 2006). The second example demonstrated a similarity with our results but the inhibitory concentration of cresol, which terminated phenol degradation was very low—0.06 g/l. Another difference consisted of the consecutive utilization of both substrates. Although the phenol concentration was much higher than the cresol concentration in the medium, cells still utilized phenol as the preferred carbon and energy source (Jiang et al. 2006). As could be seen the results from various degradation experiments with some bacterial, yeast and fungal strains that were capable to remove phenol/cresol mixtures (including ours) take us to the suggestion that despite of some common features of the phenol and cresol mixture biodegradation there are some clear differences depending on the strain biochemical potential.

Substrate interactions model for biodegradation of phenol/*p*-cresol mixture by *T. versicolor* 1

To create a kinetic model which was accounting for interactions between phenol and *p*-cresol in mixture we used the idea for "sum kinetics with interaction parameters" (SKIP) model (Reardon et al. 2002; Saravanan et al. 2008a, b). In SKIP models, the effect of the presence of one substrate S_1 (S_2) on the degradation of another substrate S_2 (S_1) is given by $S_1 I_{1/2}$ ($S_2 I_{2/1}$) terms.

In this study, the parameter identification problem was turned to estimation of values of the interaction parameters ($I_{ph/cr}$ and $I_{cr/ph}$) and the metabolic coefficients (k_{ph} and k_{cr}). The interaction coefficients ($I_{cr/ph}$, $I_{ph/cr}$) indicated the degree to which *p*-cresol affected the phenol biodegradation and vice versa. The larger values of interaction coefficient correspond to stronger inhibition.

The growth kinetic model describing biodegradation of binary mixture of phenol and *p*-cresol can be presented as follows:

$$\frac{dX(t)}{dt} = \mu(S_{ph}, S_{cr})X(t)$$

$$\frac{dS_{ph}(t)}{dt} = -k_{ph}\,\mu(S_{ph}, S_{cr})X(t)$$

$$\frac{dS_{cr}(t)}{dt} = -k_{cr}\,\mu(S_{ph}, S_{cr})X(t),$$

where X was the biomass concentration; k_{ph} and k_{cr} were the metabolic coefficients; $\mu(S_{ph}, S_{cr})$ was the specific growth rate for mixed substrates which was given in the following equation:

$$\mu(S_{ph}, S_{cr}) = \frac{\mu_{max(ph)} S_{ph}}{k_{s(ph)} + S_{ph} + S_{ph}^2 / k_{i(ph)} + I_{cr/ph} S_{cr}} + \frac{\mu_{max(cr)} S_{cr}}{k_{s(cr)} + S_{cr} + S_{cr}^2 / k_{i(cr)} + I_{ph/cr} S_{ph}}.$$

The SKIP model prediction was compared with experimental data. It was found that the designed model described the trend of experimental data satisfactorily. Computer simulations and experimental data are shown in Fig. 3.

The kinetic parameters characterizing the binary mix degradation were determined. They were estimated equal to the obtained parameters for the single substrate experiments. The only exception observed referred μ_{max} for phenol utilization which dropped twice (Table 2). According to our experimental data *p*-cresol and phenol degraded simultaneously but it was obvious that cells were unable to utilize the growth substrates in a way to produce as much as the sum of established biomasses (X) in single substrate experiments. Such data have been reported by others (Dapaah and Hill 1992). The values of metabolic and interaction coefficients are given in Table 2.

The obtained inhibitory coefficients (k_i) in single substrates experiments for phenol and *p*-cresol corresponded to the better phenol degradation compared with *p*-cresol. The lower value of $k_{i(cr)}$ showed the stronger toxic effect of *p*-cresol on the *T. versicolor* 1 strain development. In the experiments with mixed substrates we observed that the presence of phenol accelerated the *p*-cresol degradation. On the contrary, even low concentration of *p*-cresol led to a strong negative effect on the phenol utilization. The time for phenol degradation was almost doubled when the degradation of *p*-cresol was realized much faster in the dual mixture. The higher value of interaction coefficient $I_{cr/ph} = 7.46$ in contrast with $I_{ph/cr} = 4.72$ demonstrated the stronger influence of the *p*-cresol on phenol degradation.

Enzyme analyses

It was mentioned above that *T. versicolor* strains possessed a notable capability to produce laccase as a rule (Han et al. 2004; Stoilova and Krastanov 2008). Because of that fact in all our experiments we

Table 2 Parameters* for SKIP model of biodegradation of phenol/*p*-cresol mixture by *Trametes versicolor* 1

Parameters	Growth substrates		
	p-Cresol	Phenol	*p*-Cresol/phenol
S_0 (*g/l*)	0.2	0.3	0.2/0.30
μ_{max} (h^{-1})	0.1	0.1	0.1/0.05
k_s(*g/l*)	0.28	0.27	0.28/0.27
k_i(*g/l*)	0.05	0.15	0.05/0.15
k	9.95	11.5	7.59/9.45
$I_{ph/cr}$	N/A	N/A	4.72
$I_{cr/ph}$	N/A	N/A	7.46

* Mean values of three replicates are shown

measured the laccase activity with the purpose to be sure for the reason of phenolic compounds disappearance. It appeared that our experimental conditions did not favor laccase production and practically no laccase activity was detected. That is why we consider that the investigated aromatics were really degraded instead of being oxidized by laccase.

The enzyme activities of the first three enzymes accomplishing the *ortho*-mechanism of aromatics compound biodegradation were determined in *T. versicolor* 1 cells grown on phenol, *p*-cresol and mixture of phenol and *p*-cresol as a sole carbon source in the medium. The degradation medium was inoculated with prepared in advance mycelium of the *T. versicolor* strain 1. We established that the intracellular activities of the investigated enzymes were constantly high in the beginning of logarithmic growth phase of culture. That's why all samples for enzyme assays were taken at the 24th hour of growth. The activity of phenol hydroxylase, catechol 1,2-dioxygenase and cis,cis-muconate cyclase in cell free extracts obtained from cells grown on phenol and p-cresol, suggested that aromatics were oxidized by the *ortho*-type of ring fission (Table 3). The data showed that in tests with *p*-cresol as a single substrate all enzyme activities were much lower by comparison with those obtained with phenol as a single substrate. The presence of phenol leaded to enhancement of enzyme activities values. These results proved once more the favorable effect of phenol on p-cresol degradation and were in good correlation with the established interaction coefficients (Table 2).

The influence of enzyme substrate specificity on the phenol hydroxylase activity in *T. versicolor* 1

Each of the extracts of phenol, p-cresol and phenol + *p*-cresol grown mycelia were incubated with different phenol derivatives as substrates in the enzyme reactions and tested for phenol hydroxylase activity (Table 4).

In the experiments with extracts of mycelia grown on single carbon source medium (phenol or *p*-cresol) the enzyme activities values varied depending on the enzyme substrate specificity. It was interesting to find that the phenol hydroxylase activity obtained in cells cultivated in a phenol containing medium was higher than that observed in the reactions with phenol, *p*-cresol and *p*-methyl-catechol correspondingly when hydroquinone was used as an enzyme substrate in the reaction mixture. At the other hand the enzyme activity in cells grown on *p*-cresol as a carbon source during cultivation was the highest when *p*-methyl-catechol was applied as an enzyme substrate. It could be due to the existence of some phenol hydroxylase isoenzymes which have a preference for some phenol hydroxyl derivatives as hydroquinone. Some authors suggest the existences of different enzymes responsible for the oxidation of such substrates (Moonen et al. 2002).

There were differences observed as well between enzyme activities measured in the probes from single substrates grown mycelia and those grown with the mixture of both substrates. The results demonstrated a reduction of the obtained activities in the experiments carried out with dual substrates grown mycelia extracts compared with values obtained in mycelia extracts received after cultivation with phenol as a single carbon source. The only exception could be observed in the enzyme reaction with phenol used as a substrate in enzyme reaction. The comparison of the enzyme activities determined in the dual substrate experiment with those obtained with *p*-cresol as a single carbon source showed some significant differences. The phenol hydroxylase activity measured towards phenol used in the enzyme reaction was higher in mycelia grown with both compounds as a mixture with regard to the enzyme activity detected in mycelia grown with p-cresol as a single source of carbon. On the contrary, in the experiments with *p*-methyl-catechol

Table 3 Phenol hydroxilase, catechol-1,2- dioxygenase and cis,cis-muconate cyclase activities* in *Trametes versicolor* 1 cells grown on phenol, *p*-cresol and mixture of phenol and *p*-cresol as a sole carbon source in the medium

Carbon source	Phenol (0.3 g l^{-1})	*p*-Cresol (0.2 g l^{-1})	Mixture of phenol (0.3 g l^{-1}) and *p*-Cresol (0.2 g l^{-1})
Enzymes			
Phenol hydroxylase (Units/mg protein)	0.31	0.128	0.33
Catechol-1,2-dioxygenase (Units/mg protein)	0.19	0.1	0.165
Cis,cis-muconate cyclase (Units/mg protein)	0.4	0.23	0.283

* Mean values of three replicates are shown

Table 4 Phenolic substrates influence on phenol hydroxylase activity* in *Trametes versicolor* 1 cells grown in the culture media comprising single carbon source (phenol or *p*-cresol) as well as both compounds mixed

Carbon source in the growth medium	Phenol hydroxylase activity (Units/mg total protein)		
	Phenol (0.3 g l^{-1})	*p*-Cresol (0.2 g l^{-1})	Mixture of phenol (0.3 g l^{-1}) and *p*-Cresol (0.2 g l^{-1})
Enzyme substrate			
p-Cresol	0.20	0.128	0.17
Hydroquinone	0.35	0.145	0.14
p-Methyl-catechol	0.1	0.193	0.083

* Mean values of three replicates are shown

as a substrate for the investigated enzyme the opposite effect was observed (Tables 3, 4).

Conclusions

The results obtained demonstrated that the strain *T. versicolor* 1 possessed the potential to degrade and assimilate *p*-cresol as sole carbon sources in the culture medium. This compound was used through the experiments with *p*-cresol/phenol mixture degradation. We developed the SKIP model on the basis of well known Haldane kinetics, which used model parameters from single substrate experiments to predict the outcome of the two substrates mixture degradation experiment. The combined data received from both real and computer simulated experiments gave us a reason to conclude that phenol exert a positive influence on *p*-cresol degradation. In contrast the effect exerted by *p*-cresol on phenol degradation was rather negative. Although complete phenol depletion was observed the degradation rate was significantly slowed down. It could be concluded that the applied model gave a good description of the phenol/*p*-cresol mixture degradation by the investigated *T. versicolor* 1.

The influence of enzyme substrate specificity on the phenol hydroxylase activity in *T. versicolor* 1 demonstrated a good correspondence of enzyme activity variations with the kinetic parameters characterizing the studied biodegradation processes.

The investigations on specificity of interaction between different compounds are meaningful for the invention of effective remediation technologies for industrial wastes where the mixed substrates are common occurrence.

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