GROWTH OF TRAMETES VERSICOLOR IN NITRO AND HYDROXYL PHENOL DERIVATIVES

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ABSTRACT
This is the first report on the ability of Trametes versicolor to utilize hydroxyl and nitro aromatic compounds as a sole carbon source. The cultivation was carried out in Czapek salt medium supplemented with 0.5 g/l 2,3,4-nitrophenol as well as benzene-1,3-diol (resorcinol), benzene-1,2-diol (catechol) and benzene-1,4-diol (hydroquinone), respectively. The hydroxylated phenols were completely utilized for 80-96 hours. The best degradation of the investigated nitrophenols was registered with 2-nitrophenol. The strain was able to degrade it completely in 216 hours.

The dynamics of all degradation processes were investigated and characterized by specific growth rate ($\mu_{\max}$), metabolic coefficient (k) and rate of degradation (Q). The kinetic parameters established for the best utilized compounds were as follows: benzene-1,3-diol – $\mu_{\max} = 0.065$ h⁻¹, k = 9.0, Q = 0.075 h⁻¹; and 2-nitrophenol – $\mu_{\max} = 0.048$ h⁻¹, k = 8.5, Q = 0.033 h⁻¹. The intracellular phenol hydroxylase [EC 1.14.13.7], catechol-1,2-dioxygenase [EC 1.13.11.1], and cis,cis-muconate lactonizing enzyme [EC 5.5.1.5] activities were determined in cells cultivated in medium complemented with the best utilized compounds – resorcinol and 2-nitrophenol. The obtained data showed that the substrate specificity of the first enzyme – phenol hydroxylase, is decisive for the rate of degradation of the studied toxic aromatic compounds.


Keywords: Trametes versicolor, phenol hydroxylase, catechol-1,2-dioxygenase, cis,cis-muconate lactonizing enzyme, nitro-and hydroxy-phenols

Nomenclature:
X: biomass concentration (optical density at $\lambda = 610$ nm)
S: substrate concentration (g l⁻¹)
k: saturation constant for cell growth (g l⁻¹)
k: inhibition constant for cell growth (g l⁻¹)
k: metabolic coefficient (S/X)
Q: specific degradation rate (h⁻¹)
$\mu$: specific growth rate (h⁻¹)
$\mu_{\max}$: maximum specific growth rate (h⁻¹)

Introduction
Wastewaters from petroleum refineries, coal mining and a variety of industrial chemical syntheses contain many aromatics such as phenol, cresols, nitrophenols, etc (14). The metabolism of aromatic compounds, particularly phenol and its derivatives are intensively studied in prokaryotic microorganisms (1, 8, 11). Some members of the yeast genera Candida, Rodotorula, Trichosporon and others that can metabolize phenolic compounds as a sole carbon and energy source have been described (8, 14). Mycelial fungi such as Fusarium flocicferum, Graphium sp., and Aspergillus awamori have been cited for their potential for phenol degradation (4, 17, 18, 25).

The ability of white rot fungi to degrade a variety of toxic industrial pollutants of the environment is well known. This group of microorganisms is capable of completely degrading polymers of phenolic origin, including lignin (5, 19). One of the members of the white-rot fungi family with proven bioremediation and degradation capacity is Trametes versicolor. The successful bioremediation of a phenolic wastewater by T. versicolor was found to be dependent on fungal growth, laccase and other ligninolytic activities production as well as some inductors (13, 16). Strains of T. versicolor have also been described as capable of degrading phenol and some phenolic compounds (20, 23).

The enzyme phenol hydroxylase catalyzes the first reaction of phenol degradation in aerobic microorganisms (7, 12, 15). Catechol, the product of this reaction, 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 depending on the catechol dioxygenase specificity (1). The cycloisomerases catalyze the conversion of cis,cis-muconate to (+)-muconolactone in the ortho-mechanism of phenol degradation (10, 25).

Recently we reported that the mycelium of the basidiomycete fungus Trametes versicolor 1 was able to grow on phenol and methylated phenols used as sole carbon and energy sources. The investigated strain, cultivated in salt
medium was able to utilize 0.5 g/l phenol or p-cresol in 5 to 6 days. The intracellular activities of the first three enzymes involved in the phenol catabolism were established during the degradation processes (3, 23).

The microbial strains used for decontamination of different origin wastewater should not only be highly active to one of the contaminants but they should also be resistant enough to the remainder. Their resistance can be ensured by the degradation activity of the strains used towards most of the waste products present in the wastewater (2).

Nitro aromatic compounds are widely spread contaminants generated by industrial production of insecticides, herbicides, fungicides and explosives. These compounds accumulate in the environment due to their high toxicity and very good water solubility.

Hydroxyl aromatic compounds like resorcinol, catechol and hydroquinone are widely used for production of various chemical reagents. Resorcinol is used for production of dyes, plasticizers, textile, resin products etc. Catechol is used as raw material for production of pharmaceutical agents, insecticides and antioxidants while hydroquinone is mainly as photographic reactant and in medicine. These compounds can be utilized by many organisms at low concentration, but at high concentrations they can also act as toxic growth inhibitors.

The aim of this study was to examine the ability of *Trametes versicolor* strain 1 to utilize some nitro- and hydroxyl-phenol derivatives as a sole carbon and energy source and to characterize the dynamics of degradation processes. In this study the effect of the different substrates on the enzyme activities of phenol hydroxylase, catechol 1,2-dioxygenase and *cis,cis*-muconate lactonizing enzyme were investigated.

### Materials and Methods

#### Strain and culture medium

*Trametes versicolor* strain 1 was isolated from rot wood wastes in the University of Food Technology, Dept. Biotechnology, Plovdiv, Bulgaria, in 2007 and was found to be able to utilize phenol and decolorize some textile dyes (23, 24).

Biodegradation was conducted on the carbon free Chapek-Dox medium containing 0.5 g/l benzene-1,2-diol (catechol), benzene-1,3-diol (resorcinol), benzene-1,4-diol (hydroquinone), 2-nitrophenol, 3-nitrophenol or 4-nitrophenol as a single carbon and energy source (18).

#### Cultivation conditions

The flasks containing 100 ml inoculated culture medium were agitated on a New Brunswick rotary shaker (240 rpm) at 28°C. The sampling volumes of 100 ml each were taken at 24-hour intervals and centrifuged at 5000 rpm for 20 min to settle the cells down. The dry weight of the cells was determined by ULTRA X apparatus for drying (23).

#### Analytical methods

The content of phenols in the supernatant was determined by the HPLC analyses performed in C18 10 μm Bondapac BIOTECHNOL. & BIOTECHNOL. EQ. 26/2012/1 Column (3.9 mm × 300 mm) and Waters 484UV detector at 260 nm (Waters Corp., Milford, MA 01757 USA). The mobile phase was methanol – water (70:30), flow rate 0.2 ml/min and 22°C (18). The total protein concentration was determined by Lowry’s method (9).

The heat-killed culture controls were used to account for abiotic losses of phenols or sorption of phenols to fungal biomass.

The enzyme activities were determined in cell free extracts obtained by quartz sand mechanical grind in a buffer containing 0.06 M KH2PO4 and 0.06 M Na2HPO4 (pH = 7.0). The obtained lysates were clarified by centrifugation (1 min, 7000 rpm, 4°C).

The phenol hydroxylase (EC 1.14.13.7) activity was determined spectrophotometrically (LKB UV-Vis Ultraspec 1000), measuring the oxidation of NADPH in the presence of substrate (12). The activity of catechol 1,2-dioxygenase (EC 1.13.11.1) was determined by measuring the rate of *cis,cis*-muconic acid accumulation at 260 nm (21). The activity of *cis,cis*-muconate lactonizing enzyme (EC 5.5.1.5) was analyzed spectrophotometrically at 260 nm, measuring the decrease of *cis, cis*-muconic acid concentration (6).

All the experiments for biodegradation capacity and enzyme activities determination were performed in triplicates. The average values of the obtained data were shown in corresponding figures and tables in the text.

All chemicals were of the highest purity grade available (Sigma-Aldrich and Merk).

### Results and Discussion

#### Degradation of nitro substituted phenols by *Trametes versicolor*

The growth of the strain and degradation dynamics of 2-, 3- and 4-nitrophenol were examined in a medium containing from 0.1 to 0.5 g/l of each of these compounds as a single carbon source. The curves of growth and degradation obtained as a result from the strain cultivation in carbon-free Chapek Dox salt medium containing separately the three compounds mentioned above are shown in Fig. 1. The kinetic characteristics of the investigated processes were calculated by the Haldane model equation:

\[
\mu = \frac{\mu_{\text{max}} S}{k_s + S + S^2/k_i}
\]

This is a modified Monod relation to allow for the inhibitory effects of substrates. The value of *k_i* is equal to the lowest concentration of the inhibitor which causes a decrease in the microbial growth rate. It is proved that high values of *k_i* correspond to a low effect of substrate inhibition (22). It could be seen from the low values of the obtained inhibitory coefficients that the investigated nitro derivatives of phenol exerted a highly toxic effect on the strain development (Table 2727).
The fastest degradation was observed in the experiments with 2-nitrophenol utilization – 216 hours. The 68% of 3-nitrophenol was removed from the medium in 168 hours. After that period of time the strain could not grow. The worst degradation was observed in the experiments with 4-nitrophenol. Only 32% were removed from the culture medium in 120 hours and the strain growth was extremely slow. Both 3-nitrophenol and 4-nitrophenol were not completely degraded in 240 hours. The obtained substrate affinity constants (k_{s}) and maximum specific growth rates (\mu_{\text{max}}) are presented in Table 1.

The inhibition coefficients were calculated according to the Haldane-kinetics and their values are very similar (Table 1). That observation showed the comparably similar dynamics of the process of degradation of these compounds. The inhibitory effects on the Trametes versicolor 1 growth were significantly lower than those obtained in the experiments with nitro-phenols. The high inhibition coefficients’ values were in agreement with the period of time in which we observed complete substrate utilization. The obtained saturation constants (k_{s}) and maximum specific growth rates (\mu_{\text{max}}) were also comparable to each other for all investigated hydroxyl phenols degradation processes (Table 1). The resorcinol could be pointed out as the best assimilated compound among these three hydroxyl phenol derivatives.

There were not meaningful changes of any phenol compounds concentration or biomass growth observed in the heat-killed culture controls used.

Analyses on the first three enzymes of the catabolic pathway of phenol in Trametes versicolor 1 cells
In this set of experiments resorcinol and 2-nitrophenol were used as single carbon sources in the cultivation media and as enzyme substrates in the enzyme reaction mixtures. These compounds were chosen as representatives of both groups of phenol derivatives investigated in this study because of their good degradability by Trametes versicolor strain 1. All the samples for enzyme assays were taken at the middle of the culture logarithmic growth phase.

Each of the intracellular extracts of resorcinol and 2-nitrophenol grown mycelia was tested for phenol hydroxylase activity. Phenol or corresponding phenol derivatives were used as substrates in the enzyme reactions for determination of enzyme activity. As could be expected from the data presented above, the phenol hydroxylase activity was higher in the cells cultivated with resorcinol in the medium than in those cultivated with 2-nitrophenol. The best activity was observed in the reactions with resorcinol applied as a substrate for phenol hydroxylase (Table 2).

The obtained enzyme activities of the next two enzymes – catechol 1,2-dioxygenase and cis,cis-muconate cyclase, showed similar values irrespective of the compounds included as a carbon source in the cultivation medium (Table 2). The

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**Degradation of hydroxyl substituted phenols by Trametes versicolor**

In the present study the growth of the strain and degradation dynamics of resorcinol, catechol and hydroquinone in concentrations varying from 0.1 to 0.5 g/l were studied. Fig. 2 shows the curves of growth and degradation obtained as a result from strain cultivation in carbon-free Chapec Dox salt medium containing separately the three compounds mentioned above. The demonstrated results indicated very well the ability of the investigated strain to utilize these compounds and allowed a good parallel of the growth dynamics to be made. The time necessary for complete degradation of 0.5 g/l of these phenol derivates was found to be within 80-96 h including an adaptation phase.

The inhibition coefficients were calculated according to the Haldane-kinetics and their values are very similar (Table 1). That observation showed the comparably similar dynamics of the process of degradation of these compounds. The inhibitory effects on the Trametes versicolor 1 growth were significantly lower than those obtained in the experiments with nitro-phenols. The high inhibition coefficients’ values were in agreement with the period of time in which we observed complete substrate utilization. The obtained saturation constants (k_{s}) and maximum specific growth rates (\mu_{\text{max}}) were also comparable to each other for all investigated hydroxyl phenols degradation processes (Table 1). The resorcinol could be pointed out as the best assimilated compound among these three hydroxyl phenol derivatives.

There were not meaningful changes of any phenol compounds concentration or biomass growth observed in the heat-killed culture controls used.

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**Fig. 1. Biodegradation of mono-hydroxylated phenols by T. versicolor 1.** Symbols indicate: biomass (\textcircled{1} – resorcinol, \textcircled{\Delta} – hydroquinone, \textcircled{•} – catechol); concentrations (\bullet – resorcinol, \textcircled{\Delta} – hydroquinone, \bullet – catechol). Mean values of three replicates are shown.

**Fig. 2. Biodegradation of mono-nitrophenols by T. versicolor 1.** Symbols indicate: biomass (\textcircled{1} – 2-nitrophenol, \textcircled{\Delta} – 3-nitrophenol, \textcircled{•} – 4-nitrophenol); concentrations (• – 2-nitrophenol, \textcircled{\Delta} – 3-nitrophenol, • – 4-nitrophenol). Mean values of three replicates are shown.
Model kinetic parameters of growth and phenolic derivatives degradation by T. versicolor strain 1

<table>
<thead>
<tr>
<th>Kinetic parameters*</th>
<th>Catechol</th>
<th>Resorcinol</th>
<th>Hydroquinone</th>
<th>2- Nitrophenol</th>
<th>3-Nitrophenol</th>
<th>4-Nitrophenol</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\mu_{\text{max}}$ (h⁻¹)</td>
<td>0.05</td>
<td>0.065</td>
<td>0.07</td>
<td>0.048</td>
<td>0.07</td>
<td>0.04</td>
</tr>
<tr>
<td>$k_i$ (gl⁻¹)</td>
<td>0.4</td>
<td>0.5</td>
<td>0.4</td>
<td>0.13</td>
<td>0.05</td>
<td>0.045</td>
</tr>
<tr>
<td>$k_s$ (gl⁻¹)</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.68</td>
<td>0.68</td>
<td>0.85</td>
</tr>
<tr>
<td>$Q$ (h⁻¹)</td>
<td>0.064</td>
<td>0.075</td>
<td>0.06</td>
<td>0.033</td>
<td>0.035</td>
<td>0.030</td>
</tr>
</tbody>
</table>

* Mean values of three replicates are shown

TABLE 2

Enzyme activities determined in the cell-free extracts of T. versicolor strain 1 cultivated with resorcinol and 2-nitrophenol as single carbon substrates in the carbon-free medium

<table>
<thead>
<tr>
<th>Enzymes*</th>
<th>Growth Substrates</th>
<th>Resorcinol</th>
<th>2-Nitrophenol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With phenol added in the enzyme reaction</td>
<td>0.24</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>With resorcinol added in the enzyme reaction</td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td>Phenol hydroxylase</td>
<td>U/mg protein</td>
<td>0.15</td>
<td>0.12</td>
</tr>
<tr>
<td>Catechol 1,2-dioxygenase</td>
<td>U/mg protein</td>
<td>0.15</td>
<td>0.33</td>
</tr>
<tr>
<td>cis,cis-Muconate cyclase</td>
<td>U/mg protein</td>
<td>0.33</td>
<td>0.34</td>
</tr>
</tbody>
</table>

* Mean values of three replicates are shown

activity of catechol 1,2-dioxygenase could be considered as a process rate-determining step.

The data obtained in the described enzyme analyses combined with our previous experiments related to the substrate specificity of the investigated enzymes in Trametes versicolor strain 1 gave us a reason to conclude that the strain is with high potential for complete assimilation of a wide spectrum of mono-aromatic compounds (3, 23).

Conclusions

The analyses carried out with the first three enzymes of the phenol catabolism confirmed the ability of the strain Trametes versicolor 1 to utilize hydroxyl and nitro phenol derivatives applied as sole carbon sources in the media. By analysis of the obtained data it could be concluded that the investigated strain demonstrated selective degradation ability with regard to the level of aromatic compounds’ inhibitory effect and substrate’s affinity expressed by saturation coefficient.

The influence of enzyme substrate specificity on the phenol hydroxylase activity in Trametes versicolor 1 was investigated. We established a good correspondence of enzyme activity variations with the kinetic parameters characterized the studied biodegradation processes.

Acknowledgements

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REFERENCES


