GROWTH KINETICS AND BIODEGRADATION OF HIGH INHIBITORY CONCENTRATION OF PHENOL, CATECHOL AND 2,4-DICHLOROPHENOL BY TRAMETES VERSICOLOR 1

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ABSTRACT
Biodegradation of high concentration of phenol (1g/l), catechol (2 g/l) and 2,4-dichlorophenol (2g/l) by Trametes versicolor is studied. Modified kinetic model of Verhulst/Aiba/Andrews is fitted to describe the specific growth rate at inhibitory growth type. When catechol is used as carbon source the microbial culture shows highest specific growth rate (µmax, 0.0263 h⁻¹) which correlates with zero cell death rate, (β). The value of Ks (Ks, 1.360 g/l) demonstrates that T. versicolor has the lowest catechol affinity compared with phenol (Ks, 1.064g/ l) and 2,4 dichlorophenol (Ks, 0.953 g/l). The value of Kd is the lowest for catechol degradation which correspond to high inhibition effect than phenol and 2,4-dichlorophenol. The lowest specific growth rate (µmax) and the highest cell death rate (β) are observed at 2,4-dichlorophenol as a sole carbon source. The successful degradation of studied phenolics compounds is found to be dependant on the enzymes from β-ketoadipate pathway - phenol hydroxylase and catechol 1,2-oxygenase enzymes and the enzyme laccase. Catechol is determined as a best inducer for synthesis of phenol hydroxylase (0.4 U/mg), catechol 1,2-oxygenase (0.22 U/mg) and laccase (1344 U/ml).

KEYWORDS: Trametes versicolor, biodegradation, phenol, catechol, 2,4-dichlorophenol, phenol hydroxylase, 1,2-oxygenase, laccase.

INTRODUCTION
Phenolic compounds are the most common toxic and persistent organic pollutants in the environment. Phenol is produced annually about 7 billion kg and used as a raw material in various industries such as oil refining, pharmaceuticals, pesticides, coking plants as well as leather industries. Chlorinated phenols (CPHs) can be formed indirectly during chlorination of municipal or industrial wastewater, bleaching in paper industry, or during bioconversion of low molecular weight compounds and pesticides production. Catechol has many applications in industry as a photographic developer, lubricating oil, polymerization inhibitor and in pharmaceuticals. All of these phenolic compounds are extremely toxic and they are listed as priority pollutants by EPA, USA. Phenol is also used as a raw material in the manufacturing of many products, and a large amount of it is released into the environment through industrial processes. The aerobic and anaerobic degradation of different phenolic compounds by various microorganisms has been studied extensively. Fungi successfully can be used for phenolic compound degradation since they are less sensitive to inhibition and due to active production of various enzymes, which are able to cleave cyclic ring - phenol hydroxylase, catechol 1,2- oxygenase, and achieve bioconversion- laccase. The phenol degradation ability of 15 filamentous fungal strains of the genera Fusarium sp., Aspergillus sp., Penicillium sp. and Graphium sp. has been tested. The basidiomycetes are robust organisms that are tolerant to the presence of high concentrations of various pollutants mainly due to their very powerful extracellular oxidative enzymatic system.

White-rot fungi are capable of completely degradation of phenolic polymers. The biodegradation activity toward some wide spread phenolic pollutants is studied together with the laccase production. One of the members of the white-rot fungi family with proven bioremediation and degradation capacity is Trametes versicolor.
Bioprocesses are proven to be effective approaches for fully mineralisation of toxic organic compounds even at higher inhibitory effect of phenolic compounds on microbial population.\cite{7,11,18} The pre-adaptation of microbial culture is most commonly applied to increase their tolerance to toxic phenolic substrates. This approach allows achieving biodegradation of higher initial concentration of aromatic hydrocarbons.\cite{7,11,18,20}

The biodegradation and growth kinetics are essential for the understanding the capacity of microorganisms for degradation of phenolic compounds and their efficient treatment. Good experimental design and careful mathematical interpretation of data are helpful for understanding the dynamic characteristics of phenolic compounds biodegradation at inhibitory type growth of microbial population. A variety of kinetic substrate inhibition models have been used to describe dynamics of the microbial growth on phenol. The Aiba, Andrews and Haldane kinetic models are the most used for representing the growth kinetics of inhibitory substrates, where it describes the inhibitory effect of single substrates very well.\cite{1,2,4}

This study focuses on investigation of growth kinetics and degradation abilities of *Trametes versicolor* 1 in medium contained high inhibitory concentration of phenol, catechol and 2,4-dichlorophenol as sole carbon and energy source.

**MATERIALS AND METHODS**

**Microorganisms and inoculum**

A fungal strain of *Trametes versicolor* 1 collected from hills in the city of Plovdiv, Bulgaria is used in this work. The culture belongs to the collection of the Department of Biotechnology at the University of Food Technologies in Plovdiv – Bulgaria. The culture is maintained on 2% lime bean agar plates and slants at 4 °C. For mycelial inoculum production, a 15-day old plate culture grown on 2% potato dextrose agar (PDA) is used. Mycelial inoculum is prepared by inoculation 10^7 spores of fungus from agar-sant culture to 300 cm^3 shake flask containing 50 ml beer must 7.5 °B. The pH of the media is adjusted with 1M NaOH to 6.5. The inoculated flasks are incubated at 30 °C and 220 rpm for 96 h. Biomass is separated from the cultural medium through filtration under sterile conditions and washed twice with sterile distilled water. The biomass from a single flask is used to inoculate a corresponding flask containing phenolic compounds. Under the same conditions 3 control flasks are additionally prepared and the dry weight of biomass (after its separation from the cultural medium and washing with sterile distilled water) is determined by ULTRA X apparatus for drying.

**Biodegradation media**

Biodegradation is carried out in 3 different media contained the following compounds as a sole carbon and energy source; phenol 1.0 g/l; catechol (2.0 g/l) and 2,4-dichlorophenol 2.0 g/l. Media also contains the following salts (g/l): NaNO₃ – 2.0, KH₂PO₄ – 1.0, KCl – 0.5, MgSO₄·7H₂O – 0.5, and FeSO₄·7H₂O – 0.01. 50 ml from the salt solution are poured in 300 ml flasks and pH was adjusted to 6.5.

**Biodegradation studies**

Biomass from 4-day old culture of *Trametes versicolor* 1, following the sterile filtration described above, is used to inoculate the media contained the respective phenolic compounds. The process is carried out on a shaker at 220 min⁻¹ and 30°C. At regular 12-hour time intervals the residual phenolic compounds, biomass and the laccase activity are analyzed. The dry weight of the biomass is determined by ULTRA X apparatus for drying.

Phenol hydroxylase and catechol 1,2-oxygenase are tested in the biomass at 24 hour after the beginning of the biodegradation process. For the analysis of intracellular enzymes 3g quartz sand are added to the filtered biomass taken from 1 flask and washed twice with distilled water. Cell disruption is carried out for 5 min, after which the disrupted biomass is transferred into a centrifuge shell with distilled water to a volume of 4 cm³. The shells were centrifuged at 5000 min⁻¹ for 20 min. The supernatant is decanted and the precipitate is analyzed for enzymatic activity.

**Analytical methods**

**Determination of phenolic compounds concentration**

The content of residual phenols are determined by the HPLC analyses performed in C18 10μ Bondapac Column (3.9 mm x 300 mm) and waters 484UV detector (260 nm). The mobile phase was methanol - water (70:30), flow rate 0.2 cm³/min and 22°C.

**Enzyme assay**

**Laccase activity (EC 1.10.3.2)** is assayed by spectrophotometrically measurement at 530 nm using syringaldazine as a substrate. One unit of laccase activity is defined as 0.001 ΔA₅₃₀ for 1 min, pH 4.5 and 30°C.\cite{10}

**Phenol hydroxylase (EC 1.14.13.7)** is spectrophotometrically measured at 340 nm. The oxidation of NADPH in the presence of phenol is measured.\cite{12} Under the conditions of the analysis 1 unit of enzymatic activity is equaled to the quantity responsible for the oxidation of 0.17 mM NADPH and reduction of the absorbance by 0.1 for 1 min.

**Catechol 1,2-oxygenase (EC 1.13.11.1)** is assayed spectrophotometrically at 260 nm, by measuring the concentration of *cis,cis*-muconic acid.\cite{19} One unit of enzymatic activity is defined as the amount of muconic acid [μmol] produced for 1 min by 1 cm² enzyme.

All analyzes are performed in triplicate.

**Kinetic models**

The kinetic constants are calculated using optimization procedure minimizing the deviation between
experimental data and model output according equation (1) by gradient procedure in programmed setting of Eureka.

$$\sum_{i=1}^{n} (x_{exp} - x_{mod})^2 + \sum_{i=1}^{n} (s_{exp} - s_{mod})^2 \rightarrow \min$$  \hspace{1cm} (1)

Growth and lysis of microbial culture are evaluated using a modified model of Verhulst/Aiba/Andrews. The applied kinetic model describes the relation between the specific growth rate (µ) of *Trametes versicolor* 1 population and substrate concentration phenol (S_p), catechol (S_c), 2,4-dichlorophenol (S_{DCP}) at inhibitory growth type.

The dynamic of the process is analyzed with our own modeling and simulation package. Excel is used for graphic plotting.

**RESULTS AND DISCUSSION**

Some preliminary investigations on *Trametes versicolor* 1 prove its ability to degrade aromatic compounds, such as phenol at a concentration of 0.5 g/l without pre-adaptation process.\textsuperscript{[21]} The ability of *T. versicolor* to degrade catechol and 2,4-dichlorophenol at initial concentration 1 g/l is preliminary investigated (data are not published) which is the base for the current study. The knowledge of cell growth kinetics and biodegradation of phenolic compounds is essential for their effective treatment. The dynamic of biodegradation of phenol, catechol and 2,4-dichlorophenol, used as a sole carbon and energy source by *T. versicolor* 1 in time is shown in Figure 1 A,B,C. At initial concentration 2 g/l, *T. versicolor* 1 metabolize catechol and 2,4-dichlorophenol to 0.1 g/l residual phenolic compounds in 4 and 3 days, respectively. Phenol with initial concentration 1 g/l is metabolized in 3 days after the start of biodegradation to 0.05 g/l residual phenol. Continuous cell lysis is observed and biomass decreases in all in studied biodegradation media. This suggests that phenol, catechol and 2,4-dichlorophenol are inhibitory type of substrates for *Trametes versicolor* 1.

![Figure 1. Biodegradation of phenol (A); catechol (B); 2,4-dichlorophenol (C) by *Trametes versicolor* 1.](image)

The experimental data and outputs by kinetic models of Verhulst/Aiba/Andrews for phenol(A), catechol(B) and 2,4-dichlorophenol(C) concentration changes (S_p, S_c, S_{DCP}) during the biodegradation as well as changes of *T. versicolor* 1 biomass are presented in Figure 2 A,B,C and Figure 3 A,B,C respectively.

Modified kinetic model of Verhulst/Aiba/Andrews for inhibitory growth type is used to describe the specific growth rate (equation 2).

$$\frac{dx(t)}{dt} = \frac{\mu_{max}}{k_x} \frac{s(t)}{s(t) + s(t)^2/k_x} e^{\frac{-\beta x^2(t)}{k_x}}$$  \hspace{1cm} (2)

where

- \( \mu_{max} \) is the maximum specific growth rate; \( X \) is the biomass concentration g/l; \( K_x \) is half saturation coefficient (g/L); \( K_i \) is the substrate inhibition constant; \( \beta \) is cell death rate. At low substrate concentration the model is fitted to Monod’s model.

Gradient procedure, minimizing the error between experimental and model data, is used for determination the total square deviation in substrate changes - S_p, S_c, S_{DCP} in analyzed time interval and changes in *T. versicolor* 1 biomass concentration in that time interval (Table 1).
The determined kinetic constants according Verhulst/Aiba/Andrews model are summarized in Table 1. The values of square deviation for biomass and substrate concentration changes show that the applied inhibitory kinetic model describes adequately biodegradation together with culture lysis for all studied phenolic substrates. The specific growth rate, \( \mu_{\text{max}} \), is extremely low for all of three studied substrates, because of their high inhibition effect (Fig. 1 A, B, C). It is well known that dead cells provide additional food substances for the microbial population as whole. In microbiology this process is known as “hide and curious”. This is the reason for minimal growth rate of microbial population even in condition of cultural lysis and high toxicity of the investigated phenolic substrate. The value of \( \mu_{\text{max}} \) is highest at catechol as substrate. It is 8.77 and 7.99 times higher than when phenol and 2,4-dichlorophenol are used as a substrates, respectively. This result correlates with the value of cell death rate, \( \beta \), which value is zero for catechol as a substrate. The higher value of \( K_s \) (catechol) demonstrates the lower rate of catechol utilization than that determined for 2,4-dichlorophenol and phenol. Respectively the value of \( K_s \) is the lowest for catechol degradation. The catechol shows stronger toxic effect on \( T. \text{versicolor} \) than phenol (2.17 times). The possible reason for the higher toxicity of catechol compared to phenol could be the two times higher catechol concentration. Its toxic effect is higher than that of 2,4-dichlorophenol (1.99 times), which is used at the same concentration in the biodegradation media.

Table 1: Parameters for Verhulst/Aiba/Andrews model of biodegradation of phenol, catechol, 2,4-dichlorophenol by \( T. \text{versicolor} \).

<table>
<thead>
<tr>
<th>Phenolic substrate, g/l</th>
<th>( \mu_{\text{max}} ) ( \text{h}^{-1} )</th>
<th>( K_s ) g/l</th>
<th>( K_i ) g/l</th>
<th>( \beta ) (g/l)(^{-1})h(^{-1})</th>
<th>Square error/deviation/</th>
<th>biomass</th>
<th>substrat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol</td>
<td>0.0029</td>
<td>1.064</td>
<td>1.0030</td>
<td>0.0102</td>
<td>12.6</td>
<td>0.0170</td>
<td></td>
</tr>
<tr>
<td>Catechol</td>
<td>0.0263</td>
<td>1.360</td>
<td>0.4617</td>
<td>0</td>
<td>0.917</td>
<td>0.0029</td>
<td></td>
</tr>
<tr>
<td>2,4-Dichlorophenol</td>
<td>0.0033</td>
<td>0.953</td>
<td>0.9197</td>
<td>0.0191</td>
<td>8.998</td>
<td>0.0064</td>
<td></td>
</tr>
</tbody>
</table>

According National Research Council, (1993), the toxicity of the studied substrates terminates or delays cell metabolism. Microbial cells cease to functionate when at least one of the main steps in the plurality of physiological processes occurring in them is blocked. This may result in physical disruption of the cell structure, or competitive binding of an enzyme essential for the metabolization of toxic substances. In this study, the established high toxicity of catechol probably is related with inhibition of enzyme(s) essential for the metabolite processes in \( T. \text{versicolor} \). In support of this statement could be pointed out that when catechol is used as a sole carbon source the established cell lysis is less than when other studied phenolic substrates are used. The initial biomass concentration is 50 g/l and at the end of the analyzed period of time the residual biomass is 40 g/l, which is respectively 2.4 and 2.3 time higher than that in biodegradation media contained phenol (17 g/l) and 2,
4-dichlorophenol (17.6 g/l) (Fig. 3, A, B, C). The understanding the enzymatic reactions involved in the degradation of phenolic compounds is therefore of much interest. Yemendzhiev et al., 2008 find that the strain T. versicolor 1 synthesizes enzymes phenol hydroxylase and catechol 1,2- oxygenase, which take part in the first two stages of phenolic compounds metabolization. For the first time for this fungi these authors prove the ability of T. versicolor 1 to produce intracellular phenol hydroxylase (0.333 U/mg protein) and cis,cis-muconate lactonizing enzyme (0.41 U/mg protein). In our study the ability of the same strain T. versicolor 1 to produce intracellular phenol hydroxylase and catechol 1,2- oxygenase when different phenolic compounds are used as sole carbon and energy source are examined (Table 2).

Table 2: Enzyme activity of phenol degrading enzymes by T. versicolor 1, cultivated in media with different phenolic substrates as sole carbon and energy source.

<table>
<thead>
<tr>
<th>Phenolic substrates</th>
<th>Phenol hydroxylase, U/mg protein</th>
<th>Catechol 1,2-oxygenase, U/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol (1 g/l)</td>
<td>0.21±0.004</td>
<td>0.18±0.003</td>
</tr>
<tr>
<td>Catechol (2 g/l)</td>
<td>0.43±0.01</td>
<td>0.22±0.004</td>
</tr>
<tr>
<td>2,4-Dichlorophenol (2 g/l)</td>
<td>0.14±0.003</td>
<td>0.12±0.002</td>
</tr>
</tbody>
</table>

When phenol, catechol and 2,4-dichlorophenol are used as sole carbon and energy source in biodegradation media T. versicolor 1 synthesizes the two enzymes phenol hydroxylase and catechol 1,2-oxygenase but the activity of phenol hydroxylase is higher than the activity of catechol 1,2-oxygenase. Catechol is better inductor for biosynthesis of phenol hydroxylase and catechol 1,2-oxygenase by T. versicolor 1 than phenol and 2,4-dichlorophenol.

Trametes versicolor 1 is able to synthesize also the enzyme laccase when grow on substrate phenol and catechol as sole carbon source (Fig.4). When phenol is used as carbon source, laccase activity starts to increase in a day after beginning of the process and reaches its maximum 766.6 U/ml on the third day. In media with catechol, maximal laccase activity (1344 U/ml) is detected on the 1st day after the start of the process and it is kept equal to the 4th day.

Obviously, the weaker lysis of Trametes versicolor 1 when catechol is being metabolized results in higher residual biomass concentration and determines higher potential for biosynthesis of analyzed enzymes - phenol hydroxylase, catechol 1,2-oxygenase and laccase. Despite the higher enzyme activity in media with catechol as substrate the rate of catechol degradation is not faster than rate of degradation of 2,4-dichlorophenol (Fig.2, B, C). This result could be explained by weaker inhibition effect of 2,4-dichlorophenol (Ki, 0.9197 g/l) than catechol (Ki, 0.4617 g/l) inhibition. Probably, catechol affects parallel and negative on other enzyme systems and strain cell function. The degradation process of toxic phenols is a function of entire microbial metabolisms as well as the structure of toxic substrate.

The studied by us T. versicolor 1 shows significant biodegradation capacity compared to some others fungal stains cited in the literature. Bernats et al., 2015 report about phenol reduction from 420±12 mg/l initial concentration to 29±1 mg/l in seven days by T. versicolor. The results of Tebbouche et al., 2015 shows Aspergillus niger completely degrades phenol (1000 mg/L) after 140 h. Current study shows, that owing to phenol-degrading capacity of T. versicolor 1, this strain could be successfully used in waste water treatment plant for phenolic compounds removal.

CONCLUSION
Kinetic model of Verhulst/Aiba/Andrews describes adequately the biodegradation of phenol, catechol and 2,4-dichlorophenol (sole carbon sources) by T. versicolor 1. The values of kinetic constants for a model of inhibitory type growth are determined. It is established that at 1.0 g/L phenol, 2 g/L catechol, 2 g/L 2,4-
dichlorophenol concentration, the biodegradation process runs in the presence of substrate inhibition. The ability of \textit{T. versicolor} 1 to produce the phenol-degrading enzymes – phenol hydroxylase and catechol 1,2-oxygenase as well as laccase demonstrates the capacity of the strain \textit{T. versicolor} 1 for enzyme biodegradation of phenol, catechol and 2,4-dichlorophenol in concentration higher than inhibiting ones for culture growth.

ACKNOWLEDGMENT
This work was supported by the National Council of Science Research of the Bulgarian Ministry of Education and Science.

CONFLICT OF INTEREST
The authors do not have any conflict of interest related to this research.

REFERENCES