

## BIODEGRADATION OF PHENOL, CATECHOL AND 2, 4-DICHLOROPHENOL AT HIGHER INITIAL INHIBITORY CONCENTRATIONS BY *TRAMETES VERSICOLOR* 1 IN A “FED-BATCH” PROCESS

IVANKA STOILOVA\*; GALIA DIMITROVA; GALENA ANGELOVA; ALBERT KRASTANOV  
University of Food Technologies, Department of Biotechnology, BG-4002 Plovdiv, Bulgaria

### Abstract

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Biodegradation of phenol, catechol and 2,4-dichlorophenol, at higher initial inhibitory concentrations, by *Trametes versicolor* 1 in a “fed-batch” process is investigated. The strain (2.5 g biomass) biodegrades total 7.45 g phenol for 20 days at initial phenol concentration of 1.0 g/dm<sup>3</sup> and 6 feedings with concentration 1 g/dm<sup>3</sup>. At initial concentration of catechol and 2,4-dichlorophenol 2.0 g/dm<sup>3</sup> and feedings with same concentration, the culture (2.5 g biomass) biodegrades respectively total 11.13 g catechol for 22 days at 5 feedings and 3.03 g 2,4-dichlorophenol at 1 feeding for 6 days. It is established that *Trametes versicolor* 1 synthesizes phenol-hydroxylase, catechol 1,2-oxygenase and laccase – enzymes, required for biodegradation and biotransformation of the above phenolic substrates. Laccase production depends on the nature of the substrate. Strain produces considerable amount of laccase reaching to 1343.3 U/cm<sup>3</sup> in medium containing catechol but the enzyme is not being synthesized in the medium comprised of 2,4-dichlorophenol as sole carbon and energy source. The enzyme activities of phenol hydroxylase и catechol 1,2- oxygenase are higher in media with catechol than in media with other two phenolic compounds. At the end of the biodegradation process of the phenolic compounds, the second enzyme of the catabolic pathway – catechol 1,2-oxygenase is not identified in the medium. The absence of the second enzyme of the metabolic pathway is probably the decisive factor for the termination of the biodegradation process.

*Key words:* *Trametes versicolor* 1; biodegradation; phenol; 2,4-dichlorophenol; catechol; phenol hydroxylase; catechol 1,2-oxygenase; laccase

### Introduction

Phenols, owing to their persistence in the nature, are one of the most important groups of eco-toxins. As priority pollutants (Keith and Telliard, 1979), phenolic compounds must be eliminated, within the frame of sustainable development to preserve the environmental quality. Well known physico-chemical methods for phenol removing are expensive, low effective and generate toxic by-products (Loh et al., 2000).

The process of biodegradation is a well-established and powerful technique for treating domestic and industrial ef-

fluents. Bioprocesses are proved to be effective approaches for fully mineralisation of organic compounds (Dos Santos et al., 2009; Sanchez, 2009). Additionally, biological methods are preferred over physicochemical methods because of their economic aspects as well as nontoxic by-products. The aerobic and anaerobic degradation of phenol has been studied extensively using various microorganisms, such as bacteria, yeasts, and fungi (Chandana and Sridevi, 2009; Michałowicz and Duda, 2007; Najafpoor et al., 2015; Reshma and Mathew, 2014; Tebbouche et al., 2015; Sung et al., 1997). Fungi successfully can be used for phenol degradation since they are

\*E-mail: wstoilowa@yahoo.com

less sensitive to inhibition (Santos and Linardi, 2004) and, due to active production of various enzymes, which are able to degrade cyclic ring compounds – phenol hydroxylase, catechol 1,2-oxygenase (Hadibarata and Kristanti, 2014; Katayama-Hirayama et al., 1994; Krug and Straube, 1986) and achieve bioconversion- laccase (Sanchez, 2009). Santos and Linardi (2004) have tested the ability of 15 filamentous fungal strains of the genera *Fusarium sp.*, *Aspergillus sp.*, *Penicillium sp.*, and *Graphium sp.* to degrade phenol. One of most effective cyclic compound cleaving fungi classes are basidiomycetes. They are robust organisms that are tolerant to the presence of high concentrations of various pollutants, even with a low bioavailability and this ability is mainly due to their very powerful extracellular oxidative enzymatic system (Bernats and Juhna, 2015; Leita, 2009; Rubilaret et al., 2008). Rubilar et al. (2008) analyse the degradation of chlorophenols by white rot fungi, which are a group of organisms very suitable for the removal of chlorinated phenolic compounds. The degradation rate of total phenol in batch flasks by four fungal monocultures of *Trametes versicolor*, *Phanerochaete chrysosporium*, *Gloeophyllum trabeum* in synthetic medium showed that white-rot fungus *Trametes versicolor* is the most effective of the species.

It is well known the toxicity of phenolic compounds to microbial cells. It delays or terminates the metabolic process. (National Research Council, 1993). Phenols pose several toxic cellular effects. Membrane toxicity is the most pronounced effect (The Environmental Protection Agency (EPA), 2002). Hydrocarbons toxic effect on membranes is mainly due their lipophilicity. Hydrocarbons tend to accumulate into the lipophilic layer of the cell membrane disturbing its integrity (Sik-kema et al., 1995). Strain ability to cope with the toxic effects of phenols is reflected on their growth rates. Beyond a certain inhibitory initial concentrations, the biomass growth yield and specific growth rate tend to decrease (Farooqi et al., 2008 ; Najafpoor et al., 2015; Tebbouche et al., 2015).

The pre-adaptation of microbial culture is most commonly applied to increase their tolerance to toxic phenolic substrates. This approach allows achieving biodegradation at higher initial concentration of aromatic hydrocarbons (Farooqi et al., 2008; Najafpoor et al., 2015; Wang et al., 2010).

Biodegradation involves two simultaneously processes – metabolizing of the phenolic compound and increasing the biomass. At higher initial concentration of phenolic compounds the biomass or specific growth rate decreases. Microbial cells cease to function when at least one of the essential steps in their numerous physiological processes is blocked. The blockage may result from gross physical disruption of the cell structure or competitive binding of a single enzyme essential for metabolizing the toxicant (National Research Council,

1993). The death phase of microbial cell is a longer process compared to the previous phases of the cell development. The opportunities of microbial culture to degrade phenolic compounds during the death phase as well as the factors determined the termination of the process of biodegradation have not been studied and they are subject of scientific and practical interest. This unexplored possibility poses the aim of this research to study the biodegradation of phenol, catechol and 2,4-dichlorophenol, at higher initial inhibitory concentration, by *Trametes versicolor* 1 in a “fed-batch” process.

## Materials and Methods

### Microorganism 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 belonged to the collection of the Department of Biotechnology at the University of Food Technologies in Plovdiv – Bulgaria. The culture is maintained on 2% lima 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 inoculating  $10^7$  spores of fungus from agar-slant culture to 300 cm<sup>3</sup> 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 biomass in the flasks (after its separation from the cultural medium and washing with sterile distilled water) and the initial biomass level is determined by ULTRA X apparatus for drying.

### Biodegradation media

Biodegradation is carried out in 3 different media containing the following compounds as a sole carbon and energy source: phenol 1.0 g/dm<sup>3</sup>, while for catechol and 2,4-dichlorophenol the concentrations were 2.0 g/dm<sup>3</sup>. Media also contained the following salts (g/dm<sup>3</sup>): NaNO<sub>3</sub> – 2.0, KH<sub>2</sub>PO<sub>4</sub> – 1.0, KCl – 0.5, MgSO<sub>4</sub>·7H<sub>2</sub>O – 0.5, and FeSO<sub>4</sub>·7H<sub>2</sub>O – 0.01. 50cm<sup>3</sup> from the salt solution are poured in 300 ml flasks and pH is adjusted to 6.5.

### Biodegradation studies

Biomass (2.5 g) from 4-day old culture of *Trametes versicolor* 1, following the sterile filtration described above is

used to inoculate the media containing the respective phenolic compounds. The process is carried out on a shaker at  $220 \text{ min}^{-1}$  and  $30^\circ\text{C}$ . At determined time intervals the residual phenolics and the laccase activity are analyzed. When the residual concentration of the respective phenolic compound decreased to  $0.0 \text{ g/dm}^3$  a new portion of substrate is introduced to recover the initial substrate concentration and that feeding of the media is repeatedly carried out until cessation of the biodegradation process. No feeding with salt solution is carried out. After every collection of analytical samples the detracted amount of liquid in the flasks is restored by an equivalent volume of sterile distilled water.

Biomass quantities are determined in the beginning of the biodegradation process, in the end of each feeding and in the end of the process. The dry weight of the biomass is determined by ULTRA X apparatus for drying.

Phenol-degrading enzymes are analyzed at the start and end time of the process. For the analysis of intracellular enzymes, 3 g quartz sand is added to the filtered biomass taken from 1 flask and washed twice with distilled water. Grinding is carried out for 5 min, after which the ground biomass was transferred into a centrifuge shell with distilled water to a volume of  $4 \text{ cm}^3$ . The shells are centrifuged at  $5000 \text{ min}^{-1}$  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  $10 \mu\text{m}$  Bondapac Column ( $3.9 \text{ mm} \times 300 \text{ mm}$ ) and waters 484UV detector ( $260 \text{ nm}$ ). The mobile phase is methanol – water (70:30), flow rate  $0.2 \text{ cm}^3/\text{min}$  and  $22^\circ\text{C}$ .

### Enzyme assay

*Phenol hydroxylase* (EC 1.14.13.7) is assayed spectrophotometrically at  $340 \text{ nm}$ . The oxidation of NADPH in the presence of phenol is measured (Neujahr and Gaal, 1973). Under the conditions of the analysis 1 unit of enzymatic activity equaled to the quantity responsible for the oxidation of  $0.17 \text{ mM}$  NADPH thus reducing the absorbance by 0.1 for 1 min.

*Catechol 1,2-oxygenase* (EC 1.13.11.1) is assayed spectrophotometrically at  $260 \text{ nm}$ , by measuring the concentration of *cis,cis*-muconic acid (Varga and Neujahr, 1970). One unit of enzymatic activity is defined as the amount of muconic acid [ $\mu\text{mol}$ ] produced for 1 min by 1 ml enzyme.

*Laccase* activity (EC 1.10.3.2) is assayed according to Marbah *et al.* (1985) using syringaldazine as a substrate. One unit of laccase activity is defined as  $0.001 \Delta A_{530}$  for 1 min, pH 4.5 and  $30^\circ\text{C}$ .

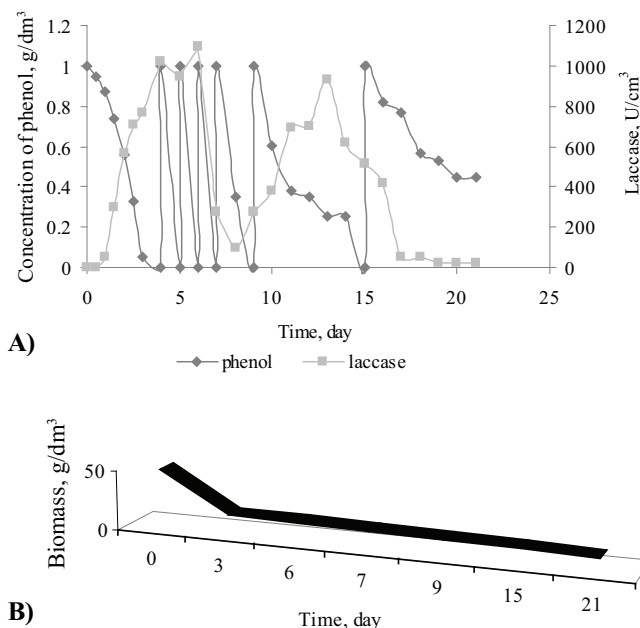
## Results and Discussion

In the current study, the possibility of a mycelial mass of the microbial culture *Trametes versicolor* 1 to degrade higher concentrations of phenolic compounds by feeding method without pre-adaptation of the microbial culture is studied.

Seven (7) cycles of degradation of a total  $7.45 \text{ g}$  phenol for 20 days at initial concentration of  $1.0 \text{ g/dm}^3$  and 6 feedings with same phenol concentration are realized. (Figure 1, A). During the metabolization of the first portion of phenol the culture is adapting to the carbon source, therefore the time of degradation is longer than that of the subsequent 4 feedings when the process is 2-times quicker. Lysis of microbial biomass is observed in the process of degrading the first dose of phenol resulting in 66 % biomass loss after 3 days (Figure 1, B). During next 6 feedings, the lysis of the culture proceeds slows and in the end of the process the analyzed biomass is  $10.2 \text{ g/dm}^3$ .

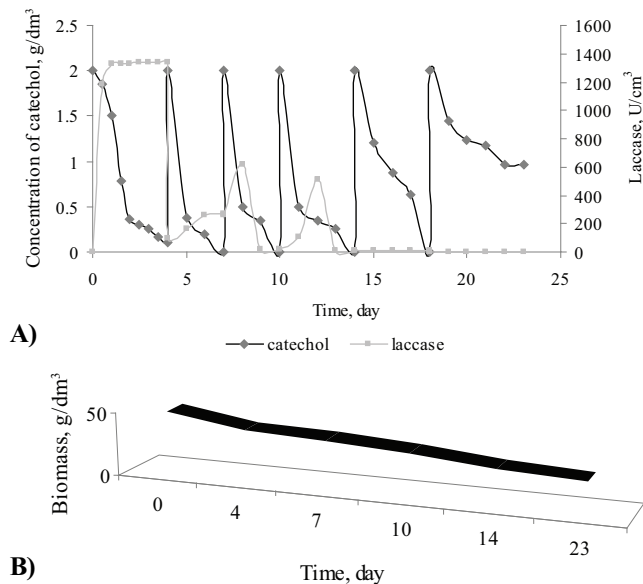
In the course of the above biodegradation process *Trametes versicolor* 1 produces the enzyme laccase which actively participates in the process of phenol transformation. The culture vigorously synthesizes laccase until the 6<sup>th</sup> day (the 3<sup>th</sup> feeding) reaching a maximum activity of  $1096.0 \text{ U/cm}^3$  (Figure 1, A).

$2.5 \text{ g}$  ( $50 \text{ g/dm}^3$ ) biomass of *Trametes versicolor* 1 is used to carry out 6 cycles of degradation of  $11.13 \text{ g}$  catechol for 22



**Fig. 1. Biodegradation of phenol (at initial concentration of  $1.0 \text{ g/dm}^3$ ) (A) and changes in the biomass (B) of *Trametes versicolor* 1 with periodic feeding**

days at initial concentration  $2.0 \text{ g/dm}^3$  and 5 consecutive feedings with same catechol concentration (Figure 2, A), while after the addition of the 5<sup>th</sup> dose only 43 % of catechol is metabolized.

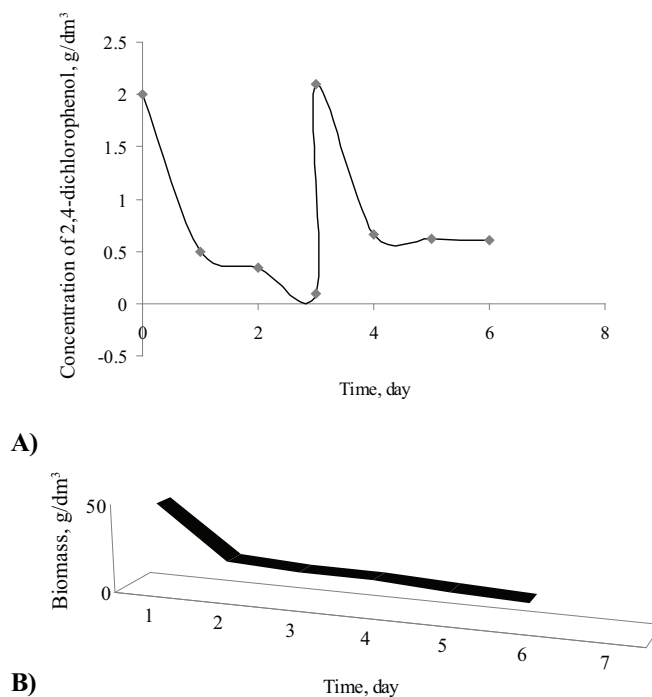


**Fig. 2. Biodegradation of catechol (at initial concentration of  $2.0 \text{ g/dm}^3$ ) (A) and changes in the biomass (B) of *Trametes versicolor* 1 with periodic feeding**

A continuous biomass lysis is observed but it did not exceed 49.6% for the entire biodegradation process i.e. the process is characterized by a considerably smaller biomass loss (Figure 2, B). In the end of the process the established amount of biomass is  $25.2 \text{ g/dm}^3$ .

At the same time, catechol turned out to be a powerful inducer of laccase biosynthesis, with the activity of the enzyme reaching its maximum of  $1343.3 \text{ U/cm}^3$  as early as the first day of the process and maintaining that level until the completion of the biodegradation of the first catechol feeding dose (Figure 2, A). In the course of degradation of the next catechol doses the activity of the enzyme decreases and increases twice in a manner similar to the mechanism of phenol biodegradation by *Trametes versicolor* 1.

The degradation pattern of 2,4-dichlorophenol by *Trametes versicolor* 1 is presented on Figure 3, A. Culture degrades  $2.0 \text{ g/dm}^3$  substrate fully but after the introduction of the subsequent substrate dose it completes only 70% degradation, i.e.  $2.5 \text{ g}$  ( $50 \text{ g/dm}^3$ ) biomass catabolizes a total of  $3.03 \text{ g}$  for 6 days. With 2,4-dichlorophenol being the substrate, 64.8% of the culture suffers lysis during the first 3 days of the process (the time needed for the catabolism of the first substrate dose) (Figure 3, B). At the end of the process the estimated biomass is  $13.5 \text{ g/}$



**Fig. 3. Biodegradation of 2,4-dichlorophenol (at initial concentration of  $2.0 \text{ g/dm}^3$ ) (A) and changes in the biomass (B) of *Trametes versicolor* 1 with periodic feeding**

$\text{dm}^3$ . *Trametes versicolor* 1 does not produce the enzyme laccase when 2,4-dichlorophenol is used as substrate.

During biodegradation of phenolic compounds *Trametes versicolor* 1 synthesizes the enzyme laccase together with the enzymes from b-ketoadipate pathway – phenol hydroxylase and catechol 1,2- oxygenase. These two enzymes catalyse the two first stages in b-ketoadipate pathway for phenolic compound biodegradation. The activity of phenol hydroxylase and catechol 1,2- oxygenase at different phenolic substrate are summarized in Table 1. The enzyme activities of phenol hydroxylase and catechol 1,2- oxygenase are higher in media with catechol than in media with other two phenolic compounds. The enzymes of the  $\beta$ -ketoadipate pathway are inducible in microorganisms (Krug and Straube, 1986; Nennekar and Vaidyanathan, 1981). The high activities of these two enzymes in medium with catechol as a sole substrate determine it more significant biodegradation compared to other two phenolic compounds.

During the biodegradation of studied phenolic compounds the enzyme activity decreases together with reduction of biomass concentration. The fully termination of biodegradation of the three studied phenolic substrate is established in absence of catechol 1,2- oxygenase independently from the final biomass concentration. Catechol 1,2- oxygenase activity is not found at

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

Phenolic substrates	Phenol hydroxylase, U/mg protein	Catechol 1,2-oxygenase, U/mg protein
Phenol (1 g/dm <sup>3</sup> )	0.21±0.004	0.18±0.003
Catechol (2 g/dm <sup>3</sup> )	0.43±0.01	0.22±0.004
2,4-Dichlorophenol (2 g/dm <sup>3</sup> )	0.14±0.003	0.12±0.002

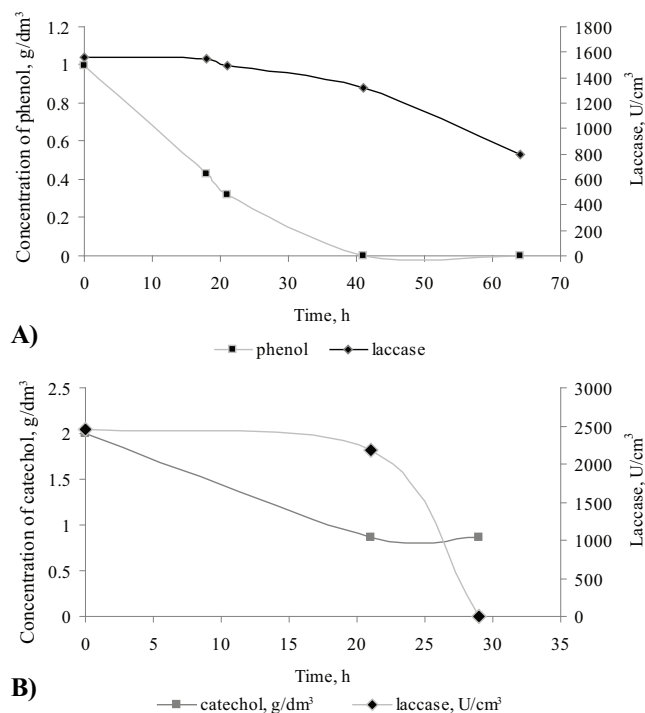
residual biomass concentration 10.2, 25.2 and 13.5 g/dm<sup>3</sup> in media with phenol, catechol and 2,4 dichlorophenol respectively. There is no found catechol 1,2-oxygenase activity even at higher residual biomass concentration in media with catechol. Obviously, the absence of this enzyme has a critical role for termination of the process of biodegradation of the three studied phenolic compounds.

Some other authors report for degradation of high concentration of phenol by *Trametes versicolor* without cell lysis but after long pre-adaptation period (Najafpoor et al., 2015; Farooqi et al., 2008; Wang et al., 2010). Yeom et al. (1997) considered the pre-adaptation mechanism of microbial population should be considered in the aspect of enzyme induction of catechol 1,2-oxygenase. According them the adaptation of microbial cultures support the better biodegradation of phenolic compounds by means of induction of catechol 1,2-oxygenase. Our results confirm the importance of catechol 1,2-oxygenase for the process of biodegradation as well as its termination. The highest biodegradation of catechol corresponds to highest catechol 1,2-oxygenase activity. The absence of this enzyme leads to termination of the biodegradation process.

In the course of the biodegradations *Trametes versicolor* 1 produces the enzyme laccase which actively participated in the process of phenol and catechol transformation but not in 2,4-dichlorophenol degradation. Several peaks of enzyme activity are observed when phenol and catechol are used as substrates. Considering the continuous lysis of the culture the appearance of these peaks could not be explained by the biosynthetic potential of the strain. Two model solutions, phenol (1 g/dm<sup>3</sup>) and highly purified laccase (1560 U/cm<sup>3</sup>) and catechol (2 g/dm<sup>3</sup>) and highly purified laccase (2460 U/cm<sup>3</sup>), are used for clarifying the changes in laccase activity (Figure 4 A, B). High enzyme activities are applied for fully oxidation of phenolic compounds. Considerable decrease (49%) of enzyme activity is established in 41 to 64<sup>th</sup> hour in a fully transformed substrate phenol and intermediate products. In a model solution of catechol and laccase at 43.5 % biotransformation of catechol, the enzyme activity decreases with 11.6 %

after which the enzyme is completely inhibited in 29<sup>th</sup> hour. That is to indicate that a strong product inhibition is occurred. The product inhibition of laccase could explain the increase of laccase activity after 6<sup>th</sup> cycle of phenol biodegradation (Figure 1,A) and after 3<sup>rd</sup> and 4<sup>th</sup> cycles of catechol biodegradation (Fig. 2,A). The increased substrate concentration decrease inhibitory effect of the products of laccase activity.

The lack of laccase in medium with 2,4-dichlorophenol confirms the inducibility of this enzyme. From other point of view probably the absence of the enzyme laccase even in case of other two metabolizing enzymes is the reason for low rate of 2,4-dichlorophenol metabolism by *Trametes versicolor* 1.



**Fig. 4. Changes in laccase activity in a model solution containing phenol (A) and catechol (B)**

Biodegradation of phenolic compounds is associated with many factors such as concentration of the phenolic derivatives, concentration of viable biomass, presence of inhibitors, temperature, pH and microbial adaptation. In the present study the used toxic concentrations of the substrates and laccase product inhibition result in reduction in viability and the lysis of *Trametes versicolor* 1. The determining factor for termination of the biodegradation of phenol, catechol, and 2,4-dichlorophenol from the test culture is the loss of enzymatic activity of catechol 1,2- oxygenase.

## Conclusions

*Trametes versicolor* 1 could be applied successfully for the degradation of high concentration of phenols. The biomass changes during the biodegradation process are common to all of the three phenolic substances – lysis of the culture throughout the entire process. The investigated aromatic compounds as well are potent disinfectants when added at very high concentrations and the lysis of the culture is inevitable. In the end of the biodegradation process of all phenolic compounds the second enzyme of the catabolic pathway – catechol 1,2 oxygenase is not identified in the medium. The absence of the second enzyme of the metabolic pathway is probably the decisive factor for the termination of the biodegradation process. *Trametes versicolor* 1 synthesizes considerable amount of the enzyme laccase during the biodegradation process. Laccase production depends on the nature of the substrate – in the media comprised of 2,4-dichlorophenol the enzyme is not being synthesized. The most suitable medium for laccase biosynthesis proves to be the one containing catechol. In the process of phenolic compounds biodegradation product inhibition of laccase is revealed.

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