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# BIODEGRADATION OF PHENOLIC MIXTURES AT HIGH INITIAL CONCENTRATIONS BY *TRAMETES VERSICOLOR* 1 IN A "FED-BATCH" PROCESS

**Galena Angelova[1*], Ivanka Stoilova[1], Kolyo Dinkov[2] and Albert Krastanov[1]**

[1]University of Food Technology, Department of Biotechnology, 26 Maritza Blvd., 4002 Plovdiv, Bulgaria.
[2]University of Food Technology, Department of Process engineering, 26 Maritza Blvd., 4002 Plovdiv, Bulgaria.

***Corresponding Author: Dr. Galena Angelova**
University of Food Technology, Department of Biotechnology, 26 Maritza Blvd., 4002 Plovdiv, Bulgaria.

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## ABSTRACT
Biodegradation of mixed substrates phenol and catechol and phenol and 2,4-dichlorophenol at high inhibitory initial concentrations (2 g/dm$^3$) by *Trametes versicolor* 1 in a "fed-batch" process is studied. It is established that *Trametes versicolor* 1 (biomass 50 g/dm$^3$) biodegrades total 6.14 g mixture of phenol and catechol for 14 d at 2 feedings and 2.57 g mixture of phenol and 2,4-dichlorophenol for 8 d at 1 feeding. It is found that *Trametes versicolor* 1 synthesizes phenol-hydroxylase, catechol 1,2-oxygenase – enzymes, required for biodegradation of substrates in the two studied phenolic mixtures. Laccase is produced in media containing substrates phenol and catechol, in the media comprised mixture of phenol and 2,4-dichlorophenol this enzyme is not being synthesized. The lysis of *Trametes versicolor* 1 during the biodegradation of phenol and catechol (79,2 %) and phenol and 2,4-dichlorphenol (76,4 % ) is weaker than the lysis during the biodegradation of phenol (96 %) at the same initial concentration (2 g/dm$^3$).

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

## INTRODUCTION
Aromatic phenol mixtures are present in wastewater from industrial and municipal sources as well as in contaminated groundwater. These are major xenobiotics,which are often found in the effluents discharged from the industries such as paper and pulp, textiles, gas and coke, fertilizers, pesticides, steel and oil refineries etc.[1,2] The occurrence of contaminants in mixtures is an important problem because the removal or degradation of one component can be inhibited by other compounds in the mixture and because different conditions may be required to treat different compounds within the mixture.[1] Researchers have noted that microbial degradation of a compound in a mixture can be strongly affected by other substituents of the mixture.[3,4] To understand mixture effects, one must consider the metabolic role each compound plays for the microorganisms. This has been observed not only for mixtures of toxic chemicals (bioremediation), but also for mixtures of pollutants and readily degradable compounds (wastewater treatment), and mixtures of sugars (fermentation). Moreover, biodegradation of individual pollutants in mixtures has been shown to be different from the degradation as single carbon source.[1, 5]

Of the several methods available for treatment of phenolic mixture, biological treatment is especially attractive because it has the potential to almost degrade phenol completely with producing innocuous end products and minimum secondary waste generation.[6, 7]

Literature sources describe a number of individual representatives of the genera *Candida*, *Rodotorula* and *Trichosporon*, which are capable of metabolizing aromatic compounds.[8, 9, 10, 11, 12]
The specific enzymes responsible for biodegradation occupy an important place in these investigations. Lignin-degrading enzymes can also degrade various organic compounds, including xenobiotics.

The potential biotechnological use of white-rot fungi has attracted considerable attention during the past few decades.[13,14] 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 *Trametes versicolor* was found to be dependent on a fungal growth, enzyme (laccase) production and some inductors.[15] A strain of *T. versicolor* isolated from paper mill effluent has been described as capable of degrading phenolic compounds.[16]

The opportunities of microbial culture to degrade phenolic mixtures during the death phase have not been studied by now and they are of scientific and practical interest. This unexplored possibility posed the aim of this research to study the biodegradation of the mixture of phenol +catechol and phenol + 2,4-dichlorophenol of higher, inhibitory initial concentrations 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 $^{o}$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 ml shake flask containing 50 cm$^3$ beer must 7.5 $^{o}$B. The pH of the media was adjusted with 1M NaOH to 6.5. The inoculated flasks are incubated at 30 $^{o}$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 initial biomass level in the flasks (after its separation from the cultural medium and washing with sterile distilled water) is determined by ULTRA X apparatus for drying.

### Biodegradation media

Biodegradation was carried out in 2 different media containing mixture of compounds: phenol + catechol and phenol +2,4-dichlorophenol as a sole carbon and energy source. Each phenolic mixture is with concentration of 2.0 g/dm$^3$, of which 1.0 g/dm$^3$ phenol and 1.0 g/ dm$^3$ the second phenolic compound. Phenol at concentration 2.0 g/dm$^3$ is used a sole substrate. Media also contained the following salts (g/dm$^3$): $NaNO_3$ – 2.0, $KH_2PO_4$ – 1.0, KCI – 0.5, $MgSO_4.7H_2O$ – 0.5, and $FeSO_4.7H_2O$ – 0.01. 50 cm$^3$ from the salt solution are poured in 300 cm$^3$ flasks and pH was 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 min$^{-1}$ and 30 ºC. 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 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, 3g 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 cm$^3$. The shells are 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 10 µm 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 cm$^3$/min and 22°C.

### Enzyme assay

*Phenol hydroxylase (EC 1.14.13.7)* is assayed spectrophotometrically at 340 nm. The oxidation of NADPH in the presence of phenol is measured.[17] Under the conditions of the analysis 1 unit of enzymatic activity equaled to the quantity responsible for the oxidation of 0.17 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 nm, by measuring the concentration of *cis,cis*-muconic acid.[18] One unit of enzymatic activity is defined as the amount of muconic acid [µmol] produced for 1 min by 1cm$^3$ enzyme.

*Laccase* activity (EC 1.10.3.2) is assayed according to Marbach et al.[19] 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$^{o}$C.

## RESULTS AND DISCUSSION

Some preliminary investigations of *Trametes versicolor* 1 proves its ability to degrade phenol, catechol and 2,4-dichlorphenol as a sole carbonic and energy source.[20] Waste waters and soils containing mixtures of phenolic contaminants are quite common for the environment. In that regard, the possibility of *Trametes versicolor* 1 to

metabolize mixtures of phenolic compounds is being investigated.

Degradation of the mixture of phenol and catechol with an initial total concentration of 2.0 g/dm$^3$ and three consecutive feedings is studied (Fig. 1A).

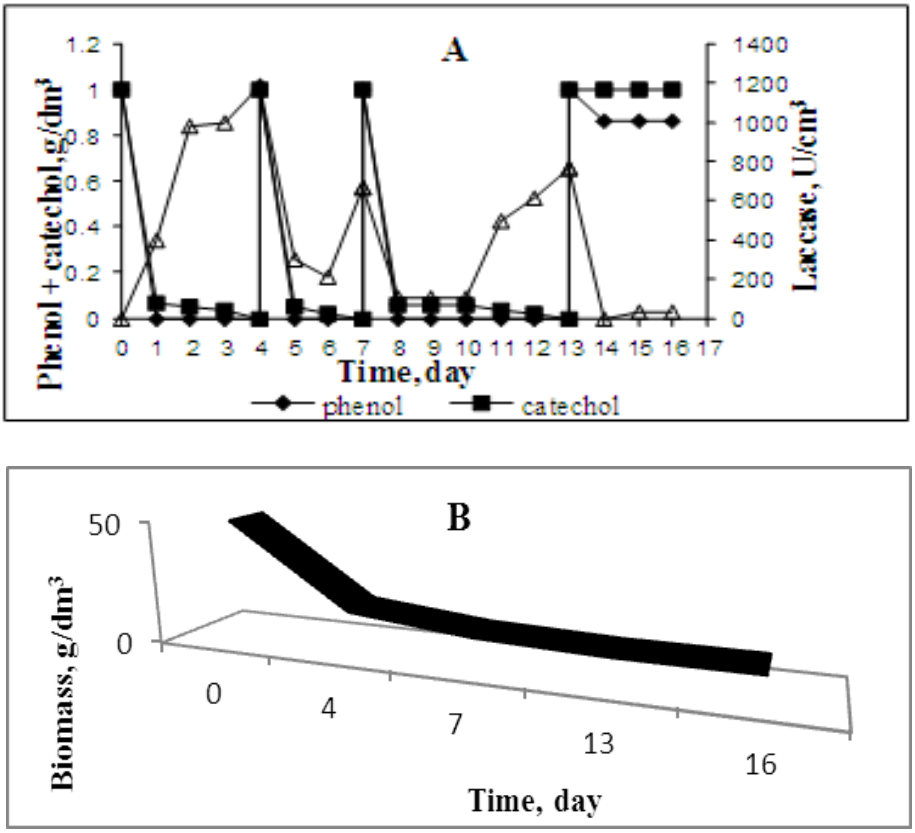


**Figure 1: Biodegradation of phenol + catechol mixture by Trametes versicolor 1(A) and changes in the biomass of Trametes versicolor 1(B) during biodegradation process.**

*Trametes versicolor* 1 is able to degrade 6.14 g of the above phenolic compounds in 14 days with 3 feedings. The initial biomass concentration is 50 g/dm$^3$. The biodegradation of both aromatic compounds occurs simultaneously. The initial phenol concentration and the phenol from the next two feedings are completely degraded for one day. After the third feeding dose only 0.14 g/dm$^3$ phenol is degraded. The degradation of catechol follows a similar pattern of degradation during of each of the three feedings. 93.2 % of the initial catechol concentration is metabolized for 1 day while the complete degradation takes 3 days; 94.7% of the first catechol feeding dose is metabolized for 1 day and the residual concentration for 2 days; 94.2% of the second dose is metabolized for 1 day and the residual catechol concentration needs 5 days. The catechol of the third feeding dose remains unaffected.

The changes in the biomass during the biodegradation process are studied. It is established that 66 % of the initial biomass is disrupted in 4 days from the beginning the biodegradation process that matched to degradation of the initial concentration of studied phenolic substrates. The process terminates at final biomass concentration of 10.4 g/dm$^3$ (Fig. 1B).

With regard to the second mixture – phenol and 2,4-dichlorophenol, 2.5 g (50 g/ dm$^3$) biomass of the culture completes biodegradation of a total of 2.57 g in 8 days (Fig. 2A).

The degradation of the two individual compounds run simultaneously, but still more intensively in the case of 2,4-dichlorophenol – for 1 day, while phenol degradation takes 6 days. Biomass changes during the biodegradation process are shown in Fig. 2B. As soon as the third day of the process biomass lysis reaches 66.4 %, and on the 6$^{th}$ day it is 75 %. The final biomass is 11.8 g/dm$^3$ on the 8$^{th}$ day, while for the previous mixture similar value of biomass concentration is established after a 14-day long process.

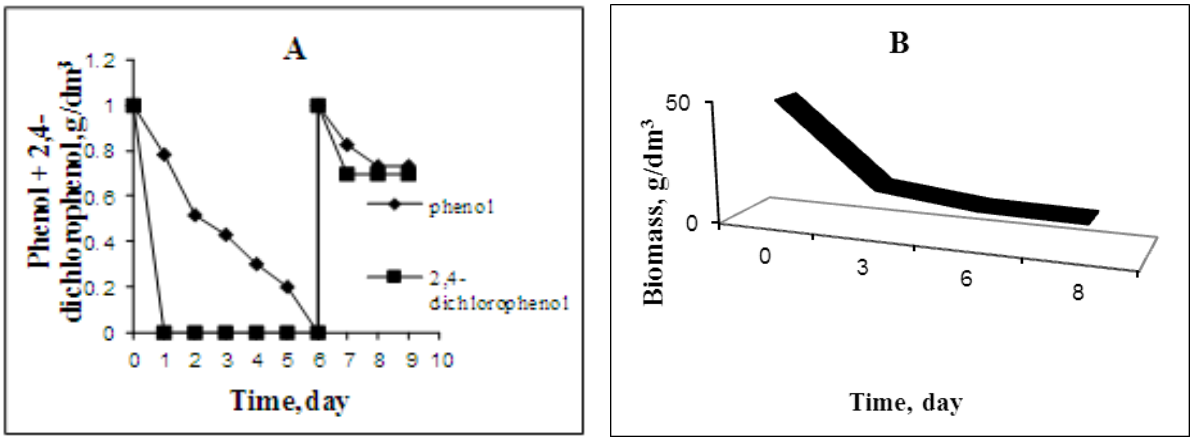


**Figure 2. Biodegradation of phenol + 2,4-dichlorophenol mixture by *Trametes versicolor* 1 (A) and changes in the biomass of *Trametes versicolor* 1 (B) during biodegradation process.**

*Trametes versicolor* 1 metabolizes mixtures of the above studied phenolic compounds with the enzymes from β-ketoadipate pathway - phenol hydroxylase and catechol 1,2- oxygenase (dates are not showed). During the biodegradation of studied phenolic compounds the enzyme activities decrease together with reduction of biomass concentration. In the end of the biodegradation process of two investigated mixtures the second enzyme of the catabolic pathway – catechol 1,2 oxygenase is not identified in the biomass.

*Trametes versicolor* 1 also synthesizes the enzyme laccase but only in the medium with phenol and catechol but does not produce laccase in the second mixture – phenol and 2,4-dichlorophenol (Fig. 2A). The curve characterizing laccase activity displays 3 peaks – the highest enzymatic activity of 1180.0 U/cm$^3$ is established upon completion of the biodegradation of the first dose of phenolic mixture, the second peak of 666.0 U/cm$^3$ is at the end of the second dose metabolization, and the third maximum of 766.0 U/cm$^3$ is identified in the end of the decomposition of the third dose of aromatic compounds (Fig. 1A).

*Trametes versicolor 1* (2.5 g biomass) biodegrades 2.4 times less phenolic compounds from mixture phenol and 2,4-dichlorophenol than mixture phenol and catechol. Probably the lack of laccase in the mixture phenol and 2,4-dichlorophenol is the reason for the weaker biodegradation. In previous studies of *Trametes versicolor 1* is established that the enzyme laccase is induced only in the medium containing phenol and catechol as sole carbon sources, but not in medium with 2,4-dichlorophenol.[20] In accordance to Arvin et al.[21], the stimulation of the biodegradation of one compound by another in a mixture can be accomplished by induction of catabolic enzymes required for digestion of the second pollutant. In a first mixture of phenolic compounds phenol in the presence of catechol is digested for 1 day. The second mixture phenol in the presence of 2,4-dichlorophenol is digested for 6 days by *Trametes versicolor 1*. Probably in the second mixture exhibit substrate interactions when used together, as evidenced in research of Saéz and Rittmann.[4] These authors find that 4-chlorophenol is a non-competitive inhibitor of phenolic oxidation at high concentrations of phenol but a competitive inhibitor of low phenol concentrations when these substrates are utilized simultaneous. According Reardon et al.[1] in mixture experiments, the rate of consumption of one substrate is found to be affected by the presence of the others, although the degree of influence varied widely.

The toxicity of the two mixtures, containing phenolic compounds with initial concentration 2 g/dm$^3$ is compared to the toxicity of phenol as sole substrate at the same initial concentration (Fig. 3 A). *Trametes versicolor* 1 (biomass 50 g/ dm3 ±0.1) catabolizes a total of 2.0 g phenol for 16 days and almost fully loss of biomass is observed- 96% (Fig. 3 B).

For comparison the lysis of *Trametes versicolor* 1 during the biodegradation of phenol and catechol reaches 79.2 % and 76.4 % for phenol and 2,4-dichlorphenol. During the process of biodegradation of this high phenolic concentration the laccase activity reach its maximum of 257 U/cm3 at first day and second peak with less activity at 10 th days. The catabolizing enzymes phenol hydroxylase and catechol 1,2- oxygenase are found in the biomass. It is obvious the higher toxicity of phenol as a sole substrate in studied concentration compared to the studied phenolic mixtures.

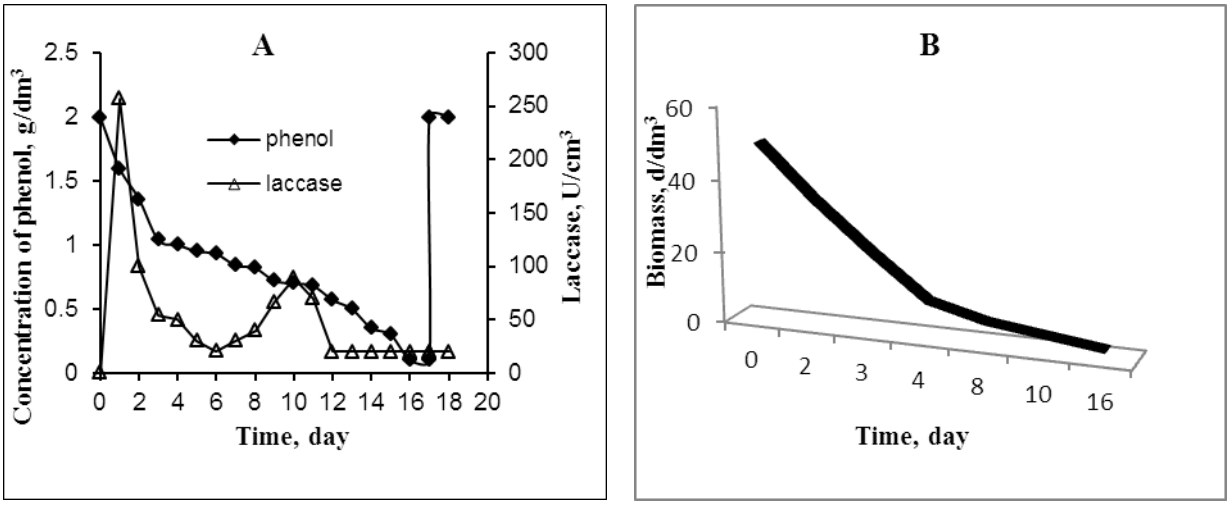


**Figure 3. Biodegradation of phenol (at initial concenration of 2 g/dm3 by Trametes versicolor 1 (A) and changes in the biomass of *Trametes versicolor* 1 (B) during biodegradation process.**

The development of strategy for future researches with *Trametes versicolor* 1 for phenolic compounds biodegradation could be stimulated by increasing the growth of culture using lower concentrations of substrate (the initial ones together with feeding dose)..

## CONCLUSIONS

Co-contamination of natural environments with mixtures of pollutants is an important problem. In biodegradation or bioremediation investigations and projects, it is important to understand and be able to model the fate of specific chemicals. Development of treatment strategies for soil or water contamination requires consideration of interactions among substrates to control the concentration of individual pollutants. It is important therefore to predict the biodegradation of pollutant mixtures in a given system. In this relation *Trametes versicolor* 1 shows significant ability to degrade phenolic mixtures in higher, initial concentrations during the death phase. Degradation of phenolic mixtures depends on the combination of individual aromatic compounds. The chemical nature of the individual compounds in the mixture is possible cause for the substrate interactions between them, and for induction of laccase. It is possible prerequisites for varying degrees of biodegradation of two mixtures of phenolic compounds. The biomass changes during the biodegradation of studied phenolic mixture are common to the two phenolic mixtures – lysis of the culture throughout the entire process. The investigated aromatic compounds as well as their mixtures are potent disinfectants when introduced at very high concentrations and the lysis of the culture is inevitable. The received results are basis for future studies concerning substrate interactions, the starting concentrations of the tested substrates, their relative amounts and conditions for the development of culture.

## ACKNOWLEDGMENTS

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