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RESEARCH ARTICLE

Biodegradation of natural phenolic compounds as single and mixed substrates by *Fusarium flocciferum*

Elsa Mendonça
Instituto Nacional de Engenharia e Tecnologia Industrial
Estrada do Paço do Lumiar
1649-038 Lisboa, Portugal
Tel: 351 21 7165141
Fax: 351 21 7166966
E-mail: elsa.mendonca@ineti.pt

Aida Martins
Instituto Nacional de Engenharia e Tecnologia Industrial
Estrada do Paço do Lumiar
1649-038 Lisboa, Portugal
E-mail: aida.martins@ineti.pt

Ana Maria Anselmo*
Instituto Nacional de Engenharia e Tecnologia Industrial
Estrada do Paço do Lumiar
1649-038 Lisboa, Portugal
Tel: 351 21 7165141
Fax: 351 21 7166966
E-mail: ana.anselmo@ineti.pt

http://www.ineti.pt

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The mycelium of *Fusarium flocciferum* was assayed for its ability to degrade aromatic compounds, namely, gallic, protocatechuic, vanillic, syringic, caffeic, and ferulic acids and syringic aldehyde, commonly found in agro-industrial wastes. The biodegradation assays were performed in liquid medium with the phenolic compounds as single substrates and as a synthetic mixture containing the seven aromatic compounds. The results with single substrates indicated that in 24 hrs of incubation the fungus was able to reduce the phenolic concentration from 200 mg/l to below detection limits, except for syringic acid, being the lowest degradation rates found for this acid and its aldehyde. The biodegradation experiments with the mixture of phenolic compounds showed that after 8 hrs the total phenolic concentration was reduce from 350 mg/l to below the detection limits of all the tested compounds. In all the experiments a rise in the pH and an effective detoxification of the phenolic solutions were also observed.

Environmental pollution due to the release of natural phenolic compounds from agro- industrial operations has become widespread in the world. The structure of the compounds present is similar in many industrial effluents and residues like those produced in wine-distillery, olive oil extraction, green olive debittering, cork preparation, wood debarking and coffee production (Field and Lettinga, 1991; Borja et al. 1993; Brand et al. 2000; Lesage-Meessen et al. 2001; Minhalma and de Pinho, 2001; Aggelis et al. 2002).

The biological treatment of industrial wastewaters usually depends upon the oxidative activities of microorganisms. Filamentous fungi can be an important source of phenol-degrading species as they grow frequently in wood where phenolic structures are present. Nevertheless filamentous fungi are not frequently used due to difficulties in their cultivation in liquid media and their slow growth rate in comparison with most of the other microbial species (Righelato, 1975). Moreover, organic compounds like phenol and its derivatives have toxic effects that limit the biological treatment, because they can be growth-rate inhibitory even to species that have the metabolic capability of using it as a substrate for growth (Evans, 1963).

The purpose of this study was to investigate the ability of the fungus *Fusarium flocciferum* to degrade some phenolic

* Corresponding author

compounds namely, gallic, protocatechuic, vanillic, syringic, caffeic and ferulic acids and syringic aldehyde, usually present in agro-industrial effluents. The results of the chemical and ecotoxicological monitoring during biodegradation experiments are presented and discussed having in mind the development of a bio-treatment capable of degrading these compounds and detoxifying these wastes.

MATERIALS AND METHODS

Organism

Fusarium flocciferum was isolated from an industrial effluent containing phenol and is able to grow on this compound as the sole carbon source (Anselmo and Novais, 1984).

Medium

Growth and degradation experiments liquid medium was Yeast Nitrogen Base (YNB) without amino acids and ammonium sulphate (Difco, Detroit, U.S.A.), supplemented with potassium nitrate, 5g/l (Merck, Darmstad, Germany).

Chemicals

Seven phenolic compounds, 4 benzoic acids, 2 cinnamic acids and 1 benzaldehyde, were used: gallic acid (Acros Organics, New Jersey, USA), protocatechuic acid (Acros), vanillic acid (Sigma Chemicals, St. Louis, USA), syringic acid (Sigma), caffeic acid (Sigma), ferulic acid (Acros) and syringic aldehyde (Acros). Solutions of 200 mg/l of the phenolic compound were prepared in YNB. A mixture of the 7 compounds was also studied. A solution of the different compounds was prepared by dissolving 50 mg/l of each compound in YNB. All other chemicals used were from Merck (p.a.).

Analytical procedures

Biomass was determined by dry weight measurement.

Chemical Oxygen Demand (COD) was made by the dichromate COD Method, (Hach Company, Loveland, USA) with a Hach DR 2000 Direct reading spectrometer.

pH determination was made by potentiometry (Clesceri et al. 1998).

Phenolic compounds determination was made by High-Performance Liquid Chromatography (HPLC). The HPLC analyses were performed with a Beckman System Gold chromatograph with a diode detector (System Gold 168 detector). The HPLC column used was a Chrompak INERTSIL C8 (5 μm, 4.6 x 250 mm). Two solvents were used for elution, A: H_2O-H_3PO_4 (999:1) and B: methanol-H_3PO_4 (999:1). The gradient profile was 0-5min, 90% A; 5-12.5 min, 80% A; 12.5-40 min, 60% A; 40-50 min 30% A; 50 min, 90% A. The flow rate was 0.8 ml/min, and the temperature of the chromatographic oven was 45ºC. Detection was carried out at 254 nm and 280 nm. Each determination was obtained by the arithmetic mean of two independent analysis. The detection limits for the phenolic compounds were lower than 0.5 mg/l.

Ecotoxicity was assessed by measuring the inhibition of the luminescence of *Vibrio fischeri* exposed for 5 minutes (Microtox® Test, Microbics, Carlsbad, U.S.A.). The test determines the EC_{50}-5 min, the effective concentration that reduces the bacteria light emission by 50% (Microbics, 1992).

Inoculum

Small portions of vegetative mycelium culture on the surface of YM-agar (Difco) supplemented with phenol 1g/l were used to inoculate 200 ml YNB with 500 mg/l phenol in 1000 ml flasks in a rotary shaker at 150 rpm and 25ºC. The medium was changed daily by filtering the growing mycelium under aseptic conditions. The cells were filtered, resuspended and incubated in the described conditions during 5 days. The biomass was then filtered and resuspended in the solutions for the biodegradation experiments.

Biodegradation experiments

The biodegradation experiments were performed with a fungal biomass concentration of 200 mg/l dry weight in 200 ml of YNB in 1000 ml flasks in a rotary shaker for 24 hrs at 150 rpm and 25ºC. A control flask with no inoculation was also prepared. Samples were taken after 1, 2, 4, 6, 8 and 24 hrs for analysis.

In order to interpret the disappearance of the phenolic compounds from the solution, a non-linear regression model was fitted using the original data, applying Sigmaplot SPW7 for Windows® (2001- SPPS Inc.).

The initial degradation rate of each compound, expressed in mg/l.h, was calculated by dividing 50% of its initial concentration by the time required to degrade this amount. The data reported are the average of the values obtained from duplicate experiments.

RESULTS

Results of the single substrate biodegradation experiments with the seven phenolic compounds are presented in Figure 1 and Table 1. The depletion of the phenolic compounds seems to follow a Hill sigmoid curve ($0.986 \leq r^2 \leq 0.999$),

which has been accepted as a mathematical model to describe biodegradation processes in similar test conditions. Comparing the degradation profiles of the aromatic compounds obtained it is possible to establish two types of behaviour:

- A group of compounds that includes, protocatechuic, vanillic, caffeic, ferulic and gallic acids which exhibit very similar initial degradation rates, 45±5 mg/l.h, and no evidence of a lag induction phase greater than one hour.

  Gallic acid presents a slightly different profile with a lower degradation rate (30 mg/l.h), being the only compound of this group still detectable at 6 and 8 hrs of incubation, with concentrations of 11.0 and 0.6 mg/l, respectively.

- A second group comprises syringic aldehyde and syringic acid, which clearly exhibit lag induction phases. In fact, after one hour of incubation the concentrations of both substrates remain at the initial value. These two compounds present lower degradation rates, 17 mg/l.h for syringic aldehyde and 14 mg/l.h for syringic acid.

These results are in agreement with the values obtained for the COD (Table 1). The percentage of COD reduction obtained after 6 hrs, for the first group of compounds, are above 50% and similar to those obtained after 24 hrs, with the exception of ferulic acid. Syringic aldehyde and syringic acid exhibit lower COD removal rates, with values of 16.0% and 30.6%, respectively, after 6 hrs of incubation. Concerning the pH of the phenolic compounds solutions, initial values were between 3.8-4.4 and after 24 hrs the pH increased to values between 4.8-5.7 (Table 1).

The results of the ecotoxicological analysis of samples during the biodegradation experiment period show increasing values of EC_{50}-5 min in all cases, reflecting the decrease in acute toxicity to the bacteria *Vibrio fischeri* (Table 1).

The results obtained in the mixed substrate experiments are presented in Figure 2 and Figure 3, and Table 2. The Hill sigmoid biodegradation model seems to describe successfully the depletion profile of each phenolic compound ($0.986 \leq r^2 \leq 0.999$) and of the mixture ($r^2 = 0.998$).

Again, it is possible to observe the two types of behaviour described above. The group of phenolic compounds, which includes protocatechuic, vanillic, caffeic, ferulic and gallic acids, exhibit very similar initial degradation rates. In this group ferulic acid presents the highest degradation rate, 20 mg/l.h. For the remaining 4 substrates the degradation rates calculated were between 13 – 15 mg/l.h. None of these compounds were detected after 4 hrs of incubation. The initial degradation rates for syringic aldehyde and syringic acid were clearly lower, 9 and 6 mg/l.h respectively.

The results obtained in the mixed substrate experiments are presented in Table 2, where COD, pH and ecotoxicity values are displayed. The analysis of these values shows that although total depletion of the seven aromatic compounds occurs after 6 hrs, decrease in both COD and toxicity and increase in pH were observed throughout the incubation period. This fact is probably related to the formation of intermediate degradation products and their temporary accumulation in the growth medium.

The observation of ecotoxicity results shows that after an initial decrease of toxicity from an EC_{50} value of 4.1% to 10.4% corresponding to the first 2 hrs of incubation, a slight increase in this parameter to a value of 5.9% was observed after 4 hrs incubation time.

The value obtained for the total phenol initial degradation rate, 80 mg/l.h, is clearly superior to that obtained in the single experiments, with an initial concentration of 200 mg/l, suggesting that the initial mixed substrate concentration of 350 mg/l has a stimulatory effect in the *F. flocciferum* mycelium.

DISCUSSION

In this work, the ability of *F. flocciferum* to degrade, as single and mixed substrates, several monocyclic natural aromatic compounds, commonly present in agro-industrial effluents, was studied. To our knowledge, this is the first study concerning the biodegradation of natural aromatic compounds by *F. flocciferum*.

In fact, though the microbial degradation of phenolic compounds is widely reported in the literature, only a few studies involving *Fusarium* strains have been published. Bilton and Cain, 1968 described the growth of *Fusarium solani* on protocatechuate as sole carbon source. Later, Nazareth and Mavinkurve, 1986 reported the degradation by *Fusarium solani* (Mart.) Sacc. of ferulic acid and 19 other related phenolic compounds, including vanillic, protocatechuic, syringic acids and syringic aldehyde also studied in this work.

The same authors identified vanillin, vanillic and protocatechuic acids as catabolic products of ferulic acid degradation. In our degradation experiment with ferulic acid, the analysis of the chromatograms of the sample obtained after two hours, allowed the identification of a peak corresponding to vanillic acid and suggesting that a similar metabolic pathway is present in *F. flocciferum*. In fact, this metabolic pathway is one of the major pathways

for the microbial transformation of ferulic acid (Rosazza et al. 1995).

Another fungus, the *Penicillium* strain Bi 7/2 can use a wide range of phenolic compounds as sole source of carbon and energy, including protocatechuic and gallic acids (Hofrichter and Scheibner, 1993).

Bacterial degradation of gallic and syringic acids has also been published. Tack et al. 1972 reported the isolation of a *Pseudomonas putida* strain, which oxidized gallate readily to completion after growth at the expense of syringic acid. More recently, the metabolization of gallic acid by *Leuconostoc oenos* and *Lactobacillus hilgardii* was reported by Vivas et al. 1997 and Alberto et al. 2001, respectively.

Mixed substrate degradation studies with phenolic compounds have been limited, involving in general two or three substrates and a specific bacterial strain (Reardon et al. 2000; Rogers and Reardon, 2000). Recently, Di Gioia et al. 2001 assayed the ability of two bacterial strains, *Ralstonia* sp. LD35 and *Pseudomonas putida* DSM 1868 to degrade a mixture of 9 monocyclic aromatic compounds which included vanillic, caffeic and syringic acids, among others. The authors reported that the co-culture of the two strains was able to biodegrade seven of the nine components of the mixture.

The mycelium of *F. flocciferum* was able to utilize as carbon source, the seven tested compounds either as single or mixed substrate, detoxifying the solutions. For both experiments a Hill sigmoid model was applied successfully to describe the experimental data. This model is currently accepted to fit biodegradation kinetics, which results were obtained in experimental conditions similar to those described in the present work (Struijs and van den Berg, 1995; Painter, 1997; Ahtiainen et al. 2003).

These results together with the fact that this strain is able to grow on phenol, catechol and resorcinol at concentrations up to 1 g/l (Anselmo and Novais, 1984; Anselmo and Novais, 1992), confer to this fungus a remarkable potential for its application in bioremediation and wastewater treatment, especially in detoxification of phenolic wastes.

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APPENDIX

Tables

Table 1. Results obtained in the single substrate biodegradation experiments with the different phenolic compounds for COD reduction (%), pH and ecotoxicity (EC_{50}-5 min. (%). (The values are the arithmetic mean of at least two independent analyses).

	Gallic acid	Protocate-chuic acid	Vanillic acid	Syringic acid	Caffeic acid	Ferulic acid	Syringic aldehyde
				COD reduction (%)			
6 hours	68.6	86.9	86.2	30.6	83.3	51.1	16.0
Final	67.1	83.6	81.0	79.6	81.5	77.9	59.5
				pH			
Initial	3.8	4.0	3.7	3.9	4.1	3.9	4.4
Final	4.8	5.1	5.7	5.5	5.5	5.3	4.9
				EC_{50}-5 min (%)			
Initial	8.6	4.9	7.3	8.1	11.7	5.3	11.2
Final	27.4	42.0	45.9	53.3	48.9	26.5	27.5

Table 2. Values obtained in the biodegradation experiments with the mixture of seven phenolic compounds for COD (mg/l O_2), total phenols (mg/l), pH and ecotoxicity (EC_{50}-5 min (%). (The values are the arithmetic mean of at least two independent analyses).

Parameters Time (hours)	**COD** (mg/l O_2)	**pH**	**Ecotoxicity (EC_{50}-5 min %)**
0	520	3.9	4.1
1	515	4.0	11.3
2	390	4.0	10.4
4	210	4.2	5.9
6	100	4.7	31.0
8	55	4.9	41.4
24	45	5.2	51.9

Figures

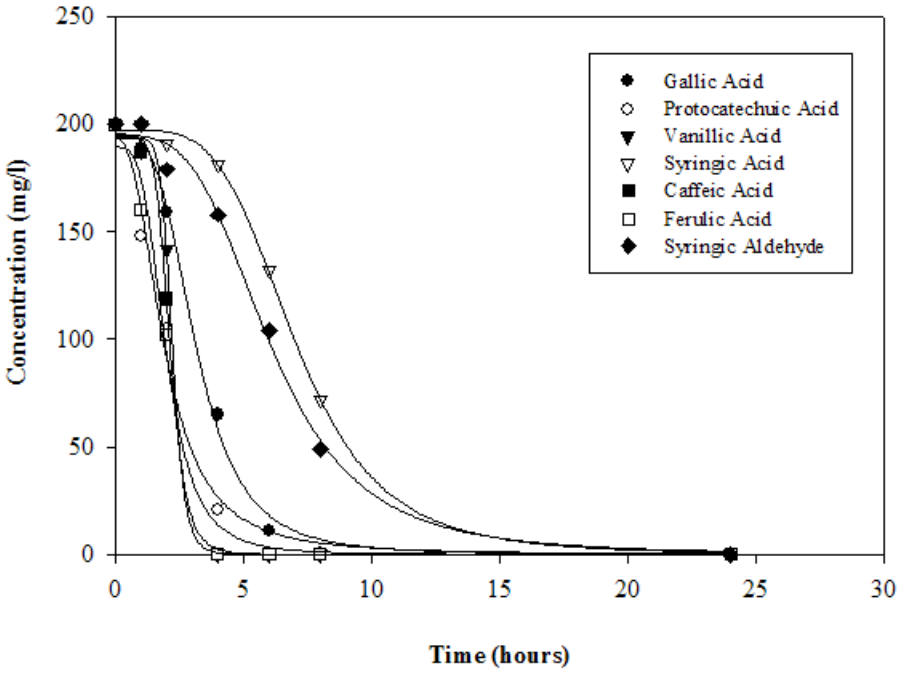

Figure 1. Depletion profiles of the seven monocyclic aromatic compounds, obtained in the single substrate experiments. The values presented are the average of duplicate experiments.

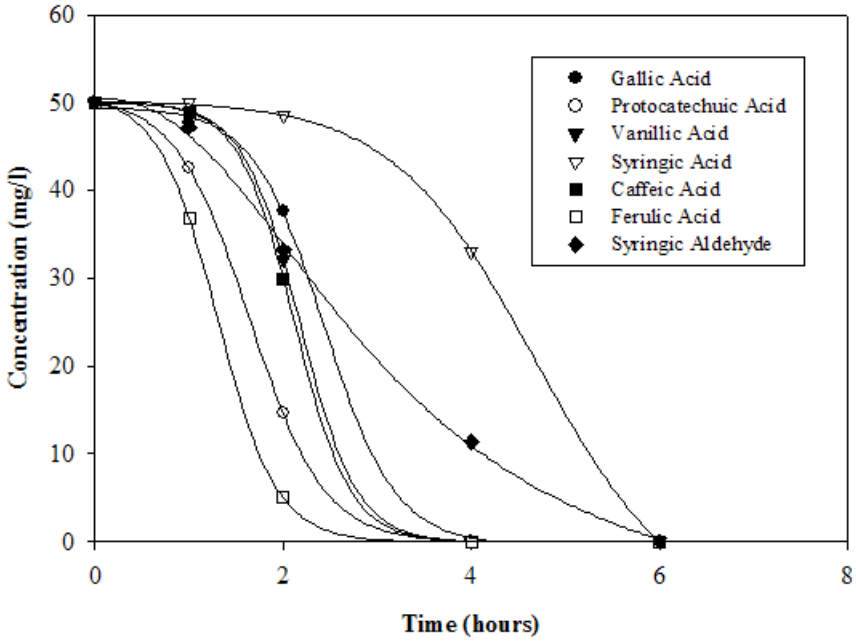

Figure 2. Depletion profiles of the seven monocyclic aromatic compounds, obtained in the mixed substrate experiments. The values presented are the average of duplicate experiments.

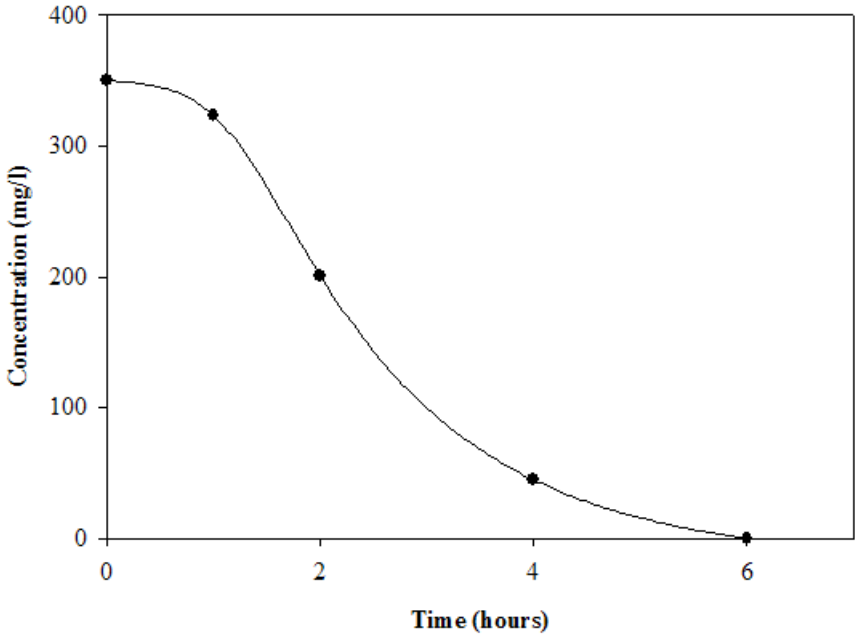

Figure 3. Depletion profile for the mixture of the seven aromatic compounds as total phenols (mg/l). The presented values are the sum of each compound concentration obtained at the corresponding time