# Fungal Biomass-Based Processing of Phenolics-Rich Wastewaters

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A variety of phenolic compounds commonly contained in many vegetal processing wastewaters complicate their biological treatment since such compounds are tipically characterized by a biorecalcitrant nature, generally leading to an overall increased disposal cost.
In this work, the capability of the fungus *Trichoderma viride* to actively grow on phenolics-containing substrates was tested. Gallic and tannic acid were adopted as model pollutants of the phenolic fraction contained in most wastewaters, while glucose was representative of readily metabolizable COD. *T. viride* was shown not to be inhibited by concentrated gallic acid and to be able to use it as a substrate for its growth. Tannic acid was not removed but did not inhibit *T. viride* growth.

## 1. Introduction

Meeting local environment protection rules requires that a wastewater stream be treated until compliance with the organic load allowance before being discharged into surface waters. When the organic load is mainly constituted by easily biodegradable substrates, a biological treatment process is often preferred due to economical reasons.
However, when the organic load also includes biorecalcitrant compounds, a significant toxicity is generally experienced by the microorganisms making up the mixed populations of biological treatment processes, in turn requiring the deployment of non-biological treatments (e.g. oxidizing agents, membranes, adsorbent resins, etc). Many wastewaters from vegetal-based productions exhibit such a character, e.g. those of olive oil extraction (Borja et al., 2006), winemaking (Beltrán et al., 1999) and cork processing (Gonzáles et al., 2006) since they contain phenolic compounds. Indeed, phenolic compounds, tipically exhibit toxicity (Paixão et al., 1999) toward microbial activity. Phenolics-rich wastewater (PRW) treatment is the target of the present work. The present research work reports about the results obtained in the treatment of PRWs by utilizing a pure culture of the fungus of the species *T. viride*.
Fungi feature the general ability of growing on wood and other lignocellulosic materials thanks to a pool of intra- and extracellular phenyl-, glucan- and xylan-degrading or modifying enzymes (Hammel, 1997): *T. viride* is a cellulase high producer (Domingues et al., 2000); besides, fungi like *T. viride* also grow at very low pH values (Brown and Halsted, 1975), thus being able to survive in acid wastewaters. In the present work, the ability of *T. viride* to grow on PRWs was investigated through model systems.

## 2. Materials and Methods

### 2.1 Microorganism

The *Trichoderma viride* strain used in the present research was kindly provided by Prof. Lo Curto (University of Messina, Italy) and originally supplied by Centraalbureau voor Schimmelculters (CBS) of Baarn (Holland). The microorganism was conserved as a pure culture on glucose agar medium and carefully propagated therefrom in the growth medium.

### 2.2 Biomass Production

A small inoculum taken from the agar slant culture was put in a sterilized reactor (maximum volume 2 l) containing the growth medium reported by Domingues et al. (2000) in which nutrient concentrations were modified according to the actual COD due to carbon source compounds in order to keep them balanced. The carbon sources used were: glucose (10 g $l^{-1}$) and gallic acid (2 g $l^{-1}$) together, glucose (10 g $l^{-1}$) and tannic acid (2 g $l^{-1}$) together and gallic acid alone (2 g $l^{-1}$). The initial pH of the growth medium value was regulated to 4.5 without further control during the growth phase. Temperature was not controlled either. The reactor was magnetically stirred while air was supplied by a sparging device after sterilization by a dry filter unit. All the growth phases were considered completed when the carbon sources were depleted but for tannic acid (which was not removed).

### 2.3 Batch Tests

The produced biomass was recovered through a settling phase and subsequently centrifuging the supernatant. Next, the biomass was resuspended in a medium having the same proportion between nutrients and total COD as that used in the growth phase but a different concentrations of carbon sources. All tests were performed at a controlled value of pH (4.5) and temperature (25 °C). The length of such tests was 10 hours.

### 2.4 Analytical Methods

The determinations of Total Suspended Solids (TSS) and Sludge Volume Index (SVI) were carried out according to Standard Methods (APAT, IRSA-CNR, 2003).

OUR determination was performed according to the method of Kappeler and Gujer (1992). The determination of phenolic compounds was performed by the Folin-Ciocalteau method (Singleton and Rossi, 1965), while the determination of glucose was performed with the Neocuproine method (Dygert et al., 1965).

Substrate analysis was performed daily during the growth phases and at regular time intervals during batch tests; the liquor was sampled, filtered and then stored at –4 °C until the moment of the analysis.

## 3. Results and discussion

### 3.1 Batch Tests

Three types of batch removal tests were performed: glucose/gallic acid mixture, gallic acid alone and glucose/tannic acid mixture. A summary of the obtained results are reported in Table 1. The *T. viride* biomass for all tests was obtained as reported in the "Material and Methods" section.

A typical plot of the results obtained in the kinetic analyses of batch tests performed on the mixture glucose/gallic acid is reported in Figure 1. The initial conditions of the test

were: concentration of biomass 300 mg $l^{-1}$; concentration of glucose 1000 mg $l^{-1}$; concentration of gallic acid 1200 mg $l^{-1}$.

The OUR profile in Figure 1a-left shows three characteristic zones. During the first hour, just the endogenous OUR was monitored. After the substrate spike addition a first exponential increase of the OUR and a simultaneous removal of glucose (Fig. 1a-right) were recorded. When glucose became limiting, and after a period (about 1 h) of acclimatization, further growth of the biomass on gallic acid was observed, confirmed by the time profiles of both substrates and of the OUR. Besides, an analysis performed the day after revealed that gallic acid was completely removed. It can be concluded that the biomass was indeed able to use both substrates for its growth, with a diauxic growth mechanism (Bailey and Ollis, 1986).

These tests pointed out that high initial values of gallic acid are not inhibitory for the growth of *T. viride*. On the contrary, *T. viride* is able to grow on it. Further tests (result data not shown) revealed that a concentration up to 3 g $l^{-1}$ is not inhibitory for the microorganism growth.

Next, the behavior of *T. viride* was investigated on a medium containing gallic acid as only carbon source. A typical plot of the results is reported in Figure 1b. The initial conditions of the test were: concentration of biomass 350 mg $l^{-1}$; concentration of gallic acid 200 mg $l^{-1}$.

Figure 1b-left shows two typical OUR zones. Initially, the endogenous OUR was monitored. Then, gallic acid was spike-wise added. The meaning of the remainder of the OUR profile is not immediately clear: the data show a gradual increase, but not really an exponential profile. Accordingly, the removal of gallic acid showed a linear profile (Figure 1b-right), most likely indicating a slow growth. Again, an analysis performed the day after showed that gallic acid was completely removed. After this treatment, however, *T. viride* was not able to sustain further growth cycles on a medium containing gallic acid as the only carbon source (result data not shown). Further investigation on the "irreversibly toxic boundary" during the diauxic and monoauxic growth might provide further insights into its causes and information of a significant practical value.

Finally, the behavior of *T. viride* on the glucose/tannic acid mixture was investigated. The growth phase pointed out that *T. viride* is not able to remove tannic acid, even though it is not inhibited by its high concentration (2 g $l^{-1}$). A more precise characterization of this phenomenon was provided through batch tests. The initial conditions of the test were: concentration of biomass 350 mg $l^{-1}$; concentration of glucose 1000 mg $l^{-1}$; concentration of tannic acid 1000 mg $l^{-1}$.

After the spike-wise addition of substrates, OUR profile immediately increased with respect to the endogenous value (Figure 1c-left). In the first 4 h, the OUR increased, clearly indicating a growth phase. Subsequently, the OUR profile rapidly decayed marking the depletion of the growth substrate (glucose) reported in Fig. 1c-right. No removal of tannic acid, for growth or other purposes, was observed, thus confirming the behavior observed during the growth phase. After that, the OUR showed a decaying profile.

Tannic acid is a large molecule (Molecular Formula = $C_{76}H_{52}O_{46}$; FW = 1701.20) with a structure close to that of an oligomer, featuring gallic acid (Molecular Formula = $C_7H_6O_5$; FW = 170.12) as repetitive element. Seemingly, *T. viride* is not able to produce

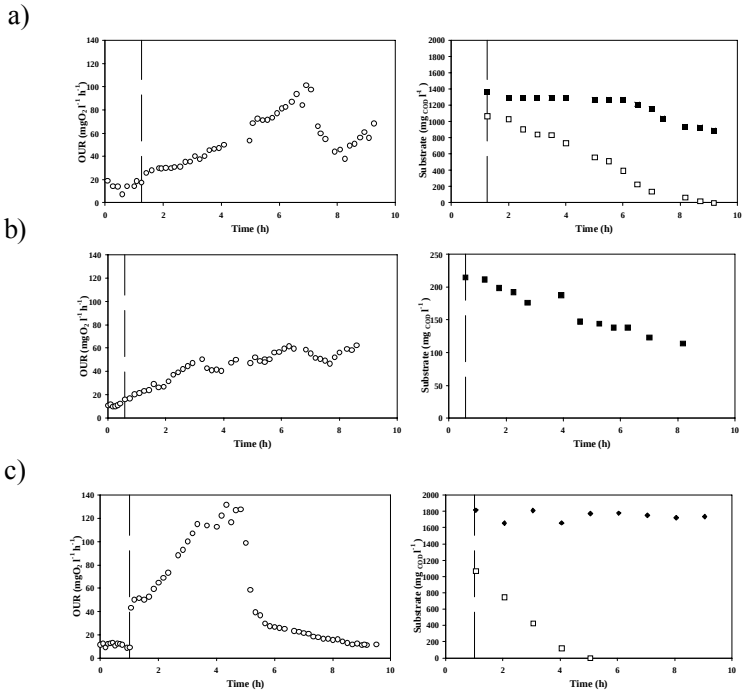


Figure 1 – Kinetic tests of *T. viride*: left figures show OUR profiles while right ones show substrate(s) concentration profiles; a) glucose/gallic acid; b) gallic acid; c) glucose/tannic acid. The dashed line indicates the time of substrate spike addition.
○ = OUR; □ = glucose; ■ = gallic acid; ♦ = tannic acid.

tannase whereby tannic acid would be degraded to glucose and gallic acid, which it was shown to be able to grow on. Glucose, gallic acid and tannic acid were chosen here as representatives of, respectively, readily metabolizable COD, low and high molecular weight polyphenolic compounds. Thus, it can be roughly concluded that the readily metabolizable COD can be removed without any inhibition from phenolic substances. Furthermore, even though no specific test for *T. viride* growth on a mixture of tannic and gallic acid was carried out in the frame of the current work, it is worth to recall here that Bajpai and Patil (1997) found gallic acid to significantly enhance tannase production by one specific strain of *T. viride*.

With regard to sterility requirement, it can be observed that, owing to the high inhibitory properties and low pH exhibited by wastewaters, external microbial contaminations are easily avoided.

**3.2 Morphology**

During the performed investigation the morphological properties of *T. viride* were clearly affected by the metabolized substrates. While many interesting implications of this dependence are out of scope here, biomass settling properties are of the uppermost

Table 1 – Substrate removal rates during *T. viride* growth.

| Growth substrates | Initial specific removal rates ($g_{substrate}$ / $g_{biomass}$ / h) | | |
|---|---|---|---|
| | **glucose** | **gallic acid** | **tannic acid** |
| glucose and gallic acid | 383 | 0<br>(203) | / |
| gallic acid | / | 40 | / |
| glucose and tannic acid | 924 | / | 0 |

/ = not measured; (number) = not initial speed

Table 2 – Morphological characteristic of biomasses of *T. viride*

| Growth substrates | Colour | SVI (ml $g^{-1}$) |
|---|---|---|
| glucose | white | 116 |
| glucose / gallic acid | light brown | 387 |
| glucose / tannic acid | dark brown | 96 |
| gallic acid | black | 2860 |

engineering relevance, e.g., for the design of recovery operations. Biomass settling ability was characterized here by the SVI, usually employed for activated sludges.
Although depending on the actual growth conditions, such as stirring rate, substrate concentration and inoculum size, *T. viride* generally grows on glucose as white-coloured pellet-shaped agglomerates (Domingues et al., 2000). This morphologic state improves the settling properties by lowering the recovery time and energy requirements. However, the exposition to phenolic substances seems to change this morphology. Biomass grown on glucose/gallic acid exhibited a SVI value three times higher than glucose-grown biomass. Gallic acid-grown biomass, which appeared in a highly dispersed form, exhibited an even higher SVI value (about 24 times higher than glucose-grown one). On the other hand, biomass grown on glucose/tannic acid showed an SVI value which was very close to that of glucose-grown biomass. Since tannic acid was not removed from the medium by *T. viride*, we argue that settling properties are worsened when a polyphenolic compound enters the cellular metabolism compared to when it remains unaffected.

## 4. Conclusions

*T. viride* can withstand high concentrations (up to 2 g $l^{-1}$) of polyphenolic substances and is able to remove gallic acid for growth, even though not for repeated cycles. Gallic acid also affects *T. viride* morphology in term of settling properties.
Tannic acid does not exhibit any inhibitory activity on *T. viride* but is not removed from a system containing only glucose as an additional substrate.

A preliminary conclusion which can be drawn is that this kind of method can be applied to many phenolics-rich wastewaters, like olive oil mill wastewaters, cork processing wastewaters and wine distillery wastewaters. Co-processing of wastewaters containing tannic acid might even be possible, as well as the simultaneous treatment of wastewaters and production of enzymes, owing to the enhancement of this latter in the presence of polyphenolic compounds pointed out by Arrieta-Escobar and Belin (1982).

## 5. References


APAT and IRSA-CNR, 2003, Analytical methods for waters. APAT, Rome (in italian).

Arrieta-Escobar, A. and J.M. Belin, 1982, Effects of Polyphenolic Compounds on the Growth and Cellulolytic activity of a Strain of *Trichoderma Viride*, Biotech. and Bioeng., 24, 983-989.

Bailey, J.E. and D.F. Ollis, 1986, Biochemical Engineering Fundamentals. International Edition, McGraw Hill Book-Co. Singapore.

Bajpai, B. and S. Patil, 1997, Induction of tannin acyl hydrolase (EC 3.1.1.20) activity in some members of fungi imperfecti, Enz. Microb. Technol., 20 (8), 612-614.

Beltrán, F.J., García-Araya, and M.P. Álvarez, 1999, Wine Distillery Wastewater Degradation. 1. Oxidative Treatment Using Ozone and Its Effect on the Wastewater Biodegradability, J. Agric. Food Chem., 47, 3911-3918.

Borja, R., B. Rincón and F. Raposo, 2006, Review – Anaerobic biodegradation of two phase olive mill solid wastes and liquid effluents: kinetic studies and process performances, J. Chem. Technol. Biotech., 81, 1450-1462.

Brown, D.E. and D.J. Halsted, 1975, The effect of Acid pH on the Growth of *Trichoderma viride*, Biotech. and Bioeng., 17, 1199-1210.

Domingues, F.C., J.A. Queiroz, J.M.S. Cabral and L.P. Fonseca, 2000, The influence of culture conditions on mycelial structure and cellulose production by *Trichoderma reesei* Rut C-30, Enzyme Microb. Technol., 26, 394–401

Dygert, S., L.H. Li, D. Florida and J.A. Toma, 1965, Determination of reducing sugars with improved precision, Anal. Biochem., 13, 367-374.

Paixão, S.M., E. Mendonça, A. Picado and A.M. Anselmo, 1999, Acute Toxicity Evaluation of Olive Oil Mill Wastewaters: A Comparative Study of Three Aquatic Organisms, Envir. Toxicol., 14(2), 263-269.

González, T., J.R. Domínguez, J. Beltrán-Heredia, H.M. García and F. Sanchez-Lavado, 2006, "Aluminium sulfate as coagulant for highly polluted cork processing wastewater: Evaluation of settleability parameters and design of a clarifier-thickener unit". J. Hazar. Mater. (in press).

Hammel, K. E., 1997, Fungal degradation of lignin. In: Driven by Nature: Plant Litter Quality and Decomposition. Eds. Cadisch and Giller. CAB International, Wallingford.

Kappeler, J. and W. Gujer, 1992, Estimation of kinetic parameters of heterotrophic biomass under aerobic conditions and characterization of wastewater for activated sludge modelling, Wat. Sci. Technol., 25(6), 125-139.

Singleton, V.L. and J.A.J. Rossi, 1965, Colorimetry of total phenolics with phosphomolybdicphoshotungstic reagents, Am. J. Enol. Vitic., 16, 144–58.