

Model Investigation of Fungal Activity on a Synthetic Biorecalcitrant Wastewater

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The remediation of those effluents containing pollutants which are hard to be metabolized (biorecalcitrant) is becoming an increasingly important environmental problem, due to the complex nature of many wastewaters. An example is the class of polyphenols. In this work, the capability of the fungus *Trihoderma viride* to act as bioremediation agent for the treatment of a synthetic wastewater containing glucose, acetic acid and gallic acid (chosen as phenolic model pollutant) was characterized. The investigation was carried out in a Sequencing Batch Reactor (SBR) with a Hydraulic Retention Time of 5 d. No settling phase was operated as this work is intended to be only a model study focused on the metabolic biomass performance (i.e., the removal of pollutant from the liquor). After few days, the organic load was completely removed, including the depletion of gallic acid from the medium. Furthermore, a 0.57 yield (C-mmol based) was achieved, thus indicating the absence of inhibition phenomena.

1. Introduction

One of the most important resources of humanity is fresh water. In fact, although water is very abundant on the Earth, only a small amount is easily accessible and can be used for antropic purposes, such as domestic (drinking and washing) utilization, irrigation and industrial applications.

After being used in both domestic and agro-industrial cases, the outcoming water contains an excess of organic matter that has to be removed prior to be discharged in order to avoid polluting effect on the receiving water bodies and to preserve the integrity of the existing micro- and macro- aquatic systems.

To remove part of the organic load, activated sludge processes are usually deployed. In these processes, the removal of pollutants is due to a bacterial biomass suspension which is confined into a biological reactor. The main reactions which take place are the mineralization (conversion into CO₂ and H₂O) and the conversion of the organic soluble matter into new biomass (which is solid and then gravimetrically separable through a settling phase). Whenever it is possible, biological processes are used as they are more economic than chemical processes. In some cases, however, due to the high organic load, toxicity or presence of biorecalcitrant compounds, biological processes could not be used, since no chemical oxygen demand (COD) removal is achieved biologically.

Thus, being a biological treatment infeasible, a chemical pre-treatment can represent an adequate way to reduce the COD prior to biological treatment, as for example the advanced oxidation process (San Sebastian Martínez et al., 2003).

A typical example of great concern in industrialized countries is the treatment of the class of polyphenols, which are both bio-recalcitrant and inhibitory (Paixao et al., 1999). They arise from petroleum refining, coking and coal conversion, chemical plants, pulp and paper plant and agro-industrial processes, like olive oil extraction (Borja et al., 2006) and cork manufacturing (Mendonça et al., 2007).

The removal of this class of pollutants enjoys a lot of attention from researchers because it usually represents a significant fraction of many wastewaters coming from diffusely formed European (Table 1) wastewaters. In Europe, the main responsible for the emissions of phenols is the metallurgic industry, which in 2004 released about 240 tons as direct emission and 637 tons of phenols as indirect emissions (2004 EPER data).

Table 1 - Emission of Phenols from agro-industrial activities in Europe (EPER, 2004)

ActivityDescription	Emission in water (tons/year)	
	Direct	Indirect*
Basic inorganic chemicals or fertilisers	39.51	0.652
Basic organic chemicals	135.22	510.02
Biocides and explosives	0.366	7.55
Coke ovens	5.17	823.00
Combustion installations > 50 MW	9.62	0.642
Industrial plants for pulp from timber or other fibrous materials and paper or board production	13.30	0.879
Installations for surface treatment or products using organic solvents	0.1218	0.7445
Installations for the disposal of nonhazardous waste and landfills	2.62	7.22
Installations for the disposal or recovery of hazardous waste or municipal waste	2.22	9.18
Installations for the production of carbon or graphite	0.5099	-
Installations for the production of cement klinker, lime, glass, mineral substances or ceramic products	0.1151	2.68
Metal industry and metal ore roasting or sintering installations, Installations for the production of ferrous and non-ferrous metals	239.84	637.61
Mineral oil and gas refineries	45.99	85.45
Pharmaceutical products	4.17	83.15
Plants for tanning of hides and skins	-	2.71
Plants for the pre-treatment of fibres or textiles	0.03	1.95
Slaughterhouses, plants for the production of milk, other animal raw materials or vegetable raw materials	0.6347	3.34
(*) transfer to an off-site waste water treatment		

A recent development in wastewater technology consists in the enhancement of the fungal fraction of microbial consortia owing to their interesting characteristics. For example, they are usually more resistant to low pH, they are able to degrade substrates which are commonly considered as recalcitrant or inhibitory and they have the ability to form pellets (in the appropriate process conditions). Furthermore, previous investigations have been carried out by these authors as batch studies with a particular kind of fungal biomass, *Trichoderma viride* (D'Urso et al, 2007), on the capability to degrade polyphenols in a binary (the carbon sources were glucose and gallic acid) synthetic wastewater. Thus, an investigation of the bio-remediation ability of such a fungus (strain 8/90) in a continuous reactor fed with a more complex model phenol-containing wastewater was performed.

Activated Sludge Models (ASMs) are the most widely used model systems for simulating the biological processes occurring in activated sludge plants (Henze et al., 2000). In (ASMs), the soluble organic matter is divided in three fractions:

- Readily biodegradable material (RBM);
- Fermentation products (FPs).
- Slowly biodegradable (i.e. inert and recalcitrant) material (SBM);

On this basis, a synthetic mixture was formulated. RBM was modelled by glucose, while FPs, as customary, by acetate.

Gallic acid was adopted to represent SBM,. In this way, the presence effect of one compound of the class of phenols in high concentration (3 g/l) on the removal process performance was investigated. Besides, macro- (N and P) and micronutrients (trace elements) were dosed in excess. Particulate matter was not included in the model substrate formulation. Only the growth which takes place by degradation of soluble substrates based on the consumption of oxygen was considered, where ammonia nitrogen is incorporated into cell mass.

Autotrophic nitrogen removal, storage phenomena and hydrolysis of particulate matter were not targeted by this work.

2. Materials and Methods

2.1 Biomass

For this work, *Trichoderma viride* Pers:Fr. Isolate 8/90 has been used. It was kept on Petri dishes as a pure culture and stored at 26° C.

2.2 Analytical Methods

Glucose analysis was performed through the Somogyi-Nelson (Somogyi, 1926) method while gallic acid was analysed by the Folin-Ciocalteu (Singleton and Rossi, 1965) one. Acetic acid was analysed by gas chromatography (see Table 2). The dissolved carbon content was determined using an Elemental HiTOC (Germany) analyser and auto-injector: a filtered (0.45µm) sample was injected into a catalyst furnace where all carbon was oxidised to carbon dioxide.

Another sample was injected into a reactor containing dilute acid, where the inorganic component, assumed to be carbonate, was converted into carbon dioxide. The carbon dioxide was removed from each reaction in two carrier gas streams to separate infra red detectors. The output of the detectors was integrated and expressed as elemental carbon.

Organic carbon was calculated as the difference between the total and inorganic carbon readings.

Standard solid content analyses for wastewater treatment systems (TSS, VSS and SVI as reported by APAT and IRSA-CNR, 2004) were also performed.

Table 2 - Gas Chromatograph Conditions for acetic acid analysis

Injector temperature	170°C
Liner	Gooseneck with glass wool
Injection technique	Purge splitless
Purge on time	0.5 minutes
Oven temperature profile	Initial temperature 30°C (2 min) Ramp 10°C/min to 110°C (10 min) Ramp 10°C/min to 150°C (5 min)
Run length	31min (45min total - includes cooling to 30°C)
Detector	FID (190°C)
Headpressure	50kPa
Carrier gas (Helium)	20 mL/min over a 0.5% formic acid reservoir
Hydrogen	18 mL/min
Oxygen	32 mL/min
Nitrogen	40 mL/min

2.3 Reactor Management

The study was performed as a continuous treatment in a Sequencing Batch Reactor (SBR) system (volume = 2 l).

The SBR was fed with a solution containing glucose (10 g/l), gallic acid (3 g/l) and acetic acid (2 g/l) and the required nutrients.

A Hydraulic Retention Time (HRT) and Solid Retention Time (SRT; no settling phase) of 5 days was adopted for modelling purposes. The pH and temperature set points were fixed, respectively, at 3.5 (aim of this investigation was also identifying the biomass behaviour in extremely acidic conditions) and 25°C.

The reactor was managed as a batch one during the first three days, to allow for *Trichoderma viride* biomass growth: a diluted (1:4) substrate mixture was introduced as growth medium. After three days, the above mentioned medium was continuously fed to the system.

The phases of the reactor were the following:

- Load phase: 5 min
- Reaction phase: 340 min
- Discharge phase: 15 min

The feeding consisted on a synthetic solution with the following composition (g/L): glucose (10), gallic acid (3), acetic acid (2), KH_2PO_4 (1), $(\text{NH}_4)_2\text{SO}_4$ (5), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1.23), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.00271), $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (0.0016), $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$ (0.0014), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.0036), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.8). The organic load rate was equal to 3240 mgCOD/d (or 1260 mg/l in term of Dissolved Organic Carbon, DOC).

3. Results and discussions

The feeding described in Section 2.3 was initially diluted (1:4) and used as growth medium for the *T. viride* biomass. Such a phase lasted three days during which the reactor was managed in batch mode. Afterwards, feeding was continuously provided to reactor.

Glucose and acetic acid were immediately utilized by the biomass, while gallic acid was not metabolised (see Figure 1). After 24 h from the start-up (time $d = 1$) it is possible to observe an increase in the substrates' concentration due to water loss in the outflowing gas. Subsequently, the reactor volume was adjusted daily with the addition of some distilled water.

During reactor feeding, both glucose and acetic acid were depleted (see Figure 2). Interestingly, gallic acid didn't provide any inhibition to the growth of the fungal biomass as usually can be expected in the case of bacterial biomass (Caffaz et al., 2007). However, an increase of gallic acid concentration was recorded till $t = 10$ d. Thereafter gallic acid concentration decreased and from $t = 16$ d gallic acid was completely removed from the biomass.

A possible explanation for this behavior could be the following: the biomass was able to remove the above mentioned compound but with a slower specific removal rate with respect to glucose and acetic acid thus leading to its accumulation in the liquor. With the biomass concentration becoming higher and higher, the overall removal rate became sufficient to provide a depletion of gallic acid from the medium.

This explanation can be confirmed looking at the profiles in Figure 3. The actual gallic acid concentration profile was lower than that calculated with the hypothesis of no removal, thus indicating a partial gallic acid utilization. As the biomass concentration become higher enough, a decrease in gallic acid concentration in the outflowing effluent and, later on, a complete removal were observed.

Furthermore, Dissolved Organic Carbon (DOC) content was measured as well. this quantity exhibited a profile similar to the gallic acid one, . i.e. it raised while gallic acid removal was not complete and then decreased to a constant but lower value when gallic acid was almost depleted.

After $t = 16$ d, all the substrates were completely removed. However, even though there was a 96% removal, on DOC basis, a stable DOC value was always recorded, likely because of the formation of by-products.

In the end, it must be observed that the low pH conditions didn't affect the biomass growth.

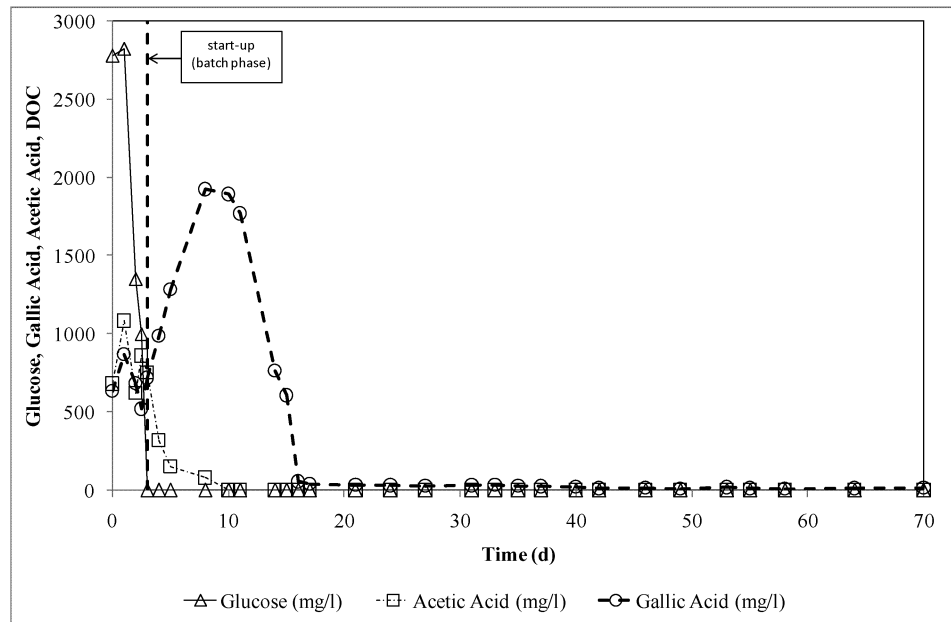


Figure 1 – Substrates' profiles in the reactor effluent.

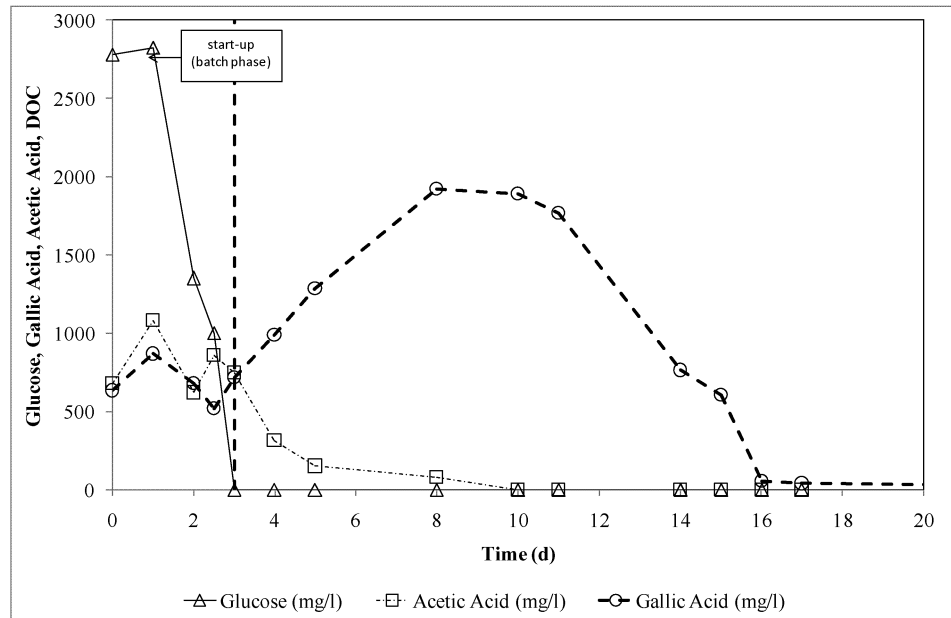


Figure 2 – Substrates' profile in the reactor effluent (detail on the first 20 days).

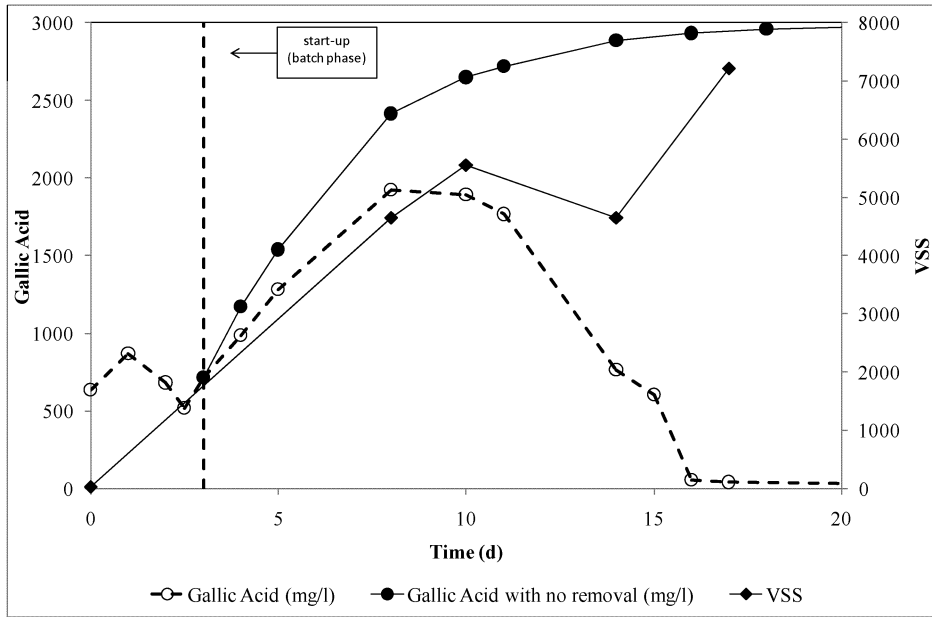


Figure 3 – Comparison between gallic acid concentration in the outgoing effluent and the calculated concentration under the hypothesis of no removal (accumulation) during the first 20 days.

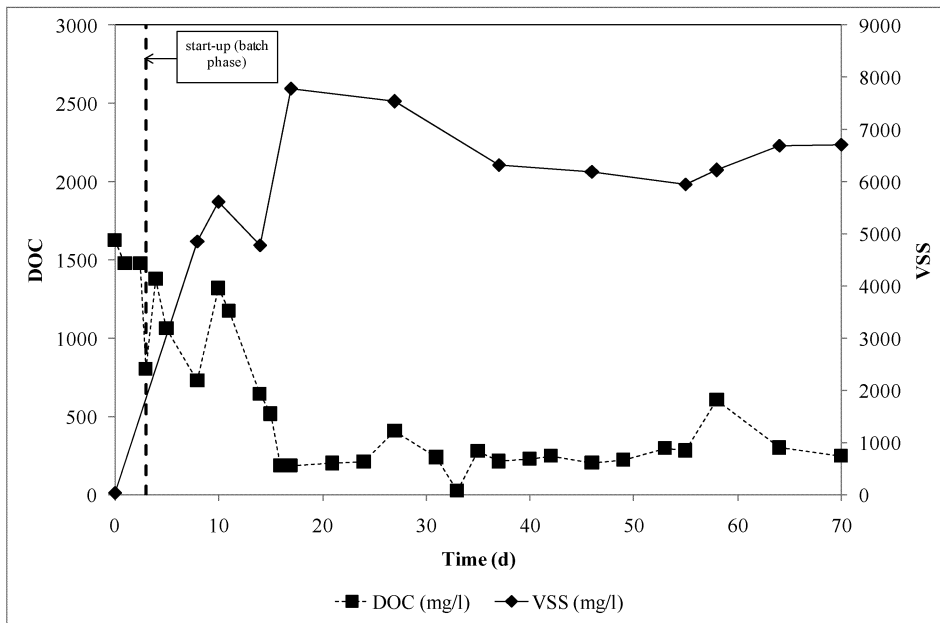


Figure 4 – Dissolved Organic Carbon (DOC) and biomass content (as Volatile Suspended Solids, VSS) content.

4. Conclusions

The treatment of wastewaters containing phenols-like compounds usually represents a problem because of the inhibitory effects exhibited on microorganisms. Instead, fungi can withstand high concentrations of these compounds and even remove them. In this work, unacclimatised *T. viride* was challenged in a slightly modified continuous biological treatment model treatment; the biomass showed the ability to completely and simultaneously remove the RBM, FB and SBM components of a low (3.5) pH substrate fed to the reactor (being the 24.6% made up of gallic acid on DOC basis) from the very beginning.

These results suggest that the ability of fungi to contribute essential steps of the carbon cycle occurring in nature can also be exploited in biological treatments of biorecalcitrant wastewaters, where their resistance to high concentrations of SBM provide tolerance to concentration fluctuations of this latter and therefore reduce the requirements on the equalisation basin.

5. References

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