# Bio-degradation ability of three bacterial strains isolated from a Jacto reactor used for the treatment of an agro-industrial effluent

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## Introduction

Jet-loop reactors (Jacto) developed and scaled-up at INETI have been successfully applied for biological treatment of agro-industrial effluents (wine, olive oil and other agro-industries wastewaters) using an improved inoculum (Eusébio *et al.*, 2004; Eusébio *et al.*, 2005).

The degradative ability of microbial communities depends on the stability of the constituent members as well as on their ability to degrade or mineralise the target compounds. The most of the effluent bio-treatments referred in the literature were successfully carried out using native microbial consortia or biological sludges, instead of defined or characterised consortia. Nevertheless, it is of major importance to identify the microorganisms belonging to the involved consortium in order to achieve better degradation yields and the consequent optimisation of the treatment.

The aim of this work was to characterise a microbial consortium previously developed during a winery wastewater JACTO bio-treatment and to assess the relationship between treatment efficiency and microbial consortium composition.

Isolation and identification of all cultivable microorganisms present in the inoculum were carried out using conventional methods. In order to investigate the role of a determine sub-population with treatment efficiency, the three most predominant bacterial isolates were grown separately and their degradation ability evaluated in terms of COD and phenol reduction.

Profile of microbial consortium present in the effluent is being determined by TGGE analysis and preliminary results are herein presented.

**Characterisation of the microbial population in the JACTO bio-reactor**

At INETI, jet-loop like bio-reactors (Jacto) were developed for an efficient bio-treatment of winery effluents, using the native microbial consortia (Eusébio *et al.*, 2004).

The microbial characterisation of the effluent was performed at the final of the bio-treatment process using the conventional methodologies usually applied on microbiology.

Microbial populations were isolated by spread plate method on Tryptone Soy Agar culture medium (TSA), incubated at 30ºC for up to 48h. Identification of the isolates was performed on the basis of cell and colonial morphology, Gram staining, motility, presence/absence of endospores, catalase and citochromium oxidase tests. Biochemical tests (API 20 NE, API 20 E, API 50 CHB and API staph from API system, Analytical Profile Index, bioMérieux vitek, Inc. France) were applied to isolates.

Table 1 - Microbial characterisation of adapted inoculum obtained after winery effluent treatment in Jacto bio-reactor.

| Isolates | Identification / Typification |
|---|---|
| AS | *Brevibacillus brevis* |
| CA | *Pseudomonas fluorescens* |
| BM | *Staphylococcus epidermidis* |
| SV | $Gram^+$ non-sporulated bacilli |
| AG | $Gram^+$ non-sporulated bacilli |
| I | $Gram^+$ coccus |

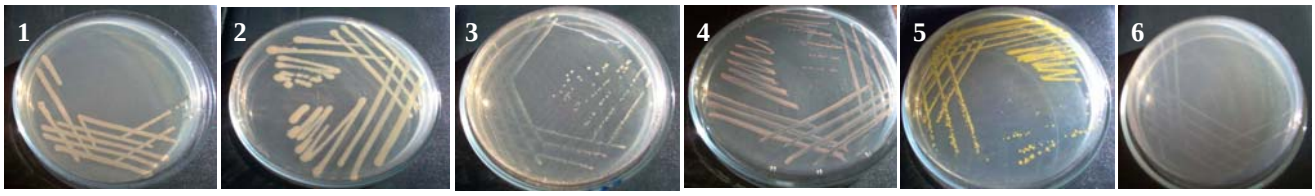



Fig. 1 - Petri plates isolations in TSA medium obtained from the adapted inoculum collected after winery effluent treatment in JACTO bio-reactor. **1 -** AS; **2** - CA; **3 -** BM; **4** - SV; **5** - AG; **6** - I.

Microbial characterisation carried out at the final of the winery effluent bio-treatment revealed a predominance of $Gram^+$ microorganisms. The presence of these microorganisms at the final phase of the JACTO treatment indicates that this

microflora must be well adapted to the high shear conditions occurring inside the bio-reactor, and suggests their possible significant role on the biodegradation process.

**Biodegradation studies**

In order to detect if the degradation ability of this microbial consortium could be attributed to one (or more than one) of the cultivable sub-populations present, the individual activity of the three most predominant bacterial isolates (AG and SV, typified as Gram$^{+}$ non-sporulated bacilli, and BM, identified as *Staphylococcus epidermidis*) was determined by measuring the COD and total phenols removal.

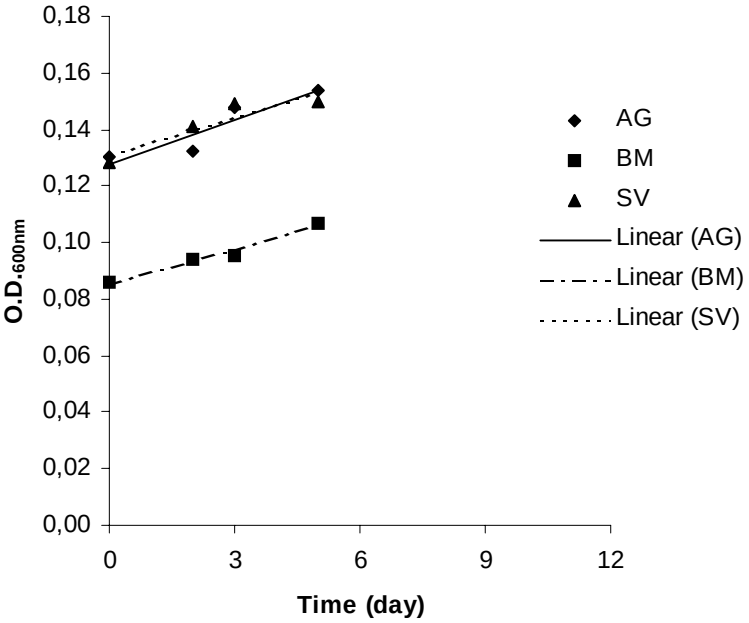
Pre-inocula of the isolates AG, BM and SV were prepared in Tryptone Soy Broth (TSB) liquid medium incubated at 30ºC for and 150 rpm, for 24 h. 20 ml of each of the cultures were used to inoculate 200 ml of sterilised (autoclaved at 120ºC, 1 atm) winery effluent in 1L-Erlenmeyer flasks, in duplicates, and incubated at 30ºC and 150 rpm, for 10 days. Sterilised winery effluent incubated under the same conditions was used as control.

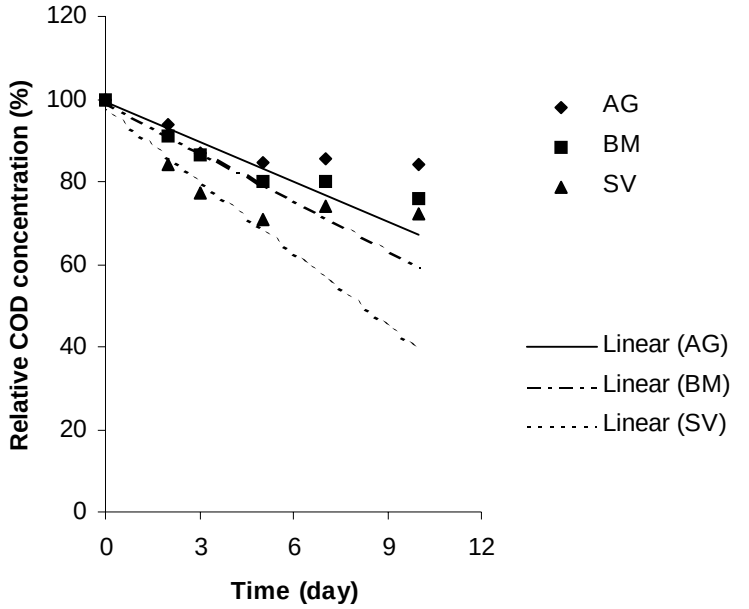
Microbial growth of each isolate was measured by reading optical density at 600 nm ($O.D._{600nm}$). Chemical oxygen demand (COD), total suspended solids (TSS) and volatile suspended solids (VSS) were determined according to Standard Methods (APHA, 1998). Total phenols content was determined according Singleton and Rossi (1965).

Physical-chemical main parameters of crude winery effluent were determined and results are shown in Table 2.

Table 2 - Analytical characterisation of the crude winery effluent.

| Character | Unit | Value |
|---|---|---|
| pH | - | 5,00 |
| COD | $g.l^{-1}$ | 10,40 |
| Total phenols | $mg.l^{-1}$ | 54,04 |
| TSS | $g.l^{-1}$ | 0,05 |
| VSS | $g.l^{-1}$ | 0,03 |



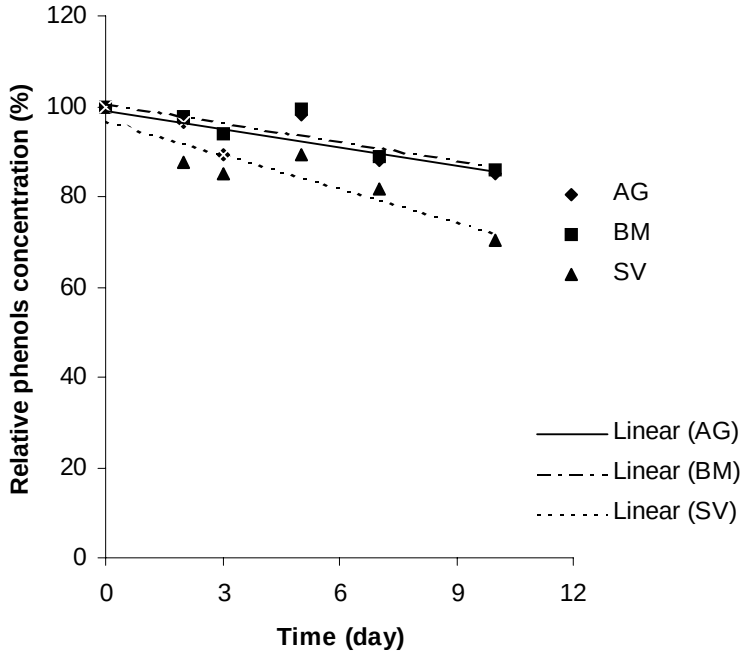




Fig.2 - Microbial growth and relative COD and phenols concentration for each isolate AG, BM and SV, in 1L- shaking flasks under batch conditions. Cultures were performed in duplicate. In the case of relative COD concentration, linearization was made for the first five days of bio-treatment.

Time course of bacterial growth and relative removal of COD and total phenols achieved by the cultures of each one of bacterial isolates AG, BM and SV, under the tested conditions is shown in Fig. 2. Since the rate for COD removal stabilised after the fifth day of bio-treatment, linearisation of the obtained results was only made for the first five days.

The three inocula tested were able to grow in crude sterilised winery effluent without any nutrient supplementation which is of major importance for their future application to improve effluent treatment efficiency. In terms of $O.D._{600nm}$ readings, similar specific growth rates of 0,0045 $day^{-1}$, 0,0052 $day^{-1}$ and 0,0041 $day^{-1}$ were obtained for isolates AG, BM and SV, respectively. The cultures also evidenced a potential degradation in terms of COD removal and total phenols removal. At the $5^{th}$ day of effluent treatment process, a removal rate of 0.37, 0.44 and 0.71 g COD $l^{-1}.d^{-1}$ was obtained for isolate AG, BM and SV, respectively. At the $10^{th}$ day of effluent treatment, a total phenol removal rate of 1.05, 0.63 and 1.34 $g.l^{-1}.d^{-1}$ was obtained for isolate AG, BM and SV, respectively.

Table 3 summarises the performance of each bacterial culture and of the total microbial consortium under the tested experimental conditions.

Table 3 - COD and total phenols removal efficiency (%) determined after the winery effluent bio-treatment, in 1000-ml shaking flasks, under batch conditions. Assays were performed in duplicate.

| Condition | COD (%) | Phenols (%) |
|---|---|---|
| Consortium | 10 | 24 |
| AG | 15 | 11 |
| BM | 20 | 6 |
| SV | 29 | 15 |

COD and total phenols removal was higher in cultures inoculated with SV isolate, a $Gram^{+}$ non-sporulated bacillus, indicating that this microorganism could have an important role in the winery effluent biodegradation. In relation to the AG isolate, *Staphylococcus epidermidis* (BM isolate) presents a good COD removal rate but it seems that it plays a minor role in phenols biodegradation. *Staphylococcus epidermidis* is found normally on skin and in mucous membranes. It is non-motile,

gram-positive cocci, arranged in irregular clusters. This bacterium is an opportunistic pathogen that does not cause problems unless it enters the bloodstream via cuts, catheters, or needles, for example. Stevens et al. (2002) identified *Staphylococcus* among several heterotrophic bacteria with nitrification potential isolated from a BNR installation (wastewater treatment process using biological nutrient removal). The identified heterotrophic nitrifiers directly oxidized ammonium to nitrate with little or no nitrite accumulation. A strain of *Staphylococcus epidermidis* was isolated from a bacterial consortium used for the remediation of a chromate-contaminated constructed wetland system (Vatsourina *et al.*, 2005). During the winery wastewater treatment using the JACTO bio-reactor, and herein referred, the supplementation with ammonium (as nitrogen source) seems to favour the presence of *Staphylococcus epidermidis* in the microbial consortium.

Furthermore comparing the performance of each one of the three tested different inocula with the consortium performance, it can be observed that the consortium efficiency was lower in terms of COD removal but higher in terms of phenol removal. Therefore, phenol degradation rates may be dependent of the synergy among several microorganisms present in the consortium. It might be probable that these three isolates may participate in this "co-metabolism" but the presence of other species in the consortium that may be relevant for this activity cannot be excluded.

Tabela 4 - Biomass formation (VSS) vs. COD consumption, at the 5$^{th}$ day of treatment in 1L-shaking flasks, under batch conditions. Assays were performed in duplicate.

| Condition | Loading charge (g COD.l$^{-1}$) | COD removal (%) | CODc (g COD.l$^{-1}$) | VSS$_{f-i}$ (g.l$^{-1}$) | VSS / CODc |
|---|---|---|---|---|---|
| Consortium | 8,71 | 10 | 0,87 | 0,025 | 0,029 |
| AG | 11,40 | 15 | 1,71 | 0,037 | 0,022 |
| BM | 10,85 | 20 | 2,17 | 0,023 | 0,011 |
| SV | 12,15 | 29 | 3,52 | 0,018 | 0,005 |

In terms of biomass formation as a function of COD removal, AG isolate, a Gram$^{+}$ non-sporulated bacilli, has shown the highest performance that was quite identical to the one achieved by the whole microbial consortium. Since this culture also represents a good percentage of COD and phenol removal, in near future it will be

developed an inoculum to be added to the whole microbial consortium and assay the survival and degradation ability for winery wastewater treatment.

The non-sporulated bacilli, not yet identified, are probably Lactic acid bacteria (LAB). In fact, their presence in this microbial consortium is expected since LAB are part of the natural microbiota of the winemaking process. Four LAB genera present in must and wine (Lactobacillus, Leuconostoc, Pediococcus and Oenococcus) were referred by Blasco et al. (2003).

**PCR-TGGE Analysis**

In order to obtain a molecular profile of the microbial consortium, it was performed a PCR-TGGE analysis using a temperature gradient between 42ºC and 49ºC, at a constant voltage of 200 V (figure 3). DNA was extracted from samples according to Zhou *et al.* (1996) with some modifications.

Fig.3 - PCR-TGGE analysis of effluent after treatment process. **1**, 100 bp DNA ladder marker; **2**, microbial consortium.

Although running TGGE conditions as well as further purification of effluent samples are still under course, the molecular profile of microbial consortium analysed by PCR-TGGE revealed at least six distinct bands. The complete molecular profiling of the microbial consortium along the bio-treatment, that detects and identifies all cultivable and non-cultivable microorganisms, will provide a very fast and reliable tool to monitor JACTO bio-treatment.

**References**


- Blasco, L, Ferrer, S, and Pardo, I (2003) Development of specific fluorescent oligonucleotide probes for in situ identification of wine lactic acid bacteria. *FEMS Microbiology Letters*, 225: 115-123.
- Eusébio, A, Petruccioli, M, Lageiro, M, Federici, F, and Duarte, JC (2004) "Microbial characterisation of activated sludge in jet-loop bioreactors treating winery wastewaters". *J. Ind. Microbiol. Biotechnol.* 31: 29-34.
- Eusébio, A, Mateus, M, Baeta-Hall, L, Sàágua, MC, Tenreiro, R, Almeida-Vara, E, and Duarte, JC (2005) *Int. Biodet. Biodeg.* (submitted).
- Singleton, VL and Rossi, JA, Jr (1965) "Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents". *Amer. J. Enol. Viticult.* 16: 144-58.
- APHA (1998) Standard Methods for the Examination of Water and Wastewater, 20th Edition, Amer. Public. Health Assoc., Washington, DC.
- Stevens, WE, Drysdale, GD, Bux, F (2002) Evaluation of nitrification by heterotrophic bacteria in biological nutrient removal processes. *South African Journal of Science* 98 (5-6): 222-224.
- Vatsourina, A, Vainshtein, M, Kuschk, P, (2005, eFirst) Anaerobic co-reduction of chromate and nitrate by bacterial cultures of *Staphylococcus epidermidis* L-02. *J Ind Microbiol Biotechnol*.
- Zhou, J, Bruns, MA and Tiedje, JM (1996) DNA Recovery from Soils of Diverse Composition. *Appl. Environ. Microbiol.*, 62 (2): 316 - 322.