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# Biotreatment of olive washing wastewater by a selected microalgal-bacterial consortium

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ABSTRACT

This purpose of the study was the development of a microalgal-bacterial consortium to degrade phenolic compounds. Two microalgae were isolated and characterized from an olive wash water (OWW) storage basin and identified as *Scenedesmus obliquus* and *Chlorella vulgaris* according to their 18S rRNA gene sequences. The two strains were cultured in synthetic olive washing water medium containing phenolic compounds, which showed they were capable of growth in the presence of these substances, although they were sensitive to phenolic compounds and their growth decreased compared to controls grown in the absence of the compounds. Complementary experiments were carried out using a microalgal-bacterial consortium containing the two microalgae and two bacterial strains able to degrade phenolic compounds (*Raoultella terrigena* and *Pantoea agglomerans*). The results showed that the microalgal-bacterial consortium actively metabolized phenolic compounds with more that 99% of phenolic compounds removed at 48 h. The consortium also removed significant amounts of N and P from the liquid medium. The selected microalgal-bacterial consortium appeared to be a promising candidate for the bioremediation of OWW.



## 1. Introduction

The olive oil industry is one of the major food sectors for Mediterranean countries which is fast spreading to other areas such as Australia, Chile and the USA (Cerrone et al., 2011) and generates a large amount of wastewater and other wastes (Khatib et al., 2009). Two and three-phase systems are used for the extraction of olive oil, however, for both systems, preliminary washing of the olives with potable water is necessary. This washing generates an effluent with a composition similar to olive mill wastewater (OMWW) produced during the three-phase milling process. For these reasons, olive washing water (OWW) disposal is becoming a worldwide environmental issue (Guardia-Rubio et al., 2008) as the effluent requires specific treatment prior to disposal (Roig et al., 2006).

OWW composition is similar to OMWW, but contains lower concentrations (from 1/10 up to 1/50) of pollutants such as chemical oxygen demand (COD) and phenolic compounds (Pozo et al., 2007; Cerrone et al., 2011). Phenols and their derivatives are considered important pollutants since they are harmful to organisms (microorganisms and plants) even at very low concentrations. The most effective techniques for degrading organic compounds are advanced oxidation processes (AOPs) which are based on the generation of reactive species such as hydroxyl and sulphate radicals (Liotta et al., 2009). Heterogeneous photocatalysis has been used for phenol degradation (Das et al., 2005; Parida et al., 2006) and the Fenton reaction involving hydrogen peroxide and Fe ion has also been extremely popular, though is major disadvantage is requiring a low pH (~3).

Biological treatment may be an environmentally-friendly and economical alternative for the removal of phenol when compared with the traditional methods of chemical-physical degradation. To date, the only published works attempting the biological treatment of OWW are those of Pozo et al. (2007) using a submerged bacterial biofilter and by Cerrone et al. (2011) using the white fungus *Trametes versicolor* in a continuous bubble-column bioreactor process. Recently, Maza-Márquez et al. (2013) isolated two phenol-

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degrading strains affiliated with *Raoultella terrigena* and *Pantoea agglomerans* based the partial 16S rRNA gene. The biological treatment of OWW with this strains generated an effluent complying with the EU legislation for its reuse in ferti-irrigations and/or its direct disposal.

Algae are a logical choice for water treatment due to their ubiquitous distribution, the production of *in situ* oxygen via photosynthesis and their removal of nutrients, serving to reduce the number of treatment steps required (Garrett and Allen, 1976; Fallowfield and Garrett, 1985). The assimilation of $CO_2$ by microalgae contributes to a higher nutrient uptake, which improves the removal of nutrients by assimilation when compared to mechanically-aerated bacterial systems. An additional advantage to using photosynthetic oxygenation is that no $CO_2$ is being released into the atmosphere, which contributes to the mitigation of greenhouse effects.

Heterotrophic microalgae can be out-competed by heterotrophic bacteria in continuous open systems, as microalgae often exhibit lower specific growth rates than bacteria (Semple et al., 1999; Lee, 2001). The practical applications of pollutant biodegradation by algae remain uncertain and should be further investigated. However, in recent years, the capability of these microorganisms to biotransform and biodegrade phenol (Semple et al., 1999) and other pollutants (Safonova et al., 2004) has been reported, suggesting these organisms could potentially be used in the biotreatment of polluted soil and water.

It is apparent that a system which includes several microorganisms would be preferable for bioremediation processes, as it is nearly impossible to find a single microorganism that can degrade a mixture of different pollutants (Abeliovich and Weisman, 1978). In this context, studies on microbial consortia of bacteria and cyanobacteria have been initiated, while consortia between bacteria and microalgae still remain mostly unexplored (Safonova et al., 2004). Microalgae and cyanobacteria play an important role in consortia since they supply molecular oxygen to heterotrophic partners and support the initial steps of degradation (Cerniglia, 1992) while the heterotrophic microorganisms supply $CO_2$ to photosynthetic partners. Thus, the combination of phototrophic and heterotrophic organisms is able to enhance the degradation potential of the whole consortium.

This approach was used in the present study to remediate OWW polluted with high concentrations of phenolic compounds. The major aims of the present work were the isolation of microalgal strains resistant to phenolic compounds, selection of bacterial strains which were able to degrade phenolic compounds and evaluation of the batch remediation process of OWW by the consortium.

## 2. Materials and methods

### 2.1. Isolation and identification of microalgal species from olive washing water (OWW)

Several microalgae were collected from the OWW storage basin located outside the olive oil factory "Nuestra Señora de los Desamparados" (Puente Genil, Córdoba, Spain). For the isolation of strains, olive washing water (OWW) samples (1, 2, 3 ml) were serially-diluted in 1 L Erlenmeyer flasks which contained 300 mL of modified Rodriguez-López medium as described by Stainer et al. (1971). The medium contained $NaNO_3$ (1.5 g $L^{-1}$), $K_2HPO_4$ (0.04 g $L^{-1}$), $MgSO_47H_2O$ (0.075 g $L^{-1}$), $CaCl_22H_2O$ (0.036 g $L^{-1}$), citric acid (0.01 g $L^{-1}$), ferric ammonium citrate (0.006 g $L^{-1}$), $Na_2EDTA$ (0.001 g $L^{-1}$), $Na_2CO_3$ (0.02 mg $L^{-1}$) and 1 mL of trace metal solution per litre. The trace metal solution contained $H_3BO_3$ (61.0 mg $L^{-1}$), $MnSO_4$ (169.0 mg $L^{-1}$), $ZnSO_4 \cdot 7H_2O$ (287 mg $L^{-1}$), $CuSO_45H_2O$ (2.5 mg $L^{-1}$), and $(NH_4)_6MoO_44H_2O$ (12.5 mg $L^{-1}$). The Erlenmeyer flasks were incubated at 25 ± 1 °C, 160 rpm rotation, and 200 μmol $m^{-2}$ $s^{-1}$ illumination with a 16:8 h light dark cycle (Jeiotech,GC-300TLH,Lab Companion, Des Plaines, Il, USA) for 48–72 h.

Samples (0.1 mL) were serially diluted and spread on modified Rodriguez-López medium plates containing 4% agar and incubated as previously described. Individual colonies were examined microscopically, isolated and a partial 18S rRNA sequence obtained. The primers used to amplify the partial 18S rRNA gene of the selected isolates were forward primer Euk1 (5′-CTGGTTGA TCCTGCCAG-3′) and reverse primer Euk 516r (5′-ACCAGACTTGC CCTCC-3′) (Diez et al., 2001). Sequences were analysed on-line by the European Bioinformatics Institute biocomputing tools (htpp://www.ebi.ac.uk). The BLASTn program (Altschul et al., 1997) was used for analysis of sequences similarity and Clustal X version 2.0.3 software (Jeanmougin et al., 1998) was used for sequences alignment. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 1 (Kumar et al., 2001); the p-distance based evolutionary tree was inferred using the Neighbour-Joining algorithm.

### 2.2. Microalgae tolerance of different concentration phenolic compounds

Microalgae (isolated from OWW) were grown on synthetic OWW medium composed of 20 mg $L^{-1}$ $NaNO_3$, 10 mg $L^{-1}$ $K_3PO_4$, and trace minerals (Kotturi et al., 1991). Culturing was accomplished in 1 L Erlenmeyer flasks containing 300 mL of medium in a shaking incubator (HB 201 SF-L Hanbaek-Scientific Co, Seoul,

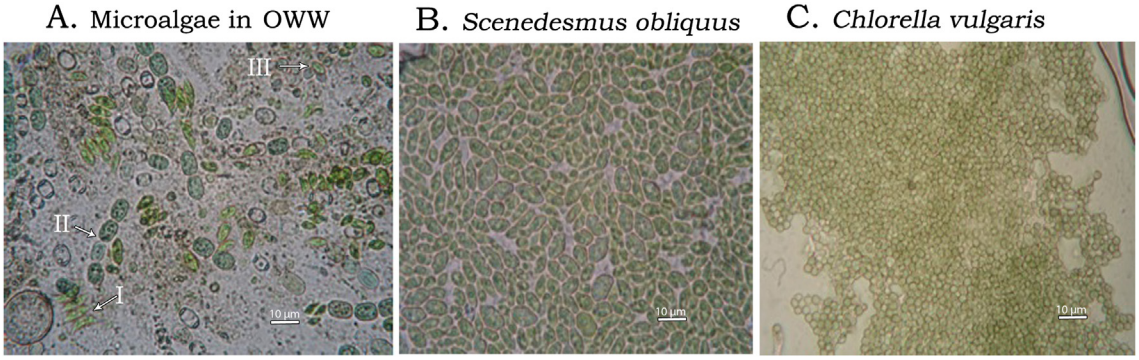


**Fig. 1.** Microscopic images of microalgae (A) and the major microalgal strains (B and C) isolated from OWW (x1000). (A) Complex morphological types found in OWW. (B) *Scenedesmus obliquus* and (C) *Chlorella vulgaris* growing on Rodriguez-Lopez medium.