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Combined bioremediation and enzyme production by *Aspergillus* sp. in olive mill and winery wastewaters

José Manuel Salgado [a], Luís Abrunhosa [a], Armando Venâncio [a], José Manuel Domínguez [b], Isabel Belo [a, *]

[a] CEB-Centre of Biological Engineering, University of Minho, Braga, Portugal
[b] Department of Chemical Engineering, Sciences Faculty, University of Vigo (Campus Ourense), As Lagoas s/n, 32004 Ourense, Spain

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ABSTRACT

Olive mill wastewaters (OMW) and vinasses (VS) are effluents produced respectively by olive mills and wineries, both sectors are of great economic importance in Mediterranean countries. These effluents cause a large environmental impact, when not properly processed, due to their high concentration of phenolic compounds, COD and colour. OMW may be treated by biological processes but, in this case, a dilution is necessary, increasing water consumption. The approach here in proposed consists on the bioremediation of OMW and VS by filamentous fungi. In a screening stage, three fungi (*Aspergillus ibericus*, *Aspergillus uvarum*, *Aspergillus niger*) were selected to bioremediate undiluted OMW, two-fold diluted OMW supplemented with nutrients, and a mixture of OMW and VS in the proportion 1:1 (v/v). Higher reductions of phenolic compounds, colour and COD were achieved mixing both residues; with *A. uvarum* providing the best results. In addition, the production of enzymes was also evaluated during this bioremediation process, detecting in all cases lipolytic, proteolytic and tannase activities. *A. ibericus*, *A. uvarum* and *A. niger* achieved the highest value of lipase (1253.7 ± 161.2 U/L), protease (3700 ± 124.3 U/L) and tannase (284.4 ± 12.1 U/L) activities, respectively. Consequently, this process is an interesting alternative to traditional processes to manage these residues, providing simultaneously high economic products, which can be employed in the same industries.

1. Introduction

Liquid wastes from agro-industrial processes like wineries and olive oil industries are sources of local water pollution. Generally, these industries are nearby and generate their wastes at the same time of the year. For this reason, a common process to treat both wastes is of great interest. Vinasses (VS) and olive mill wastewaters (OMW) are the main liquid wastes generated in wineries and olive mills, respectively. These effluents are harmful to sewage treatment plants due to the large amounts of organic and suspended matter (Rytwo et al., 2013).

VS are liquid wastes generated by the distillation process of lees and low quality wines. Like other winery wastes, VS present acidic pH values, high biochemical oxygen demand (BOD) and chemical oxygen demand (COD). These wastes contain dead microorganisms and other compounds of wine (e.g. phenols, acids, carbohydrates, mineral salts) (Salgado et al., 2010). Commonly, VS are released to surface waters causing significant environmental problems (Beltrán et al., 1999). The amount of VS generated is 8–15 times greater than the ethanol production (Strong, 2009). In addition, the VS are produced in large quantities in a short season, making its treatment a harder challenge (Vlyssides et al., 2010). In the past few years, multiple applications have been investigated to manage their proper treatment. Among other applications, VS have been proposed as a nutritional supplement for microorganism growth (Salgado et al., 2009), for the production of protein-rich fungal biomass (Nitayavardhana and Khanal, 2010) or as a co-product in grape marc composting (Paradelo et al., 2010).

OMW are stable emulsions composed of water, olive pulp remains and residual oil (Lanciotti et al., 2005). Due to their high content on phenolic compounds (PC), COD and long-chain fatty acids, OMW are toxic to microorganisms and plants, and cannot be directly disposed into the environment (Paraskeva and Diamadopoulos, 2006; Sierra et al., 2007; Iamarino et al., 2009; Zirehpour et al., 2014). A common use of OMW is as soil

* Corresponding author.
E-mail address: ibelo@deb.uminho.pt (I. Belo).

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amendment, but it can cause phytotoxic problems and change physicochemical characteristics of soil (Mekki et al., 2007). To meet the environmental standards, aerobic and anaerobic biological processes, including anaerobic co-digestion with other effluents and composting, are predominantly used to treat OMW (Paraskeva and Diamadopoulos, 2006). Bioremediation is environmentally friendly and cost competitive in comparison with chemical decomposition (España-Gamboa et al., 2011). This treatment can reduce COD and phenolic content, thus facilitating further management before its disposal (Ntougias et al., 2012). Nevertheless, phenols can act as inhibitors of many microorganisms, when present at low concentrations, and may reduce the performance of biological treatments. Therefore, in many cases, it is necessary to dilute OMW in order to reduce its toxicity to microorganisms, increasing water consumption (Öngen et al., 2007; Mann et al., 2010; Jarboui et al., 2013; Lakhtar et al., 2010).

The mixture of OMW with other agro-industrial effluents, which have lower pollutant load, may be a way to improve biological treatment performances. With this approach, initial phenolic content and COD can be reduced, avoiding the inhibition of microorganisms without using water. For example, cheese whey's addition to OMW in a proportion of 90/10 (v/v) was necessary to achieve the highest colour and phenolic reduction during the biological treatment of OMW with *Lactobacillus paracasei* (Aouidi et al., 2009).

Aerobic treatments for OMW detoxification can be performed by bacteria and fungi (Aouidi et al., 2009; Gonçalves et al., 2009; Mann et al., 2010). Three types of fungi are commonly used: white rot fungi, *Aspergillus* spp. and several different yeasts (McNamara et al., 2008). Among the *Aspergillus* genera, *Aspergillus niger* and *Aspergillus terreus* have been the most studied species to reduce PC, COD and colour of OMW (Garcia-Garcia et al., 2000; Garrido-Hoyos et al., 2002). *A. niger* belongs to the so called black aspergilli group, which include presently 19 accepted taxa (Samson et al., 2007). New species belonging to this group have been isolated from grape berries as *Aspergillus uvarum* and *Aspergillus ibericus* (Perrone et al., 2008; Serra et al., 2006). Production of enzymes in OMW and their detoxification by *A. ibericus* have already been tested (Abrunhosa et al., 2012), but *A. uvarum* has not been studied yet.

The aim of the present work was to select novel strains of *Aspergillus* spp., which could decolourise as well as improve the bioremediation of OMW, and study their suitability to treat OMW and VS mixtures while producing enzymes.

2. Materials and methods

2.1. Characterisation of effluents

The OMW were collected from olive mill (Region of Trás-os-Montes) and VS were collected from winery (Region of Minho) in 2012. Both effluents were separately homogenized and stored at −20 °C until use.

Total nitrogen (TN) was determined by the test kit Hach Lange LCK 338. For COD determination, the test kit Hach Lange LCK914 was used according to the manufacturer method. Total organic carbon (TOC) in liquid residues was quantified by the test kit Hach Lange LCK 387. Reducing sugars were determined by the dinitrosalicylic acid (DNS) method, and protein was analysed according to the Bradford method (Bradford, 1976). Total phenols were assessed by the Folin–Ciocalteau method (Commission Regulation (EEC) No. 2676/90), using caffeic acid as standard. Total solids were analysed by oven-dried to constant weight at 105 °C.

2.2. Screening of fungi for OMW bioremediation on agar plates

Table 1 shows the strains of *Aspergillus* assayed in this work, all of them supplied by MUM culture collection (University of Minho, Braga, Portugal). Fungi were cultivated to observe mycelium growth and decolourisation of OMW in agar media formulated with undiluted OMW, OMW diluted in the proportion 1:1 and 1:10 (v/v) with a nutrient supplemented medium (3 g/L $NaNO_3$, 1 g/L K_2HPO_4, 0.5 g/L KCl, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L $CaCl_2 \cdot 2H_2O$, 1 mL/L metal solution (10 g/L $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$)) or diluted 1:1 and 1:3 (v/v) with VS. Fungi were pre-incubated on malt extract agar (MEA) (2% malt extract, 2% glucose, 0.1% peptone and 2% agar) plates at 25 °C and stored at 4 °C before inoculation on OMW agar plates. Inoculum was placed into the centre of the corresponding dishes, the growth and the halo of OMW decolourisation were followed during five days by measuring diameters of mycelium growth and decolourisation halos by visual inspection.

2.3. Bioremediation process by submerged fermentation

Submerged fermentations were performed in 250 mL Erlenmeyer flasks containing 100 mL of culture medium. The three media studied were 100% OMW without supplementation; OMW:nutrient supplementation medium (1:1, v/v) and OMW:VS (1:1, v/v). Flasks with media were sterilised during 15 min at 121 °C. Spores stored at 4 °C in slant tubes were suspended in a solution (0.01% peptone and 0.01% Tween 80) by strong agitation. Once spores were suspended, all media were inoculated with 2 mL of spores' suspension (10^7 spores/mL, counted with Neubauer chamber). The flasks were then incubated at 25 °C without agitation. Samples were collected during fermentation. All experiments were performed in duplicate.

2.4. Analytical methods

Decolourisation of media in submerged fermentations was determined by measuring absorbance at wavelengths of 395 and 525 nm. The biomass dry weight was determined at the end of fermentation after filtration using common laboratory filters (Whatman Nº 1) followed by drying at 100 °C. COD and total phenols were measured by the methods previously described. Statistical analyses were performed using Statistica 5.0 software.

Protease activity was quantified by a spectrophotometric method (Charney and Tomarelli, 1947), which is based on the reaction of 0.5 mL of SSF extracts with 0.5 mL of 0.5% (w/v) azocasein in acetate buffer 50 mM (pH 5) at 37 °C during 40 min. After incubation, 10% (w/v) trichloroacetic acid was added to precipitate residual protein not hydrolysed by the proteolytic enzymes. The sample was centrifuged (3000 g, 5 min) and a solution of potassium hydroxide 5 N was added to the supernatant, producing a pinky-orange colour. The intensity of this coloration was measured at a wavelength of 428 nm. The blank was performed under the same conditions, but adding trichloroacetic acid before incubation. One unit of proteolytic activity was defined as the amount of enzyme that produced an increase of 0.01 in absorbance relatively to the blank per minute, under the assay conditions.

Lipolytic activity was determined by spectrophotometric method, which was carried out using *p*-nitrophenyl-butyrate (*p*-NPB) in sodium acetate buffer 50 mM (pH 5.6) as substrate. The reaction mixture was incubated at 37 °C for 15 min and was stopped by adding acetone (Gomes et al., 2011). The absorbance was measured at a wavelength at 405 nm. One unit of activity was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol per minute, under the assay conditions.

Tannase activity was analysed by spectrophotometric method