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# Production of polyhydroxyalkanoates from dephenolised and fermented olive mill wastewaters by employing a pure culture of *Cupriavidus necator*

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ABSTRACT

The feasibility of producing polyhydroxyalkanoates (PHAs) by feeding a pure culture of *Cupriavidus necator* with a pre-treated olive mill wastewater (OMW) was demonstrated at 500 mL shaken flask scale. The OMW was previously dephenolised and then fermented to produce an effluent rich in volatile fatty acids (VFAs). The latter stream ($OMW_{Acid}$) was then employed as the carbon source for PHAs production.

Firstly, pre-grown cells were fed with different dilutions of $OMW_{Acid}$, namely: 25, 50, 75 and 100% (v/v). Significant inhibitory effects were observed when $OMW_{Acid}$ concentration was 75 and 100%. Thereafter, experiments with laboratory prepared solutions, simulating the $OMW_{Acid}$, allowed to demonstrate that polyphenols significantly contributed to the observed inhibition. Furthermore, The copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (poly(HB-co-HV)), containing 11% of hydroxyvalerate, was accumulated up to 55% of the cells dry weight when two consecutive accumulation batch processes were carried out with 25% of $OMW_{Acid}$ and without adding any exogenous carbon source.

The obtained results are promising in the perspective of continuing the production study at a bench-top bioreactor scale and thereafter analysing the possibility of developing a biotechnological PHAs production process as a part of an integrated OMW valorization process.



## 1. Introduction

Polyhydroxyalkanoates (PHAs) are well known aliphatic polyesters naturally produced by many microorganisms [1,2]. Different materials with new properties can be obtained by combining different PHAs monomers, including bioplastics with similar or even better physicochemical and mechanical properties than those exerted by their petrochemical-based homologues polyolefins. As an example, a polymer with a lower oxygen permeability, which can be exploited to enhance the material features of food packaging, can be obtained [3–6]. Besides, PHAs can be produced from renewable resources, therefore representing one of the most promising biopolymers for replacing the petrochemical-based plastomers, elastomers, latexes or even high-performance polymers [7].

Nevertheless, nowadays, PHAs industrial production is carried out from expensive carbon sources, resulting in a hardly economically competitive product with respect to that of petrol-based polymers. The production costs are mainly associated to those of carbon source procurement and downstream process (recovery and purification), representing both 30% (approximately) of the final product cost [8]. Thus, new alternatives and strategies are being studied in order to lower PHAs production costs. To this aim, the application of alternative inexpensive carbon sources, usually represented by agro-industrial wastes [9], was widely studied. Fried oil [10], effluents from the palm mill [11], molasses [12], cheese whey [13], olive mill wastewater (OMW) [14] and biodiesel waste glycerol [13,15], among others, were tested with pure or mixed cultures. Pure cultures (wild type or genetically modified) allow getting higher productivities and PHAs content. Conversely, the employment of mixed cultures has the economic advantage

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that they do not need to work under sterile conditions, since the microbial selection of PHAs producer strains can be carried out under selective pressure by repeated feast and famine processes [16].

On the other hand, most of the mentioned alternative substrates have moderate to low carbon source concentration (around 80 g $L^{-1}$ and 10–40 g $L^{-1}$, respectively), this leading to a low PHAs productivity potential [17], which hence negatively affects process costs. Pure cells allow high cell densities and PHAs contents, which conversely contribute moderating downstream costs. Furthermore, the employment of a single bacterial strain allows the obtainment of a well-defined single type of polymer, while a mixture of polymers would be obtained from microbial consortia.

The present work was dedicated to evaluate the possibility of producing PHAs by employing a pure culture and an inexpensive substrate. In particular, *Cupriavidus necator* (formerly *Ralstonia eutropha*) was chosen as the biocatalyst since it is a well-known and robust PHAs producer strain capable of high accumulation potential. OMW was selected as the alternative carbon source because it is an abundant biowaste mostly produced within the Mediterranean region. Particularly, the experimental wastewater was produced in Italy, where about 1 $Mm^3$ of OMWs are generated per year. OMW is a typical effluent of the olive oil industry, which applies the conventional three phases extraction procedure. Compared to the biowaste obtained from the two phase extraction one (mainly generated in Spain and conventionally called "alperujo"), which includes the solid fraction from processed olives, OMW is a much more homogeneous and liquid effluent. It is considered an environmental harmful waste because of its typical acidity (pH 3–6), high organic content (40–200 g COD $L^{-1}$), occurrence of polyphenolic compounds (1–20 g $L^{-1}$), and seasonality [18]. Polyphenols are known to exert antimicrobial activity; on the other hand, they are natural antioxidants, which could be exploited in several industrial fields [19]. All this considered, the development of integrated OMW valorization processes would allow combining its treatment to the obtainment of added value products (e.g., polyphenols, PHAs and biogas).

In this work, the experimental OMW was previously dephenolised, in order to couple the recovery of added value molecules to a detoxification of the effluent. Then, it was digested under acidogenic condition to obtain a volatile fatty acids (VFAs)-rich stream ($OMW_{Acid}$), which was used as the carbon source for PHAs accumulation. More in details, the main aims of the present work were: (a) to verify the possibility of producing PHAs, by using a pre-grown culture of *C. necator,* from $OMW_{Acid}$; (b) to determine what type of polymer can be produced from $OMW_{Acid}$; (c) to study the occurrence of inhibitory effects due to $OMW_{Acid}$ concentration and determine the potential inhibitors by employing laboratory prepared OMW simulating solutions; and (d) to verify the possibility of increasing PHAs content by applying consecutive accumulation batch processes, with the perspective of developing a cell-recycling strategy for achieving a high cell density. All experiments were carried out in 500 mL shaken flasks.

To the very best of our knowledge, this work represents the first attempt to produce PHAs within a pure culture of *C. necator* by employing digested OMW as alternative carbon source.

## 2. Materials and methods

### 2.1. Chemicals and olive mill wastewater

The standard volatile fatty acids (VFAs) mixture (Supelco), poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (Poly(HB-co-HV)) (12 mol% PHV; natural origin), salts (BioReagent) for the mineral medium, single VFAs and fructose (BioReagent) were purchased from Sigma–Aldrich. The OMW was kindly supplied by the "Sant'Agata d'Oneglia" Italian olive mill, which is located in the Liguria northern region, and it had a COD of about 55 g $L^{-1}$, partially due to polyphenolic compounds (about 2.55 g $L^{-1}$). It underwent a pre-treatment procedure, which consisted in a solid phase extraction (SPE), which was carried out by using a non-polar resin (Amberlite XAD16, Sigma–Aldrich) as the adsorbent and ethanol (96% grade, Sigma–Aldrich) as the desorption solvent [19]. The SPE procedure enabled an extended removal of the polyphenolic fraction; however, a significant amount of total polyphenols still occurred (Table 1). The dephenolised wastewater was processed under anaerobic acidogenic conditions in order to obtain an effluent rich in VFAs ($OMW_{Acid}$), which was employed as the carbon source for the biological accumulation of PHAs. The anaerobic acidogenic digestion of the OMW was carried out in a 2.5 L packed bed bioreactor, whose configuration was reported elsewhere [20]. In brief, it was an up-flow glass column, which was packed with ceramic material and was operating under continuous mode at 35 °C with a hydraulic retention time (HRT) of 7 days. The main features of the $OMW_{Acid}$ are shown in Table 1; it contained different short chain VFAs, mainly (g $L^{-1}$): acetic (7.22 ± 0.16), propionic (1.25 ± 0.05), butyric (1.75 ± 0.03), valeric (0.22 ± 0.04) and caproic (0.35 ± 0.01) acids.

**Table 1**
$OMW_{Acid}$ main features.

| | |
|---|---|
| COD (g COD $L^{-1}$) | 31.4 ± 0.5 |
| Total VFAs (g $L^{-1}$) | 11.43 ± 0.6 |
| Total polyphenols (g $L^{-1}$)[a] | 1.20 ± 0.20 |
| pH | 6.5 ± 0.1 |
| N-$NH_4$ (mg $L^{-1}$) | 60 ± 1 |
| Proteins (g $L^{-1}$) | 1.56 ± 0.12 |
| Lipids (g $L^{-1}$) | 3.24 ± 0.34 |

[a] Expressed as gallic acid equivalents.

### 2.2. Bacterial strain, inoculum and culture media

*C. necator* DSMZ 545 (DSMZ, Germany) was used as the PHA producer strain. Pre-culture was started from agar plates and pre-grown within 24 h in 500 mL Erlenmeyer flask containing 150 mL of Luria Bertani (LB) medium without any extra carbon source [21]; incubation conditions were 30 °C and 150 rpm. The experiments were performed according to a dual-phase strategy (as reported in Section 2.3). Therefore, the same medium was always employed in the first process step dedicated to cell growth (balanced growth phase), while different culture media were utilised in the second process steps dedicated to PHAs production (accumulation phase), depending on specific experimental aims. All these accumulation media exerted a $NH_4$ limitation, which did not allow cell growth (essential nutrient limitation). At the same time, this limitation triggered the biopolymer accumulation.

In particular, the growth medium consisted of a slightly modified E2 mineral medium [22] and it was employed for the cell growth phase. It contained 1.5 instead of 1.1 g $L^{-1}$ of $NH_4HPO_4$. Fructose (5 g $L^{-1}$) was added as the sole carbon source. Two types of ammonia free-media were employed for the subsequent PHAs accumulation phase: they included (a) the actual $OMW_{Acid}$, or (b) laboratory prepared solutions, which simulated $OMW_{Acid}$ by containing target chemicals occurring in the actual acidogenic effluent ($SimOMW_{Acid}$). The former media were prepared by filtering the $OMW_{Acid}$ with Whatman N11 (11 μm) filters, adding E2 salts (except for $NH_4HPO_4$) by respecting their concentration in the E2 medium, and autoclaving the amended $OMW_{Acid}$ using special Beckman flasks, which allowed to perform a subsequent centrifugation (8000 RPM, 4 °C and 25 min) under sterile conditions; finally, when necessary, it was diluted with a sterilised distilled water