PRODUCTION OF POLYHYDROXYALKANOATES
BY Cupriavidus necator FROM TREATED OLIVE MILL WASTEWATER

Extended abstract

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Background

Nowadays the development and production of new polymers is of great interest, either when looking for a new property or a more eco-compatible material. In this sense, polyhydroxyalkanoates (PHAs) are one of the newcomer polymers, since they can exert similar or even better physicochemical properties than those of petrochemical-based polymers.

However, it is not easy to achieve an economic feasible process. Generally, main costs associated to PHA production processes are those due to the carbon source for the fermentation process and to the downstream step (biopolymer separation and purification), which affect total PHA production costs for approximately 30% each other (Choi and Lee, 1999).

The exploitation of a largely available organic agro-waste such as olive mill wastewater (OMW) -instead of costly refined sugars- could be persecuted for PHAs production. OMW is a dark brown-greenish effluent, with high phenols content, which abounds within the Mediterranean region. The first step of a hypothetical bio-refinery of OMWs should be represented by the waste dephenolization, which would both decrease its antimicrobial activity and allow the recovery of added-value natural antioxidants. Thereafter, in the perspective of developing a biotechnological PHA production process, the dephenolized OMW should undergo to an anaerobic acidogenic digestion dedicated to the production of a volatile fatty acids (VFAs)-rich stream, since VFAs are suitable substrates for PHA producing microorganisms.

The production of PHAs from acidified OMW (OMW\textsubscript{Acid}) was already reported by using a mixed culture (Beccari, et al., 2009). However, low PHAs content were obtained, as typically happens when mix cultures are employed, this implying high separation and purification costs for an industrial scale production. At the same time, studies on PHAs production from different treated wastewater with pure cultures were reported (e.g., OMWs were employed with Azotobacter strains (Cerrone et al., 2010).

Objectives

The present work was dedicated to evaluate the feasibility of employing a pure culture of Cupriavidus necator in the development of a PHA production process fed with an OMW\textsubscript{Acid}. This microorganism was chosen since it can accumulate till 80% of PHAs with respect to the cell dry weight and also has the capacity to degrade phenols.

Specifically, two particular aims were defined: the use of OMW as solvent for preparing the growing culture medium and the use of OMW\textsubscript{Acid} as carbon source for the PHA accumulation phase. To the very best of our knowledge, this work represents the first attempt of singularly testing those strategies.

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Methods

Standards, salts and carbon sources

Fructose, acetic, propionic, butyric, valeric and caproic acids, salts for the E2 mineral medium and poly (3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (12wt.% PHV; natural origin) were purchased from Sigma Aldrich.

Two kinds of OMWs were employed: a dephenolized OMW (OMWDeph) and an OMWAcid, which was the product stream of an anaerobic acidogenic immobilized cells process developed in our laboratories to obtain a volatile fatty acids (VFAs)-rich effluent (Scoma, et al., 2011). The latter stream contained different short chain volatile fatty acids, namely (g/L): acetic (7), propionic (1.3), butyric (1.8), valeric (0.2) and caproic (0.3) acids. Cultural media were prepared by filtering OMWDeph and OMWAcid (Whatman N11, 11µm), dissolving the corresponded amount of E2 or E2 (ammonia free) salts into the filtered OMWDeph and OMWAcid, respectively, and sterilizing them in autoclave. Thereafter, centrifugation was performed (10,000 rpm and 4°C for 20 minutes) maintaining sterilization in order to obtain a clear sterilized solution.

Bacterial strain and inoculum

Cupriavidus necator (DSMS 545) inoculum was started from LB-Agar plates and grown within 24 hours in 500 mL Erlenmeyer flask containing 150 mL of LB medium; incubation conditions were 30ºC and 150RPM.

Analytical techniques

Sampling was performed periodically. The optical density (OD) measurement at 600nm, using a Cary-100 UV-Vis spectrophotometer, was implemented to detect cellular concentration variations. Cellular concentration, in terms of cell dry weight (CDW), can be determined by employing an OD vs. CDW calibration curve (data not shown). Thereafter, samples were centrifuged; the supernatant and pellet were separated and stocked for analyses.

Fructose was determined by HPLC-IR analysis, using a Varian Hi-Plex H column (300 x 7.7 mm); the mobile phase was sulfuric acid 5 mM at an elution rate of 0.6 mL/min and the operating temperature was 65°C.

VFAs and PHAs were determined by GC-FID analysis (Agilent 7890A). For the VFAs, it was employed a HP-INNOWAX column (ID 0.25 mm, length 30 m and film thickness 0.25 µm) and the method described in (Scoma, et al., 2011). The PHAs were determined according to the methanolysis method described by (Braungeg, et al., 1978) using a CP-Sil 5 CB column (ID 0.25 mm, length 30 m and film thickness 0.25 µm) with the temperature program described by (Bengtsson, et al., 2008). The validity of this method was checked with thermo gravimetric analysis (TGA).

Experimental approach

OMWDeph as solvent for C. necator growth

Balanced growth was carried out in different experimental E2 mineral media, which were prepared by using different relative proportions of OMWDeph and distilled water, in order to determine the maximal OMW volume quantity that can be used as solvent before the occurrence of inhibition effects. To this aim, five different conditions were tested depending on the percentage of OMWDeph used for preparing the culture media, namely: 0, 25, 50, 75 and 100%. The incubation conditions were the same previously described. Unique batch tests were started by inoculating 500 mL Erlenmeyer flasks containing 150 mL of E2 mineral medium prepared with the corresponded percentage amount of OMWDeph and 10g/l of fructose, so that the initial absorbance (at 600nm) was 0.150.

OMWAcid as carbon source for PHA accumulation

Balanced growth was carried out in about 24h under the same incubation conditions previously described, by using distilled water as the solvent and by adding 5 g/L of fructose as the carbon source, so that the initial absorbance (at 600nm) was 0.400. When the growth phase finished, the growth broth was centrifuged (6000 rpm for 6 minutes). The accumulation phase was started by re-suspending the obtained pellet in 150 mL of accumulation medium, which was composed by E2 (ammonia free) and OMWAcid (source of VFAs, which represented the PHA precursors). In order to determine the maximal amount of OMWAcid that could be used before detecting accumulation inhibition, four different conditions were tested depending on the OMWAcid content in the accumulation medium, namely (%v/v): 25, 50, 75 and 100. A control test was carried out but using a water solution representing the mixture of VFAs inside OMWAcid (SYN-OMWAcid). It contained only the corresponding VFAs and it allowed to determine whether eventually observed inhibition effects were due to VFAs themselves or the wastewater matrix. Each condition was tested in triplicate. The experiments lasted about 50 hours.

Results and discussion

The results of the growth inhibition test are shown in Fig. 1. When the culture media was prepared using only distilled water (0% OMWDeph) the maximal absorbance was 3.75 (A.U), with a specific growth rate of 0.219 h⁻¹. For 25% OMWDeph, a smaller specific growth rate of 0.128 h⁻¹ without any lag phase detection was calculated. The final OD for this condition was 3.66 (A.U.), which is a comparable value with respect to the former experimental control
condition. At the contrary, a strong inhibition was observed when culture media contained 50% or more of OMW. On the other hand, it was observed that after 80 hours the OD related to 50% OMW_{Deph} experiment started to increase at a similar exponential rate to the previous mentioned. A possible explanation of this evidence is represented by the fact that the employed strain requires a critical cell concentration, which can be estimated in an OD of 0.6 (A.U.), approximately, to rich the exponential growth in the presence of inhibitors. In particular, polyphenols can be responsible for the growth inhibition, since about 1 g/L of total phenols (TPs) were found in the employed dephenolized wastewater (Scoma et al., 2011). Therefore, about 0.25 g/L and 0.50 g/L of TPs occurred in 25% and 50% OMW_{Deph}, respectively. Interestingly, C. necator was found to growth without inhibition effects at least below 0.25 g/L of polyphenols.

Fig. 1. Growth trend: a strong inhibition was detected when the culture media contains 50% of OMW_{Deph}

As for the feasibility test of PHAs production from OMW_{Acid}, PHA accumulation was detected for 25% OMW_{Acid} immediately after the accumulation phase started (Fig. 2); 40% of PHAs content in a cell dry weight base was obtained at the end of the experiment. When using 50% OMW_{Acid}, a significant “accumulation lag phase” (constant optical density), which lasted 23 hours, was observed before accumulation started. Both conditions represent, either because of the scarce final PHAs content or dilatory accumulation rate, low productivity and thus a difficulty to obtain a feasible industrial biotechnological process.

Fig. 2. Biomass (triangles) and total PHAs content (squares) during the sequential batch experiment. This is for an accumulation culture media containing 25% of OMW_{Acid}

On the contrary, when using SYN-OMW_{Acid}, neither completely inhibition nor “accumulation lag phase” were detected; however, the same PHA content of about 40% of PHAs (on dry weight bases) was detected. Finally, VFAs were observed to contribute to the strain inhibition, since it was observed that the more SYN-OMW_{Acid} content was in the media the lowest was the accumulation rate. From this it can be inferred that something in the OMW_{Acid} matrix cause the observed inhibition.

Concluding remarks

The production of PHAs from treated OMW with C. necator is proposed as a strategy to achieve an economical feasible process.
Growth inhibition results suggested that 25% of the water required for preparing the growing culture media, can be saved by replacing it with OMW$_{Deph}$. Furthermore, if increasing the amount of inoculum it could be saved at least up to 50%.

As for the PHAs production from OMW$_{Acid}$, compromising results were obtained either because of the scarce final PHAs content (in the case 25% OMW$_{Acid}$) or dilatory accumulation rate (in the case 50% OMW$_{Acid}$).

All this considered, and still trying to obtain an economical feasible process, the implementation of three sequential batches is proposed: one dedicated to the cell growth (using 50% OMW$_{Deph}$) and two for PHAs accumulation (using 25% of OMW$_{Acid}$). In this way, the resulting increase in PHAs content would allow avoiding high separation and purification costs that compromise the economic feasibility.

**Keywords:** *Cupriavidus necator*, Olive mil wastewater, Polyhydroxyalkanoates, Pure culture, *Ralstonia eutropha*

**References**


