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Biological Treatment of Two-Phase Olive Mill Wastewater (TPOMW, alpeorujo): Polyhydroxyalkanoates (PHAs) Production by *Azotobacter* Strains

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***Azotobacter chroococcum* H23 (CECT 4435), *Azotobacter vinelandii* UWD, and *Azotobacter vinelandii* (ATCC 12837), members of the family *Pseudomonadaceae*, were used to evaluate their capacity to grow and accumulate polyhydroxyalkanoates (PHAs) using two-phase olive mill wastewater (TPOMW, alpeorujo) diluted at different concentrations as the sole carbon source. The PHAs amounts (g/l) increased clearly when the TPOMW samples were previously digested under anaerobic conditions. The MNR analysis demonstrated that the bacterial strains formed only homopolymers containing β-hydroxybutyrate, either when grown in diluted TPOMW medium or diluted anaerobically digested TPOMW medium. COD values of the diluted anaerobically digested waste were measured before and after the aerobic PHA-storing phase, and a clear reduction (72%) was recorded after 72 h of incubation. The results obtained in this study suggest the perspectives for using these bacterial strains to produce PHAs from TPOMW, and in parallel, contribute efficiently to the bioremediation of this waste. This fact seems essential if bioplastics are to become competitive products.**

Keywords: Two-phase olive mill wastewater (TPOMW), polyhydroxyalkanoates (PHAs), *Azotobacter* spp., chemical oxygen demand (COD)

Polyhydroxyalkanoates (PHAs) are polyesters synthesized by several microorganisms under limiting nutrient conditions in the presence of an excess of carbon source being accumulated as storage material inside microbial cells [8, 32, 36]. They offer similar characteristics to conventional plastics and they can be degraded at high rates to carbon dioxide and water by numerous bacterial strains, making PHAs attractive substitutes to petrochemical thermoplastics [6, 30].

The PHAs-producing microorganisms are included in two categories, depending on the culture requirements for the production of PHAs. The first group requires a limitation in some of the nutrients, such as N, P, Mg, K, or S, for an effective accumulation of bioplastic, being the carbon source in excess. The second group does not require any limitation for the PHA synthesis, being capable of accumulating this material while it grows. In this group belongs, for example, the members of *Azotobacter* genus, aerobic nitrogen-fixing bacteria [26, 28].

Since the first report on PHA biodegradability, numerous studies have focused both on the isolation of new microorganisms with PHA synthesis capacities and the use of substrates cheaper than glucose as a source for PHA production. In this sense, substrates such molasses, whey, hemicellulose, and palm oil have been used successfully for this purpose [2, 32], but the polymer concentration and content have always been lower than those obtained with purified carbon sources. The use of wastes or subproducts for PHAs production has not been investigated in depth, and only a few studies have focused on the use of organic wastes from agriculture or sludge from treatment wastewater plants for PHAs synthesis [12, 15, 31, 37, 41]. From an economical point of view, the cost of substrate for PHAs production (mainly glucose, which contributes most significantly to the overall production cost of PHAs) can be decreased if a waste product is used [9]. This strategy could make PHAs production more economical, which at the same time treating wastes without extra disposal cost.

The olive oil industry is very important in the Mediterranean basin and is one of the agro-industries that generate more amounts of liquid, solid, and semisolid wastes with high environmental pollution risk. Two-phase olive mill waste (TPOMW, alpeorujo) is a semisolid waste generated in the

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olive oil mill industry by the two-phase extraction system that was implemented in Spain at the beginning of the 90's decade to replace the traditional three-phase extraction system. This semisolid waste is then dried and further treated with a second centrifugation to extract the residual oil. About 0.8 ton of solid waste is generated per ton of processed olives by this extraction system. TPOMW is characterized by a low pH value (4–6) and high phytotoxicity, which is mainly due to its content in polyphenols, lipids, salt, and organic matter [18].

The olive mill wastes generated in the olive oil production process display suitable characteristics to be considered as substrates in the bacterial synthesis of biopolymers: a high concentration of organic matter and elevated hydrosoluble carbohydrate content [12]. Moreover, these substances can be utilized by the same PHA-producing bacteria for acidogenic metabolism, generating volatile fatty acids (VFAs) considered as precursors for copolymer synthesis [4]. However the antimicrobial character that exhibits this by-product restricts its use as a microbial culture medium at high concentrations.

In parallel, the treatment of TPOMW has been the aim of numerous investigations: physicochemical treatments [3], direct soil application [38], or biotechnological transformations like composting [7] or anaerobic digestion [5], but an adequate and environmentally friendly solution of their disposal problem has not been found yet.

To date, the investigations on the bacterial production of PHAs from olive mill by-products such as olive oil mill wastewater (OOMWW, alpechín) are few [11, 12, 31], and there are none so far, according to our knowledge, on the PHAs bacterial production using TPOMW as the sole carbon and energy source.

In this paper, we describe the growth and production of PHAs by microorganisms belonging to the *Pseudomonadaceae* family in both fresh and digested anaerobically TPOMW samples, diluted at different concentrations as the sole carbon and energy source, as well as the capacity of these microorganisms to reduce the organic matter content of this waste in parallel to PHAs production.

Materials and Methods

Microorganisms

The bacterial strains *Azotobacter chroococcum* H23 (Spanish Type Culture Collection, CECT 4435, originally isolated from the rhizosphere of *Zea mays* [22]), *Azotobacter vinelandii* UWD [25], and *Azotobacter vinelandii* ATCC 12837 were used in this study to determinate their PHAs storing capacity in different growth media. The microorganisms were maintained in Burk's N-free medium slants [40]. The composition of Burk's N-free liquid medium (1 l) was the following: K_2HPO_4, 6.4 g; KH_2PO_4, 1.6 g; NaCl, 2.0 g; $MgSO_4 \cdot 7H_2O$, 2.0 g; $CaSO_4 \cdot 2H_2O$, 0.5 g; $NaMoO_4 \cdot 2H_2O$, 0.01 g; ferric citrate, 0.02 g; glucose, 10 g; distilled water, 1 l. The pH was adjusted to 7.2 using 0.1 N NaOH. Burk's solid medium was formulated by adding 16.0 g/l of agar-agar (Difco).

Two-Phase Olive Mill Wastewater Samples

TPOMW (alpeorujo) samples were supplied by "Olivarera Los Desamparados", S.C.A. (Puente-Genil, Córdoba, Spain). The samples were maintained under refrigeration (4°C) until use (48 h maximum). The characterization of the waste was the following: pH 4.5; chemical oxygen demand (COD) 175 g/l; biological oxygen demand at five days (BOD_5) 8 g/l; N total (%) 15.0; total phenolic content (%) 2.5; total reduced sugars (as glucose) 9.1 (%).

TPOMW Culture Media

In this study, we used two different TPOMW culture media: TPOMW medium and anaerobiacally digested TPOMW medium.

The TPOMW medium was prepared at different concentrations [20%, 40%, and 60% (v/v)] of fresh TPOMW in autoclaved distilled water (121°C for 1 h). In order to remove solid particles, the TPOMW medium was centrifuged (10,000 rpm for 15 min) and sterilized by filtration (Millipore, 0.45 μm) prior to inoculation with the *Azotobacter* strains. The sterilized pH medium was adjusted to pH 7.2 with pure NH_3. NH_3 was used for a double purpose: to give a right pH value and to give a nitrogen source to the bacterial culture.

The anaerobically digested TPOMW medium was prepared at different concentrations [20%, 40%, and 60% (v/v)] of fresh TPOMW, as previously described, but digested under anaerobic conditions in a bioreactor designed and constructed at laboratory scale. After the anaerobic phase, the digested samples were centrifuged (10,000 rpm for 15 min) and the supernatants were recovered and sterilized by filtration (Millipore, 0.45 μm). The pH value of the digested sterilized samples was adjusted to pH 7.2 with pure NH_3.

Anaerobic Bioreactor at Laboratory Scale

Fig. 1 shows the diagram of the bioreactor constructed at laboratory scale. Erlenmeyer flasks (1 l of total volume), sealed with silicon stoppers and connected to a glass tube (U shape) filled with water to guarantee the anaerobic conditions, were incubated with the diluted TPOMW samples [20%, 40%, and 60%, (v/v)] during 14, 24, 27, 30, and 36 h at 35°C. Previously, helium gas was bubbled for 15 min to prevent the presence of oxygen in the system.

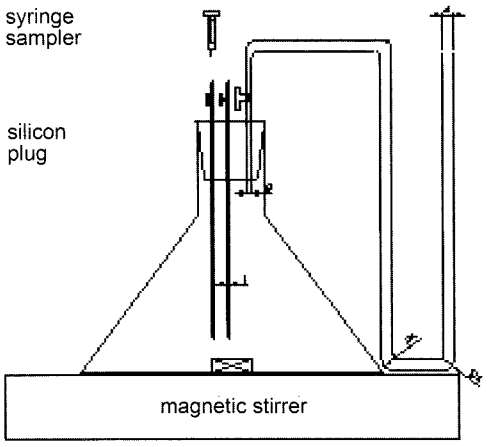

Fig. 1. Anaerobic bioreactor scheme.

Volatic Fatty Acids (VFAs) Determination

The VFAs produced in the anaerobic bioreactor at 14, 24, 27, 30, and 36 h of incubation were determined by gas chromatography–mass spectrometry (GC–MS) using a GC–MS Agilent 6890 Series GS System gas chromatograph and 5973 mass selective single quadrupole detector (Agilent technologies), with previous extraction with a modification of the SPE (solid phase extraction) method for extraction of organic acids from wine-BIOTAGE IST 0400C utilizing the ENV+ ISOLUTE SPE column; ENV+ ISOLUTE cartridges were chosen because of no selective interaction with the solute, and for their suitability for solute of both ionic charges, as previously suggested [10, 21] for use in complexed organic substrates.

PHAs Extraction and Chemical Characterization

PHAs extraction. The PHAs produced in each experiment was extracted as previously described [17]. The lyophilized biomass was dissolved with pure chloroform and heated at boiling point. Then, the samples were sonicated for 10 min and centrifuged (20,000 rpm) for 15 min to separate the organic fraction (where the PHAs were contained) from the pellet; the supernatant was taken and cooled at room temperature in glass Petri dishes. Then, the PHAs were collected, weighed, and stored for further analyses.

Magnetic nuclear resonance analysis. Nuclear magnetic resonance (NMR) technique was utilized to identify the polymers synthesized by the strains; the utility of this technique for the identification of the specific biopolymer has been demonstrated previously [29, 35]. For this, a Varian Bruker AM-300 (Inova model) spectrometer in the pulse-Fourier transform mode, at a frequency of 250 MHz, using glass tubes with CD_3Cl solutions was employed, with a pulse width of 4.95 μS and a spectral width of 4,803.1 Hz, being the number of accumulations depending on the quality of the sample and was comprehended between 16 and 64. Chromatograms of pure commercial products (homo- and heteropolymers) supplied by Sigma–Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) were obtained and used to compare with the sample chromatograms.

Results and Discussion

Growth and PHAs Production by *Azotobacter* Strains in Burk's Glucose Medium

The growth of the bacterial strains in liquid Burk's glucose medium during 72 h of incubation under controlled temperature (28°C) and agitation (100 rpm) was measured. At each incubation time, an aliquot of the growth medium was used for optical density (OD) measurements (λ=640 nm) and the rest of the medium was centrifuged (10,000 rpm, 15 min) and the pellet picked up. There after, the biomass was frozen (−80°C) and lyophilized for PHA extraction following the methodology previously described.

Table 1 shows both the biopolymer amounts (g/l) extracted and the biomass (g/l) produced by the *Azotobacter* strains both in Burk's N-free medium and Burk medium amended with NH_4Cl (0.12%) after 72 h of incubation. As is shown, the biopolymer amounts were very small (only 0.5 g/l after 72 h of incubation) when the strains were grown in Burk's N-free medium.

Azotobacter strains are free-living nitrogen fixers that use a considerable amount of energy in two correlated processes: the biosynthesis of alginate and the dinitrogen fixation, a high endothermic reaction. Moreover, the synthesis of bioplastics (PHAs) is a process that requires energy, and consequently, under diazotrophic conditions, the PHAs accumulation will only be possible when a deviation of part of the energy used in the atmospheric N_2 fixation process occurrs [24, 41].

Hence, when the strains were grown in Burk's medium amended with NH_4Cl (0.12%), under such adiazotrophic conditions, the biomass and the amounts of PHAs produced at each incubation time increased markedly. These effects were detected when the microorganism used was *Azotobacter vinelandii* UWD, which showed significant increases of the PHA yield (5-fold), so the addition of a nitrogen source had a helpful effect on the PHAs production. Similar results have been previously reported by other authors [16, 31].

Nuclear Magnetic Resonance (NMR) Analysis

The Fig. 2A shows the NMR spectrum of the bioplastic (PHAs) obtained from *Azotobacter vinelandii* UWD grown in chemically defined medium containing 0.5% glucose as carbon source. The spectrum was exactly the same as the NMR spectrum of the pure commercial product supplied by Sigma–Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) (Fig. 2B). Under our experimental conditions, no heteropolymers (poly [HB-co-HV]) were synthesized, independently of the *Azotobacter* strains assayed and the presence or absence of nitrogen source in the growth medium.

Table 1. Biomass yield (g/l) and PHAs production (g/l) by *A. vinelandii* UWD, *A. chroococcum* H23, and *A. vinelandii* ATCC 12387 grown in Burk's medium amended or unamended with 0.12% NH_4Cl after 72 h of incubation.

	Burk's medium without N source		Burk's medium with N source	
Strain	Biomass (g/l)	PHAs (g/l)	Biomass (g/l)	PHAs (g/l)
A. *vinelandii* UWD	0.8	0.455	3.4	2.3
A. *chroococcum* H23	2.16	0.56	3.68	1.518
A. *vinelandii* ATCC 12387	1.25	0.20	3.2	1.043

Values are the mean of three experiments.

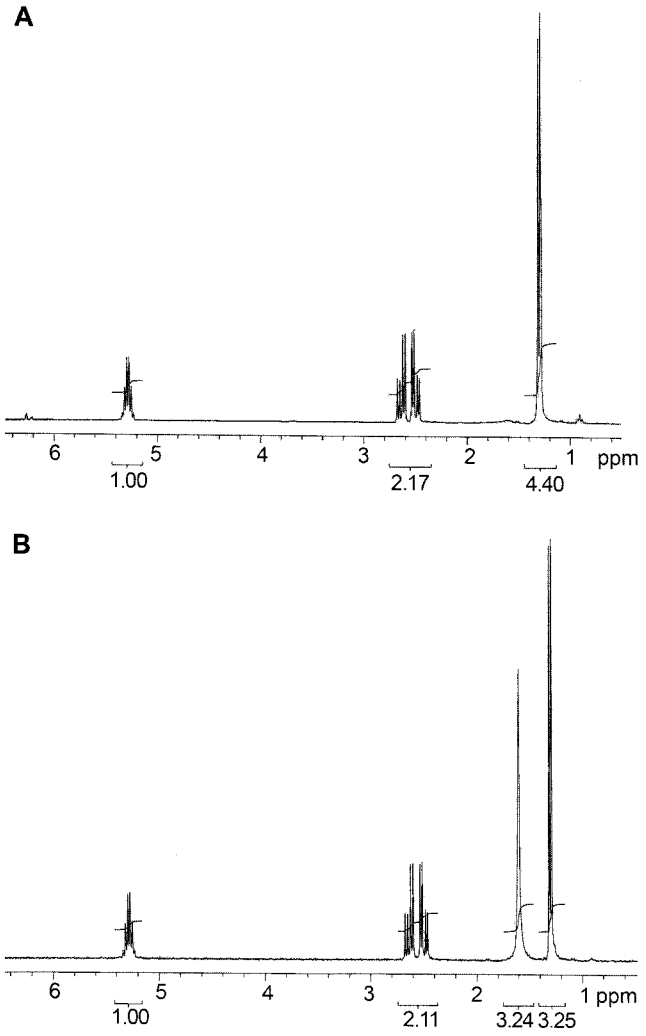

Fig. 2. NMR chromatograms of PHB produced by *A. chroococcum* H23 in anaerobically digested TPOMW (40%) after 72 h of incubation (**A**) and of pure commercial PHB supplied by Sigma (**B**).

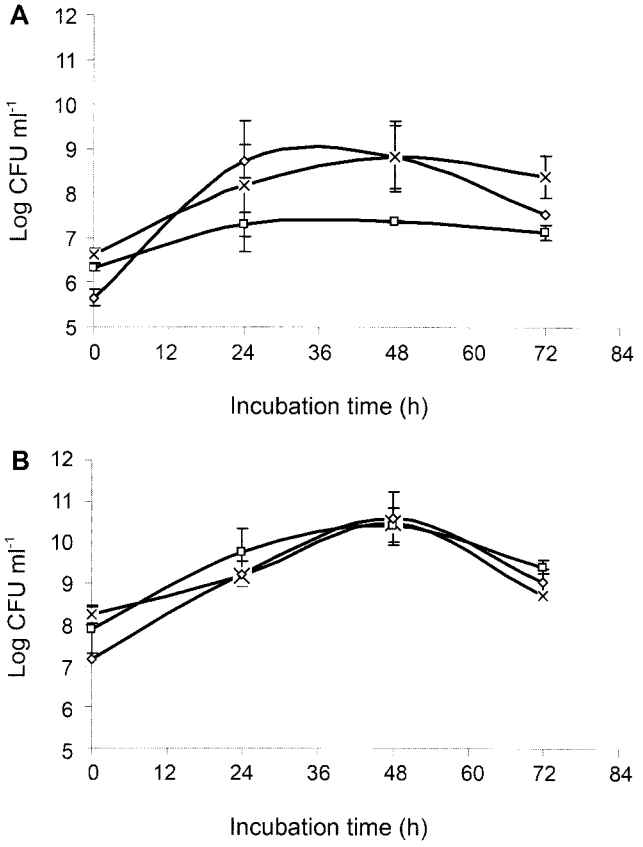

Fig. 3. Bacterial growth (as log CFU/ml) of (**A**) *A. chroococcum* H23 and (**B**) *A. vinelandii* UWD on TPOMW media at 20% (◇), 40% (□), and 60% (×).
Values are means of three experiments±SD.

Growth and PHAs Production by *Azotobacter* Strains in TPOMW Media

Once we had evaluated the capacity of the different *Azotobacter* strains to produce PHAs in chemically defined media (Burk's media with and without addition of nitrogen source) as well as the homopolymer nature of the bioplastic synthesized, we evaluated the possibility of the use of TPOMW as substrate for bacterial growth and PHAs production. Numerous studies [14, 16, 20, 23, 31] have reported the antimicrobial effect of the polyphenol compounds present in the olive oil mill wastewaters. For this, diluted centrifuged and sterilized TPOMW media, prepared as described above, were inoculated with cell suspensions (10^6 cells/ml) pregrown in the same liquid medium and maintained at 28°C for 24 h under continuous agitation. The inoculated TPOMW media were incubated under agitation (150 rpm) and controlled temperature (28°C) for 72 h. After 24, 48, and 72 h, aliquots (0.1 ml) were spread on Burk's N-free solid media and incubated for 3 days at 28°C. Colony-forming units were countered and expressed as log CFU/ml. For PHA determination, the remaining growth media were centrifuged and the pellets frozen (−80°C) for lyophilization. The extraction of PHA from lyophilized biomass was achieved as described in the Material and Methods section.

A. vinelandii UWD and *A. chroococcum* H23 were able to grow in culture media containing TPOMW at different concentrations [20%, 40%, and 60% (v/v)] as carbon and energy sources (Fig. 3A and 3B, respectively). Consequently, it could be suggested that *Azotobacter* exhibits abundent growth without any toxic effect by the phenolic compounds present at the waste concentrations used in our study. However, when the PHAs content of the *Azotobacter* cells was studied, the data showed that under these culture conditions the accumulation of PHAs was low (average 0.12 g/l after 72 h incubation; data no shown) compared with the PHAs accumulated in cells grown in chemically defined media (Table 1).

Growth and PHAs Production by *Azotobacter* Strains in Anaerobically Digested TPOMW Media

Several authors [12, 13, 33] have focused on the study of new processes for the production of PHAs from wastes, by the combination of anaerobic and aerobic steps. In this study, we evaluated the feasibility of PHAs production using anaerobic and aerobic steps. Thus, diluted [20%, 40%, and 60% (v/v)] TPOMW samples were anaerobically digested in a bioreactor (see Materials and Methods section). After the incubation time (27 h), the waste was used for the PHAs production by *Azotobacter* strains under aerobic conditions.

The anaerobic biotransformation of this waste products is a complex process that begins with the hydrolysis of high molecular weight compounds (hydrolytic phase) to generate other compounds more easy and more susceptible, hypothetically, to biodegradation. After the hydrolytic phase, short-chain VFAs such as acetic, propionic, butyric, or valeric acids are produced (acidogenic phase). These VFAs are considered as precursors for the synthesis of hydroxybutyric-co-hydroxyvaleric acid (HB-co-HV), heteropolymers with special thermoplastic characteristics [26, 27].

The objective of the anaerobic step was to subject the TPOMW samples to hydrolytic and acidogenic phases, in which organic compounds are transformed, and following fermented to VFAs comprising mainly from acetic, propionic, and butyric acids. Some authors [11, 42] demonstrated in their investigations how the number of carbon atoms of VFAs released could affect the composition of the biopolymer produced in the following aerobic step. Hence, VFAs containing an even number of carbon atoms (acetic and butyric acids) induce the formation of hydroxybutyric monomers, whereas VFAs with an odd number of carbon atoms (propionic or valeric acids) induce the formation of hydroxyvaleric monomers, and consequently the copolymer poly(HB-co-HV) formation. In our study, acetic, butyric, and valeric acids were only detected in the TPOMW samples under anaerobic conditions after 27 h of incubation, contrary to the results obtained under other different retention times (14, 24, 30, and 36 h), where no or more reduced amounts of VFAs were detected. Therefore, this incubation time was sufficient for VFAs production, and preventing their transformation to methane by the methanogenic bacteria.

To test the viability of the digested TPOMW samples to hold the bacterial growth and PHAs storing, the total reduced hydrosoluble carbohydrate content (as glucose) was determined at different times. As Fig. 4 demonstrates, the total reduced carbohydrate amount after 27 h of incubation under anaerobic conditions was equal to that presented in N-free Burk's medium (10 g/l), and consequently, the sugar content was sufficient to support the bacterial growth in the aerobic PHAs storing phase.

The digested, centrifuged, and sterilized TPOMW samples (pH value adjusted at 7.2 with pure NH_3) were inoculated with the bacterial strains, as described previously. The inoculated samples were incubated under aerobic conditions and controlled temperature (28°C) for 72 h. At 24 h, 48 h, and 72 h, the samples were centrifuged and the pellets frozen (−80°C) for lyophilization.

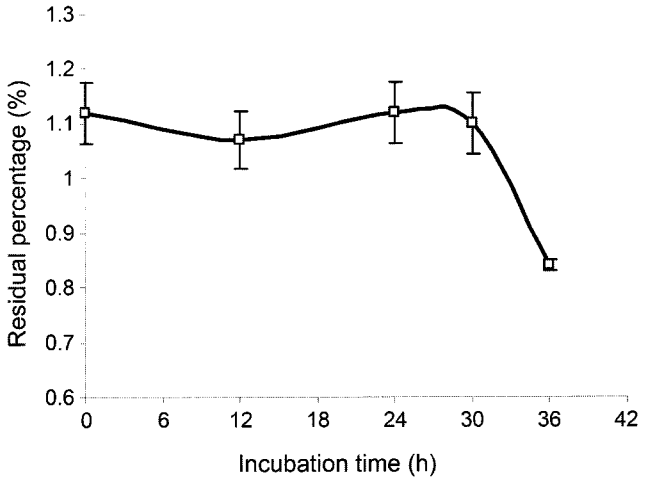

Fig. 4. Evolution of the residual content (%) of hydrosoluble reduced carbohydrates (as glucose) in diluted (60%) TPOMW samples under anaerobic digestion.
The values are means of three experiments±SD.

Table 2 shows the lyophilized biomass and PHA yield (as g/l) from the *Azotobacter* strains after 72 h of incubation in anaerobically digested TPOMW media amended with Burk's salts. The maximum bioplastic production (1.063 g/l) was reached when the bacterial strain *A. vinelandii* UWD grew on anaerobically digested TPOMW diluted (60%) media, under aerobic conditions. In the spite of that, the best result of PHAs production was with the bacterial strain *A. vinelandii* UWD, and a higher percentage (48%) of accumulated PHAs was reached by *A. chroococcum* H23, showing a promising storing capacity.

The results from NMR analysis demonstrated that *A. chroococcum* H23 and *Azotobacter vinelandii* UWD formed only homopolymers (poly-β-hydroxybutyrate, PHB) when they grew in anaerobically digested TPOMW media. A previous study [31] demonstrated that *A. chroococcum* H23

Table 2. Biomass yield and PHAs production by *Azotobacter* strains grown in diluted (40% and 60%) anaerobically digested TPOMW media after 72 h of incubation under aerobic conditions.

	40% (v/v)		60% (v/v)	
Strain	Biomass (g/l)	PHAs (g/l)	Biomass (g/l)	PHAs (g/l)
A. vinelandii UWD	2.23	0.79	3.22	1.06
A. chroococcum H23	2.39	0.74	1.16	0.55
A. vinelandii ATCC 12387	1.3	0.09	0.87	0.09

Values are the mean of three experiments.

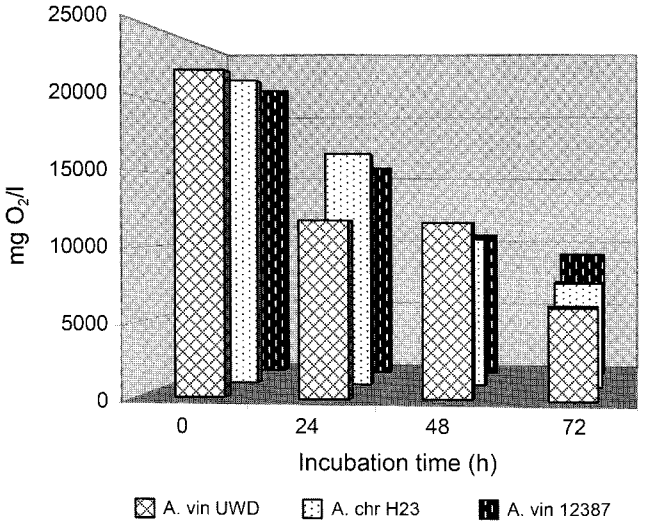

Fig. 5. Evolution of COD (mg O_2/l) values into diluted (60%) anaerobically digested TPOMW media after aerobic growth of *Azotobacter* strains.
The values are means of three experiments.

produced copolymers (β-hydroxybutyrate-co-hydroxyvalerate, HB-co-HV) when grown in diluted (60%) olive oil mill wastewater (alpechín) media supplemented with valerate as the copolymer precursor (100 mM). However, the PHA synthesized by *Azotobacter* strains in this study always was PHB, even in the presence of VFAs; in fact, it was demonstrated [1] that when a carbohydrate as source of C is metabolized, with acetylCoA being the main intermediate, the pathway leads to the production of PHB with the employment of three sequential enzymes; it was the case of our substrate, where carbohydrates were in higher quantity (around 10 g/l) than volatile fatty acids (30 mg/l, as total VFAs), and so were metabolized first.

Chemical Oxygen Demand Reduction

Fig. 5 shows the evolution of COD values (as mg O_2/l) of anaerobically digested TPOMW diluted (60%) samples inoculated with the *Azotobacter* strains after 24 h, 48 h, and 72 h of incubation under aerobic conditions. After 72 h of incubation, the COD reduction was obvious, with the samples inoculated with *Azotobacter vinelandii* UWD being those that showed the lowest COD value: 6,130 mg O_2/l.

The COD evolution pattern was different between the bacterial strains; *A. chroococcum* and *A. vinelandii* showed the lowest COD reduction after 24 h of incubation (25%) whereas the COD value in media inoculated with *A. vinelandii* UWD decreased about 46% (11,792 mg O_2/l) at the same time. However, this COD value was maintained until 72 h of incubation, at which that the COD value dropped markedly.

Many biological treatments have been applied to reduce the COD value of several agriculture wastes. These treatments [19, 34] are based on cultivation of microorganisms, but those involve long fermentation cycles as well as very diluted samples to minimize the waste toxicity.

In our study, although we have used diluted TPOMW samples (60% TPOMW), the COD reductions in a short period of time (72 h) have been clear, corresponding to the lowest COD value to the maximum PHAs bacterial synthesis, suggesting that a biological treatment of this waste could be combined with production of a useful product: PHB.

The experimentation at laboratory scale has provided data that conclude that *Azotobacter* sp. strains (in particular, *Azotobacter chroococcum* H23 CECT4435 and *Azotobacter vinelandii* UWD) are bacterial strains with a good PHAs production capacity, as much in a chemically defined medium as in anaerobically digested TPOMW diluted media. The anaerobically digested TPOMW diluted medium was shown as an adequate bacterial growth medium, with enough C content (as reduced hydrosoluble sugars) to support growth and later PHA storing phase.

The data on PHAs production using TPOMW diluted media as a carbon and energy source showed that the yield increased markedly when the TPOMW diluted samples were subjected to a previous anaerobic digestion phase.

Finally, the COD reduction (72%) observed in the incubated anaerobically digested TPOMW samples used as substrate for microbial PHAs production demonstrates that *Azotobacter* strains exhibit a significant bioremediation potential. Hence, the bioplastic production from a waste like TPOMW could be linked to its treatment and it could stand for its implementation as a suitable method in a production plant at pilot scale.

Acknowledgments

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