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Review

Wastewater valorisation of olive oil production: A biotechnological approach for biopolymer production

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ABSTRACT

A global increase in the demand for olive oil has led to a current increase in the levels of by-products released from its production. The limited presence of these by-products over a short time period of the year and their large volumes, especially in Mediterranean countries, causes a challenge in terms of treating such substrates. Their eco-toxicological potential lies in their phytotoxicity and high organic charge, due in particular to the presence of polyphenols. Land disposal of such substrates is strictly limited or directly not permitted by law. Furthermore, the different composition of the substrates coming from the different systems of olive oil extraction makes it difficult to treat such substrates in an eco-friendly and definitive way. This review examines, in particular, the biotechnologically driven treatment of these by-products; especially in the production of biopolymers from the by-products released in the olive oil extraction process. These biopolymers could be either exopolysaccharides or polyhydroxyalkanoates. Moreover, such biopolymers can be produced by both the system of two-phase and three-phase olive oil production. In order to give a potential valorisation to these kinds of wastes in a biotechnological way, one of the most promising applications is in the production of bioplastics. This environment-friendly bioprocess, based on fermentation technology, could be the most suitable approach for competitive optimisation and produce an alternative to the thermoplastic polymers derived from crude oil.

KEYWORDS: olive mill wastewaters, polyhydroxyalkanoates, fermentation technology, bioplastics, waste valorisation

1. INTRODUCTION

An increasing global demand for olive oil is a common issue in many countries. It originally started in the Mediterranean countries, but has spread across medium-latitude nations. This demand is due to olive oil being an essential component of a healthy diet. By-product volumes have increased in parallel with the increased production of olive oil [1]. There are two methods of obtaining olive oil: two-phase and three-phase extraction. Both use a centrifugation system and are essentially variations of the traditional method based on “press olive cake”. The two-phase system uses a dry centrifugation in the first phase, followed by the addition of water to separate the olive oil via liquid-to-liquid separation. The three-phase system involves the addition of water twice for the first centrifugation and for the liquid-to-liquid separation that leads to the extraction of olive oil. Depending on the system used, different by-products are generated. The two-phase system can result in the production of large amounts of a semi-solid wet waste, the two-phase olive mill wastewater (TPOMW or “alpeorujo”). The three-phase system yields olive oil mill wastewater (OOMW) that is essentially liquid wastewater. In spite of sharing the same origin, these by-products

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are very different in composition and aspect. The TPOMW has a predominantly high organic load, giving a high chemical oxygen demand (value of 150 g/L of O_2 consumed), a notable polyphenol content (up to 25g/L), a high hydrosoluble sugar content (up to 19%) and high values of NPK nutrients (especially of N that can reach values of about 180 g/L). The OOMW has a lower organic content (COD 43 g/L), but a higher polyphenol content (up to 350 g/L) and a lower hydrosoluble sugar content, with respect to TPOMW (only 0.5%). These by-products could represent a hazard, especially for the environment and in particular for the watershed and land fertility [2, 3, 4]. The main reason for their potential to be harmful is the fact that the generation of such by-products is limited to a period of at least two months a year (harvesting season: November to December). The need to find an environment-friendly treatment of such waste is therefore an important issue. One of the most promising ways of treating olive mill wastewater is to convert them into a (bio) resource. This achieves two objectives: an effective treatment of waste and the biotechnological production of an added value. The application of biotechnology to treat wastewater is not new and offers many kinds of possible bioproducts. They range from lipase [5], pharmaceutical compounds [6], food additives [7], biogas production [8] and, as discussed in greater detail in this review, biopolymers [9, 10, 11]. This review will attempt to show the state-of-the-art approaches for the biotechnological valorisation of olive mill wastewater, primarily for the production of biopolymers, with different kinds of olive mill wastewaters.

2. Biopolymers

The definition of biopolymers states that they are polymers produced by living organisms linked by covalent bonds. There is a strict regulation for identifying which polymers should be considered biopolymers and biodegradable. This regulation is stated by the European Committee for Standardization and is named EN 13432. Under this law, the biopolymers should fulfil the following requirements:

- be degradable to at least 90% in nine months if in a saturated carbon dioxide environment (such values need to be tested with an EN 14046 standard method)
- when in contact with organic materials, for three months, the whole mass of the material should be composed of 90% of not more than 2-mm fragments (to be tested with an EN 14045 standard method)
- the material must NOT have negative effects on the composting process.
- an agronomic assay of the resulting compost should not present any ecotoxicological effect on target plants
- low concentrations of additional heavy metals in the material
- pH values within established limits
- salt content within established limits
- VSS (Volatile Suspended Solids) within established limits
- nitrogen, phosphorus, magnesium and potassium levels within established limits

The biopolymers can be of different origin and composition. They can all have the same nature, i.e., repetition of equal monomers in chains of varied length, or they could have a mixed nature, i.e., with repetition and alternation of monomers of different composition, more or less varied among them. The first case refers to homopolymers and the latter to heteropolymers. Furthermore, they can also be composed of molecules of different composition, with a covalent bond linking the different parts, e.g., a lipopolysaccharide or a lipoprotein. These are biopolymers that living organisms regularly produce for their metabolism. Many of them could be interesting because of their biotechnological potential.

2.1. Main biopolymers with plastic properties

Different biopolymers with plastic properties share their biodegradable nature, even if they are of different chemical origin or are produced from different substrates. The main substrates that are employed for the synthesis of biobased materials are starch, cellulose (of wood origin) and different organic substrates that are easy to metabolise microbially. When we talk about microbially-

produced biopolymers with plastic properties, we refer to a polyesteric and aliphatic biopolymer, called polyhydroxyalkanoate. This type of biopolymer is detailed below and typically comes from the bacterial metabolism of simple carbon sources like glucose or more complex substrates with variable compositions (like organic wastewaters).

The main biopolymer (with plastic properties) currently on the market is only polylactic acid (PLA), formed by the condensation of lactic acid. PLA is usually produced from starch obtained from crops like corn. Many industrial branches share similar technologies to generate products of such a biopolymer with different degrees of mixing with thermoplastic polymers. Other well-known biopolymers are: anhydride biopolymers, polybutylene succinate (PBS), cellulose esters (e.g., cellulose acetate) and polylactone (e.g., polycaprolactone). The polyanhydrides contain a repetition of acyl groups that bond the same oxygen atom (the ester bond). They have a variety of applications and are especially used for the synthesis of other organic polymers. One of the most well-known biopolymers produced using polyanhydride is cellulose acetate. The substrate of such a biopolymer is evident from the name cellulose, which, when condensed with anhydride, yields cellulose acetate. It is a renewable resource-based biopolymer because it comes from wood material. It is therefore highly interesting in the petrol oil world. Polycaprolactone comes from the condensation of the lactone ring; it needs specific catalysts and is the base for producing special polyurethanes. One of the emerging biopolymers is polybutylene succinate. This biopolymer is composed of a repetition of the ester of the succinic acid monomer. Interest in it is due to the fact that succinic acid is easily obtained from biomass. One of the disadvantages of this polymer is that its chemical backbone (butylene), even though it is completely biodegradable, is derived from petrochemicals. Apart from the different chemical compositions, all the biopolymers share a common crop origin that makes them different from polyhydroxyalkanoates, which are derived from microbial metabolism and can be produced by biotechnological fermentation without the need of agricultural biomass.

2.2. Polyhydroxyalkanoates (PHAs)

Polyhydroxyalkanoates are one of the most interesting biopolymers and are produced by many microorganisms (in particular bacteria). Their uniqueness resides in the fact that they share many features with thermoplastic polymers (obtained from petrol oil) (Table 1) and can be regarded as a potential substitute of plastics, especially when plastics need to be rapidly biodegradable. Two aspects contribute to the increasing interest in such biopolymers. First of all, it is easy to obtain different compositions of this biopolymer by varying the substrate used. This reflects a metabolic response to changes in environmental conditions. Second, the ease of cultivating bacteria in a fermentative way can facilitate the production of biopolymers on a larger scale.

The chemical structure of such a biopolymer is depicted in Figure 1.

It is a linear molecule composed of a repetition of a basic monomer that has an ester bond within it, with an organic side chain differing in length (this gives different physico-chemical properties). Variations in different monomer composition and chain length of PHAs depend on the type of bacterial metabolism and the composition of the media where the bacteria are growing. Bacteria store these biopolymers in the cytoplasm in a specific cellulosome that is enlarged or degraded depending on bacterial requirements. These biopolymers are a source both of reducing power and extra energy for the bacteria [12]. The challenging aspect of the biotechnological valorisation of a substrate (especially if using waste as the main resource) is matching the bacterial requirements with an effective production of the biopolymers of interest.

3. Wastewater valorisation through biopolymer production

Regarding wastewater valorisation for biopolymer production, many efforts have been made using a huge variety of substrates. The most effective approach is the fermentative way, i.e., with fermentors. During the course of time, many scientists have tried it using various substrates located in their socio-economic area as a starting substrate. Many different treatments have been

Table 1. Comparison between the main biopolymer and thermoplastic polymer properties. Modified and updated adaptation from the source: "Polímeros biodegradables" CIPP-CIPEM Centro Investigación de Procesamiento de Polímeros de la Universidad de los Andes. 2007.

Parameter	**Polymer**									
	LDPE	**HDPE**	**PP**	**PS**	**PHB**	**Mazin (PLA)**	**CDP (PLA)**	**BAK 1095**	**BAK 2195**	**PLLA**
Film Formation								Yes		Yes
Injection moulded									Yes	Yes
Melting point T_m [°C]	115	130	171	230	171-182	150	120-170	125	177	170-180
Glass transition temperature T_g [°C]	-120		-15	80-100	5-10	63.8	60			58
Crystallinity (%)			65-70		65-80					
Density [g cm $^{-3}$]	0.92	0.95	0.94	1.05	1.24		1.25			1.25
Mol Weight Mw ($x10^{-5}$)			2.2-7		1-8					
Mol Weight distribution			5-12		2.2-3					
MFI (g/10 min)	0.1-22	0.02-1.5	0.3-40	1.2-2.5	5-20					
Hardness (Rockwell H scale)					56-67	20.6				
Transparency (%)	T	T	T							94
Flexural Modulus[GPa]			1.7	62-76	3.5-4					
Tensile strength [MPa]	3000	3350	39	40-48	40	32.22		220	550	0.49
Tensile strength at break [MPa]	8-10		23	35	15-27	21-87				70
Extension to break (%)	150-600	>800	400-900	1.0-2.5	4-18	30.72	35	>400	120	2.4
UV resistance	Poor	Poor	Poor		Good					

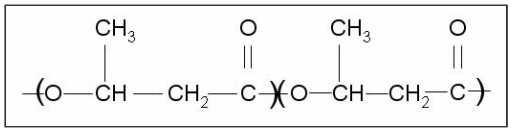

Figure 1. Chemical structure of a polyhydroxyalkanoate chain.

used depending on the preferred substrate. Results from wastewater valorisation assays combined with those from a simple carbon source use tests have helped optimise biopolymer production by developing, as much as possible, a practical application. The increase in petrol oil price and the necessity of wastewater treatment have promoted the application of wastewater in biopolymer production. Many substrates have been used with the intention of wastewater valorisation, in particular: phosphorous-enriched urban wastewater [13], palm-oil mill wastewater [14], sugarcane bagasse wastewater [15], sewage sludge [16], whey waste [17], paper mill wastewater [18], brewery wastewater [19] and even carbon dioxide effluent [20].

4. Polyhydroxyalkanoate production as means of olive oil mill wastewater valorisation

An interesting and effective substrate for PHA production is olive oil mill wastewater, predominantly due to its abundance in Mediterranean countries, it is easily fermentable, it is a low-cost resource, and has a high organic load. It is worth highlighting one of these aspects: abundance. Approximately 5 million cubic metres in the main world producer (Spain) is responsible for 70% of the olive oil produced globally. Therefore, it is evident that the available substrate is not limiting, particularly when considering that olive oil production is increasing worldwide. The real problem of fermenting such a substrate is its varied composition. As discussed before, the two mainstream systems of olive oil production generate two different substrates: TPOMW, a semi-solid waste with a higher hydrosoluble sugar content and important polyphenol content, and OOMW, a liquid waste with a high polyphenol concentration. An effective and sustainable way of producing PHAs from these substrates needs to make the most of both substrates. One of the possible ways of obtaining optimum PHA production is by using a combination of both TPOMW and OOMW. Even if many studies have concentrated their efforts on optimising olive oil wastewater treatment [21, 22, 23, 24], few have found an effective way of using this substrate specifically for PHA production with a bacterial population [25, 26, 11]. Basically, when an olive oil mill wastewater (TPOMW or OOMW) is used for PHA production, there are two available options: choose a mixed and substrate-adapted bacterial population [26] or selective inocula [10] with a proven ability of PHA accumulation. Both share a common intent, but differ in the strategy to be applied.

4.1. Polyhydroxyalkanoate production with a mixed population using olive oil mill wastewater

The main task in using a mixed bacterial population is choosing one that has a high global PHA potential as a result of its metabolism. This could be achieved by leading a mixed population to conditions that promote massive PHA production or, regardless of the substrate conditions, fine tuning the relationships among the microbial communities so that a mixed population can prevail with selected PHA production ability. Both strategies are based on neglect sterility exigency because bacterial selection is determined by the environment. This is the strength of the strategy, but also its weakness. In fact, it is more difficult in this way to drive the microbial population to generate PHAs on a massive scale, basically due to the presence of many variables at the same time. Notable examples of these are the studies reported by [26-28], focusing on the anaerobic digestion of the substrate to better achieve an easily available source of carbon for PHA production. Every strategy like this shares an acidogenic anaerobic step that is useful for treating and optimising olive oil mill wastewater for the purpose. This step is explained below in detail.

4.1.1. Anaerobic digestion of TPOMW as a key step for optimisation of the substrate

Even among the different compositions, TPOMW has two main characteristics that distinguish it

with respect to other substrates: high organic load (with an important contribution of hydrosoluble sugars) and high polyphenol content. Both features, mixed, contribute to making this substrate easy to biodegrade, mainly because of the organic load as well as the high polyphenol content that is very difficult to metabolise. Polyphenols very quickly become the recalcitrant fraction of the substrate or even worse, inhibit total bacterial metabolism. To prevent this inhibition, it is important to treat the substrate in such a way that it can be easily metabolised and optimised for PHA production. Anaerobic digestion could be used for such a purpose. In fact, in anoxic conditions, the C-rich substrate goes through acidogenic metabolism, which leads quite quickly to the production of diffused volatile fatty acids (VFAs). These compounds, are mainly produced by acidogenic bacteria, like *Firmicutes*, particularly *Clostridium* in the absence of oxygen [28]. The same compounds, in the process of the anoxic phase, feed metanogenic bacteria that generate biogas as a result of the reducing environment, especially when the OOMW is co-digested with TPOMW [29]. The main objective of the anaerobic digestion of OOMW/TPOMW is to maximise VFA production (acidogenic origin) and also optimise the hydraulic retention time (HRT) to prevent VFAs from triggering metanogenic metabolism. Furthermore, VFA production should be varied as much as possible in order to be useful for both PHA whole production and PHA chemical heterogeneity.

4.1.2. Metabolic pathways involving VFAs as biopolymer precursor in PHA production

When sugars are metabolised by bacteria, one of the most important metabolic intermediate generated is AcetylCoA. When it accumulates, it can follow different pathways. The more direct pathway involves only three enzymatic reactions that lead to PHA production. They are, in order, condensation, reduction and the final polymerisation into PHAs. However, it is different in the case of VFA metabolism. The VFAs that the bacteria can get, will give chemically different PHAs. In general terms, following the β-oxidation of the original VFAs with an even C number, an even plus-two C biopolymer is produced with a sequential addition of two C each time. Two aspects can be added to this general rule: the biopolymer elongation chain and the *de-novo* fatty acid synthesis. The first reflects that for merely thermodynamic implications, generation of long-chain PHAs from a short VFA (number of C<6) is improbable because it is unstable. The second demonstrates that even in a reduced quantity, PHAs with an odd C number can be produced by a VFA source with an even C number. This can be possible because of *de-novo* fatty acid synthesis. It is therefore evident that optimising the digestion of OOMW/TPOMW by maximising either the VFA or the hydrosoluble sugar content could be key for an effective production of PHAs.

4.1.3. Microbial dynamics in the anaerobic digestion of TPOMW

To optimise the acidogenic yield of VFA production, it is important to know the dynamics involved in such conditions. These dynamics could vary depending on the configuration of the anaerobic bioreactor used. Only a few authors [27, 28] have focused their attention on the different configuration of anaerobic bioreactors for OOMW acidification. Only one [28] has also conducted a biodiversity analysis of the microbial population present within. It is evident that efficient COD removal (for VFAs achievement) results in an effective change to the population. Optimised conditions for VFA production with the best OLR (Organic Load Retention) reduction [28] showed a selection for the acidogenic population, which led to the prevalence of *Firmicutes* (as *Clostridium sp* and *Bacillus sp*). This is consistent with the fact that optimised acidogenesis is occurring and the methanogenesis is inhibited. It is important to note that the best conditions for VFA production do not always represent the most efficient conditions for generating a variety of VFAs. For example [25], more propionic acid is produced with low hydraulic retention times. This precursor compound for PHA production is not produced at the same time as that of the maximum yield of VFAs [28].

5. Polyhydroxyalkanoate production with selected inocula using olive oil mill wastewater

The complementary strategy for PHA production using TPOMW/OOMW is the use of a selected population with a great potential for generating PHAs. This selective inoculum could be an engineered strain with a knock-out gene to enhance intracellular storage of PHAs or a strain that shows increased PHA storage. Despite many studies demonstrating good results with engineered strains, especially *E. coli* [30, 31], there are two drawbacks: poor biomass yield (and consequently PHA ratio) and increased operational costs. The best option is therefore to use a selected population with "wild type" potential for yielding PHAs. Many authors have shown the use of selected inocula for PHA production. Most of them also considered the selected C source used. A typical example is the use of easily available sugars (sucrose and glucose) or short-chain fatty acids (acetate and butyrate). On the contrary, very few authors have used selective inocula for PHA production using a waste-based substrate, particularly TPOMW/OOMW. Starting from such state of the art our effort was directed towards adopting such a strategy of selective inocula for TPOMW/OOMW substrate for PHAs production. In particular our study was focused on the use of *Azotobacter* sp.

5.1. *Azotobacter* sp and their potential for PHA production

Azotobacter sp are free nitrogen fixing bacteria, ubiquitous in soil samples. They belong to the *Pseudomonadaceae* family. Their metabolism is aerobic and involves efficiently fixing atmospheric nitrogen. They can metabolise many organic compounds and survive in microaerophilic conditions. They show great potential in PHA production [32].

5.2. Optimised results for PHA production with selected inocula of *Azotobacter* sp using TPOMW

Our efforts in terms of applied research have demonstrated the feasibility of using *Azotobacter* strains as PHA producers, especially from TPOMW at a laboratory scale. Our designed process consists of the following steps:

- An anaerobic digestion to maximise VFA production and polyphenol degradation
- Downstream use of effluent coming from the anaerobic process for a selective inoculation and PHA production from this substrate in aerobic conditions.

Both steps are optimised, particularly for the parameters of HRT (anaerobic process) and for increasing TPOMW concentration (aerobic process).

These steps are analysed in greater detail below.

5.2.1. Anaerobic digestion of TPOMW substrate with laboratory-scale specifically designed bioreactors

To match three desired results (greater VFA production with a concurrent polyphenol reduction and the maintenance of hydrosoluble sugar content), we set specific experiments to optimise the anaerobic process.

These basically aimed to achieve the best HRT to gain the three targets. The configuration of our anaerobic bioreactor is shown in Figure 2.

Its use and operation comes from quite a simple design, but is still efficient for biodegrading polyphenols whilst maintaining the hydrosoluble sugar content of 1 g/L. In fact, the strictly sealed plug with a U-shape tube guarantees perfect anaerobiosis. Filling the U-shape tube with water is an easy method to check the production of VFAs, that as long as they are produced will

Figure 2. Anaerobic laboratory-scale bioreactor.

displace the water along the section. Their lack of sterility is cost-saving and permits the autonomous development of the anaerobic community, in particular the acidogenic bacteria. Similar to [25, 28], we had the same HRT values; the HRT was 27 hours for optimised VFA production. On the contrary, our results on hydrosoluble sugar content were higher and showed that even after the anaerobic step, there was a sufficient amount of hydrosoluble sugars that could support the aerobic growth of selected inocula. This is ascribed to the substrate used. TPOMW, in fact, is characterised by a high hydrosoluble sugar content that is possibly still present after the anaerobic digestion. This is due to their high solid content and organic load [11]. Indeed, the situation is very different for the OOMW substrate. It is a liquid with a relatively lower OLR and hydrosoluble sugar content, but with a higher polyphenol content; therefore, anaerobic digestion permits a decrease in the total polyphenol content [26], but its initially low hydrosoluble sugar content is also extremely affected by this process.

5.2.2. Aerobic PHA production from an anaerobically digested TPOMW substrate with a selective inoculum of *Azotobacter* sp.

The downstream effluent from anaerobic bioreactors (with a subsequent sterilisation process by filtration and an addition of ammonia as buffer) is a very efficient substrate for *Azotobacter* growth and concurrent PHA production. This is achieved in parallel with the growth of the selected strain, with no need for stress induction like in other strains [33, 34]. The best conditions for maximised PHA production are: a growth of 72 hours and the use of 60% (w/v) TPOMW. PHA production reaches a maximum value of 49% PHA/CDW for a total yield of 1.06 g/L. This is the same that can be achieved from a simulated media of TPOMW. This medium contains the expected concentrations of the three most representative VFAs plus the addition of 1% of the most representative hydrosoluble sugars present in TPOMW: glucose and mannitol [7].

The selective inocula is cultivated in a simulation of TPOMW media that reflects a highly variable composition and tries to make the most of the VFA content to generate PHAs. It is important to note that the high hydrosoluble sugar content is not affected by the anaerobic step. Thus, in the subsequent aerobic phase, this primary C source is first choice for the bacteria. The VFAs altogether act as the chemical precursors for the heteropolymerisation of PHAs [35]. This happens in the second phase concomitantly with the growth and parallel storage of PHAs. In fact, these bacteria, especially when a limitless nitrogen source is present, are able to dedicate most of their energy to the biosynthesis of PHAs. They do not need to use this energy to fix nitrogen. Thus, along with the chemically defined media that simulates the composition of TPOMW, we can efficiently grow the selective inocula, transform waste into a resource and use it as the main C source.

5.2.3. Optimizing PHA production from anaerobically digested TPOMW with selective inocula of *Azotobacter* sp.

The really challenging part of the applied research for trying to transform waste into a resource is the optimisation step and all the following steps up to the scale-up process.

From our results [11], it is evident that increasing the C concentration enhances PHA production [36]. The difficulty is reaching a compromise of not increasing too much and not having a limiting C source, which is damaging for the bacteria. Our results demonstrated that 60% w/v of TPOMW represents the best substrate for our selected strain and after a period of growth of 72 hours, elicits a 25% higher PHA production than the flask laboratory assay. The key step of optimising PHA production through TPOMW biodegradation is using a bioreactor. This is the common and easiest way of giving shape to the preliminary step at the pilot plant scale. Its strength resides in the fact that the multi-parameter way of operation can both adjust the variables as desired in a very dynamic way and also represent a model of the process that is occurring. According to many studies [37, 38, 39], the best way of operating is with the sequence batch reactors (SBR) that combines ease with viability. In fact, as the reactors are put in sequence, this reduces the

amount of waste and saves on costs. Our model was intended to follow that way. Along with the batch reactor, an efficient process uses a fed-batch bioreactor. Although this maximises the biomass used, it also means an increase in costs and a possible source of contamination of the substrate (especially when it is a complex one). Due to that, we chose to operate only with batch reactors. This meant optimising the substrate at the beginning or during the course of the process and controlling the changing variables. It excluded an external addition of substrate or nutrients. Thus, our results could go in two directions: set dynamic conditions that made the selective strain hyperproductive (if possible) or optimised parameters that caused the hyperproducing ability of the PHAs (in our case, this was a drop in the dissolved oxygen).

5.2.4. Strategies for PHA production with anaerobically digested TPOMW in bioreactors (with selected inocula)

The dynamic conditions that we are referring to are well described in the literature as *feast* and *famine* conditions [40]. They consist of:

1. an addition of a rich C substrate
2. addition of fresh new media with limited C
3. addition of fresh media rich in a C source

The first step is to obtain a high bacterial population, the second is intended to cause stress in the population and the third is intended for an increased PHA yield. These steps can vary depending on the intentions and the population metabolism. Our results showed that a double concentration of PHAs was obtained after the third step compared to the first one. This was only half of the best PHA yield for this waste substrate. Therefore, it was necessary to scour for other strategies. This was proven to be useful as the highest PHA yield of 1.32 g/L was achieved by reducing the amount of dissolved oxygen in the media. This was obtained by two concomitant controls: reducing the agitation rate as well as the headspace in the bioreactor. In our particular case, this helped the PHA storage ability of the selected strain for two reasons: they did not have to protect their nitrogenase against the harmful oxygen and they could use their energy to store PHAs. It is important to underline a particularity of this strain. They are able to produce a protective biopolymer (alginate) with a tuned biomolecular/genic control, especially in the presence of a high concentration of dissolved oxygen [41]. They do that at the expense of PHA production (that is, in general terms, produced when is present a low dissolved oxygen environment). In fact, alginate production occurs in an extracellular capsule that the bacteria synthesise in stressful conditions (in this case, a high oxygen flux). Hence, our strategy was rewarded by the high PHA yield and reasonably supported by the literature [41]. These results were the basis for establishing the next step of the pilot plant bioprocess, starting from the optimisation achieved at the bioreactor scale. The challenge of transferring the results to a greater scale was further complicated by the different compositions of the olive oil mill wastewater. Nevertheless, this was beneficial as it broadened the application of this strategy to waste of different origins.

Widening the possible substrates that could be used (and its geographic origin), was intended as a possible biorefining integrated process (to further reduce the costs).

5.2.5. Pilot-plant PHA production with OOMW (with selected inocula)

From the bioreactor results at the laboratory scale, we moved on to the pilot-plant scale. We used a waste substrate that was as raw as possible and selected inocula to reduce costs. The waste in use had a low hydrosoluble sugar content (0.1%) and higher polyphenol content (350g/L), both with respect to TPOMW. These features indicated that anaerobic digestion was inapplicable, since this hydrolytic process is useful for degrading macromolecular polyphenols but is highly damaging for the desired hydrosoluble sugars. Thus, work with fresh wastewater was preferred, deleting the anaerobic step. This compromise in maintaining the hydrosoluble sugar content was rewarded by good growth of the selected strains in the raw substrate, representing a further reduction in costs. The pilot plant process gave a PHA yield of 1.31 g/L within the same time period as that of the laboratory scale, mirroring the same results as

that from the latter scale even with a completely different substrate. Most importantly, OOMW was mainly useful in the raw form compared to TPOMW, which needed a preliminary step. In addition, the nitrogen content in OOMW was sufficient to guarantee both growth and PHA production for the selected strain. This means that it is not necessary to add external nitrogen (which was applied as ammonia to the TPOMW substrate). Even if the PHA yields indicate OOMW/TPOMW as a viable substrate that can be used in a reasonable time period, there are some features that need to be considered for its possible use.

5.3. Critical points for PHA production with an OOMW/TPOMW substrate in a fermentative way (chemical and physical aspects)

The physicochemical composition can vary depending on the weather condition and the fertility of the soil. Consequently, it is hard to model a standard treatment for such a variable substrate and this obviously affects PHA production. Nonetheless, this could also be turned into something positive if a defined biopolymer is desired; however, biotechnological/biochemical expertise is probably needed to support this possible bioprocess. This expertise could add cost for the olive oil producer. Furthermore, OOMW/TPOMW chemical composition also affects the downstream process after PHA production. In fact, the law is very strict about land disposal of this type of waste and, varying among different countries, a limited amount can be spread on soil but in an intermittent way. However, our results showed that there was a direct correlation between the best substrate for PHA production and a reduction in its ecological-toxicological impact on target organisms. The toxic potential of the OOMW/TPOMW substrate decreased by about 25% following its use in PHA production. Thus, this could be an added value to the process, but is still far from being the definitive solution. Additionally, it is worth considering the extraction method used to obtain the PHAs from intracellular storage [42]. Even if many possibilities are considered in the scientific literature to be more eco-friendly, the most efficient way is to use mixed organic solvents, but the only way of doing that at an industrial scale will require a constant reuse of them with minimal loss. This is due essentially to two reasons: not to increase costs and not to compromise the entire process, which would unnecessarily contaminate the overall process.

5.3.1. Critical points for PHA production with an OOMW/TPOMW substrate in a fermentative way (general aspects)

Apart from its physicochemical features, there are other aspects that need to be considered. The reduced seasonality of the waste generation limits its use to that particular time period or immediately thereafter. This could be an advantage, due to the fact that a concentrated period of time could turn out to be useful for disposing the total amount and have sufficient substrate for fermentation purposes. Its regionalism limits its use to those countries where it is mainly produced, although olive oil production is increasing worldwide and is spreading to new emerging countries where treatment will become important. The increasing demand for land use, either for agriculture or for raising biofuel production from crops, makes it urgent to find options to escape from the biofuel vs food debate for land use. Waste valorisation for PHA production in a fermentative way, in theory, has the advantage of not threatening land use for other purposes that are not agricultural. This is very crucial if a complete life cycle analysis (LCA) of the valorisation of OOMW/TPOMW is desired. In fact, LCA is an effective way of becoming conscious of whether and in which way a type of waste can actually be useful [43]. Furthermore, the easier the OOMW/TPOMW valorisation process, the less would be the overall cost. For that, it is important to take the narrow path to try and guarantee effective treatment/valorisation using cheaper technology. In a biotechnological approach, this entails having the desired microbial population for PHA production and preventing possible external contamination (of non-PHA producers). This external contamination directly correlates with the size of the fermentors, mainly due to the difficulty of maintaining sterile conditions,

which also increases costs. Biotechnologists place a lot of importance on sterility, especially when using selected inocula. There are ways of preventing contamination, apart from the sterilisation process, which consist of adjusting the conditions of the media (for example, in our case, we reduced oxygenation to prevent fungal contamination when our selected inocula were in the first phases of growth) or reducing the lag phase that produces an exponential growth of the selected inocula so that they are the predominant population and minimise the effects of contamination.

5.4. Future perspectives for PHA production with an OOMW/TPOMW substrate in a fermentative way

There are two different approaches that could be considered when analysing future perspectives; they consist of two opposite ways of thinking. It could be useful to concentrate efforts for optimising PHA production, using TPOMW and OOMW separately from one another. On the other hand, an integrated approach that is more adaptable could be employed, which tries to valorise either a mixing of the substrates or using each substrate independently but with shared steps where possible. Something similar can be seen with the cooperation among olive oil producers. This cooperation could also be useful for achieving a sufficient amount of fermentable substrate for PHA production. An even more integrated process could be possible, where different types of waste can be used at the same time for a more effective way of valorising them together. This is the basis for a fermentative biorefining-driven approach. Public opinion is always more demanding in terms of environment-friendly choices, but there is also a widespread consciousness to achieve real answers to meet those needs. The current bioplastic sector is dominated by biopolymers of crop origin and even if it is an emerging sector, its production yields are increasing exponentially. Therefore, without alternatives to prevent the clash between food and bioproducts for land use, the moment where the biopolymer sector raises food prices could be very soon. Hence, with an applied research approach, an effective waste valorisation for PHA production might also become a social added value. This is the intention for the biotechnological approach of producing PHAs from TPOMW/OOMW.

CONCLUSION

A biotechnological way of using TPOMW/ OOMW for the production of biopolymers with properties similar to thermoplastic polymers has demonstrated to be interesting in terms of saving costs and being eco-friendly. Its strength is due to the use of a fermentative way with a selected PHA-producer population of bacteria. Its economic feasibility is compensated by the valorisation of this waste. Further improvement of this biotechnology is needed, especially to increase PHA yields and developing the most eco-friendly method of extraction.

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REFERENCES

1. Caputo, A., Scacchia, F., Pacifico, M., and Pelagagge. 2003, Appl. Therm. Eng., 23(2), 197-214.
2. Aranda, E., Garcia-Romera, I., Ocampo, J. A., Carbone, V., Mari, A., Malorni, A., Sannino, F., De Martino, A., and Capasso, R. 2007, Chemosphere, 69(2), 229-239.
3. Albuquerque, J. A., Albuquerque, J., Gonzalvez, J., Garcia, D., and Cegarra, J. 2004, Bioresource Technol., 91, 195-200.
4. Ramos-Cormenzana, A., Monteoliva-Sánchez, M., and López, M. J. 1995, Int. Biodet. and Biodeg., 35, 249-268.

5. Brozzoli, V., Crognale, S., Sampedro, I., Federici, F., D'Annibale, A., and Petruccioli, M. 2009, Bioresource Technol., 100, 3395-3402.
6. Garcia-Castello, E., Cassano, A., Criscuoli, A., Conidi, C., and Drioli, E. 2010, Wat. Res., 44, 3883-3892.
7. Ghoreishi, S. M., Gholami. and Shahrestani, R. 2009, J. Food Eng., 93, 474-481.
8. Gonçalves, M. R., Costa, J. C., Marques, I. P., and Alves, M. M. 2011, Energy, 36(4), 2138-2141.
9. Morillo, J. A., Aguilera, M., Ramos-Cormenzana, A., and Monteoliva-Sanchéz, M. 2006, Curr. Micr., 53, 189-193.
10. Pozo, C., Martinéz-Toledo, M. V., Rodelas, B., and González-López, J. 2002, J. Biotechnol., 97, 125-131.
11. Cerrone, F., Sánchez-Peinado, M., Juárez-Jimenez, B., González-López, J., and Pozo, C. 2010, J. Microbiol. Biotechn., 20(3), 594-601.
12. Sudesh, K., Abe, H., and Doi, Y. 2000, Progr. Polym. Sci., 25, 1503-1555.
13. Randall, A. A. and Liu, Y. H. 2002, Wat. Res., 36, 3474-3478.
14. Mumtaz, T., Yahaya, N. A., Abd-Aziz, S., Rahman, A. N., Lai Yee, P., Shirai, Y., and Hassan, M. A. 2010, J. Clean Prod., 18, 1393-1402.
15. Hu, J. and Stahl, K. 2008, Bioresource Technol., 99, 8042-8048.
16. Liu, Z., Wang, Y., He, N., Huang, J., Zhu, K., Shao, W., Wang, H., Yuan, W., and Li, Q. 2011, J. Hazard. Mater., 185, 8-16.
17. Bosco, F. and Chiampo, F. 2010, J. Biosci. Bioeng., 109(4), 418-421.
18. Bengtsson, S., Werker, A., Christensson, M., and Welander, T. 2008, Bioresource Technol., 99, 509-516.
19. Law, K. H., Cheng, Y. C., Leung, Y. C., Lo, W. H., Chua, H., and Yu, H. F. 2003, Biochem. Eng. J., 6, 203-208.
20. Ishizaki, A., Tanaka, K., and Taga, N. 2001, Appl. Microbiol. Biot., 57, 6-12.
21. Cayuela, M. L., Millner, P. D., Meyer, S. L. F., and Roig, A. 2008, Sci. Total Environ., 399, 11-18.
22. Mekki, H., Anderson, M., Benzina, M., and Ammar, E. 2008, J. Hazard. Mater., 158, 308-315.
23. Altieri, R. and Esposito, A. 2010, Int. Biodet. Biodeg., 64, 124-128.
24. Aviani, I., Laor, Y., Medina, S., Krassnovsky, A., and Raviv, M. 2010, Bioresource Technol., 101, 6699-6706.
25. Ntaikou, I., Kourmentza, C., Koutrouli, E. C., Stamatelatou, K., Zampraka, A., Kornaros, M., and Lyberatos, G. 2009, Bioresource Technol., 100, 3724-3730.
26. Dionisi, D., Carucci, G., Petrangeli, Papini, M., Riccardi, C., Majone, M., and Carrasco, F. 2005, Wat. Res., 39, 2076-2084.
27. Beccari, M., Bertin, L., Dionisi, D., Fava, F., Lampis, S., Majone, M., Valentino, F., Vallini, G., and Villano, M. 2009, J. Chem. Technol. Biotech., 84, 901-908.
28. Bertin, L., Lampis, S., Todaro, D., Scoma, A., Vallini, G., Marchetti, L., Majone, M., and Fava, F. 2010, Wat. Res., 44, 4537-4549.
29. Fezzani, B. and Ben Cheikh, R. 2008, Desal., 228, 159-167.
30. Jae Park, S., Choi, J. I., and Lee, S. Y. 2005, Enzyme Microb. Tech., 36(4), 579-588.
31. Li, R., Zhang, H., and Li, Q. 2007, Bioresource Technol., 98(12), 2313-2320.
32. Martínez-Tolédo, M. V., González-López, J., Rodelas, B., Pozo, C., and Salmerón, V. 1995, J. Appl. Bact., 78, 413-418.
33. Wen, Q., Chen, Z., Tian, T., and Chen, W. 2010, J. Env. Sci., 22(10), 1602-1607.
34. Liu, Q., Luo, G., Zhou, X. R., and Chen, G. 2011, Metab. Eng., 13(1), 13-17.
35. Lemos, P. C., Serafim, L. S., and Reis, M. A. M. 2006, J. Biotechnol., 122(2), 226-238.
36. Lemos, P. C., Viana, C., Salgueiro, E. N., Ramos, A. M., Crespo, J. P. S. C., and Reis, M. A. M. 1998, Enzyme Microb. Tech., 22(8), 662-671.
37. Nogueira, R., Alves, C., Matos, M., and Brito, A. G. 2009, Bioresource Technol., 100(7), 2106-2110.
38. Johnson, K., Kleerebezem, R., and Van Loosdrecht, M. C. M. 2010, Wat. Res., 44(7), 2141-2152.

39. Villano, M., Beccari, M., Dionisi, D., Lampis, S., Miccheli, A., Vallini, G., and Majone, M. 2010, Process Biochem., 45(5), 714-723.
40. Albuquerque, M. G. E., Martino, V., Pollet, E., Averous, L., and Reis, M. A. M. 2011, J. Biotechnol., 151(1), 66-76.
41. Segura, D., Guzmán, J., and Espín, G. 2003, Appl. Microbiol. Biot., 63, 159-163.
42. Jaquel, N., Lo, C. W., Wei, Y., Wu, H., and Wang, S. S. 2008, Biochem. Eng. J., 39, 15-27.
43. Harding, K. G., Dennis, J. S., von Blottnitz, H., and Harrison, S. T. L. 2007, J. Biotechnol., 130(1), 57-66.