Production of PHAs with variable HV content from agricultural residues

C. Magonara[1], E. Montagnese[1], G. Pesante[1], C. Vona[2], M. Villano[2], F. Battista[1], D. Bolzonella[1]

[1]Department of Biotechnology, University of Verona, Verona, 37134, Italy
[1]Department of Chemistry, Sapienza University of Rome, Roma, 00185, Italy
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Presenting author email: claudia.magonara@univr.it

The production of agricultural and food waste – leftovers from the cultivation and the processing of agricultural goods – is estimated worldwide at roughly 998 million tons a year (Raut *et al.*, 2023), while in the European Union over 58 million tons of food waste are generated annually (Eurostat, 2023). Agricultural waste, by-products and co-products are usually defined as plant or animal residues that are not (or not further processed into) food or feed (Gontard *et al*., 2018), and are thrown away, generating costs and being responsible for health and environment-related problems when they are not appropriately managed (Abdel-Shafy & Mansour, 2018). In the framework of a circular economy approach, these wastes should instead be seen as resources and turned into biobased, high added-value compounds that have the potential to substitute – at least partially – precursors or chemicals that at present are produced using non-renewable materials and processes with a high environmental impact.

Both biofuels and biochemicals can be recovered from agricultural residues. Producing polyhydroxyalkanoates (PHAs) is a possible way to valorise these streams. PHAs are biodegradable polymers characterized by tunable physicochemical properties which make them suitable for the synthesis of (bio)materials alternative to the traditional fossil-based plastic (Freches & Lemos, 2017). PHAs are produced by more than 300 species of microorganisms that are endowed with enzymes for the synthesis of polyesters, which are accumulated in granules in the cell cytoplasm as energy and carbon stocks to be used in case of a lack of external substrate (Cabrera *et al*., 2021). Industrial production of PHAs can take advantage of mixed microbial cultures (MMC) since these are more resilient than pure cultures, and they don't require sterilization procedures (Koller & Mukherjee, 2022). Moreover, pure bacterial cultures require synthetic substrates, with high operation costs associated (Freches & Lemos, 2017), while using MMC allows the use of waste and residues from agricultural and industrial sectors as feedstock for the bioreactor, by previously fermenting it to obtain a volatile fatty acids (VFAs)-rich mixture. However, using MMC requires a preliminary step to select PHA-accumulating microorganisms starting from an activated sludge. This selection can be done through the establishment of the feast/famine (F/F) conditions, which consists of alternating periods of availability and lack of substrate (Pinto-Ibieta *et al*., 2021).

The aim of this work was to find the best conditions for the production of PHAs using an agricultural fermentate as feedstock for the growth of MMC, which were selected for the accumulation of PHAs through the F/F method. This work is part of the AgriLoop project, whose purpose is to develop safe-and-sustainable-by-design bioconversion processes integrated into a cascading biorefinery approach to convert a range of agri-residues into plant and microbial proteins, polyesters and other bio-based chemicals (https://www.agriloop-project.eu/about/).

The agricultural fermentate used during several experimental runs was characterized by different concentrations of acetic, propionic and butyric acid. The addition of different sources of macronutrients (nitrogen and phosphorous) and micronutrients to the VFAs mixture was tested, from synthetic ones during the initial runs to micro-filtered agricultural digestate during the following ones (Table 1).

Table 1. Composition of the feedstock used for the selection reactor. The trace elements solution was prepared following Cabrera *et al*., 2021.

<table>
<tr><th>Set-up n°</th><th>VFAs (total)</th><th>Acetic acid</th><th>Propionic acid</th><th>Butyric acid</th><th rowspan="2">Nitrogen source</th><th rowspan="2">Phosphorous source</th><th rowspan="2">Other (micro)nutrients source</th></tr>
<tr><th></th><th>gCOD/L</th><th>gCOD/L</th><th>gCOD/L</th><th>gCOD/L</th></tr>
<tr><td>1</td><td>9.70</td><td>7.32</td><td>1.23</td><td>1.15</td><td rowspan="4">Ammonium bicarbonate 0.485 gN/L</td><td rowspan="4">Phosphoric acid 0.097 gP/L</td><td>-</td></tr>
<tr><td>2</td><td>9.70</td><td>7.32</td><td>1.23</td><td>1.15</td><td>-</td></tr>
<tr><td>3</td><td>9.70</td><td>7.32</td><td>1.23</td><td>1.15</td><td>Trace elements solution</td></tr>
<tr><td>4</td><td>9.70</td><td>6.26</td><td>2.46</td><td>0.98</td><td>Trace elements solution</td></tr>
<tr><td>5</td><td>9.70</td><td>6.26</td><td>2.46</td><td>0.98</td><td colspan="3">Micro-filtered agricultural digestate</td></tr>
</table>

A sequencing batch reactor (SBR) of 28 L was used for the selection of the MMC, while a batch reactor of 5 L was used for the accumulation phase. The first one was fed with the feedstock described in Table 1, with four feeding cycles at intervals of six hours, for a total of four F/F cycles per day. The dissolved oxygen (DO) was constantly monitored in the SBR, as this parameter is useful to understand the duration of the feast and famine phases (Cabrera *et al*., 2021). Other parameters monitored included suspended solids, soluble COD and VFA concentrations. The accumulation phase was carried out once a day by filling the reactor with the effluent of the selection reactor and feeding it with the same VFA mixture of the selection reactor, for a final concentration of 1 gCOD/L. In this case, the feedstock was given every time the DO increased (suggesting the depletion of the available COD), to maximize the accumulation of PHAs inside the cells.

Results show that it was possible to reach a PHA content between 20% and 40% (w/w), with a hydroxyvalerate (HV) content up to 50% of the total PHA (Table 2). HV contents between 20 and 30% - which are the desired ones according to the AgriLoop project - were obtained by using a feedstock characterized by 7.32 gCOD/L (75% w/w) of acetic acid, 1.23 (13%) gCOD/L of propionic acid and 1.15 (12%) gCOD/L of butyric acid. HV reached its maximum value of 50% when propionic acid was increased in the feedstock: 6.26 gCOD/L (65% w/w) of acetic acid, 2.46 gCOD/L (25%) of propionic acid and 0.98 gCOD/L (10%) of butyric acid.

During that experimental run, in order to maintain the F/F equal to 0.2, which has been demonstrated optimal for the selection of the biomass (Freches & Lemos, 2017), the organic loading rate (OLR) was periodically switched from a maximum of 4 kgCOD/m^{3}d to a minimum 2 kgCOD/m^{3}d by reducing the time of feeding and discharge. As a consequence, the hydraulic retention time was switched from 3 to 6 days.

Further tests are required to investigate the optimal ratio between carbon (VFAs), nitrogen and phosphorous of the feedstock which can ensure optimal growth and selection of the PHA-accumulating microorganisms in the selection reactor. Furthermore, PHA extraction tests will be conducted using sodium hydroxide (NaOH) following the protocol described by Rodrigues *et al*. (2022), in order to recover these polymers without using excessive chemicals, while preserving the polymer integrity and the molecular weight needed for downstream applications. The results gathered from these laboratory-based experiments will be utilized for running a pilot plant adjacent to an agricultural factory and comprehensive of both the fermentation reactor and the PHA-producing reactors.

Table 2. Range of PHA amount (%w/w) and composition in HV obtained from the accumulation phases during each set-up of the experimental period.

Set-up n°	**PHA (%, w/w)**	**HV (%, w/w)**
1	16.6 – 28.7	22.0 – 25.7
2	20.7 – 38.4	30.6 – 36.6
3	21.0 – 32.0	19.0 – 27.8
4	29.0 – 32.2	49.2 – 53.1
5	21.9 – 36.0	18.0 – 30.8

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