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Production and characterisation of PHAs by pure culture using protein hydrolysates as sole carbon source

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ABSTRACT

Protein hydrolysates obtained from discarded biomass can be further upgraded into high market value products, in the optic of a circular bioeconomy. In this work, residues from the cultivation of alfalfa, soybean and rice, and bovine wet blue shavings were fermented with mixed microbial cultures obtaining high concentrations of volatile fatty acid (up to 50 times compared to the original hydrolysate), mainly butyric and acetic acid. This rich medium was used for growing the bacterium *Thauera* sp., a known producer of polyhydroxyalkanoates (PHAs), biodegradable polymers with potential to replace petrol-based plastics. The overall process resulted in the production of 1.4 gPHAs/L, with a conversion rate of 32% for the alfalfa hydrolysates when considering the COD given by the initial VFAs. The obtained biopolymer was poly(3-hydroxybutyrate-co-3-hydroxyvalerate), as confirmed by the presence of characteristic peaks and by the melting temperatures and thermo-oxidative degradation in the expected range; the polymer has a high degree of purity, being without inorganic residues. This work showed the feasibility of a process aimed at the valorisation of protein hydrolysates into high-market value products such as bioplastics.

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1. Introduction

Waste is a burden for our society. Industries, mainly the three sectors of agriculture, food and oil production, produce a large volume of organic waste (Sharma et al., 2022). In particular, it was estimated that the global agricultural industry each year produces five billion metric tons of biomass (Bharathiraja et al., 2017), with 1.3 billion tons of agro-industrial waste generated from the non-edible parts of plants (Gaur et al., 2020); solid waste produced by the agricultural sector is increasing by around 7.5% each year (Adejumo and Adebiyi, 2020). Agricultural residues, defined as plant and animal by-products which are not used for food or feed, generated during crop cultivation and processing or livestock breeding (Girelli et al., 2020), accounted for 50% of the fresh weight of harvests in 2012 (Gontard et al., 2018). In the European Union, Italy, France and Spain are the main producers of agricultural residues, with a total of 367 million tons produced annually (Correddu et al., 2020).

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The management and disposal of these large quantities of waste, from its inception to its final disposal, generate high economical costs, as well as ecological, health-related and logistical problems (Abdel-Shafy and Mansour, 2018). In 2018 a revised legislative framework came into force in the EU, which aimed at reducing waste by settling a long-term plan with recycling at its forefront (EU, 2018). However, a more radical change is needed for a successful management, where waste of any type is valorised and seen as a resource, rather than considered just as an inconvenience and disposed of with the concomitant generation of costs and greenhouse gases. In March 2020 the European Union introduced a new Circular Economy Action Plan (EU, 2020), part of the European Green Deal, which looks at generating high-quality waste streams for re-use and recovery of waste by introducing innovative circular solutions into the value chain of economies (Núñez Ferrer and Stroia, 2020).

Agricultural residues therefore represent a pool of biomass which should be seen as a resource, and used for the generation of renewable bioenergy as well as the synthesis of bio-based products with high market value (Bharathiraja et al., 2017; Gontard et al., 2018; Ravindran et al., 2018). The nature and composition of this waste is heterogeneous, dependent on the type of process it is generated from, but it is mostly biodegradable, and it contains nutritious elements, such as carbohydrates, proteins, lipids, vitamins, oils, fibres, lignocellulose and pigments (Panesar et al., 2015). Agricultural residues can be tuned by means of different processes into value-added products such as biofertilisers, biostimulants, biofuels, bioplastics, animal feed and bioactive molecules, while offering an effective reduction of waste and of health and environmental threats (Gaur et al., 2020). This circular economy approach for the valorisation of biomass waste is not new, but it has only partially been exploited and it needs implementing on a larger scale (Cho et al., 2020). In particular, biomass biorefineries (structures where biomass is converted into multiple renewable bio-based products) have the potential to produce more type of products than the current petroleum-based manufacturing, reducing dependence on petroleum resources (Isikgor and Becer, 2015).

Protein hydrolysates (PHs) and biodegradable polymers are some of the products that can be generated from agricultural residues. Protein hydrolysates are a mixture of free amino acids, oligopeptides and polypeptides, which can be obtained by hydrolysis of the protein fraction contained in discarded biomass; they are used as ingredients of foods and pharmaceuticals for human consumption, since they have been reported to exhibit antioxidant properties, as well as antimicrobial, hypotensive, anticoagulant and many other (Nasri, 2017). Other PHs sources are animal and fish waste or leftovers, which are used to generate products with similar properties on human health to those produced from crops residues (LeBlanc et al., 2002; Sachindra and Bhaskar, 2008; Suetsuna et al., 2004; Tsuruki et al., 2003; Wu and Lin, 2004). PHs of both plant and animal origin have also well-established biostimulant effects, increasing germination, productivity and quality of horticultural and agronomic crops (Colla et al., 2015) and are therefore produced commercially and available in the market, exploiting the potential of their bioactive peptides (Hartmann and Meisel, 2007).

Biodegradable polymers in the form of polyhydroxyalkanoates (PHAs) are a form of intracellular storage for carbon and energy, produced by *Eubacteria* and *Archaea*, which gained high interest in recent years as an ecological solution to the problem of plastic pollution (Raza et al., 2018). Agricultural residues, being rich in many nutritious elements, constitute a perfect starting material to be converted by acidogenic fermentation into volatile fatty acids (VFAs), the main precursors for the cultivation of PHAs producing bacteria, which under excess of substrate can accumulate PHAs up to more than 90% of their cell dry weight (Pagliano et al., 2021; Rodriguez-Perez et al., 2018). PHAs can subsequently be extracted and purified and used as substitutes of petroleum-based plastic products in many applications, from packaging to the food, agricultural, medical and pharmaceutical industries (Kourmentza et al., 2017).

In this contest, the present study aimed at the valorisation of agricultural and animal leftovers into PHAs. Previous research worked on the conversion of vegetal residues and animal by-products into PHs, highlighting the properties making them valuable as food and feed additives. This paper's approach was different, since PHs were not used as final products, but as feedstocks, by fermenting them with mixed microbial cultures and evaluating the most efficient hydrolysate in terms of VFAs production. The VFA-rich fermentation fluid obtained was used for growing a pure culture of the PHA-accumulating bacterium *Thauera* sp. Sel9, and the quantity and quality of PHAs produced was evaluated. An alternative use of PHs was therefore demonstrated, by showing that PHAs can be successfully produced from protein hydrolysates derived from agricultural and animal by-products.

2. Materials and methods

2.1. Experimental set-up

The experimental set-up is illustrated in Fig. 1. Four different types of PHs normally used as biostimulants (three of vegetal and one of animal origin) were kindly donated to the University of Verona (Laboratory of chemical engineering for the environment and bio-processes) by the company ILSA Spa (https://www.ilsagroup.com/en/home/), based in Arzignano, Vicenza, Italy. The hydrolysates were first characterised for their physico-chemical characteristics (chemical oxygen demand (COD), density, solids, total Kjeldahl nitrogen (TKN), *ammoniacal nitrogen* ($N\text{-}NH_3$), total phosphorus (TP), VFA, pH) and amino acid composition, and then subjected to acidogenic fermentation trials in batch using as inoculum anaerobic digestate obtained from a local biogas plant, to assess their potential for VFAs production and to identify the most favourable conditions for the process. The sterilised VFA-rich fermentation fluid was used for growing the PHA-accumulating bacterium *Thauera* sp. Sel9 in batch tests. After a growth of four days, PHA accumulation tests were

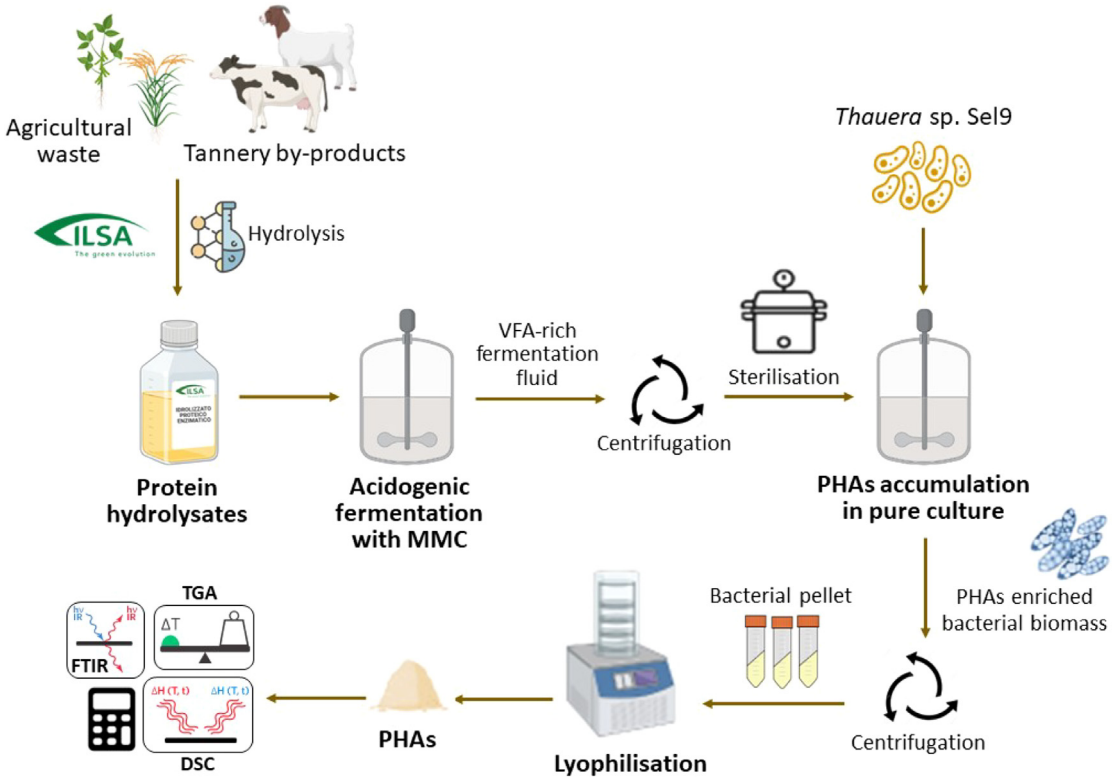


Fig. 1. Experimental set-up.

performed with a multi-spike feeding strategy (Valentino et al., 2019) in a fed-batch system for six hours. The obtained PHAs were extracted from the bacterial cells, and their amount, composition and physical properties were determined at the University of Padova, Centre for Mechanics of Biological Materials, to evaluate yields and quality and therefore assess the feasibility of the overall process.

Details on each step are given in the following Sections 2.2–2.7 and the steps are summarised here:

1. Production of the PHs by ILSA Spa, and their characterisation (physico-chemical properties, amino acid composition) at the University of Verona.
2. Fermentation trials with anaerobic digestate to test the VFAs production potential.
3. Growth tests in batch with the PHA-producing bacterium *Thauera* sp. Sel9, using the VFA-rich fermentation fluid produced in point 2.
4. PHAs accumulation process of the best performing PHs (alfalfa hydrolysate), based on the results from point 3.
5. Extraction of PHAs from the bacterial cells and polymer characterisation.

2.2. Analytical methods

Routine parameters such as soluble and total chemical oxygen demand (sCOD and tCOD), pH, total and volatile suspended solids (TSS and VSS), TKN and N-NH_3 were measured according to standard methods (APHA, 1998; IRSA-CNR, 2003).

VFAs were quantified with a Dionex ICS-1100 ion chromatographer (Thermo Fisher Scientific, USA) fitted with the column IonPac ICE-AS1 System, using methods previously described (Conca et al., 2020). Concentration of VFAs was expressed as grams of COD per litre (gCOD $_{VFA}$/L) and was calculated as follow:

$$VFA = \sum(HAc + HPr + HBt + iso - HBt + HPt + iso - HPt + HHe)$$

where HAc is acetic acid, HPr is propionic acid, HBt is butyric acid, iso-HBt is isobutyric acid, HPt is pentanoic or valeric acid, iso-HPt is isopentanoic acid, HHe is hexanoic or caproic acid.

The amount of phosphorus (TP) was measured with the LCK350 kit (Hack), following the procedure indicated by manufacturers.

The analysis of the PHA content was performed with a gas chromatograph (GC), as described previously (Braunegg et al., 1978). Briefly, 10 mg of the freeze-dried pellet were finely ground and dissolved in 1 ml of chloroform, 2 ml of methanol containing 3% (v/v) sulphuric acid and 1 ml of a 0.5% (w/v) benzoic acid solution in chloroform. The samples were then heated at 100 °C for 4 h, 1 ml of distilled water was added, and the solution was mixed for 15 min and centrifuged for 2 min at 2,100 rpm. The pellet (organic phase) was used for GC analysis (Braunegg et al., 1978). Standards for the calibration curve were prepared with pure poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (Sigma, 403105) alongside the samples and treated following the same protocol.

2.3. Protein hydrolysates

The vegetal PHs used in this study derived from agricultural residues obtained from the cultivation of alfalfa (*Medicago sativa,* family Fabaceae), soybean (*Glycine max,* family Fabaceae) and rice (*Oryza sativa,* family Poaceae), and are defined as biostimulants according to the Italian Legislative Decree n. 75 (29/4/2010) and under the Regulation EU 2019/2009. Rice by-products consisted in the bran, which is the outer layer of the cereal grain and is separated during the milling process (Rathna Priya et al., 2019). The animal PH was obtained from "wet blue shavings" of bovine hides and skins, an intermediate stage of the leather manufacturing process; this by-product is free from sanitary risks according to the Italian regulation CE n.142/2011 and is defined as "nitrogen organic fertiliser in liquid form — liquid hydrolysed animal epithelium" by the Italian Legislative Decree n. 75 (29/4/2010); this hydrolysate is not an environmental hazard because it contains the non-toxic form of Chromium (Cr(III)) and not the toxic Cr(IV) (Ciavatta et al., 2012). All four hydrolysates can be used in organic farming according to the regional Italian regulation CE n. 834/2007.

All PHs were prepared at the ILSA plant by FCEH® (Fully Controlled Enzymatic Hydrolysis, https://www.ilsagroup.com/en/pages/6/liquid-fertilizer-process.htm), which involves heating the samples at low temperatures (40–60 °C) and extracting the proteins using endo-proteases (Ciavatta et al., 2012). This process is recognised to free amino acids and peptides mainly in a L-form, which is more biologically active than the R-form and therefore more useful for plants (Cavani et al., 2017; Colla et al., 2015).

The amino acid content was determined by HPLC, following the protocol published on the Gazzetta Ufficiale G.U. 10/04/06 n° 84, DM 15/03/06 Supplement n°9.

The fraction of biodegradable COD was determined with a BM-EVO2 multi-purpose double respirometer system (Surcis s.l.) in R mode. One litre of mixed microbial culture sludge obtained from the anaerobic digestion plant "La Torre" located in Isola della Scala, Verona, Italy, was aerated and mixed at 20 °C until a stable oxygen consumption rate was reached, and then 1 ml of PH was added. The analysis lasted until the oxygen uptake rate (OUR) reached the null value. The obtained data was exported in an excel file to create the graphs and to calculate the bCOD values.

2.4. Fermentation trials

Fermentation trials were carried out in duplicates in batch systems of 125 mL at 37C̊ and in anaerobiosis, with a substrate/inoculum ratio of 10 gCOD/gTVS (Da Ros et al., 2020), in order to assess VFA production. The inoculum consisted of the liquid phase of the digestate obtained from the anaerobic digestion plant "La Torre" located in Isola della Scala, Verona, Italy. The pH was kept at the value of 8.0 by regulating it every 24 h by the addition of sodium hydroxide 30% (w/v). The fermentation continued for three days with daily measurements of pH and sCOD.

The yield of the fermentation process was calculated as:

$$VFAyield(\%) = \frac{gCODVFAfin}{gCODinit} \bullet 100$$

where gCOD $_{VFAfin}$ is the amount of VFAs in g of sCOD recorded at the end of the fermentation, and gCODinit are the g of sCOD of the protein hydrolysates before the fermentation.

2.5. Bacterial strain for PHAs production

The strain used in this study for the production of PHAs was originally selected and isolated from a mixed microbial culture producing PHAs (Conca et al., 2020). The genomic analysis revealed a 99% similarity with *Thauera butanivorans* NBRC 103042T, a betaproteobacterium of the order Rhodocyclaceae, known to produce PHAs (Botturi et al., 2020; Frison et al., 2021; Sabapathy et al., 2020). The strain will be from here referred to as "Thauera sp. Sel9". Inocula of this strain were obtained from glycerol stocks by plating on nutrient broth plates, selecting a colony and growing it for 3 days at 27 °C and 180 rpm in the medium described in Table 1, a modification of Brunner's mineral medium. After centrifugation, the pellet containing the bacterial cells was washed and resuspended in saline solution (NaCl 0.9% w/v) to obtain the required concentration of inoculum for the growth tests.

Table 1
Medium used for the growth of Thauera sp. Sel9.

Thauera sp. Sel9 medium	g/L	Trace elements	g/L
Sodium acetate	1.10	Iron (III) chloride	0.090
Sodium propionate	0.43	Boric acid	0.015
Ammonium bicarbonate	0.55	Copper (II) sulphate pentahydrate	0.003
Dipotassium phosphate	0.05	Potassium iodide	0.003
Magnesium sulphate heptahydrate	0.20	Sodium molybdate dihydrate	0.006
Calcium chloride dihydrate	0.66	Manganese (II) chloride tetrahydrate	0.012
Yeast extract	2.50	Zinc sulphate heptahydrate	0.012
Peptone	0.50	Cobalt (II) chloride hexahydrate	0.017
Trace elements	2.00 ml		

2.6. Growth tests

Growth tests in duplicates with the strain *Thauera* sp. Sel9 were carried out using the fermentation fluid obtained in Section 2.4. The fermented hydrolysates were diluted to obtain a concentration of 5 g sCOD/L (Botturi et al., 2020), the pH was regulated at 8.0 for optimal bacterial growth and sterilisation by autoclaving was performed to avoid contamination with other bacterial strains. The tests were carried out in flasks filled with 150 mL of substrate and incubated at 27 °C on an orbital shaker (200 rpm) for four days. Growth was evaluated by measuring suspended and volatile solids and sCOD consumption every day. The growth yield (Y_H) of the microorganisms was calculated as:

$$Y_H = \frac{\Delta VSS}{\Delta COD} \bullet 1,42$$

where ΔVSS and ΔCOD were respectively the variation in VSS and sCOD compared to day 0. 1,42 is the factor for the conversion of VSS into COD (Metcalf et al., 2014).

2.7. PHAs production

PHAs accumulation tests were performed with the same respirometer used for the determination of the PHs bCOD (Section 2.3). Pure cultures of *Thauera* sp. Sel9 were grown in 1 litre of the alfalfa hydrolysate at the concentration of 5 gCOD/L at 27 °C and constant agitation. A multi-spike feeding strategy (Valentino et al., 2019) was adopted to avoid substrate inhibition, with spikes of fermented hydrolysate at the final concentration of 1 g COD/L provided at each decrease of oxygen consumption. Samples were collected before each spike to determine VSS, SS, VFAs, COD and PHAs concentration, for a total of four hours.

For the analysis of the PHAs content and amounts, the samples obtained at each spike were centrifuged for 20 min at 3.900 rpm, the supernatant was removed, and the pellet was frozen at −80° C for 2 h and lyophilised for 48 h with a Lio 5P freeze-dryer (5Pascal Srl, Italy).

The PHAs values were calculated according to the following equation:

$$PHA\ (\%) = \frac{gPHA}{gVSS} \bullet 100$$

where gPHA and gVSS were, respectively, the grams of PHAs and of the volatile suspended solids of the biomass. Overall PHA production was calculated as gPHA/L.

The PHAs production performance from protein hydrolysate was calculated with the following equation when considering the initial COD of the hydrolysate:

$$YPHA\ (\%) = \frac{gPHA}{gCOD} \bullet 100$$

Or as follows when considering the VFA obtained after the fermentation:

$$YPHA\ (\%) = \frac{gPHA}{gVFA} \bullet 100$$

Qualitative chemical analysis and the identification of functional groups in the PHA samples were performed using a Fourier transform infrared (FT-IR) instrument (Thermo Scientific™ Nicolet™ iS™50 FT-IR Spectrometer), which utilised a diamond crystal as the internal reflection element. The attenuated total reflection (ATR) FT-IR spectra were obtained by plotting the transmittance [%] of the material against the wavenumber [cm^{-1}], in the range of 4.000–450 cm^{-1}.

Differential scanning calorimetry (DSC - TA Instruments DSC Q200) allowed to investigate physical changes of the material in response to temperature by measuring the heat flow absorbed or released by the material against temperature variation. The samples (about 5 mg), placed in an aluminium pan, were cyclically heated and cooled between −80 °C and 200 °C under a nitrogen atmosphere, using 10 °C/min as heating rate and 5 °C/min as cooling rate; the first heating cycle

Table 2
Physico-chemical characterisation of the protein hydrolysates.

HYDROLYSATE	pH	DENSITY (kg/L)	TVS (g/g)	TKN (g/L)	NH_3 (g/L)	TP (g/L)	tCOD (g/L)	bCOD (g/L)	bCOD/tCOD (%)
Soybean	5,5	1,18	476,21	48,0	5,8	1,79	593,35	75,06	12,65
Alfalfa	5,0	1,16	255,36	20,7	8,6	2,45	366,84	218,25	59,49
Rice bran	4,0	1,26	625,82	27,3	<1,0	1,29	924,79	261,70	28,30
Animal epithelium	5,5	1,20	582,48	101,8	<1,0	–	783,16	88,96	11,36

TVS = total volatile solids, TKN = total Kjeldahl nitrogen, NH_3 = ammonia, TP = total phosphorus, tCOD = total COD, bCOD = biodegradable COD. The amount of phosphorous was not possible to record for the animal epithelium due to interferences with the kit. Total and soluble COD (sCOD) had the same values, therefore the sCOD is not reported.

Table 3
VFAs detected in the protein hydrolysates.

HYDROLYSATE	ACETIC (gCOD/L)	Butyric (gCOD/L)	Isobutyric (gCOD/L)	Pentanoic (gCOD/L)	TOTAL VFAs (gCOD/L)
Soybean	14.258,12	427,44	0,00	492,95	15.178,51
Alfalfa	2.628,91	108,80	0,00	0,00	2.737,71
Rice bran	2,099,01	0,00	55,07	0,00	2.154,08
Animal epithelium	918,46	225,11	79,53	0,00	1.223,10

was used to exclude the effect of the prior thermal history of the samples. The melting temperatures (T_m) of the crystalline fraction of the samples were determined as corresponding to the maximum of the endothermic peak in the heating cycle.

Thermogravimetric analysis (TGA - TA Instruments SDT Q600) provides a thermal characterisation of the material by measuring its change in mass as a function of temperature. The decomposition temperatures of the organic fraction of the sample and the inorganic residue are recognisable in the resulting thermogram. The analysis was carried out in an oxidising atmosphere (in air), obtaining a thermo-oxidative decomposition. The samples (about 10 mg) were heated from room temperature up to 1000 °C, using 20 °C/min as heating rate.

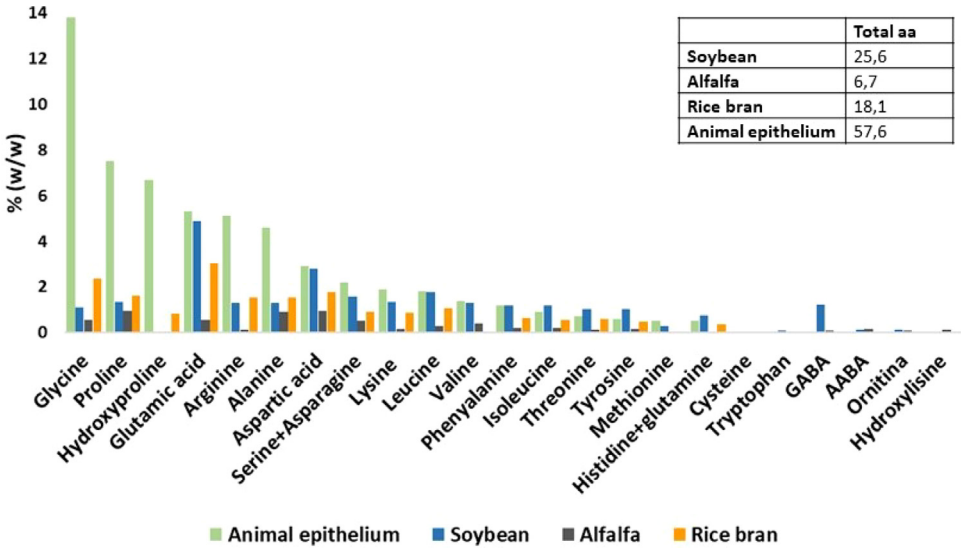
3. Results and discussion

3.1. Protein hydrolysates characterisation

The protein hydrolysates of vegetal and animal origin, before any fermentation process, were characterised for their physico-chemical values as shown in Table 2.

The pH of all the hydrolysates resulted acidic in the range between 4,0 and 5,5, with the lowest value recorded for the rice bran and the highest for the soybean. Density was similar between the samples, the rice bran having the highest value of 1,26 kg/L. TVS values indicate the presence of useable organic material in the hydrolysates, especially for the animal epithelium (582,48 g/g) and the rice bran (625,82 g/g). The TKN was much higher in the hydrolysate obtained from the animal epithelium (101,8 g/L), a result that was predictable due its origin, as PHs derived from by-products of the tanning process are known to be rich in *N* (Ciavatta et al., 2012; Ertani et al., 2009); the low level of N-NH_3 in this sample (<1,0) however indicates that most of the *N* is found in organic form and therefore ammonification has only partially occurred. The soybean hydrolysate had nitrogen amounts higher than the other vegetal hydrolysates (48,0 g/L), while expected lower values were found for alfalfa and rice bran (20,7 and 27,3 g/L respectively). The N-NH_3 fraction was however higher in alfalfa and soybean than in the other two PHs. It was not possible to record the value of TP for the animal epithelium due to interferences with the kit used for the analysis; however tannery by-products are reported to normally contain moderate amounts of P (Ciavatta et al., 2012) and a TP of 30 mg/g has been previously reported for animal PHs (though of different nature from those produced from wet blue shavings), and of 10 mg/g for alfalfa hydrolysates (Ertani et al., 2009). The recorded values for the sCOD indicate that all the hydrolysates were rich in organic compounds and therefore represented good substrates to produce VFA by fermentation, with rice bran having the highest sCOD (924,79 g/L). Soluble and total COD had the same values indicating that all the organic matter was present in a solubilised form and therefore readily available. The biodegradable COD (bCOD) was evaluated via respirometry and the calculation of the bCOD/sCOD ratio (Table 2) suggests that the most appropriate substrate for a rapid VFA production was alfalfa (59,49%), followed by rice bran (28,30%). VFAs were present in the PHs before the fermentation trials (Table 3), with soybean showing the highest concentration (15.178 g/L). Acetic acid was by far the most abundant VFA for all the PHs, butyric acid was found in much lower quantities in all the hydrolysates excluding rice bran, isobutyric acid was detected only in the animal epithelium and rice bran hydrolysates, while pentanoic acid was found only for soybean. Propionic, caproic and isocaproic were not recorded in any PH and are therefore not reported in Table 3.

The amino acidic profile was determined for the four hydrolysates and is shown in Fig. 2. The animal epithelium was the richest in amino acids (57,6% of the dry weight), as normally reported for animal-based PHs (Ertani et al., 2009). Glycine was the most abundant amino acid (13,4%), being almost double than proline (7,5%) and hydroxyproline, a result



	Total aa
Soybean	25,6
Alfalfa	6,7
Rice bran	18,1
Animal epithelium	57,6

Fig. 2. Amino acids profile of the four hydrolysates. The percentage shown is calculated as g of amino acids over 100 g of dry sample. GABA $= \gamma$-aminobutyric acid, AABA $= \alpha$-aminobutyric acid, aa $=$ amino acid.

that is in line with what reported for other animal PHs and in particular for those produced from by-products of the leather industry (Colla et al., 2017). The vegetal hydrolysates instead had a lower content of amino acids (from 6,7% in alfalfa to 25,6% in soybean), but they all had glutamic and aspartic acid among the most represented amino acids, as normally reported for legume derived PHs (Colla et al., 2015; Ertani et al., 2009).

3.2. Fermentation trials

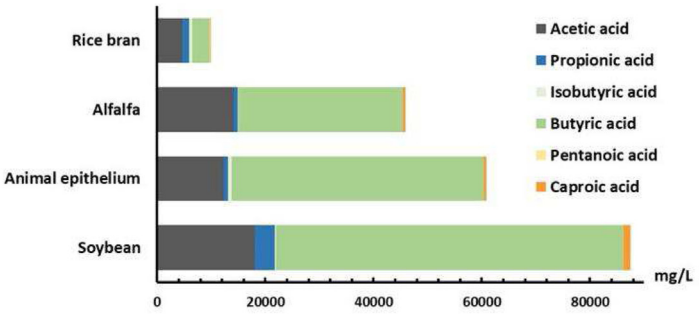
Fermentation trials were performed with the four PHs, to assess if those substrates are suitable to be converted by microbial action into easily assimilable organic material, such as VFAs. The fermentation with MMC in anaerobiosis lasted three days, and the amount and composition of VFAs in the fermented PHs at the end of the trials is presented in Fig. 3. The fermentation process resulted in a significant increase in VFAs amount for all the PHs, up to almost 50 times for the animal epithelium and 17 times for alfalfa (Fig. 3, last column in the table). This result is not surprising as these two PHs had high amount of amino acids (see table in Fig. 2), in particular methionine; indeed, previous works have shown how bacterial growth and fermentative yields are higher when supplements such as amino acids and vitamins are provided, since amino acid production is energy intensive and can be a burden for the cell, with methionine being the amino acid which consumes the most amount of ATP (Tripathi et al., 2019).

The VFAs composition also changed, with a shift from acetic to butyric acid as the most abundant VFA, to the appearance of propionic and caproic acids, which were not detected before the fermentation took place. When comparing the yield of the process in term of VFAs produced over sCOD consumed (Fig. 3, table), the best performing PH resulted soybean and alfalfa, with a yield of 33.4% and 23.6% respectively.

In order to assess whether the obtained fermented PHs were appropriate for the growth of PHA-producing bacteria, the PHs' composition in carbon (C) and nitrogen (N) was investigated, with particular attention to the C/N ratio, which is important for allowing bacterial PHAs production. The results (Table 4) indicate that all the samples had a high *N* percentage, and in turn a low C/N ratio when considering both total *N* and organic N, with animal epithelium having the highest nitrogen amounts and lowest C/N ratio. The most favourable conditions for the accumulation of PHAs are reported to be those under nutrients limitation, however recent research suggests that extreme or prolonged nitrogen deficiency could instead suppress PHAs production (Johnson et al., 2010) and that good PHAs accumulation can be obtained with C/N similar to those recorded for the fermented PHs of this study (Ahn et al., 2015; Valencia et al., 2021).

3.3. Growth test

The growth's ability on the PHs for the PHA-accumulating bacterium *Thauera* sp. Sel9 were assessed by inoculating with this strain the fermented PHs after sterilisation and growing the cultures in batch for four days. The three plant hydrolysates provided useable sCOD for the growth of the bacterial cultures (Fig. 4, MLVSS on top figure and sCOD middle figure), while there was no significant growth for the animal epithelium in the evaluated timeframe. The best growth yield (Fig. 4, bottom figure) was obtained for both the Fabaceae hydrolysates, with a maximum value of 0,52 gCOD/gCOD on day one, which is not far off the maximum theoretical value of 0,67 gCOD/gCOD (Metcalf et al., 2014). This probably reflects



HYDROLYSATE	ACETIC (mg/L)	PROPIONIC (mg/L)	ISOBUTYRIC (mg/L)	BUTYRIC (mg/L)	PENTANOIC (mg/L)	CAPROIC (mg/L)	TOTAL (mg/L)	VFA YIELD (%)	VFA INCREASE (times)
Soybean	18.039,90	3.666,55	258,28	64.344,80	0,00	1.164,14	87.473,68	33,40	5,76
Alfalfa	14.067,96	856,72	86,72	30.501,92	0	397,55	45.910,86	23,60	16,77
Rice husk	4688,38	1186,27	593,71	3305,85	72,24	142,99	9989,43	4,00	4,64
Animal epithelium	12261,77	820,17	739,18	46591,83	0,00	490,08	60.903,03	21,70	49,79

Fig. 3. VFA amount and composition obtained after the fermentation of the four protein hydrolysates.

Table 4
Characterisation of the hydrolysates after fermentation with MMC.

HYDROLYSATE	TOTAL C (%)	TOTAL N (%)	$N\text{-}NH_3$ (%)	$N\text{-}nO_3^-$ (%)	ORGANIC N (%)	C/N_{tot}	C/N_{org}
Soybean	9,1	2,1	0,7	<0,1	1,4	4,3	6,5
Alfalfa	6,4	1,1	0,6	<0,1	0,5	5,8	12,8
Rice bran	8,3	0,9	0,4	<0,1	0,5	9,2	16,6
Animal epithelium	9,8	3,6	0,6	<0,1	3,0	2,7	3,3

the utilisation of the biodegradable component of the COD, while a yield decrease was recorded in the following days once this fraction was exhausted. The yield for the animal epithelium was low as a consequence of the little COD used and of the minimal cell growth recorded. Rice bran's yield instead peaked at day two and then remained mostly stable.

The absence of growth for the animal epithelium is surprising, since it was the most promising PH, being rich in amino acids and proteins and having high values of tCOD; it also showed increased amounts of VFAs after fermentation. It is important to valorise the animal epithelium, since the leather industry is one of the most polluting industries worldwide (Ozgunay et al., 2007; Thanikaivelan et al., 2005), producing high amount of liquid and solid wastes; being able to obtain value-added products from the by-products of this process would be a way to exploit the leather industry waste (Stefan et al., 2012). While we exclude any toxic effect caused by the presence of chromium as a residue of the tanning process can be excluded, since it is present in traces and only in its safe state as Cr(III) (Ciavatta et al., 2012), possible inhibiting effects should be investigated such as those caused by too high nitrogen concentration (Mozumder et al., 2014), given that TKN values were indeed the highest in the animal epithelium. Particular attention to the preferred growing conditions of *Thauera* sp. Sel9 should be given, which at the moment are not available in the published literature. The growth of a different species of PHA-producing microorganism, or of MMC, could be an alternative to overcome the difficulties in growing *Thauera* sp. Sel9.

3.4. PHAs production and characterisation

PHAs production was assessed for the alfalfa hydrolysate, since it was the one that performed better in the overall process, from its starting characteristics (best bCOD/tCOD ratio of the four PHs, therefore more COD readily available for fermentation) to the productivity during the fermentation trials (second best VFA yield) and the growth with *Thauera* sp. Sel9 (good growth yield at short growing time). PHAs accumulation was performed in a respirometer, where oxygen consumption by the *Thauera*'s culture was used to determine the correct timing for a feeding spike with the fermented alfalfa hydrolysate, in order to guarantee an excess of VFAs that could favour the accumulation of PHAs. Fig. 5 (top) illustrates the variation in dissolved oxygen and in the oxygen uptake rate (OUR) during the 250 min of the experiment, which involved nine different feeding spikes every 30 to 21 min due to the fast OUR (up to 63.02 mgO_2/Lxh). The experiment was halted once the OUR decreased to the value of 24.54 mgO_2/Lxh).

The analysis of the samples collected at each spiking point (Fig. 5, bottom) allowed the detection of a decrease in the percentage of PHAs in the bacterial cells, probably a consequence of the excess of nitrogen and phosphorous in the feed,

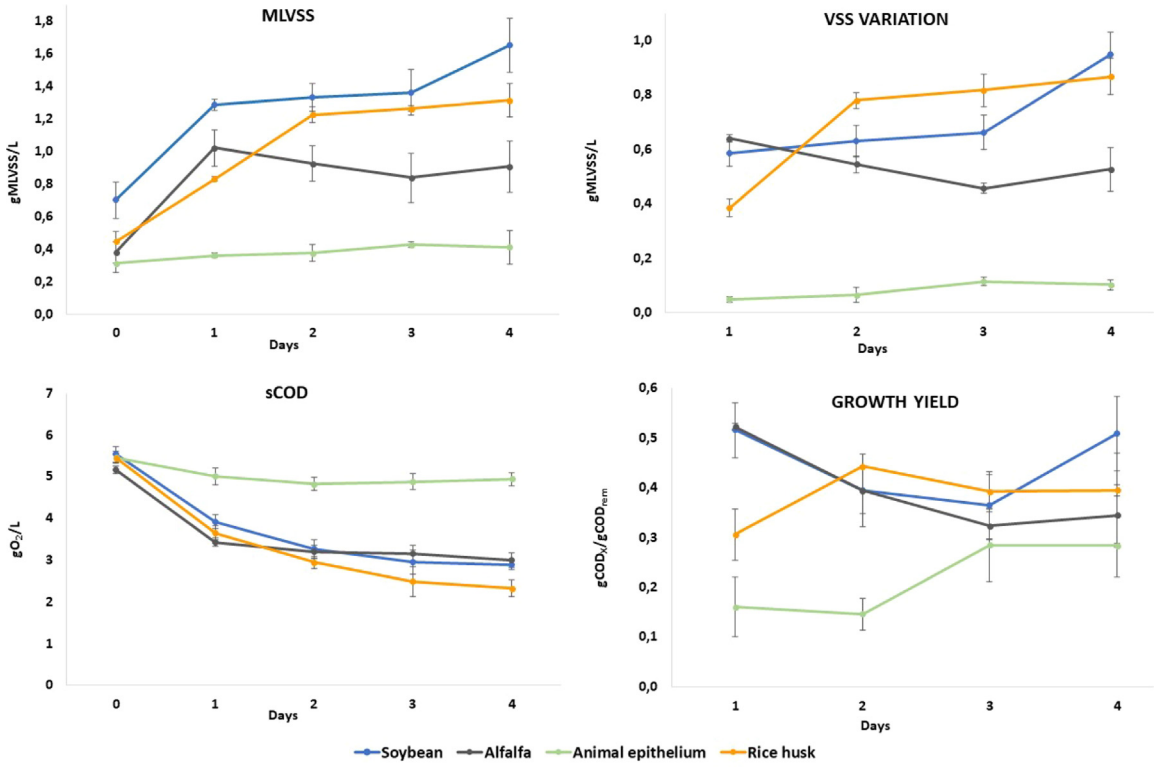


Fig. 4. Growth test on the four PHs with *Thauera* sp. Sel9. Growth was recorded as variation of the volatile suspended solids (VSS) in the top left graph, while the sCOD utilisation was reported in the bottom left graph. The right graphs illustrate on the top the cumulative variation of VSS in the culture, while at the bottom the growth yield. The bars represent the standard deviation.

which did not allow the best conditions for PHAs accumulation but on the contrary were optimal for bacterial growth (Tan et al., 2014). As a consequence, the culture productivity increased throughout the experiment (from 0.8 to 3.6 gSS/L), with the PHA productivity reaching 1.4 g/L at the end of the 250 min, showing that PHAs can be accumulated not only by increasing their amount in the bacterial cells, but also by simple bacterial growth. The maximum productivity of PHAs was not reached, however this project's aims were to evaluate the overall process feasibility from the initial PHs to the final PHAs production, rather than obtaining the highest amount of PHAs possible. When considering the overall process, from the Alfalfa protein hydrolysate to the production of PHAs, the productivity over the COD available in the hydrolysate is of 8%. However, since only a part of the COD present in the hydrolysates is available for the biological process, a more realistic calculation is obtained when considering the COD given only by the VFAs. In this case, the productivity reached with this project is of 32%, which is in line with similar processes, where PHAs were however obtained from wastewaters and using MMC (Conca et al., 2020).

The biomass collected at the end of the PHAs accumulation experiment was freeze-dried and subjected to analysis by gas chromatography (GC), Fourier-transform infrared spectroscopy (FT-IR), thermogravimetry (TGA) and differential scanning calorimetry (DSC), to assess the PHAs composition and characteristics.

The infrared spectrum of the extracted PHAs sample is shown in Fig. 6 A. The transmittance band located at 1720 cm^{-1} is attributed to the stretching vibration of the C=O group (ester carbonyl) in the PHA polyester, whereas C–O stretching bands were in the spectral region from 1055 to 1280 cm^{-1}. The peaks at 2976 and 2918 cm^{-1} correspond to stretching vibration of C–H bonds of methyl (CH_3) and methylene (CH_2) groups, besides C–H bending vibrations which appeared at 1452 and 1379 cm ^{1}. The peak at 3379 cm^{-1} revealed the intramolecular polymeric hydrogen bond (O–H), related to the presence of residual humidity in the sample. These results are consistent with previous studies (Bai et al., 2015; Shamala et al., 2009). The collected FT-IR spectrum was compared with the ones of the standard PHB-HV (Aldrich FT-IR Collection Edition II library) at the concentration of 14% and 22% (Fig. 6B) and the result showed a high match between the PHA obtained from the alfalfa protein hydrolysate and the polyester of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHB-HV).

Fig. 6C shows the thermogram of the second heating cycle and the cooling cycle of the DSC analysis. A bimodal endothermic melting peak (152 °C, 163 °C) was detected in the second heating cycle; this behaviour suggests that the poly(3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV) sample contains two crystalline domains with different sizes of

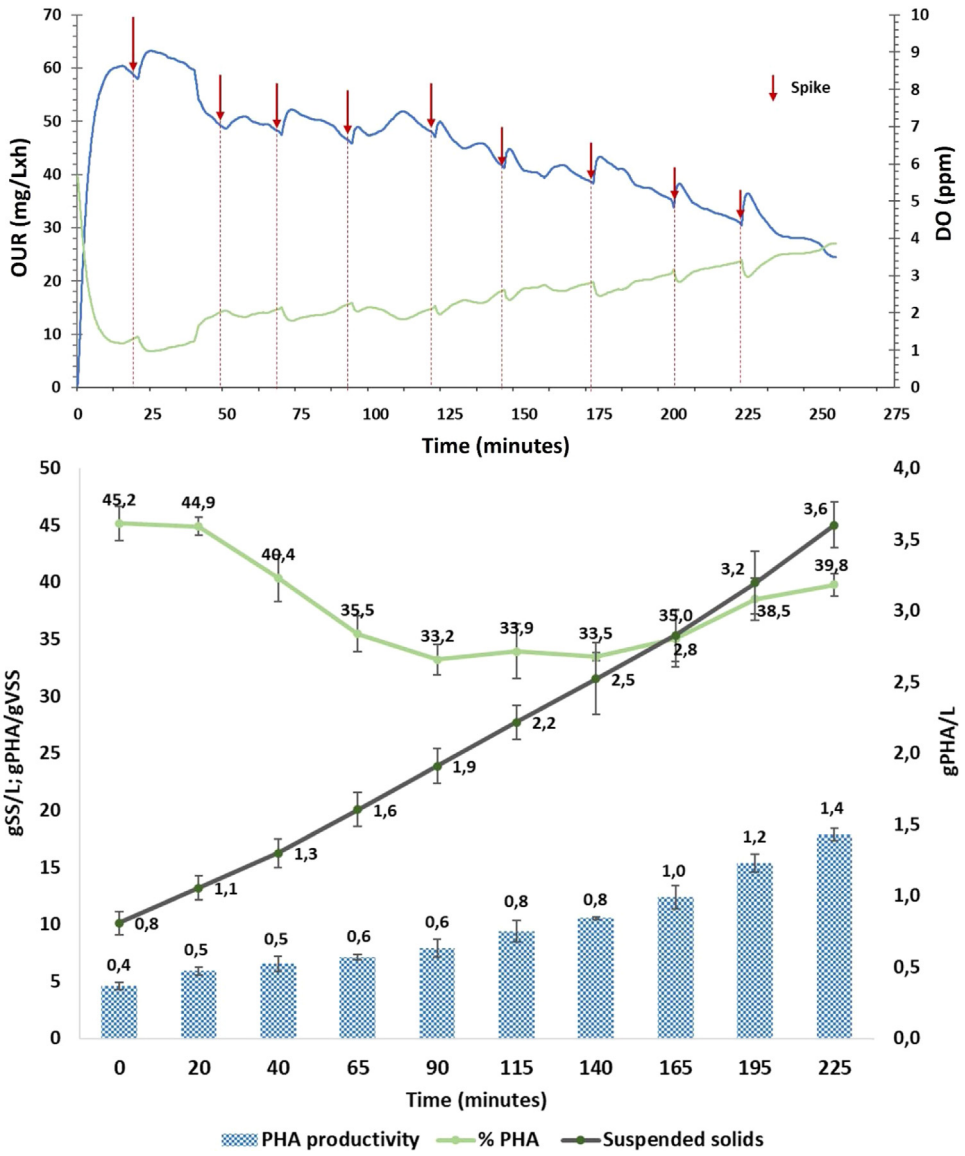


Fig. 5. PHAs accumulation for the alfalfa PH fermented with *Thauera* sp Sel9. Top figure, respirometry results. OUR = oxygen uptake rate, DO = dissolved oxygen. The red arrows indicated the time points at which a feeding spike was performed. Bottom figure: PHA accumulation results. The lines show the trend of the suspended solids (grey line) and of the percentage of PHA calculated on the dry mass (gPHA/gVSS, green line), while the bar illustrates the variation of the amount of PHAs in the culture (gPHA/L). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

lamellae of varying thickness, having different melting temperatures (Kuntanoo et al., 2015; Ponnusamy et al., 2019; Takahashi et al., 2012).

The thermo-oxidative degradation, detected with TGA (Fig. 6D), began at 230 °C (considering a weight loss of the sample of 2% at the beginning of the degradation) and was almost complete at 275 °C (with a residual weight of 6%), consistent with previous studies (Muniyasamy et al., 2016).

At the end of the analysis there were no inorganic residues, suggesting that the sample was entirely made of organic material. The absence of residues suggests a high level of purity of the organic extracted PHA.

4. Conclusions

Agricultural residues and animal by-products of various origins can be transformed into protein hydrolysates and used as end products in the form of food and feed additives, pharmaceuticals and biostimulants (Colla et al., 2015; Nasri, 2017).

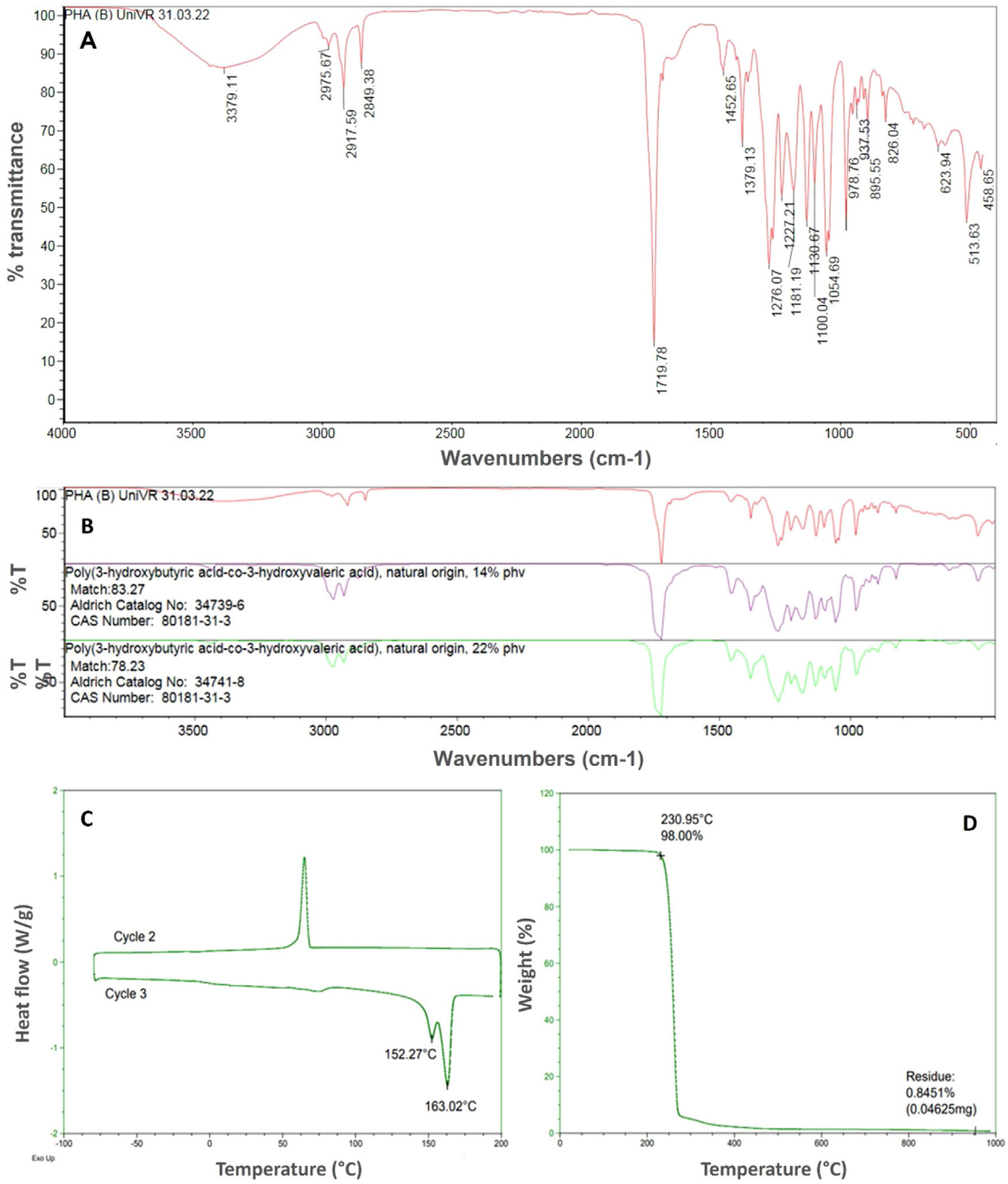


Fig. 6. Characteristics of the PHAs samples. (A) FT-IR spectrum. (B) FT-IR spectrum compared to that of the standard at the concentration of 14% and 22%. (C) DSC curve, first heating cycle. (D) TGA thermogram.

With this work we have demonstrated that it is possible to further valorise these protein hydrolysates and use them as feedstocks to produce bioplastics via fermentation with MMC and PHAs-producing bacteria. This alternative pathway for the use of PHs (and originally of agricultural residues) can be an option for industries willing to diversify their portfolio and to invest in products with low environmental impact and with the potential to reduce the volume of waste. The work presented in this paper is only preliminary and is intended to show the feasibility of the process, while it does not include a necessary economic assessment and a comparison of the value of the final product (PHAs) to those already produced from PHs. The produced PHBV is interesting in light of its purity, but also for its biocompatibility and biodegradability, making it an ideal eco-friendly substitute to fossil-based plastics.

CRediT authorship contribution statement

Paola Critelli: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualisation. **Giovanna Pesante:** Validation, Formal analysis, Curation, Writing – original draft, Writing – review & editing, Visualisation, Supervision. **Stefania Lupinelli:** Conceptualization, Resources, Writing – review & editing. **Michele Modesti:** Investigation, Data curation, Writing – review & editing. **Silvia Zanatta:** Investigation, Data curation, Writing – review & editing. **Federico Battista:** Supervision, Project administration, Writing – review & editing. **David Bolzonella:** Supervision, Project administration, Writing – review & editing. **Nicola Frison:** Conceptualization, Methodology, Validation, Resources, writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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