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Polyhydroxyalkanoated-Rich Microbial Cells from Bio-Based Volatile Fatty Acids as Potential Ingredient for Aquaculture Feed

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Abstract: In this study, the production of polyhydroxyalkanoated PHA-rich microbial biomass as a novel feed additive in aquaculture was investigated at a lab-scale. Bio-based volatile fatty acids (VFAs), obtained from the acidogenic fermentation of agricultural residues in existing anaerobic digestion plants, were used as carbon and energy to cultivate the PHA-rich microbial biomass. The experimental activities were carried out using *Thauera* sp. Sel9 as pure strain, which was grown in a continuous stirred-tank reactor (CSTR) operated at three different hydraulic retention times (HRT). The highest productivity obtained of biomass cells was 0.69 g/L day, operating at one day HRT while the observed PHAs production yield was 0.14 gPHA/g soluble COD removed. At these conditions, the PHA concentration in the microbial cells was 41%. Although the sulfur amino acids were available at high concentrations and above the typical concentration found in fishmeal, the amino acids profile of the obtained biomass revealed a lack of histidine and threonine. A preliminary economic analysis showed that the production of a novel source of feed additive from the conversion of agro-residues could give higher benefits in terms of revenues compared to the production of biogas production through anaerobic digestion.

Keywords: polyhydroxyalkanoates; feed; aquaculture; anaerobic digestion; single cell protein

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1. Introduction

The world population is expected to increase up to 9.8 billion human beings in 2050 [1], which will generate inevitable anthropogenic pressure on Earth to satisfy the increase of food demand. The United Nations Food and Agriculture Organization (FAO) estimated that the global demand for animal-derived protein will double by 2050 [2], resulting in general concern for protein-rich food security. Moreover, agriculture contributes to climate change because it is responsible for 10.3% of the GHG emissions (mainly methane and nitrous oxide) emitted in the European Union (EU) and nearly 70% of those come from the animal sector [3]. The Carbon Trust document [4] reported a land occupation for soy protein production of 6.7 m^2/kg of protein produced which results in 68% of the total agricultural land used for animal production [5]. Therefore, plant-based proteins cannot be the solution in the present scenario.

In order to reduce the environmental and climate impact of animal protein-based production, a transition towards more sustainable livestock farming is required. This can be done by placing on the market sustainable and innovative feed additives, besides a transition from animal to plant-based diets [6].

Microorganisms have always been closely related to food processing, associated with several benefits. For example, in the fermentation processes for dairy product manufacturing, the lactic acid bacteria are the main actors. Microorganisms, such as bacteria, fungi,

yeast, and algae can also be used directly as a feed or food additive [7,8]. These proteins produced with dead microorganisms (such as yeast, bacteria, fungi, or algae) are known as microbial proteins (MPs) or single-cell proteins (SCPs). The Imperial Chemical Industries (ICI) was the first to produce a full scale and commercialized MP called Pruteen® [9]. A whole range of other possibilities was further investigated; however, the low price of conventional proteins for animal feed (such as soybean and fishmeal) prevented development in the market of alternative proteins. In recent years, conversely, the increase of the fishmeal price, together with the environmental concerns about the use of land for soybean, has justified the renewed interest in research and development around SCPs [10]. A well-studied example of bacteria able to produce high-quality protein, both for human and animal consumption, is represented by hydrogen-oxidizing bacteria [11,12]. Whereas soybeans have a protein content in the range of 45–57% on dry matter (DM) [13], microorganisms can have comparable or higher values. For instance, seaweed, spirulina, fungi, and bacteria have 40–60%, 50–60%, 30–70%, and 50–80% of protein on DM, respectively [14–18].

Nowadays, the agricultural residues represent a consistent amount of waste generated in the EU which can be rethought and reused as a resource to generate new products. These agro-wastes are mainly animal and vegetal residues that cannot be further processed into food or feed and need some kind of treatment to reduce their environmental burden. The typical practice adopted at the EU level is the treatment of these materials via anaerobic digestion for biogas and renewable energy production, so that 15,000 farm-based anaerobic digestion (AD) plants are in operation in Europe [19]. However, new approaches aim at shifting the use of agro-residues and livestock byproducts for the production of biogas to the upgrade of new building blocks for the chemical and food industry [20]. In the last decades, the production of bio-based volatile fatty acids (VFAs) combined with their biological conversion to polyhydroxyalkanoates (PHAs) was deeply suggested and investigated as a potential sustainable route to replace fossil-based plastic [21–24]. However, the main impediment in the cost-effective production of PHAs is the downstream recovery and purification step, which could account for up to 50% of the total process cost [25]. With this in mind, in this work, the production of PHA-rich microbial cells was considered as a potential ingredient for aquaculture feed in order to avoid expensive downstream processes for PHAs extraction and purification. PHA-rich microbial cells were produced from an isolated strain using bio-based volatile fatty acids (VFAs) obtained by the acidogenic fermentation of agro-residues and livestock effluents. The production rate of PHA-rich microbial biomass was evaluated under three different hydraulic retention times (HRT), while the related content of proteins and amino acid composition were compared with the typical protein source available in the market for aquaculture. Finally, a preliminary economic assessment was carried out to evaluate a scenario where existing biogas plants are upgraded into advanced biorefineries that produce a novel source of proteins instead of biogas by the valorization of agro and zootechnical residues.

2. Materials and Methods

2.1. Experimental Periods

The strain used in this study for the production of PHA-rich microbial biomass was selected and isolated from a mixed microbial culture (MMC) producing PHAs as described in Conca et al. [22]. The microbiological analysis revealed a 99% similarity with *Thauera butanivorans* NBRC 103042T (from here referred to as "*Thauera* sp. Sel9"). The 16S rRNA gene (1392 bp) of the bacterial strain was amplified by using primers fD1 and rp2 [26], sequenced by Eurofins Genomics, and further screened using the EzBioCloud database [27]. The sequence analysis revealed a 99% similarity with *Thauera butanivorans* NBRC 103042T (from here referred to as "Thauera sp. Sel9").

The growth rate and productivity of *Thauera* sp. Sel9 was evaluated in a Continuous Stirred-Tank Reactor (CSTR) with 1 L as working volume, equipped with an air supply system in order to keep the oxygen concentration (DO) above 2 mg/L. During the experiments, the CSTR was kept in an incubator at 20 °C and fed with fermentation liquid enriched

with bio-based VFAs produced by the acidogenic fermentation of agricultural residues and cattle manure according to Righetti et al. [20]. Briefly, grass silage and cattle manure were mixed and then diluted with the processed water in order to obtain a final total solid concentration of around 8%. The mixture was homogenized in a storage tank and then fed to the acidogenic fermentation unit for the production of VFAs. More details related to the acidogenic fermentation process were reported in Righetti et al., [21]. The fermentation liquid was filtrated at 0.20 µm in order to remove all the suspended solids and sources of biological contaminations. Then, the fermentation liquid was diluted with distilled water in order to achieve a concentration of soluble COD of 4–5 gCOD/L where up to 20% of this fraction were bio-based VFAs. The experimental set-up of the CSTR is presented in Figure 1.

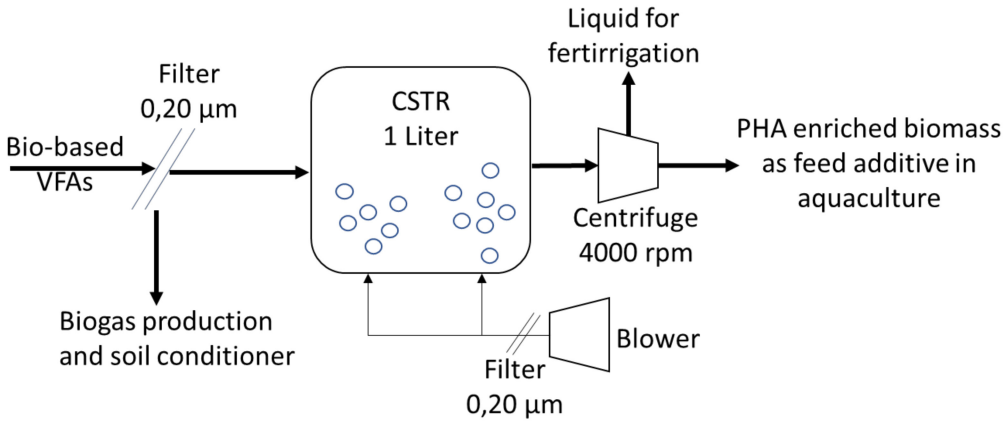

Figure 1. Experimental set-up used for the production of PHA-rich microbial biomass.

The overall experimental period lasted for 60 days, where the productivity of PHA enriched cell was evaluated under three different hydraulic retention times (HRTs), four days for Period 1, two days for Period 2, and one day for Period 3. Steady-state conditions were considered when the variations of the MLSS concentration were less than 5% for approximatively 3× solid retention time (HRT) of the bioreactor.

2.2. Calculations

The concentration of the active biomass was calculated by subtracting the MLVSS value with the PHA concentration. Each operating condition was compared based on biomass productivity (P_{MLVSS}) and yields of the active biomass and PHA production calculated according to the Equations (1)–(5) reported in Table 1:

Table 1. The calculation for production.

Acronym	Calculation	Unit	Equation
P_{MLVSS}	$\frac{MLVSS}{HRT}$	$\frac{g}{L\ day}$	(1)
$Y_{X/sCOD}$	$\frac{MLVSS - PHA}{CODs\ removed}$	$\frac{gX}{gCOD}$	(2)
$Y_{X/VFAs}$	$\frac{MLVSS - PHA}{VFAs\ removed}$	$\frac{gX}{gCOD_{VFA}}$	(3)
$Y_{PHA/sCOD}$	$\frac{PHA\ produced}{COD\ removed}$	$\frac{gPHA}{gsCOD}$	(4)
$Y_{PHA/VFAs}$	$\frac{PHA\ produced}{VFAs\ removed}$	$\frac{gPHA}{gCOD_{VFA}}$	(5)

2.3. Characterization of the PHA-Rich Microbial Biomass

The effluent from each operating condition was evaluated for mixed liquor suspended solids (MLSSs), mixed liquor volatile suspended solids (MLVSSs), and soluble COD (sCOD) according to the standard methods [28,29]. The VFA concentrations included the sum of acetic, propionic, butyric, isobutyric, pentanoic, isopentanoic HPt, iso-HPt, HHe, and hep-

tanoic acid. The concentration of each type of acid was determined by ion chromatography through a Dionex ICS-1100 (Thermo Fisher Scientific, USA) equipped with IonPac ICE-AS1 as the column, as presented in Conca et al. [22].

The biomass cells effluent from the bioreactor were analyzed for their PHA content. Each sample of biomass cell (5.0 mL) was pretreated using a sodium hypochlorite solution (1.0 mL; 5% active chlorine) and placed in a freezer at −20 °C. The samples containing PHAs were treated with 2.0 mL of the acidified methanol solution containing benzoic acid (0.05 g/L) as an internal standard with 1.0 mL of $CHCl_3$ and digested for 4 h at 100 °C. After cooling, two separate phases (i.e., aqueous and organic) were obtained by the addition of 1.0 mL of distilled water. The quantification of the PHAs was performed by the injection of 1.0 µL of organic phase in the gas chromatograph based on the method illustrated in Braunegg et al. [30]. During the best operating conditions with the highest productivity, the PHA-rich microbial biomass was freeze-dried after centrifugation (4000 rpm) and characterized for Total Kjeldahl Nitrogen (TKN). Then, the obtained value was multiplied by the factor 6.25 in order to indirectly calculate the content of proteins [28,29] Finally, the amino acid composition of the whole mixture (freeze-dried samples) was estimated following the procedure described by Silva et al. [31].

3. Results and Discussion

3.1. Production of PHA-Rich Microbial Biomass

The effect of different growth rates applied to *Thauera* sp. Sel9 were evaluated at the steady-state conditions which were observed after three times the applied HRT.

In period 1, after 20 days of operation, the concentration of MLSS effluent was less than 0.5 g/L (Table 2), which resulted in the lowest productivity of biomass obtained during the experiments (0.42 g/L day).

Table 2. Concentration of soluble COD, VFAs, MLSS, and PHAs in the effluent of the CSTR.

Parameter	Unit	Period 1 (Days 1–22)	Period 2 (Days 23–48)	Period 3 (Days 48–60)
Soluble COD	gCOD/L	0.64	1.42	2.14
VFAs	mgCOD/L	<5	<5	190 ± 10
MLSS	g/L	0.43	0.92	0.69
PHAs	mg/L	64	285	283

The removal efficiency of the sCOD was around 82% which corresponded to a concentration of 0.64 gCOD/L, while the VFAs were almost completely removed (99%). In period 1, the relatively low substrate loading rate promoted more endogenous conditions, which resulted in a relatively low yield in terms of biomass growth (0.17 gX/g soluble COD removed). However, the growth yield of the active biomass could be affected also by the high COD/N ratio influent, which limited the available nutrients required for the growth of the active biomass. A previous study [32] reported high acetate uptake rates with carbon-limited conditions (medium C/N ratios 6–13.2 Cmol/Nmol), while nitrogen-limited SBRs (medium C/N ratios 15–24 Cmol/Nmol) were characterized by high ammonia uptake rates. Moreover, the bacteria cell growth in strongly nitrogen-limited SBRs showed higher baseline PHA contents, while carbon-limited conditions resulted in biomass cells with higher maximal PHA storage capacities.

In periods 2 and 3, the productivity of MLSS increased to 0.92 and 0.69 g/L per day, respectively (Table 2), although the observed growth yields of active biomass did not change significantly compared to Period 1. In these periods, the removal efficiency of soluble COD decreased to 66% and 56%, respectively, by the decrease of the HRT from two days to one day.

In all the experimental periods, nutrient limiting conditions allowed the conversion of VFAs into PHAs, which were stored at different concentrations according to the applied HRT (Table 3).

Table 3. Summary of the main results obtained during the HRT tested with *Thauera* sp. Sel9.

Parameter	Unit	Period 1 (Days 1–22)	Period 2 (Days 23–48)	Period 3 (Days 48–60)
Influent				
Total COD [g/day]	gCOD/day	0.87 ± 0.01	2.10 ± 0.2	4.90 ± 0.2
COD_{VFA} [g/L day]	gCOD/day	0.20 ± 0.01	0.51 ± 0.09	0.75 ± 0.16
Effluent				
Soluble COD [g/L day]	gCOD/day	0.16 ± 0.02	0.71 ± 0.3	2.14 ± 0.3
VFA [g/L day]	gCOD/day	<0.01	<0.01	0.19 ± 0.02
MLSS [g/L day]	gMLSS/day	0.107 ± 0.02	0.460 ± 0.14	0.690 ± 0.13
Removal efficiency				
sCOD [%]	%	82%	66%	56%
COD-VFAs [%]	%	99%	99%	74%

Period 1 resulted in the lowest concentration of PHA in the bacteria cells (15% based on MLSS) due mainly to the longer HRT applied, which allowed more time for the degradation of storage compounds under low substrate availability. On the other hand, in this period, the observed production yield of PHA was 0.08 g PHA/g CODs removed, which is rather low compared with the findings from a similar study [23]. In period 2 and period 3, the concentration of PHAs increased up to 31 and 41% (based on MLSS), respectively, while the concentration in the effluent did not change significantly (Table 2). On the other hand, the observed PHA production yield increased up to 0,14 gPHA/g soluble COD removed, which was seven times more than period 1 and 1.4 times more than period 2 (Table 4). Considering that in period 3 the VFAs fraction was around 20% of the soluble COD influent and the VFAs removal efficiency was 74%, the observed PHA production yield based on the VFAs removed (74% removal efficiency) was 0.39 $gPHA/gCOD_{VFAs}$. Diniz et al. [33] found a PHA production yield ranging from 0.15 and 0.19 g PHA/g carbohydrate consumed by a pure culture of *Pseudomonas putida* fed with glucose and fructose as carbon source, under limitation of nitrogen and phosphorus. However, in this work, the carbon source used was bio-based VFAs, which are considered the main chemical precursors for the production of PHAs.

Table 4. Yields of X, PHAs, and %PHA based on MLSS.

Period	Y_X	$Y_{PHA/VFA}$	PHAs
	g X/gCODs	$gPHA/gCOD_{VFAs}$	%MLSS
Period 1 (days 1–22)	0.13	0.08	15
Period 2 (days 23–48)	0.22	0.28	31
Period 3 (days 49–60)	0.17	0.39	41

3.2. Nutritional Value of the PHA-Rich Microbial Biomass

The PHA-rich microbial biomass obtained in Period 3 contained around 6.5% nitrogen, which correspond to around 41% of microbial protein based on MLSS. This value is in line with the protein contained in the soybean and fungi [13]. However, this value is a bit lower than the range of values reported by Matassa et al. [10] as the typical concentration of crude protein in a bacterial cell, which is 50–83% based on dry matter.

On the other hand, in this work, the lower protein concentration founds was due to the fact of the relatively high PHA concentration stored in the biomass. Besides the PHA are well known as a precursor in the sector of bioplastics [23], several studies demonstrated

their reported prebiotic effect on animals. For instance, Sugunaa et al. [34] designed a study to assess the immunostimulatory effect of poly-β hydroxybutyrate–hydroxyvalerate (PHB–HV) extracted from *Bacillus thuringiensis B.t.A102* on the immune system of fishes like *Oreochromis mossambicus*. Laranja et al. [35] demonstrated the protective effect of PHB accumulating bacteria on *Penaeus monodon* larvae on exposure to pathogenic *V. campbelli*. Figure 2 shows the pattern of the amino acids contained in the PHA-rich microbial biomass, which were compared with the composition of amino acid taken from commercial fishmeal, soymeal (Figure 3) and the actual demand for the growth of trouts.

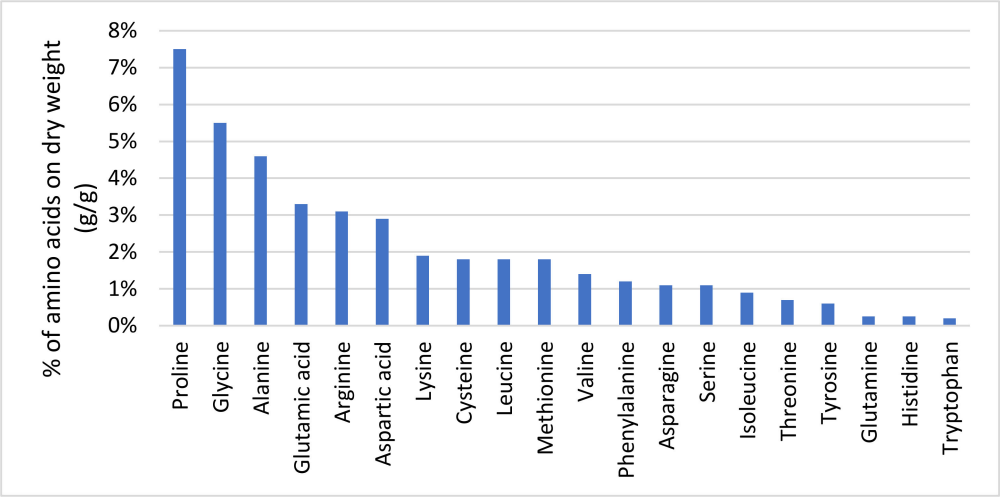

Figure 2. The amino acids profile of the PHA enriched biomass obtained in Period 3.

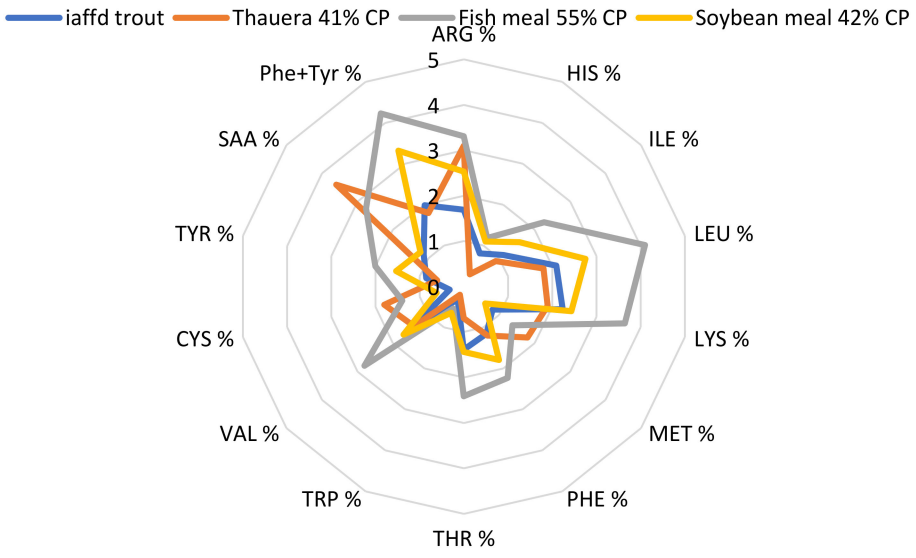

Figure 3. Comparison of the amino acids composition of the PHA enriched biomass compared with the commercial protein source such as soybean and fish meal. The protein content is in relation to the actual demand of amino acids of the trouts. ARG: Arginine; HIS: Histidine; ILE: Isoleucine; LEU: Leucine; LYS: Lysine; MET: Methionine; PHE: Phenylalanine; THR: Threonine; TRP: Tryptophan; VAL: Valine; CYS: Cystine; TYR: Tyrosine; TSAA (Met + Cys): sulfured amino acid; Phe+Tyr: Phenylalanine + Cystine.

The PHA-rich microbial biomass showed a lack of histidine and threonine compared with the soybean and fishmeal, but the remaining amino acids were in the right composition for the demand required by trouts. In particular, the sulfur amino acid (SAA),

like methionine and cysteine, were available at high concentrations and above the typical concentration contained in fishmeal. Zhang et al. [36] reported that *Thauera* sp., as well as other bacterial strains like *Paracoccus* sp., may increase the content of tryptophan and protein-like substance when the C/N ratio is decreasing. So, further investigation should be carried out in order to link the operating growth condition with the nutritional value of the biomass for different feed applications. On the other hand, the presence of PHAs in the biomass may give prebiotic properties, thus a higher market price compared with the current source of animal protein, like fishmeal. Ongoing research is evaluating PHAs as potential antibiotics as thus a potential feed supplement of interest for aquaculture [37,38].

3.3. Preliminary Economical Consideration

Currently, agro-residues are valorized by feeding anaerobic digesters to produce renewable energy (heat and electricity) from the biogas. Around 20–25 tons of agro-residues dry matter produce a daily biogas flow rate of 12,000 m^3 of biogas which could be further valorized to 24 MWh of electricity through a combined heat and power unit (CHP) with an electrical yield around 40%. The price of electrical energy in the market without incentives can be considered 30–50 €/MWh produced, so the total revenue could be estimated around 24–36 €/ton of dry matter of agro-residues fed to the anaerobic digester.

In the upgraded scenario, the acidogenic fermentation of the same agro-residues could produce up to 0.13 $gCOD_{VFA}$/kg of volatile solids [21] which resulted in a total daily VFAs production of around 2.5 tons COD_{VFA} or 10 tons of soluble COD. Considering the production yields obtained in this study, the potential production of dry PHA-rich microbial biomass from the utilization of the soluble COD and the VFAs could achieve around 1.5 tons of dry matter per day. As an innovative product for aquaculture, the initial selling price of the PHA-rich microbial biomass could be estimated similar to animal protein sources (around 2000 €/ton of dry matter), which results in total revenue of up to 115 €/ton dry matter of treated agro-residues. The latter is 3–5 times higher than the current scenario based on biogas production, so it is evident that product revenues that can be obtained from PHA-rich microbial biomass production are significantly higher than those obtained from anaerobic digestion.

3.4. Description of the Farm to Fork Model

In this work, the scenario envisages the upgrading of existing biogas plants based on agro-residues treatment, into a biorefinery aiming at the valorization of agro-residues into VFAs and further conversion into PHA-rich microbial biomass [39,40]. In line with the recent targets fixed by the Farm to Fork strategy [5], the production of such PHA-rich biomass could enable the transition to a more sustainable ingredient feed for aquaculture, through the valorization of agricultural residues and livestock effluents (solid and liquid manure). Indeed, the aquaculture sector represents a valid alternative due to the projected growth in the market in the next years [32]. Seafood, wild-catch, and aquaculture represent the largest animal protein industry in the world; the protein sector for aquaculture has grown faster if compared with other animal feed sectors (it is reported to have an annual growth of around 7%) [41]. Moreover, the development of new value chains covering primary and feed/food sectors could be a more profitable alternative compared with the current biogas produced from the anaerobic digestion of industry or agricultural residues and byproducts.

4. Conclusions

The valorization of agriculture residues and livestock effluents into novel sources of feed could increase the resilience of agriculture, promote new market value chains, and farm to fork models.

In this work, PHA-rich microbial biomass produced from the biorefinery of agro-residues and zootechnical effluents has been considered as novel ingredients for aquaculture feed. The growth of *Thauera* sp. Sel9 as a pure strain was evaluated in a CSTR under

three different HRT. The highest productivity in terms of MLSS produced was 0.69 g/L day, operating at one day of HRT. At this condition, the observed PHAs production yield was 0.14 gPHA/g soluble COD removed from the fermentation liquid with a PHA concentration of 41% based on MLSS and protein content in line with soybean and fungi. The related aminogram showed the right composition of the amino acid with a lack of histidine and threonine, although the SAA were available at high concentration and above the typical concentration contained in fishmeal. The preliminary economic analyses showed that the conversion of agro-residues to PHA-rich microbial biomass could increase 3–5 times the benefits in terms of revenues compared with the current practices based on biogas production through anaerobic digestion. It should be noted here that this observation does not mean that the production of PHA-rich microbial biomass is economically viable. More research is needed in order to further evaluate the capital expenditure for the upgrade of the existing biogas facilities together with the associated cost of the bioprocess operation and the downstream process for purification and green incentives for biogas production.

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Conflicts of Interest: The authors declare no conflict of interest.

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