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Enhancement of PHA Production by a Mixed Microbial Culture Using VFA Obtained from the Fermentation of Wastewater from Yeast Industry

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Abstract: Wastewater from the yeast production industry (WWY) is potentially harmful to surface water due to its high nitrogen and organic matter content; it can be used to produce compounds of higher commercial value, such as polyhydroxyalkanoates (PHA). PHA are polyester-type biopolymers synthesized by bacteria as energy reservoirs that can potentially substitute petrochemical-derived plastics. In this exploratory work, effluent from WWY was used to produce PHA, using a three-step setup of mixed microbial cultures involving one anaerobic and two aerobic reactors. First, volatile fatty acids (VFA; 2.5 g/L) were produced on an anaerobic batch reactor (reactor A) fed with WWY, using a heat pretreated sludge inoculum to eliminate methanogenic activity. Concurrently, PHA-producing bacteria were enriched using synthetic VFA in a sequencing batch reactor (SBR, reactor C) operated for 78 days. Finally, a polyhydroxybutyrate (PHB)-producing reactor (reactor B) was assembled using the inoculum enriched with PHA-producing bacteria and the raw and distilled effluent from the anaerobic reactor as a substrate. A maximum accumulation of 17% of PHB based on cell dry weight was achieved with a yield of 1.2 g PHB/L when feeding with the distilled effluent. Roche 454 16S rRNA gene amplicon pyrosequencing of the PHA-producing reactor showed that the microbial community was dominated by the PHA-producing bacterial species *Paracoccus alcalophilus* (32%) and *Azoarcus* sp. (44%). Our results show promising PHB accumulation rates that outperform previously reported results obtained with real substrates and mixed cultures, demonstrating a sustainable approach for the production of PHA less prone to contamination than a pure culture.

Keywords: acidogenic fermentation; Baker's yeast wastewater; biopolymer; mixed microbial culture; pyrosequencing; waste valorization

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1. Introduction

The European yeast industry produces 1 million tons annually; approximately 30% is exported globally [1]. Wastewater from yeast production industries (WWY) is highly polluted as it contains organic matter with chemical oxygen demand (COD) of up to 130 g/L [2], non-biodegradable organic pollutants, nitrogen (250–1450 mg of total nitrogen L^{-1}), phosphorus (17–100 mg of total phosphorus L^{-1}), and sulfate (485–5300 mg/L) [3]. The presence of molasses in the WWY generates its bad odor and dark brown color, which are its most problematic parameters [3]. The treatment of WWY has been mainly addressed through anaerobic biological processes as it is less costly than aerobic, physical, and chemical treatments [4], but it has proven to be insufficient to fulfill the discharge

requirements for the industry [5]. WWY has mainly been used to generate methane via anaerobic digestion; this gas [6] can be stored or burned and converted to heat in the factory. Treated WWY can be used for agricultural irrigation [7], and resources such as fertilizer [8,9], protein for animal feed, and pigments can also be recovered [10]. Finding effective and sustainable methods to treat WWY is an important aspect of the development of a more circular bioeconomy.

Organic carbon contained in WWY can be converted into compounds of higher commercial value, such as volatile fatty acids (VFA) or biopolymers such as polyhydroxyalkanoate (PHA). Biopolymers have a wide range of industrial applications due to their biodegradability and biocompatibility [11]. PHA are polyester-type biopolymers synthesized by bacteria as carbon and energy reservoirs in nutrient-limited stress conditions such as deficiency of nitrogen, phosphorus, or magnesium in the culture media [12]. PHAs include different polymers with structural properties similar to polypropylene [13], such as polyhydroxyvalerate, polyhydroxyhexanoate, or polyhydroxybutyrate (PHB), with the latter being the most common and best characterized. The presence and proportion of the different types of PHA depend on the microorganisms involved and the carbon compounds used as a substrate [11]. Raw materials for PHA production are two to three times more expensive than those used for synthetic polymers [11]; therefore, their use at an industrial scale is limited.

Routinely, biopolymers have been produced using pure cultures and have achieved concentrations up to 80% of PHA accumulation [14]. Uncovering more affordable raw materials is therefore crucial for the large-scale production of PHA. Mixed cultures have been proposed [15] as a source of PHA production due to low production and maintenance costs; they can transform a wide variety of raw materials, including organic wastes, and do not require sterilization processes [11]. PHA-accumulating bacteria can be naturally found in activated sludge treating wastewater [12]; this type of system is characterized by its dynamic conditions [16], with PHA being a key part of the carbon transformation metabolism [17].

Research over the past 15 years on PHA production at a laboratory scale using mixed microbial culture has mainly consisted of a three-step process involving: (1) VFA production from acidogenic fermentation, (2) biomass production of a microbial-mixed culture with superior capability to produce PHA and (3) PHA production [17]. VFA has been obtained from the anaerobic transformation of a wide range of organics wastes including: olive and palm oil, sugar molasses and vinasses [18], starch, and glycerol from the biodiesel industry [19], and industrial and domestic wastewaters [17,20].

The stability of the bacterial cultures and the accumulation rate of the biopolymer are two essential challenges for the efficient production of PHA and can be achieved by modifying the operational conditions of the reactors [15]. The most efficient technique to select and enrich PHA-producing bacteria is the feast/famine regime in aerobic sequencing batch reactors (SBR) under nutrient-excess conditions [21]. Specific nitrogen (2 to 15 mg g^{-1}) and phosphorus (0.5 to 3 mg g^{-1}) to COD ratios have proven to favor PHA production, preventing the growth of non-PHA-accumulating bacteria [22]. The success of this feast/famine technique when using waste as a substrate is the VFA fraction of the wastewater as its carbon fraction serves as a PHA precursor [23]. Other factors affecting the successful enrichment of PHA-accumulating biomass are neutral pH and temperature (20 to 35 °C) [21]. Acidic growing conditions can be avoided by increasing the buffering capacity of the feedstock [24]. The addition of allylthiourea (3.4 to 10-mg/L) [25] to inhibit nitrifying bacteria in PHA-producing reactors is a common practice as these bacteria can specifically compete with PHA-accumulating bacteria for ammonium and oxygen [24,25].

This work evaluated PHA production by a mixed microbial culture obtained from a tropical domestic wastewater sludge using VFA derived from residues of the Colombian yeast production industry. We demonstrated the importance of pretreating the sludge with heat and distilling the wastewater from the yeast production industry for boosting PHA production in a three-step coupled biotechnological process. To this end, we have directly estimated VFA production rates of the acidogenic reactor, quantified the PHA

production capacity of the microbial-mixed culture in the aerobic reactor, and characterized the microbial community that developed within the reactors. Our results show promising PHA accumulation rates that outperform previously reported results and prove that our system had the capacity to enrich the biomass with PHA-accumulating bacteria.

2. Materials and Methods

2.1. Phase I: Inoculum Adaptation for VFA Production

VFA production was evaluated under three different treatments in anaerobic batch reactors inoculated with sludge obtained from a WWY treatment plant (4°4′10.92″ N 76°11′52.08″ W, Colombia) and fed with WWY (Figure 1). Each treatment used a different WWY COD concentration with values of 5, 10, and 15 g/L. The reactors were assembled in 1 L containers and operated at room temperature under constant magnetic stirring (110 rpm) for 18 days (Table 1). Previous to inoculation, the WWY sludge was heated at 90 °C for 10 min to eliminate methanogenic bacteria (which compete with the acetogenic bacteria) and thus boost VFA production in the reactor [26]. The WWY had the following characteristics: 68.45 g/L of COD, 771.90 mg/L of volatile suspended solids (VSS), and a pH of 7.65 (Table 1). Each reactor was inoculated with 30 mL of WWY sludge. The WWY COD concentration from the reactor with the highest yield of VFA production was selected to operate the larger acidogenic reactor.

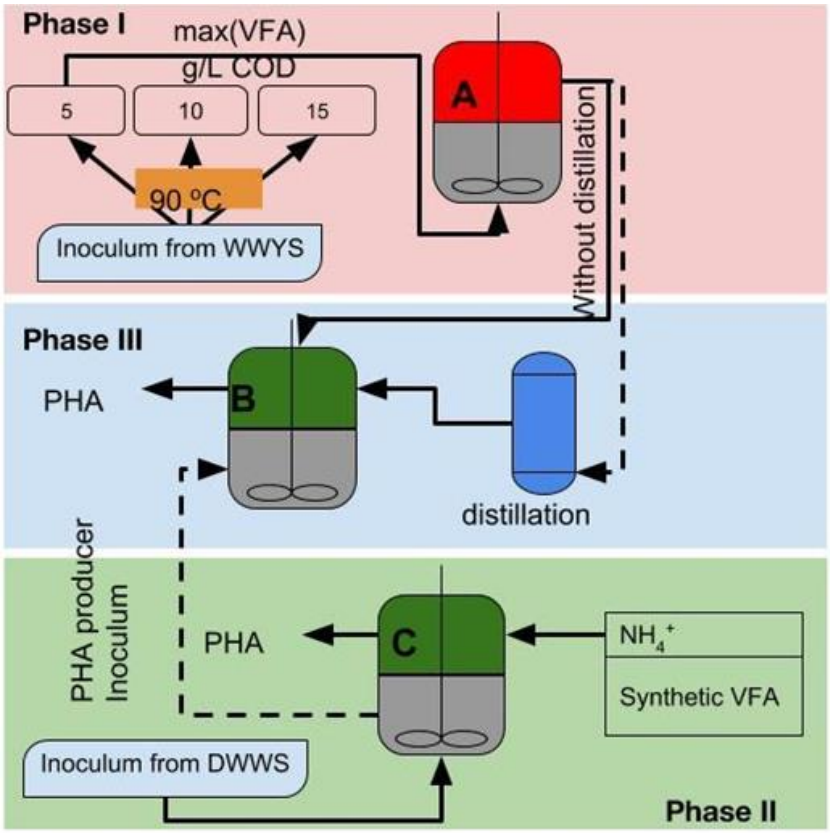


Figure 1. Phase I: In order to evaluate optimal VFA production, small reactors using WWY with different COD concentrations were operated. Sludge obtained from a WWY treatment plant was heated at 90 °C for 10 min and used as inoculum. The reactor with COD concentration with the higher VFA production rate (5 g COD L^{-1}) was scaled up (reactor A). **Phase II:** Adaptation of PHB production inoculum was achieved by inoculating an SBR (reactor C) with domestic wastewater sludge (DWWS); it was fed for 78 days with increasing concentrations of synthetic VFA. **Phase III:** A coupled system from reactors A (distilled effluent) and C (inoculum enriched with PHA-producing bacteria) were combined to assemble a PHA-producing reactor (reactor B).

Table 1. Operational conditions of reactors.

Conditions	Preliminary Tests	Reactor A	Reactor C	Reactor B	Control Reactor
Type or reactor	Anaerobic Batch	Anaerobic SBR	Aerobic SBR	Aerobic SBR	Aerobic SBR
Type of wastewater	Yeast wastetwater	Yeast wastetwater	Mineral salts medium	Raw and distilled VFA	Raw VFA
Working Volume (L)	0.7	1.7	1	0.2	0.2
Volume of inoculum (L)	0.03	0.1	0.5	0.1	0.1
COD (g/L)	5, 10 or 15	5	-	-	-
VFA in feed (g/L)	-	-	0.416–4.16	2–2.5	2–2.5
VSS (mg/L)	56–170	56.4	2.4	2.6	2.8
pH	7.65	7.65	8.9	7.8	8.7
DO (mg/L)	-	-	4	4.6	4.2
HRT (days)	18	7	2	2	2
Total length of operation (days)	18	70	78	70	70
Air flow (L/min)	-	-	6	6	6
Stirring speed (rpm)	110	110	-	-	-

The acidogenic SBR was also inoculated with pre-heated WWY sludge and fed with 1.6 L of WWY with a COD concentration of 5 g/L (bioreactor A) but was operated for 70 days with a hydraulic retention time (HRT) of 7 days (Figure 1 and Table 1). This reactor was assembled in 2 L container using 0.1 L of WWY sludge and operated at room temperature under constant magnetic stirring (110 rpm) (Table 1). At the end of each cycle, the stirrer was turned off for 1 day to settle down the biomass. In order to start the new cycle, supernatant was replaced with 1.6 L of WWY. VFA from the effluent of the acidogenic reactor A (Figure 1) were extracted by distillation following method 5560 C from APHA [27] and stored at 4 °C until further use.

2.2. Phase II: Adaptation of Inoculum for PHA Production

The microbial inoculum for PHA production was sourced from an activated sludge obtained from the aerobic tank of a domestic wastewater treatment plant (DWWS) (3°23′16.8″ N, 76°30′46.8″ W, Colombia). The adaptation process took place in an aerobic SBR (reactor C) fed with Mineral Salts Medium (pH 7) supplemented with synthetic VFA as a carbon source and 0.05 g/L of NH_4Cl (Figure 1 and Table 1) [28]. Acetic, propionic, and butyric acids were added in equal proportions, and the concentration was periodically increased during four phases of operation as follows; 0.416 (day 1 to 7), 2.080 (day 8 to 21), 4.16 (day 22 to 49), and 6.24 g VFA L^{-1} (day 50 to 78). The SBR aerobic reactor was operated at room temperature (25 °C) in a 2 L container with an initial working volume of 1 L and biomass/culture media ratio of 1:1 (Table 1). The VSS of the reactor on day 0 was 2.4 g/L. The reactor was maintained at room temperature (25 °C) and had an HRT of 48 h. Air was pumped into the bottom of the reactor to achieve complete mix using a ceramic diffuser with an airflow of 6 L/min. At the end of each cycle, the air tab was turned off for 30 min to settle down the biomass. In order to start the new cycle, clear supernatant was replaced with 500 mL medium of fresh medium. Dissolved oxygen and pH were monitored at the end of each cycle with average values of 4.0 ± 2.5 mg O_2 L^{-1} and 8.9 ± 0.5, respectively (Table 1).

2.3. Phase III: PHA Production in a Coupled System

Two aerobic SBR reactors (reactor B and control reactor) were inoculated with the PHA-producing biomass from reactor C and operated for 10 weeks under the same conditions described previously, using different feed sources (Figure 1). Reactor B was fed with raw (week 1–5) and distilled effluent (week 6–10) from reactor A. The pH of distilled effluent was previously adjusted to 7, using small amounts of NaOH, and was supplemented with Mineral Salts Medium [28] and 0.05 g NH_4Cl L^{-1}. The control reactor was fed with Mineral Salts Medium [28] supplemented with 2 g of pure synthetic VFA L^{-1} (as described previously for reactor C). Both reactors had a total volume of 500 mL and a workload of

200 mL with a biomass/culture media ratio of 1:1. After each cycle, 100 mL of supernatant was removed and later replaced with fresh media (Table 1). Dissolved oxygen and pH were monitored at the end of each cycle. The average pH values of the reactor B and control were 7.8 ± 0.2 and 8.7 ± 0.3, respectively. Meanwhile, the average dissolved oxygen concentration was 4.6 ± 1.4 mg O_2 L^{-1} for reactor B and 4.2 ± 1.8 mg O_2 L^{-1} for the control reactor (Table 1).

2.4. Physicochemical Analysis

Total VFA concentration was measured according to protocols described elsewhere [27]. COD was measured using a Shimadzu UV-1800 spectrophotometer according to the methodology described in American Standard Test Methods [29]. PHB was quantified by HPLC following the protocol described by Moreno et al. [30] using an Agilent Technologies Series 1100 liquid chromatograph equipped with a SUPELCOGELTM C-610H column and a diode array detector at 210 nm for PHB. HPLC measurements were performed under an isocratic elution regime using an H_2SO_4 aqueous solution (0.005 M) as the mobile phase, a flow rate of 0.7 mL/min, and an injection volume of 25 µL. The column and the detector were held at 60 and 65 °C, respectively. External calibration standard curves with six points were used for the quantification of PHB.

2.5. Microbiological Quantification of Anaerobic Bacteria

Anaerobic bacteria inoculated in reactor A were characterized and quantified using culture-dependent assays based on the most probable number method (MPN) [31]. This technique was used to estimate the number of total anaerobic bacteria, glucose and lactate fermenting bacteria, acetate syntrophic bacteria, butyrate syntrophic bacteria, and propionate syntrophic bacteria. Media composition for the cultivation of each bacterial group is described elsewhere [31]. Serial dilutions in Hungate tubes with five replicates each, were incubated at 35 ± 2 °C for 15 days.

2.6. Molecular Techniques for Diversity Analysis

Genomic DNA was extracted from 0.25 g of mixed liquor from reactor C (days 0, 44 and 72) and reactor B (week 10) using DNA isolation PowerSoil kit (MoBio, Carlsbad, CA, USA). Results were confirmed by electrophoresis using a 1.6% agarose gel stained with ethidium bromide with a run time of 20 min at 100 V. Extracted DNA quality and quantification were accessed using a Spectrophotometer (NanoDrop 2000C UV-Vis, Thermo scientific). The concentration of the DNA extracts was determined using a Qubit dsDNA HS assay kit (Invitrogen, Life Technologies, Paisley, UK). The microbial community within reactors was assessed by next-generation amplicon sequencing of the 16S rRNA V3-V4 region through bTEFAP pyrosequencing [32] utilizing Roche 454 FLX titanium instruments and reagents, following manufacturer's guidelines (MR DNA, Shallowater, TX, USA). The eubacteria primer set 27F (5′GAGTTTGATCNTGGCTCAG-3′)/519R (5′GTNTTACNGCGGCKGCTG-3′) used in this experiment amplified a fragment of approximately 492 bp. Sequence data were processed using a proprietary analysis pipeline (MR DNA, Shallowater, TX, USA), where Operational Taxonomic Units (OTUs) were defined by clustering at 97% of similarity and singleton sequences, and chimeras were removed. Final OTUs were taxonomically classified using BLASTn against a curated database derived from the Ribosomal Database Project (RDPII) [33] and the National Center for Biotechnology Information (NCBI) [34]. The observed richness and Simpson's diversity index values were calculated similarly to Janarthanan et al. [20].

3. Results and Discussion

3.1. Dark Fermentation of WWY

Dark fermentation is a type of biological production of hydrogen carried out by obligate anaerobes and facultative anaerobes in the absence of light and oxygen [35]. Treatment of anaerobic sludge inoculum with heat is a common practice in biohydrogen

production as it prevents methanogenesis. More recently, the same method was shown to improve the VFA production yields [23,26,36]. VFA can be used as a carbon source for a wide range of processes such as power generation and fuel cells [37]. The reactor with a WWY COD of 5 g/L produced up to 45% more VFA than the reactors with a WWY COD of 10 and 15 g/L, hence this COD concentration was used to feed reactor A.

Total COD from the effluent of reactor A decreased by 20% from 4.96 g COD L^{-1} on day 1 to 3.98 g COD L^{-1} on day 7. The COD from the VFA produced by reactor A increased from 1.36 on day 1 to 1.98 g COD L^{-1} on day 7; the latter concentration corresponds to 49% of the total COD on day 7. We believe that the reduction in the COD concentration of reactor A could be related to the production of CO_2, a byproduct of dark fermentation. The biomass, which had an initial value of 21.22 mg of VSS L^{-1}, showed an increase of 7.94% after 7 days of incubation. The total concentration of VFA in the effluent ranged between 2 to 2.5 g/L. A similar range was obtained by Zampol et al. [18] and Buitrón and Carvajal [38], which used sugarcane and tequila vinasses, respectively, to generate hydrogen. Compounds such as formate, acetate, propionate, and butyrate can be expected in fermented WWY [39], considering the high concentration of sugars, proteins, and glutamic acids present [4].

3.2. Assessment of Anaerobic Bacteria in the Acidogenic Reactor

Anaerobic digestion consists of four main steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis [37]. Dark fermentation comprises the first three aforementioned steps. MPN cultivation of the anaerobic bacteria present in the acidogenic reactor A showed a positive result for the total anaerobic bacteria ($4 \pm 0.3 \times 10^6$ MPN mL^{-1}) and for the glucose and lactate fermenting bacteria ($7 \pm 0.2 \times 10^6$ MPN mL^{-1}) after 8 days of culture. These results confirm the occurrence of the first two-step of the anaerobic process (hydrolysis, acidogenesis) and the presence of VFA-producing bacteria. In contrast, no bacteria grew when acetate, butyrate, and propionate were used as a carbon source, demonstrating the absence of acetate, butyrate, and propionate syntrophic bacteria. Furthermore, the absence of these bacteria implies that there were no acetogenic methanoclastic bacteria consuming the acetic acid produced in reactor A during the acetogenesis. These results lay out the efficiency of the inoculum heating treatment to eliminate methanogenic bacteria. Subsequent studies should further confirm the inhibition of methane production in the acidogenic reactors as well as to measure hydrogen and carbon dioxide production [36].

3.3. Adaptation of the Inoculum for PHA Production

PHA-accumulating bacteria were adapted to VFA consumption in reactor C, which was operated during four stages defined by the increase in the concentration of synthetic VFA in the feed. Figure 2a shows the correlation between VFA concentration and biomass yield (Y_{bio}) in the reactor. The average Y_{bio} values for each stage fluctuated between 85 ± 14 to 167.6 ± 45 during the first three stages of the adaptation process. During stage four, the average Y_{bio} value decreased to 18 ± 8. The highest PHB accumulation percentage in reactor C was obtained during stage two with 10.3%; this value corresponded to a PHB concentration of 0.8 g/L (Table 2). In the other stages of operation, the PHB concentrations were lower than 0.4g PHB/L, and the accumulation percentage rate varied from 0 to 6%. The increase in the PHB accumulation at stage two hints at the adaptation of the microbial community to the VFA feed regimen in the reactor, whereas the decrease in the PHB accumulation rate in steps three and four could correspond to an inhibition of the biopolymer production due to the high concentrations of VFA as observed in Tamang et al. [40]. Morgan-Sagastume et al. [41] observed that there is no direct correlation between an increase in the VFA concentration and the average biomass yield concentration of PHA-producing reactors. The VFA concentration used at stage two gives an estimate of the ideal range of substrate concentration that should be used to feed and enrich the PHA-producing bacterial mixed culture.

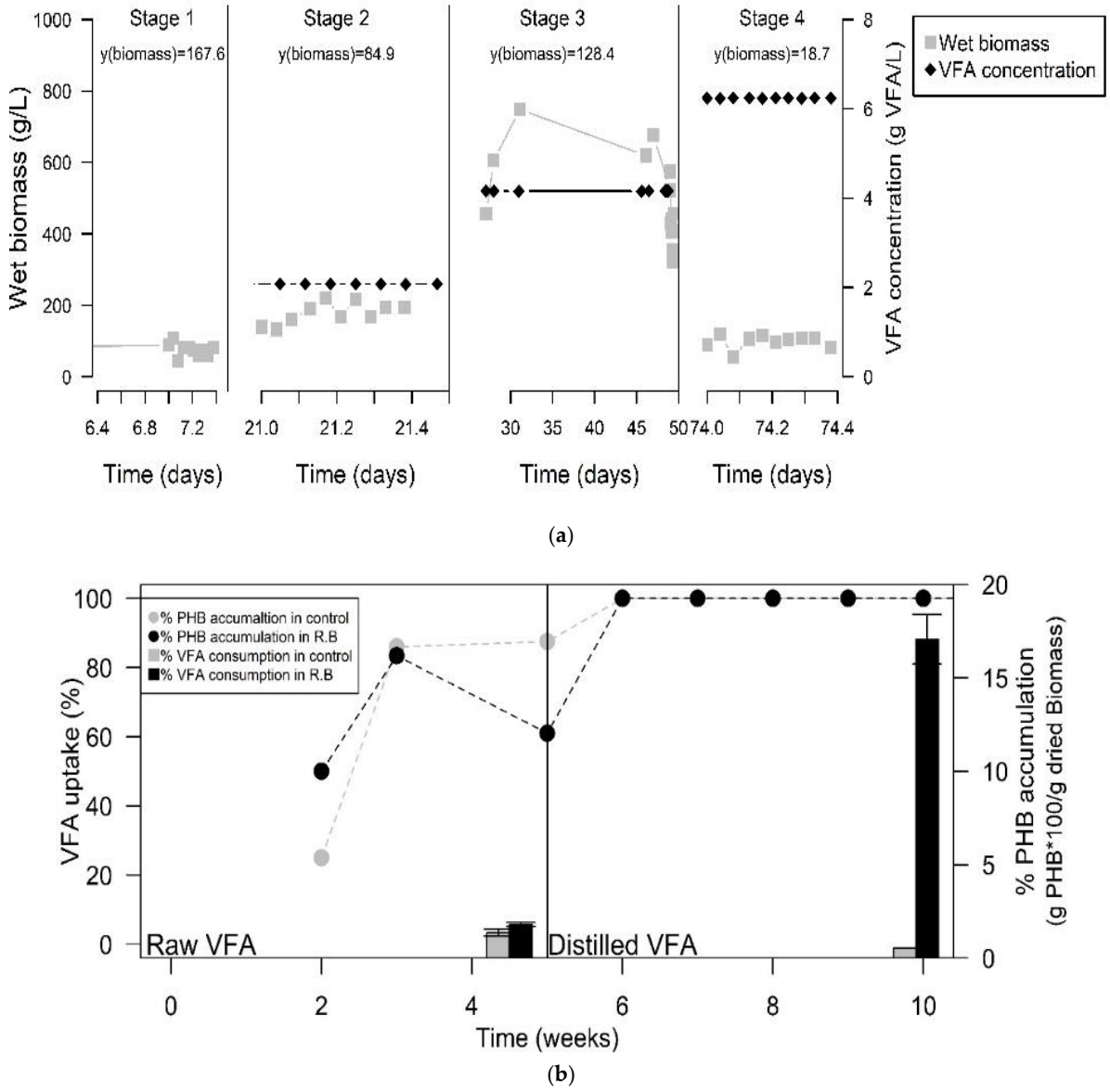


Figure 2. (**a**) Concentration of biomass (gray) and pure synthetic VFA (black) in the feed solution from reactor C. (**b**) Percentage of VFA consumption (dots) and PHB composition (bars) in reactor B (RB) (gray) and control reactor (black).

Table 2. Percentage of PHB accumulation and PHB concentration in reactor C during its four stages of operation.

Stage	Time (Days)	VFA Concentration (g/L)	% PHB Accumulation (g PHB·100/g dry biomass^{-1})	PHB Concentration (g/L)	y(PHA)
1	**0**	**0.416**	5.2	0.12	0.0011
2	**21**	**2.08**	10.3	0.80	0.0070
3	**31**	**4.16**	0.6	0.09	0.0005
3	**49**	**4.16**	1.0	0.09	0.0008
4	**74**	**6.24**	2.9	0.32	0.0180

3.4. PHA Production in a Coupled System

The raw and distilled effluent from reactor A, used as the carbon source during different stages of operation of reactor B, had a similar concentration of VFA with values ranging between 2 to 2.5 g VFA L^{-1}. The VFA consumption in reactor B increased from

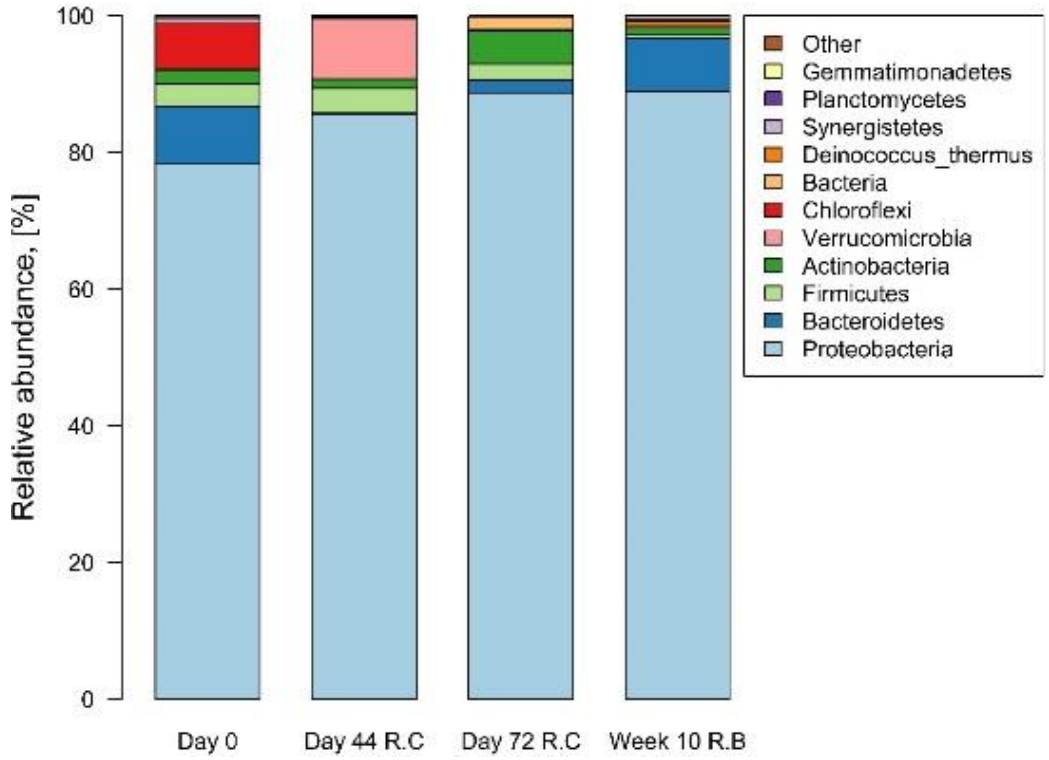
61 to 100% when the feed was distilled (Figure 2b). This change also had an effect on the biopolymer composition of the sludge, as it increased from 0.06 g PHB/L on week 4 to 1.2 g PHB/L on week 10 (Figure 2b). These numbers correspond to 1.5 and 17% of the dry biomass as PHB, respectively. The control reactors, fed with pure synthetic VFA, also achieved a 100% consumption of VFA after 5 weeks of operation. Nevertheless, the biopolymer accumulation on weeks 4 and 10 was lower than 1.8% of the dry biomass (Figure 2b). The biopolymers composition in reactor B was higher than that reported by Albuquerque et al. [13], which obtained biopolymer compositions of 10% using VFA from fermented molasses in concentrations of 1.8 g VFA L^{-1} (30 mmol of C L^{-1}). In a similar experiment, Shen et al. [19] obtained 1 g PHA L^{-1} using sugar molasses with a VFA concentration of 4 g/L. In our research, it was possible to obtain similar PHB concentrations when feeding with half of the VFA concentration (2.0–2.5 g VFA L^{-1}). The characterization of the VFA present in the raw and distilled effluent of reactor A should be a subject of investigation in future studies.

3.5. Characterization of the PHB-Producing Microbial Community

The evolving composition of the microbial community of reactors C and B was elucidated using 16S RNA pyrosequencing (Figure 3). Bacterial species reported in the literature as PHA producers and extracellular PHA decomposers were identified. Proteobacteria had the highest relative abundance (Figure 3a), representing between 78 and 88% of the phyla identified in the samples from reactor C; meanwhile, in reactor B, it had an abundance of 88%. The most abundant classes on day 72 of reactor C were Alpha and Beta proteobacteria, covering 30 and 35% of the relative abundance, respectively. These two classes were also the most abundant in reactor B, with a relative abundance of 42% and 45%, respectively. These results agree with those obtained by Queirós et al. [42], which obtained a PHB-producing culture from an activated sludge originating from a wastewater treatment plant using hardwood spent sulfite liquor timber as a substrate. The authors explored the microbial community using fluorescence in situ hybridization (FISH) and observed that the most abundant classes were identified as Alpha-(72.7 $\pm$ 4%) and Beta-(11.1 $\pm$ 0.37%).

The PHA-producing species with the highest abundance in reactor C were *Pseudoacidovorax* sp. with 8%, *Rhizobium* sp. with 11%, and *Azoarcus indigenous* with 36% during days 0, 44, and 72, respectively (Figure 3b). On reactor B, the two most abundant PHA-producing species were *Paracoccus alcalophilus* and the genus *Azoarcus* sp., with abundances of 32% and 44%, respectively. *P.alcalophilus* increased its relative abundance significantly from 4% on day 72 in reactor C to 32% on week 10 in reactor B. This bacteria is a non-spore-forming Gram-negative wall with coccoid or short rod morphology, which grows in pH between 7.0 to 9.5 [43]. *Azoarcus* sp. is a genus of Gram-negative bacteria, non-spore former, with a straight or slightly curved rod morphology; it can be found individually or in pairs and grows at neutral pH [35]. This genus is also known for its capacity to fix nitrogen-fixing and plant growth promotion traits [44]; therefore, the PHA-producing sludge could potentially be used as a fertilizer [45]. Wijeyekoon et al. [46] reported that most species identified as PHA producers are Gram-negative Proteobacteria.

The increase or decrease in different phyla and classes along the operation of the bioreactors is natural in the adaptation of the community to different operating conditions (for example, the carbon source), where, in some cases, certain species can be favored or affected. The boost in the abundance of *P.alcalophilus* could be a consequence of the inhibition of its antagonistic species caused by toxic phenolic or alcohols compounds usually contained in the raw effluents from anaerobic reactors [18,38]. Future studies should evaluate the presence of phenolic compounds in the raw, alcohols, and distilled VFA obtained from the effluent of anaerobic reactors.



(**a**)

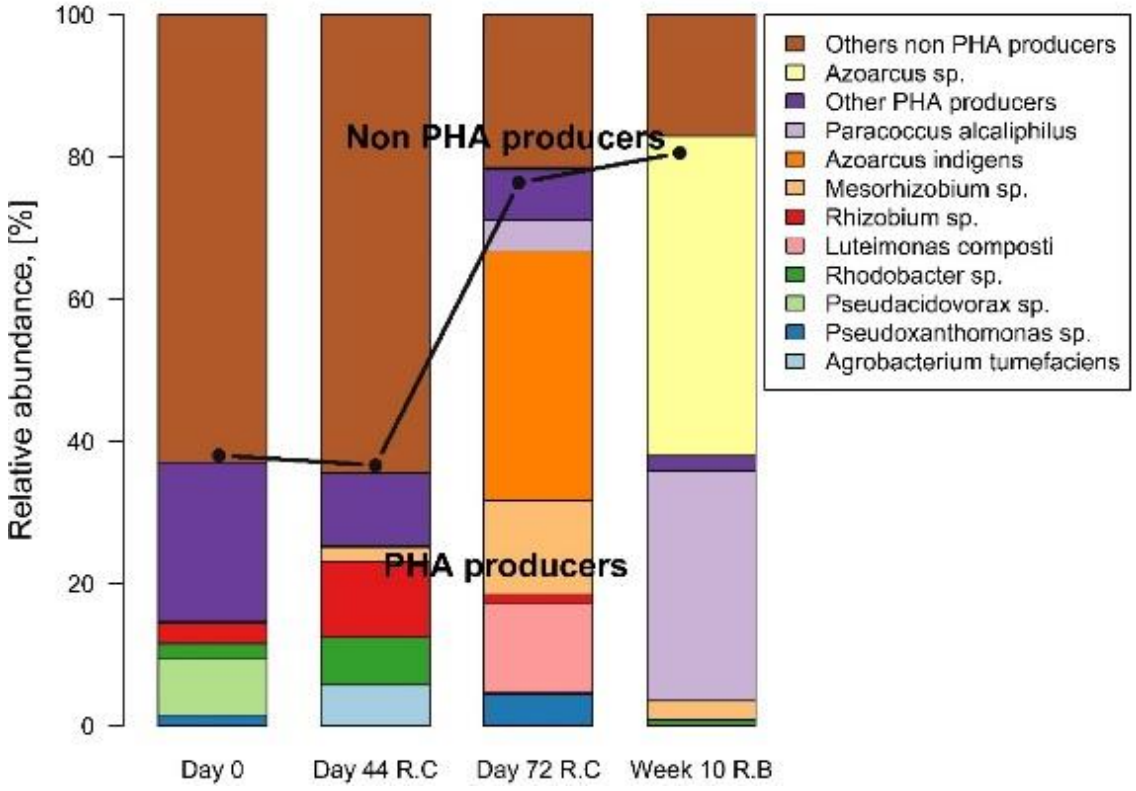


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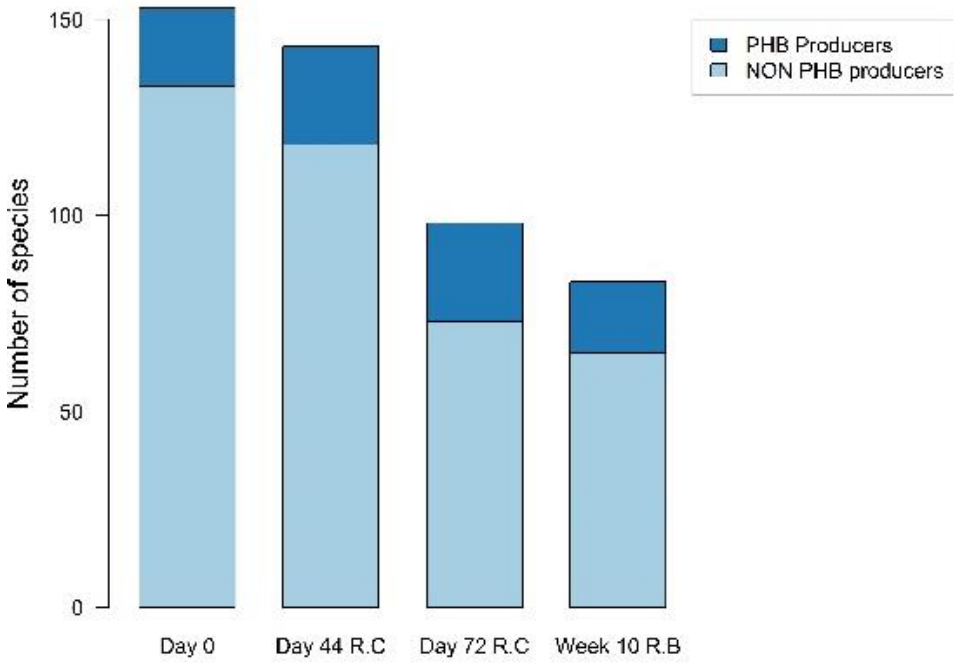
Figure 3. *Cont.*



(**c**)

Figure 3. (**a**) Relative abundance of phyla, (**b**) species and PHA producers in reactor C (days 0, 44 and 72) and reactor B (R.B, week 10). (**c**) Comparison of the total number of PHA-producing species against total species in reactor C (days 0, 44 and 72) and reactor B (R.B, week 10).

3.6. Analysis of Abundance of PHA-Producing Species

PHA-producing microorganisms can be selected by using cycles with a phase of carbon excess followed by a phase of carbon depletion in the substrate. There was a reduction in the total number of species of reactor C which began with 153 species on day 0 and ended with 98 on day 72. Furthermore, the number of total species on week 10 of reactor B was 83. The number of PHB-producing species on day 72 of reactor C and week 10 of reactor B were 25 and 18, respectively (Figure 3c). The relative abundance of PHA-producing bacteria increased from 37% to 78.52% between days 0 and 72 of operation of reactor C, whilst in reactor B, the bacterial group presented an enrichment of 83% (Figure 3b).

3.7. Biodiversity Indexes

Simpson diversity and observed richness of species indexes combine richness and evenness, but they differ in the fact that the former takes into account the dominant species (evenness) while the latter evaluates the number of present species [47]. During operation days 0 and 72 of reactor C, the observed richness and diversity of Simpson indexes decreased from 40.55 to 14.38 and from 0.93 to 0.84, respectively (Table 3). On reactor B, the diversity for both indexes were lower, with an observed richness index of 6.38 and a Simpson diversity index of 0.69. The decline in evenness and species richness over time is explained because there was a selection of certain species over time. This finding coincides with the principle of bacterial enrichment using selective culture media. In this particular case, only certain species would be able to metabolize the substrate and survive famine cycles during the selection of PHA-producing bacteria t, although there would be species that live off the symbiosis of PHA-producing bacteria. Morgan-Sagastume et al. [41] also observed a decrease in the species evenness when bio-enriching a PHA accumulating inoculum using VFA from the fermentation of waste-activated sludge (WAS) in SBR reactors [41]. In their study, the species' evenness was measured by Lorenz curves.

Table 3. Heat map of the richness of species observed and Simpson diversity index.

	Time	Observed Richness	Simpson Diversity Index	Total Sequences	OTUs
Reactor C	**Day 0**	40.55	0.93	5637	166
	Day 44	22.95	0.93	11,001	151
	Day 74	14.43	0.84	11,755	107
Reactor B	**Week 10**	6.38	0.69	6282	87

4. Conclusions

Developing alternative methods for the production of biopolymers based on a circular economy contributes to the achievement of the United Nation's Sustainable Development goals. In this study, we demonstrated the importance of pretreating the sludge and wastewater from the yeast production industry for PHA production in a three-step coupled biotechnological process. The elimination of methanogenic bacteria in the acidogenic reactor together with the distillation of its effluent favored the availability of VFA used as a substrate for PHA production in the SBR. Further studies should focus on scaling up this technology for industrial use at a pilot-scale level seeking to further optimize PHA production.

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