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Effect of ozone pre-treatment on polyhydroxyalkanoate production from dark fermentation effluents

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ABSTRACT

This study examines the biosynthesis of polyhydroxyalkanoates (PHAs) by a *Cupriavidus necator* pure culture using permeates obtained from the acidogenic dark fermentation of ozonized and non-ozonized organic wastes. The influence of ozone pre-treatment on permeate composition, and its subsequent impact on PHA production, was systematically assessed by analyzing PHA yield, volatile fatty acid (VFA) uptake, and microbial growth dynamics. From the results obtained, it was observed that ozonation significantly enriched the permeate, increasing dissolved organic carbon by 59.43% and dissolved nitrogen by 47.61%, resulting in a C/N ratio of 6.55—close to the optimal ratio (C/N = 6) for efficient PHA fermentation. Additionally, ozone pre-treatment shifted the VFA profile, increasing butyric acid concentration by 88%, a preferred substrate for *C. necator* during PHA biosynthesis. The ozone pre-treatment led to a 15% increase in microbial growth and a 60% improvement in PHA production, raising the final accumulation from 0.85 g/L to 1.35 g/L.

Kinetic and stoichiometric modeling corroborated the advantageous role of ozonation, demonstrating a marked enhancement in carbon conversion efficiency, with PHA yields reaching 0.91 g PHA/g VFA and a maximum biomass-specific accumulation of 4 g PHA/g biomass, corresponding to an intracellular PHA content of 80%. These results underscore ozone pre-treatment as a powerful strategy for optimizing VFA-to-PHA bioconversion, revealing its capacity to offer a promising route toward more efficient and sustainable biorefinery processes.

1. Introduction

The depletion of fossil resources and growing concerns about the environmental impact of conventional fossil fuel-based plastics are driving research toward biodegradable and biocompatible alternatives. As a result, various polymers such as polylactides, aliphatic polyesters, polysaccharides and polyhydroxyalkanoates (PHAs) have been developed as promising options since they are monomers used in the synthesis of bioplastics [1]. The significance of the global biopolymer market can be highlighted by indicating that, according to the report by European Bioplastics (EUPB) and the Nova Institute, global bioplastics production was expected to increase from 2.11 million tons in 2019 to 2.43 million tons by 2024 [2].

Among the various groups of bio-polymers, PHAs are the most well-known ([3]; Zaini [4,5]). These bio-polymers are synthesized by bacteria as intracellular carbon and energy stores ([6]; Zaini [5]). Traditionally, PHA accumulation is thought to be related to nutrient limitations, such as nitrogen or phosphorous, and an excess of a carbon substrates for growth [7]. However, in the literature it has been described that a carbon limitation strategy can be favorable for the enrichment and long-term cultivation of a PHA-producing community [8]. Sufficient nutrients as nitrogen are necessary for the successful

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enrichment of PHA-producing cultures with high PHA storage capacities [8].

The PHA production can be carried out using either mixed microbial cultures (MMC) or pure culture fermentation processes. One advantage of producing PHA with MMC, as opposed to pure cultures, is the significant cost reduction [9]. This is because there is no need for reactor sterilization and fewer process controls are required [10,11]. In the case of pure cultures, the main advantage is the higher productivity, being able to accumulate PHAs up to 90% of their cell dry weight [10,12]. Pure cultures used for PHA production include strains such as *Alcaligenes latus* and *Burkholderia sacchari*, as well as genetically modified strains such as recombinant *Escherichia coli* and recombinant *Wautersia eutropha* [10]. In the literature, Gram-negative, facultative bacterium *C. necator* (formerly known as *Ralstonia eutropha*) has been the subject of extensive research for nearly five decades, making it one of the PHA producers cultures most thoroughly studied [13,14].

Up to four natural PHA synthesis routes starting from different carbon sources have been described. These include sugars, 'unrelated' carbon sources (such as glucose, fructose, glycerol, gluconate, ethanol and acetate), volatile fatty acids (VFA) and a specific route for butyric acid [15]. The VFAs are preferred over alternative substrates such as glycerol or carbohydrates, because the latter tend to produce glycogen rather than PHA [11]. In addition, starting the synthesis from VFAs is energetically advantageous in terms of ATP molecules compared to oxidation of an equivalent amount of glucose [16].

Among the wide spectrum of existing VFAs, mixed and pure cultures, including *C. necator*, show specific preferences for certain acids. In the literature, it has been described that the MMC show a preference for butyric acid and valeric acid as carbon sources for the PHA production over shorter chain acids such as propionic acid or acetic acid [17]. Similarly, experimental results have consistently shown that *C. necator* prefers butyric acid [18–20].

The VFAs required for the PHA generation can be obtained from waste with high levels of biodegradable material through a biological process known as Dark Fermentation (DF), also known as acidogenic fermentation. The DF is a specific stage within the anaerobic digestion process in which hydrolysis intermediates are metabolized by acidogenic bacteria to produce end products such as VFAs, carbon dioxide (CO_2), and hydrogen (H_2), which is of particular interest due to their energy potential. The production of VFA from carbonaceous wastes, to be used for PHA generation is of great importance as nearly 30% of the total production cost of PHA is attributed to the acquisition of carbon substrates [21]. In addition, this procedure provides a solution for these waste materials within the circular economy scenario. This possibility brings PHAs closer to becoming renewable chemical platforms within the framework of biorefineries based on biological systems [22].

Nowadays, biosolids from wastewater treatment plants (WWTPs) is one of the waste types with a very high organic load, and it is generated in large quantities, reaching 1,200,000 tons of sewage sludge per year in Spain. [23]. Unfortunately, the biosolids present a very low C/N ratio and a high particulate substrates content. This is very relevant, because in literature it has been described that C/N ratio is a crucial factor in the DF process [24]. Therefore, biosolids co-fermentation with substrates presenting high soluble organic load and high C/N ratios, such as the vinasse, a by-product from alcohol production, stands out as an interesting option to fit the C/N ratio to the optimum values [25].

Focused on the production of rich VFAs effluents, efforts have been centered on the enhancement of the DF process. In this sense, it is well known that hydrolysis is the limiting step [26], so the application of pre-treatments can improve the particle solubilization and, hence, increase the acidogenic activity and the subsequent VFA production. In this sense, the enhanced VFA production could lead to an increased PHA production, since the VFA can be metabolized and stored as PHA [17]. The pre-treatment techniques include physical, chemical and biological methods [27]. Among physical methods, thermal treatments offer high solubilization but involve high energy costs and the potential formation of soluble inert fractions. Similarly, ultrasonic treatment can improve biodegradability but often impairs sludge filterability. Regarding chemical methods, acid or alkali pre-treatment are common but present operational challenges, such as equipment corrosion and the need for subsequent pH neutralization [28]. In contrast, ozone pre-treatment offers a more sustainable footprint as it can be generated on-site, eliminating the risks associated with the transport and storage of hazardous chemicals. Although ozone generation entails an energy demand and requires monitoring for potential by-products like bromates under specific conditions [29], it has been described as an effective technique for improving the VFA production [30,31]. However, its influence on subsequent PHA biosynthesis remains largely unexplored. This study addresses this gap by investigating how ozone-induced changes in the VFA profile affect the final PHA accumulation, providing an integrated perspective of the entire process.

Unfortunately, DF effluent is not only rich in VFAs but also contains high levels of particulate substrates and biomass, which can negatively affect the efficiency of PHA fermentation [32]. To overcome this, filtration systems commonly used in anaerobic membrane bioreactors (AMBRs) make it possible to obtain a permeate rich in solubilized materials, such as the desired VFAs, but free of biomass and particulate substrates.

Mathematical modeling has become an essential tool in understanding, predicting, and optimizing polyhydroxyalkanoates (PHA) production processes, which face challenges related to efficiency, scalability, and process control [33]. Currently available models can be broadly classified into two main categories: structured and unstructured, each with distinct characteristics and applications.

On the one hand, unstructured models focus on macroscopic variables such as biomass, substrate, and product concentrations, without focusing on the internal cellular mechanisms. These models are typically based on empirical kinetics and are particularly useful for describing overall process behavior under controlled conditions. Their simplicity and low computational requirements make them especially suitable for industrial applications, where process optimization often relies on controlling macroscopic variables [34]. This is particularly relevant when dealing with complex multi-compound substrates, such as substrates from waste effluents [35]. For most standard cases, formal-kinetic and low-structured models are sufficient to accurately describe the kinetics of PHA production in a satisfactory manner [36].

On the other hand, structured models incorporate intracellular components, including enzymes, metabolites, and regulatory pathways, offering a mechanistic representation of the biological system. These models reflect the real biochemical metabolic situation of living microbes and can range from simple pathways involving a few enzymatic reactions to comprehensive metabolic networks representing major catabolic and anabolic pathways [34]. Structured models provide deeper insights into metabolic fluxes and cellular responses, making them particularly valuable for strain design and metabolic engineering. However, they require more detailed data and involve higher computational complexity.

In this context, the present study evaluated the PHA production, by means of a pure culture (*C. necator*), from permeate obtained from thermophilic acidogenic DF membrane bioreactor co-fermenting vinasses mixed with ozonized or non-ozonized biosolids as co-substrates. The effect of ozone pre-treatment on VFA concentration profiles and its influence over PHA production was studied and modelled with the aim of determining the main kinetic and stoichiometric parameters.

2. Material and methods

2.1. Dark fermentation

In order to generate a VFA enriched effluent, required for the PHA production, a dark co-fermentation process of wine vinasses and

biosolids was developed. Once the DF process finished, the enriched VFA effluent was filtered by using a membrane module with the aim to avoid particulate substrates and biomass.

2.1.1. Substrates characterization

The substrates used in this work were characterized before their use in the anaerobic fermentation experiments. On the one hand, the biosolids was taken from an activated sludge system, which was thickened before being combined with primary sludge from the Guadalete municipal WWTP placed in Jerez de la Frontera. On the other hand, the vinasse, a by-product of the wine distillation process, was taken from Bodega Gonzalez-Byass, S.A. located in Jerez de la Frontera. The characteristics of these substrates (Table 1) were consistent with those reported in previous studies carried out by our research group using the same materials [25,37]. The variability of biosolids is primarily linked to seasonal fluctuations in WWTP operations. However, it has been previously reported that such variability—specifically in solids content—does not significantly impact VFA production yields when using sludge from this specific source [38]. Regarding wine vinasses, their origin from an industrial process ensures highly reproducibility and homogeneous characteristics. To ensure experimental reproducibility, a single batch of both substrates was used. Nevertheless, further research at a larger scale considering seasonal fluctuations would be beneficial for full-scale implementation.

Both wastes, biosolids and vinasses, were mixed in a 50:50 volume ratio, following the recommendations described in the literature [39], to feed the reactor. The reactor was operated with two different configurations: one configuration where the biosolids and vinasses were blended without ozone pre-treatment (named Feed Control, F—C), and another configuration where the biosolids underwent ozone pre-treatment prior to blending (named Feed Ozone, F—O). In the latter experiment, the vinasses were not subjected to ozonation in accordance with the recommendations reported in the literature [40] [41]. In Table 2, the main characteristics of the influents fed to the DF experiments are presented.

2.1.2. Ozone pre-treatment

As previously stated, only the biosolids were ozonated before its mixing with vinasses. Previous studies [40] have confirmed that this method was the optimal approach to enhance the anaerobic biosolids and vinasses co-fermentation process in thermophilic DF focused to the H_2 and VFA production. Thermophilic conditions were selected in accordance with the results previously reported in the literature [42,43]. In addition, ozonation of only biosolids ensures that only 50% of the feed is ozonated, providing operational and economic advantages.

The ozonation was carried out in the Ozonation Technology Pilot Plant at the Wine and Agrifood Research Institute of the University of Cádiz. The ozone was generated by pre-concentration of the atmospheric oxygen using a commercial ozone generator manufactured by ZonoSistem, model GZ20 PROY 1536. The generated ozone was fed to an open column reactor, containing the biosolids, through a porous diffuser. This was achieved at a flow rate of 0.6 L/min, delivering a concentration of 35 mg O_3/L, using a dose of 0.018 g O_3/g $TS_{initial}$. This value was previously optimized and described in the literature [44].

Table 1
Characteristics of substrates used: biosolids and vinasses.

Parameter	Biosolids Mean (SD %)	Vinasses Mean (SD %)
pH	6.45 (1.86)	3.20 (9.68)
TS (g/kg)	45.05 (3.41)	19.61 (0.50)
VS (g/kg)	30.84 (4.89)	18.10 (3.92)
TCOD (mgO_2/L)	64,831.00 (15.60)	40,270.00 (3.72)
SCOD (mgO_2/L)	15,580.25 (39.9)	39,860.00 (0.80)
DOC (mg/L)	1103.26 (67.27)	8286.07 (4.06)
DTN (mg/L)	321.86 (23.61)	3.20 (9.68)

TS = total solids; VS = volatile solids; T_{COD} = total chemical oxygen demand; S_{COD} = soluble chemical oxygen demand; DOC = dissolved organic carbon; DTN = dissolved total nitrogen; SD = Standard deviation (%).

Table 2
Characteristics of feeds used: without pre-treatment (F—C) and pre-treated with ozone (F—O).

Parameter	F-C Mean (SD %)	F-O Mean (SD %)
pH	5.26 (1.52)	5.08 (1.57)
TS (g/kg)	29.44 (16.30)	30.93 (2.23)
VS (g/kg)	26.88 (8.14)	25.90 (1.85)
TCOD (mgO_2/L)	68,048.68 (10.26)	56,771.62 (17.53)
SCOD (mgO_2/L)	34,142.11 (17.66)	30,524.40 (14.57)
DOC (mg/L)	5142.63 (25.72)	4745.42 (29.42)
DTN (mg/L)	404.63 (22.91)	366.39 (23.55)

TS = total solids; VS = volatile solids; T_{COD} = total chemical oxygen demand; S_{COD} = soluble chemical oxygen demand; DOC = dissolved organic carbon; DTN = dissolved total nitrogen; SD = Standard deviation (%).

2.1.3. Dark fermentation process

The substrates mentioned above, biosolids and vinasses, were subjected to co-fermentation in a Continuous Stirred Tank Reactor (CSTR) with a volume capacity of 5 L. This reactor was equipped with a jacketed system connected to a thermostatic bath that kept the substrate at 55 °C, within the thermophilic range. The reactor was inoculated with 4 L of inoculum from a single-stage anaerobic sludge digester operating under steady-state conditions in the thermophilic range, with a hydraulic retention time (HRT) of 20 days, pH maintained at 7.5 and constant stirring at 40 rpm. After achieving stability, the HRT was reduced to 15 days, and the pH of the reactor was gradually reduced to 5.5, the optimum value for DF [45], by adding hydrochloric acid into the feed. Subsequently, the HRT was further reduced to 6 days (organic loading rate (OLR) of 4.48 gVS/L·d). These conditions were specifically selected to maximize the hydrolysis rate of the complex substrates and to prevent the consumption of the generated VFAs by methanogenic archaea, which would be more active under basic pH conditions [46]. The reactor was operated in semi-continuous mode with an active volume of 4 L, fed on a daily base with the biosolids and vinasses mixture. These settings were maintained for at least three HRTs to ensure steady-state operation. Additionally, process stability was monitored through daily pH measurements and a comprehensive effluent characterization performed twice per week. After completion of the control test, the effect of the biosolids ozonation pre-treatment was evaluated. With this aim, the systems operated with two different biosolids and vinasse mixtures: non ozonized (F—C) and ozonized (F—O).

In both scenarios, with and without the ozone pre-treatment, one liter of effluent was taken and then filtered through an external membrane filtration module consisting of a multi-channel ceramic membrane manufactured by Atech Innovations GmbH with a specific pore size of 1.2 μm and a surface area of 593 cm^2. The retentate was continuously returned to the original sample. The membrane filtration was operated for 2 h and 20 min, the time required to obtain the necessary permeate volume. During this period, a slight decline in permeate flux was noted; however, the pressure at the inlet and outlet of the membrane remained constant, suggesting that no significant fouling occurred. Therefore, no cleaning was necessary during the operation, and a standard backwash with water was only performed upon completion of the filtration process. Working in this way, the membrane module separated microorganisms and other particles, generating a clear and sterilized permeate.

From this operation, two different permeates were obtained. The permeate obtained when the reactor was operated without ozone pre-treatment (Permeate Control, P—C) and the permeate obtained when the reactor was operated with ozone pre-treatment (Permeate Ozone, P—O). The main characteristic of each one of these permeates are presented on Table 3.

Table 3
Characteristics of the permeate obtained from the reactor without pre-treatment (P—C) and the reactor pre-treated with ozone (P—O).

Parameter	P-C Mean (SD %)	P-O Mean (SD %)
pH	5.75 (0.50)	5.57 (0.60)
S_{COD} (mgO_2/L)	7568.92 (0.79)	6624.93(4.67)
DOC (mg/L)	3601.48 (0.75)	5741.83 (0.92)
DTN (mg/L)	549.75 (0.71)	811.50 (0.87)
Total acidity (g acetic equivalent/L)	5.60 (5.04)	6.00 (7.55)

S_{COD} = soluble chemical oxygen demand; DOC = dissolved organic carbon; DTN = dissolved total nitrogen; SD = Standard deviation (%).

2.2. PHA fermentation

2.2.1. Microorganism and inoculum

In this study, *C. necator* (CECT 4635) served as the microorganism responsible for the PHA production. To maintain the strain, it was stored at −70 °C in glycerol stocks containing 50% *v*/v glycerol and nutrient medium in accordance with the literature [47]. The nutrient medium consisted of 5 g/L beef extract, 10 g/L peptone and 5 g/L NaCl. For inoculum preparation, 0.6 mL of frozen bacterial stocks were added to 20 mL of nutrient medium in 100 mL Erlenmeyer flasks. These flasks were then sealed with a cotton stopper and incubated at 30 °C for 16 h with shaking at 250 rpm. This specific incubation time was chosen on the basis of its correlation with the achievement of the exponential growth phase under these conditions [47].

2.2.2. PHA fermentation medium

Before its use, the permeates obtained from DF were supplemented with the following macronutrients: 0.2 g/L $MgSO_4{\cdot}7H_2O$, 1.5 g /L KH_2PO_4, and 9 g /L $Na_2HPO_4{\cdot}12H_2O$. The pH was then adjusted to 7.2 using NaOH micro pearls. Then the solutions were centrifuged at 10,000 rpm for 10 min and the supernatant was sterilized in an autoclave at 121 °C for 20 min in order to ensure its sterilization. Before *C. necator* inoculation, 1 mL/L of trace elements was added to the medium. The trace elements solution contained: 10 g/L $FeSO_4{\cdot}7H_2O$, 2.25 g/L $ZnSO_4{\cdot}7H_2O$, 1 g/L $CuSO_4{\cdot}5H_2O$, 0.5 g/L $MnSO_4{\cdot}5H_2O$, 2 g/L $CaCl_2{\cdot}2H_2O$, 0.23 g/L $Na_2B_4O_7{\cdot}7H_2O$, 0.1 g/L $Na_2MoO_4{\cdot}2H_2O$ and 10 mL/L of a 13% HCl solution. Subsequently, the permeates were fermented under the specific conditions previously described in the literature [47].

100 ml of fermentation medium were dispensed into 500 mL Erlenmeyer flasks and inoculated with 5 mL of *C. necator*. Each flask was closed with a cotton plug, in order to avoid microbial contamination, and incubated at 30 °C, shaking at 250 rpm in a Thermo Scientific MaxQ 6000 rotary shaker for 72 h. Aliquots were withdrawn at regular intervals to analyze VFA, biomass and PHA concentrations in accordance with the procedures described in the literature [47]. In order to ensure the reproducibility of data, all the tests were performed by triplicate.

2.3. Analytical methods

2.3.1. Physicochemical characterization

To characterize the main parameters of substrates and permeates, the following parameters were analyzed: pH, using a Crison pH meter; total solids (TS) and volatile solids (VS), determined by the gravimetric method with a self-calibrating balance; total chemical oxygen demand (T_{COD}) and soluble chemical oxygen demand (S_{COD}), using the colorimetric method; dissolved organic carbon (DOC) and dissolves total nitrogen (DTN), analyzed using a total organic carbon analyzer (Shimadzu TOC-L CSH/CSN). All analysis were performed according to the standard methods described by APHA-AWWA-WPFC [48].

2.3.2. Volatile fatty acids determination

Individual VFAs, including acetic, propionic, butyric, valeric, caproic, heptanoic, isobutyric, isovaleric and isocaproic acids were determined in substrates and permeates by using a Shimadzu GC-2010 gas flame ionization detector (FID) chromatograph equipped with a Nukol-packed capillary column was used for the analysis accordingly to the literature [41]. Hydrogen was used as the carrier gas at 42.1 mL/min and 75.5 kPa, providing a 45 cm/s linear velocity and 1.43 mL/min flow rate in the column. Nitrogen was also used as a carrier gas. The FID flame was produced with synthetic air (400 mL/min, 50 kPa) and hydrogen (40 mL/min, 60 kPa). The temperature protocol for the oven initiated at 115 °C for 30 s, then increased at a rate of 30 °C per minute until reaching 150 °C. From there, the temperature continued to rise at 15 °C per minute until it reached 180 °C, where it was maintained for 4 min. To ensure accurate quantification, phenol was introduced as an internal standard at a precisely determined concentration ranging between 500 and 600 mg/L. These analyses were performed according to the methods described in Methods Standard [48] and by Zahedi et al. [49].

2.3.3. Biomass concentration

The biomass concentration during the PHA fermentation processes was determined. Biomass concentration in terms of cell dry weight (CDW) was indirectly measured by optical density (OD) at 600 nm using a UV–visible spectrophotometer (UV-VIS DR 5000, Hach, Düsseldorf, Germany). To establish the calibration curve correlating OD with CDW, samples were taken periodically from different *C. necator* fermentations. The OD of these samples and the corresponding CDW were measured. These independent fermentations were carried out using a synthetic glucose-based medium. To determine CDW, samples were centrifuged (Eppendorf 5810R) at 10,000 rpm for 10 min. The supernatant was discarded, and the precipitate was washed with distilled water and centrifuged again at the same conditions. Finally, the precipitate was dried at 60 °C to a constant weight. The dry weights obtained were then correlated with their respective optical densities at 600 nm [47].

2.3.4. Polyhydroxyalkanoate determination

Samples taken for PHA quantification (1 mL) were treated by mixing with 0.2 mL sodium hypochlorite (10% *w/v*) to stop cell growth. The samples were then centrifuged at 10,000 rpm for 10 min to separate the pellet from the supernatant. The pellet was reconstituted in a screw-capped test tube containing 2 mL of methanol acidified with 3% *v*/v sulfuric acid (H_2SO_4) and 1 mL of chloroform. The samples were incubated at 100 °C for 4 h. Upon cooling to ambient temperature, 1 mL of distilled water was introduced, and the tubes were vortexed for 30 s to promote phase separation. Then 1 µL of the organic phase at the bottom was extracted and injected into a Flame Ionization Detector (FID) gas chromatograph (GC-2014, Shimadzu Europe GmbH, Duisburg, Germany) at 250 °C and using nitrogen as the carrier gas. The temperature program was as follows: 5 min at 70 °C, an increase of 8 °C per minute to 120 °C, held for 1 min, followed by a ramp of 50 °C per minute to 240 °C, held for 5 min (total time 19.65 min). A calibration curve was established by injecting a Sigma Aldrich poly (3-hydroxybutyric acid-*co*-3-hydroxyvaleric acid) standard (8% PHV), which was subjected to the same procedure as the biomass samples. Heptadecane, at a concentration of 0.1 g/L dissolved in chloroform, was used as an internal standard in accordance with the literature [47].

2.4. Mathematical modeling

Regarding mathematical modeling, it is important to highlight that no single model type can capture all the characteristics of the diverse combinations of microbial production strains and substrates. Additionally, modeling requirements differ significantly between pure and mixed microbial cultures. Therefore, the choice of modeling strategy must be adapted to the specific characteristics of each process. In the literature, the optimization of PHA production processes is usually conducted using unstructured models. This preference from the operational nature of industrial procedures, which primarily relies on the control of

macroscopic variables such as biomass and substrate concentration, as well as product yield. Consequently, in this study, unstructured models were employed to describe the PHA production process. Specifically, various formal kinetic models were selected, focusing on extracellular molecular species—namely substrates and products—as well as biomass. These models do not incorporate intracellular metabolic reactions, aligning with the study's objective to capture the overall process dynamics in a simplified yet effective manner. In this work three different unstructured models were tested to fit the PHA production process studied. Some of the formal kinetics models described in the literature are presented in Table 4.

Based on these unstructured models, a model including the most relevant aspects of them was merged in a new model describing the three main processes taking place: biomass growth, substrate consumption and PHA accumulation.

Usually, the optimization of the PHA production processes is carried out by using unstructured models. This can be explained in the operational procedure of the industrial processes, mainly based on the control of macroscopic variables.

In the model proposed in this work, the exponential growth rate of *C. necator* was related to the residual biomass (X_R), see Eq. 1. In the literature, it has been described that the cell components of *C. necator* consist of two main parts, the PHA, which is an intracellular biopolymer, and the X_R, which consists of the rest of the cellular components [53]. This X_R is the catalytically active component of the cell and it is responsible for the metabolic activity of the cells. Because of that, the usual term X in growth rate equations was therefore replaced by X_R to represent the autocatalytic growth of the cells [54]. In order to include the cellular lysis, a decay term was also included in the equation used in the model proposed in this work, see Eq. (1).

$$\frac{dX_R}{dt} = \mu_m \cdot \frac{S}{k_S + S} \cdot X_R - k_d \cdot X_R \tag{1}$$

where X_R is the residual biomass (g/L), t is the reaction time (d), S is the substrate- in this case VFA- concentration (g/L), μ_m is the maximum specific growth rate (h^{-1}) and k_d is the decay rate (h^{-1}).

Regarding the PHA synthesis by the *C. necator*, in the literature it has been described that PHA is synthesized both in the growth and stationary phases [50,51,55]. This phenomenon of growth and non-growth associated synthesis can be represented by the Luedeking-Piret model [56].

$$\frac{dP}{dt} = k_1 \cdot \frac{dX_R}{dt} + k_2 \cdot X_R \tag{2}$$

where P is the product concentration -in this case PHA- (g/L), k_1 and k_2 are constants of proportionality to the instantaneous rate of biomass growth, and to the actual biomass concentration.

Finally, substrate consumption during the PHA biosynthesis process was described as a function depending on the biomass growth, product generation and cell maintenance (including cell mobility, enzyme turnover, osmotic work, nutrient storage and other processes).

$$\frac{dS}{dt} = -\frac{1}{Y_{X/S}} \cdot \frac{dX_R}{dt} - \frac{1}{Y_{P/S}} \cdot \frac{dP}{dt} - k_e \cdot X_R \tag{3}$$

where $Y_{X/S}$ is the biomass yield (g/g), $Y_{P/S}$ is the PHA yield (g/g), and k_e is the substrate used for maintenance.

The simultaneous estimation of model parameters was conducted by initializing them with a predefined set of values and computing the corresponding theoretical Biomass PHA and VFA profiles, as described by Eqs. (1)-(3). The Gauss–Newton optimization algorithm was employed to iteratively solve the model equations by minimizing the objective function, *X(p)*, defined as the sum of squared errors (SSE) between experimental and simulated data. The parameter set yielding the global minimum of the objective function was identified as the optimal solution. Through successive iterations, the initial estimates were refined until convergence was achieved, thereby providing the most accurate parameter values consistent with the observed data [57,58]. The mathematical SSE expression used in the estimation process, is provided in Eq. (4).

$$\chi(\mathrm{p}) = \frac{\sqrt{\sum_{i=1}^{n} \left(x_{meas,i} - x_i(\mathrm{p})\right)^2}}{\bar{x}} \tag{4}$$

where *x(p)*, is the SSE, $x_{meas,i}$, is the i^{th} experimental measurement of the parameter, $x_i(p)$, is the calculated value of the model corresponding to the i^{th} measurement, *n* is the number of data points and $\bar{x}$, is the mean the measurements. PHA productivities were calculated based on volatile fatty acid (VFA) consumption, expressed as g PHA/g VFA, and on intracellular polymer accumulation, expressed as g PHA/g biomass.

Table 4
Unstructured models used to describe the PHA production process.

Substrate consumption	Biomass growth	PHA production	Reference
$\frac{dS}{dt} = -\frac{1}{Y_{X/S}} \cdot \frac{dX}{dt} - \frac{1}{Y_{P/S}} \cdot \frac{dP}{dt} - K_e \cdot X$	$\frac{dX}{dt} = \frac{\mu_m}{k_S + S} \cdot S \cdot X \cdot \left(1 - \frac{X}{X_m}\right)$	$\frac{dP}{dt} = \alpha \cdot \frac{dX}{dt} + \beta \cdot X$	[50]
$\frac{dS}{dt} = -K_A \cdot S \cdot (X \cdot A)$	$\frac{dX}{dt} = \frac{\mu_m}{k_S + S} \cdot S \cdot X$	$\frac{dP}{dt} = \alpha \cdot \mu \cdot X \cdot A + b \cdot X \cdot A$	[51]
$\frac{dS}{dt} = -k_7 \cdot \frac{dX_R}{dt} - k_8 \cdot \frac{dP}{dt} - k_6 \cdot X_R$	$\frac{dX}{dt} = \mu_m \cdot \frac{S}{k_S + S} \cdot \left(1 - \left(\frac{S}{S_m}\right)^n\right) \cdot X_R$	$\frac{dP}{dt} = k_1 \cdot \frac{dX_R}{dt} + k_2 \cdot X_R$	[52]

3. Results and discussion

The influence of ozone pre-treatment on the growth of *C. necator* and on the PHA production was evaluated. Ozone pre-treatment was applied to the biosolids before its blending with vinasses in the dark fermentative process. The application of the ozone pre-treatment resulted in permeates production with different VFA profiles.

3.1. Characterization of raw feeding

The permeate obtained from the effluent of the DF reactor presented different characteristics due to the ozone pre-treatment (P—O) versus (P—C), see Table 3.

When ozone pre-treatment was carried out, the permeate presented a lower S_{COD} content but higher DOC (59.43%) and DTN (47.61%). This can be explained because ozone pre-treatment facilitates particle solubilization and improves hydrolysis in DF [59,77], resulting in a reduced concentration of particulate substrates but increased DOC concentrations. The increase in DTN may also be explained by the solubilization of different nitrogen forms caused by the ozonation process. Consequently, these trends were also observed in the permeate.

A permeate with a higher carbon content facilitates carbon uptake by the biomass, leading to enhanced cell growth and PHA storage [10]. As previously stated, the C/N ratio is crucial for the PHA storage. In the literature, a C/N ratio ranging from 6 to 13 has been described as the optimum to provide the highest PHA content, leading higher or lower C/N ratios to poorer PHA accumulations [60,61]. In this context, the two permeated obtained in this work presented C/N ratio within the optimal range described in the literature: 7.07 for P—O and 6.55 for P—C. The C/N ratio of the permeate obtained using P—O falls within the optimal range, ensuring effective substrate uptake and PHA production, whereas the C/N ratio of the permeate obtained without pre-treatment (P—C) is closer to the lower limit of the optimal range but still effective.

With regard to total acidity, as can be observed in Table 3, the permeate shows an increase of 7.16% in total acidity when P—O was

applied. It has been shown that ozone treatment improves the VFAs production in samples of biosolids under DF [41,62,63]. This increase in VFA production can be attributed to the increased hydrolysis of organic matter in the biosolids due to the harsh oxidation' conditions caused by the ozone treatment [30,31]. This aspect is important as the yield of PHA production is influenced by the initial concentration of total volatile acidity as well as its profile [17].

The VFA profile present in each permeate is shown in Fig. 1. The use of ozone as a pre-treatment influenced the VFA profile produced in the DF reactor and, therefore, in the permeate obtained from its effluent. The permeate obtained from the experiment carried out with ozonized biosolids was characterized by high VFAs concentrations with a carbon number equal to or greater than 4 ($n \geq 4$) (Fig. 1.A). In particular, ozone promoted the production of a VFA profile with a significant increase in the butyric acid content, which increased by 88.14%, see Fig. 1.B.

The available VFA profile directly dictates the composition of the synthesized PHA. According to the literature, even-chain VFAs such as acetate (C_2) and butyrate (C_4) serve as precursors for hydroxybutyrate (HB) monomers. In contrast, odd-chain VFAs like propionate (C_3) and valerate (C_5) are essential for the formation of hydroxyvalerate (HV) monomers, resulting in the copolymer P(HB-co-HV) [64]. Consequently, the predominance of even-chain VFAs in the dark fermentation permeate aligns with the production of PHB as the sole polymer observed in this study.

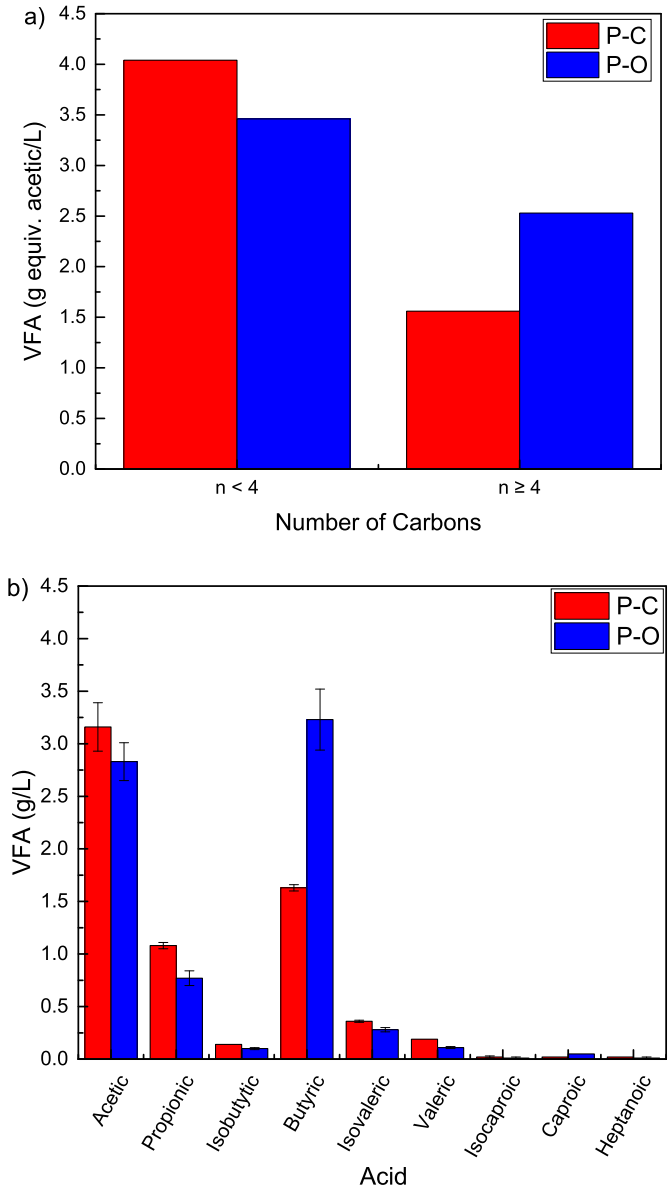

Fig. 1. VFAs concentrations with carbon number less than 4 ($n < 4$) and with carbon number greater than or equal to 4 ($n \geq 4$) (A) and individual VFAs (B) in the permeate of non-ozonized sample (P—C) and in the permeate of ozonized sample (P—O).

3.2. VFA consumption during PHA fermentation

The VFAs present in the DF permeates were metabolized by *C. necator*, resulting in the production of PHA. Fig. 2 shows the evolution of total VFA concentration throughout the PHA fermentation process. At the beginning of the cultivation, the initial total VFA concentrations were 4.68 g equiv. acetic/L and 5.69 g equiv. acetic/L for P—C and P—O, respectively. In both systems, a progressive decrease in VFA concentration was observed as the carbon source was assimilated by *C. necator*. For P—C, the VFAs were rapidly consumed, reaching a minimum value of 0.29 g equiv. acetic/L at 24 h. In contrast, for the P—O medium, this minimum concentration was reached at 48 h.

The higher initial concentration of total VFA obtained when dealing with P—O samples, see Table 3, extended the PHA production process even after 24 h. This extension of the growth phase is directly correlated to the higher initial substrate load, requiring a longer period for substrate consumption. In contrast, the lower total VFA obtained when dealing with P—C samples leads to a reduced PHA production process. At the end of the PHA production process, a very similar total VFA removal were achieved when operating with both the P—C and P—O permeates, being their values 95.05% and 95.48%, respectively.

Fig. 3 shows the evolution of the concentration of every VFA analyzed during PHA fermentation. This evolution provides an insight into the VFA consumption patterns and, consequently, the metabolic behavior of *C. necator*.

In the first scenario (Fig. 3.A), where the fermentation was carried out with the DF permeate obtained from samples without ozone pre-treatment (P—C), acetic acid was the predominant VFA, followed by butyric acid. During the first 12 h, the butyric acid curve was steeper than that of the acetic acid curve, indicating a faster consumption rate.

This trend was also observed in the second scenario (Fig. 3.B), where fermentation was carried out with the DF permeate obtained from ozonized samples (P—O). In this case, butyric acid was the dominant VFA, followed by acetic acid. Similar to the first scenario, but even more

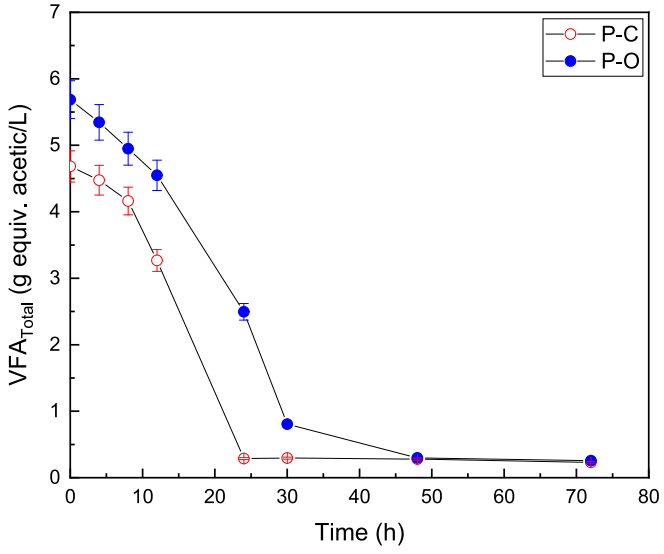

Fig. 2. Evolution of total VFA concentration during PHA fermentation using DF permeate obtained from untreated (P—C) and ozone-treated (P—O) samples.

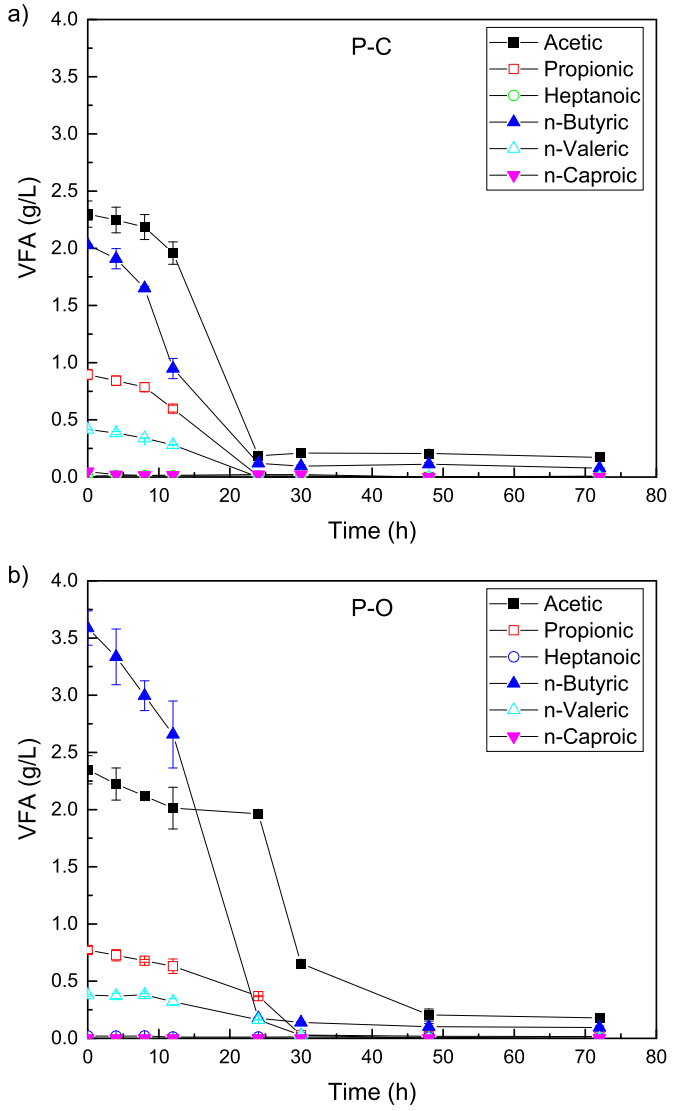

Fig. 3. Evolution of the concentration of individual VFAs throughout PHA fermentation, using a) DF permeate obtained from untreated (P—C) and b) ozone-treated (P—O) samples.

pronounced, the slope of the butyric acid concentration trend was much steeper than that of the acetic acid curve during the first 12 h. By 24 h, butyric acid was completely consumed, and acetic acid started to be metabolized.

This trend is supported in the literature by a study investigating the kinetics of PHA production and VFA consumption by *C. necator*, which showed a higher initial consumption rate of butyric acid compared to acetic and propionic acids. In addition, fast utilization of butyric acid within the first 12 h of cultivation resulted in a slight increase in poly-hydroxybutyrate (PHB) concentration [65]. Further studies confirm that butyrate serves as a preferred substrate among different VFAs for *C. necator* in PHA production [19,20]. This metabolic preference is further supported by stoichiometric efficiency; theoretically, the PHB yield from butyric acid (0.94 Cmol PHB/Cmol HBu) is approximately 40% higher than that from acetic acid. From a biochemical perspective, the conversion of butyrate into 3-hydroxybutyrate monomers involves a more direct pathway with lower energy requirements compared to shorter-chain VFAs [66]. Consequently, the metabolic behavior of *C. necator* observed in this study was strongly influenced by the initial VFA profile differences. In the P—O medium, the high initial availability of butyric acid allowed the culture to prolong the consumption of this energetically preferred substrate. This extended availability contributed to the higher biomass and PHA yields observed compared to the P—C medium, where the early depletion of butyrate forced a quicker metabolic shift to the less efficient acetic acid consumption. The PHA productivity when dealing with ozonized samples was about 4.3 g PHA/(L·d).

The VFAs with a carbon number greater than 4 ($n > 4$) were completely metabolized (valeric, caproic and heptanoic) whereas acetic, propionic and butyric acids ($n \leq 4$) were not completely metabolized. Fig. 4 shows the final percentage removal of acetic, propionic and butyric acids during fermentation for PHA production. This shows that *C. necator* has a stronger preference for VFAs with carbon numbers greater than 4. Studies by Vu and collaborators (2022) have also observed that in addition to common VFA such as acetic, butyric and propionic acids, *C. necator* has a remarkable ability to assimilate long-chain carboxylate compounds, including valeric, isovaleric, isobutyric and caproic acids.

In addition, Fig. 4 shows that the use of ozone as pre-treatment promotes the subsequent metabolism of butyric acid for PHA production.

3.3. Biomass growth during PHA production

Bacteria consume the VFAs as a carbon source for their growth and for intracellular storage of carbon and energy, which would be accumulated as PHA.

Fig. 5 shows the evolution of cell biomass concentration throughout the PHA production experiment. Bacterial growth trend perfectly follows the evolution of VFA consumption during the process (Fig. 2). In the PHA fermentation carried out using the DF permeate obtained with substrates without pre-treatment (P—C), the total VFA concentration was minimal after 24 h, (0.29 g equiv. acetic/ L) see Fig. 2, obtaining at that moment the maximum biomass concentration (2.74 g /L in terms of CDW) see Fig. 5a. In contrast, in the PHA fermentation using the DF permeate obtained with ozone pre-treated samples (P—O), VFAs were still available after 24 h (2.50 g equiv. acetic/ L), see Fig. 2, obtaining the maximum biomass concentration after 30 h of operation (3.15 g /L in terms of CDW), see Fig. 5a. This biomass concentration is about 15% higher than that obtained with P—C. From that moment on, 24 h for

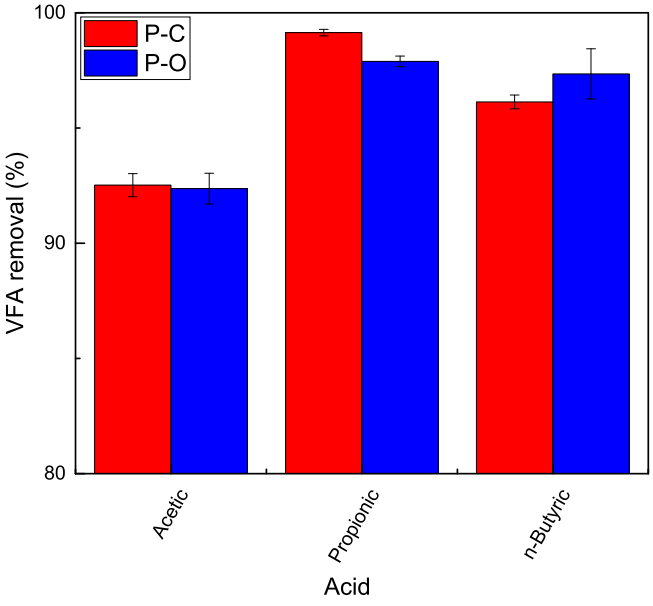

Fig. 4. Final removal of acetic, propionic, and n-Butyric acids during the PHA production process, using DF permeate obtained from untreated (P—C) and ozone-treated (P—O) samples.

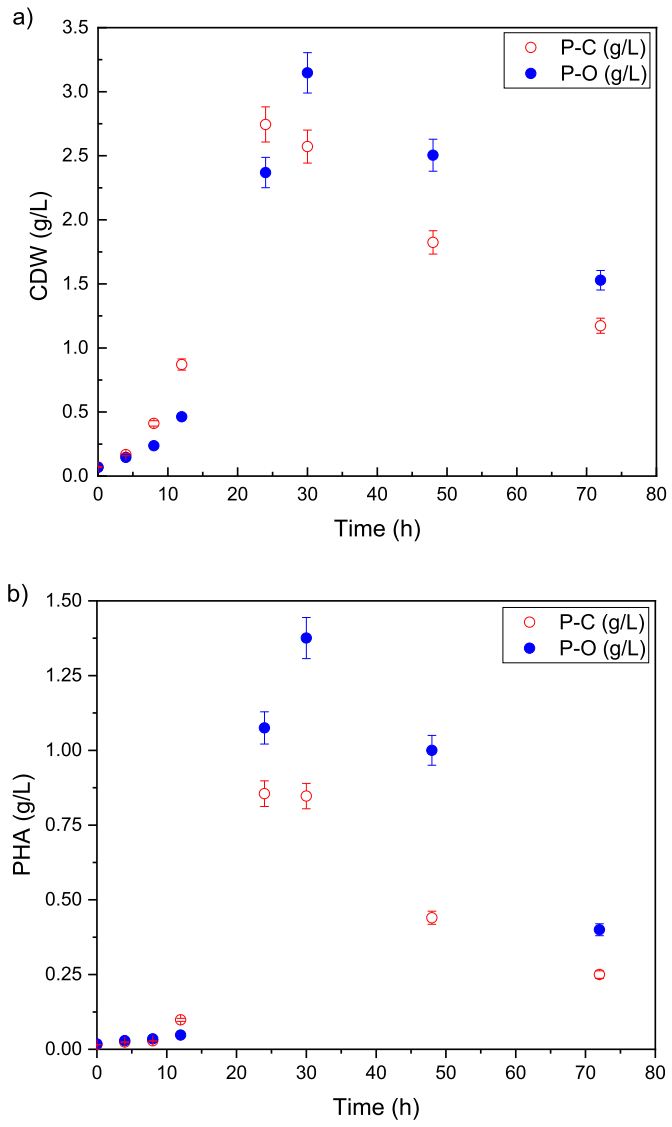

Fig. 5. a) Evolution of cell biomass concentration in terms of Cell Dry Weight (CDW) during the PHA fermentation process, using DF permeate untreated (P—C) and ozone-treated (P—O) samples. b) PHA stored during PHA fermentation, using permeate obtained from untreated (P—C) and ozone-treated (P—O) AMBR.

P—C and 30 h for P—O, a significant decrease in cell biomass concentration was observed due to the endogenous decay, see Fig. 5a. Despite this behavior, acetic acid (0.66 g/L) was still present in the PHA fermentation from P—O substrates after 30 h, see Fig. 3. This trend has been also observed in other studies Haga clic o pulse aquí para escribir texto.using synthetic VFAs, where a significant decrease in the biomass concentration was observed after 36 h, although a small amount of acetic acid (0.7 g/L) was still present in the medium after 48 h [67]. This behavior can be explained by substrate mass transfer limitations at the end of the PHA production process, or due to its scarce consumption for cell maintenance rather than cell growth [68,69]. This behavior continued until all available carbon sources were completely consumed, after which the bacteria underwent cell lysis or, having depleted their stored PHA reserves, began the endogenous decay, as indicated by the drop in OD, see Fig. 5a.

The literature also reports that, although VFAs possess a high potential for microbial metabolism, certain VFA concentrations can exert an inhibitory effect on bacterial growth [13,70]. In this study, based on the previous results and the results reported in the literature it was verified that the VFA concentration reached in the DF permeate does not reach the threshold concentrations causing inhibitory effects on the growth of *C. necator*. The absence of an extended lag phase, together with the increased specific growth rate observed in the P—O medium (which contains a higher proportion of butyric acid), further indicates that the substrate concentrations remained within the non-inhibitory range for this strain.

3.4. PHA production

During fermentation, *C. necator* consumes the carbon present in VFAs for cell growth and PHA storage. As it is shown in Fig. 5b, the PHA concentration during PHA production matches the cell biomass trends previously presented in Fig. 5a. Initially, the PHA accumulation from the DF permeate obtained without pre-treatment (P—C) and pre-treated with ozone (P—O) was similar up to 20 h. After this time, PHA production from P—O exceeded that from P—C.

This trend is consistent with the fact that the cell biomass concentration peak was obtained after 22 h of operation in the fermentation carried out with P—C, coinciding with the complete consumption of available VFAs, see Fig. 2, and the highest PHA concentration observed for this experiment (0.86 g/L), see Fig. 5b. In contrast, fermentation with P—O showed a peak in cell biomass concentration after 30 h of operation. At this peak, VFAs were still available, resulting in a remarkable PHA concentration of 1.38 g/L.

After 24 h for P—C and 32 h for P—O, the available VFAs were depleted, see Fig. 2, resulting in a decrease in bacterial concentration (Fig. 5a) and PHA concentration (Fig. 5b) due to cellular decay and the endogenous metabolisms. The difference in timing underscores the close relationship between fermentation duration and metabolic state. The higher initial substrate availability in the P—O medium delayed the onset of substrate limitation, thereby extending the active PHA-accumulation phase by approximately 6–8 h relative to the P—C medium. As a result, the ozone pretreatment not only supplied a greater abundance of carbon precursors but also prolonged the period of anabolic activity before the culture transitioned to endogenous polymer consumption. In a study using a synthetic mixture of acetic, butyric and propionic acids, a decrease in PHA concentration was observed after 36 h of cultivation due to substrate depletion by *C. necator*. This suggests that the cells may use the intracellularly stored PHA as an energy source to maintain their bioactivity as previously reported in the literature [65].

At peak production, the PHA yield in grams per gram of biomass (in terms of CDW) was 0.325 and 0.449 g PHA/g CDW for P—C and P—O, respectively. Resulting the ozone pre-treatment in a 38.15% increase in the PHA yield. In the literature, when treating other substrates, the conversion of VFAs into PHAs, measured in grams of PHA per gram of biomass (in terms of volatile suspended solids, VSS), reached values of 0.4–0.5 g PHA/g VSS for cheese whey and organic fraction of municipal solid wastes (OFMSW), 0.6–0.7 g PHA/g VSS for winery wastewater, and 0.2–0.3 g PHA/g VSS for olive mill wastewater [71]. In this work, the maximum PHA cell content ranged from 4.99% to 27.16% of cell dry weight for the cases without pre-treatment and with ozone pre-treatment, respectively. Therefore, the use of ozone led to an improvement in this ratio of about 544.25%.

At the PHA peak, an accumulated PHA concentration of 0.85 g/L was obtained from P—C and 1.35 g/L from P—O (Fig. 5a). From these results, the use of ozone as a pre-treatment in the biosolids resulted in a 59.35% increase in the accumulated PHA concentration from the DF permeate.

In studies with a similar experimental approach [72], where pre-treatments such as hydrodynamic cavitation were evaluated on a mixture of biological sludge from a WWTP with fruit and vegetable waste, the VFAs production by fermentation and the suitability of the effluent for PHA production by *C. necator* were assessed. The best result obtained was 2.90 g/L of accumulated PHA, which is higher than the

1.35 g/L obtained in this study using ozone pre-treated biosolids and vinasse as initial substrates, in contrast to the mixture with food waste. This lower value could be explained by the lower biomass concentration reached during the PHA fermentation.

Ozone pre-treatment has also been utilized in other studies to produce PHA from complex oily bilge water (OBW). Under optimized conditions (ozone dose of 2 g/L, pH 6, and contact time of 75 min), PHA production was enhanced by 4.5-fold [73]. This value is higher than that obtained in this study (1.59-fold increase).

Although the final PHA concentration obtained in this study (1.35 g/L for P—O) was lower than values reported in previous works using macroalgal hydrolysates—which reached up to 2.65 g/L [74] — This discrepancy is primarily due to the dilute composition of the dark-fermentation permeate, rather than reflecting any intrinsic metabolic constraint. In fact, the conversion efficiency of the ozone-treated VFA stream was superior. The yield obtained here ($Y_{P/S} = 0.91$ g PHB/g VFA) significantly exceeded the yields observed in sugar-based fermentations from *Rugulopteryx okamurae* or carrageenan waste, which ranged from 0.21 to 0.77 g PHB/g sugar [74–76]. This confirms that short-chain fatty acids are metabolically more favorable precursors for PHA biosynthesis using *C. necator* than algal sugars, provided that the initial concentration is sufficient.

3.5. Modelization of the PHA production

The model proposed in this work was fitted to the experimental data set obtained with and without ozone pre-treatment of the wastes.

In Fig. 6.a, the fitting of the experimental results obtained without ozone pre-treatment is presented. In this Figure, it can be observed that a very good fitting of the model to the experimental results was obtained. As can be seen in Fig. 6.a, the VFA were consumed in about 22.0 h, reaching an X_R concentration of about 1.9 g/L, and a PHA accumulation of 0.85 g/L. In the case of the fitting to the ozone pre-treated samples, see Fig. 6.b, a very good fitting was also obtained. In this case, the VFA consumption took about 32 h, which can be explained because of the higher initial VFA concentration reached during the DF of ozonized sampled, which was about a 20% higher when the biosolids were ozonized before the DF experiments. The maximum X_R concentration reached was about 1.9 g/L, a value very similar to that obtained when no pre-treatment was performed, but the maximum accumulated PHA was about 1.35 g/L. In this sense, it is important to highlight that the ozonized samples lead to a significantly higher PHA accumulation which increased from 0.85 to 1.35 g/L, an increase higher than 55% when the VFA concentration only increased 20%.

As can be observed in Fig. 6, a very good fitting was obtained when dealing with both ozonized and non-ozonized samples. From the mathematical fitting, the values of the main stoichiometric and kinetic parameters of the model proposed in this work were determined. The results obtained are presented in Table 5.

Analyzing the results presented in Table 5, it can be highlighted the very similar values were obtained in most cases, obtaining significant differences in the PHA yield which increases from 0.67 to 0.91 g PHA/g VFA. This increase indicates that a defined quantity of VFA derived from ozonized samples yields a significantly higher production of PHA compared to the same quantity obtained from non-ozonized samples. A similar increase was observed in the K_1 parameter, which increases from 1.53 to 4.16 g PHA/g X_R when the ozonation was performed, which corresponds to an 80% of PHA accumulation in the biomass, being the accumulation when dealing with non-ozonized substrates about an 60%. The K_1 parameter is the PHA constant of proportionality to the instantaneous rate of biomass growth. In this case, the ozone treatment increases the value of this constant significantly whereas the proportional constant to residual biomass concentration K_2, remained unaltered, around 0.001 1/d. These results indicate that ozonation facilitates the PHA accumulation during *C. necator* growth but does not significantly affect to the PHA accumulation during maintenance of existing biomass.

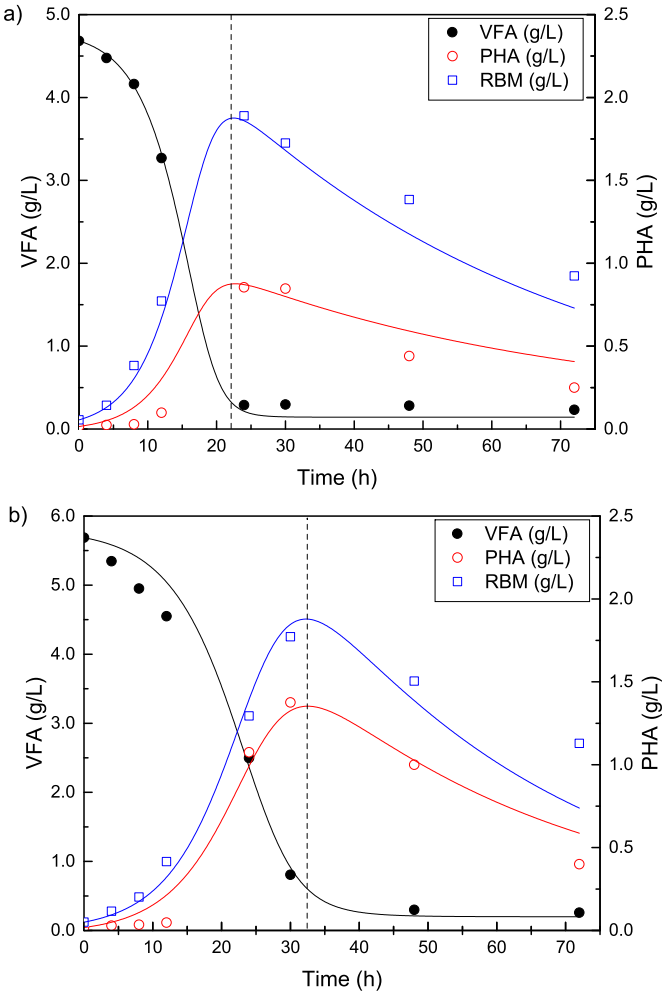

Fig. 6. VFA, Biomass and PHA concentration profiles when operating with a) non-ozonized samples and b) Ozonized samples. Lines correspond to model fitting.

Table 5
Values of the kinetic and stoichiometric parameters of the model.

Model's parameter	Non-pretreated	Ozonation pre-treatment
μ_m (d^{-1})	0,45	0,45
K_s (g/L)	3,05	6,37
K_d (d^{-1})	0,04	0,04
K_e (d^{-1})	0,04	0,05
$Y_{X/S}$ (g X_R/g VFA)	0,05	0,05
$Y_{P/S}$ (g PHA/g VFA)	0,67	0,91
K_1 (g PHA/g X_R)	1.53	4,16
K_2 (d^{-1})	0,001	0,001

4. Conclusions

Ozone pre-treatment of organic waste markedly enhances polyhydroxyalkanoate (PHA) production by *C. necator* by improving both substrate quality and metabolic performance. Ozonation increased dissolved organic carbon and nitrogen availability, resulting in a more favorable C/N ratio for acidogenic fermentation and subsequent PHA biosynthesis. Moreover, the selective enrichment of butyric acid—a preferred carbon source for *C. necator*—promoted higher microbial growth rates and intensified PHA accumulation. Kinetic and stoichiometric modeling confirmed the efficiency gains achieved through ozone conditioning, with PHA contents reaching up to 80% of the biomass. Overall, these findings demonstrate that ozone pre-treatment is a robust

and effective strategy for upgrading waste-derived feedstocks, significantly advancing the conversion of organic residues into high-value bioplastics and strengthening the sustainability and performance of integrated biorefinery systems. This approach aligns with circular economy principles by transforming low-value waste into high-added-value biopolymers, thereby enhancing the overall sustainability and economic viability of integrated biorefinery platforms.

CRediT authorship contribution statement

Encarnación Díaz-Domínguez: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Agustín Romero-Vargas:** Investigation, Formal analysis. **Luis Alberto Fernández-Güelfo:** Methodology, Formal analysis. **Francisco Jesús Fernández-Morales:** Writing – review & editing, Writing – original draft, Methodology, Data curation. **María Eugenia Ibañez-López:** Investigation, Formal analysis. **James Lyng:** Writing – original draft, Supervision, Methodology. **José L. García-Morales:** Writing – review & editing, Supervision, Project administration, Conceptualization.

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.

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Data availability

Data will be made available on request.

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