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Polyhydroxybutyrate Production from the Macroalga *Rugulopteryx okamurae*: Effect of Hydrothermal Acid Pretreatment

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Abstract: This study focuses on mitigating the socio-economic and environmental damage of the invasive macroalga *Rugulopteryx okamurae* and counteracting the pollution from petroleum-based plastics by using the alga as a feedstock for polyhydroxybutyrate (PHB) production. The enzymatic hydrolysis of *R. okamurae*, non-pretreated and hydrothermally acid-pretreated (0.2 N HCl, 15 min), was carried out, reaching reducing sugar (RS) concentrations of 10.7 g/L and 21.7 g/L, respectively. The hydrolysates obtained were used as a culture medium for PHB production with *Cupriavidus necator*, a Gram-negative soil bacterium, without supplementation with any external carbon and nitrogen sources. The highest yield (0.774 g PHB/g RS) and biopolymer accumulation percentage (89.8% cell dry weight, CDW) were achieved with hydrolysates from pretreated macroalga, reaching values comparable to the highest reported in the literature. Hence, it can be concluded that hydrolysates obtained from algal biomass hydrothermally pretreated with acid have a concentration of sugars and a C/N ratio that favour PHB production.

Keywords: macroalgae; pretreatment; enzyme hydrolysis; fermentation; polyhydroxyalkanoates

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1. Introduction

Polyhydroxyalkanoates (PHAs) as precursors of bioplastics represent a promising, biodegradable, sustainable, and eco-friendly alternative for counteracting the negative environmental impact of conventional petrochemical-based plastics [1]. In addition to their high functionality, they show the advantage of reducing the landfill requirements for plastic waste disposal and reducing the environmental impact [2,3].

PHA are bio-polymers synthesised by different micro-organisms as lipids inclusions and accumulated in the form of intracellular granules as energy storage materials, ranging in size from 0.2 to 0.5 µm [4]. The micro-organism *Cupriavidus necator* has been widely studied for PHA production, given its ability to accumulate significant amounts of PHAs from renewable biomass [5]. It has been demonstrated that the wild-type *C. necator* can accumulate poly(3-hydroxybutyrate) [P(3HB)], one of the most representative members of PHAs, in around 80% of its cellular dry weight (CDW). This bacterium does not accumulate PHA in the growth phase, initiating its production in the presence of an excess carbon source and some nutrient limitations, such as nitrogen, phosphorous, oxygen, or magnesium [4].

PHA production at full scale is limited, given the high production cost compared to the major fossil-fuel-based plastics [6]. Raw materials are estimated to account for about 45% of the total production cost, representing the carbon source with 70–80% of the total expense [7,8]. To overcome this limitation, renewable feedstock seems promising for achieving their economically feasible production with the future circular economy. Many studies have been carried out on the PHA production from lignocellulosic biomass [9–11], including wood wastes, agricultural residues, and energy crops, with cellulose, hemicellulose, and lignin as

their main structural components [12,13]. However, the use of lignocellulosic biomass shows some limitations, such as the competition for land occupation with edible crops and the high cost of breaking the natural recalcitrance of its structure [14]. To address these problems, the use of macroalgae biomass as a raw material for PHA production has recently received much attention, given the fact that it does not require freshwater and arable land for its cultivation, so it does not compete with conventional food sources [13,15,16]. Furthermore, seaweed biomass has a high carbohydrate content, with a low proportion of lignin, which facilitates its processing in biorefinery processes [17].

One of the limitations of the use of seaweed for PHA production is the lack of enough algal biomass to supply the market for its exploitation in some countries. In this regard, the high availability of underutilised beach-cast seaweed biomass makes it an attractive and sustainable alternative to satisfy the demand [18]. This biomass is accumulated in large quantities on the coast, causing serious environmental and social problems, and affecting the economy of the region [18,19]. An example of algae causing this type of problem is the invasive macroalga *Rugulopteryx okamurae* used in this work. Only in the first summer after its occurrence in the Strait of Gibraltar (Tarifa and Ceuta) did 5000 tonnes of macroalgae deposits have to be removed from 15.5 km of the coastline of Ceuta [20]. The high rate of spread of this alga causes changes in the marine habitat, seriously affecting benthic communities, which are being displaced. Thus, the competition for space is affecting *Astroides calycularis* (orange coral), one coral species threatened by the algal invasion [21].

Moreover, this invasive alga affects not only the environment but also the economy of the affected regions. Affected beaches have to be restored, which is costly. It also sometimes results in the closure of popular beaches during the tourist beach season. To this must be added the economic losses for the fishing sector, which is harmed by the accumulation of algae in fishing nets [20,21]. According to the latest *R. okamurae* strategy and control document published by the Spanish Ministry for Ecological Transition and Demographic Challenge (MITECO) approved on 28 July 2022, this alga continues to spread throughout the Mediterranean. Moreover, this invasive macroalga has already been included in the EU list of alien species of concern since 2019.

In order to achieve the sustainable exploitation of macroalgae, it is necessary to convert their polymeric carbohydrates into monomeric sugars through saccharification processes, which can be subsequently fermented to PHAs [22]. Hydrothermal acid pretreatment is one of the most common methods applied for the pretreatment of lignocellulosic biomass [23] and it has been widely used for algae biomass [24,25]. For this reason, this type of pretreatment was applied here to *R. okamurae*.

Biomass from food waste, and the agricultural and forest industries has been widely studied for the production of PHAs. In the case of macroalgae biomass, the studies are more recent and there is little literature related to this topic. Studies for this purpose have been carried out with *Sargassum* sp. and *Laminaria japonica,* which are among the few examples that can be cited [26,27]. In the first case, the best hydrothermal acid pretreatment condition was studied (reaction time and acid concentration) using a 10% solid biomass (*w*/*v*) of *Sargassum* sp., obtaining the best reducing sugar (RS) concentration (12 g/L) at 0.15 N H_2SO_4 for 30 min and 121 °C. With the biomass pretreated, in situ, the best enzymatic hydrolysis condition (pH and temperature) was studied, obtaining the highest RS release (8 g/L) at pH: 5 and 50 °C. The final hydrolysed medium obtained (20 g/L RS between the two stages) was used for fermentations with *C. necator* PTCC 1615 (derived from ATCC17699), with which the addition of different nitrogen sources (yeast extract, urea, NH_4Cl, and $(NH_4)_2SO_4$) at a concentration of 1 g/L, and the addition of NaCl at different concentrations (4 g/L, 8 g/L, 12 g/L, 16 g/L, and no addition) were evaluated. The best concentration of PHB obtained (3.93 g/L), yield (0.47 g PHB/g RS consumed), and PHB accumulation (73.4%) was achieved with 1 g/L $(NH_4)_2SO_4$ and 8 g/L NaCl after 36 h of fermentation, and then started to decrease. In the second case, 10% (*w*/*v*) of *L. japonica* was exposed to hydrothermal acid pretreatment using different concentrations of H_2SO_4 and HCl (from 0.05 N to 0.3 N). The pretreated liquids with 0.3 N H_2SO_4 for 20 min at 120 °C

were selected for fermentation with *C. necator* NCIMB 11599, which had to be concentrated (from 6.1 g/L to 20 g/L RS), and 1 g/L $(NH_4)_2SO_4$ was added as a nitrogen source. Thus, a PHB concentration of 1.58 g/L and an accumulation rate of 32% was reached after 60 h of fermentation, and then started to decrease. In this work, the brown alga *Rugulopteryx okamurae* was used for the first time for the production of PHB. In addition, for the first time, changes in the amount of the carbon and nitrogen source (C/N ratio) contained in the algal hydrolysate medium (depending on whether the alga was pretreated or not) were evaluated for their influence on *C. necator* growth and biopolymer accumulation.

In this context, the main objective of this study was to assess PHB production by fermentation with *Cupriavidus necator* using the macroalga *Rugulopteryx okamurae* as raw material, developing the following two comparisons to evaluate its influence in the PHB yield: (1) the use of hydrolysed media enriched in fermentable sugars from pretreated and non-pretreated macroalga; and (2) the use of synthetic media prepared with different C/N ratios to compare fermentations with hydrolysed media.

2. Material and Methods

2.1. Sampling and Conditioning of Seaweed Biomass

Beach-cast seaweed of the brown macroalga *Rugulopteryx okamurae* was collected in the coastal waters of Punta Camorro (Tarifa, Spain) during low tides in the spring. Salts and residues were removed from the macroalgae by placing them in 25 L polyethylene drums and washing them with tap water until the conductivity was less than 600 µS/cm. Then, washed macroalga was dried in a greenhouse for 24 h and milled using a cutting mill to obtain a 1 mm particle size. They were subsequently stored in hermetically closed drums at room temperature until usage.

2.2. Thermochemical Pretreatment Process

The hydrothermal acid pretreatment was adopted to enhance the release of sugars from *R. okamurae* in the subsequent enzymatic hydrolysis stage. The pretreatment conditions were 15 min of reaction time and 0.2 N of HCl. These conditions were selected according to previous work in which optimal values for the variables' reaction time and acid concentration were studied for *R. okamurae* [28]. Then, 125 mL of acid suspensions with 10% (*w*/*v*) dried biomass was included in 250 mL Pyrex® bottles and introduced in the autoclave at 121 °C. Subsequently, the solid biomass was separated by vacuum filtration with a Whatman No. 1 filter paper and washed with tap water until it showed a pH close to 6.0. This solid biomass had a high moisture content, with the subsequent risk of contamination and degradation of the sugars. Therefore, the biomass was dried in a forced convection oven at 40 °C for 24 h and stored at room temperature until its use in the enzymatic hydrolysis stage. The filtrate was not used for the following steps but it was stored at −20 °C. The procedure described was based on previous studies for *R. okamurae* [28].

2.3. Enzymatic Saccharification

Enzymatic hydrolysis was carried out in 250 mL Erlenmeyer flasks containing 45 mL of 50 mM sodium phosphate buffer (pH 5.0). The biomass was added to the buffer and autoclaved for 20 min at 121 °C before the addition of the commercial enzyme preparation Cellic Ctec2 (Novozymes, Denmark). Following enzyme addition, the flasks were tightly sealed with silicone stoppers and incubated on a rotary shaker at 50 °C. The hydrolysis was performed for 72 h at the following conditions: 10% (*w*/*v*) loading biomass, 50 FPU/g biomass of enzyme preparation, and 250 rpm of stirring rate. A unit of filter paper activity (FPU) was defined as the amount of enzyme that produces 1 µmol of reducing sugars per min under the specified conditions of pH and temperature. These specific conditions and the enzyme preparation were chosen following Romero-Vargas et al. [29]. Samples were collected and analysed for RS content at the end of hydrolysis and stored at −20 °C until they were used for fermentation.

2.4. Fermentation of Sugar Hydrolysates for PHB Production

2.4.1. Micro-Organism and Culture Media

Cupriavidus necator (CECT 4635) was used for PHB production. The rehydration and growth of lyophilised cells were carried out following the instructions provided for the recovery of lyophilised cultures of the CECT (Spanish collection of type cultures). Then, 0.3 mL of nutrient broth consisting of 5 g/L beef extract, 10 g/L peptone, and 5 g/L NaCl was added to the lyophilised vial, and then it was incubated for 30 min at room temperature. After rehydration, the entire volume was transferred to a conical centrifuge tube with 5 mL of nutrient broth and incubated in a rotary shaker (MaxQ6000, Thermo Fisher, Waltham, MA, USA) at 30 °C and 250 rpm for 48 h. Next, the grown culture was transferred to a 100 mL Erlenmeyer flask with 20 mL of nutrient broth and incubated at 30 °C and 250 rpm for 24 h. The culture finally obtained was recollected in 2 mL cryotubes with nutrient broth in 50% glycerol and stored at −70 °C until usage.

2.4.2. PHB Production by *C. necator* on Synthetic Media

For inoculum preparation for the fermentations, the bacteria from −70 °C stocks were reactivated in a 100 mL flask containing 20 mL of nutrient broth (3% (*v*/*v*) inoculum) and incubated at 30 °C and 250 rpm for 16 h (optical density value between 4–5). Subsequently, the fermentations were carried out in 500 mL Erlenmeyer flasks with 100 mL of synthetic medium consisting of 0.2 g/L $MgSO_4 \cdot 7H_2O$, 1.5 g/L KH_2PO_4, 9 g/L $Na_2HPO_4 \cdot 12H_2O$, and 1 mL of trace element per litre. The trace element 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. Glucose was selected as carbon source (C) and ammonium sulphate as nitrogen source (N), which were used at different concentrations to evaluate cell growth and PHB productions, specifically, 10, 20, and 30 g/L for glucose (C10, C20, and C30, respectively), and 0, 0.1, and 1 g/L for $(NH_4)_2SO_4$ (N0, N0.1, and N1 respectively). Before fermentation, the pH of the synthetic media was adjusted to 7.2 with NaOH 1 M and sterilised at 121 °C for 20 min in an autoclave. Finally, 1%, 5%, or 10% *v*/*v* inoculum was added to each flask and incubated at 30 °C and 250 rpm for 96 h. During fermentation, samples were withdrawn for the analysis of biomass (cell dry weight, CDW), reducing sugars (RSs), and polyhydroxybutyrate (PHB) accumulation. The fermentations were carried out in triplicate.

2.4.3. PHB Production by *C. necator* on Algal Hydrolysate

The sugar hydrolysate obtained from the non-pretreated and pretreated seaweed was used for PHB production with *C. necator*. Fermentations were carried out under the same conditions as the synthetic media. For this purpose, the algal hydrolysate harvested after hydrolysis was centrifuged (Eppendorf 5810R, Barkhausenweg, Hamburg, Germany) at 10,000 rpm for 10 min. The supernatant was collected and supplemented by adding the following salts at a concentration of 0.2 g/L $MgSO_4 \cdot 7H_2O$, g/L KH_2PO_4, and 9 g/L $Na_2HPO_4 \cdot 12H_2O$ (neither glucose nor $(NH_4)_2SO_4$ was added). Then, the pH of the hydrolysates was adjusted to 7.2 using NaOH micropearls. Finally, it was sterilised in an autoclave at 121 °C for 20 min. Before *C. necator* inoculation, 1 mL/L of trace element (same composition as used for synthetic media) was added to the medium. During fermentation, samples were withdrawn for the analysis of biomass, RS, and PHB accumulation. The fermentations were carried out in triplicate.

2.5. Analytical Techniques

RS concentration of hydrolysates obtained after enzymatic saccharification and of samples extracted during PHB fermentation was measured by the dinitrosalicylic acid (DNS) method adapted to microplates [30,31]. Before analysis, the samples were centrifuged at 10,000 rpm for 10 min and the supernatant was used for analysis.

The pH was measured in a pH-meter Basic20 (Crison®, Barcelona, Spain).

For cell concentration in terms of dry cell weight (DCW, g/L), samples taken during fermentations were centrifuged at 10,000 rpm for 10 min and the supernatant was discarded. The biomass pellets were washed with sterile distilled water and centrifuged twice. The biomass pellets were then dried in a hot air oven at 60 °C until the weight remained constant. The DCW concentration was based on a standard calibration curve of optical density measured at 600 nm at different DCWs.

The extraction and quantification of PHB were based on the methodology described by Simona et al. [32]. First, 1 mL of non-filtered culture broth was mixed with 0.2 mL of 10% sodium hypochlorite, for cell lysis and to stop PHB microbial consumption, and stored at –20 °C for the following analysis. The previous esterification into 3-hydroxybutyric esters was necessary for PHB determination by gas chromatography [33]. For this purpose, samples were thawed and centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and the pellet was resuspended in 2 mL of acidic methanol (sulphuric acid 3% *v*/*v*), then transferred to a 10 mL Pyrex® tube and mixed with 1 mL of chloroform with heptadecane (0.1 g/L) as internal standard. The tube was incubated in a thermoblock ACCUBLOCK D-1032 (LabNet Biotecnica, Madrid, Spain) at 100 °C for 4 h. The tube was then cooled to room temperature and 1 mL of distilled water was added and mixed in a vortex for 30 s. The tube was conserved at 4 °C until the aqueous phase and organic phase were separated. Finally, the organic phase was collected and transferred to a chromatography vial for PHB analysis. The determination was performed through gas chromatography (GC-2014, Shimadzu, Kyoto, Japan), equipped with a hydrogen flame ionisation detector (FID) and a Chrompack CPSIL-5CB (25 m × 0.25 mm × 0.25 µm), using nitrogen as the carrier gas. The temperature of the injection port and the detector were 220 °C and 250 °C, respectively. The temperature program used for a total time of 19.65 min was: initial stage, 70 °C for 5 min; intermediate stage, 120 °C (rate 8 °C /min) for 1 min; and final stage, 240 °C (rate 50 °C /min) for 5 min. The monomer 3-hydroxybutyrate (HB) concentration in fermented samples was determined using a calibration curve obtained with the commercial standard of poly (3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (8% *w*/*w* PHV; Merck KGaA, Darmstadt, Germany).

The experimental C/N ratio was measured according to the content of dissolved organic carbon and dissolved nitrogen in the fermentation media, using an Analytik-Jena® multi N/C 3100 carbon analyser (Jena, Germany) with a chemiluminescence detector (CLD) according to the combustion-infrared method (5310B) of the Standard Methods [34].

3. Results and Discussion

3.1. Fermentation for PHB Production with Synthetic Media

Fermentations with different concentrations of $(NH_4)_2SO_4$, N0, N0.1, and N1 were carried out using an inoculum size of 1% *v*/*v* and 10 g/L of glucose (C10). The results obtained for the substrate (RS), biomass (CDW), and product (PHB) profiles are shown in Figure 1. It can be seen how reducing the ammonium sulphate concentration impaired the growth of the bacteria and the PHB production. *C. necator* was only able to grow in fermentation when 1 g/L of $(NH_4)_2SO_4$ was used. In this case, a lag phase was observed for 24 h before PHB accumulation (Figure 1c). This period is associated with the time needed for the micro-organism to enter nitrogen-deficient conditions and its duration will depend mainly on the C/N ratio, but also the carbon and nitrogen source [26,35,36]. Although nitrogen deficiency results in the accumulation of PHB, this nutrient is key to the biosynthesis of proteins, amino acids, nucleic acids, and cofactors [37]. Without nitrogen, or with concentrations of it that are too low, bacteria cannot thrive, which would explain the effect observed in the N0 and N0.1 fermentations. Another work with *Pseudomonas putidas* reports the same effect observed here, where no bacterial growth or PHB accumulation was observed without a nitrogen source [38].

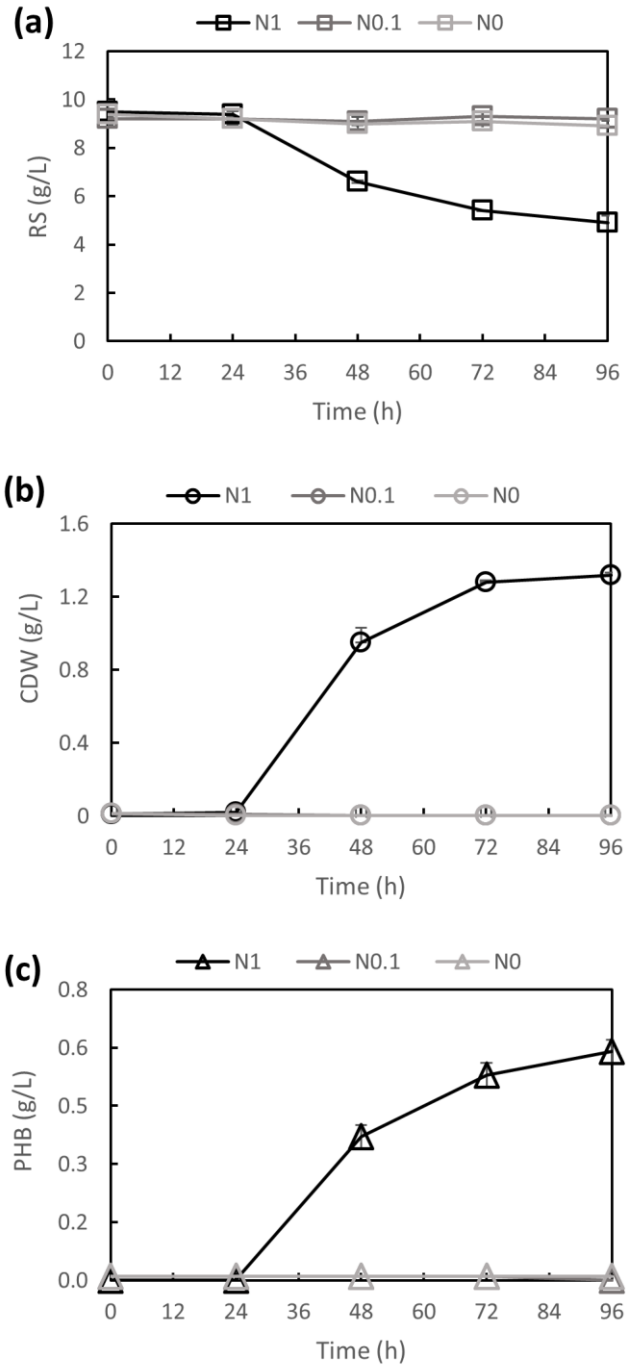

Figure 1. (**a**) substrate (RS), (**b**) biomass (CDW) and (**c**) product (PHB) profiles of the fermentations with 1, 0.1, and 0 g/L of $(NH_4)_2SO_4$ (N1, N0.1, and N0, respectively) with 10 g/L glucose and an inoculum size of 1% *v*/*v*.

According to the literature, PHA production with *C. necator* can be enhanced when the inoculum size is increased from 1% to 5%. Beyond 5%, PHA biosynthesis is impaired due to the depletion of nutrients in the medium [39]. In order to test whether an increase in inoculum size could improve fermentation, a 5% and 10% *v*/*v* inoculum was tested in fermentation with the same synthetic medium conditions (N1 and C10). The results are shown in Figure 2. It can be seen that a similar PHB concentration was obtained whether a 1% or 5% inoculum was used (Figure 2c). However, when a 5% inoculum was used, the lag phase was not observed and 72 h less was needed to reach the same PHB concentration. When a 10% inoculum was used, the growth profile was similar to that obtained with the 5% inoculum (Figure 2b), but a 56.4% decrease in PHB accumulation was observed. This

was in line with the literature results discussed above, consuming PHB at these conditions. Based on the results obtained, a 5% inoculum was chosen for the following fermentations.

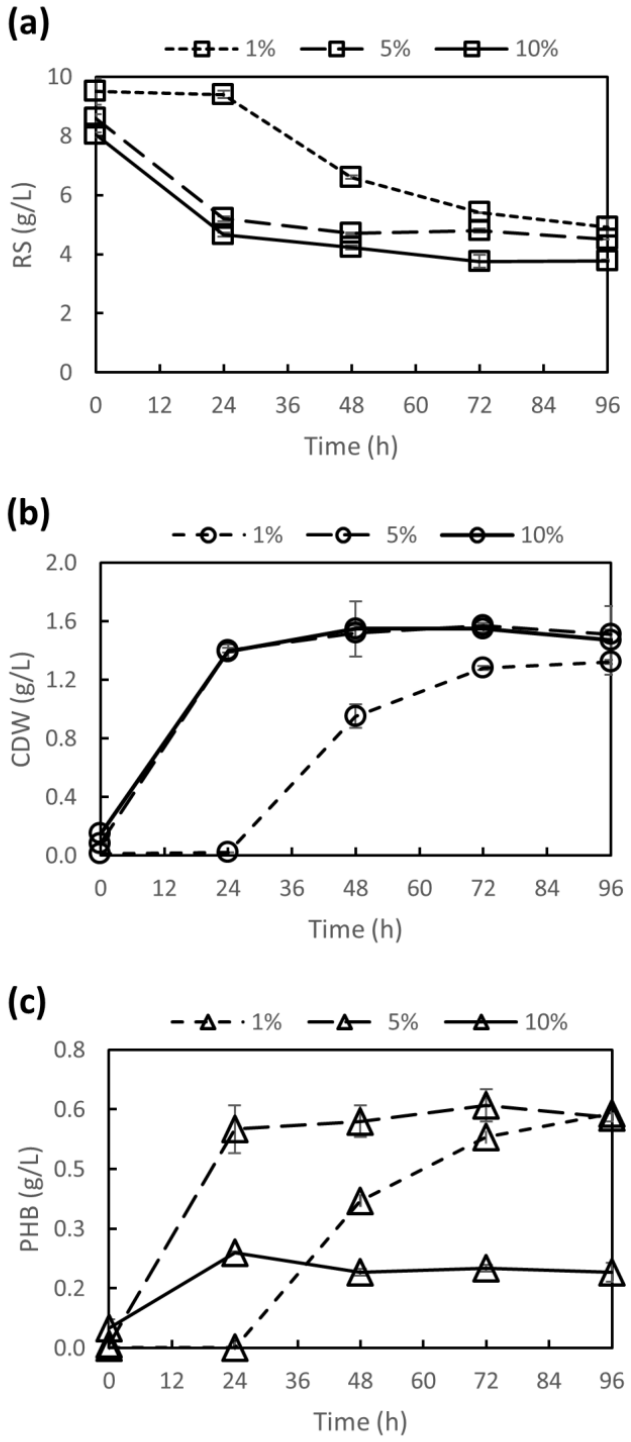

Figure 2. (**a**) substrate (RS), (**b**) biomass (CDW), and (**c**) product (PHB) profiles of the fermentations with an inoculum size of 1% *v*/*v*, 5%, and 10% with 10 g/L glucose and 1 g/L $(NH_4)_2SO_4$.

Figure 3 shows the results obtained in the fermentations carried out with different glucose concentrations (C10, C20, and C30) and 1 g/L of ammonium sulphate. A marked increase in PHB concentrations obtained at the end of each fermentation was observed when the initial glucose concentration used in the synthetic medium was doubled (C20) or tripled (C30), compared to the standard growth concentration with 10 g/L of glucose (C10), reaching 2.67 g/L, 3.06 g/L, and 0.57 g/L PHB, respectively. However, the lag phase

also increased, reaching 48 h in the case of the C30 fermentation in terms of CDW. As mentioned above, this period is associated with the time needed for the micro-organism to enter nitrogen-deficient conditions and its duration will depend mainly on the C/N ratio (higher in C30). One effect that can be observed is the difference in the concentration of glucose initially prepared and that observed at the moment of starting the fermentation. The sterilisation of the culture media was carried out by autoclave, which could give rise to caramelisation reactions [28]. This could explain the differences observed. Even so, the initial glucose concentrations in the C20 (16.6 g/L) and C30 (25.6 g/L) fermentations were practically double and triple that of C10 (8.6 g/L), which is in line with the objective of the experiment. The C20 and C30 fermentation profiles were very similar, indicating that, above about 20 g/L glucose, the fermentation did not improve substantially in terms of PHB production. Another effect observed is that glucose showed a very slow consumption profile, and was not depleted in any of the fermentations. This is in line with the results obtained in other works, in which the consumption profile of different substrates (fructose, acetate, 1,4-butanediol, and glucose) were studied, with glucose being the substrate that is consumed more slowly [36]. Only 19% of 50 g/L glucose was used after 120 h of fermentation in a 1 L Erlenmeyer flask with 200 mL of culture medium (the same working volume ratio as in the present study) with *C. necator* DSM 545 inoculated in the exponential phase and under similar conditions (200 rpm, 30 °C). Here, glucose was consumed by 51%, 44%, and 29% in the C10, C20, and C30 fermentations, respectively, after 144 h.

3.2. Fermentation of Algal Hydrolysate

PHA production was performed in a batch culture in 500 mL containing 100 mL of hydrolysate algae medium from non-pretreated (NPM) and pretreated (PM) macroalga with an initial reducing sugar concentration of 10.72 g/L and 21.71 g/L, respectively. This would be the equivalent of reproducing the C10 and C20 fermentation media but with a real (non-synthetic) substrate. These RS concentrations are in line with those obtained in a previous study, where it was observed that, of the RS concentration achieved in the hydrolysates obtained from *R. okamurae* (under the same pretreatment and enzymatic hydrolysis conditions applied in the present work), glucose is the major component (around 80–98%) [28]. Based on these results, it was assumed that the substrate available for fermentation was mainly glucose. As mentioned above, both the pretreatment and hydrolysis conditions used on the macroalgae were selected according to the results of the optimisation carried out in a previous study [28,29]. In those works, the variables biomass loading, enzyme dose, stirring rate, and operation mode were studied in the enzymatic hydrolysis stage to obtain the highest concentration of fermentable sugars, and a kinetic study was also performed. The results determined that the conditions defined above were the ones that allowed the best release of sugars in enzymatic hydrolysis. In Romero-Vargas et al. [28], it was established that the commercial enzyme preparation Cellic Ctec2 is the most appropriate enzyme for a saccharification process using macroalgae, as it shows better yields than other typically used enzymes. For the hydrolysis time, the authors also established that, above 24 h, the saccharification process does not seem to be cost-effective, but that, if it is stopped at that time, part of the substrate is not used. Therefore, for the better utilisation of the biomass, in this study, the hydrolysis process was carried out for 72 h. In the aforementioned study, the pretreatment stage was evaluated according to the combined severity factor (CSF), and the enzymatic hydrolysis stage according to the concentration of sugars that were released. It was established that, at the reaction time and acid concentration conditions mentioned, the pretreatment is the one that generates the lowest possible CSF value, allowing the highest concentration of fermentable sugars to be obtained in the enzymatic hydrolysis process. Furthermore, it is established that HCl is the most suitable of the other acids commonly used in this type of process, as it is easier to recover, shows better process efficiency, and is also about 40% cheaper compared to H_2SO_4. At these conditions, a saccharified medium with a glucose concentration similar to that of the synthetic medium used in this work for

the C20 fermentation was also obtained, which was the one that showed the most desirable results within the conditions tested, as discussed above.

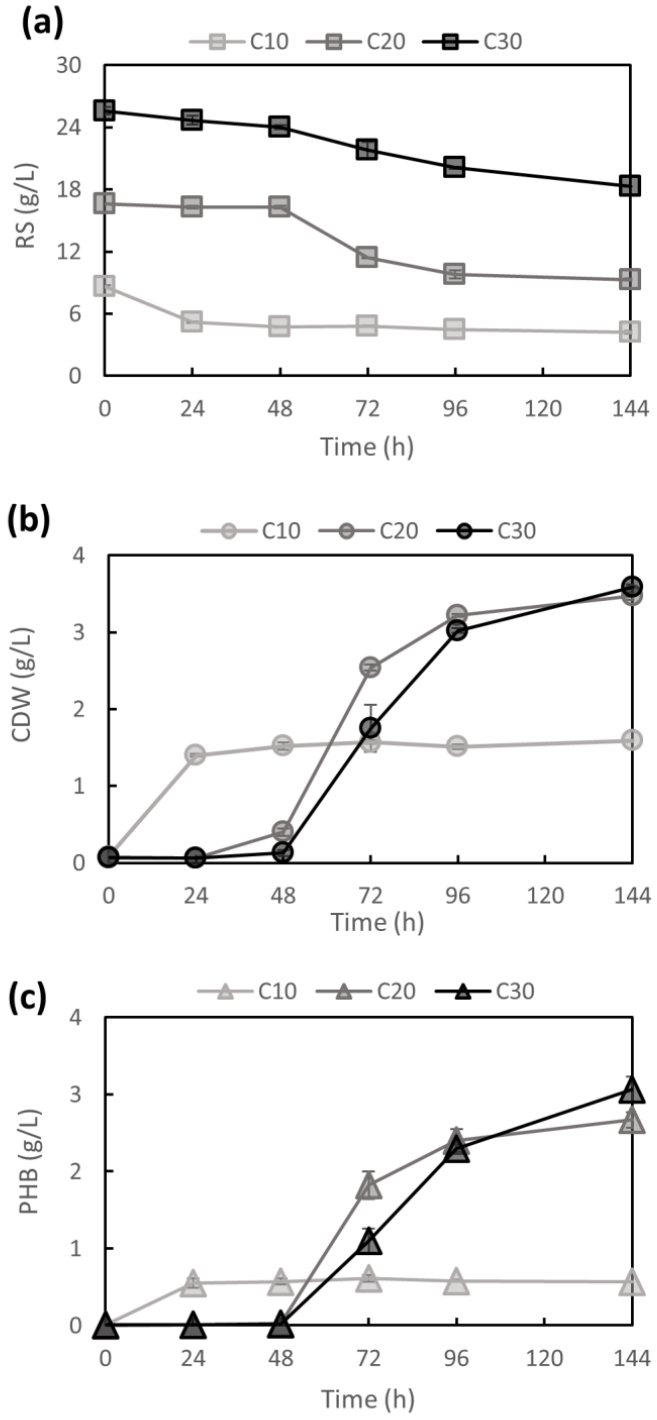

Figure 3. (**a**) substrate (RS), (**b**) biomass (CDW), and (**c**) product (PHB) profiles of the fermentations with 10, 20, and 30 g/L of glucose (C10, C20, and C30, respectively) with 1 g/L $(NH_4)_2SO_4$ and an inoculum size of 5% *v*/*v*.

Figure 4 shows the results of the fermentations carried out with the seaweed hydrolysates, comparing the profiles with those obtained in the C20 fermentation. *C. necator* was able to grow using as the sole C source the sugars contained in the seaweed hydrolysate, reaching 2.24 g/L and 2.95 g/L of CDW in the NPM and PM fermentation after 48 h and 240 h of fermentation, respectively. The PHB concentrations reached were 1.33 g/L in

NPM fermentation, and 2.65 g/L in PM fermentation, after 48 h and 240 h, respectively, although, in the latter case, the PHB concentration could have increased further after 240 h. Moreover, in PM fermentation, the concentrations were similar to those obtained in the C20 fermentation; however, four more days were needed. It is reasonable to consider that, if doubling the PHB production means going from 48 h to 240 h of fermentation time, performing the pretreatment step may not compensate for this. Therefore, if the results obtained in this study were to be transferred to an industrial process to produce PHB from algal biomass, it would be advisable to carry out the process in two stages: a first stage of biomass growth, using a non-pretreated seaweed hydrolysate (NPM fermentation), and a second stage of PHB accumulation, using a pretreated seaweed hydrolysate (PM fermentation). This production strategy has already been tested to reduce (about 20%) the process time due to the long lag phases at the beginning of each bath, and, therefore, biopolymer production costs [40]. In the mentioned study, the fermentations were carried out with a mutant strain of *C. necator* in a bioreactor with a working volume of 0.5 L, using a synthetic medium with 10 g/L fructose (C source) and 2.2 g/L urea (N source) for the first stage, and 135 g/L rapeseed oil for the second stage, leading to a significant increase in productivity (1.45 g/L.h).

The highest percentage of PHB accumulation among the fermentations carried out in this study was achieved in the one performed with the hydrolysate obtained from pretreated macroalgae (89.8%) after 240 h, which is among the highest accumulation percentages reported in the literature (up to 90%) [36,41,42]. In the NPM fermentations, the highest percentage of PHB accumulation was 64%, which was achieved at 96 h. In those carried out with the synthetic C10 medium, the highest percentage was 39.3% at 24 h, and, with the C20 and C30 media, 76.9% and 85.2%, respectively, both after 144 h.

Regarding the use of the substrate, both fermentations with hydrolysates from NPM and PM reached a similar consumption, 4.09 g/L (38% of the initial concentration) and 5.09 (26% of the initial concentration), respectively. However, only in the first case (NPM fermentation), from 48 h, did the RS concentration remain practically constant, and CDW and PHB concentrations start to decrease. This effect has also been reported by other authors and has been attributed to the utilisation of the biopolymer by the bacteria for cell maintenance. This would occur at some point during fermentation, due to a lack of nutrients [27]. It was observed that, when the synthetic medium contained relatively low initial concentrations of C and N, at the same C/N ratio (2 g/L glucose and 0.1 g/L NH_4Cl versus 20 g/L glucose and 1 g/L NH_4Cl), the decrease in CDW and PHB concentrations was produced when less glucose and NH_4Cl were used [35]. In this respect, the results for the C/N ratio analysis (Table 1) showed similar values for the NPM and PM fermentation media (17 and 21, respectively). However, comparing the concentrations of each element (C and N) for each medium (Table 1), it can be seen that the NPM medium contained a slightly higher concentration of total carbon (C_T, 11.07 g/L) than the PM medium (9.50 g/L), by 16.5%, but almost twice the concentration (42%) of total nitrogen (N_T, 0.64 g/L and 0.45 g/L, respectively). When compared to the synthetic media, both media (NPM and PM) were richer in N_T. Moreover, the NPM medium showed a similar C_T to the C30 medium. Therefore, the NPM medium would not have nutrient limitations. The decrease in CDW and PHB concentration could be explained if it is considered that, unlike the PM medium, fermentable monosaccharides (RS) represented a smaller proportion of the C_T, with the rest of the unreacted polysaccharides, as well as other organic and inorganic molecules. In this regard, although the NPM medium had a similar RS concentration to that of glucose in the C10 medium, the experimental C_T concentration of the latter was much lower (3.75 g/L), a 66.1% of difference. As for the comparison between the PM medium, whose RS concentration was similar to the glucose concentration of the synthetic C20 medium, the experimental C_T concentration of the latter (7.65 g/L) indicates that only 19.5% of the C_T contained in the hydrolysate did not correspond to fermentable monosaccharides, making it a more suitable medium for fermentation than the NPM one. On the other hand, as already reported, the pretreatment results in an impoverishment of nitrogen compounds,

which are transferred to the acid solution used for pretreatment [28]. This is in line with the results obtained here. Thus, the pretreatment would favour the bioconversion from polysaccharides contained in the macroalgae to fermentable monosaccharides as well as the nitrogen source deficit, and, as a result, the accumulation of PHB. Based on the results, although the C/N ratio is useful for fermentations in synthetic media, in the case of media obtained from biomass, the richness in fermentable monomers must also be considered.

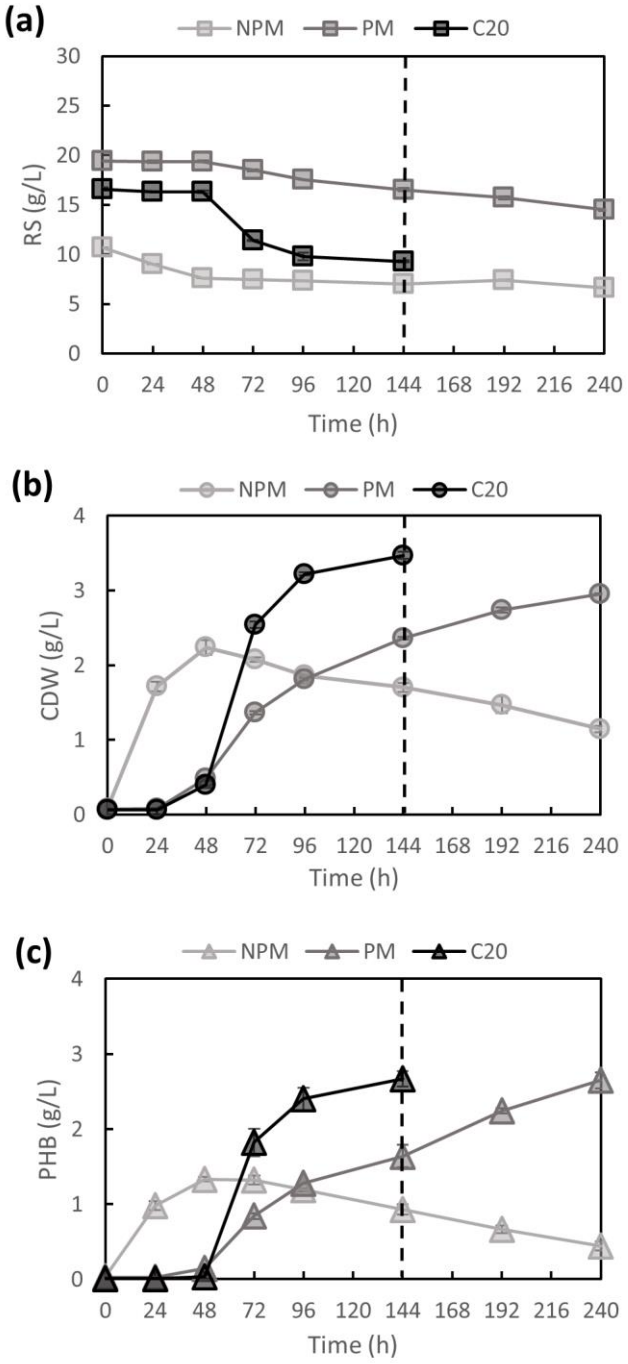

Figure 4. (**a**) substrate (RS), (**b**) biomass (CDW), and (**c**) product (PHB) profiles of the fermentations with hydrolysate media from non-pretreated macroalga (NPM) and from pretreated macroalga (PM) and with synthetic medium containing 20 g/L glucose and 1 g/L $(NH_4)_2SO_4$ (C20), with inoculum size of 5% *v*/*v*. The vertical dotted line in each graph indicates the time at which the fermentations in NPM and PM medium were extended.

Table 1. C/N ratio, total carbon (C_T), and total nitrogen (N_T) concentration of the fermentation media C10 (10 g/L glucose and 1 g/L $(NH_4)_2SO_4$), C20 (20 g/L glucose and 1 g/L $(NH_4)_2SO_4$), C30 (20 g/L glucose and 1 g/L $(NH_4)_2SO_4$), NPM (hydrolysate from non-pretreated macroalgae), and PM (hydrolysate from pretreated macroalgae). The theoretical values were calculated as a function of the amount of glucose and $(NH_4)_2SO_4$ added to the different synthetic media.

	Fermentation Medium				
C/N ratio	**C10**	**C20**	**C30**	**NPM**	**PM**
Experimental	21:1	42:1	61:1	17:1	21:1
Theoretical	19:1	38:1	57:1	n/d	n/d
C_T (g/L)	**C10**	**C20**	**C30**	**NPM**	**PM**
Experimental	3.75	7.65	11.13	11.07	9.50
Theoretical	4.00	7.99	11.99	n/d	n/d
N_T (g/L)	**C10**	**C20**	**C30**	**NPM**	**PM**
Experimental	0.18	0.18	0.18	0.64	0.45
Theoretical	0.21	0.21	0.21	n/d	n/d

n/d: not detailed.

Regarding yields, in terms of the amount of biopolymer produced with respect to the substrate consumed (g PHB/g RS), the fermentation with hydrolysates from PM showed the highest value among all the performed media (Figure 5). The C20 and C30 fermentations, which showed the best yields in the synthetic medium, reached the highest values after 144 h of fermentation, 0.366 g PHB/g RS and 0.419 g PHB/g RS, respectively. At this time, 0.536 g PHB/g RS was obtained for the hydrolysates from PM. This medium also showed the best yield value after 72 h of fermentation, 0.774 g PHB/g RS, and 0.700 g PHB/g RS after 48 h. Taking into account the average yields, the natural media (algae hydrolysates) showed better yields compared to the synthetic media. Specifically, in the best case, the average yields of the fermentations carried out with PM (0.466 g PHB/g RS) were 135.4% better than the average yields of the fermentations performed with C20 (0.198 g PHB/g RS), after 144 h of the process. This could be higher if the averages are calculated for up to 72 h of fermentation. According to the data collected in the literature, comparing fermentations carried out by a diverse range of micro-organisms and with hydrolysates obtained from lignocellulosic biomass of different origins, exposed to different types of pretreatment, these values would be in the range of the best yields achieved [41]. Regarding fermentations carried out with macroalgae, as mentioned before and to the best of our knowledge, from the little literature found, the best yield data had been achieved using *Sargassum* sp., 0.47 g PHB/g RS after 36 h of process, with an accumulation percentage of 73.4% and a PHB concentration of 3.93 g/L [26]. Although the concentration values obtained in the present study are lower compared to those, they are higher than those reported in other studies using pretreated *L. japonica* hydrolysates, where 1.58 g/L PHB and an accumulation percentage of 32% were obtained [27]. One of the advantages of the present study over those compared is that it was not necessary to add ammonium sulphate or any other additional nitrogen source to the hydrolysed medium. Other chemicals such as NaCl were also not added. Furthermore, for pretreatment, HCl (40% cheaper than H_2SO_4) was used here and a shorter reaction time was applied, which contributes to lower process costs. On the other hand, the high percentage of PHB accumulation achieved here results in a lower cell biomass residue than in the cases found in the literature, favouring the extraction and purification stages of the biopolymer in the subsequent processing (downstream).

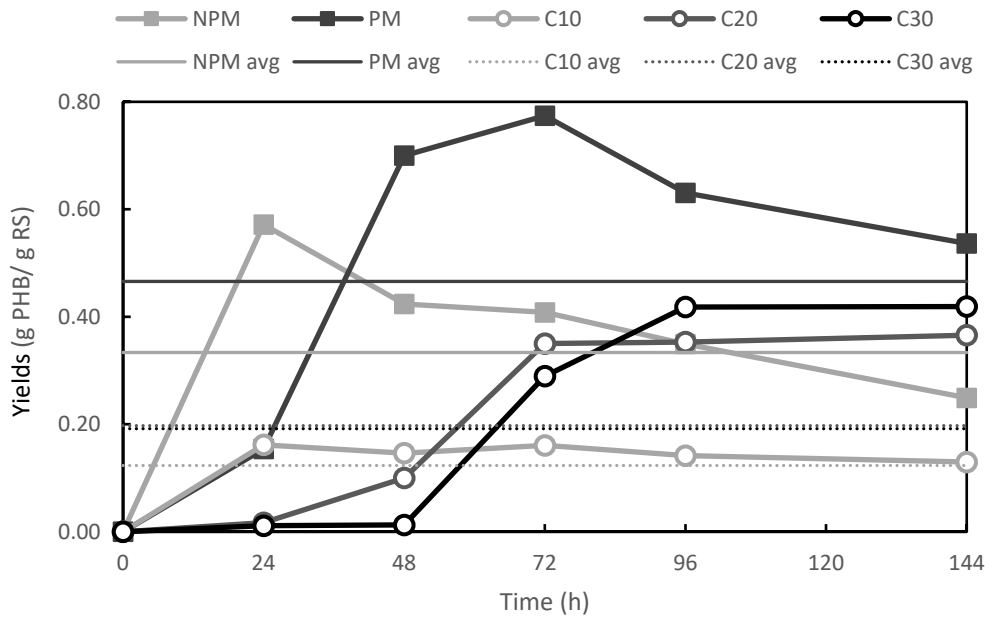

Figure 5. Yields achieved in the fermentations carried out with the natural media (hydrolysate of non-pretreated algae, NPM, and pretreated algae, PM) and synthetic media (with 10 g/L, 20 g/L, and 30 g/L glucose, C10, C20, and C30, respectively, and 1 g/L $(NH_4)_2SO_4$, N1), with inoculum size of 5% *v*/*v*. Average of the yields in each case (avg).

Based on the data obtained, it is possible to estimate the amount of biopolymer that can be produced depending on the amount of seaweed used in a production process of the characteristics studied here. If non-pretreated seaweed is used, 9.33 kg PHB/ t dry seaweed could be obtained. On the other hand, 18.59 kg PHB/ t dry pretreated seaweed could be obtained, twice as much. Taking as a reference the 5000 tonnes of algae deposit collected on 15 km of the coast of Ceuta in 2016, when the spread of macroalga through the Strait of Gibraltar was still incipient, and taking into account that approximately 90% of its weight is water [43], 9.3 tonnes of PHB could be generated just with the amount of macroalga collected in that area. Taking into account that the production costs of PHA from waste ranges between 1390–10,500 €/t [44], producing the 9.3 tonnes of the example would have an estimated cost of between 12,927–97,650 €. Producing the same amount of petroleum-derived plastic would cost 9300–13,950 €. Thus, according to these estimates, producing PHA could be up to seven times more expensive than producing conventional plastics. However, in the best-case scenario, it could even result in a lower cost. It should also be considered that, according to the latest studies, only on the coasts of Tarifa (Spain) does the invasion of *R. okamurae* generate losses of 3.3 million euros per year [45]. Thus, the use of this macroalga to produce PHB could contribute to reducing these economic losses.

4. Conclusions

This study demonstrates that polyhydroxybutyrate can be synthesised utilising a saccharified medium obtained from the invasive macroalga *Rugulopteryx okamurae* as the sole fermentation substrate, achieving yield values (0.774 g PHB/g RS) and biopolymer accumulation (89.8%) comparable to the highest values observed in the literature to date using lignocellulosic biomass or other macroalgae. A C/N ratio analysis revealed that the hydrolysates were rich in the N source, which was detrimental to PHB production. However, hydrothermal acid pretreatment contributed to reducing the N_T in the hydrolysates (increasing the C/N ratio) and to increasing the ratio of fermentable monomers with respect to C_T.

Likewise, in this work, an estimation of the potential production of PHB under the given conditions and the associated costs has been made. For this purpose, a real case of the disposal of invasive algae waste from the coast of Ceuta (Spain) was used. The estimation

indicated that approximately 9.3 tonnes of PHB could be produced with a production cost of 12,927 € in the best case, which is even lower than the cost of producing conventional plastics. However, in the worst-case scenario, it would cost up to seven times more. It is important to note that the exploitation of the algae could contribute to reducing the significant economic losses caused by the invasion of *R. okamurae* in Spain (3.3 million euros per year along the coasts of Tarifa). Thus, it would be beneficial to conduct a techno-economic analysis for a PHB production from *R. okamurae*, since the production costs depend mostly on the raw material, and could be cost-effective in this case.

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References

1. Al-Battashi, H.S.; Annamalai, N.; Sivakumar, N.; Al-Bahry, S.; Tripathi, B.N.; Nguyen, Q.D.; Gupta, V.K. Lignocellulosic Biomass (LCB): A Potential Alternative Biorefinery Feedstock for Polyhydroxyalkanoates Production. *Rev. Environ. Sci. Bio/Technol.* **2019**, *18*, 183–205. [CrossRef]
2. Ilyas, R.A.; Sapuan, S.M.; Kadier, A.; Kalil, M.S.; Ibrahim, R.; Atikah, M.S.N.; Nurazzi, N.M.; Nazrin, A.; Lee, C.H.; Faiz Norrrahim, M.N.; et al. Properties and Characterization of PLA, PHA, and Other Types of Biopolymer Composites. In *Advanced Processing, Properties, and Applications of Starch and Other Bio-Based Polymers*; Elsevier: Amsterdam, The Netherlands, 2020; pp. 111–138.
3. Saratale, R.G.; Saratale, G.D.; Cho, S.K.; Kim, D.S.; Ghodake, G.S.; Kadam, A.; Kumar, G.; Bharagava, R.N.; Banu, R.; Shin, H.S. Pretreatment of Kenaf (*Hibiscus Cannabinus* L.) Biomass Feedstock for Polyhydroxybutyrate (PHB) Production and Characterization. *Bioresour. Technol.* **2019**, *282*, 75–80. [CrossRef] [PubMed]
4. Raza, Z.A.; Abid, S.; Banat, I.M. Polyhydroxyalkanoates: Characteristics, Production, Recent Developments and Applications. *Int. Biodeterior. Biodegrad.* **2018**, *126*, 45–56. [CrossRef]
5. Sohn, Y.J.; Son, J.; Jo, S.Y.; Park, S.Y.; Yoo, J.I.; Baritugo, K.A.; Na, J.G.; Choi, J.-i.; Kim, H.T.; Joo, J.C.; et al. Chemoautotroph *Cupriavidus necator* as a Potential Game-Changer for Global Warming and Plastic Waste Problem: A Review. *Bioresour. Technol.* **2021**, *340*, 125693. [CrossRef] [PubMed]
6. Gholami, A.; Mohkam, M.; Rasoul-Amini, S.; Ghasemi, Y. Industrial Production of Polyhydroxyalkanoates by Bacteria: Opportunities and Challenges. *Minerva Biotecnol.* **2016**, *28*, 59–74.
7. Choi, J.; Lee, S.Y. Process Analysis and Economic Evaluation for Poly(3-Hydroxybutyrate) Production by Fermentation. *Bioprocess Eng.* **1997**, *17*, 335. [CrossRef]
8. Du, C.; Sabirova, J.; Soetaert, W.; Ki Carol Lin, S. Polyhydroxyalkanoates Production from Low-Cost Sustainable Raw Materials. *Curr. Chem. Biol.* **2012**, *6*, 14–25. [CrossRef]
9. Cesário, M.T.; Raposo, R.S.; de Almeida, M.C.M.; van Keulen, F.; Ferreira, B.S.; Telo, J.P.; da Fonseca, M.M.R. Production of Poly(3-Hydroxybutyrate-Co-4-Hydroxybutyrate) by *Burkholderia sacchari* Using Wheat Straw Hydrolysates and Gamma-Butyrolactone. *Int. J. Biol. Macromol.* **2014**, *71*, 59–67. [CrossRef] [PubMed]
10. Davis, R.; Kataria, R.; Cerrone, F.; Woods, T.; Kenny, S.; O'Donovan, A.; Guzik, M.; Shaikh, H.; Duane, G.; Gupta, V.K.; et al. Conversion of Grass Biomass into Fermentable Sugars and Its Utilization for Medium Chain Length Polyhydroxyalkanoate (Mcl-PHA) Production by Pseudomonas Strains. *Bioresour. Technol.* **2013**, *150*, 202–209. [CrossRef]
11. Getachew, A.; Woldesenbet, F. Production of Biodegradable Plastic by Polyhydroxybutyrate (PHB) Accumulating Bacteria Using Low Cost Agricultural Waste Material. *BMC Res. Notes* **2016**, *9*, 509. [CrossRef]

12. Adeleye, A.T.; Odoh, C.K.; Enudi, O.C.; Banjoko, O.O.; Osiboye, O.O.; Toluwalope Odediran, E.; Louis, H. Sustainable Synthesis and Applications of Polyhydroxyalkanoates (PHAs) from Biomass. *Process Biochem.* **2020**, *96*, 174–193. [CrossRef]
13. Yun, E.J.; Kim, H.T.; Cho, K.M.; Yu, S.; Kim, S.; Choi, I.G.; Kim, K.H. Pretreatment and Saccharification of Red Macroalgae to Produce Fermentable Sugars. *Bioresour. Technol.* **2016**, *199*, 311–318. [CrossRef]
14. Öhgren, K.; Bura, R.; Saddler, J.; Zacchi, G. Effect of Hemicellulose and Lignin Removal on Enzymatic Hydrolysis of Steam Pretreated Corn Stover. *Bioresour. Technol.* **2007**, *98*, 2503–2510. [CrossRef] [PubMed]
15. Fernand, F.; Israel, A.; Skjermo, J.; Wichard, T.; Timmermans, K.R.; Golberg, A. Offshore Macroalgae Biomass for Bioenergy Production: Environmental Aspects, Technological Achievements and Challenges. *Renew. Sustain. Energy Rev.* **2017**, *75*, 35–45. [CrossRef]
16. Jiang, R.; Ingle, K.N.; Golberg, A. Macroalgae (Seaweed) for Liquid Transportation Biofuel Production: What Is Next? *Algal Res.* **2016**, *14*, 48–57. [CrossRef]
17. Greiserman, S.; Epstein, M.; Chemodanov, A.; Steinbruch, E.; Prabhu, M.; Guttman, L.; Jinjikhashvily, G.; Shamis, O.; Gozin, M.; Kribus, A.; et al. Co-Production of Monosaccharides and Hydrochar from Green Macroalgae *Ulva* (Chlorophyta) sp. with Subcritical Hydrolysis and Carbonization. *BioEnergy Res.* **2019**, *12*, 1090–1103. [CrossRef]
18. Harb, T.B.; Chow, F. An Overview of Beach-Cast Seaweeds: Potential and Opportunities for the Valorization of Underused Waste Biomass. *Algal Res.* **2022**, *62*, 102643. [CrossRef]
19. Lymperatou, A.; Engelsen, T.K.; Skiadas, I.V.; Gavala, H.N. Different Pretreatments of Beach-Cast Seaweed for Biogas Production. *J. Clean. Prod.* **2022**, *362*, 132277. [CrossRef]
20. Carrillo, J.A.; Ocaña, Ó.; Ballesteros, E. Massive Proliferation of a Dictyotalean Species (Phaeophyceae, Ochrophyta) through the Strait of Gibraltar (Research Note). *Rev. Acad. Canar. Cienc.* **2016**, *28*, 165–170.
21. García-Gómez, J.C.; Sempere-Valverde, J.; González, A.R.; Martínez-Chacón, M.; Olaya-Ponzone, L.; Sánchez-Moyano, E.; Ostalé-Valriberas, E.; Megina, C. From Exotic to Invasive in Record Time: The Extreme Impact of *Rugulopteryx okamurae* (Dictyotales, Ochrophyta) in the Strait of Gibraltar. *Sci. Total Environ.* **2020**, *704*, 135408. [CrossRef]
22. Wang, D.; Kim, D.H.; Kim, K.H. Effective Production of Fermentable Sugars from Brown Macroalgae Biomass. *Appl. Microbiol. Biotechnol.* **2016**, *100*, 9439–9450. [CrossRef] [PubMed]
23. Ko, J.K.; Um, Y.; Park, Y.-C.; Seo, J.-H.; Kim, K.H. Compounds Inhibiting the Bioconversion of Hydrothermally Pretreated Lignocellulose. *Appl. Microbiol. Biotechnol.* **2015**, *99*, 4201–4212. [CrossRef] [PubMed]
24. Ding, L.; Cheng, J.; Lin, R.; Deng, C.; Zhou, J.; Murphy, J.D. Improving Biohydrogen and Biomethane Co-Production via Two-Stage Dark Fermentation and Anaerobic Digestion of the Pretreated Seaweed *Laminaria digitata*. *J. Clean. Prod.* **2020**, *251*, 119666. [CrossRef]
25. Sivagurunathan, P.; Kumar, G.; Kobayashi, T.; Xu, K.; Kim, S.-H. Effects of Various Dilute Acid Pretreatments on the Biochemical Hydrogen Production Potential of Marine Macroalgal Biomass. *Int. J. Hydrogen Energy* **2017**, *42*, 27600–27606. [CrossRef]
26. Azizi, N.; Najafpour, G.; Younesi, H. Acid Pretreatment and Enzymatic Saccharification of Brown Seaweed for Polyhydroxybutyrate (PHB) Production Using *Cupriavidus necator*. *Int. J. Biol. Macromol.* **2017**, *101*, 1029–1040. [CrossRef] [PubMed]
27. Muhammad, M.; Aloui, H.; Khomlaem, C.; Hou, C.T.; Kim, B.S. Production of Polyhydroxyalkanoates and Carotenoids through Cultivation of Different Bacterial Strains Using Brown Algae Hydrolysate as a Carbon Source. *Biocatal. Agric. Biotechnol.* **2020**, *30*, 101852. [CrossRef]
28. Romero-Vargas, A.; Fdez-Güelfo, L.A.; Blandino, A.; Díaz, M.J.; Díaz, A.B. *Rugulopteryx okamurae*: Effect of Hydrothermal Acid Pretreatment on the Saccharification Process. *Bioresour. Technol.* **2023**, *388*, 129721. [CrossRef] [PubMed]
29. Romero-Vargas, A.; Fdez-Güelfo, L.A.; Blandino, A.; Romero-García, L.I.; Díaz, A.B. *Rugulopteryx okamurae*: Assessment of Its Potential as a Source of Monosaccharides for Obtaining Bio-Products. *Chem. Eng. J.* **2023**, *468*, 143578. [CrossRef]
30. Gonçalves, C.; Rodriguez-Jasso, R.M.; Gomes, N.; Teixeira, J.A.; Belo, I. Adaptation of Dinitrosalicylic Acid Method to Microtiter Plates. *Anal. Methods* **2010**, *2*, 2046–2048. [CrossRef]
31. Miller, G.L. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Anal. Chem.* **1959**, *31*, 426–428. [CrossRef]
32. Simona, C.; Laura, L.; Francesco, V.; Marianna, V.; Cristina, M.G.; Barbara, T.; Mauro, M.; Simona, R. Effect of the Organic Loading Rate on the PHA-Storing Microbiome in Sequencing Batch Reactors Operated with Uncoupled Carbon and Nitrogen Feeding. *Sci. Total Environ.* **2022**, *825*, 153995. [CrossRef] [PubMed]
33. Braunegg, G.; Sonnleitner, B.; Lafferty, R.M. A Rapid Gas Chromatographic Method for the Determination of Poly-β-Hydroxybutyric Acid in Microbial Biomass. *Eur. J. Appl. Microbiol. Biotechnol.* **1978**, *6*, 29–37. [CrossRef]
34. Marzo, C.; Díaz, A.B.; Caro, I.; Blandino, A. Conversion of Exhausted Sugar Beet Pulp into Fermentable Sugars from a Biorefinery Approach. *Foods* **2020**, *9*, 1351. [CrossRef] [PubMed]
35. Ahn, J.; Jho, E.H.; Nam, K. Effect of C/N Ratio on Polyhydroxyalkanoates (PHA) Accumulation by *Cupriavidus necator* and Its Implication on the Use of Rice Straw Hydrolysates. *Environ. Eng. Res.* **2015**, *20*, 246–253. [CrossRef]
36. Fereidouni, M.; Younesi, H.; Daneshi, A.; Sharifzadeh, M. The Effect of Carbon Source Supplementation on the Production of Poly(3-Hydroxybutyrate-Co-3-Hydroxyvalerate) by *Cupriavidus necator*. *Biotechnol. Appl. Biochem.* **2011**, *58*, 203–211. [CrossRef] [PubMed]
37. Morlino, M.S.; Serna García, R.; Savio, F.; Zampieri, G.; Morosinotto, T.; Treu, L.; Campanaro, S. Cupriavidus Necator as a Platform for Polyhydroxyalkanoate Production: An Overview of Strains, Metabolism, and Modeling Approaches. *Biotechnol. Adv.* **2023**, *69*, 108264. [CrossRef] [PubMed]

38. Goff, M.; Ward, P.G.; O'Connor, K.E. Improvement of the Conversion of Polystyrene to Polyhydroxyalkanoate through the Manipulation of the Microbial Aspect of the Process: A Nitrogen Feeding Strategy for Bacterial Cells in a Stirred Tank Reactor. *J. Biotechnol.* **2007**, *132*, 283–286. [CrossRef] [PubMed]
39. Arumugam, A.; Senthamizhan, S.G.; Ponnusami, V.; Sudalai, S. Production and Optimization of Polyhydroxyalkanoates from Non-Edible *Calophyllum inophyllum* Oil Using *Cupriavidus necator*. *Int. J. Biol. Macromol.* **2018**, *112*, 598–607. [CrossRef] [PubMed]
40. Santolin, L.; Waldburger, S.; Neubauer, P.; Riedel, S.L. Substrate-Flexible Two-Stage Fed-Batch Cultivations for the Production of the PHA Copolymer P(HB-Co-HHx) with *Cupriavidus necator* Re2058/PCB113. *Front. Bioeng. Biotechnol.* **2021**, *9*, 623890. [CrossRef]
41. González-Rojo, S.; Díez-Antolínez, R. Production of Polyhydroxyalkanoates as a Feasible Alternative for an Integrated Multiproduct Lignocellulosic Biorefinery. *Bioresour. Technol.* **2023**, *386*, 129493. [CrossRef]
42. Sousa, B.V.; Silva, F.; Reis, M.A.M.; Lourenço, N.D. Monitoring Pilot-Scale Polyhydroxyalkanoate Production from Fruit Pulp Waste Using near-Infrared Spectroscopy. *Biochem. Eng. J.* **2021**, *176*, 108210. [CrossRef]
43. Sudhakar, K.; Mamat, R.; Samykano, M.; Azmi, W.H.; Ishak, W.F.W.; Yusaf, T. An Overview of Marine Macroalgae as Bioresource. *Renew. Sustain. Energy Rev.* **2018**, *91*, 165–179. [CrossRef]
44. Ganesh Saratale, R.; Cho, S.K.; Dattatraya Saratale, G.; Kadam, A.A.; Ghodake, G.S.; Kumar, M.; Naresh Bharagava, R.; Kumar, G.; Su Kim, D.; Mulla, S.I.; et al. A Comprehensive Overview and Recent Advances on Polyhydroxyalkanoates (PHA) Production Using Various Organic Waste Streams. *Bioresour. Technol.* **2021**, *325*, 124685. [CrossRef] [PubMed]
45. Mogollón, S.L.; Zilio, M.I.; Buitrago, E.M.; Caraballo, M.Á.; Yñiguez, R. Economic Impact of *Rugulopteryx okamurae* (Dictyotales, Ochrophyta) along the Andalusian Coastline: The Case of Tarifa, Spain. *Wetl. Ecol. Manag.* **2024**, *32*, 19–32. [CrossRef]