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Integrated biorefinery strategy for poly(3-hydroxybutyrate) accumulation in *Cupriavidus necator* DSM 545 using a sugar rich syrup from cereal waste and acetate from gas fermentation

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ABSTRACT

Poly(3-hydroxybutyrate) (PHB) is one of the most well-known biodegradable and biocompatible biopolymers produced by prokaryotic microorganisms. It belongs to the family of polyhydroxyalkanoates (PHAs), and it has gained significant attention in recent years due to its potential as a sustainable alternative to traditional petroleum-based plastics. *Cupriavidus necator* has been identified as a potential producer of PHB for industrial applications due to its ability to produce high amounts of the polymer under controlled conditions, using a wide range of waste substrates. In this study, the ability of *Cupriavidus necator* DSM 545 strain to produce PHB was tested in a fed-batch strategy providing two different organic substrates. The first is a sugar-based syrup (SBS), derived from cereal waste. The second is an acetate-rich medium obtained through CO_2 -H_2 fermentation by the acetogenic bacterium *Acetobacterium woodii*. The carbon sources were tested to improve the accumulation of PHB in the strain. *C. necator* DSM 545 proved to be able to grow and to perform high accumulation of biopolymer on waste substrates containing glucose, fructose, and acetate, reaching about 10 g/L of PHB, 83% of biopolymer accumulation in cell dry mass, in 48 h of fed-batch fermentation in 0.6 L working volume in a bioreactor. Moreover, a Life Cycle Assessment analysis was performed to evaluate the environmental impact of the process converting the sugar syrup alone and the integrated one. It demonstrated that the integrated process is more sustainable and that the most impactful step is the PHB production, followed by the polymer extraction.

1. Introduction

The awareness of the pollution from the accumulation of plastics into the environment led to increasing efforts to tackle this issue by replacing traditional polymers with biodegradable ones. Moreover, due to climate change, the replacement of fossil materials with alternatives to produce polymers is more advisable. Accordingly, biomass is an alternative renewable source deeply used to make different kinds of bio-based polymers like polylactic acid (PLA) [1] and polyhydroxyalkanoates (PHAs) [2,3]. PHAs are polyesters of hydroxyalkanoic acids that share several chemicals, physical, thermal, and mechanical features with synthetic petrochemical-based polymers, but they are completely biodegradable and biocompatible [4]. PHAs production relies on the metabolism of several microorganisms that accumulate it in the form of intracellular granules to store carbon and energy during bacterial stress conditions such as nitrogen shortage and carbon excess. The granules can consist of molecules of 3–5 carbon atoms named short-chain length (scl), or 6–14 carbon atoms named medium chain length (mcl). Poly (3-hydroxybutyrate) (PHB) is composed of a C4 basic unit and is the most common and best-studied among PHAs. Even though PHAs are very promising environment-friendly materials, their large-scale industrial production still needs to be improved. One of the main criticalities

Abbreviations: AWM, Acetate rich waste medium; cAWM, Concentrated acetate-rich waste medium; CCU, Carbon-Capture-Utilization; CDW, Cell dry weight; CED, Cumulative energy demand; DO, Dissolved Oxygen; GWP, Global warming potential; HPLC, High Pressure Liquid Chromatography; OD, Optical Density; PHAs, Poly-hydroxyalkanoates; PHB, Poly(3-hydroxybutyrate); PLA, Polylactic acid; GSB, Glucose-based syrup; SBS, Sugar-based syrup; BPR, Back Pressure Regulation valve; MFC, Mass Flow Controllers.

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is their high production costs. The production expense is estimated to be 3–4 times higher than polypropylene and polyethylene polymers (about 2.4–5.5 USD/kg), impairing their industrialization and commercialization [5,6]. Currently, PHAs production is mainly based on the bioconversion of refined pure sugars and these sources represent about 40% of the total PHAs production cost [7]. Thus, one of the main attempts to make the production cheaper is to reduce the impact of the carbon sources provided as feedstock for microorganisms. Waste substrates such as molasses and cellulosic material have been employed allowing valorization of by-products and savings of economic resources leading to an improvement in the cost of production and environmental impact [8]. Therefore, there is an increasing interest for prokaryotes able to accumulate biopolymers valorizing waste products.

Among PHB producing microorganisms, one of the most studied is *Cupriavidus necator,* formerly also known as *Ralstonia eutropha, Wautersia eutropha* or *Alcaligenes eutrophus* [9]. It is a very versatile bacterium, able to grow both autotrophically and heterotrophically [10] and perform denitrification in anoxic conditions [11]. This bacterial strain can metabolize several organic carbon sources during heterotrophic growth, including sugar, fatty acids, amino acids, alcohols, aromatic compounds, and TCA cycle intermediates [12].

Wild-type *C. necator* metabolizes fructose as a preferential carbon source, and N-acetylglucosamine exclusively via the Entner–Doudoroff pathway by its key enzyme 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (EC 4.1.2.14) [13,14]. In this study, C. *necator* DSM 545 strain was used for its ability also to utilize glucose better than the wild-type strain. *C. necator* DSM 545 owns the constitutive expression of the gene coding for the enzyme glucose-6-phosphate dehydrogenase [15]. This enzyme catalyzes the conversion of glucose-6-phosphate to 6-phosphogluconate, during which a $NADP^+$ molecule is reduced to NADPH, a pivotal cofactor for PHB synthesis [16]. For PHB accumulation two molecules of acetyl-CoA are condensed by 3-ketothiolase (PhaA) to acetoacetyl-CoA. The latter is reduced by the NADPH-dependent acetoacetyl-CoA reductase (PhaB) to (R)− 3-hydroxybutyryl-CoA; then polymerization to PHB is carried out by the PHA synthase (PhaC). The synthesis of PHB can be consistent only if a carbon source is available in excess and if one essential nutritional element like N, O, S, Mg, K, or P limits cell growth [17].

The current study combines two organic carbon by-products for *C. necator* DSM 545 growth and PHB accumulation. Following a strategy supporting the circular economy, the first substrate is a syrup from a Piedmont cereal factory containing about 50% of glucose and 50% of fructose. The second organic carbon soruce is acetate, synthesized through carbon dioxide (CO_2) based bacterial fermentation. CO_2 is abundant as waste from several industrial processes and could be recycled as a cheap carbon source [18]. Anaerobic acetogenic bacteria synthesize acetic acid reducing CO_2 through the Wood–Ljungdahl pathway (WLP), using H_2 as an electron donor [19]. Due to increasing interest in green hydrogen production, several studies on sustainable H_2 production technologies are ongoing [20], so the Carbon-Capture-Utilization (CCU) approach is a promising way to produce acetic acid sustainably [21]. The biological method for acetate production allows using a low process temperature (30–37 °C) with respect to the thermo-chemical processes, favoring energy cost savings [21,22]. Bacterial synthesis also benefits from the high specificity of the enzymes involved in the microbial WLP pathway, which usually leads to improved acetate yields with respect to other processes [23]. Among these bacterial species, *Acetobacterium woodii* is one of the most studied and suitable for this work. It grows heterotrophically on different organic carbon substrates [24]. Moreover, it is among the best-performing acetogen on the CO_2-H_2 blend [25]. Acetic acid is the main metabolic product, and autotrophic culturing boosts its synthesis. In the present work, *A. woodii* was grown under a CO_2-H_2 atmosphere in a pressurized bioreactor. Performing the fermentation at pressures higher than the atmospheric one increases gaseous substrate availability in the liquid broth, leading to an increased CO_2 conversion by the biocatalyst [26]. The acetate-rich medium (AWM) could be supplied as a feed in *C. necator* DSM 545 cultures for PHB production.

Eq. 1 shows the first step for indirectly using carbon dioxide to accumulate PHB: the H_2-mediated reduction of the CO_2 into acetate performed by acetogenic bacteria [27].

$$CO_2 + 4\ H_2 \rightarrow C_2H_4O_2 + 2\ H_2O \quad (1)$$

Acetic acid in the AWM is then provided to *C. necator*. It metabolizes the organic substrate to allow both biomass and PHB production, as shown in (Eqs. (2) and (3)).

$$C_2H_4O_2 + 0{\cdot}79\ O_2 + 0{\cdot}22\ NH_4^+ \rightarrow 1{\cdot}14\ CH_{1{\cdot}74}O_{0{\cdot}46}N_{0{\cdot}19} + 1{\cdot}33\ H_2O + 0{\cdot}86\ CO_2 + 0{\cdot}22\ H^+ \quad (2)$$

$$1{\cdot}5\ C_2H_4O_2 + 0{\cdot}75\ O_2 \rightarrow 0{\cdot}5\ C_4H_6O_2 + 1{\cdot}5\ H_2O + CO_2 \quad (3)$$

C. necator can also directly convert H_2 and CO_2 into PHB in presence of oxygen. To allow cell growth avoiding gas starvation, the composition ratio of H_2:O_2:CO_2 should be 7:2:1, which might cause an explosive atmosphere [28]. According to the mass balance, the indirect conversion of CO_2 to PHB through acetic acid is an attractive alternative regarding safety, CO_2 fixation, H_2 consumption, and substrate cost [27].

Garcia-Gonzalez and colleagues [27] previously assessed the PHA production by *C. necator* DSM 545 cultured with acetic acid in the growing medium. Al Rowaihi and coworkers [22] furnished acetic acid produced by *A.woodii* to *C. necator* grown on pure commercial fructose. This study aims to synthesize PHB through *C. necator* DSM 545 by converting a real syrup from a cereal factory and an acetate-rich medium from gas fermentation. Thus, the first novelty of the current work relies on the design and test of an integrated biorefinery strategy that valorized two waste substrates. The sugar syrup was supplied alone or in combination with acetic acid from CO_2-H_2 gas fermentation. The latter feedstock was synthesized by *A.woodii* in a pressurized bioreactor with a continuous gas supply to avoid gas depletion from the culture medium. The second novelty is a Life Cycle Assessment (LCA) analysis to compare the PHB production through the integrated biorefinery system and real syrup alone. The evaluation considered the environmental impacts of 1 kg of pure PHB production, according to ISO 14040–44, and relied on the parameters applied and results obtained in the current experimental work.

2. Materials and methods

2.1. PHB biosynthesis through C. necator fermentation

2.1.1. C. necator growth media and cultivation in shake flasks

Cupriavidus necator DSM 545, was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Germany). A glycerol stock, stored at – 20 °C in 15% v/v glycerol, was cultured in 0.05 L of Luria–Bertani broth at 30 °C, 200 rpm for 18 h (ES-20/60, Orbital Shaker Incubator, Biosan, Latvia) in a flask of 0.250 L total volume. LB medium was prepared using 10 g/L NaCl, 10 g/L tryptone and 5 g/L yeast extract. Then, 4% of the culture volume was used to inoculate 50 mL of a seeding medium at pH 6.8 containing: $(NH_4)_2SO_4$ 4 g/L; KH_2PO_4 1.5 g/L; Na_2HPO_4 3.74 g/L; $MgSO_4{\cdot}7H_2O$ 0.2 g/L; and trace elements solution 10 mL/L [29]. The sugar substrate was a syrup with 250 g/L of sugars, mainly glucose, named in this work as glucose-based syrup (GBS) (composition: 96.97% glucose, 2.12% maltose, 0.62% triose, 0.20% other polysaccharides and 0.1% fructose). It was provided by Sedamyl® company, a Piedmont cereal factory and it was used at a final concentration of 10 g/L.

The trace elements solution contained $FeSO_4{\cdot}7H_2O$ 10 g/L, $ZnSO_4{\cdot}7H_2O$ 2.25 g/L, $CuSO_4{\cdot}5H_2O$ 1 g/L, $MnSO_4{\cdot}5H_2O$ 0.5 g/L, $CaCl_2{\cdot}2H_2O$ 2 g/L, $Na_2B_4O_7{\cdot}10H_2O$ 0.23 g/L, $(NH_4)_6Mo_7O_{24}$ 0.1 g/L, HCl 35% 10 mL/L.

Except for the trace element, $MgSO_4$ solution and sugars, culture

media were sterilized by autoclaving. $MgSO_4$ and trace element solution were filtered through a 0.22 μm sterile PES filters. The syrup was provided ready to use by the company.

2.1.2. C. necator growth in bioreactor

C. necator DSM 545 growth in the bioreactor was carried out in a Biostat A bioreactor (Sartorius®, DE) with a total volume of 1.3 L. The fermentation medium contained $(NH_4)_2SO_4$ 3 g/L; KH_2PO_4 13.3 g/L; citric acid 1.87 g/L; $MgSO_4{\cdot}7H_2O$ 1.2 g/L; trace element (see Section 2.1.1) 10 mL/L. The feedstock was a demineralized and isomerized syrup (SBS) with 280 g/L total sugars (45.6% glucose, 47% fructose and the remaining percentage of maltose, triose, and other polysaccharides) supplied at a final concentration of 12 g/L. Again, this syrup was provided by Sedamyl® as ready to be used. Biostat A bioreactor (Sartorius®) is equipped with a glass vessel. Sterilization of the reactor vessel filled with the fermentation medium, without sugars, trace metals and magnesium, was done in an autoclave. After autoclaving, the broth was completed with SBS, and the filtered solutions of magnesium and trace elements. If not otherwise stated, 0.6 L of fermentation medium was inoculated with 8 mL of *C. necator* DSM 545 grown in the seeding medium (see Section 2.1.1) to have an initial optical density at 600 nm ($OD_{600\ nm}$) of ≈ 0.1.

The head plate of the vessel was fitted with temperature/DO and pH probes (Endress+Hauser). All fermentations were performed providing sterile air at 12 L/h rate through a micrometric sparger placed at the vessel's bottom. The dissolved oxygen (DO) was set at 40% using a cascade in the agitation mode, triggered when the dissolved oxygen decreased in the bioreactor due to bacterial growth. The agitation was carried out using Rushton turbines. pH was controlled at 6.8 by adding 2 M NaOH. Fermentations were performed at atmospheric pressure. The vessel temperature was maintained at 30 °C thanks to an outer thermoblanket. Samples were withdrawn from the reactor at 15, 18, 21, 24, 36, 40, 45 and 48 h after the inoculation to analyze the content of sugars, biomass and PHB. After 15 h of fermentation, a fed-batch strategy feeding SBS was conducted to avoid sugar shortages. Sugars consumption was monitored through HPLC. During the fed-batch, feeding was conducted manually using a sterile syringe to supply a volume of SBS to reach a concentration of sugars in the culture medium of 4, 5, or 6 g/L.

2.2. Acetate production through gas-fermentation

2.2.1. A. woodii growth media and cultivation in serum bottles

Acetobacterium woodii DSM 1030 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DE). The medium used for the heterotrophic cultivation in serum bottles contained: KH_2PO_4 1.76 g/L; K_2HPO_4 8.44 g/L; fructose 3.6 g/L; $KHCO_3$ 6 g/L; NH_4Cl 1.0 g/L; NaCl 2.9 g/L; yeast extract 2 g/L; cysteine hydrochloride 0.5 g/L; $MgSO_4$ 0.180 g/L; resazurin 1 mg/L; trace element solution SL9 1 mL/L; selenite-tungstate solution 1 mL/L; and vitamin solution DMSZ 141 2 mL/L. The vitamins solution contained: biotin 0.002 g/L; folic acid 0.002 g/L; pyridoxine-HCl 0.010 g/L, thiamine-HCl.$2H_2O$ 0.005 g/L; riboflavin 0.005 g/L; nicotinic acid 0.005 g/L; D-Ca-pantothenate 0.005 g/L; vitamin B12 0.0001 g/L; p-Aminobenzoic acid 0.005 g/L; lipoic acid 0.005 g/L. The selenite-tungstate solution contained: NaOH 0.5 g; $Na_2SeO_3{\cdot}5H_2O$ 3 mg; $Na_2WO_4{\cdot}2H_2O$ 4 mg. The trace element solution contained: nitrilotriacetic acid 12.8 g/L; $FeCl_2{\bullet}4H_2O$ 2.0 g/L; $ZnCl_2$ 0.070 g/L; $MnCl_2{\cdot}4H_2O$ 0.1 g/L; H_3BO_3 0.006 g/L; $CoCl_2 \cdot 6H_2O$ 0.19 g/L; $CuCl_2{\bullet}2\ H_2O$ 0.002 g/L; $NiCl_2{\bullet}6\ H_2O$ 0.024 g/L; $Na_2MoO_4{\cdot}2H_2O$ 0.036 g/L. The trace elements solution, the vitamins solution, the selenite-tungstate solution, and the fructose were sterilized separately through 0.22 μm PES sterile filters. The other components of the medium were sterilized by autoclaving.

30 mL of sterile heterotrophic medium lacking cysteine were injected into a sterile glass bottle (total volume 160 mL) plugged with a rubber cap. Each bottle was then gassed with N_2. pH was adjusted to 7.2–7.3 with 100 μL of H_2SO_4 1.5 M. Subsequently, anaerobic bottles were stored at room temperature for around 24 h. 3 mL of a frozen 10% DMSO- stock culture, stored at − 80 °C, was used as inoculum. Cultures were grown in an orbital shaker (Biosan, LV) at 200 rpm at 30 °C until they attained an $OD_{600\ nm} \approx 1$.

2.2.2. A. woodii gas fermentation in bioreactor

Gas fermentations were conducted in a pressurized custom-adapted bioreactor manufactured by the H.E.L company (UK). Details of reactor hardware are provided elsewhere [30]. Briefly, it consists of an oil-jacketed 2 L stainless steel vessel. Rushton turbines performed the stirring at 400 rpm. Pressure, temperature, pH, and redox probes (Sentek, UK) are placed in the head plate of the vessel. A proportional Back Pressure Regulation (BPR) valve (Norgren, USA) controls the pressure inside the vessel to the stated level. A micrometric sparger placed at the vessel's bottom allows the sparging of the gas. Mass Flow Controllers (MFC) (Vögtlin Instruments, CH; Bronkhorst High-Tech BV, NL) allow the control of the in-flow gas rate. The oil jacket controls the temperature at 30 °C during the fermentation and allows the sterilization of the vessel in the preliminary operations and cleaning phase of the fermentation. During the fermentation, pH was controlled at 7 using NaOH 3.5 M provided through a piston pump (Eldex, USA).

Growth medium in the bioreactor contained: KH_2PO_4 0.176 g/L; K_2HPO_4 0.844 g/L; NH_4Cl 1.0 g/L; NaCl 2.9 g/L; yeast extract 2 g/L; cysteine hydrochloride 0.5 g/L; $MgSO_4$ 0.180 g/L and resazurin 1 mg/L. 0.8 L of the medium was loaded into the vessel and sterilized through in situ autoclaving. After cooling, trace element solution SL9 1 mL/L, selenite-tungstate solution 1 mL/L, and vitamin solution DMSZ 141 2 mL/L were added. The composition and sterilization methods of trace element solution, selenite-tungstate solution, and vitamin solution were the same described in Section 2.2.1. Then, the complete autotrophic medium was gassed first with nitrogen, to remove oxygen, and then with a 70% H_2 and 30% CO_2 mix at 4.5 L/h for 3 h at a constant pressure of 1.5 bar before inoculation [30]. A heterotrophic preculture of *A. woodii* was inoculated such that the starting $OD_{600\ nm}$ in the vessel was ≈ 0.2.

The gas fermentation was conducted in liquid batch and continuous gas supply. The fermentation lasted 169.5 h and was performed by augmenting the pressure inside the reactor when the $OD_{600\ nm}$ rose. The inoculation was performed with slight overpressure (1.1 bar). Furthermore, pressure increase was combined with two in-flow gas rates. The following steps in sequence were applied: i) 1.1 bar and 0.6 L/h in-flow gas rate; ii) 1.7 bar and 0.6 in-flow gas rate; iii) 2.25 bar and 0.6 L/h in-flow gas rate; iv) 2.25 bar and 1.2 L/h in-flow gas rate; v) 2.7 bar 1.2 in-flow gas rate, and vi) 3.3 bar 0.6 in-flow gas rate (Fig. 3). During the fermentation, samples were withdrawn from the reactor to check the performance, measuring $OD_{600\ nm}$ and analyzing metabolites through HPLC.

2.3. Fed-batch fermentation using sugars syrup and acetate

2.3.1. Fermentation on sugars and sodium acetate

A fermentation using SBS and commercial sodium acetate (Sigma-Adrich, USA) was conducted using the same settings parameters described in paragraph 2.1.2. As mentioned, the bioreactor was first sterilized containing the fermentation medium without sugars, trace metals, and magnesium solution. In parallel, a 300 g/L sodium acetate solution was sterilized through filtration using 0.22 μm sterile PES filters. After autoclaving, the cool fermentation medium was complemented with 12 g/L SBS syrup, magnesium solution and trace elements as described above (Section 2.1.2). After 15 h of fermentation, the fed-batch feeding mode started, providing SBS as described in paragraph 2.2.2. Moreover, an additional feeding of sodium acetate was performed. It started after 24 h of fermentation. The acetate feeding was done through a calibrated pump (Sartorius®), injecting a concentrated acetate solution volume such that 2 g/L of acetate were provided in the vessel each feeding. Acetate was fed at 30, 33, 39, 42, 44 h. During the test, pH was controlled at 6.8 by adding 2 M NaOH. Samples to analyze

the content of sugars, acetate, biomass and PHB were taken at 15, 18, 21, 24, 36, 40, 45, and 48 h.

2.3.2. Fermentation on sugars and acetate-rich waste medium (AWM) from gas fermentation

After *A. woodii* fermentation, as described in paragraph 2.3.2, the broth was collected and stored at − 20 °C. Then, the medium was centrifuged to discard bacterial cells and filtered through 0.22 μm PES sterile filters. The acetate concentration was measured through HPLC, and it was 12.5 g/L. Subsequently, the AWM was concentrated using a rotavapor (Laborota 4000 – Heidolph Instruments, DE) at 50 mbar, 40 °C, 150 rpm up to an acetate concentration of 130 g/L. Before addition into the fermentation medium for PHB production, the concentrated AWM medium (cAWM) was sterilized using 0.22 μm sterile PES filters. *C. necator* experiment for biopolymer production providing SBS and cAWM as substrates were conducted in the fed-batch mode using the same strategy described in paragraph 2.3.1.

Also in this case, samples were withdrawn from the reactor to measure the content of sugars, acetate, biomass and PHB at 15, 18, 21, 24, 36, 40, 45 and 48 h.

2.4. Analytical methods and calculation

2.4.1. Biomass measurements

Bacterial growth was estimated by measuring the optical density at 600 nm ($OD_{600\ nm}$) using the Jasco V730 STR-773 spectrophotometer (Jasco, UK). Distilled water was used as blank and, if needed, samples were diluted with distilled water before measurements. 3 mL of culture medium was collected at each sampling time and centrifuged at 6931 xg for 5 min (CAPPRondo Mini CR1512, CAPP, DK) to evaluate the biomass growth (g/L) of *C. necator* during fermentations. The pellet was washed twice with 1 mL of deionized water and centrifuged again at 6931 xg for 5 min. Then, the wet biomass was dried in an oven (Memmert, DE) at 90 °C for at least 20 h and weighed.

2.4.2. Substrates and PHB quantification

Sugars, acetate, and metabolites (e.g. formic acid) concentration in the culture medium were measured through HPLC analysis. Briefly, a sample of 3 mL was collected from the reactor and filtered using 0.22 μm PES filters into vials for HPLC analysis. To evaluate the amount of PHB produced by *C. necator* DSM 545, the same dried pellet used to estimate the cell dry weight (CDW) was hydrolyzed in thermoresistant glass tubes using 1 mL of 96% H_2SO_4. The tubes were placed in a silicon oil bath and kept at 90 °C in mixing conditions at 200 rpm for one hour. At the end of the reaction, samples were diluted 1000 times, and dilutions were analyzed through HPLC, detecting the crotonic acid peak. It is the monomer derived from PHB hydrolysis and is used as a reference standard [31].

HPLC analyses were done using a Prominence HPLC System (Shimadzu, JP) equipped with a photodiode array detector (PDA) and a refractive index detector (RID). Separation of compounds was achieved using a ROA-organic acid H+ (8%) column (Phenomenex) under an isocratic flux of 0.7 mL/min of 5 mM of H_2SO_4 at 50 °C.

2.4.3. Theory/calculations

2.4.3.1. PHB content and yield. PHB content was estimated by first relating the PHB concentration obtained by HPLC analysis to the biomass weight from which the extraction was done. Then, the amount was related to the total biomass obtained from that sample.

$$\text{Total PHB (g/L)} = \text{g PHB / volume of sample (L)} \quad (4)$$

The % of PHB content was calculated as:

$$\text{PHB (\%)} = (\text{g PHB}_{\text{weighted biomass}} / \text{g weighted biomass}) \bullet 100$$

The g $PHB_{weighted\ biomass}$ is quantified by HPLC analysis and multiplied for the dilution factor of the analyzed sample.

The yield (Y_{PHB}) was calculated as follows:

$$Y_{PHB}\ (-) = \text{g PHB /g consumed substrate (S)}$$

2.4.3.2. C. necator growth. The growth was estimated by measuring $OD_{600\ nm}$ and drying the samples (CDW) as described in paragraph 2.4.1. The maximum growth rate (μmax) was estimated by calculating the ln (X/X0) between two sampling points collected at different time (hours) during the exponential phase.

X = biomass (g/L) of a sample from 15 h to 36 h of the fermentation.

X0 = biomass (g/L) at 0 h of the fermentation.

The logarithmic scale was plotted, and the slope of the linear equation was obtained to calculate the μ.

2.5. Environmental evaluation

Life cycle assessment (LCA) was performed with SimaPro 9.4 software and database Ecoinvent 3.8. LCA was performed according to ISO 14040–14044 (2006).

2.5.1. Goal and scope

The study's goal was the environmental comparison of the production of 1 kg of pure PHB through two processes: the fermentation of the real syrup alone and the integrated biorefinery system (paragraphs 2.1.2 and 2.3.2). The scope of the study was to understand if PHB production could be environmentally sustainable at an industrial scale.

The functional unit (FU) was 1 kg of pure PHB since it was the product for which the fermentation systems were tailored. In this study, the consumed reagents, the required energy, and the produced emissions were referred to the FU. The boundary conditions were depicted in Fig. 5, and according to [32] the adopted approach was from gate (bin) to cradle.

The processes described in paragraphs 2.1.2 and 2.3.2 were scaled to produce 1 kg of pure PHB.

The sugar-waste substrate, SBS, came from a Piedmont plant, so the study was geo-contextualized in Piedmont, a region in the North-West of Italy. In accordance with [32] only the direct consequences of PHB production were considered, and the environmental impacts of the infrastructures were not included, because they were less important to the overall results of scenario evaluation.

The PHB production system included a foreground and background system. The foreground system directly involved with the reference flow. The background system linked with the foreground system included energy production and chemical supply [32].

Following the zero-burden assumption, it was assumed that SBS and cAWM did not contain any credits related to the impacts produced during its previous stages of the life cycle [33] but only the one related to the transport to the treatment plant, reported in the Life Cycle Inventory (LCI) in paragraph 2.5.2 and in the supplementary materials in a detailed form (Tables S1 and S2).

2.5.2. Life cycle inventory (LCI)

The LCI defined all inputs and outputs involved in the PHB production by fermenting the real syrup alone (Table S1, Supplementary materials) and the real syrup plus the acetate-rich medium (Table S2, Supplementary materials). The primary data came from the experimental tests reported in paragraphs 2.1, 2.2, and 2.3. For the PHB extraction step, evaluation is based on data reported by [31]. Until 2015, the most adopted PHB extraction technique was based on halogenated organic solvents as chloroform. Nonetheless, it is expensive, environmentally unfriendly, and may cause biopolymer degradation [34].

The study of [35] investigated environmentally low impact techniques to recover PHB from *C. necator*, by testing butyl acetate and ethyl acetate to replace the conventional halogenated solvents. The authors proved that butyl acetate is a suitable alternative to replace halogenated solvents as chloroform to recover PHB. The advantage is that the PHB remains in the organic phase, separated from cell debris in the aqueous phase. Instead, in the conventional chloroform-based recovery, PHB remains in the same phase of the cell debris [35].

Table S3 (Supplementary materials) compares the environmental impacts of chloroform and butyl acetate (based on Ecoinvent 3.8 database), proving that butyl acetate is more environmentally friendly than chloroform.

The secondary data were taken from Ecoinvent 3.8. According to [36] attributional LCA was applied. In detail, attributional LCA means a modeling approach by which inputs and outputs are attributed to the FU of a product system, linking the unit processes of the system according to a normative rule. The energy required by the equipment was calculated according to [37] considering Eq. 5:

$$Q_{sub}=m_{sub} \bullet c_p \bullet (T_{in}-T_{reac}) \quad (5)$$

where *Qsub* is the thermal power required for heating the substrate from an inlet temperature of 20 °C to incubation and fermentation processes; m_{sub} is the mass substrate flow rate; T_{in} and T_{reac} are the inlet and incubator/reactor temperatures, respectively, and c_p is the specific heat capacity. Since fermentation broths could be considered as water, water c_p is used.

2.5.3. Life cycle impact assessment (LCIA)

Life cycle impact assessment was performed with the ReCiPe 2016 Midpoint (H) and the cumulative energy demand (CED) methods. The analyzed impact category with ReCiPe 2016 Midpoint (H) was climate change (kg $CO_{2\ eq}$). It is the indicator of the global warming potential (GWP) caused by the emissions of greenhouse gases into the atmosphere. We selected this category to understand the contribution of the biorefinery systems studied in the current work to climate variation. Moreover, several studies that evaluate this category are available in the scientific literature for comparison. Furthermore, the environmental impact of each impact category is detailed in Table S4 of supplementary materials to provide a general overview of the environmental impact of the two investigated processes.

The non-renewable energy of the CED represents the direct and indirect energy used throughout the life cycle, including the energy consumed during the extraction, manufacturing, and disposal of the raw and auxiliary materials. Biological processes (acetate and PHB production) were carried out at low temperatures (around 30–35 °C) but for a long time (acetate fermentation lasted 169.5 h and PHB synthesis 48 h). CED method was employed to quantify the impacts related to energy consumption of the two production processes.

Bio-based plastics are completely or partially produced from biomass feedstock. Atmospheric CO_2 converted into carbon compounds through photosynthesis is defined as biogenic carbon in LCA studies. Accordingly, there are two approaches to managing biogenic carbon within LCA studies: 1) temporary carbon storage and 2) carbon neutrality [38]. Among them, climate neutrality is often assumed, which considers that the carbon that is sequestered by the feedstock is released back into the environment in a closed loop with no net climate forcing effect. The present study adopted carbon neutrality according to [39].

2.5.4. Interpretation data and sensitivity analysis

The last step of LCA is the interpretation of the results to evaluate the goal achievement. A sensitivity analysis was performed to measure and detect possible variations in the biorefinery systems response to environmental impacts. The sensitive analysis was performed by varying the PHB yield production ± 5% w/w for all the units included in the processes.

3. Results and discussion

3.1. C. necator DSM 545 fed-batch fermentation using sugar-based syrup (SBS) as carbon source

C. necator can metabolize several sugars to produce PHB. Therefore, *C. necator* DSM 545 was tested in a fed-batch fermentation providing the sugar syrup SBS as feedstock, containing mainly fructose and glucose. During the fermentation, a fed-batch strategy was performed to avoid sugar shortage and to achieve the carbon-to- nitrogen ratio suitable for PHB synthesis, as described in paragraph 2.1.2. Bacteria growth and the PHB accumulation were monitored (Fig. 1), and obtained results were compared with literature data.

C. necator DSM 545 exponential phase started after 12 h from the beginning of the fermentation, showing a high growth slope from 12 to 24 h, which is also the fermentation phase during which the bacteria started to accumulate the biopolymer.

Total sugars furnished were 35.5 g/L and 34.5 g/L were consumed. Thus, 97% of the whole amount of SBS was utilized. The maximum CDW achieved was 9.9 g/L, and the specific growth rate (μ) was 0.161 h^{-1}. 7.1 g/L PHB were synthesized (Fig. 1), obtaining a PHB yield (g PHB/g consumed substrate) of 0.207 $g_{PHB}/g_{cons.subs}$. The percentage of accumulation (g PHB/g biomass) was 75%. The latter value is consistent with the percentage of polymer accumulation reported in the literature for experiments performed using the same sugars (Table 1).

As shown in Table 1, sugar-based waste feedstock has been extensively studied for the growth and PHB production in *C. necator*.

The table shows works in which waste-substrates with simple sugars were used. They differ in working volumes, feeding strategies, and, in some cases, duration. The studies [42,43], and [44] used broken rice, molasses, and red apple waste, respectively. Thus, they valorized regional food wastes (Piedmont – Italy), like the current study, that valorized the SBS. Other studies using the same strain highlighted differences or similarities in yields and percentage of biopolymer accumulation. In the study [40], saccharified potato starch was used to achieve a high biomass and PHB concentrations (g/L), in a bioreactor. Using a phosphate limiting strategy for 72-hour fermentation, the yield of PHB of [40] was similar to the one obtained in the present work. In the study [43], molasses were used to accumulate PHB using a nitrogen limitation strategy in a working volume of 4 L in the bioreactor. In this case, the process lasted 48 h, and the same strain of the present work was used. These studies by Haas et al., 2008 [40] and Dalsasso et al. [43] reported a similar content of PHB (%). However, the % of PHB accumulation was lower than the one achieved in the present work, suggesting that either the substrates or the feeding strategies might lead to a higher accumulation. In the study [45], the same strain was used, the fermentation lasted the same hours (48 h), the starvation was induced by limiting nitrogen, and a similar accumulation percentage of PHB and productivity were achieved, even though the yield of produced PHB over consumed substrate was not reported. However, in study [45] the fermentation was conducted in flasks (0.150 L working volume), suggesting that the fermentation set up in the present work could be further enhanced to achieve higher concentrations of biomass and PHB. The works of [41,42] and [44] were conducted in flasks, as well. The studies of Brojanigo et al. [42] and Costa et al., 2018 [44] were performed using the same *C. necator* strain of the current work. Both these investigations achieved a similar biomass concentration but lower PHB accumulation, which could be due to the performing the fermentation in a less controlled system than a bioreactor. The presented flask fermentations also lasted much longer than the present study (168 h and 96 h, respectively). The work of Koutinas et al. [41] shows interesting results for *C. necator* DSM 259 fermentations in flasks using wheat hydrolysates as carbon sources. A higher biomass and PHB yield were achieved than the current study, nonetheless the fermentation process was longer (167.5 h).

The comparisons presented in Table 1 display *C. necator* capability to

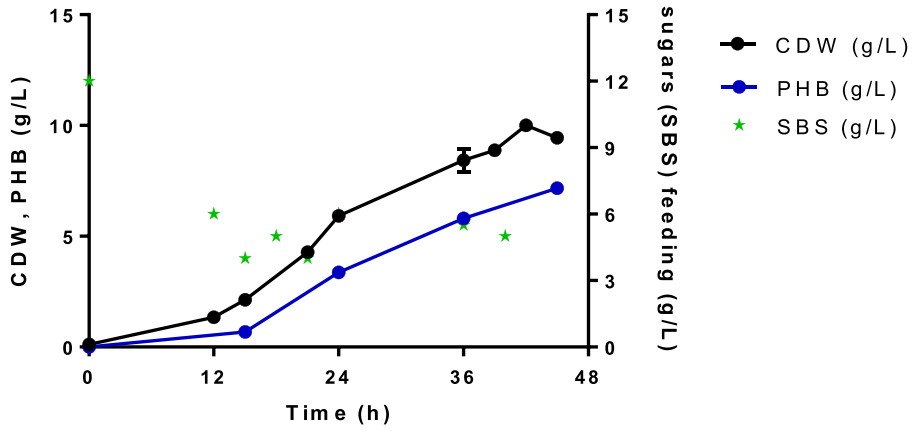

Fig. 1. Growth and PHB accumulation using *C. necator* DSM 545 in a fed-batch fermentation using SBS as only carbon source.: SBS feeding (g/L).★.

Table 1
sugar-rich waste substrates used for different strains of *C. necator* to accumulate PHB.

Waste substrates	Strain	Cell dry weight (g/L)	PHB content (g/L or %)	Productivity (g L^{-1} h^{-1})	Yield (g PHB/g consumed substrate)	Working volume	Duration (h)	Source
Saccharified potato starch	*C. necator* NCIMB 11599	179	94 g/L - 52.5%	1.47	0.22–0.46	3.4 L	72	[40]
Wheat hydrolysate	*C. necator* DSM 529	29.9	60%	0.29	0.43	< 0.5 L	167.5	[41]
Broken rice	*C. necator* DSM 545	13.32	5.18 g/L - 44.10%	/	/	0.1 L	168	[42]
Molasses	*C. necator* DSM 545	/	11.7 g/L - 56%	/	/	4 L	48	[43]
Red apple extract (food waste)	*C. necator* DSM 545	10.9	7.4 g/L - 68%	/	/	0.1 L	96	[44]
Wheat straw	*C. necator* DSM 545	15.1	12.1 g/L - 80.1%	0.252	/	0.150 L	48	[45]
SBS (cereals)	*C. necator* DSM 545	9.9	7.1 g/L – 75%	0.148	0.207	0.6 L	45	This study
SBS (cereals) & AWM	*C. necator* DSM 545	12	10 g/L – 83%	0.208	0.270	0.6 L	48	This study

accumulate high PHB content, underlying the versatility of this strain in metabolizing sugar-based waste substrates.

The differences presented in the reported studies can be due to i) the different substrates provided and the availability of their derived simple sugars ii) the feeding strategy, iii) PHB accumulation triggering strategies (e.g., nitrogen/phosphorus limitation, dissolved oxygen) during the accumulation phase, or iv) the fermentation system used (either flasks or bioreactors). Nevertheless, as Table 1 shows, the results obtained in the current work are consistent with previous literature data. This result engages further studies to valorize SBS as a substrate using *C. necator* DSM 545 for next-generation biopolymer production in a circular economy approach.

3.2. *C. necator DSM 545 fed-batch fermentation using sugar-based syrup (SBS) and acetate*

After the fed-batch fermentation using only the SBS as substrate, an integrated biorefinery system using *A. woodii* and *C. necator* DSM 545 as biocatalysts was performed. The integrated approach allows the production of the biopolymer exploiting the sugar-based waste, as described in Section 3.1, and the acetic acid produced through a CO_2-H_2 gas fermentation performed by *A. woodii*. CO_2-based polymers look promising, as this approach benefits the environment: i) the use of CO_2 for the manufacturing of bioplastics does not compete with food production or soil usage; ii) in an economy approach, CO_2 is an abundant source of carbon and the ultimate sustainable resource for the plastic industry; iii) the indirect use of CO_2 could theoretically reduce equal amounts of CO_2 (2.84 ton CO_2/ton PHB) while consuming 50% less H_2 than the completely autotrophic production of PHB by *C. necator* [27].

3.2.1. *C. necator DSM 545 fed-batch fermentation using SBS and commercial acetate*

C. necator DSM 545 was grown on SBS and commercial acetate as an additional carbon source. This test was a reference to evaluate the performance of the strain in terms of growth and PHB synthesis using the two different carbon sources (Fig. 2). During the fermentation, SBS and acetate were separately provided following the fed-batch strategy described in Section 2.3.1.

The total amount of SBS fed (25 g/L sugars) was consumed at the end of the experiment. Sodium acetate was provided five times (at 30, 33, 40, 43, 45 h) and was also completely consumed during the fermentation (Fig. 2 and Fig. 1S in supplementary material). Results showed a lower PHB yield (0.162) with respect to the fermentation providing only SBS (see Section 3.1). Nevertheless, the maximum biomass of 11.6 g/L and a maximum PHB content of 8.7 g/L after 48 h was reached, leading to the same percentage of PHB accumulation (75%) as providing sugar-feed only. Moreover, a slightly higher μ was achieved (0.177 h^{-1}). Thus, according to the observed results, adding commercial acetate to SBS allowed the achievement of similar performances in terms of bacteria growth or PHB accumulation with respect to only SBS fermentation, but providing about 30% less sugar feedstock.

3.2.2. *Acetate production through gas-fermentation by A. woodii*

Acetate production through gas-fermentation was performed using

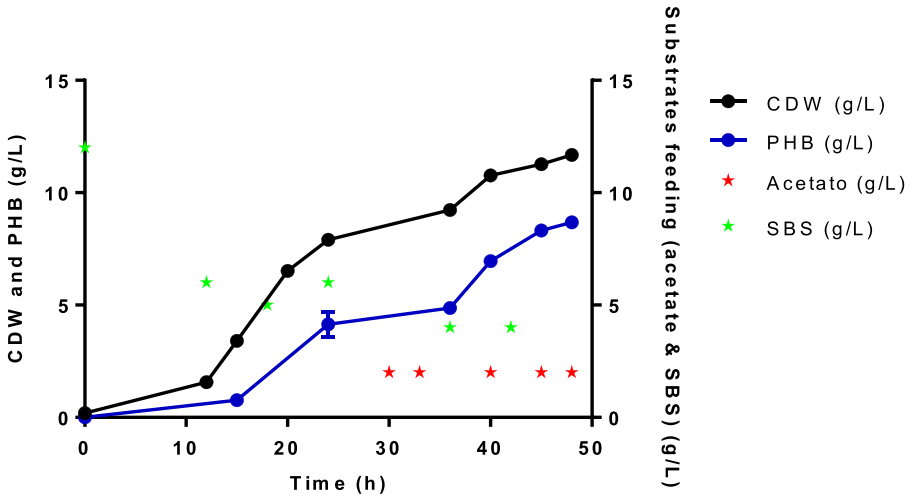

Fig. 2. Growth and PHB accumulation (g/L) using *C. necator* DSM 545 in a fed-batch fermentation providing SBS and pure commercial sodium acetate as carbon sources. ★: acetate solution feeding during the fed-batch (up to a concentration in the fermentation broth of 2 g/L). ★: SBS feeding (g/L achieved in the fermentation broth after the feeding).

the acetogenic bacterium *A. woodii* grown in a pressurized bioreactor. It was fed with a continuously provided 70% H_2 and 30% CO_2 blend with an in-flow gas rate ranging from 0.6 to 1.2 L/h in a liquid batch (Fig. 3). To avoid gas-limited growth, the fermentation was performed by increasing in-flow gas rate and/or the pressure inside the reactor when the $OD_{600\ nm}$ increased. The process started at 1.1 bar pressure and finished at 3.3 bar. The culture in the reactor reached a maximum $OD_{600\ nm}$ of 1.37 after 121 h. Bacteria produced acetate during the experiment, reaching a final concentration of 12.5 g/L at the end of the process. However, formic acid was synthesized at the higher pressure applied (final concentration 3 g/L), probably due to an impairment of the bacteria metabolism as previously described [26,46] (Fig. 3).

At the end of the fermentation, the medium was centrifuged to separate bacterial cells from the supernatant containing acetate. Then, the supernatant was concentrated through a rotavapor and sterilized through filtration as described in paragraph 2.3.2. The concentration step was needed in order to feed a low volume of acetate-rich broth in the fermentation medium of *C. necator* DSM 545, as described in the following section. In the concentrated AWM the acetate reached 130 g/L while formate 25.7 g/L.

3.2.3. *C. necator DSM 545 fed-batch fermentation using SBS and A. woodii gas fermentation acetate-rich medium (AWM)*

The concentrate AWM from *A. woodii* gas fermentation was provided as the supplemental acetate feedstock in a fed-batch fermentation using SBS as sugar-feed, as previously described for the reference test using commercial acetate (Section 3.2.1). The fermentation was conducted for 48 h, during which *C. necator* DSM 545 was cultivated by applying a fed-batch strategy with SBS and concentrated AWM separately provided (see Section 2.3.2). The whole amount of sugar (33 g/L) was consumed at 48 h, while half of the furnished acetate was consumed (5 g/L). The exponential phase lasted about 24 h (from 12 h to 36 h from the beginning of the fermentation), during which *C. necator* started accumulating PHB. The maximum biomass concentration was reached at 48 h and was 12 g/L, while the μ was 0.151 h^{-1}. PHB production was 10 g/L after 48 h of fermentation, with a biopolymer accumulation of 83% and a yield equal to 0.270. As described above, *A.woodii* produced a low amount of formic acid besides acetic acid during gas fermentation. Thus, also formic acid was transferred into the *C. necator* medium during the fed-batch with cAWM, furnishing 0.38 g/L of formate at each feeding (up to 1.9 g/L). Nevertheless, unlike acetate, the formic acid accumulated in the medium, suggesting that *C. necator* did not consume it. The literature reports that the growth of this bacterium on formate is particularly demanding of reducing agents, like H_2 [47]. Therefore, it is

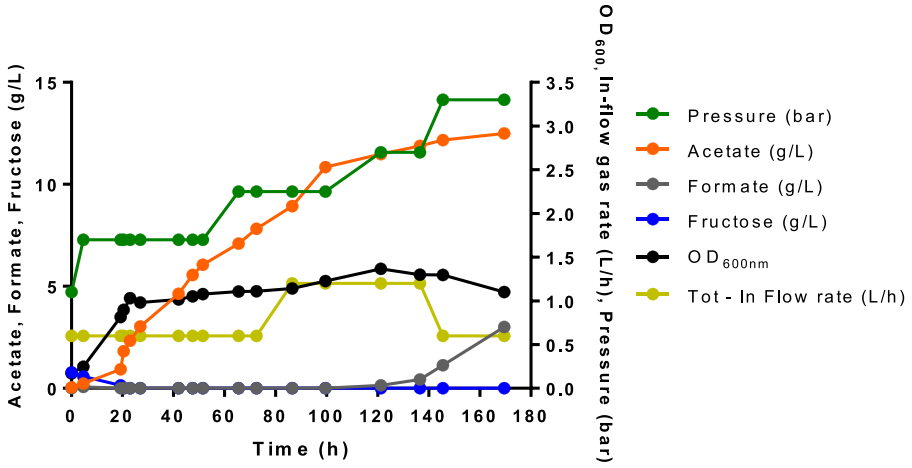

Fig. 3. Growth and acetate production of *A. woodii* grown in a liquid batch and continuous gassing (70:30 H_2:CO_2) mode in a pressurized bioreactor.

possible that under the process conditions applied, the bacterium preferentially utilized the more easily metabolized substrates (SBS and acetate).

Results obtained in this test showed that *C. necator* DSM 545 can efficiently grow on the SBS and a concentrated acetate-rich culture broth from gas fermentation. The utilization of waste fermentation media to make value-added products was studied for several applications in recent years. To the best of our knowledge, the only other study in which acetate from gas fermentation was fed as a carbon feedstock to produce PHB by *C. necator* is the work of Al Rowaihi and coworkers [22]. In that study, the CO_2 conversion into PHB was achieved in a two-stage biological process like the one in the current study. Nevertheless, some differences in bacteria strains and processes occur. Table 2 details the main differences between the two protocols.

As listed in Table 2, Al Rowaihi and coworkers [22] performed the gas fermentation in a pressurized bioreactor up to 2 bar but applying a gas-fed-batch operative mode. The authors did not report formic acid synthesis besides acetate synthesis, as in the present study (Section 3.2.2). This difference could be explained by the different mode of gas feeding. *A. woodii* products' spectrum is influenced by the amount of dissolved CO_2 in the liquid medium. A high concentration of dissolved CO_2 led to a metabolic impairment and formic acid synthesis in *A. woodii* [48]. Results suggested that the dissolved CO_2 concentration in the investigation of Al Rowaihi et al. did not overcome the threshold amount of carbon dioxide, while the present study did. Nevertheless, in the present study *A. woodii* produced a higher titer of acetate. The PHB cell content rose to 83%, achieving 10 g/L of PHB and 12 g/L of biomass and a max growth rate of 0.261 h^{-1}. The percentage of PHB accumulation in [22] was lower, suggesting that the feeding strategy and the conditions applied in this study improved PHB synthesis. Nonetheless, in [22], the AWM concentration step is avoided. This step is energy-consuming. Thus, other experiments should be carried out providing a not concentrated AMW, like Al Rowaihi and coworkers did, to make the current process more energetically sustainable.

The reported findings demonstrated the potential of utilizing two waste streams, SBS and concentrated AWM to make valuable bioproducts.

3.3. Environmental evaluation

The present section describes the comparison between the environmental evaluation of the PHB production process based on SBS alone (Section 3.1) and the integrated bioprocess (Sections 3.2.2 and 3.2.3). The work of [31] was the reference considered for reagents and data applied to evaluate the extraction step of PHB production.

The environmental assessment of 1 kg of pure PHB was studied through Life Cycle Assessment (LCA).

The two impact categories were climate change (kg CO_2 eq./1 kg PHB) and fossil energy demand (MJ eq./kg PHB). They were investigated with ReCiPe 2016 MidPoint (H) and cumulative energy demand (CED), respectively. As indicated by [49], the analysis detailed these two impact categories because they are the most studied in the LCA of bioplastic. Thus, considering these parameters, the present study is more comparable to other literature studies. Moreover, according to Green Deal Europe, energy is the key parameter to evaluate performance and system efficiency in the productive processes. The quantification of climate change and the emission of CO_2 eq. has gained attention due to the necessity to achieve carbon neutrality in the productive system by 2050.

The investigated processes consisted of four sequential steps: i) the pre-culture of *C. necator* DSM 545; ii) the culture of *C. necator* DSM 545, iii) the fermentation to produce PHB (considering the SBS alone or in combination with cAWM as substrates), and iv) the extraction to obtain the pure PHB.

The environmental impacts of the investigated processes are depicted in Fig. 5. Fig. 5, from A to D, displays the integrated processes. Fig. 5 E – F displays the single-feedstock process. The environmental impacts of the process converting SBS alone were 7.19 kg CO_2 eq./1 kg PHB (Fig. 5E) and 119 MJ eq./1 kg PHB (Fig. 5F). The impacts of the integrated process were 5.05 kg CO_2 eq./1 kg PHB (Fig. 5A) and 83.01 MJ eq./1 kg PHB (Fig. 5C).

The global warming potential impact category and the energy consumption achieved a higher impact in the single-feedstock process than the integrated one. This is because PHB yield was lower when providing the sugar syrup alone than with cAWM. Hence, the process exploiting SBS alone required a higher amount of feedstock and reagents. Moreover, a higher fermentation working volume is required for the pre-culture and fermentation steps. Table S4 in the supplementary materials depicts the global overview of all the other impact categories. It shows that the integrated process reached a lower environmental emission.

The outcomes of comparing the two processes led to a focus on the integrated one. In particular, bottlenecks were investigated to further improve its performance. The global warming potential impact category and the energy consumption had the same trend. The process step with the highest environmental impact was the PHB production (step iii) (3.02 kg CO_2 eq./1 kg PHB and 20.98 MJ eq./1 kg PHB) mainly due to the addition of cAWM from *A. woodii* (1.12 kg CO_2 eq./1 kg PHB and 14.50 MJ eq./1 kg PHB), followed by the extraction of PHB (1.81 kg CO_2 eq./1 kg PHB and 39.75 MJ eq./1 kg PHB).

The process items with the highest environmental impact were reagents used for bacteria growth and PHB extraction, followed by AWM production by *A. woodii*, and energy. Each one of these process items were analyzed in detail.

The reagents were necessary to carry out bacteria growth and biochemical reactions. Their contribution to non-renewable energy was 50.09% and in climate change was 52.7% to the total impact of step iii). This result is consistent with the study of [50], which proved the impacts of nutrients and reagents that constitute the culture broth.

In the extraction step (step iv) the highest impact was due to the use of butyl acetate and acetone in both the investigated impact categories. Nevertheless, the impacts were limited due to their recycling and reintroduction in the extraction process according to [31]. Butyl acetate was chosen as the solvent for this evaluation because it has a higher recovery level (96%) and it allows higher product purity (up to 99%) than ethyl acetate at 103 °C. As shown in [35], this method has a heating incubation time of 30 min. PHB recorded the highest molecular weight of 1.4×10^6 compared with the standard procedure, which involves

Table 2
Comparison between the work of Rowaihi et al., 2021 [22] and the present work. The table highlights the differences for the bacterial strains *C. necator* and *A. woodii*.

	Al Rowaihi et al. 2021 [22]	This study
A. woodii		
Pressurized bioreactor	yes	yes
Operative mode	Gas fed-batch	Continuous gassing
Gas mix ratio H_2:CO_2	85:15	70:30
Pressure (bar)	2	From 1.1–3.3
Duration (h)	212	169.5
Final acetate content (g/L)	3.2	12.5
Acetate productivity (g L^{-1} d^{-1})	0.36	1.77
C. necator		
Strain	DSM 259	DSM 545
Cultivation mode	Shaking flasks	Bioreactor
Sugar feeding	Commercial fructose	SBS
Acetate feeding	Filtered AWM - One feed	Concentrated AWM - Fed batch
Total acetate uptake (g/L)	3	5
PHB cell content	33.3%	83%
Duration (h)	217	48
Productivity (g L^{-1} d^{-1})	-	4.99
PHB tier (g/L)	0.5	10

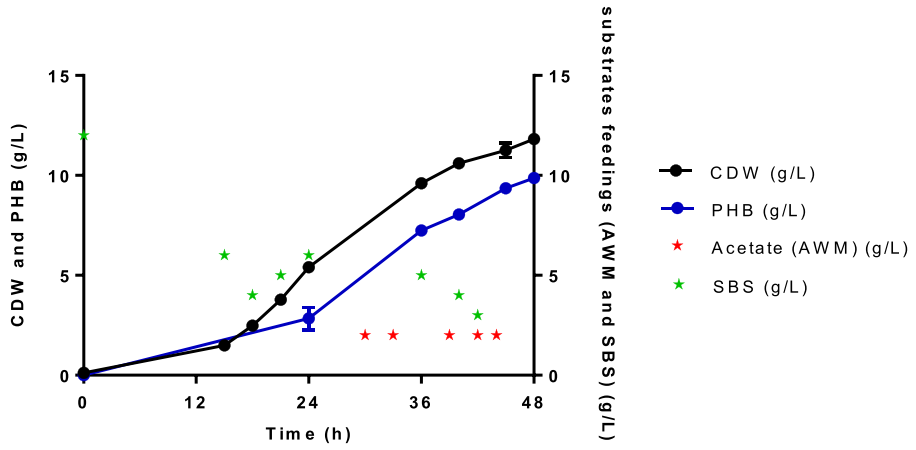

Fig. 4. Growth and PHB accumulation (g/L) using *C. necator* DSM 545 in a fed-batch fermentation providing SBS and concentrated AWM as carbon sources. ★: concentrated AWM feeding during the fed-batch (up to a concentration of 2 g/L in the fermentation broth); ★: SBS feeding (g/L).

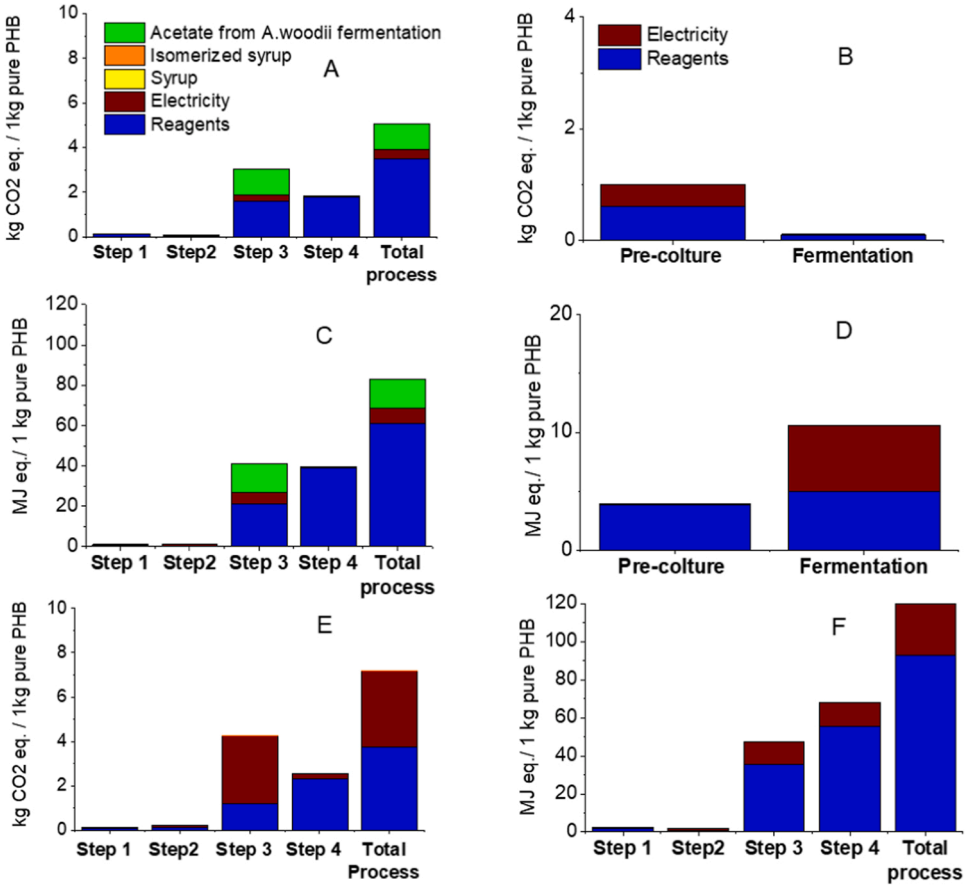

Fig. 5. detailed contribution of the environmental impacts of the process unit considering the climate change impact and non-renewable energy category. A) climate change impact of the whole integrated biorefinery, B) climate change impact of AWM produced from *A. woodii* fermentation, C) non-renewable energy category of the whole integrated biorefinery, D) non-renewable energy category of AWM produced from *A. woodii* fermentation; E) climate change impact of the fermentation of SBS alone, F) non-renewable energy category of the fermentation of SBS alone.

chloroform. Therefore, according to the literature, butyl acetate seems to be a good alternative to halogenated solvents such as chloroform for the recovery of PHB [35].

Moreover, employing fossil-based or bio-based reagents can lead to a completely different environmental footprint and climate change values [51]. The LCA performed by [50] proved that the type of solvent employed in extracting products (organic or inorganic solvents) affects the results significantly.

The second highest impactful item was the production of AWM by *A. woodii* through gas fermentation. It contributed 1.12 kg CO_2 eq./1 kg PHB and 14.50 MJ eq./1 kg PHB. A focus on *A. woodii* process was depicted in Fig. 5 (B and D). For the acetate from the fermentation with *A. woodii,* the main impacts were due to the reagents in the culture medium and for the biochemical conversion of CO_2 into acetate (in

particular H_2). They represented 64% in climate change category and 61% in non-renewable energy to the total impact related to acetate production. The energy required by *A. woodii* fermentation was due to the process run time (169.5 h).

The last highest impactful process item was energy which contributed 9% to the total impact of the integrated biorefinery, both in climate change and non-renewable energy categories.

One of the findings of the review of [52] is that the impact of bio-based products strongly depended on the type of energy source that could be used to replace the fossil one in biorefineries and the conversion process of complex feedstock (e.g. rice straw) into high-added value products. Several LCA studies evidenced that the environmental impact of PHB production can be even higher than that of conventional petroleum-based polymers [53,54].

The climate change and non-renewable impacts achieved in the present study were in line with the ones related to the production of PHB from corn sugars which were 3.95 kg CO_2 eq./ 1 kg of PHB and 79.85 MJ eq./ 1 kg of PHB [55].

The study of [49] produced PHB from glucose using *C. necator* DSM 428. The final impact was 1.9 kg CO_2 eq./ 1 kg of PHB, but the downstream process was neglected.

To prove the key role of the feedstock conversion into the target product and to validate our study, the sensitivity analysis was performed by varying the PHB yield in the production process by 5% w/w.

The sensitivity analysis confirmed that the most impactful step was the PHB production step, and it highlighted that the increase of SBS conversion into PHB reduced the environmental impacts of − 6.7% climate change and − 12.4% non-renewable energy. Hence the results of our LCA analysis could be considered like the optimized study about PHB of [55] and [49].

According to the LCA analysis, the integrated bioprocess for PHB production is more sustainable than the process that consumes SBS alone. Nevertheless, further improvements to the integrated process should be made. Concerning the fermentation processes, LCA evaluation pointed out that the items with the highest environmental impact were reagents used for bacteria growth, the energy demand due to the long fermentation process of *A. woodii*, and AWM concentration. Thus, the first challenge should be *A. woodii* and *C. necator* media optimization. Removing not strictly necessary reagents, considering the impact of this removal in terms of bacteria growth and acetate or PHB production should be faced. Moreover, the *A.woodii* gas fermentation could be further optimized to shorten the duration. An alternative could be exploiting faster microorganisms to reduce CO_2 to acetate. As an example, the literature describes the thermophilic bacterium *Thermoanaerobacter kivui* as a promising biocatalyst that performs well on CO_2: H_2 blend to produce acetate [56]. Furthermore, tests using the AWM without the concentration step should be performed. As above-mentioned, this step is energy-consuming: avoiding it will improve the sustainability of the process in terms of energy required.

4. Conclusions

In this study, we used two different organic substrates for PHB production in *Cupriavidus necator* DMS 545. The two substrates were a sugar-based syrup (SBS) from cereal waste and an acetate-rich medium (AWM) obtained through CO_2-H_2 gas fermentation by *Acetobacterium woodii*. The SBS was provided alone or in combination with the AWM, reaching a PHB accumulation of 75% and 83%, respectively.

The sugar syrup was furnished by a local cereal factory. The syrup is derived from cereal waste, making it a less expensive resource than pure commercial sugars. Moreover, the valorization of local waste allowed the application of the circular economy approach. The tested syrup resulted in being a suitable carbon source to boost *C. necator* DSM545 growth and biopolymer accumulation.

The acetate-rich medium, obtained through *A. woodii* gas fermentation and furnished to *C. necator* DSM 545, induced a higher growth rate and PHB production than those obtained by providing the sugar syrup alone or by adding the commercial acetate. This result suggested that acetate, salts and other minerals in the *A. woodii* culture medium could boost the *C. necator* growth and PHB production. The process parameters and results obtained in the experimental campaign were used to evaluate the environmental impacts of the PHB production process supplying SBS alone or with cAWM through an LCA analysis. The process with SBS alone impacted 7.19 kg CO_2 eq./1 kg PHB and 119 MJ eq./1 kg PHB, while the integrated biorefinery process impacted 5.05 kg CO_2 eq./ 1 kg PHB and 83.01 MJ eq./1 kg PHB. Thus, according to LCA analysis, the integrated bioprocess is more sustainable. Nevertheless, the gas fermentation performed by *A. woodii* was one of the most impactful steps of the integrated PHB production (1.12 kg CO_2 eq./1 kg PHB and 14.50 MJ eq./1 kg PHB). These outcomes suggest that further improvement could be done to reduce the impact of the CCU strategy, and that an improved and greener energy storage system could decrease the overall impact for each step of the process.

CRediT authorship contribution statement

Silvia Bellini: Investigation, Data curation, Writing – original draft, Writing –Visualization. **Francesca Demichelis**: Conceptualization, Methodology, Writing – original draft, Writing – review & editing, LCA analysis. **Tonia Tommasi:** Writing – review & editing. **Loredana Tarraran:** Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Debora Fino:** Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests, Debora Fino reports financial support was provided by Piedmont Region.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2023.111661.

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