fermentation

MDPI

Article

Production of Polyhydroxyalkanoates through Soybean Hull and Waste Glycerol Valorization: Subsequent Alkaline Pretreatment and Enzymatic Hydrolysis

Zulma Sarmiento-Vásquez, Luciana Porto de Souza Vandenberghe *, Susan Grace Karp and Carlos Ricardo Soccol

Department of Bioprocess Engineering and Biotechnology, Federal University of Paraná, Centro Politécnico, Curitiba 81531-980, PR, Brazil
* Correspondence: lucianapsv@gmail.com

Abstract: Alkaline pretreatment and sequential enzymatic hydrolysis of soybean hull were investigated to obtain fermentable sugars for polyhydroxyalkanoates production along with residual glycerol as low-cost carbon sources. Soybean hull is composed of approximately 32% cellulose, 12% hemicellulose, 6% lignin, and 11% protein. Alkaline pretreatment was carried out with 2% NaOH concentration, 10% (*w/v*) biomass loading, and 60 min incubation time in an autoclave at 120 °C. The response surface methodology (RSM) based on the central composite design (CCD) tool was employed to optimize the enzymatic hydrolysis process, where the variables of biomass loading, enzymes' concentration, and time were considered. The maximum total reducing sugars concentration obtained was 115.9 g·L^{-1} with an enzyme concentration of 11.5 mg protein/g dry substrate for enzyme preparation B1, 2.88 mg protein/g dry substrate for XylA, and 57.6 U/g dry substrate for β-glucosidase, after 42 h at 45 °C, and pH was 4.5. Subsequently, the saccharification step was conducted by increasing the processing scale, using a 1 L tank with stirring with a controlled temperature. Implementing the same enzyme concentrations at pH 4.5, temperature of 45 °C, 260 mL working volume, and incubation time of 42 h, under fed-batch operation with substrate feeding after 14 h and 22 h, a hydrolysate with a concentration of 185.7 g·L^{-1} was obtained. Initially, to verify the influence of different carbon sources on *Cupriavidus necator* DSMz 545 in biomass production, batch fermentations were developed, testing laboratory-grade glucose, soybean hull hydrolysate, and waste glycerol (a by-product of biodiesel processing available in large quantities) as carbon sources in one-factor-at-a-time assays, and the mixture of soybean hull hydrolysate and waste glycerol. Then, the hydrolysate and waste glycerol were consumed by *C. necator*, producing 12.1 g·L^{-1} of biomass and achieving 39% of polyhydroxyalkanoate (PHB) accumulation. To the best of our knowledge, this is the first time that soybean hull hydrolysate has been used as a carbon source to produce polyhydroxyalkanoates, and the results suggest that this agro-industrial by-product is a viable alternative feedstock to produce value-added components.

Keywords: soybean hull; alkaline pretreatment; saccharification; biomass hydrolysate; fermentable sugars; polyhydroxyalkanoates; biorefinery

Citation: Sarmiento-Vásquez, Z.; Vandenberghe, L.P.d.S.; Karp, S.G.; Soccol, C.R. Production of Polyhydroxyalkanoates through Soybean Hull and Waste Glycerol Valorization: Subsequent Alkaline Pretreatment and Enzymatic Hydrolysis. *Fermentation* **2022**, *8*, 433. https://doi.org/10.3390/fermentation8090433

Academic Editors: Teresa Gervasi and Giuseppina Mandalari

Received: 5 August 2022
Accepted: 26 August 2022
Published: 1 September 2022

1. Introduction

Agro-industrial activities around the world generate enormous quantities of waste material. However, considering environmental, productivity, and sustainability trends, these materials are now seen from a new perspective. Currently, due to their interesting physical–chemical composition, they can be used and reintegrated in productive processes that guarantee their valorization. Agricultural sectors produce lignocellulosic waste materials (straw, cob, stalks, bagasse, hulls), which have as main structural compounds cellulose (40–60%), hemicellulose (20–40%), and lignin (10–25%) [1]. Given that around 200 billion metric tons of these wastes are generated annually [2], there has been a remarkable

increase in research on applications that allow the reuse of these structural compounds. These polysaccharides have a high potential for transformation and valorization, as they are used as feedstock for the generation of biofuels, organic acids, and various high-cost molecules [3].

Worldwide, Brazil stands out in agribusiness, which represents one of the main pillars of the national economy. It is worth mentioning important examples in this area, such as soybean production. In 2020–2021, Brazil produced 135.409 million tons of soybean (*Glycine max*), which corresponds to 37.3% of world production, being the world's leading producer of this grain [4]. It is estimated that around seven million tons of soybean hulls were generated in Brazil by 2020. This material has low recalcitrance due to its low lignin content (2–13%), which facilitates processing for the utilization of hemicellulose (19–34%) and cellulose (29–52%). Various physical, chemical, and enzymatic pretreatments have been implemented for the saccharification of these compounds to obtain fermentable sugars that allow the production, through biotechnological processes, of medium- to high-value-added molecules such as enzymes, organic acids, and biofuels [5].

On the other hand, in biodiesel production industry, for every 10 kg of biodiesel, 1 kg of glycerol is generated as a by-product [6]. In 2020, approximately 600,000 m^3 of glycerol was generated in Brazil, representing an increase of 140% over the past decade [7]. The wide availability of glycerol outlines its application on an industrial scale. Thus, it has been widely used in the cosmetics, food, pharmaceutical, and renewable energy industries. The prospects for future environmentally friendly applications are of great interest in the R&D areas of the industry. Thus, high-value-added molecules (i.e., 1,3-propanediol, malic acid, lipids, biopolymers, among others) have been produced through microorganisms [8,9].

In addition to the problem of the excessive generation of waste materials because of the production processes that currently support human needs (food, fuel, clothing, construction), there is also the excessive accumulation of millions of tons of plastic. It is estimated that more than 8.3 billion tons of plastic have been produced since 1950, and about 60% ends up in landfills or in the natural environment, and approximately eleven million tons are dumped into the sea each year [10]. In 2020, global plastics production reached 367 million tons [11], of which less than 1% were bioplastics. In the United States, for example, only 9% of this material was recycled [12]. A plausible alternative to this situation focuses on the production of biodegradable polymers, which are generated from renewable raw materials and can be degraded by biological processes. Polyhydroxyalkanoates (PHA) belong to this group of biopolymers and could be produced intracellularly by microorganisms. They have characteristics of conventional plastics and, most importantly, are biodegradable [13]. However, the marketing value is affected by the high cost of raw materials within the production process, which has been a major obstacle to large-scale production [14]. Thus, as an alternative for the use of available waste material in the region and to mitigate the environmental problem generated by the use of polymers produced from petroleum, this work explores the use of agro-industrial by-products, such as soybean hulls and wasted glycerol, as raw materials to produce PHAs by *Cupriavidus necator*. To the best of our knowledge, this is the first time that soybean hull hydrolysate has been used as a carbon source to produce polyhydroxyalkanoates, and the results suggest that both this agro-industrial by-product and glycerol are viable alternative feedstocks to produce value-added components.

2. Material and Methods

2.1. Characterization of Soybean Hull

The soybean hulls (SBH) used in this study were supplied by the company Imcopa S.A. (Araucária, Paraná, Brazil). Following the NREL/TP-510-42620 standard on sample preparation for compositional analysis [15], the SBH were subjected to drying processes at 45 ± 3 °C for 24 h (Thot drying oven, mod. 510.480, Thoth Equipments, Piracicaba, SP, Brazil), milling (Marconi-MA 580/E electric knife mill, Marconi Equipments, Piracicaba, SP, Brazil), and sieving (USS/ASTM no. 20 mesh, Marconi Equipments, Piracicaba, SP, Brazil).

In the analyses described below, particle size of 0.84–2.00 mm was employed [16–18]. The total solids and moisture of the SBH were analyzed using a balance for moisture determination by infrared spectroscopy (Top Ray, BEL, BEL Equipments, Piracicaba, SP, Brazil). Ash content was determined by applying the methodology established in NREL/TP-510-42622 [19] using a muffle furnace (Nova Instruments, Piracicaba, SP, Brazil) at 575 ± 25 °C to calcine the material for 6 h. For the determination of extractables in water and ethanol, the methodology established in the standard NREL/TP-510-42619 [20] was adopted. The extraction was carried out during 8 h with the addition of 190 ± 5 mL of each solvent (water and ethanol consecutively) in the Soxhlet equipment. The determination of structural carbohydrates and lignin was performed following the methodology established in NREL/TP-510-42618 [21].

For the determination of reducing sugars (RS) and ions analyses, it was necessary to prepare an aqueous extract of the SBH. Thus, in an incubator water bath with circular orbital stirring (Ethiktechnology, Labstore, Curitiba, PR, Brazil), a mixture of 1 g of SBH and 50 mL of deionized water was heated at 100 °C for 10 min. This content was carefully transferred to a 100 mL volumetric flask and the volume was completed with deionized water. The sample was filtered using qualitative filter paper and stored at 4 °C until analysis. The followed stages in this work are summarized in Figure 1.

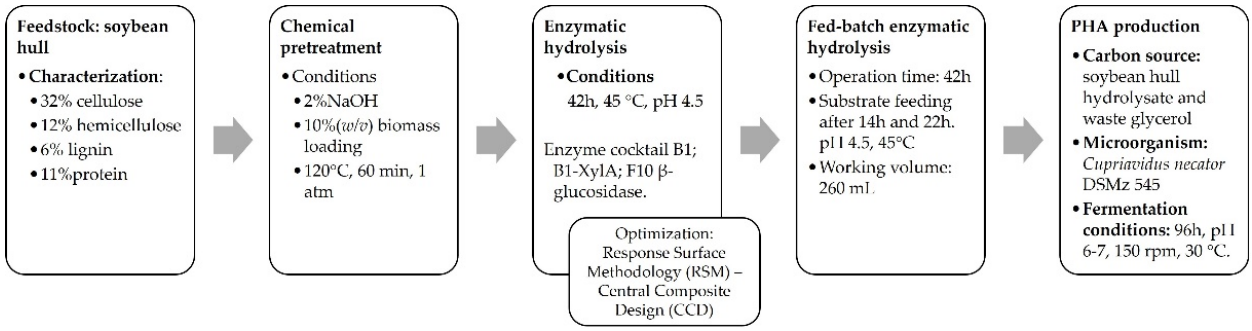

Figure 1. Stages followed for the biotechnological production of PHB through the valorization of soybean hulls and residual glycerol.

2.2. Pretreatment Conditions of Soybean Hulls

Considering the physicochemical characteristics of SBH, it was determined that chemical pretreatment followed by enzymatic hydrolysis should be used to obtain a sugars-rich hydrolysate to be employed in further fermentation processes [22]. A chemical pretreatment using 2% (*w/v*) of NaOH, SBH in a ratio of 10% (*w/v*) and at 121 °C for 1 h was developed [16,17]. After pretreatment, once the material reached room temperature, it was neutralized with HCl, filtered, washed with distilled water to remove soluble components, and filtered again [23]. The resulting wet solid material was reserved for the following steps once the total solids and moisture were analyzed using an infrared moisture determination balance (Top Ray, BEL, BEL Equipments, Piracicaba, SP, Brazil).

2.3. Optimization of Enzymatic Hydrolysis

2.3.1. First Step of SBH Enzymatic Hydrolysis Optimization

For the saccharification step of alkaline pretreated SBH, an enzyme cocktail (produced at the Federal Research Centre "Fundamentals of Biotechnology" at Lomonosov Moscow State University, Moscow, Russia) composed of cellulase and xylanase complexes of *Penicillium verruculosum* (B1 host preparation), xylanase B1-XylA preparation, was used. This was obtained through the recombinant expression of *Penicillium canescens* xylanase A in the *P. verruculosum* B1 host strain, and β-glucosidase F10 preparation, which was obtained by the recombinant expression of *Aspergillus niger* β-glucosidase in the B1 host strain [16]. SBH saccharification conditions through enzymatic hydrolysis were optimized using the response surface methodology (RSM). Following a central composite design (CCD) approach,

eighteen assays were performed (with 6 axial points and 2 center point runs), using the software Statistica® 5.0 (Statista Inc., New York, NY, USA) for data analysis. The substrate loading (% *w/v*), saccharification time (h), and enzyme cocktail concentration (*w/w*) with B1, β-glucosidase F10 and XylA, were studied, keeping pH at 4.5 and temperature constant at 45 °C. The independent factors in the design were studied at five different levels ($-\alpha$, -1, 0, +1 and $+\alpha$) as shown in Table 1. The analysis of variance was evaluated at a significance level of 95% ($p < 0.05$) for all compounds. In an orbital agitated water bath (Ethiktechnology-Labstore, Curitiba, PR, Brazil) samples were incubated at 100 rpm, from 12 h to 72 h, using 20 mL as working volume in 125 mL Erlenmeyer flasks. Then, heat shock treatment was performed to stop the enzyme activity. Samples of enzymatically treated SBH were filtered through qualitative filter paper and stored under refrigeration until further use.

Table 1. The independent and coding levels employed in the CCD.

Independent Variable	–1.682	–1	0	+1	+1.682
Substrate loading (% *w/v*)	15.0	24.5	38.5	52.5	62.0
Saccharification time (h)	12.0	24.2	42.0	59.8	72.0
Enzyme concentrations (g of enzyme/g SBH)					
B1	3.28	6.62	11.52	16.42	19.76
β-glucosidase F10	16.40	33.10	57.59	82.08	98.78
XylA	0.82	1.66	2.88	4.10	4.49

Coded levels for studied factors from (−1.682) to (+1.682), with 0 corresponding to the central point.

2.3.2. Second Step of SBH Fed-Batch Enzymatic Hydrolysis Optimization

Additional optimization of SBH enzymatic hydrolysis assays were performed in a 1L bioreactor (mod. TE-054-MAG, Tecnal, Piracicaba, SP, Brazil) adapted to a mechanical stirrer with a spiral propeller blade (mod. TE-139, Tecnal, Piracicaba, SP, Brazil). The SBH enzymatic hydrolysis was conducted with the use of a reaction volume of 260 mL of a sodium acetate buffer (1M, pH 4.5) [16], incubated at 45 °C with constant stirring for 42 h. The previously optimized enzyme cocktail was employed, which was composed of 11.52 mg·g^{-1} dry substrate of B1, 2.88 mg·g^{-1} dry substrate of B1-XylA, and 56.7 U/g of dry substrate of F10 β-glucosidase. In this case, the final substrate concentration (in dry basis) was set at 38.5% (*w/v*) by applying a fed-batch strategy, with the addition of alkaline-pretreated SBH for the saccharification reaction. The saccharification process began with 12.9% (*w/v*) solids content and 12.8% and 12.8% of substrate were fed at 14 h and 22 h to achieve 38.5% (*w/v*) of total solids content. Then, heat shock treatment was performed to disactivate the enzymatic reaction, the hydrolyzed SBH material was filtered through qualitative filter paper, and the hydrolysate was stored under refrigeration until further use (directly for PHB production without detoxification) and/or analysis. The glucose yield (*g/g*) was calculated considering the glucose concentration quantified by HPLC, for 1 g of SBH biomass. The degree of conversion from cellulose to glucose (C_{CG}%) was determined as the ratio of the glucose obtained to the theoretical yield based on the amount of cellulose in soybean hulls, where Y_G is the glucose yield (*g/g*) and C is the amount of cellulose in 1 g SBH [24].

$$C_{CG} = \frac{Y_G}{C \times 1.1} \times 100 \quad (1)$$

2.4. PHA Production

The *C. necator* strain DSMz 545 was purchased from DSMz (Braunschweig, Germany) and used throughout this study. This bacterial strain was selected for its capacity for PHA production, which has been widely studied [25,26]. Culture stocks were generated from the lyophilized cells and stored at −80 °C in glycerol (20% *v/v*). Initially, to improve the rate of glycerol consumption by *C. necator*, successive cultures were carried out in Petri dishes at gradually increasing glycerol concentrations (5–25 g·L^{-1}). Besides glycerol, the medium

was composed of ($g \cdot L^{-1}$) yeast extract 10, peptone 5, and agar 15. The inoculum for the shake flask was prepared by transferring bacterial cells to a liquid medium containing ($g \cdot L^{-1}$) carbon source 20 (according to the experiments described as follows), yeast extract 10, and peptone 5. The maintenance medium was prepared on slant agar containing the described medium and 15 $g \cdot L^{-1}$ agar [27].

The culture medium implemented to produce the biopolymer salt mineral medium (MSM) [28] was composed of the following elements ($g \cdot L^{-1}$): $(NH_4)_2SO_4$, 4; KH_2PO_4, 13.3; $MgSO_4 \cdot 7H_2O$, 1.2; carbon source 20; yeast extract 1; citric acid 1.7 and 10 $mL \cdot L^{-1}$ of trace element solution, consisting of ($g \cdot L^{-1}$) $FeSO_4$, 10; $ZnSO_4$, 2.25; $CuSO_4$, 1; $CaCl_2$, 2; $Na_2B_4O_7$, 0.23; $MnSO_4$, 0.5; $(NH_4)_6Mo_7O_{24}$, 0.1 and 10 $mL \cdot L^{-1}$ of HCl (35%). For PHA production, different carbon sources were tested: laboratory-grade glucose, laboratory-grade glycerol, waste glycerol (WG), and reducing sugars from the obtained hydrolysate of SBH. For each case, the final concentration was calculated based on 8 g/mol of C (considering that 20 g L^{-1} of glucose is equivalent to 8 g/mol of C). When used simultaneously, 50% of SBH hydrolysate (SBHH) and 50% of WG were added to the medium. Carbon sources and components susceptible to precipitation under sterilization conditions were sterilized separately (20 min at 120 °C, 1 atm). Prior to inoculation of the medium, the pH of 6.0–7.0 was checked and, when necessary, adjusted with KOH solution. In order to avoid variable results, the experiments were performed in triplicate. Standard deviation was calculated from the mean of the three values.

For the inoculum, the effect of different C:N ratios (1:1, 5:1, and 11:1), nitrogen source (yeast extract, ammonium sulfate, and urea), and laboratory-grade glucose on biomass production was studied. In these cases, the inoculated flask (10% *v/v*), with a total volume of 50 mL of the growth media, was added to 250 mL flasks and incubated in an orbital shaker (Excella E24 Incubator Shaker Series, New Brunswick Scientific, Enfield, CT, USA) for 24 h at 30 °C with 100 rpm agitation. Batch fermentation for biopolymer synthesis was performed in 250 mL Erlenmeyer flasks: 5 mL of bacterial suspension, obtained in the exponential phase of inoculum culture (10% *v/v*) was added to 45 mL of MSM, with an initial pH between 6.0 and 7.0, and incubated in an orbital shaker at 30 °C and 150 rpm for 96 h. The kinetics of PHA and biomass production and substrate consumption were monitored periodically.

2.5. Analytical Procedures

The elemental composition parameters of SBHH and fermented medium were determined prior to the use of HPLC (Agilent, Santa Clara, CA, USA) and ion chromatography, with the following conditions. A Metrohm CH-9101 ion analyzer was used to determine the SBH composition of ions. A Metrosep C3 250/4.0 column (250 × 4.0 mm ID; no. 5607002) was employed to quantify cations, under the following conditions: eluent 5.0 mM HNO_3; flow rate 1.0 $mL \cdot min^{-1}$; detector CD; temperature 40 °C; injection volume 20 µL. For anion analysis, a Metrosep-A Supp 5 250/4.0 column (250 × 4.0 mm ID; No. 7610789) was used, with the following analysis conditions: eluent 3.2 mM Na_2CO_3 + 1.0 mM $NaHCO_3$; flow rate 0.7 $mL \cdot min^{-1}$; detector suppressed CD; room temperature 25 °C; injection volume 20 µL. Quantification of sugars, organic acids, glycerol, and determination of WG purity were performed in an Agilent 1260 Infinity HPLC equipment of the Analytical Chemistry Laboratory of Bioprocess and Engineering Department of Federal University of Paraná-UFPR. Analytical conditions were: Hi-Plex H column (300 × 7.7 mm) operated at 60 °C, 0.005 MH_2SO_4 as the mobile phase at 0.6 $mL \cdot min^{-1}$ flow rate (Ajinomoto Brazil Ltd.a, Limeira, SP, Brazil). Supplied WG was filtered through qualitative filter paper and, using a densimeter, the density was determined. The purity and elemental composition parameters of WG were established before use in the processes, using HPLC and ion chromatograph (Shimadzu Europe, Duisburg, Germany), under the conditions described above.

The 3,5-dinitrosalicylic acid (DNS) methodology was implemented for the determination of reducing sugars [29] for both SBH aqueous extract and hydrolyzed SBH. Cells' dry weight was determined by adding 2 mL of culture medium in a previously weighed

Eppendorf tube and after centrifugation at 6000 rpm, the pellet was washed with deionized water and taken to the drying oven (80 °C) for 12 h. After 1 hour in the desiccator, the material was weighed on an analytical balance and calculations were performed. All analyses were done in triplicate.

The accumulated PHB content was estimated through gas chromatography (GC) analysis [30], in which approximately 10 mg of lyophilized cells was subjected to methanolysis in the presence of methanol, 3% (*v*/*v*) sulfuric acid, and chloroform. The mixture was incubated at 100 °C for 140 min (Thermoreaktor TR300, Merck, Darmstadt, Germany). After cooling, 1 mL of Na_2CO_3 solution was added. The organic layer was separated, and 1 mL of a solution was obtained. The resulting hydroxyacyl-methyl-esters were analyzed by GC (GC2010 Plus Capillary GC, Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with a flame ionization detector (FID), an autoinjector (AOC-20i), and SH-RTX ™-Wax capillary column (Shimadzu, 30 m, 0.32 mm ID, 0.25 µm). The injection volume was 1 µL and the split ratio 1:75. The carrier gas (helium) was controlled at a linear speed of 45 cm/s. The injector and FID detector temperature were maintained at 240 °C and 250 °C, respectively. The column oven temperature was set to start at 100 °C for 2 min, then increased to 280 °C at a rate of 40 °C/min and maintained for 4 min. The chromatogram data were analyzed using GCsolution Workstation Version 2.32 software (Shimadzu, LabSolutions, Columbia, MD, USA). The internal standard was benzoic acid, and the external standard was poly((R)-3-hydroxybutyric acid) (Sigma-Aldrich). For identification and quantification, the standards were also subjected to methanolysis as described above, and the methyl esters were analyzed.

The extraction and purification of the biopolymer were performed using solvents as described below. Six percent sodium hypochlorite and chloroform (1:1) were added to the freeze-dried biomass, maintaining a temperature of 37 °C at 1500 rpm for 2 h. Subsequently, the material was centrifuged (3000 rpm, 10 min) and the top layer was removed. The solution obtained was filtered to remove cellular debris and poured into a tube of known mass. By adding methanol (1:9), the biopolymer was precipitated and separated by centrifugation (3000 rpm, 10 min), and the methanol–chloroform mixture was decanted. The precipitated polymer was again dissolved in chloroform and precipitated with methanol and then dried to obtain a highly purified polymer [31].

3. Results and Discussion

3.1. Characterization of Soybean Hull and Waste Glycerol

The SBH was characterized to identify the structure and composition of the material to determine the best pretreatment strategy to be implemented. The selection of the most suitable pretreatment is of most importance because it allows proper exposition of cellulose structure before enzymatic action, thus obtaining the highest concentration of fermentable sugars.

The composition of the analyzed SBH was determined as follows. The amount of cellulose 31.0 ± 1.6%, hemicellulose 11.8 ± 1.3%, and lignin 6.18 ± 0.8% are close to values reported by other authors related to SBH analysis [22]. The low lignin content showed that mild pretreatment conditions could be employed without reducing the cellulose-rich fraction loss and favoring higher enzymatic hydrolysis conversion to fermentable sugars [32,33]. Other components, such as protein (10.5%), extractives (9.3 ± 1.0%), ash (3.6 ± 0.03%), moisture (6 ± 0.10%), and others (≈21.62%) mostly represented by pectin, lipids, organic acids, were determined.

The determination of soluble sugars showed a concentration of 6.02 $mg \cdot g^{-1}$ $SBH_{(bs)}$ in the aqueous extract, while for the hydrolysate, the concentration reached 115.9 $g \cdot L^{-1}$. Thus, the results show an increase of more than 100% in the availability of fermentable sugars for application in subsequent processes. The composition of ions present in SBHH was compared with the composition of the aqueous extract obtained directly from the non-treated SBH (in natura), (Table 2).

Table 2. Quantification of ions in the aqueous extract of SBHH and in the WG.

Ions	Concentration ($mg \cdot L^{-1}$)		
	Aqueous Extract SBH	**SBH Hydrolysate**	**Waste Glycerol**
Soluble anions			
Cl^-	34	135	12,150
F^-	2	278	nd
SO_4^{-2}	9	84	170
PO_4^{-3}	6	nd	nd
Br	nd	nd	nd
NO_x^-	nd	nd	nd
Soluble cations			
Na^+	2	630	10,350
Ca^{+2}	6	110	nd
Mg^{+2}	6	60	nd
K^+	58	nd	nd
NH_4^+	nd	nd	nd

nd = not determined.

For both anions and cations, in all cases (except for phosphate and potassium, respectively) a significant increase in the concentration of each analyzed component was observed. In addition, WG density was 1.12 $g \cdot mL^{-1}$, the purity corresponded to 39 ± 1%, ion content of WG was also determined where a high amount of anion Cl^- (≈12 $g \cdot L^{-1}$) and the cation Na^+ (≈10 $g \cdot L^{-1}$) stood out. These elements may be present in high concentrations given that they are used as catalysts in production and downstream processes where glycerol is generated as waste [34]. Taking these parameters into account, the formulation of the culture medium was possible for the subsequent fermentative processes.

3.2. Alkaline Pretreatment of Soybean Hull

Alkaline pretreatment has been considered a viable method for the bioconversion of lignocellulosic biomass [32] and is recommended when the substrate has a low lignin content [35]. Moreover, it reduces the formation of inhibitory compounds, and it is useful even when lower temperatures are implemented [36]. Thus, considering that the structural compounds of the SBH used in this research corresponded to 6.18% lignin, a mild alkaline pretreatment was selected. Qing et al. [22] analyzed acid and alkaline solutions to compare their performance during pretreatment and subsequent enzymatic hydrolysis. They found that alkaline pretreatment at 100 °C, for 120 min, with a solid to liquid ratio set at 1:20 and NaOH 1%, was more effective in delignification and improved the enzymatic digestibility of pretreated substrate, when compared with acid pretreatment.

After the alkaline pretreatment of SBH, a mass loss of 48% was quantified, which reflects the structural effect caused by sodium hydroxide. NaOH promotes the opening of hydrogen bonds between cellulose and hemicellulose, and between the latter and lignin stimulates the cleavage of ester bonds [37]. In consequence, lignin, small portions of hemicellulose, and other components such as proteins, extractives, and ash were removed. Thus, the hydrolysis of other SBH constituents could also occur. In addition, during the procedures, a loss of the pretreated biomass was observed when the material was transferred to the flasks or on filtration membranes, and this material was not directly quantified. Saha and Cotta [38], after an alkaline pretreatment for the conversion of rice hull cellulose and hemicellulose to simple sugars, quantified a mass loss of 53%. Karp et al. [16] described similar results in SHB, where after an alkaline pretreatment with a substrate concentration of 10% (*w/v*) of solids, NaOH 2%, at 121 °C for 1 h, a mass loss of 51.3% was quantified. Comparatively, in the present work, the NaOH concentration was lower (1%), being the main difference that could have influenced the slightly lower mass loss of 48% compared to 51% when NaOH at a concentration of 2% was used.

Biomass swelling of biomass and surface alteration after the alkaline pretreatment (data not shown) was observed, which was also previously reported by Camiscia et al. [39]. The swelling of lignocellulosic biomass represents the degradation of the ester and glycosidic chain and suggests an increase in the internal surface area, and solvation effect on the structural composition of the material [40,41]. The swelling effect promotes a larger contact surface for higher efficiency of the enzymatic action. Therefore, with the subsequent enzymatic treatment of the resultant solid material, which is the cellulose-rich fraction, monomeric fractions are obtained for further utilization in fermentation processes [22,42].

3.3. Optimization of Enzymatic Hydrolysis of Soybean Hull

3.3.1. Batch Enzymatic Hydrolysis Process

The CCD tool of RSM methodology was used to analyze the effects and the interactions between the selected factors that influence the enzymatic hydrolysis of SBH, as is shown in Table 3 and represented in Figure 2. R^2 was 0.7941, indicating that the model could explain 79.4% of the response variability. This model is presented below: $z = -16.539586971434 + 6.9365911630221 \times x - 0.065286327941407 \times x^2 + 0.45024633546848 \times y - 0.0041949471036503 \times y^2 - 0.025583940331375 \times x \times y - 0.086229983660131 \times 42 \times x + 0.02828167597832 \times 42 \times y + 21.6274308$. According to the results, linear substrate loading and linear interaction between enzyme cocktail loading and time were significantly positive factors in the release of RS from SBH, as shown in the Pareto chart (Figure 2a), while the other variables and their possible interactions do not represent statistical significance. Important concentrations of RS were observed when the interaction between processing time and enzymatic cocktail loading are considered, as shown in the plotted response surface of Figure 2b, which shows an optimum region near the established range. During the experimental processes it was observed that once in the reaction medium, the substrate (SBH) presented a swollen appearance, and with the passage of time and thanks to the enzymatic action, there was a reduction in the size of the material until a relatively homogeneous mixture was obtained in cases where the substrate concentration was around 38.5%. When the substrate concentration was higher, this effect was not achieved, and the reaction medium was not completely homogenized, due to the low agitation rate during this stage (100 rpm). These observations may be related to the results obtained in the RSM for the interaction between the concentration of the substrate and the enzymatic cocktail (Figure 2c), and between substrate concentration and processing time (Figure 2d), in which a possible inhibition of the substrate on the enzymatic action can be suggested, which would require additional studies to be confirmed.

Table 3. Central composite design matrix for the SBH enzymatic hydrolysis optimization.

Standard Run	Enzyme Cocktail			Substrate (d.m.) (% (*w*/*v*))	Time (h)	Reducing Sugars ($g \cdot L^{-1}$)	Yield (g Glucose/g SBH)
	B1 (mg of Protein/g of Substrate)	β-Glucosidase (U/g of Substrate)	Xylanase A (mg of Protein/g of Substrate)				
1	6.62	33.10	1.66	24.5	24.2	60.7	0.15
2	6.62	33.10	1.66	24.5	59.8	64.9	0.16
3	6.62	33.10	1.66	52.5	24.2	61.9	0.07
4	6.62	33.10	1.66	52.5	59.8	99.3	0.12
5	16.42	82.08	4.10	24.5	24.2	91.9	0.23
6	16.42	82.08	4.10	24.5	59.8	63.9	0.16
7	16.42	82.08	4.10	52.5	24.2	89.9	0.11
8	16.42	82.08	4.10	52.5	59.8	84.2	0.10
9	3.28	16.40	0.82	38.5	42.0	76.0	0.12
10	19.76	98.78	4.94	38.5	42.0	72.7	0.12
11	11.52	57.59	2.88	15.0	42.0	42.3	0.17
12	11.52	57.59	2.88	62.0	42.0	115.9	0.12
13	11.52	57.59	2.88	38.5	12.0	71.8	0.12
14	11.52	57.59	2.88	38.5	72.0	78.6	0.13
15 (C)	11.52	57.59	2.88	38.5	42.0	86.6	0.14
16 (C)	11.52	57.59	2.88	38.5	42.0	82.7	0.13
17 (C)	11.52	57.59	2.88	38.5	42.0	79.4	0.13
18 (C)	11.52	57.59	2.88	38.5	42.0	79.2	0.13

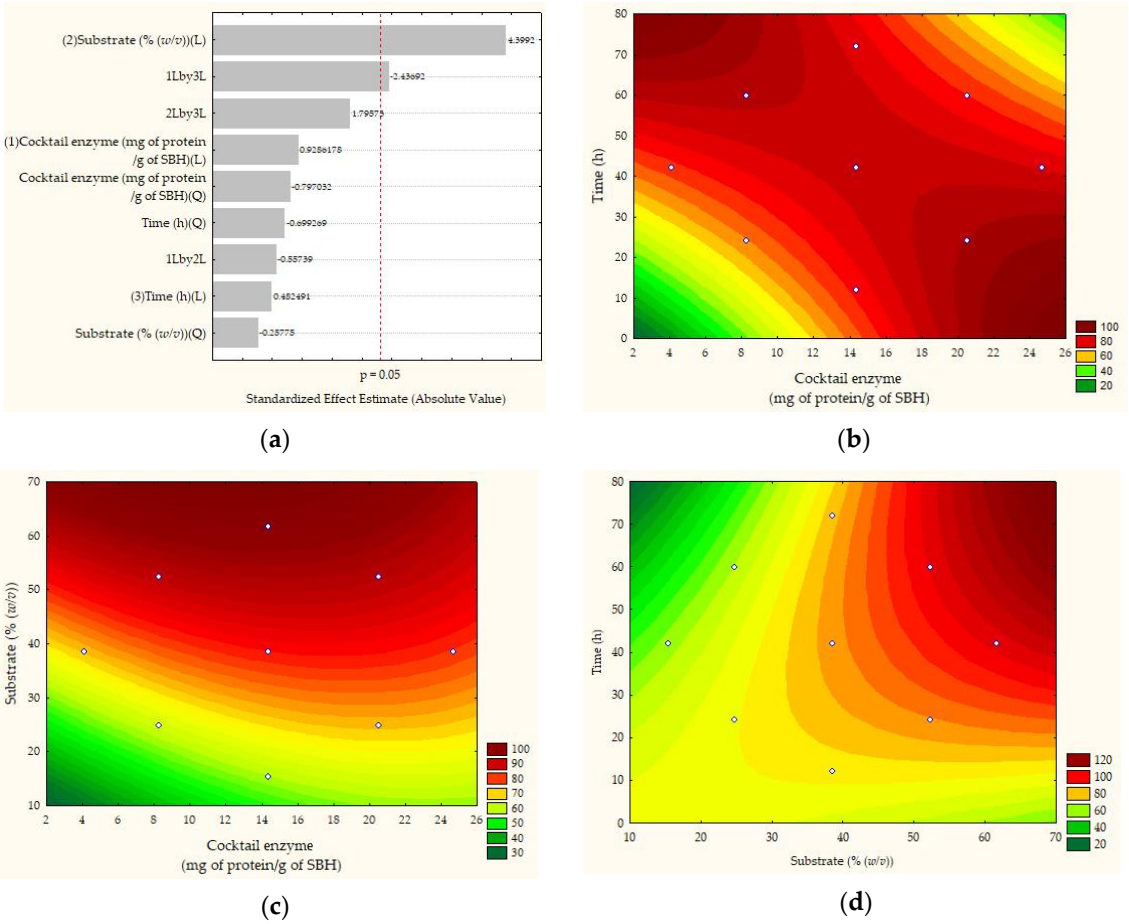

Figure 2. Influence of enzyme cocktail loading, substrate loading, and process time on saccharification of SBH. Response surface plot for optimal concentration for reducing sugars ($g \cdot L^{-1}$) showing interactive effects of: (**a**) Pareto chart of standardized effects on reducing sugars production; (*p*-value = 5%); (**b**) cocktail enzyme loading (mg of protein/g of substrate) vs. time (h); (**c**) cocktail enzyme loading (mg of protein/g of substrate) vs. substrate (% *w/v*); (**d**) substrate (% *w/v*) vs. time (h).

The highest concentration of reducing sugars (115.9 $g \cdot L^{-1}$) was reached by adding 62% (*w/v*) of pretreated SBH (d.m.) after 42 h of process and using the enzyme cocktail composed of B1 enzyme with 11.52 mg protein g^{-1} dry substrate, B1-XylA enzyme, with 2.88 mg protein g^{-1} dry substrate, and F10 β-glucosidase enzyme, with 57.59 U/g of dry substrate (standard run 12). It should be noted that since it was a non-commercial enzymatic cocktail resulting from the drying of a raw fermentation broth, residual sugars (not quantified) remained and they could possibly have interfered with the value mentioned above. In this case, the glucose yield was 0.12 g/g_{SBH}, which corresponded to 18% cellulose conversion. Meanwhile, in the central point (where the substrate loading was 38.5%), using the same concentration of the enzyme cocktail and in the same period, an average glucose yield was 0.13 g/g_{SBH} and cellulose conversion corresponded to 21%, obtaining an average concentration of reducing sugars of 81 $g \cdot L^{-1}$. Considering these results, the next stage of scaling under these processing conditions was developed.

Chemical pretreatment followed by enzymatic hydrolysis of SBH has been previously tested by different authors to recover the greatest amount of reducing sugars from lignocellulosic biomass. Karp et al. (2020) [16] analyzed the application of the enzyme combination of B1 host and B1-XylA to hydrolyze SBH, the same enzymes used in the present work. They described that after an alkaline pretreatment with NaOH followed by enzymatic hydrolysis with this preparation, the SBH was efficiently saccharified, yielding a concentration of reducing sugars between 92–96 $g \cdot L^{-1}$; the glucose recovery corresponded

to 81 $g \cdot L^{-1}$, 74 g/100 g; glucose and reducing sugar yields from initial substrate mass were 36% and 42–47%, respectively. On the other hand, Dall Cortivo et al. [43] carried out the pretreatment of SBH by dilute acid hydrolysis using 1% (mass concentration) of sulfuric acid and heat treatment for 40 min in an autoclave (121 °C), using a solid–liquid ratio of 1:10. The solid fraction was subsequently enzymatically hydrolyzed by the commercial enzyme Celluclast® 1.5 L (Novozymes, Curitiba-PR, Brazil) and the resultant hydrolysate had a final concentration of 25.7 ± 0.7 $g \cdot L^{-1}$ of glucose.

In other experiments, Yoo et al. [24] performed an alkaline pretreatment of SBH (10%) in which 1% sodium hydroxide solution was added, and the mixture was processed at 121 °C for 30 min and 1 atm. For the subsequent enzymatic saccharification, a combination of commercial enzymes (cellulase, Celluclast 1.5 L; β-glucosidase, Novozyme 188, and Viscozyme® L, a cell wall degrading enzyme complex) was implemented and incubated at 50 °C for 72 h in 0.05 M sodium acetate buffer at pH 5. As a result, after enzymatic hydrolysis of alkaline pretreated SBH, glucose yield was 0.36 g/g, which corresponded to 93.3% cellulose conversion. In the present work, where a non-commercial enzyme cocktail was implemented, at the same time of processing (72 h) glucose yield was ≈0.13 g/g, which corresponded to ≈20% cellulose conversion.

3.3.2. Fed-Batch Enzymatic Hydrolysis Process

After validation of the results of the optimization of enzymatic hydrolysis in a batch process, a fed-batch strategy was implemented, as described in the previous section, in a 1L stirred enzymatic bioreactor. Hence, a considerable increment in reducing sugars concentration (37.6%) was obtained, ranging from 115.9 $g \cdot L^{-1}$ to 185.7 $g \cdot L^{-1}$. As well, glucose yield was 0.38 g/g_{SBH}, which corresponded to 60% cellulose conversion. In addition to the fed-batch approach, one of the main factors that improved the efficiency of SBH enzymatic hydrolysis was the stirring with the help of a propeller, which contributed to a better homogenization of the material and better contact of the enzymes with the substrate. Hernández-Beltrán and Hernández-Escoto [44] developed the enzymatic hydrolysis of sorghum straw biomass (pretreated with an alkaline–oxidative medium) at high-solids loading (20% *w/v*·) through a fed-batch operation, using a stirred tank bioreactor (150 rpm), and applying a commercial enzyme complex (Cellic® CTec2, Novozymes®). The produced hydrolysate after 10 h reached a reducing sugars concentration of 126 $g \cdot L^{-1}$.

The higher loading of the SBH substrate used in this study, 38.5%, with lower enzymes' concentrations, in the same system and volume of bioreactor would promote lower process costs, which is certainly the main bottleneck of enzymatic processes. Makasekuru et al. [45] implemented a high-solids fed-batch enzymatic saccharification method, in alkaline-catalyzed atmospheric glycerol organosolv pretreated sugarcane bagasse. They began with 10% (*w/v*) of solids content and 6%, 6%, and 8% of substrates were fed at 6, 12, and 24 h to achieve 30% of the total solids content. The saccharification process mediated by the cellulase enzyme was carried out at 50 °C and 180 rpm. In this study, they used additives (Tween 80, tea saponin, BSA) and accessory enzymes (xylanase and AA9 enzyme) to facilitate cellulase activity. As a result, the saccharification process released 180 $g \cdot L^{-1}$ of fermentable sugars with 70% glucose yield after 72 h. On the other hand, González-Rios et al. [46] studied the quick fed-batch saccharification strategy of pretreated wheat straw (by autohydrolysis followed by a steam explosion stage) at high solid loadings, developed to achieve high sugar concentrations. After optimization of variables (pretreatment time, number of feedings, and the time between them), total sugar concentrations after 60 h with 30% (*w/w*) of solids were 163.5 $g \cdot L^{-1}$, 42% higher than its batch saccharification counterpart.

Other authors have previously tested the performance of high-solids fed-batch enzymatic saccharification, in different lignocellulosic matrices but we are not aware of references that applied this method to SBH. In the previous examples, the authors used various factors independently, such as optimization tools, additives to enhance the enzymatic activity, and stirred tank bioreactors applying commercial enzyme complexes during the process, achieving similar results for RS to those obtained in the present work. Thus, the

optimization of the enzymatic hydrolysis of SBH was relevant to achieving a high release of sugars to be further used as substrate in PHB production. This operational alternative allowed the saccharification process to be made viable, reducing operating costs, with possible process scale-up.

3.4. PHA Production from Alternative Substrates

3.4.1. Inoculum Development and Preparation

C. necator strain DSMz 545 was first cultivated using the medium described for inoculum development using laboratory-grade glucose, where the C:N ratio was tested (1:1, 5:1, and 11:1). In this case, the concentration of yeast extract and peptone was fixed in the inoculum medium, varying glucose concentrations between 5 and 50 $g{\cdot}L^{-1}$. The highest biomass concentration, 5.6 ± 0.3 $g{\cdot}L^{-1}$, was achieved at a C:N ratio of 5:1, corresponding to 20 $g{\cdot}L^{-1}$ of glucose, with a consumption of 42% of the carbon source. At a C:N ratio of 1:1 corresponding to 5 $g{\cdot}L^{-1}$, and a C:N ratio of 11:1 corresponding to 50 $g{\cdot}L^{-1}$, we obtained 4.9 ± 0.4 $g{\cdot}L^{-1}$ and 4.5 ± 0.2 $g{\cdot}L^{-1}$ of biomass, respectively. Thus, in the following step the 5:1 ratio was maintained. Annamalai and Sivakumar [31] studied the effect of different C:N ratios (20:1–20:5) on cell growth of mutant strain *R. eutropha* NCIMB 11599, finding that at a C:N ratio of 20:5 the cell growth was the maximum obtained, reaching 6.23 $g{\cdot}L^{-1}$, a value close to that of the cell growth obtained in this work.

Then, the effect of three different nitrogen sources, yeast extract, ammonium sulfate, and urea, in the biomass development was studied, using 20 $g{\cdot}L^{-1}$ of glucose. The best results were obtained with yeast extract that was added to the culture medium allowing the production of 7.6 ± 0.1 $g{\cdot}L^{-1}$ of biomass, with 100% consumption of the carbon source. Using ammonium sulfate 5.4 ± 0.3 $g{\cdot}L^{-1}$ of biomass was obtained, while 4.6 ± 0.4 $g{\cdot}L^{-1}$ was produced using urea. In both cases, lower consumption of the carbon source was observed with 63% and 47%, respectively. In the same study, Annamalai and Sivakumar [31] analyzed the effect of both inorganic and organic nitrogen sources (NH_4Cl, NH_4NO_3, $(NH_4)_2SO_4$, yeast extract, beef extract, peptone, and tryptone) on cell growth. They found that the ammonium sulfate permitted a higher amount of biomass, corresponding to 6.14 $g{\cdot}L^{-1}$, and using yeast extract, biomass production reached 5.52 $g{\cdot}L^{-1}$, contrarily to what was found in this study, where yeast extract was shown to be the best source of nitrogen for the microorganism.

On the other hand, Cavalheiro et al. [47] evaluated the mechanisms of cell membrane adaptation during the various stages involved in the production of PHA when different carbon sources, glucose (30 $g{\cdot}L^{-1}$) or waste glycerol (30 $g{\cdot}L^{-1}$), were used in the seeding medium and in the basal mineral medium. They used a stock culture of *C. necator* to inoculate the pre-culture for the fed-batch experiments. They found that the degree of saturation of the fatty acids of the phospholipids in the cell membranes of *C. necator* varied during the time course of the fermentation, and cell membranes adapted to the environment by increasing their fluidity, which facilitated substrate uptake and cell division.

As a tentative step to better adapt *C. necator* strain DSMz 545 cells during inoculum development, for subsequent PHB production, the influence in biomass generation of different carbon sources was studied. Laboratory-grade glucose and laboratory-grade glycerol were used as the control/reference carbon sources. SBHH, WG, and their combinations were evaluated as presented in Table 4. In all cases, as it was previously indicated, the initial concentration was calculated based on 8 g/mol of C (considering that 20 g L^{-1} of glucose is equivalent to 8 g/mol of C). When used simultaneously, 50% of SBHH and 50% of WG were added to the medium. Laboratory-grade glucose provided the best biomass production (10.3 ± 0.2 $g{\cdot}L^{-1}$) followed by SBHH (9.0 ± 0.4 $g{\cdot}L^{-1}$), showing consumption of 81% and 91%, respectively. The combination of SBHH and WG was promising with a biomass production of 6.7 $g{\cdot}L^{-1}$ and total consumption of 48%. In this case, the microorganism showed a preference for SBHH sugars consumption (80%) and a lower consumption rate of WG, which reached only 21% after 24 h of culture (data not shown). As a low-cost by-product and because this carbon source is available in large quantities, waste glycerol

can be used as a substrate to produce biomolecules, solving an environmental problem and at the same time reducing the cost of production. When used simultaneously with the SBHH, it is intended to make the production of this type of biopolymer even more economically viable, considering that the cost of the raw material represents more than 50% of the total production cost [48]. Although the microorganism metabolized glycerol (both commercial and waste), biomass production was only 4.5 $g \cdot L^{-1}$ and the consumption of this carbon source reached 17%. SBHH was shown to be a very good alternative for inoculum preparation, which may reduce the lag phase of the microorganism in the following step of biopolymer production.

Table 4. *C. necator* strain DSMz 545 with different carbon sources (inoculum and production media).

Carbon Source	Inoculum		PHB Production		PHB
	Biomass ($g \cdot L^{-1}$)	Carbon Source Consumption (%)	Biomass ($g \cdot L^{-1}$)	Carbon Source Consumption (%)	
SBHH + WG	6.7 ± 0.4	48	8.7 ± 0.1	50	39.0 ± 0.8
SBHH	9.0 ± 0.4	91	7.8 ± 0.2	77	31.8 ± 0.2
WG	4.5 ± 0.2	17	6.9 ± 0.2	44	25.4 ± 1.6
LGG	10.3 ± 0.2	81	9.2 ± 0.2	90	39.4 ± 1.1

SBHH: soybean hull hydrolysate; WG: waste glycerol; LGG: laboratory-grade glucose.

3.4.2. PHB Production

It has been reported in the literature that different carbon sources for polyhydroxyalkanoates production show good prospects for implementation and improvement of yields [48]. Specifically, the use of hydrolysates from different lignocellulosic feedstocks (e.g., rice bran and straw, wheat bran, sunflower hull, sugar cane, among others), have been implemented, obtaining results of accumulation between 20 and 80% of PHA using non-genetically modified strains [31,49–52]. To the best of our knowledge, this is the first time that SBHH has been used as a carbon source to produce polyhydroxyalkanoates. Initially, to verify the influence of different carbon sources on *C. necator* DSMz 545 in biomass production, batch fermentations in MSM medium were developed over 96 h, testing laboratory-grade glucose, SBHH, and WG as carbon sources in one-factor-at-a-time assays, and the mixture of SBHH and WG (Table 4). Then, all samples were analyzed after performing the acid methanolysis by gas chromatography. The retention time of the obtained methyl esters, both from the commercial PHB standard and the samples obtained after fermentation, coincided, allowing the conclusion that the generated biopolymer belonged to the polyhydroxybutyrate (PHB) type.

Biomass production reached 9.2 ± 0.1 $g \cdot L^{-1}$ with the use of laboratory-grade glucose, as well as the best accumulation percentage of 39.4, which was then used as a reference value. When Sen et al. [53] implemented the same strain, in 400 mL MSM culture medium with glucose as carbon source (10 $g \cdot L^{-1}$) in batch fermentation at 30 ± 1 °C, 72 h, 250 ± 10 rpm, they achieved 2.81 $g \cdot L^{-1}$ of biomass and PHB accumulation of 27%. For SBHH used individually, although a high consumption was observed (77%), biomass concentration reached 7.8 ± 0.2 $g \cdot L^{-1}$ and was found to lead to an accumulation of 31.8%. Silva et al. [50] tested sugarcane bagasse hydrolysate (after adsorption treatment with 20% active charcoal for the elimination of toxic compounds) as a carbon source for *Burkholderia sacchari* for PHB production. The microorganism was cultured in batch fermentation using MSM medium (50 mL, 150 rpm, 30 °C), reaching a final cell dry weight of 6.13 $g \cdot L^{-1}$ and 23.33% PHB accumulation. Superior results were attained by Annamalai and Sivakumar [31] using the mutant strain *C. necator* NCIMB 11599, which reached 25 $g \cdot L^{-1}$ and 63% of PHB accumulation using an alkaline pretreated wheat bran hydrolysate (incubated at 150 rpm for 72 h at 30 °C). In this case, a genetically modified strain was employed, it allowed verification that the hydrolysate could be used by microorganisms later in the production of biopolymers.

The low consumption of WG (44%) when used independently, was observed, which directly influenced a lower biomass growth (6.9 ± 0.2 $g \cdot L^{-1}$) and lower accumulation

of the biopolymer (25.4%) when compared with the other results. Similarly, Gahlawat and Soni [54] also reported the use of WG as the sole carbon source (20 $g \cdot L^{-1}$) for PHB production by *C. necator* DSM 545 in batch fermentation, reaching 5.7 $g \cdot L^{-1}$ of biomass and 3.42 $g \cdot L^{-1}$. As pointed out by these authors, the content of contaminants, mainly methanol and sodium ions, affects the growth rate of the microorganism during the fermentation. In this study, the WG used had a considerable content of Na^+ and Cl^- ions, which could have an adverse impact on the growth rate of the strain and PHB production [55].

When SBHH and WG were used simultaneously (4 g/mol C each one), the consumption of the total carbon source was 50% with a biomass production of 8.7 $\pm$ 0.1 $g \cdot L^{-1}$, which was higher than the value achieved when SBHH or WG were used independently. In this case, when this mixture of carbon sources for inoculum development was used, biomass generation (6.7 $\pm$ 0.4) was lower than that observed during the biopolymer production stage (8.7 $\pm$ 0.1). According to Wang et al. [56], glucose is a highly preferred carbon source for some microorganisms, and that also allows it to support relevant growth rates. For the PHB production process, this variety of carbon sources can allow the microorganism to initially consume the most accessible carbon source during the lag and exponential phase and once this is exhausted, the cell will adapt to the consumption of the other available sources to continue with its development [57].

Finally, and meeting expectations, this combination of carbon sources enabled the generation of PHA content of 39%. Figure 3 shows an example of mass balance that exemplifies the process presented in this work. This is a very good result when a large-scale PHB production is projected, bringing important alternatives that allow overcoming the problem of high process costs of biopolymers' production [58]. García et al. [27] developed a study exploring the production of PHAs by *C. necator* DSM 545 in rapeseed meal hydrolysates with MSM medium using a free amino nitrogen solution (400 $mg \cdot L^{-1}$) and crude glycerol (25 $g \cdot L^{-1}$). Bach fermentation was carried out at 180 rpm, 30 °C, pH 6.7–6.9, for 57 h, reaching a total cell dry weight of 13.7 $g \cdot L^{-1}$ with total consumption of glycerol and 46.2% of PHB accumulation. The authors concluded that the hydrolysate, rich in amino acids, acted as a nutrient supplement to achieve desirable microbial growth and PHA production.

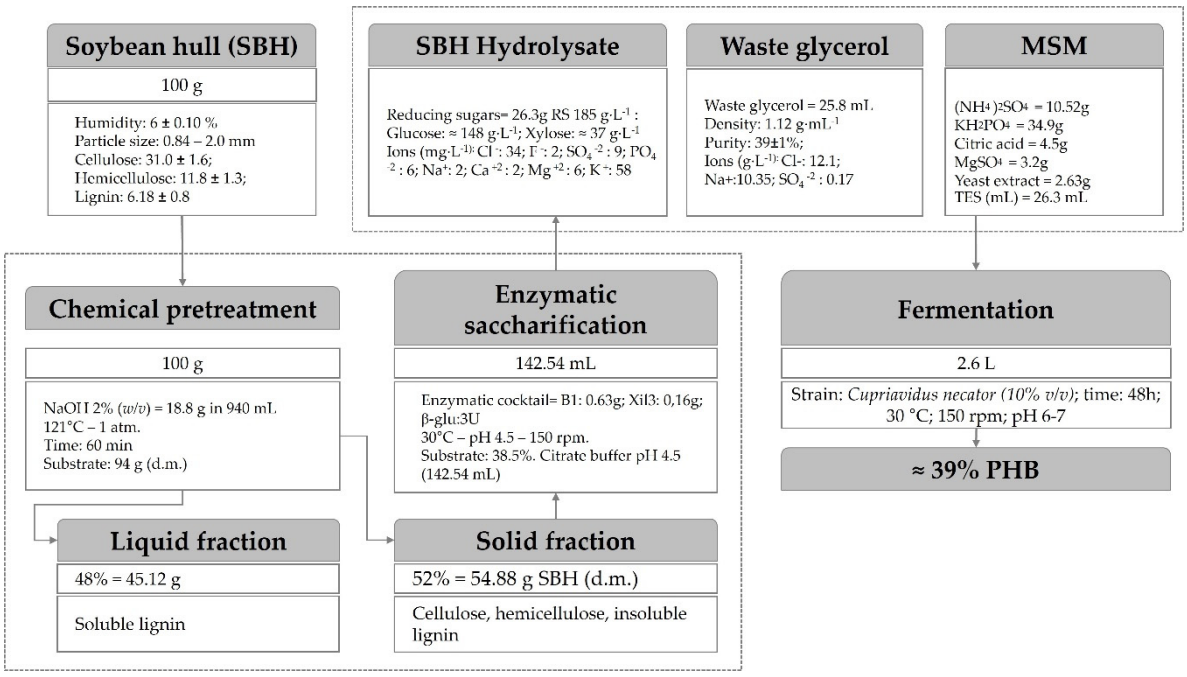

Figure 3. Mass balance of the production process of PHB by *C. necator* DSMz 545 in MSM medium containing SBHH and WG as carbon sources.

3.4.3. PHB Production Kinetics

Considering the results obtained in the previous phase, the kinetics of PHB production using simultaneously SBHH and WG as carbon sources were analyzed as shown in Figure 4. It was observed that the cells of *C. necator* were in the lag phase during the initial 6 h of fermentation, since although they showed slow cell growth and the consumption of carbon sources was null, their metabolism was adapting to the new conditions and were active. The consumption of available carbon sources only started after 12 h of the process. Then, it was observed that up to 48 h of fermentation, the microorganism showed exponential growth until it reached 5.3 ± 0.23 $g \cdot L^{-1}$ of biomass, with productivity of 0.110 $g \cdot L^{-1} \cdot h^{-1}$ to then enter the stationary phase.

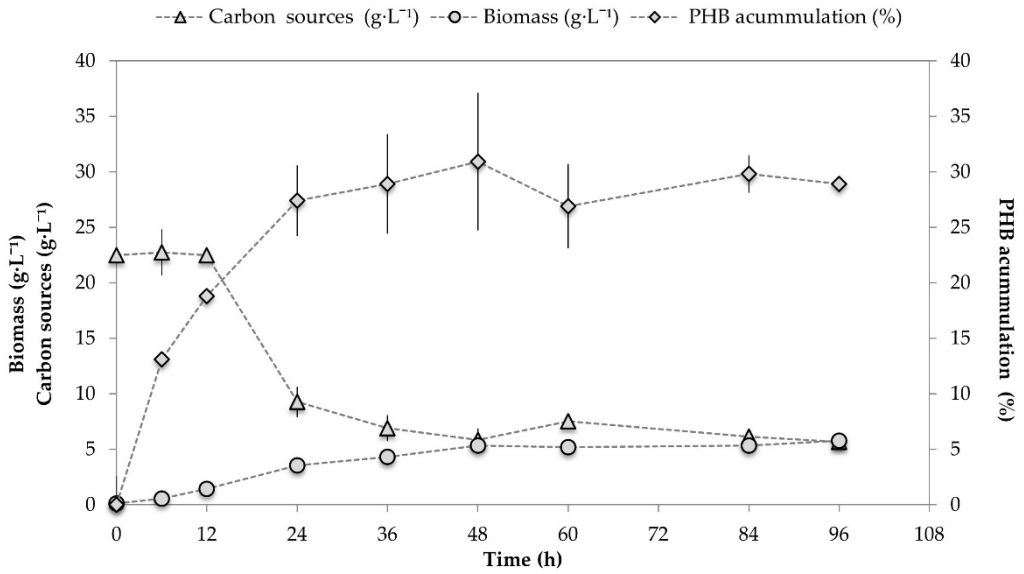

Figure 4. Kinetics of PHB production by *C. necator* DSMz 545 in MSM medium containing SBHH and WG as carbon sources, performed in 250 mL flask.

The consumption profiles for SBHH and WG were followed (Figure 5). For SBHH, glucose and xylose were the main sugars, which were metabolized by the microorganism. Glucose was completely consumed during the first 24 h of the process, during which time the microorganism simultaneously used 53% of the xylose, of which it had a total consumption of 66% at the end of the process. Since the alkaline pretreatment of SBH preserves the hemicelluloses and promotes the generation of pentoses, the fact that the microorganism implemented in the process has the capacity to metabolize these sugars represents a great advantage, because in this case a greater use of this low-cost raw material is obtained. Previously, Poomipuk et al. [59] also described the consumption of this pentose by *Cupriavidus* sp. KKU38. In this case, the hydrolysate was produced from cassava starch, reaching a PHA concentration of 46%.

Figure 5 shows that *C. necator* started the consumption of WG after 24 h (coinciding with the total depletion of the glucose source), showing a total consumption of 39% after 96 h. Maximum carbon sources consumption was reached after 36 h (72%) and remained almost stable until the end of the fermentation. This behavior indicates the preference of *C. necator* for glucose compared to xylose and WG. This fact shows the need to implement optimization methods or fed-batch culture strategies, where a great imbalance of C:N would be created to favor PHB production, as previously demonstrated by Kim et al. [60]. In their study, *C. necator* ATCC 17,699 was grown in flask culture to produce PHA using fructose and γ-butyrolactone as carbon sources. Initially, they established that in batch fermentation, when fructose (20 $g \cdot L^{-1}$) as the sole carbon source was used, it reached 4.95 $g \cdot L^{-1}$ and 53.7% of biomass and PHA accumulation, respectively. Then, to improve those results, they implemented a fed-batch culture for 44 h, where cells were grown by feeding fructose using

the DO-stat method. During the polymer accumulation phase, the feeding solution was changed to a mixture of fructose and γ-butyrolactone and nitrogen limitation was applied. Under these culture conditions, the maximum biomass and PHA accumulation values were 48.5 $g \cdot L^{-1}$ and 50.2%, respectively, reaching maximum productivity of 0.55 $g \cdot L^{-1} \cdot h^{-1}$.

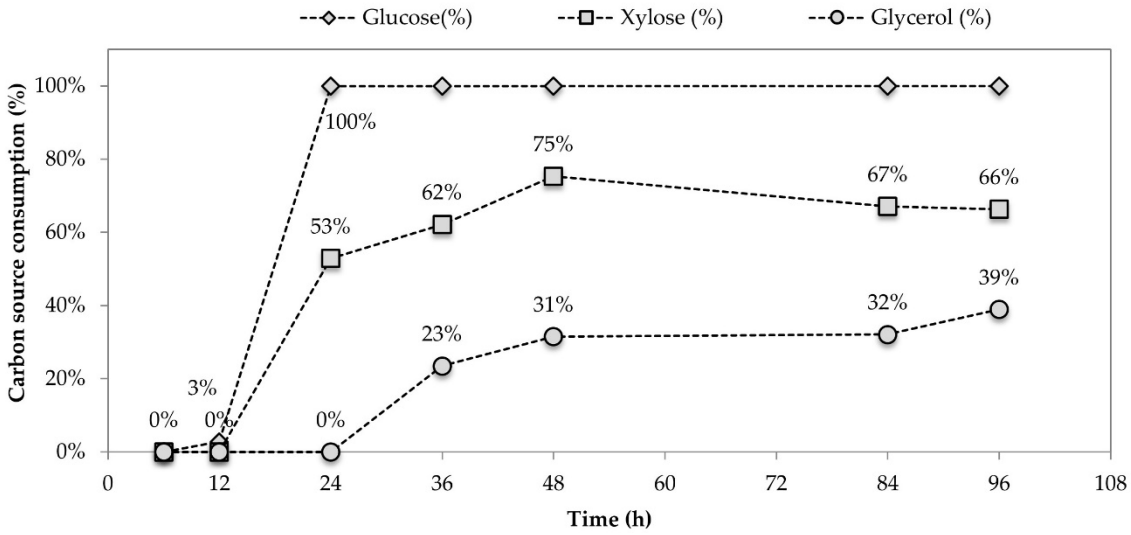

Figure 5. Kinetics of SBHH and WG consumption by *C. necator* DSMz 545 in MSM medium, performed in 250 mL flask.

Regarding the PHB accumulation (Figure 4), it was found that the production of the compound started in the exponential phase and continued until the beginning of the stationary phase. In the first 6 h of the process, 13% was accumulated with a maximum accumulation of 31% after 48 h and the rate was constant until the end of the process. Sen et al. [53] observed similar results using molasses pretreated with hydrothermal acid as a carbon source for *C. necator* (batch culture, work volume 400 mL, 30 °C, 250 rpm), where during the first 24 h of fermentation a generation of approximately 10% of PHB was quantified, and after 60 h there was maximum production of 27.3% of PHB. Although the data obtained are slightly low, they are still within the ranges reported in the literature for native strains on a laboratory scale in batch culture, for which fermentation optimization processes have not been applied. On the other hand, low agitation during the fermentation process (150 rpm) reduces the interactions between the cell and the substrate, in addition to affecting the ability of cell membranes to adapt to the conditions of the medium, reducing the absorption capacity of nutrients from the medium and consequent cell division, as it was observed [27,28]. Furthermore, some factors such as the presence of possible compounds detrimental to the growth of microorganisms, both in the hydrolysate and in the glycerol (which were not quantified), to a certain extent affect the performance of the microorganism during the PHB accumulation stage.

4. Conclusions

The present study demonstrated for the first time that the agro-industrial residues of soybean hull hydrolysate and waste glycerol are alternative carbon sources for the biotechnological production of polyhydroxyalkanoates, molecules of medium-high added value. The efficacy of alkaline pretreatment for the hydrolysis of soybean hull biomass allowed the removal of lignin and increased the subsequent enzyme accessibility. In order to reduce the future cost associated with the enzymatic saccharification of the SBH biomass, a crude enzyme cocktail was effectively used instead of a purified commercial preparation, thus improving the economic feasibility of the process. For the saccharification stage, the operational conditions were optimized by implementing the response surface methodology (RSM), through the central composite design (CCD) tool. Then, the *C. necator* DSMz

545 strain was able to metabolize the sugars (hexoses and pentoses) present in the obtained hydrolysate and waste glycerol, accumulating 39% of polyhydroxybutyrate within 96 h in batch culture.

Author Contributions: Conceptualization, Z.S.-V. and L.P.d.S.V.; methodology, Z.S.-V., L.P.d.S.V. and S.G.K.; formal analysis, Z.S.-V. and L.P.d.S.V.; investigation, Z.S.-V.; writing—original draft preparation, Z.S.-V.; writing—review and editing, Z.S.-V. and L.P.d.S.V.; supervision, L.P.d.S.V.; project administration, L.P.d.S.V.; funding acquisition, L.P.d.S.V. and C.R.S. All authors have read and agreed to the published version of the manuscript.

Funding: CAPES-PROEX and CNPq, process number 310774/2019-5.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico do Brasil (CNPq) for the research scholarship. The authors thank professors Arkady Sinitsyn, Alexandra Rozhkova, Ivan Zorov, and Olga Sinitsyna for donating the cocktail enzyme samples.

Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

1. Su, T.; Zhao, D.; Khodadadi, M.; Len, C. Lignocellulosic biomass for bioethanol: Recent advances, technology trends, and barriers to industrial development. *Curr. Opin. Green Sustain. Chem.* **2020**, *24*, 56–60. [CrossRef]
2. Nahak, B.; Preetam, S.; Sharma, D.; Shukla, S.; Syväjärvi, M.; Toncu, D.-C.; Tiwari, A. Advancements in net-zero pertinency of lignocellulosic biomass for climate neutral energy production. *Renew. Sustain. Energy Rev.* **2022**, *161*, 112393. [CrossRef]
3. Niju, S.; Swathika, M.; Balajii, M. Pretreatment of lignocellulosic sugarcane leaves and tops for bioethanol production. In *Lignocellulosic Biomass to Liquid Biofuels*; Academic Press: Cambridge, MA, USA, 2020; pp. 301–324. [CrossRef]
4. Embrapa. *Soja Em Números (Safra 2020/21); Empresa Brasileira de Pesquisa Agropecuária Embrapa Soja Ministério Da Agricultura, Pecuária e Abastecimento*; Embrapa: Brasília, Brazil, 2022.
5. Amaro Bittencourt, G.; Porto de Souza Vandenberghe, L.; Valladares-Diestra, K.; Wedderhoff Herrmann, L.; Fátima Murawski de Mello, A.; Sarmiento Vásquez, Z.; Grace Karp, S.; Ricardo Soccol, C. Soybean Hulls as Carbohydrate Feedstock for Medium to High-Value Biomolecule Production in Biorefineries: A Review. *Bioresour. Technol.* **2021**, *339*, 125594. [PubMed]
6. Abdul Raman, A.A.; Tan, H.W.; Buthiyappan, A. Two-Step Purification of Glycerol as a Value Added by Product from the Biodiesel Production Process. *Front. Chem.* **2019**, *7*, 774. [CrossRef]
7. *ANP Anuário Estatístico*; Agência Nacional Do Petróleo, Gás Natural e Biocombustíveis: Rio de Janeiro, Brazil, 2021.
8. Ju, J.-H.; Wang, D.; Heo, S.-Y.; Kim, M.-S.; Seo, J.-W.; Kim, Y.-M.; Kim, D.-H.; Kang, S.-A.; Kim, C.-H.; Oh, B.-R. Enhancement of 1,3-propanediol production from industrial by-product by *Lactobacillus reuteri* CH53. *Microb. Cell Factories* **2020**, *19*, 6. [CrossRef]
9. Goyal, S.; Hernández, N.B.; Cochran, E.W. An update on the future prospects of glycerol polymers. *Polym. Int.* **2021**, *70*, 911–917. [CrossRef]
10. UNEP Beat Plastic Pollution. Visual Feature | Beat Plastic Pollution (unep.org). Available online: https://www.unep.org/interactives/beat-plastic-pollution/ (accessed on 21 August 2021).
11. PlasticsEurope. *An Analysis of European Plastics Production, Demand and Waste Data*; PlasticsEurope: Bruxelles, Belgium, 2021.
12. Environmental Protection Agency Facts and Figures about Materials, Waste and Recycling. Plastics: Material-Specific Data. Plastics: Material-Specific Data | US EPA. Available online: https://www.epa.gov/facts-and-figures-about-materials-waste-and-recycling/plastics-material-specific-data (accessed on 21 August 2021).
13. Gahlawat, G. *Polyhydroxyalkanoates Biopolymers—Production Strategies*; Navard, P., Ed.; Springer: Berlin/Heidelberg, Germany, 2019.
14. Li, M.; Wilkins, M. Fed-batch cultivation and adding supplements to increase yields of polyhydroxybutyrate production by *Cupriavidus necator* from corn stover alkaline pretreatment liquor. *Bioresour. Technol.* **2020**, *299*, 122676. [CrossRef]
15. Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, A.; Sluiter, J.; Templeton, D. *Preparation of Samples for Compositional Analysis: Laboratory Analytical Procedure (LAP)*; National Renewable Energy Laboratory: Golden, CO, USA, 2008.
16. Karp, S.G.; Osipov, D.O.; Semenova, M.V.; Rozhkova, A.M.; Zorov, I.N.; Sinitsyna, O.A.; Soccol, C.R.; Sinitsyn, A.P. Effect of Novel *Penicillium verruculosum* Enzyme Preparations on the Saccharification of Acid- and Alkali-Pretreated Agro-Industrial Residues. *Agronomy* **2020**, *10*, 1348. [CrossRef]

17. Sarmiento-Vásquez, Z.; Vandenberghe, L.; Rodrigues, C.; Tanobe, V.O.A.; Marín, O.; de Melo Pereira, G.V.; Ghislain Rogez, H.L.; Góes-Neto, A.; Soccol, C.R. Cocoa pod husk valorization: Alkaline-enzymatic pre-treatment for propionic acid production. *Cellulose* **2021**, *28*, 4009–4024. [CrossRef]
18. Bittencourt, G.A.; Vandenberghe, L.P.D.S.; Valladares-Diestra, K.K.; Soccol, C.R. Soybean hull valorization for sugar production through the optimization of citric acid pretreatment and enzymatic hydrolysis. *Ind. Crop. Prod.* **2022**, *186*, 115178. [CrossRef]
19. Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D. *Determination of Ash in Biomass: Laboratory Analytical Procedure (LAP)*; National Renewable Energy Laboratory: Golden, CO, USA, 2008.
20. Sluiter, A.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D. *Determination of Extractives in Biomass: Laboratory Analytical Procedure (LAP)*; National Renewable Energy Laboratory: Golden, CO, USA, 2008.
21. Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D.; Crocker, D. *Determination of Structural Carbohydrates and Lignin in Biomass: Laboratory Analytical Procedure (LAP)*; National Renewable Energy Laboratory: Golden, CO, USA, 2011.
22. Qing, Q.; Guo, Q.; Zhou, L.; Gao, X.; Lu, X.; Zhang, Y. Comparison of alkaline and acid pretreatments for enzymatic hydrolysis of soybean hull and soybean straw to produce fermentable sugars. *Ind. Crop. Prod.* **2017**, *109*, 391–397. [CrossRef]
23. Karp, S.G.; Rozhkova, A.M.; Semenova, M.V.; Osipov, D.O.; de Pauli, S.T.Z.; Sinitsyna, O.A.; Zorov, I.N.; de Souza Vandenberghe, L.P.; Soccol, C.R.; Sinitsyn, A.P. Designing enzyme cocktails from Penicillium and Aspergillus species for the enhanced saccharification of agro-industrial wastes. *Bioresour. Technol.* **2021**, *330*, 124888. [CrossRef] [PubMed]
24. Yoo, J.; Alavi, S.; Vadlani, P.; Amanor-Boadu, V. Thermo-mechanical extrusion pretreatment for conversion of soybean hulls to fermentable sugars. *Bioresour. Technol.* **2011**, *102*, 7583–7590. [CrossRef]
25. Kumar, M.; Rathour, R.; Singh, R.; Sun, Y.; Pandey, A.; Gnansounou, E.; Andrew Lin, K.Y.; Tsang, D.C.W.; Thakur, I.S. Bacterial polyhydroxyalkanoates: Opportunities, challenges, and prospects. *J. Clean. Prod.* **2020**, *263*, 121500. [CrossRef]
26. Mozumder, M.S.I.; de Wever, H.; Volcke, E.I.P.; Garcia-Gonzalez, L. A robust fed-batch feeding strategy in-dependent of the carbon source for optimal polyhydroxybutyrate production. *Process Biochem.* **2014**, *49*, 365–373. [CrossRef]
27. García, I.L.; López, J.A.; Dorado, M.P.; Kopsahelis, N.; Alexandri, M.; Papanikolaou, S.; Villar, M.A.; Koutinas, A.A. Evaluation of by-products from the biodiesel industry as fermentation feedstock for poly(3-hydroxybutyrate-co-3-hydroxyvalerate) production by *Cupriavidus necator*. *Bioresour. Technol.* **2013**, *130*, 16–22. [CrossRef]
28. Cavalheiro, J.M.B.T.; de Almeida, M.C.M.D.; Grandfils, C.; da Fonseca, M.M.R. Poly(3-hydroxybutyrate) production by *Cupriavidus necator* using waste glycerol. *Process Biochem.* **2009**, *44*, 509–515. [CrossRef]
29. Miller, G.L. Use of Dinitrosalicyclic Acid Reagent for Determination of Reducing Sugar. *Anal. Chem.* **1959**, *31*, 426–428. [CrossRef]
30. Evangeline, S.; Sridharan, T.B. Biosynthesis and statistical optimization of polyhydroxyalkanoate (PHA) produced by *Bacillus cereus* VIT-SSR1 and fabrication of biopolymer films for sustained drug release. *Int. J. Biol. Macromol.* **2019**, *135*, 945–958. [CrossRef]
31. Annamalai, N.; Sivakumar, N. Production of polyhydroxybutyrate from wheat bran hydrolysate using *Ralstonia eutropha* through microbial fermentation. *J. Biotechnol.* **2016**, *237*, 13–17. [CrossRef]
32. Kim, J.S.; Lee, Y.Y.; Kim, T.H. A review on alkaline pretreatment technology for bioconversion of lignocellulosic biomass. *Bioresour. Technol.* **2016**, *199*, 42–48. [CrossRef] [PubMed]
33. Xu, H.; Li, B.; Mu, X. Review of Alkali-Based Pretreatment To Enhance Enzymatic Saccharification for Lignocellulosic Biomass Conversion. *Ind. Eng. Chem. Res.* **2016**, *55*, 8691–8705. [CrossRef]
34. Ganesan, R.; Manigandan, S.; Shanmugam, S.; Chandramohan, V.P.; Sindhu, R.; Kim, S.-H.; Brindhadevi, K.; Pugazhendhi, A. A detailed scrutinize on panorama of catalysts in biodiesel synthesis. *Sci. Total Environ.* **2021**, *777*, 145683. [CrossRef]
35. Singh, J.; Suhag, M.; Dhaka, A. Augmented digestion of lignocellulose by steam explosion, acid and alkaline pretreatment methods: A review. *Carbohydr. Polym.* **2015**, *117*, 624–631. [CrossRef]
36. Jönsson, L.J.; Martín, C. Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects. *Bioresour. Technol.* **2016**, *199*, 103–112. [CrossRef]
37. Wu, D.; Wei, Z.; Mohamed, T.A.; Zheng, G.; Qu, F.; Wang, F.; Zhao, Y.; Song, C. Lignocellulose biomass bioconversion during composting: Mechanism of action of lignocellulase, pretreatment methods and future perspectives. *Chemosphere* **2022**, *286*, 131635. [CrossRef]
38. Saha, B.C.; Cotta, M.A. Enzymatic saccharification and fermentation of alkaline peroxide pretreated rice hulls to ethanol. *Enzym. Microb. Technol.* **2007**, *41*, 528–532. [CrossRef]
39. Camiscia, P.; Giordano, E.D.; Brassesco, M.E.; Fuciños, P.; Pastrana, L.; Cerqueira, M.; Picó, G.A.; Valetti, N.W. Comparison of soybean hull pre-treatments to obtain cellulose and chemical derivatives: Physical chemistry characterization. *Carbohydr. Polym.* **2018**, *198*, 601–610. [CrossRef]
40. Bastos, J.A.; Remor, P.V.; Alino, J.H.L.; Frare, L.M.; Lofhagen, J.C.P.; Edwiges, T. Hydrolysate recycling improves economic feasibility of alkaline pretreatment for bioenergy production. *J. Environ. Chem. Eng.* **2021**, *9*, 105935. [CrossRef]
41. Jin, Y.; Huang, T.; Geng, W.; Yang, L. Comparison of sodium carbonate pretreatment for enzymatic hydrolysis of wheat straw stem and leaf to produce fermentable sugars. *Bioresour. Technol.* **2013**, *137*, 294–301. [CrossRef]
42. Behera, S.; Arora, R.; Nandhagopal, N.; Kumar, S. Importance of chemical pretreatment for bioconversion of lignocellulosic biomass. *Renew. Sustain. Energy Rev.* **2014**, *36*, 91–106. [CrossRef]
43. Cortivo, P.R.D.; Hickert, L.R.; Rosa, C.A.; Ayub, M.A.Z. Conversion of fermentable sugars from hydrolysates of soybean and oat hulls into ethanol and xylitol by *Spathaspora hagerdaliae* UFMG-CM-Y303. *Ind. Crop. Prod.* **2020**, *146*, 112218. [CrossRef]

44. Hernández-Beltrán, J.U.; Hernández-Escoto, H. Enzymatic hydrolysis of biomass at high-solids loadings through fed-batch operation. *Biomass-Bioenergy* **2018**, *119*, 191–197. [CrossRef]
45. Mukasekuru, M.R.; Kaneza, P.; Sun, H.; Sun, F.F.; He, J.; Zheng, P. Fed-batch high-solids enzymatic saccharification of lignocellulosic substrates with a combination of additives and accessory enzymes. *Ind. Crop. Prod.* **2020**, *146*, 112156. [CrossRef]
46. Gonzalez-Rios, J.A.; Valle-Pérez, A.U.; Amaya-Delgado, L.; Sanchez, A. A quick fed-batch saccharification strategy of wheat straw at high solid loadings improving lignocellulosic ethanol productivity. *Biomass-Convers. Biorefinery* **2021**, 1–13. [CrossRef]
47. Cavalheiro, J.M.B.T.; de Almeida, M.C.M.D.; da Fonseca, M.M.R.; de Carvalho, C.C.C.R. Adaptation of *Cupriavidus necator* to conditions favoring polyhydroxyalkanoate production. *J. Biotechnol.* **2012**, *164*, 309–317. [CrossRef]
48. Sirohi, R.; Pandey, J.P.; Gaur, V.K.; Gnansounou, E.; Sindhu, R. Critical overview of biomass feedstocks as sustainable substrates for the production of polyhydroxybutyrate (PHB). *Bioresour. Technol.* **2020**, *311*, 123536. [CrossRef]
49. Oh, Y.H.; Lee, S.H.; Jang, Y.-A.; Choi, J.W.; Hong, K.S.; Yu, J.H.; Shin, J.; Song, B.K.; Mastan, S.G.; David, Y.; et al. Development of rice bran treatment process and its use for the synthesis of polyhydroxyalkanoates from rice bran hydrolysate solution. *Bioresour. Technol.* **2015**, *181*, 283–290. [CrossRef]
50. Silva, L.F.; Taciro, M.K.; Ramos, M.E.M.; Carter, J.M.; Pradella, J.G.C.; Gomez, J.G.C. Poly-3-hydroxybutyrate (P3HB) production by bacteria from xylose, glucose and sugarcane bagasse hydrolysate. *J. Ind. Microbiol. Biotechnol.* **2004**, *31*, 245–254. [CrossRef]
51. 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]
52. Saratale, G.D.; Oh, M.-K. Characterization of poly-3-hydroxybutyrate (PHB) produced from *Ralstonia eutropha* using an alkali-pretreated biomass feedstock. *Int. J. Biol. Macromol.* **2015**, *80*, 627–635. [CrossRef] [PubMed]
53. Sen, K.Y.; Hussin, M.H.; Baidurah, S. Biosynthesis of poly(3-hydroxybutyrate) (PHB) by *Cupriavidus necator* from various pretreated molasses as carbon source. *Biocatal. Agric. Biotechnol.* **2019**, *17*, 51–59. [CrossRef]
54. Gahlawat, G.; Soni, S.K. Valorization of waste glycerol for the production of poly (3-hydroxybutyrate) and poly (3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer by *Cupriavidus necator* and extraction in a sustainable manner. *Bioresour. Technol.* **2017**, *243*, 492–501. [CrossRef] [PubMed]
55. Mothes, G.; Schnorpfeil, C.; Ackermann, J.-U. Production of PHB from Crude Glycerol. *Eng. Life Sci.* **2007**, *7*, 475–479. [CrossRef]
56. Wang, X.; Xia, K.; Yang, X.; Tang, C. Growth strategy of microbes on mixed carbon sources. *Nat. Commun.* **2019**, *10*, 1279. [CrossRef]
57. Rodriguez-Contreras, A.; Koller, M.; Dias, M.M.-D.S.; Calafell-Monfort, M.; Braunegg, G.; Marqués-Calvo, M.S. Influence of glycerol on poly(3-hydroxybutyrate) production by *Cupriavidus necator* and *Burkholderia sacchari*. *Biochem. Eng. J.* **2015**, *94*, 50–57. [CrossRef]
58. Bhatia, S.K.; Otari, S.V.; Jeon, J.-M.; Gurav, R.; Choi, Y.-K.; Bhatia, R.K.; Pugazhendhi, A.; Kumar, V.; Banu, J.R.; Yoon, J.-J.; et al. Biowaste-to-bioplastic (polyhydroxyalkanoates): Conversion technologies, strategies, challenges, and perspective. *Bioresour. Technol.* **2021**, *326*, 124733. [CrossRef]
59. Poomipuk, N.; Reungsang, A.; Plangklang, P. Poly-β-hydroxyalkanoates production from cassava starch hydrolysate by *Cupriavidus* sp. KKU38. *Int. J. Biol. Macromol.* **2014**, *65*, 51–64. [CrossRef]
60. Kim, J.S.; Lee, B.H.; Kim, B.S. Production of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) by *Ralstonia eutropha*. *Biochem. Eng. J.* **2005**, *23*, 169–174. [CrossRef]